Inducible Nitric Oxide Synthase as a Target of Anti-Inflammatory Treatment Modalities

ACADEMIC DISSERTATION
To be presented, with the permission of the Faculty of Medicine of the University of Tampere, for public discussion in the small auditorium of Building B, Medical School of the University of Tampere, Medisiinarinkatu 3, Tampere, on August 22nd, 2008, at 12 o’clock.
ACADEMIC DISSERTATION
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http://granum.uta.fi

Acta Universitatis Tamperensis 1339
ISBN 978-951-44-7421-7 (print)
ISSN 1455-1616

Acta Electronica Universitatis Tamperensis 754
ISSN 1456-954X
http://acta.uta.fi

Tampereen Yliopistopaino Oy – Juvenes Print
Tampere 2008
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This thesis is based on the following original communications, referred to in the text by their Roman numerals I-IV.


IV Hämäläinen M, Korhonen R and Moilanen E: Calcineurin inhibitors down-regulate iNOS expression by destabilising mRNA. International Immunopharmacology, in press.

In addition, some unpublished data are presented.
# Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>ARE</td>
<td>AU-rich element</td>
</tr>
<tr>
<td>BH₄</td>
<td>tetrahydrobipterin</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CBP</td>
<td>cAMP-response-element-binding protein-binding protein</td>
</tr>
<tr>
<td>cGMP</td>
<td>cyclic guanosine monophosphate</td>
</tr>
<tr>
<td>CREB</td>
<td>cAMP-response-element-binding protein</td>
</tr>
<tr>
<td>CsA</td>
<td>cyclosporin A</td>
</tr>
<tr>
<td>EMSA</td>
<td>electrophoretic mobility shift assay</td>
</tr>
<tr>
<td>GAPDH</td>
<td>glyceraldehyde phosphate dehydrogenase</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>granulocyte-macrophage colony stimulating factor</td>
</tr>
<tr>
<td>GR</td>
<td>glucocorticoid receptor</td>
</tr>
<tr>
<td>GRE</td>
<td>glucocorticoid responsive element</td>
</tr>
<tr>
<td>HAT</td>
<td>histone acetyltransferase</td>
</tr>
<tr>
<td>HDAC</td>
<td>histone deacetylase</td>
</tr>
<tr>
<td>IFN</td>
<td>interferon</td>
</tr>
<tr>
<td>IκB</td>
<td>inhibitory κB</td>
</tr>
<tr>
<td>IKK</td>
<td>IκB kinase</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>JAK</td>
<td>janus activated kinase</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun N-terminal kinase</td>
</tr>
<tr>
<td>L-NIO</td>
<td>L-N-iminoethyl-L-ornithine</td>
</tr>
<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
</tr>
<tr>
<td>NF-AT</td>
<td>nuclear factor of activated T cells</td>
</tr>
<tr>
<td>NF-κB</td>
<td>nuclear factor κB</td>
</tr>
<tr>
<td>NO</td>
<td>nitric oxide</td>
</tr>
<tr>
<td>NOS</td>
<td>nitric oxide synthase</td>
</tr>
<tr>
<td>PDE</td>
<td>phosphodiesterase</td>
</tr>
<tr>
<td>PPAR</td>
<td>peroxisome proliferator-activated receptor</td>
</tr>
<tr>
<td>PTK</td>
<td>protein tyrosine kinase</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>reverse-transcriptase polymerase chain reaction</td>
</tr>
<tr>
<td>sGC</td>
<td>soluble guanylate cyclase</td>
</tr>
<tr>
<td>STAT</td>
<td>signal transducer and activator of transcription</td>
</tr>
<tr>
<td>TGF</td>
<td>transforming growth factor</td>
</tr>
<tr>
<td>TSA</td>
<td>trichostatin A</td>
</tr>
<tr>
<td>UTR</td>
<td>untranslated region</td>
</tr>
</tbody>
</table>
Abstract

Nitric oxide (NO) is a gaseous signaling molecule with various physiological and pathophysiological properties. Three distinctive nitric oxide synthase (NOS) enzymes produce NO; endothelial NOS (eNOS), neuronal NOS (nNOS) and inducible NOS (iNOS). Of these, eNOS and nNOS are mainly responsible for the low physiological production of NO. In inflammation, bacterial products and inflammatory cytokines induce the expression of iNOS, which produces high amounts of NO over prolonged periods. Nitric oxide has regulatory and pro-inflammatory properties in inflammation. In a number of inflammatory diseases, such as rheumatoid arthritis and inflammatory bowel disease, increased iNOS expression and NO production have been shown to have detrimental effects. Selective iNOS inhibitors have proved to have anti-inflammatory effects in many experimentally-induced inflammatory reactions.

The aim of the present study was to explore how iNOS expression and NO production are regulated. This may be related to the pathogenesis of inflammation and the knowledge can be utilized in the development of novel drugs. In the present study, the effects of several anti-inflammatory compounds on iNOS expression and NO production were investigated and the more precise mechanisms of action were further clarified.

iNOS expression and NO production was induced by bacterial lipopolysaccharide (LPS) in murine macrophages, by LPS and interferon γ (IFNγ) in murine fibroblasts, and by cytokine interleukin-1β in human alveolar epithelial cells. Chemical methods, and techniques of cellular and molecular biology were applied to study the production of NO, expression of iNOS, and the activation of transcription factors important for iNOS expression.

The glucocorticoid, dexamethasone, as well as RU24858, which is a dissociated glucocorticoid, were found to inhibit iNOS expression and NO production probably by a mechanism related to increased histone deacetylation. Glucocorticoids have earlier been described to exert their effects on certain other pro-inflammatory genes via increased histone deacetylation. In the present study, histone deacetylation is proposed as a novel mechanism mediating the inhibitory effects of glucocorticoids on iNOS expression and NO production.

Eight flavonoids out of 36 naturally occurring phenolic compounds studied, were found to inhibit the activation of transcription factor nuclear factor κB (NF-κB), and four of them inhibited also the activation of signal transducer and activator of transcription 1 (STAT-1), in addition to their inhibitory action on iNOS expression and NO production. The activations of these two transcription factors are known to be important in iNOS expression. The inhibition of the
activation of NF-κB and/or STAT-1 is believed to explain the reduced iNOS expression and NO production caused by the flavonoids flavone, daidzein, genistein, isorhamnetin, kaempferol, quercetin, naringenin and pelargonidin.

Calcineurin is a serine/threonine phosphatase known to mediate several cellular signals, e.g. in response to T cell activation. Three calcineurin inhibitors, cyclosporin A (CsA), tacrolimus (FK-506) and pimecrolimus, were shown to inhibit iNOS expression and NO production, probably by enhancing the degradation of iNOS mRNA.

In the present study, different mechanisms to explain how iNOS expression is regulated in response to inflammatory stimuli were identified. Novel compounds that inhibited iNOS expression were discovered. In addition, novel mechanisms of action were clarified for compounds which were previously known to inhibit iNOS expression and NO production. These mechanisms are associated with the anti-inflammatory properties of these compounds and this knowledge may be utilized in the development of new anti-inflammatory compounds with more selective target specificities.
Typpioksidit (NO) ovat elimistön signaalimolekyylit, jotka säätelevät sekä fysiologisia että patofysiologisia toimintoja. Typpioksidisyntetät (NOS) ovat entsyymejä, jotka tuottavat typpioksidia. Niitä tunnetaan kolme erilaista; endoteliaalinen NOS (eNOS), neuronaalinen NOS (nNOS) ja induusoituva NOS (iNOS). eNOS ja nNOS tuottavat pääosin fysiologisissa olosuhteissa pieniä määriä NO:a vasteen solun reseptorivälitteiselle aktivaatiolle, kun taas tulehduskessa bakteriiperäiset tuotteet ja tulehdusta voimistavat sytykinnit lisäävät iNOS:n ilmentymistä ja NO:n tuottoa. Tulehduksessa tuotetulla NO:lla on tulehdusta voimistavia ja säätäviä ominaisuuksia. Monissa tulehdussairauksissa, kuten reumataudeissa ja tulehdussairauksissa suolistosairauksissa, typpioksidilla on havaittu olevan haitallista, tulehdusta voimistavaa vaikutusta. Selektiivisillä iNOS entsyymin estäjillä on saatu aikaan suotuisia tuloksia tulehduksissa.

Väitöskirjatyön tarkoituksena oli tutkia iNOS:n ilmentymisen ja NO:n tuoton säätelyä. Tavoitteena oli myös lisätä tietoa iNOS:n ilmentymisen säätelystä tulehduksessa, mitä voitaisiin edelleen käyttää hyväksi lääkekehityksessä. Väitöskirjatyössä tutkittiin erityisesti tulehdusta vaimentavien yhdisteiden vaikutuksia iNOS:n ilmentymiseen ja NO:n tuottoon. Tehokkaiden yhdisteiden osalta tutkittiin tarkemmin niiden molekulaarisia vaikutusmekanismeja.

Glukokortikoidi deksametasoni ja nk. dissosiaatiosteroidi RU24858 estivät NO:n tuottoa ja iNOS:n ilmentymistä, ja vaikutus näytti välittyvän histonien deasetylaation kautta. Kahdeksan 36 tutkitusta mm. elintarvikkeissa esiintyvää fenolialaa yhdisteeseen, estivät iNOS:n ilmentymistä ja NO:n tuottoa. Kaikki kahdeksan flavonoidia estivät iNOS:lle tärkeän transkriptiotekijän NF-κB:n aktivaatiota, ja näistä neljä estivät myös transkriptiotekijän STAT-1:n aktivaatiota. Vaikutukset transkriptiotekijöiden aktivaaatioon voivat selittää iNOS:n ilmentymisen ja typpioksidin tuoton estovaikutuksen. Immunosuppressiiviset yhdisteet siklosporiini A, takrolimuusi (FK-506) ja pimekrolimuusi ovat kalsineuriiniestäjiä, joita käytetään mm. estämään hylkimisreaktiota elinsirtojen yhteydessä, ja tulehdussairauksien kuten reuman ja suolistosairauksien hoidossa. Kalsineuriiniestäjien havaittiin estävän iNOS:n ilmentymistä ja NO:n tuottoa nopeuttamalla iNOS:n lähetti-RNA:n hajoamista.

iNOS:n ilmentymisen ja NO:n tuoton estoon voidaan ajatella olevan osatekijänä näiden tulehdusta lievittävien yhdisteiden vaikutusmekanismeissa. Glukokortikoidien iNOS:n ilmentymistä ja NO:n tuottoa estävän uutena mekanismina esitetään vaikutus histonien deasetylaatioon. Tiettyjen flavonoidien

Tiivistelmä
havaittiin estävän iNOS:n ilmentymistä geeniaaktivointia aikaisessa vaiheessa estämällä iNOS:lle tärkeiden transkriptiotekijöiden NF-κB:n ja STAT-1:n aktivaatiota. Kalsineuriniiestäjät vaikuttivat iNOS lähetti-RNA:n hajoamisnopeuteen, mikä puolestaan johti iNOS:n ilmentymisen ja NO:n tuoton vähennemiseen.

Tässä työssä tunnistettiin erilaisia mekanismeja, miten iNOS:n ilmentymistä ja NO:n tuottoa voidaan vähentää. Tutkimuksessa löydettiin uusia iNOS:n ilmentymistä estäviä yhdisteitä, sekä selvitettiin aiemmin tunnettuja tehokkaiden yhdisteiden tarkempia vaikutusmekanismeja. Tulokset antavat lisätietoa tutkittujen yhdisteiden tulehdusta vaimentavista mekanismeista, ja tätä tutkimustietoa voidaan hyödyntää kehitettäessä uusia entistä selektiivisempiä lääkeaineita tulehdustautien hoitoon.
Introduction

Inflammation is a defence response of the body to invading microbes and toxins as well as to its own injured cells and tissues. Repair processes closely follow the resolution of inflammation. The classical signs of inflammation are redness, swelling, heat, pain, and loss of function (rubor, tumor, calor, dolor, functio laesa).

Inflammation can be triggered by many different processes e.g. infection and microbial toxins, trauma, physical and chemical agents, tissue necrosis, foreign bodies, and allergens. Acute inflammation is characterized by an increase in blood flow, leakage of plasma proteins and fluids from microvasculature, and emigration of inflammatory cells from the blood flow to the site of inflammation. Persistent infection, prolonged exposure to potentially toxic agents and autoimmunity are the main causes of chronic, prolonged inflammation. Chronic inflammation is characterized by infiltration of mononuclear cells, tissue destruction, and angiogenesis and fibrosis. (Kumar et al. 2005)

The immune response can be divided into innate immunity and adaptive immunity. Innate immunity is an inborn defence mechanism, which does not require prior contact with the pathogen (such as bacteria and viruses), utilizes pattern recognition techniques, is rapid and linked with a low risk of autoimmunity. The response in innate immunity is constant and not increased after repeated exposures to the pathogen. In response to pattern recognition, the Toll-like receptors become activated, leading to the triggering of inflammatory transcription factors and enhanced formation of pro-inflammatory mediators. In contrast to innate immunity, adaptive immunity has a later onset, but the response is more specific and increases after repeated exposure. Specific proteins are recognized and, in theory, any protein structure can trigger the response, which confers also to a considerable risk of autoimmunity. (Kumar et al. 2005, Wollenberg and Klein 2007)

Nitric oxide (NO) is a signaling molecule with immunomodulatory properties. Macrophages are important cells in inflammatory responses, and have been shown to produce NO through the inducible nitric oxide synthase (iNOS) pathway in response to inflammatory stimulus. High levels of NO may lead to increased cytotoxicity in the inflammatory focus, and inhibitors of expression and enzymatic activity of iNOS have been shown to have anti-inflammatory effects (Vallance and Leiper 2002, Tinker and Wallace 2006). NO is one of the cytotoxic molecules targeted at destroying pathogens and cancer cells. However, in autoimmune reactions and other forms of chronic inflammation, NO and NO-derived radicals can harm also the host tissues. In addition, NO regulates many
vascular and cellular responses in the inflammatory reaction. Inhibitors of NOS, especially iNOS, have been demonstrated to have anti-inflammatory properties in various experimentally-induced inflammatory reactions. Therefore, the inhibition of iNOS expression is an interesting molecular target for anti-inflammatory compounds.
Review of the literature

1 Nitric oxide

1.1 Discovery of NO

Nitric oxide (NO) is a small and short-lived signaling molecule involved in various physiological and pathophysiological conditions in the human body (Vallance and Leiper 2002, Korhonen et al. 2005). The history of NO begins from the discovery of nitroglycerine by Ascanio Sobrero in 1847. Nitroglycerine was first used as a remedy for headache and later for the treatment of angina pectoris (Marsh and Marsh 2000). It was not until the late 1970’s when the mechanisms behind nitrate-containing compounds began to be unravelled. Ferid Murad and co-workers reported that NO and nitro compounds were capable of activating guanylate cyclase, an enzyme catalyzing the formation of a second messenger cyclic guanosine monophosphate (cGMP) (Arnold et al. 1977). A few years later, Robert Furchgott and John Zawadzki made the seminal observation that endothelial cells were key players in producing a substance evoking the acetylcholine-mediated relaxation of arterial smooth muscle (Furchgott and Zawadzki 1980). Salvador Moncada (Palmer et al. 1987) and Louis Ignarro (Ignarro et al. 1987) identified this endothelium-derived relaxing factor (EDRF) as NO in 1987. Mouse macrophages were found to produce nitrite in response to bacterial lipopolysaccharide (Stuehr and Marletta 1985). It was soon realized that nitrite and nitrate produced by activated macrophages had been oxidized from NO (Hibbs et al. 1988). Nitric oxide produced by macrophages has been implicated in the cytotoxic, antimicrobial and antitumor activity of these cells (MacMicking et al. 1997).

Furchgott, Ignarro and Murad were awarded the Nobel Prize in Physiology or Medicine in 1998 …”for their discoveries concerning nitric oxide as a signaling molecule in the cardiovascular system”.

1.2 Biosynthesis of NO

Nitric oxide is produced from the amino acid L-arginine by nitric oxide synthase (NOS, EC1.14.13.39) enzymes. NO, citrulline and nicotinamide adenine dinucleotide phosphate (NADP) are formed from L-arginine, molecular oxygen and NADPH in a two-step oxidation reaction catalyzed by NOS (Figure 1).
NOS enzymes are only active as homodimers and require cofactors (6R)-5,6,7,8-tetrahydrobiopterin (BH$_4$), flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), iron protoporphyrin IX (haem) as well as tightly bound calmodulin (CaM) in order to express their activity. The domain structure of human inducible NOS (iNOS) is represented in Figure 2A and the proposed model of the iNOS dimer in Figure 2B. (Siddhanta et al. 1998, Alderton et al. 2001)
Three NOS isoenzymes, which share ~50% sequence homology have been characterized (NOS I, II and III), and they catalyze the formation of NO via the L-arginine pathway. NOS I (neuronal NOS, nNOS) and NOS III (endothelial NOS, eNOS) are constitutively expressed enzymes and responsible for the short-term production of low levels of NO in response to physiological stimuli. nNOS and eNOS were first characterized in neuronal and in vascular endothelial cells, respectively, but they were later also found in several other cells. The production of NO by nNOS and eNOS is regulated by the cytosolic free calcium concentration $[\text{Ca}^{2+}]$. Elevation of $[\text{Ca}^{2+}]$, enables tight binding of CaM and results in activated enzyme. NOS II (inducible NOS, iNOS) is an inducible form of NOS and its activity is independent of cytosolic calcium concentration due to its high affinity to CaM. The production of NO through the iNOS pathway is regulated at the level of iNOS expression. iNOS expression is regulated at transcriptional and posttranscriptional levels and once induced e.g. by proinflammatory cytokines and/or bacterial products it can produce high amounts of NO for prolonged periods. The main features of the three distinctive NOS enzymes are presented in Table 1. (Alderton et al. 2001, Korhonen et al. 2005)

**Table 1. Main characteristics of nitric oxide synthase enzymes**

<table>
<thead>
<tr>
<th>NOS</th>
<th>Type</th>
<th>Genomic localization</th>
<th>Cellular localization</th>
<th>MW/ kDa (monomer)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>constitutive/ $\text{Ca}^{2+}$-dependent</td>
<td>12q24.2-12q24.3</td>
<td>cytosol</td>
<td>160</td>
</tr>
<tr>
<td>II</td>
<td>inducible/ $\text{Ca}^{2+}$-independent</td>
<td>17cen-17q11.2</td>
<td>cytosol</td>
<td>130</td>
</tr>
<tr>
<td>III</td>
<td>constitutive/ $\text{Ca}^{2+}$-dependent</td>
<td>7q35-7q36</td>
<td>membrane/cytosol</td>
<td>135</td>
</tr>
</tbody>
</table>

nNOS, with a chromosomal location of 12q24.2-12q24.3 was first characterized in neuronal cells but it has also been found in several nonneuronal cell types. This so called constitutively expressed NOS has been shown to be regulated not only at the level of enzyme activity by $[\text{Ca}^{2+}]$, but also at the level of expression. Multiple alleles of nNOS have been described. nNOS is regulated post-transcriptionally by alternative mRNA splicing and the activity of the enzyme is regulated by phosphorylation. (Forstermann et al. 1998, Alderton et al. 2001, Kavya et al. 2006)

Endothelial NOS (eNOS) was first identified in bovine aortic endothelial cells and thereafter it has been found to be expressed in several endothelial and nonendothelial cell types. The eNOS gene is located at 7q35-7q36. Post-translational modifications are important in targeting eNOS to Golgi membranes.
and plasmalemmal caveolae. Expression of eNOS is regulated by several factors and some eNOS polymorphisms have been linked to the increased risk for coronary artery disease and some other diseases. It has also been shown that there is also post-transcriptional regulation for eNOS. (Forstermann et al. 1998, Alderton et al. 2001, Kavya et al. 2006)

iNOS gene is located at 17cen-17q11.2, and it is expressed in variety of immune and nonimmune cells and regulated at multiple levels (Korhonen et al. 2005). Nitric oxide production via the iNOS pathway is highly regulated at the level of iNOS expression. The regulation of iNOS expression is discussed in more detail in chapter 2.

NO has been shown to have a role in the function of mitochondria (Erusalimsky and Moncada 2007). It has been proposed that mitochondria produce NO through an alpha isoform of nNOS which has implications in energy metabolism, oxygen consumption and free radical production in mitochondria (Haynes et al. 2004).

Nitric oxide may be formed also non-enzymatically in addition to the above described NOS dependent L-arginine pathway. The dietary intake of nitrate and nitrite is an important factor in the non-enzymatic production of NO. Spinach and lettuce are especially rich in nitrate, and in some countries also drinking water contains elevated levels of nitrate. Nitrite is a preservative used in meat products. Commensal bacteria in the human gastrointestinal tract are able to reduce nitrate to nitrite which may be further reduced to NO by different pathways (Lundberg et al. 2008). Non-enzymatic NO production has been described to occur in stomach (Lundberg et al. 1994), in ischaemic heart (Zweier et al. 1995) and in skin (Weller et al. 1996). Non-enzymatic production of NO has been shown to be enhanced in acidic conditions and hypoxia (Lundberg et al. 2008).

1.3 Inhibitors of nitric oxide production

1.3.1 NOS inhibitors

Excessive nitric oxide production by iNOS has been detected in several inflammatory diseases, raising the possibility of beneficial effects of NOS inhibitors in the treatment of inflammatory disorders, though yet no single compound has entered clinical use. Inhibition of eNOS is mainly harmful leading to vasoconstriction and hypertension whereas inhibition of nNOS has both beneficial (e.g. in neurological disorders) and detrimental effects. Therefore, in the development of NOS inhibitors, it is important to consider isoform selectivity as well as potency. (Vallance and Leiper 2002, Tinker and Wallace 2006)

Inhibition of NOS at early stages of experimentally induced vascular injury in rat intestine was found to be detrimental whereas at later time-points, administration of NOS inhibitors was beneficial (Laszlo et al. 1994). A highly
iNOS selective inhibitor, GW273629, prevented indomethacin-induced intestinal injury in rats (Evans and Whittle 2001), and another iNOS selective inhibitor, 1400W, ameliorated experimentally induced colitis in rats (Kankuri et al. 2001). In animal models of osteoarthritis and rheumatoid arthritis N-(iminoethyl)-L-lysine (L-NIL), which is a partially iNOS selective NOS inhibitor, suppressed disease progression (Connor et al. 1995, Pelletier et al. 1998). In contrast to these studies, McCartney-Francis and co-workers found L-NIL to make arthritis worse in rats (McCartney-Francis et al. 2001).

NO production has been shown to be increased in septic shock. Case reports from the early 1990’s seemed to indicate that treatment of severe septic shock with a non-selective NOS inhibitor N°-monomethyl-L-arginine (L-NMMA) would be beneficial (Petros et al. 1991), but a multi-centre, randomized, placebo-controlled, double-blind study with nearly 800 patients revealed increased mortality in patients treated with L-NMMA as compared to patients in the placebo group (Lopez et al. 2004). In both groups, refractory shock and multiple organ failure were the two most common causes of death (Lopez et al. 2004). NOS inhibitors have been studied in several diseases with beneficial outcomes e.g. in the treatment of colon cancer (Rao 2004) as well as headache (Ashina et al. 1999) but in some other diseases they have appeared to be ineffective, e.g. in the treatment of psoriasis with L-NMMA (Ormerod et al. 2000). It must also be kept in mind that iNOS derived NO is important in host defence and there are several studies showing that administration of NOS inhibitors can worsen the course of an infection (MacMicking et al. 1997). Inhibition of NOS had also detrimental effects in wound healing, e.g. administration of L-NIL impaired re-epithelialization process in wounded mice (Stallmeyer et al. 1999).

Most of the investigated NOS inhibitors are analogues of L-arginine, which is the substrate of NOS enzyme. Despite the fact that arginine binding sites are very similar between different NOS enzymes, isoform selectivity has been attained with these types of NOS inhibitors. Inhibition of NO production by inhibiting NOS enzyme may also be achieved by inhibition of flavoproteins, by antagonizing calmodulin, by competing with tetrahydrobiopterin, or inhibitors can act as ligands for the haem iron or inhibit NOS dimerization. Here, selectivity for a specific NOS isoform uses the scheme devised by Alderton et al. (2001) i.e. non-selective with less than 10-fold selectivity against other isoforms, partially selective with 10-50 –fold selectivity and finally highly selective (>50 –fold selectivity). (Alderton et al. 2001, Tinker and Wallace 2006)

L-NMMA is a non-selective NOS inhibitor, which competitively inhibits the enzyme by inhibiting binding of L-arginine. L-NIL, which has been used in several experimental settings as a selective iNOS inhibitor, has 23-fold and 49-fold selectivity for iNOS vs. nNOS and eNOS, respectively, and thus it may best be characterized as a partially selective iNOS inhibitor. N-(iminoethyl)-L-ornithine (L-NIO) is an acetamidine analogue of arginine and a partially selective iNOS inhibitor like L-NIL. (Figure 3) (Alderton et al. 2001, Tinker and Wallace 2006)
Bis-isothioureas were first described as highly iNOS selective inhibitors with 190-fold selectivity for iNOS vs. eNOS but in practice with no preference for inhibiting iNOS over nNOS (Garvey et al. 1994).

![Chemical Structures](image)

**Figure 3. Structure of L-arginine and some NOS inhibitors**

Pharmacological utilization of bis-isothioureas was hindered due to their poor tissue and cell penetration as well as their toxicity. N-substituted amidine N-(3-(aminomethyl)benzyl)acetamidine (1400W) has been characterized as a highly selective iNOS vs. eNOS (>4000-fold) inhibitor with partial (32-fold) selectivity of iNOS against nNOS (Garvey et al. 1997). 1400W penetrates tissues and cells.
but its acute toxicity at high doses has limited its use in humans. Sulphur-substituted acetamidine acids GW274150 and GW273629 have proven efficacy in inhibiting iNOS not only in vitro but also under in vivo conditions (Alderton et al. 2005). GW274150 and GW273629 are highly selective iNOS inhibitors against both constitutive isoforms of NOS. (Figure 3)

Recently, an imidazopyridine derivative BYK191023 has been characterized, which inhibits competitively binding of L-arginine and is structurally unrelated to any of the previously known inhibitors. BYK191023 showed 200-fold selectivity for iNOS vs. nNOS and over 1000-fold selectivity for iNOS vs. eNOS with no apparent toxicity even at high concentrations and is thereby a promising candidate for clinical development (Strub et al. 2006). Also coumarin-based molecules have shown selectivity towards iNOS (Jackson et al. 2005).

FR260330 is an example of a compound which has been reported to inhibit iNOS probably through inhibition of iNOS dimerization (Chida et al. 2005). It should be noted that dimerization inhibitors are unlikely to be capable of inhibiting already active dimeric enzyme. Two L-arginine analogues, N-(1-imino-3-butenyl)-L-ornithine (L-VNIO) and N-propyl-L-arginine (L-NPA), are examples of highly nNOS selective inhibitors since they have poor capabilities of blocking either eNOS or iNOS (Paige and Jaffrey 2007).

1.3.2 Pharmaceuticals inhibiting iNOS expression

Nitric oxide production can also be inhibited by down-regulating iNOS expression. Several pharmaceuticals have been shown to exert their effects on NO production by inhibiting iNOS expression. This mechanism differs from the NOS inhibitors which impair the enzymatic activity of the NOS enzyme. Inhibition of the inflammatory transcription factors nuclear factor-κB (NF-κB) and signal transducers and activators of transcription (STAT) are important mechanisms to achieve inhibition of iNOS mediated NO production.

Glucocorticoids are widely used anti-inflammatory drugs, and they have been shown to inhibit iNOS expression and NO production in different cell types and after different stimuli (Di Rosa et al. 1990, Radomski et al. 1990, Geller et al. 1993, Kleinert et al. 1996, Salzman et al. 1996, Saura et al. 1998). The action of glucocorticoids on iNOS expression and NO production will be discussed in more detail in chapter 3.

Calcineurin inhibitors cyclosporin A and tacrolimus have been shown to inhibit NO production e.g. in macrophages (Kim et al. 2004b), and are discussed in more detail in chapter 5.

Non-steroidal anti-inflammatory drugs (NSAIDs) are characterized as inhibitors of cyclooxygenase (COX) and they have also been shown to inhibit iNOS expression. Ibuprofen inhibited iNOS expression in rat glia cells (Stratman et al. 1997) and in murine macrophages (Ogawa et al. 2000) but inhibition was achieved in both studies only at relatively high concentrations of ibuprofen. Salicylates have been shown to inhibit iNOS expression already at clinically
achievable concentrations (10 µM) in macrophages stimulated with bacterial endotoxin lipopolysaccharide (LPS) and interferon γ (IFNγ), probably through their inhibitory effect on CAAT/enhancer-binding protein β (C/EBPβ) (Cieslik et al. 2002). In rat cerebellar slices, LPS-induced iNOS expression was reduced after treatment with lysine clonixinate and indomethacin (Di Girolamo et al. 2003), and the methyl ester derivative of indomethacin inhibited LPS-induced iNOS expression in murine macrophages (Chao et al. 2005). In alveolar macrophages, nimesulide suppressed iNOS expression both in vivo and in vitro (Khanduja et al. 2006).

Disease modifying antirheumatic drugs (DMARDs) aurothiomalate and hydroxychloroquine have been shown to inhibit interleukin-1β (IL-1β) induced iNOS expression and NO production in chondrocytes and osteoarthritic cartilage, respectively (Vuolteenaho et al. 2005a), and sulfasalazine at relatively high concentrations inhibited iNOS expression in LPS and IFNγ-stimulated macrophages (Hasko et al. 2001).

Inhibitors of the janus activated kinase (JAK) – STAT signaling cascade have shown some promise in anti-inflammatory drug development. Two JAK inhibitors, AG-490 (Blanchette et al. 2003, Sareila et al. 2006) and WHI-P154 (Sareila et al. 2006, Sareila et al. 2008b), have been shown to inhibit iNOS expression and NO production in macrophages. Another group of molecules with an inhibitory effect on iNOS expression is the peroxisome proliferator-activated receptor (PPAR) α and PPARγ agonists. PPARα agonists have been shown to inhibit iNOS expression in macrophages (Colville-Nash et al. 1998) and in a study by Paukkeri et al. (2007) PPARα agonists were shown to enhance the degradation of iNOS protein through the proteasome pathway in macrophages stimulated with LPS (Paukkeri et al. 2007). The inhibition of iNOS expression by PPARγ agonists has been suggested to occur via the inhibition of NF-κB (Castrillo et al. 2000) and JAK-STAT (Chen et al. 2003) signaling cascades. The thiol-modulating compound, orazipone, has been shown to inhibit iNOS expression and NO production by inhibiting the activation of transcription factors NF-κB and STAT-1 (Sareila et al. 2008a). Transforming growth factor β (TGFβ) has been proposed to inhibit NO production by enhancing the degradation of iNOS protein (Vuolteenaho et al. 2005b).

1.4 Molecular effects of NO

Nitric oxide is a diatomic, highly diffusible gas which easily reacts with transition metals and with radicals, its effective concentration in vivo has been estimated to range between 5 nM to 4 µM. The amount of NO is buffered by red blood cells as NO reacts with oxyhemoglobin forming nitrosyl-heme-hemoglobin and nitrite. The signaling events mediated by NO in the cells depend on the amount of NO produced and on the localization where it is produced i.e. the molecular environment. NO mediated signaling can be divided based on the
amount of NO produced to the effects of a low NO level ([NO]_low) and to the effects of high NO concentration ([NO]_high). (Hanafy et al. 2001)

Characteristically [NO]_low targets transition metals like iron, copper and zinc, with iron being the most abundant transition metal as the target of NO. The main target of [NO]_low is the enzyme soluble guanylate cyclase (sGC), which catalyses the conversion of guanosine 5’-triphosphate (GTP) to cyclic guanosine monophosphate (cGMP) and pyrophosphate, and cGMP mediates many of the physiological effects of NO. In the sGC, NO targets the haem iron and after binding, the molecular conformation and structure of the enzyme changes resulting in approximately 400-fold increase in the catalytic activity of sGC. cGMP has four major targets: cGMP-dependent protein kinase (PKG), cyclic nucleotide-gated channels, cAMP dependent protein kinases and phosphodiesterases (PDEs) (Hanafy et al. 2001). PKG is a serine/threonine kinase, which is activated upon binding of cGMP. PKG phosphorylates several targets with the main overall result of vascular relaxation. For example, the NO used to relieve angina pectoris or to treat pulmonary hypertension in premature infants exerts its beneficial effects mainly through this pathway. PKG seems to be the main effector of cGMP, but cGMP has also been shown to activate protein kinase A (PKA) to some extent. cGMP also affects the activities of some PDEs. The mechanism of action of the drug used in the treatment of erectile dysfunction, sildenafil, is based on the inhibition of cGMP-specific PDE (PDE 5). PDE5 is selectively inhibited by sildenafil, which leads to an increase of the concentration of cGMP. Increased cGMP levels result in vasodilatation of penile vessels augmenting erection through PKG signaling. (Hanafy et al. 2001)

[NO]_low has also other targets in addition to sGC. Activities of some iron or zinc containing transcription factors are affected by NO. Also other haem-containing enzymes in addition to sGC are affected including heme-oxygenase 1, cytochrome c oxidase and catalase. Overall these effects of [NO]_low can also be cited as direct effects of NO, and the targets of [NO]_low mediated signaling are summarized in Figure 4. (Hanafy et al. 2001)
When the concentration of NO is high ([NO]_{\text{high}}), nitrosylation and nitrination are the main mechanisms of NO mediated signal transduction, which can also be defined as indirect effects of NO.

During S-nitrosylation, NO is covalently attached to the thiol side chain e.g. in cysteine and this post-translational protein modification affects the function of several enzymes, receptors and also transcription factors. The rate-limiting step is the formation of nitrogen dioxide (NO_2), which oxidizes NO to dinitrogen trioxide (N_2O_3), which subsequently decomposes to the nitrosonium ion (NO^+) and the nitrite ion (NO_2^-). Several enzymes and other regulatory proteins are known to promote S-nitrosylation or de-nitrosylation reactions (Stamler et al. 2001). NOS promoted S-nitrosylation can affect several substrates, their function, location and interactions with other proteins. In addition, NOS auto-S-nitrosylation can occur, leading to inhibition of NO production (Ravi et al. 2004). Copper, zinc superoxide dismutase (SOD) catalyses the S-nitrosylation of haemoglobin by NO, and ceruloplasmin catalyses the formation of S-nitroso glutathione (GSNO), which is the main non-protein S-nitroso thiol in vivo. De-nitrosylation and trans-nitrosylation also occur. Caspases (Mannick et al. 2001), phosphatases (Li and Whorton 2003), JNK (c-Jun N-terminal kinase) (Park et al. 2000), some membrane receptors (Nozik-Grayck et al. 2002) and transcription factors (Matthews et al. 1996) are examples of substrates for S-nitrosylation. Nitrosylation has also been shown to be involved in transcriptional silencing via an increase in the amount of methylated DNA (Hmadcha et al. 1999).
interesting example of S-nitrosylated transcription factor is nuclear factor κB (NF-κB) where p50 subunit has been shown to be S-nitrosylated at Cys62 resulting in the decrease of NF-κB activity (Matthews et al. 1996), and in addition, S-nitrosylation of Cys179 in IKKβ (IκB kinase β) has been shown to inhibit IκB (inhibitory κB) phosphorylation and subsequent proteasomal degradation (Reynaert et al. 2004). Reactions leading to S-nitrosylation and some examples of substrates are illustrated in Figure 4. (Hanafy et al. 2001, Hess et al. 2005)

Tyrosine nitration by [NO]_{high} has been recognized as another important post-translational protein modification that can affect cell signaling, cytoskeletal organization and the catalytic activity of some enzymes. In tyrosine nitration, superoxide (O_2^-) reacts with NO forming peroxynitrite (ONOO'). ONOO' is rapidly decomposed to NO_2 and hydroxyl radical (OH), and the reaction between tyrosyl radical (Tyr) and NO_2 yields NO_2Tyr (nitrotyrosine). These radicals can also react with other molecules and radicals. For example, NO_2 may form N_2O_3 in a reaction with NO. ONOO' can form ONOOCO_2^- in a reaction with CO_2, which can decompose to CO_3^- and NO_2. Excessive production of peroxynitrite is toxic in a variety of ways to the cells, causing protein nitration and oxidation, damage in the DNA, apoptosis, and inhibition of mitochondrial respiration. In addition to the toxicity of peroxynitrite, the tyrosine nitration has been shown to modify the activity or function of compounds like prostacyclin synthase, prostaglandin H synthase, Mn superoxide dismutase, ribonucleotide reductase, iNOS, histones and actin. (Figure 4) (Hanafy et al. 2001, Schopfer et al. 2003, Szabo et al. 2007, Vuolteenaho et al. 2007)

1.5 NO in inflammation

Nitric oxide regulates broad spectrum of physiological and pathophysiological processes. From immunological point of view, NO has been shown to affect the differentiation, proliferation and apoptosis of immune cells, production of cytokines and other inflammatory mediators, expression of costimulatory and adhesion molecules, synthesis and deposition of extracellular matrix components, adhesion of platelets and leukocytes to endothelium, and tumor growth. In addition, NO is one of the molecules that activated macrophages can utilize to kill microbes and tumor cells. However, in inflammatory diseases, NO and its derivatives target also host cells. NO has been shown to have both detrimental and beneficial effects in inflammation (Bogdan 2001, Korhonen et al. 2005) which have also been demonstrated in studies with iNOS deficient mice (Mashimo and Goyal 1999, Kolios et al. 2004).

NO production and iNOS expression have been shown to be elevated in many inflammatory disorders (Kolios et al. 2004, Vuolteenaho et al. 2007). Several studies implicate the usefulness of NOS inhibitors in resolving experimental inflammation such as rheumatoid arthritis, osteoarthritis, and
1.5.1 Rheumatoid arthritis and NO

Rheumatoid arthritis (RA) is a chronic autoimmune disease which has been estimated to affect 1% of world’s population. It is characterized as a progressive disease, potentially leading to joint destruction, functional disability and decreased life expectancy. The etiology of RA is not known but during the early course of the disease, inflammatory cells accumulate into the inflamed synovium and the production of inflammatory mediators and destructive agents is increased. Four of the following criteria need to be fulfilled for the diagnosis of RA: morning stiffness, arthritis in three of more joint areas, arthritis of typical hand joints, symmetric arthritis, rheumatoid nodules, serum rheumatoid factor and typical radiographic changes. There are many different drugs used in the treatment of RA e.g. NSAIDs, DMARDs such as methotrexate, sulfasalazine, hydroxychloroquine, and cyclosporin A, biological drugs such as tumor necrosis factor α (TNFα) blockers infliximab and etanercept, and glucocorticoids. (Kumar et al. 2005, Brunton et al. 2006)

Increased NO production has been reported in RA patients (Farrell et al. 1992, Grabowski et al. 1996a, Holm 2000), and the increased urinary nitrite (metabolite of NO) concentrations have been shown to diminish after prednisolone treatment (Stichtenoth et al. 1995). Elevated levels of NO were shown to correlate with several RA disease activity parameters (Onur et al. 2001). Increased iNOS expression and NO production have also been detected in animal models of arthritis (Stefanovic-Racic et al. 1994, Weinberg et al. 1994, Cuzzocrea 2006, Vuolteenaho et al. 2007), and the use of NOS inhibitors have been shown to have beneficial effects in experimentally induced arthritis (McCartney-Francis et al. 1993, Stefanovic-Racic et al. 1994, Weinberg et al. 1994, Connor et al. 1995). In mice genetically manipulated to lack iNOS, the autoantibody mediated arthritis was found to be milder with reduced cartilage destruction as compared to wild type mice (Kato et al. 2003). Based on experimental data, inhibitors of iNOS expression or activity might have disease-modifying properties in RA, but so far no clinical data is available.

1.5.2 Inflammatory bowel disease and NO

Inflammatory bowel disease (IBD) consists of Chron’s disease and ulcerative colitis. IBD affects up to 1 in 200 individuals in western countries but the prevalence is much lower in developing countries. Chron’s disease may be localized to any part of the gastrointestinal tract whereas ulcerative colitis is limited to the area from colon to rectum. In IBD, there is a chronic uncontrolled immune response against commensal microbes in the gut. Medical therapy for
IBD includes 5-aminosalicylic acid, sulfasalazine, immunosuppressants including cyclosporin A, anticytokine therapy against TNFα, and glucocorticoids. (Kumar et al. 2005, Brunton et al. 2006)

NO seems to have a significant role in intestinal barrier function and dysfunction as well as in inflammatory bowel diseases (Kolios et al. 2004). Elevated production of NO and the expression of iNOS have been demonstrated in patients with IBD (Boughton-Smith et al. 1993a, Middleton et al. 1993, Singer et al. 1996, Kimura et al. 1997). Increased NOS activity has been detected in experimentally induced colitis in animals (Boughton-Smith et al. 1993b), and several studies have reported NOS inhibition to have beneficial effects in animal models of intestinal inflammation (Miller et al. 1993, Rachmilewitz et al. 1995, Whittle et al. 1995, Kiss et al. 1997, Zingarelli et al. 1998, Evans and Whittle 2001, Kankuri et al. 2001, Menchen et al. 2001). In endotoxin-induced intestinal inflammation, NO was found to regulate the response in a biphasic manner (Laszlo et al. 1994). In the early stages of the response, low levels of NO were reported to be protective and anti-inflammatory whereas a few hours later when the inflammatory response had proceeded and high amounts of NO were produced, inhibitors of NOS suppressed extravasation and reduced the inflammatory response (Laszlo et al. 1994). In iNOS deficient mice, the experimentally induced colitis was less severe with a later onset as compared to wild type mice (Zingarelli et al. 1999, Hokari et al. 2001, Krieglstein et al. 2001). Pharmaceuticals targeting the inducible NOS pathway are predicted to be valuable in the treatment of disorders of the gastrointestinal tract (Whittle 2005).

2 Regulation of iNOS expression

2.1 Transcriptional regulation

Depending on the cell type and species in question, iNOS expression can be induced by different stimuli. In many murine cell types, lipopolysaccharide (LPS), which is a glycolipid component of the outer membrane of gram-negative bacteria, or a proinflammatory cytokine such as IFNγ or TNFα are capable of inducing transcription and expression of iNOS, whereas in human cells, a mixture of cytokines is usually required. In general, the most important transcription factors for the induction of iNOS expression are nuclear factor κB (NF-κB) and signal transducer and activator of transcription 1 (STAT-1). In all mammalian iNOS genes, promoter binding sites for NF-κB, octamer factors and factors induced by TNFα have been found near the TATA box. Also factors induced by IFNγ, and C/EBPβ and activator protein 1 (AP-1) have been shown to be involved in the regulation of iNOS transcription. Figure 5 shows the binding sites for the best known transcription factors involved in the regulation

**Figure 5.** 1kb and 16 kb promoters of murine and human iNOS gene, respectively. Approximate binding sites for certain transcription factors are indicated. NF-κB = nuclear factor κB, GAS = gamma-activated site, ISRE = interferon-stimulated response element, AP-1 = activator protein 1, C/EBPβ = CAAT/enhancer-binding protein β, IL-6RE = interleukin-6 responsive element, TNFα-RE = tumor necrosis α responsive element, Oct = octamer factor. (Modified from Kleinert et al. 2003)

### 2.1.1 Nuclear factor κB

Nuclear factor κB (NF-κB) is a transcription factor regulating the expression of antimicrobial products, proinflammatory cytokines and chemokines, and enzymes especially important in innate immunity. NF-κB has also been shown to regulate expression of genes outside the immune system such as in embryonic development, development of mammary gland, bone, skin and the central nervous system.

Major signaling pathways that lead to the activation of NF-κB originate from Toll/IL-1 receptor, TNF receptor or from B cell receptor. All these signaling cascades lead to the activation of NF-κB –mediated transcription by the activation of IKK kinase (IkB kinase) and subsequent degradation of IkB (inhibitory κB). Here, the Toll/IL-1 receptor activated signaling cascade, and especially the LPS-induced NF-κB activation through Toll-like receptor (TLR) 4 signaling will be discussed in more detail.

Both IL-1 receptors and Toll-like receptors belong to the TIR (Toll/interleukin-1 receptor) superfamily, and eleven subgroups of TLRs have been characterized. TLRs 1-6 are localized in the plasma membrane of most cells
whereas TLRs 7-9 are endosomal receptors in some cell types. TLRs are pattern-recognizing receptors that detect conserved pathogen-associated microbial patterns (PAMPs) present in various microbes. PAMPs include LPS, products of gram-positive bacteria, dsDNA produced by viruses, non-methylated CpG DNA present in bacteria, and flagellin. After ligand binding and dimerization of the receptors, the TLR signaling cascade in the cytosol is triggered by the TIR domain of TLR. In the MyD88-dependent pathway, this adaptor molecule associates with the TIR domain of TLR and interacts with the death domains of serine/threonine IL-1 receptor-associated kinase-4 (IRAK-4). After binding to MyD88, IRAK-4 activates IRAK-1 by phosphorylation, which subsequently recruits tumor necrosis factor receptor-associated factor-6 (TRAF6) to the MyD88/IRAK-4/IRAK-1 complex. IRAK-1 and TRAF6 dissociate from the complex and TRAF6 activates transforming growth factor β activated kinase 1 (TAK-1) by phosphorylation. The IKK complex is activated by the TAK-1 dependent phosphorylation. The most common form of the IKK complex consists of the catalytic subunits of IKKα and IKKβ, and the regulatory subunit of IKKγ. This activated IKK complex catalyzes the phosphorylation, polyubiquitination and subsequent degradation of IκB via the 26S proteasomal pathway. IκBs retain NF-κB dimers in the cytoplasm by binding to their Rel-homology domains, which interferes with their nuclear localization signal. After IκBs are degraded, the released NF-κB dimers are able to translocate to the nucleus and bind to the promoter sequences in the target gene. Activation of NF-κB triggers the transcription of several of the genes involved in immune and inflammatory responses. (Bonizzi and Karin 2004, Hayden and Ghosh 2004, West et al. 2006)
Trace amounts of LPS have been shown to activate NF-κB, and LPS is the most potent known stimulator of TLR ligands. TLR4 in a complex with CD14 and MD-2 is homodimerized and thereby activated after LPS binding. Activation of the IKK complex and the subsequent degradation of IκB leads to the activation and nuclear translocation of NF-κB (Figure 6). (Bonizzi and Karin 2004, West et al. 2006)

The NF-κB family consists of five members; NF-κB1 (p105/p50, precursor/processed form), NF-κB2 (p100/p52), RelA (p65), RelB and c-Rel. NF-κB may form several different homo- or heterodimers, which possess different transcriptional characteristics. The p50-RelA dimer is the most common form of NF-κB in the TLR-pathway. In the mouse iNOS promoter, there are two NF-κB elements critical for the transcriptional activity of iNOS; one in region I (position -76 to -85) and the other one in region II (position -962 to -971) (Lowenstein et al. 1993, Xie et al. 1993). Binding of NF-κB heterodimers p50-RelA and p50 - c-Rel to the region II NF-κB element in the mouse iNOS promoter has been demonstrated (Kim et al. 1997). Inhibition of NF-κB activity by PDTC (pyrrolidine dithiocarbamate) has been shown to lead to the inhibition of iNOS expression (Xie et al. 1994). In the human iNOS
promoter, several binding sites for NF-κB have been identified and their relative importance in iNOS inducibility has been proposed (Nunokawa et al. 1996, Marks-Konczalik et al. 1998, Taylor et al. 1998). (Bonizzi and Karin 2004, West et al. 2006)

The pathway illustrated above is also called the classical NF-κB pathway, which is thought to play a central role in innate immunity. The other, alternative pathway, proceeds via IKKα homodimers and results in the nuclear translocation of p52-RelB dimers. This alternative pathway is connected with the role of NF-κB signaling cascade in adaptive immunity. Also a MyD88-independent pathway activating NF-κB has been reported, which has been suggested as representing the late-phase of NF-κB activation in response to LPS. (Bonizzi and Karin 2004, West et al. 2006)

The regulation of the balance of NF-κB activation is important since sufficient activity is required in the battle against invading pathogens but excessive activity may lead to inflammatory disorders. Members of the TLR family have been implicated in several autoimmune and chronic inflammatory diseases as well as in severe infectious diseases. Soluble decoy receptors can be designated as the first line endogenous negative regulators in TLR signaling. There is one copy of the TLR4 gene in mammalians but a soluble isoform of TLR4 has been detected in mouse macrophages and this may also exist in humans. MyD88s, the short form of MyD88, inhibits TLR4 mediated NF-κB signaling by antagonizing MyD88. Cytoplasmic IRAK-M, SOCS-1 (suppressor of cytokine signaling) and TOLLIP (Toll-interacting protein) all affect IRAK activity in TLR4 signaling leading to reduced NF-κB activation. There are also some membrane-bound negative regulators endogenously down-regulating TLR signaling. Negative regulation occurs also through the reduction of the number of TLRs. (Liew et al. 2005, West et al. 2006)

2.1.2 STAT-1 and IRF-1

Transcription factors signal transducers and activator of transcription (STAT) and interferon regulatory factor (IRF) are activated/induced by IFNγ stimulation. IFNγ is a cytokine well known for its antiviral activity but it plays a role also in the immune response as well as being involved in cell proliferation and apoptosis. Over 200 genes are known to be regulated by IFNγ and the main cell types producing IFNγ are natural killer (NK) cells, T helper cells and cytotoxic CD8+ T cells. During the early phase of inflammation, the most important functions of IFNγ are the activation of macrophages and inhibition of Th2 cell division.

IFNγ-stimulated signaling cascades leading to the activation of STAT and IRF transcription factors are illustrated in Figure 7. Binding of IFNγ to its receptor (which is ubiquitously but not uniformly expressed) leads to receptor α-chain dimerization and subsequent association of β-chains. Janus activated kinase 1 (JAK1) is associated with the α-chain and JAK2 with the β-chain. The
JAK kinases are transphosphorylated and activated after receptor dimerization. The activated JAKs phosphorylate tyrosine residues in the α-chains of the receptor. This phosphorylation of IFNγ receptor α-chains creates binding sites for the SH2 domains of the inactive cytosolic STAT-1α. Bound STAT-1α is phosphorylated at a tyrosine residue, which leads to its dissociation from the receptor and subsequent formation of the STAT-1α homodimer (this STAT-1α homodimer is also called gamma-interferon activation factor, GAF). The STAT-1α homodimer is able to translocate to the nucleus and bind to its target sequence in DNA, the gamma-activated site (GAS). Direct activation of several genes through their GAS-elements in their promoter sequences is often referred to as the primary IFNγ response. Transcription factors which are the primary response genes regulate the secondary IFNγ response through promoters with ISRE (IFN-stimulated response elements) motifs. IRF-1 is one of these transcription factors whose production is triggered by IFNγ challenge. (Figure 7) (Boehm et al. 1997, Schindler et al. 2007)

There are four characterized members in the JAK family, JAK1, 2, 3 and Tyk 2, and the mammalian STAT family consists of seven members (STATs 1, 2, 3, 4, 5a, 5b and 6). Two splice variants of STAT-1 have been identified, STAT-1α and STAT-1β, the latter forming transcriptionally inactive homodimers. Several
cytokines induce the JAK-STAT pathway with particular combination of JAKs and STATs. JAK-STAT signaling cascade can be switched off by several mechanisms. Phosphatases are important in dephosphorylating and thus inactivating receptors, JAKs and STATs. Nuclear import-export balance of active STAT dimers is directed towards export when signal decay is desired. The SOCS protein family is involved in deactivating JAK-STAT signaling through the inhibition of JAK activation and stimulation of JAK ubiquitination and subsequent proteosomal degradation. (Murray 2007, Schindler et al. 2007)

GAS (binds STAT-1) and ISRE (binds IRF-1) are found in mouse iNOS promoter (Xie et al. 1993), and in human iNOS promoter (Kleinert et al. 1998). Binding of STAT-1α to the GAS element was noted to be necessary for optimal IFNγ/LPS-stimulated induction of iNOS promoter (Gao et al. 1997). In cells derived from STAT-deficient mice, no IFN-dependent iNOS induction was detected (Meraz et al. 1996). Accordingly, NO production was severely impaired in cells derived from IRF-deficient mice (Kamijo et al. 1994). JAK inhibitors AG-490 and WHI-P154 have been shown to inhibit iNOS expression in murine cells (Salonen et al. 2006, Sareila et al. 2006).

2.2 Post-transcriptional regulation

The post-transcriptional regulation of expression of inflammatory genes has recently gained increasing attention. The regulation of the stability of mRNA has been found to be disturbed in several inflammatory disorders. Thus, inhibition of the production or gene expression of an inflammatory mediator by destabilizing mRNA has become an important target in drug development.

2.2.1 Stability of mRNA

The most important cis-regulatory elements determining mRNA decay are the AU-rich elements (ARE) found in the 3' untranslated area (3'UTR) of the mRNA. It has been estimated that as much as 5-8% of the genes in human genome contain ARE sequences at their 3'UTR potentially affecting mRNA stability. AREs are divided into three classes; class I ARE contain multiple clustered copies of the AUUUA pentamer, class II ARE contain only a few copies of the AUUUA motif which are dispersed along the 3'UTR, and the class III ARE differ in that they contain sequences other than AUUUA elements. In particular, several mRNAs encoding inflammatory cytokines and other inflammatory mediators contain ARE at their 3'UTR. mRNA may contain several ARE, and the secondary structure of the 3'UTR is important in determining the ability of binding ARE binding proteins. (Wilusz and Wilusz 2004, Eberhardt et al. 2007)

Deadenylation of mRNA is most commonly the first step in mRNA decay. After deadenylation, the mRNA may be processed by 5’ cap removal and
subsequent 5′→3′ degradation or is broken down through the exosomal 3′→ 5′ pathway. This latter route is the most common decay pathway of ARE-containing mRNA in mammalian cells. The degradation of mRNA is a strictly regulated process, and the role of ARE binding proteins is important in modulating the function of the exosome and in regulating the decapping and deadenylation steps. Discrete cytoplasmic bodies have been characterized to be the sites of mRNA decay and storage for translationally silent mRNAs. Decay bodies and stress granules have been shown to contain several enzymes and ARE binding proteins involved in mRNA decay. (Kedersha and Anderson 2002, Cougot et al. 2004)

Known ARE binding proteins include HuR, which is a member of the embryonic lethal abnormal vision (ELAV) protein family (Brennan and Steitz 2001), heterogeneous nuclear ribonucleoproteins (hnRNPs) (Raineri et al. 2004), tristetraprolin (TTP) (Blackshear 2002) and the K homology-type splicing regulatory protein (KSRP) (Gherzi et al. 2004). HuR is a ubiquitously expressed member of the ELAV protein family. HuR has been characterized as a factor stabilizing mRNA, and although it is a protein mainly located within the nucleus, it is capable of shuttling between the nucleus and cytoplasm, and the distribution of HuR is the primary factor dictating its function. The role of altered expression of HuR has also been shown to have a role in the regulation of mRNA stability (Akool et al. 2003). Members of the Hu protein family have been shown to exert their stabilizing effect through competing with destabilizing proteins for ARE binding. hnRNPD, also known as AUF-1, is a protein capable of destabilizing mRNA after binding to class I and II AREs. AUF-1 has been shown to mediate its effect on mRNA stability through recruiting the exosome and by remodeling mRNA. Like HuR, AUF-1 also shuttles between the nucleus and cytoplasm though it is also predominantly localized within the nucleus. Another destabilizing ARE binding protein is TTP, a member of the Cys-Cys-Cys-His (CCCH)-zinc finger protein family, which has been shown to mainly affect mRNAs with class II AREs. Binding of TTP recruits several of the enzymes involved in mRNA decay resulting in enhanced degradation. TTP shuttles between the nucleus and cytoplasm, and is highly regulated by its degree of phosphorylation. TTP is phosphorylated via the p38 MAP kinase pathway, and after phosphorylation, TTP can bind the 14-3-3 proteins. 14-3-3 are small proteins interacting with phosphorylated target proteins and in the way it can regulate their activity, subcellular localization and interactions with other proteins. Upon binding to 14-3-3, TTP is transferred to cytoplasm, which leads to the stabilization of the target mRNA. It has been suggested that the exclusion of TTP from ARE enables HuR to bind and stabilize the mRNA. KSRP is another ubiquitously expressed protein capable of binding ARE (all three classes), leading to destabilization of mRNA. KSRP mediates its effects by recruiting decay enzymes and exosome. Its own activity is modified by phosphorylation through the p38 MAP kinase pathway. The main characteristics of ARE binding proteins HuR, AUF-1 (hnRNPD), TTP and KSRP are
summarized in Table 2. (Wilusz and Wilusz 2004, Eberhardt et al. 2007, Garneau et al. 2007)

**Table 2. Properties of ARE binding proteins (modified from Garneau et al. 2007)**

<table>
<thead>
<tr>
<th>Type</th>
<th>Mechanisms of action</th>
<th>Regulated by</th>
</tr>
</thead>
<tbody>
<tr>
<td>HuR</td>
<td>stabilizing</td>
<td>competition with other ARE binding proteins</td>
</tr>
<tr>
<td>AUF-1 (aka hnRNPD)</td>
<td>destabilizing</td>
<td>recruits exosome remolds mRNA</td>
</tr>
<tr>
<td>TTP</td>
<td>destabilizing</td>
<td>recruits decay enzymes</td>
</tr>
<tr>
<td>KSRP</td>
<td>destabilizing</td>
<td>recruits decay enzymes and exosome</td>
</tr>
</tbody>
</table>

2.2.2 Regulation of iNOS mRNA stability

Several factors and mediators have been reported to play a role in the post-transcriptional regulation of iNOS expression. The importance of the regulation of iNOS expression at the post-transcriptional level is supported by three findings 1) iNOS promoter has been found to have basal activity in spite of no detectable mRNA accumulation, 2) mRNA accumulation is dependent on de novo protein synthesis and 3) the expression of iNOS mRNA is transient despite the presence of a continuous stimulus (Laubach et al. 1997, Linn et al. 1997). The 3’UTRs of both human and murine iNOS contain several ARE, and the 3’UTRs along with the 5’-flanking regions are important in regulating the inducibility of the iNOS gene (Nunokawa et al. 1997, Rodriguez-Pascual et al. 2000).

By regulating gene expression at the level of mRNA stability, it is possible to down-regulate protein formation even in conditions with ongoing transcription. The glucocorticoid, dexamethasone, is an example of an anti-inflammatory drug which has been reported to destabilize (i.e. reduce the half-life of) iNOS mRNA in murine macrophages stimulated with bacterial endotoxin (Korhonen et al. 2002). In IFNγ stimulated macrophages, TGFβ was reported to mediate its effects on iNOS expression also by destabilizing iNOS mRNA (Vodovotz et al. 1993). In human articular chondrocytes (Geng and Lotz 1995), as well as in murine macrophages (Korhonen et al. 2001), an increased intracellular Ca^{2+} concentration decreased the stability of iNOS mRNA. 8-Bromo-cGMP reduced iNOS mRNA stability in cytokine-stimulated human mesangial cells (Perez-Sala
et al. 2001), whereas in IL-1β treated rat cardiac myocytes enhancement of cAMP levels were found to increase the stability of iNOS mRNA (Oddis et al. 1995), with the same phenomenon being observed when cardiac fibroblasts were treated with the β2-agonist isoprenaline (Gustafsson and Brunton 2000). Tetrahydrobiopterin, a cofactor required in the enzymatic reaction forming NO from L-arginine, was found to stabilize iNOS mRNA in rat vascular smooth muscle cells, emphasizing that several mechanisms may be behind the observed effect at the target gene (Linscheid et al. 1998). In cytokine stimulated β-cells, protein kinase C (PKC) δ mediated signaling is involved in regulating iNOS mRNA degradation rates (Carpenter et al. 2001). JNK signaling pathway has been shown to be involved in the regulation of iNOS mRNA stability. SP6000125 is an inhibitor of JNK and has been demonstrated to suppress LPS-induced iNOS expression and NO production by destabilizing iNOS mRNA in murine macrophages (Lahti et al. 2003). In human cells, a role for TTP in regulating iNOS mRNA stability via the JNK pathway has been reported (Korhonen et al. 2007).

ARE binding proteins TTP, KSRP, HuR and members of the hnRNP family have been shown to be involved in regulating iNOS expression. HuR was found to bind 3’UTR of iNOS mRNA and to stabilize it in human DLD-1 cells (Rodriguez-Pascual et al. 2000). In murine macrophages, two members of the hnRNP family, i.e. hnRNPL and hnRNPI (aka PTB) were shown to interact with 3’UTR (Soderberg et al. 2002) and to be involved in the regulation of iNOS mRNA stability (Soderberg et al. 2007a). In particular, hnRNPI was proposed to mediate the post-transcriptional iNOS mRNA destabilizing effects of dexamethasone (Soderberg et al. 2007b). On the contrary, in the human cell line DLD-1, hnRNPI was found to bind to 3’UTR of iNOS mRNA and to enhance the stability of the transcript (Pautz et al. 2006). The interplay between ARE binding proteins TTP, KSRP and HuR has been demonstrated to be involved in the regulation of the stability of human iNOS mRNA. TTP was found to stabilize iNOS mRNA, not by directly binding to it but by modulating the interaction of the destabilizing KSRP with iNOS mRNA (Fechir et al. 2005). Further studies revealed that HuR and KSRP compete for the same binding site in human iNOS 3’UTR, and after cytokine stimulation, binding of the stabilizing HuR becomes favored (Linker et al. 2005). The above experiments emphasize the multi-faceted nature of the mechanisms regulating the stability of iNOS mRNA, and furthermore differences between cell type and stimulus can add another layer of complexity.

2.2.3 Regulation of iNOS protein stability

Post-transcriptional regulation may also occur at the level of protein modifications as well as at protein decay rates, modulating the balance of protein synthesis and degradation. The degradation of iNOS protein has been shown to take place in the proteasome (Felley-Bosco et al. 2000, Musial and Eissa 2001).
TGFβ can enhance iNOS protein degradation in IFNγ-induced murine macrophages (Vodovotz et al. 1993, Mitani et al. 2005) and in IL-1β stimulated immortalized chondrocytes (Vuolteenaho et al. 2005b). Dexamethasone has been shown to have an effect also on iNOS protein stability, i.e. protein degradation was found to be enhanced after dexamethasone treatment in IL-1β stimulated mesangial cells (Kunz et al. 1996). A recent study suggested that PPARα agonists inhibit iNOS expression and NO production by enhancement of iNOS protein degradation via the proteasome pathway (Paukkeri et al. 2007).

3 Glucocorticoids

Glucocorticoids are steroid hormones, which are naturally synthesized in the human body in the adrenal cortex or are manufactured synthetically. They have anti-inflammatory, immunosuppressive and metabolic effects. The endogenous production of glucocorticoids is regulated along the hypothalamus – anterior pituitary – adrenal gland – axis, resulting in the production and secretion of cortisol. Dexamethasone is an example of a synthetic glucocorticoid. RU24858 is a dissociated steroid, a molecule thought to have anti-inflammatory properties without the metabolic effects which are believed to be mainly responsible for many of the adverse effects associated with long-term glucocorticoid treatment (Figure 8).

![Figure 8. Structures of endogenous glucocorticoid cortisol and the synthetic compounds dexamethasone and RU24858](image)

3.1 Use of glucocorticoids in inflammatory diseases

Glucocorticoids are powerful anti-inflammatory agents, and they are widely used in the treatment of asthma and other allergic diseases. Glucocorticoids are extensively used also in the treatment of other inflammatory disorders like rheumatoid arthritis, systemic lupus erythematosus, inflammatory skin disorders, and inflammatory bowel disease. In addition, glucocorticoids are given to patients after organ transplantation to suppress inflammatory responses. The
most common local adverse effects of inhaled glucocorticoids are hoarseness and weakness of voice. Adverse effects like growth retardation, osteoporosis, skin atrophy, blood vessel fragility, metabolic disorders, hypertension, immunosuppression and inhibition of wound healing may result from high and prolonged oral therapy with glucocorticoids. (Rhen and Cidlowski 2005, Barnes 2006)

3.2  Mechanisms of action of glucocorticoids

Glucocorticoids are lipid soluble molecules that readily diffuse across cell membranes. In the cytosol, glucocorticoids bind to glucocorticoid receptors (GR). Human GRs are encoded by a single gene located on chromosome 5q31-32, and α- and β- variants are generated due to alternative translation initiation sites and furthermore RNA splice variants of GR are known to exist. GRα is capable of binding glucocorticoids whereas GRβ is not activated by ligand binding but when it is present as a homodimer it can bind DNA or it can form heterodimers with GRα. Expression levels of GRβ are considerably lower than those of GRα but a role for GRβ in steroid resistance in asthma has been proposed (Sousa et al. 2000). Glucocorticoid receptors are posttranslationally modified by phosphorylation in a ligand-dependent and -independent manner leading to either GR inactivation or activation depending on which serine is phosphorylated. In addition, nitrosylation, acetylation, ubiquitination and sumoylation of GR have been reported. (Lu and Cidlowski 2004, Rhen and Cidlowski 2005, Ito et al. 2006a)

Protein complexes including two molecules of heat-shock proteins (hsp) 90, one molecule of hsp70, FK-binding protein and p23 act as molecular chaperones holding GR in the cytosol yet open to activation by a ligand. Molecular chaperones dissociate from GR upon its activation, unmasking nuclear localization signals in the receptor and enabling its nuclear translocation. Glucocorticoid-activated GR can mediate its action through transcriptional effects, post-transcriptional effects, or via nongenomic effects in target cells. The anti-inflammatory effects of glucocorticoids are believed to be mediated mainly by trans-repressional mechanisms through inhibition of pro-inflammatory transcription factors and to a lesser account by increasing the transcription and expression of some anti-inflammatory mediators. (Beato et al. 1995, Dostert and Heinzel 2004, Ito et al. 2006a, Clark 2007)

3.2.1 Transcriptional effects of glucocorticoids

Glucocorticoids mediate their effects on gene transcription by different mechanisms described as cis-activation/repression and trans-activation/repression. Regulation at the transcriptional level can be mechanistically divided into protein-DNA interactions and protein-protein
interactions. After activation, GR become transferred to the nucleus and when it is present as a dimer it can bind the glucocorticoid responsive elements (GRE) found in the promoter regions of target genes. Binding to positive GRE (+GRE) leads to increased transcription of the target gene (cis-activation) whereas binding to negative GRE (-GRE) results in decreased transcription (cis-repression). GR can also interact with other transcription factors or with coactivators leading to trans-activation or trans-repression (Figure 9). (Dostert and Heinzel 2004, Barnes 2006)

**Figure 9. Regulation of transcription by glucocorticoids. GR = glucocorticoid receptor, GRE = glucocorticoid responsive element. According to the nomenclature proposed by Dostert and Heinzel 2004.**

There are relatively few inflammatory genes regulated through positive or negative GREs as compared to the number of genes regulated through the interactions between GR and another transcription factor (e.g. NF-κB) or other proteins. Pro-opiomelanocortin, osteocalcin and prolactin are examples of genes down-regulated by glucocorticoids through negative GREs, and negative GRE is found also in the promoter region of the pro-inflammatory cytokine, IL-1β (Zhang et al. 1997, Dostert and Heinzel 2004). Annexin-1 (aka lipocortin-1), IL-10 and IκB-α are examples of anti-inflammatory genes whose transcriptional activities are enhanced by glucocorticoids (Ambrose et al. 1992, Auphan et al. 1995, van Furth et al. 1995). Glucocorticoids also increase the transcription of a glucocorticoid-induced leucine zipper protein (GILZ) and mitogen-activated protein kinase phosphatase-1 (MKP-1), which inhibit transcription factor AP-1,

The upregulation of such a small number of anti-inflammatory genes seems unlikely to account for the extensive anti-inflammatory effects achieved with glucocorticoid treatment, and indeed the indirect trans-repressional down-regulation of transcription of pro-inflammatory genes is acknowledged as the most important mechanism for the anti-inflammatory properties of glucocorticoids (Table 3). Activated GR has been shown to interfere with several transcription factors such as NF-κB, AP-1 and CREB (cAMP-response-element-binding protein), which are important for the induction of several pro-inflammatory genes (Karin 1998). In mice with mutated GR which lacked the DNA-binding ability, many of the beneficial anti-inflammatory and immunosuppressive actions of glucocorticoids were still evident (Reichardt et al. 2001). Recently, a trans-repressional action of glucocorticoids has been shown to affect chromatin structure by histone acetylation and deacetylation (Ito et al. 2006a).

Table 3. Regulation of transcription of certain inflammatory genes by glucocorticoids (Barnes 1998)

<table>
<thead>
<tr>
<th>Transcriptional down-regulation</th>
<th>Cytokines</th>
<th>IL-1, -2, -3, -4, -5, -6, -9, -11, -12, -13, -16, -17, -18</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TNFα, GM-CSF</td>
<td></td>
</tr>
<tr>
<td>Pro-inflammatory enzymes</td>
<td>iNOS</td>
<td>COX-2</td>
</tr>
<tr>
<td></td>
<td>cPLA2</td>
<td></td>
</tr>
<tr>
<td>Adhesion molecules</td>
<td>ICAM-1, VCAM-1, E-selectin</td>
<td></td>
</tr>
<tr>
<td>Chemokines</td>
<td>IL-8, RANTES, MIP-1α</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Transcriptional up-regulation</th>
<th>Annexin-1</th>
<th>β2-adrenoeceptors</th>
<th>IL-1Ra</th>
<th>IL-10</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>IL-1Ra</td>
<td></td>
<td></td>
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<td></td>
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<td>IL-1Ra</td>
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</tbody>
</table>

IL=interleukin, TNF=tumor necrosis factor, GM-CSF=granulocyte-macrophage colony stimulating factor, iNOS=inducible nitric oxide synthase, COX=cyclooxygenase, cPLA2=cytoplasmic phospholipase A2, ICAM=intercellular adhesion molecule, VCAM=vascular-endothelial cell adhesion molecule, RANTES=released by normal activated T cells expressed and secreted, MIP=macrophage inflammatory protein, IκB=inhibitory κB, GILZ=glucocorticoid-induced leucine zipper protein, MKP=mitogen-activated protein kinase phosphatase
DNA in chromosomes is packaged into chromatin and approximately 146 bp of DNA is wound almost twice around an octamer of core histones (two molecules each of histones H2A, H2B, H3 and H4) forming a nucleosome. The highly conserved N-terminal tails of core histones protrude out from the nucleosome core and are subjected to modifications such as acetylation, methylation, phosphorylation and ubiquitination. Hypoacetylation of histones is associated with transcriptionally inactive DNA whereas acetylation and some other modifications of N-terminal tails of core histones result in specific binding patterns of nonhistone proteins to chromatin, leading to the open and transcriptionally active form of DNA. The so called histone code signifies the correlation between site-specific histone modifications and consequent biological function. (Khorasanizadeh 2004, Peterson and Laniel 2004)

Histone acetyltransferases (HAT) are enzymes capable of transferring acetyl groups from acetyl-CoA to histones as well as to other targets including some transcription factors. HATs can be divided into two groups depending on their mode of action. A-type HATs are involved in catalyzing acetylation related to transcription whereas B-type HATs are involved in the transport of histones to the nucleus when new nucleosomes are formed. One of the first proteins identified as having intrinsic HAT activity was Gcn5, which has been implicated in mediating transcriptional activation by acidic activators. When it forms a complex with Ada-proteins, it was shown to be recruited to specific promoter regions where it interacted with both transcriptional activators and components of the basal transcriptional machinery (Brownell et al. 1996). Other proteins identified with HAT activity include CREB-binding protein (CBP), p300 and TAFII250. N-terminal lysine residues in histones are common targets for HATs. In vertebrates, two acetylation sites in H2A, and four in each H2B, H3 and H4 within their lysine residues in the N-terminus have been identified. (Roth et al. 2001, Glozak et al. 2005)

Histone deacetylases (HDAC) are able to remove acetyl groups from histones as well as from non-histone proteins including many transcription factors and other cellular proteins. HDACs can be divided into three classes: class I HDACs (HDACs 1, 2, 3, 8 and 11) are localized in the nucleus, class II HDACs (HDACs 4, 5, 6, 7, 9 and 10) can shuttle between the cytoplasm and nucleus, and class III includes the SIR family of deacetylases whose primary targets are thought to be others than histones. HDACs form complexes with cofactors like N-Cor (nuclear receptor co-repressor) and SMRT (silencing mediator of retinoic acid and thyroid hormone receptor), with the presence of Zn$^{2+}$ ion being essential for the occurrence of deacetylation. Inhibitors of histone deacetylase function by displacing the Zn$^{2+}$ ion thus blocking the active site of HDAC, which leads to hyperacetylation of histones and other target proteins. The most potent of the known HDAC inhibitors is trichostatin A (TSA), which is a fermentation product of Streptomyces. TSA belongs to the group of hydroxamic acids and is effective in vitro at nanomolar concentrations. In addition to the hydroxamic acids, the
structures of HDAC inhibitors can include short fatty acids, cyclic tetrapeptides/epoxides and benzamides. HDAC inhibitors have shown promise as anti-cancer drugs since the accumulation of acetylated histones by HDAC inhibitors may lead to activation/repression of selective sets of genes whose expression/repression results in the inhibition of tumor growth through cytotoxicity, differentiation, inhibition of proliferation and induction of apoptosis. There are currently several HDAC inhibitors undergoing phase I and II clinical trials. (de Ruijter et al. 2003, Marks et al. 2003, McLaughlin and La Thangue 2004, Glozak et al. 2005, Monneret 2005, Taddei et al. 2005, Adcock 2006)

Pro-inflammatory transcription factors like NF-κB regulate transcription by binding to and activating coactivators such as CBP, which have intrinsic histone acetyltransferase activity. Coactivator complexes are able to acetylate histones resulting in increased transcriptional activity (Kagoshima et al. 2003). Dexamethasone has been shown to suppress IL-1β-induced GM-CSF release in epithelial cells by a mechanism involving specific patterns of histone acetylation and recruitment of histone deacetylation activity. IL-1β was shown to induce the acetylation of lysine residues 8 and 12 in histone 4 while dexamethasone induced K5 and K16 acetylation (Ito et al. 2000). Low concentrations of dexamethasone were reported to repress IL-1β-stimulated acetylation of K8 and K12 and to inhibit CBP-associated HAT activity. Furthermore, dexamethasone was demonstrated to recruit HDAC2 to the p65-CBP HAT complex (Ito et al. 2000). p65-HAT activity has been shown to be repressed also by a partial GR agonist, mifepristone, but no recruitment of HDAC2 to the NF-κB p65 subunit complex was seen after mifepristone treatment (Ito et al. 2001). In addition to modulation of CBP-mediated HAT activity, also a role for DNA methylation has been proposed in the regulation of IL-1β-induced GM-CSF release by glucocorticoids (Kagoshima et al. 2001). TNFα-induced acetylation of K8 and K12 in H4 was also suppressed by dexamethasone in macrophage cells (Tsaprouni et al. 2002). At the eotaxin promoter, TNFα-induced acetylation of K5 and K12 in H4 was inhibited by glucocorticoids (Nie et al. 2005). HDAC2 activity and expression has been shown to be reduced in patients with chronic obstructive pulmonary disease, where glucocorticoid treatment is not effective (Adcock and Lee 2006). Cigarette smoke can increase histone 4 acetylation and decrease HDAC2 activity and this may prevent the anti-inflammatory effects of glucocorticoids (Marwick et al. 2004). Theophylline was the first drug shown to induce HDAC activity and thus to augment the anti-inflammatory actions of glucocorticoids (Ito et al. 2002). The transcription factor, Smad6, has been suggested to regulate some of the adverse effects associated with glucocorticoid treatment. It has been reported that Smad6 recruits HDAC3 to activated GR bound to DNA and deacetylates histones 3 and 4, leading to reduced transcriptional activity (Ichijo et al. 2005).

As described above, some of the effects of dexamethasone may be mediated through deacetylation of histones by modulating the HAT activity of coactivator molecules and by recruiting histone deacetylase activity to the site of
transcription, leading to suppression of transcription of inflammatory genes regulated by inflammatory transcription factors NF-κB and AP-1 (Figure 10).

![Diagram of transcription and glucocorticoid effects](image)

**Figure 10.** Suppression of inflammatory genes by glucocorticoids through increased histone deacetylation (modified from Barnes 2006)

In addition, acetylation/deacetylation may affect GR signaling at other sites. The acetylation status of GR is important since the acetylation of the glucocorticoid receptor takes place after ligand binding, and then GR deacetylation by HDAC2 enables the binding of activated GR to the NF-κB complex and subsequent down-regulation of transcription of pro-inflammatory genes (Ito et al. 2006b). HDAC6 has been shown to function as an hsp90 deacetylase, and its inactivation can lead to hyperacetylation of hsp90 resulting in deficient action of GR (Kovacs et al. 2005). Acetylation status has also been shown to control the proteosomal degradation of the coactivator p300 involved in GR signaling (Li et al. 2002).

### 3.2.2 Post-transcriptional effects of glucocorticoids

Glucocorticoids have been reported to exert their effects also post-transcriptionally by influencing mRNA stability and protein degradation. Enhanced degradation of mRNA of pro-inflammatory factors IL-1β (Amano et al. 1993), COX-2 (Lasa et al. 2001) and iNOS (Korhonen et al. 2002) by glucocorticoids has been reported. MKP-1 has been shown to be regulated by glucocorticoids at the level of transcription as well as at the level of decreased protein degradation (Kassel et al. 2001).
3.2.3 Nongenomic effects of glucocorticoids

Some of the actions of glucocorticoids occur too rapidly to fit in to the above-mentioned transcriptional or post-transcriptional effects. It has been suggested that nongenomic actions of glucocorticoids are mediated through distinct membrane receptors, through classical intracellular receptors or even without receptor involvement. Some G-protein coupled receptors and kinase pathways have been proposed to be regulated by nongenomic actions of glucocorticoids. One example is the regulation of the activity of eNOS by glucocorticoids through the PI3K-Akt pathway (Hafezi-Moghadam et al. 2002).

3.3 Regulation of iNOS expression by glucocorticoids

Glucocorticoids have been shown to inhibit NO production in e.g. vascular endothelial cells (Radomski et al. 1990), vascular smooth muscle cells (Kanno et al. 1993), macrophages (Di Rosa et al. 1990), hepatocytes (Geller et al. 1993), and epithelial cells (Salzman et al. 1996). For the mechanisms of the inhibitory action both transcriptional and posttranscriptional events have been suggested.

Several studies have demonstrated that glucocorticoids can inhibit NO production and iNOS expression at the transcriptional level by inhibiting NF-κB; by reducing its nuclear translocation or DNA binding, or by increasing the amount of inhibitory IκB (Kleinert et al. 1996, Saura et al. 1998, Katsuyama et al. 1999, Matsumura et al. 2001). The inhibitory action of the glucocorticoid, dexamethasone, on iNOS has been shown at the posttranscriptional level in C6 glioma cells (Shinoda et al. 2003), and more specifically at the level of enhanced iNOS mRNA degradation in macrophages (Korhonen et al. 2002). Increased proteolytic degradation of iNOS protein and involvement of calpain I has also been reported to mediate the inhibitory action of glucocorticoids on iNOS expression and NO production (Kunz et al. 1996, Walker et al. 1997). NO production can also be regulated by limiting the substrate availability for iNOS. Glucocorticoids have been shown to inhibit BH₄ synthesis and the transport of arginine into the cell (Simmons et al. 1996), and to induce arginase II, an enzyme which consumes L-arginine (Gotoh et al. 1996).

Although in several cell types NO production and iNOS expression have been shown to response to glucocorticoids, there are a few reports of glucocorticoid-insensitive cells e.g. chondrocytes (Grabowski et al. 1996b, Vuolteenaho et al. 2001, Vuolteenaho et al. 2005b).
4 Flavonoids

Polyphenols are naturally occurring compounds comprising over 8000 variants of which the flavonoid group is the most widely distributed. Flavonoids are compounds with two benzene rings linked together with a heterocyclic pyran or a pyrrole ring. These compounds are often subdivided into the groups of flavones, isoflavones, flavonols, flavanones, flavan-3-ols and anthocyanins. The basic structures of these groups are shown in Figure 11.

*Figure 11. Basic chemical structures of flavones, isoflavones, flavonols, flavanones, flavan-3-ols and anthocyanins*

In plant physiology and biochemistry, flavonoids have several functions e.g. as antioxidants, precursors for toxic compounds and components in color pigments. Flavonoids are nonessential constituents of our food. The daily intake of flavonoids in the Netherlands and in Finland has been estimated to be 23-24 mg/d (Hertog et al. 1993, Knekt et al. 2002). Flavonoids are found rich in certain foodstuffs e.g. tea, fruits, vegetables, olive oil, seeds, nuts, chocolate, and red wine. The flavonoid obtained most from food is quercetin, which occurs mainly in tea, onions and apples (Hertog et al. 1993, Middleton et al. 2000). Flavonoids appear usually as glycosides in food, and glycosylation has been shown to affect the water-solubility, bioavailability, and antioxidant properties of flavonoids. (Middleton et al. 2000, Tapiero et al. 2002)
4.1 Flavonoids and inflammatory diseases

Flavonoids have been shown to possess several biological activities in humans. They are known to have free radical scavenging and metal chelating properties, thus being effective antioxidants. Due to their antioxidant properties, flavonoids are thought to protect tissues against reactive oxygen species and lipid peroxidation. In addition, flavonoids have been shown to possess anti-inflammatory, antiallergic, hepatoprotective, antithrombotic, antiviral and anticarcinogenic activities. (Middleton et al. 2000)

Various enzymatic reactions in humans have been shown to be affected by flavonoids. Here a few of them will be discussed briefly. Several kinases including PKC, mitogen activated protein kinases, and protein tyrosine kinases (PTK) have been claimed to be inhibited by certain flavonoids. Genistein, which is an isoflavone, has been shown to be a selective inhibitor of PTKs (Akiyama et al. 1987). It has been reported that phospholipase A$_2$ as well as lipoxygenases (LOX) and cyclooxygenases (COX) can be inhibited by certain flavonoids. Flavones have been characterized as preferentially inhibiting COX while LOX was inhibited by flavonols (Kim et al. 2004a). Adenylate cyclase has been reported to be inhibited by flavone while several phosphodiesterases appear to be inhibited by flavonoids, leading to increased cAMP and cGMP concentrations. (Middleton et al. 2000)

A high intake of flavonoids has been suggested to lower the risk of cardiovascular diseases and cancer. In a cohort study consisting of a population of 10 000 Finnish men and women, a higher flavonoid intake was shown to correlate with a lowered risk for asthma (Knekt et al. 2002). However, in the same study patients suffering from rheumatoid arthritis were shown to have a higher intake of flavonoids. Interestingly, administration of one flavonoid, quercetin, to rats with adjuvant-induced arthritis appeared to evoke anti-arthritic effects (Mamani-Matsuda et al. 2006). Tea polyphenols have been abundantly studied, especially epigallocatechin-3-callate, which has been shown to be mainly responsible for the beneficial effects of green tea. Several cancer and cardiovascular disease models and epidemiological or case-control studies with tea and its polyphenols have shown them to exert protective effects (Khan and Mukhtar 2007). In a rat study, genistein was found to inhibit the LPS-induced septic response (Ruetten and Thiemer mann 1997).

4.2 Flavonoids and iNOS

Some polyphenolic compounds have been reported to inhibit iNOS expression and NO production in macrophages (Lin and Lin 1997, Chen et al. 2001). Flavonol quercetin has been shown to inhibit the LPS-induced NO production in macrophages (Kim et al. 1999, Liang et al. 1999, Manjeet and Ghosh 1999, Wadsworth and Koop 1999, Raso et al. 2001, Wadsworth and Koop 2001). In these studies, the effects on NO production and/or iNOS expression were
evaluated, but the precise mechanisms of action remained undefined. In the studies by Wadsworth and Koop, quercetin was found not to alter the activation of NF-κB (Wadsworth and Koop 1999) nor the half-life of iNOS mRNA (Wadsworth and Koop 2001).

Genistein, a protein tyrosine kinase inhibitor, has been shown to inhibit NO production in activated macrophages (Krol et al. 1995, Sadowska-Krowicka et al. 1998, Liang et al. 1999) whereas daidzein, another isoflavone, has been shown to either inhibit (Krol et al. 1995) or have no effect (Kim et al. 1999) on NO production. Naringenin is a member of the flavanone group which apparently can inhibit LPS-induced NO production in both RAW264.7 and J774 macrophages (Tsai et al. 1999, Raso et al. 2001).

The mechanisms of the anti-inflammatory action of flavonoids are not clearly understood. One potential mechanism to account for the anti-inflammatory properties of certain subgroups of flavonoids could be the inhibition of iNOS expression and NO production. Some references in the literature point to this possibility, but more precise mechanistic studies remain to be undertaken.

5 Calcineurin inhibitors

Cyclosporin A (CsA), tacrolimus and pimecrolimus are immunosuppressive drugs, which are known to inhibit the production of IL-2 and other cytokines in T cells. CsA was identified in 1970’s and tacrolimus in 1980’s, and they are produced by fungus species Beauveria nivea and Streptomyces tsukubaensis, respectively. Pimecrolimus is an ascomycin macrolactone derivative, closely related to tacrolimus and which is produced by Streptomyces hygroscopicus. (Figure 12) (Nghiem et al. 2002)

![Figure 12. Structures of the calcineurin inhibitors, cyclosporin A, tacrolimus and pimecrolimus](image-url)
5.1 Use of calcineurin inhibitors in inflammatory diseases

CsA and tacrolimus are used to prevent allograft rejection after organ transplantation and also have a place in the treatment of certain inflammatory diseases such as rheumatoid arthritis and Chron’s disease (Faulds et al. 1993, Encke et al. 2004). In the treatment of atopic dermatitis, tacrolimus and pimecrolimus have shown efficacy and are in clinical use (Granlund et al. 2001, Nghiem et al. 2002).

The major adverse effects of orally administered CsA and tacrolimus are nephrotoxicity, neurotoxicity, hypertension, diabetes and gastrointestinal disturbances. Increased TGFβ production by CsA has been implicated in two clinically major adverse effects i.e. fibrosis and hypertension, as well as in tumor progression in CsA treated mice (Prashar et al. 1995, Hojo et al. 1999, Martinez-Martinez and Redondo 2004). Topically administered tacrolimus and pimecrolimus are well tolerated, and pimecrolimus, which was specifically developed for the treatment of inflammatory skin disorders, has an even lower risk for systemic side effects since it is less skin-permeable than tacrolimus (Grassberger et al. 2004). Since the calcineurin inhibitors are metabolized via the CYP3A4 route, these drugs have several interactions with other drugs, a factor to be taken account in multi-drug therapy (Faulds et al. 1993).

5.2 Mechanisms of action of calcineurin inhibitors

CsA and tacrolimus were found to inhibit Ca\(^{2+}\) -dependent transcription in T cells (Mattila et al. 1990), with calcineurin being found to be an important target molecule in mediating the immunosuppressive response (Liu et al. 1991). Calcineurin is an abundantly expressed calcium-calmodulin –dependent serine/threonine phosphatase (also termed PP2B) which comprises of subunits A and B. The catalytic subunit A has an autoinhibitory domain, a binding site for calmodulin and a binding site for subunit B. Upon activation by Ca\(^{2+}\)/calmodulin, the autoinhibition is terminated, allowing binding of subunit B and subsequent activation of calcineurin. Well-described targets for calcineurin in activated immune cells are the NF-AT (nuclear factor of activated T cells) family of transcription factors. After cell activation, the rise in the cytosolic calcium concentration leads to the activation of calmodulin, which is capable of activating calcineurin. Activated calcineurin can dephosphorylate the cytosolic component of NF-AT, thus enabling its nuclear translocation. In the nucleus, NF-AT when it combines with its nuclear counterpart, binds to its target sequences in DNA and is able to regulate the transcription of several genes including cytokines IL-2, IL-3, IL-4, IL-5, IL-8, IL-13, GM-CSF, and IFN\(\gamma\) (Klee et al. 1998, Kiani et al. 2000).

CsA, tacrolimus and pimecrolimus are able to inhibit the activity of calcineurin and are therefore termed as calcineurin inhibitors. Due to their lipophilicity all three drugs readily diffuse into cytosol passing through the cell
membrane. In the cytosol, drugs bind to their respective immunophilin target molecules, which are widely expressed peptidyl-prolyl cis-trans isomerases. Cyclosporin A binds to cyclophilin, and tacrolimus and pimecrolimus bind to FK binding protein 12 (FKBP12). These drug-immunophilin complexes are able to inhibit the enzymatic activity of calcineurin thus inhibiting the activation of NF-AT in activated immune cells and subsequent induction of the expression of several pro-inflammatory genes (Figure 13) (Schreiber and Crabtree 1992, Nghiem et al. 2002).

Calcineurin inhibitors have also targets other than calcineurin and on the other hand calcineurin has other effects in addition to that mediated through NF-AT. Calcineurin has been shown to be involved in the regulation of transcription factors Elk-1, NF-κB and CREB, in the regulation of the activity of JNK, and in concert with JNK kinases, calcineurin has been shown to stabilize IL-2 and IL-3 mRNAs (Chen et al. 1998, Ming et al. 1998). With respect to the calcineurin independent effects, there seems to be involvement of the immunophilins in complexes with CsA/tacrolimus. For example, glucocorticoid-induced transcription is enhanced after CsA/tacrolimus treatment because immunophilins bind to CsA/tacrolimus instead of forming molecular chaperones for the glucocorticoid receptor (Kiani et al. 2000). In T cells CsA and tacrolimus have been shown to target JNK and p38 independently of calcineurin inhibition (Matsuda et al. 2000). In monocytes/macrophages, CsA has been shown to inhibit NF-κB activation (Holschermann et al. 1996) and inhibition of proteasome activity in RAW macrophages has been suggested as a mechanism.
for this action (Meyer et al. 1997). In oncogenic mast cells, CsA was found to inhibit IL-3 by destabilizing its mRNA (Nair et al. 1994). The activity of constitutively expressed NOS has been shown to be suppressed by tacrolimus through inhibition of the calcineurin dependent dephosphorylation of NOS (Dawson et al. 1993). Since calcineurin inhibitors have several calcineurin-dependent as well as calcineurin-independent targets, the inhibition of NF-AT is not likely to be the only mechanism to explain how their immunosuppressive and anti-inflammatory effects are mediated.

5.3 Calcineurin inhibitors and iNOS

Calcineurin inhibitors have been shown to affect NO production through the iNOS pathway e.g. in vascular smooth muscle cells (Marumo et al. 1995), in C6 glioma cells (Trajkovic et al. 1999a), in cardiac myocytes (Shindo et al. 1995), in hepatocytes (Kaibori et al. 1999), in fibroblasts (Trajkovic et al. 1999b), and in macrophages (Conde et al. 1995, Hattori and Nakanishi 1995, Attur et al. 2000). The mechanisms of action have been studied at different levels, inhibition has been reported at the level of NO production or iNOS protein and/or mRNA expression. There are few reports on more specific mode of action of how calcineurin inhibitors impair NO production in different cell types.

In peritoneal macrophages exposed to LPS/IFNγ, CsA and tacrolimus were shown to inhibit NOS activity (Conde et al. 1995) whereas in LPS-treated J774 macrophages, no effect on enzyme activity was seen even though NO production was inhibited (Hattori and Nakanishi 1995). CsA and tacrolimus have been shown to have different potencies and also different mechanisms of action have been proposed. CsA has been shown to effectively inhibit NO production in macrophages (Attur et al. 2000), fibroblasts (Trajkovic et al. 1999b), glioma cells (Trajkovic et al. 1999a), as well as in vascular smooth muscle cells (Marumo et al. 1995) though tacrolimus was not found to inhibit NO production in any of these studies. In hepatocytes, tacrolimus appeared to inhibit IL-1β-induced NO production whereas CsA had no inhibitory effect, and the inhibitory action of tacrolimus was linked to the inhibition of NF-κB activation (Kaibori et al. 1999). More specific mechanisms of action remain to be clarified.
Aims of the study

iNOS and NO are important therapeutic targets in the treatment of inflammatory disorders. iNOS expression and NO production are enhanced in many inflammatory diseases, and in several experimental models, NOS inhibitors have had beneficial effects.

The aim of the present study was to find out compounds and mechanisms to inhibit NO production and iNOS expression in relation to inflammation. Better knowledge of the regulation of iNOS expression would be valuable in understanding the role of NO in the pathophysiology of inflammation as well as in the development of novel anti-inflammatory drugs.

In the present study, the interest was focused on molecules belonging to glucocorticoids, phenolic compounds, disease modifying antirheumatic drugs and calcineurin inhibitors. Further studies were carried out to clarify the more precise mechanisms of actions of those compounds effectively inhibiting iNOS expression and NO production.

The detailed aims were:

1. To study if histone deacetylation is involved in the mechanisms by which glucocorticoids inhibit iNOS expression and NO production (I)

2. To identify phenolic compounds potentially inhibiting iNOS expression and NO production and to further study the mechanisms of action of the effective compounds (II)

3. To study the effects of drugs used in the treatment of rheumatoid arthritis and inflammatory bowel disease on iNOS expression and NO production (III)

4. To further investigate the mechanisms by which calcineurin inhibitors inhibit iNOS expression and NO production (IV)
Materials and methods

1 Materials

Dulbecco’s Modified Eagle’s Medium (DMEM) was obtained from Gibco BRL (Paisley, Scotland, UK) and from Cambrex Bioproducts Europe (Verviers, Belgium), and its supplements were from Gibco BRL (Paisley, Scotland, UK) and Invitrogen (Carlsbad, CA, USA) except for non-essential amino acids, sodium pyruvate and sodium carbonate as well as Eagle’s Minimal Essential Medium (EMEM) were from Cambrex Bioproducts Europe (Verviers, Belgium).

Cyclosporin A and tacrolimus were from Calbiochem (La Jolla, CA, USA) and pimecrolimus was supplied by Novartis Pharma AG (Basel, Switzerland). Infliximab was from Centocor (Leiden, The Netherlands), etanercept from Wyeth Lederle (Espoo, Finland), methotrexate and dexamethasone were kindly provided by Orion Corporation (Espoo, Finland) and RU24858 was kindly provided by Aventis Pharma. MC1293 and apicidin were obtained from Alexis Corporation (San Diego, CA, USA). pGL3-promoter vector, passive lysis buffer and luciferase assay reagents were obtained from Promega (Madison, WI, USA).

Luteolin, luteolin-7-glucoside, vitexin, daidzein, genistein, genistin, rhamnetin, isorhamnetin, kaempferol, myricetin, taxifolin, naringin, ferulic acid, pelargonidin, procyanidin B1 and procyanidin B2 were obtained from Extrasynthese (Lyon, France). Acacetin, cyanidin, flavone, morin and quercitrin were obtained from Carl Roth GmbH (Karlsruhe, Germany). Quercetin, rutin and benzoic acid were obtained from Merck (Darmstadt, Germany). Apigenin, chlorogenic acid, dodecyl gallate, methyl gallate, octyl gallate and sinapic acid were from Fluka (Buchs SG, Switzerland).

1400W and L-NIO were kindly donated by Dr Richard Knowles, GlaxoSmithKline, Stevenage, UK. All other reagents were from Sigma (St. Louis, MO, USA).

2 Cell culture

Murine J774 macrophages and murine L-929 fibroblasts were obtained from American Type Culture Collection (Manassas, VA, USA), and human T84 colon epithelial cells were donated by Professor Markku Mäki, University of Tampere (Halttunen et al. 1996). A549/8-Luc cells, which are stably transfected with
plasmid pXP2-16kb containing a full length human iNOS promoter (16 kb) in front of a luciferase gene (de Vera et al. 1996, Hausding et al. 2000), were kindly provided by Professor Hartmut Kleinert at the University of Mainz, Germany.

All cell lines were cultured at 37°C (in 5% carbon dioxide) and passaged up to 20 times. J774 macrophages and T84 epithelial cells were grown in DMEM with glutamax-I containing 10% (5% in T84 cells) heat-inactivated foetal bovine serum (FBS), penicillin (100 units/ml), streptomycin (100 µg/ml) and amphotericin B (250 ng/ml). L-929 fibroblasts were cultured in EMEM containing 10% heat-inactivated FBS, penicillin (100 units/ml), streptomycin (100 µg/ml) and amphotericin B (250 ng/ml) and supplemented with sodium bicarbonate (0.15%), non-essential amino acids (0.1 mM) and sodium pyruvate (1 mM). A549/8-Luc cells were grown in DMEM with sodium pyruvate (1 mM) containing 5% heat-inactivated FBS, polymyxin B (2.5 µg/ml), and G418 (0.5 mg/ml) for selection.

All cells were trypsinized with trypsin-EDTA and seeded in 10-cm dishes for the extraction of nuclear proteins, in 6-well plates for the whole cell protein extraction, in 24-well plates for nitrite measurements, RNA extraction and for transient transfection experiments, and in 96-well plates for XTT-test. Cells were grown to confluency before the commencement of the experiments except in the transient transfection experiments where the cells were grown to approximately 90% confluency before transfection was performed.

3 Determination of cell viability

Cytotoxicity of the tested compounds was controlled in conditions similar to the longer-lasting cell culture experiments. Cell viability was tested using the Cell Proliferation Kit II that measures the ability of the cells to metabolise XTT to formazan by mitochondrial dehydrogenase, a function that only can occur in living cells (Roche Diagnostics GmbH, Mannheim, Germany). Cells were incubated with the tested compounds and stimulants for 20 h before addition of sodium 3’-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis (4-methoxy-6-nitro) benzene sulfonic acid hydrate (XTT) (final concentration 0.3 mg/ml) and N-methyl dibenzopyrazine methyl sulfate (8.2 µM). Cells were incubated for another 4 h and the amount of formazan accumulating in the growth medium was assessed spectrophotometrically. Cells treated with Triton-X were used as a positive control. If treatment of the cells in experimental conditions led to lowered mitochondrial dehydrogenase activity (by more than 20% compared to cells treated with the stimulant only), then that compound was regarded as toxic at the given concentration and excluded from further studies.
4 Nitrite measurement

The NO produced by cells in culture is rapidly converted to nitrite in aqueous culture media (Laurent et al. 1996). The cells’ abilities to produce NO were assessed by measuring the accumulation of nitrite into the culture medium. Culture medium was collected at certain time-points and nitrite was measured by the Griess reaction (Green et al. 1982). Briefly, in this method 1 vol of a sample was pipetted into flat-bottom 96-well plate and 1 vol of Griess reagent was added. Under acidic conditions, nitrite in the sample reacts with sulfanilamide and N-(1-naphthyl)ethylenediamine (constituents of the Griess reagent) forming an azo dye whose absorbance at 540 nm can be detected spectrophotometrically (Figure 14). Sodium nitrate was used as the standard, and the amount of nitrite accumulated into the culture media was calculated against the standard curve defined separately for each measurement and prepared in the same serum containing medium used in the study. A non-selective NOS inhibitor, L-NIO, and a highly selective iNOS inhibitor, 1400W, were used to ensure that measured nitrite was due to the NO produced by the NOS pathway in the cell culture.

![Figure 14. Griess reaction. In an acidic environment, azo dye is formed in the reaction of nitrite, sulfanilamide and N-(1-naphthyl)ethylenediamine, and may be detected spectrophotometrically (Green et al. 1982)](image)

5 Western blot analysis

Western blot analysis was used to detect proteins from whole cell and nuclear extracts.

In the extraction of whole cell proteins, cell pellets were lysed in ice-cold extraction buffer (10 mM Tris-base, pH 7.4, 5 mM EDTA, 50 mM NaCl, 1 % Triton X-100, 0.5 mM phenylmethyl sulfonyl fluoride, 1 mM sodium orthovanadate, 20 µg/ml leupeptin, 50 µg/ml aprotinin, 5 mM NaF, 2 mM sodium pyrophosphate and 10 µM n-octyl-beta-D-glucopyranoside) after the desired time of incubation. Samples were incubated on ice for 15 min, centrifuged for 10 min at 13400 g (+4°C), and the resulting supernatant was diluted 1:4 in the sample buffer (6.25 mM Tris-HCl, 10 % glycerol, 2 % SDS
and 0.025 % 2-mercapto-ethanol). Samples were stored at -20°C until analysed. The Coomassie blue method was used to measure the protein content of the samples (Bradford 1976).

In the extraction of nuclear proteins, the cells were washed with ice-cold PBS and solubilised in hypotonic buffer A (10 mM HEPES-KOH, pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM DTT, 0.2 mM PMSF, 10 µg/ml leupeptin, 25 µg/ml aprotinin, 0.1 mM EGTA, 0.1 mM Na₂VO₄, 2 mM NaF) and incubated for 10 min on ice. Thereafter, the cells were vortexed for 30 s and the nuclei were separated by centrifugation at 4°C, 21000 g, 10 s. Samples were suspended in buffer C (20 mM HEPES-KOH, pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM DTT, 0.2 mM PMSF, 10 µg/ml leupeptin, 25 µg/ml aprotinin, 0.1 mM EGTA, 1 mM Na₂VO₄, 1 mM NaF) and incubated on ice for 20 min. Thereafter samples were vortexed for 30 s and nuclear extracts were obtained by centrifugation at +4°C and 21000 g for 2 min. The protein content of the samples was determined and samples were stored as described above.

Before loading samples (20 µg/lane) to polyacrylamide gels, they were boiled for 5 min to attain linear proteins. For iNOS Western blot, 8% and for p65 and STAT-1α Western blots, 12% polyacrylamide gels were used. Actin was used as the loading control for iNOS in the whole cell extracts, and laminA/C played a corresponding role for p65 and STAT-1α in the nuclear extracts. Proteins were separated by SDS-PAGE and thereafter transferred to a nitrocellulose membrane. Non-specific binding was blocked by incubating the membrane in 5% TBS/T-milk for 1 h in RT. Membranes were incubated with primary antibodies (Table 4) o/n at +4°C, and thereafter washed with TBS/T at RT 5 times for 5 min. Membranes were incubated with the secondary antibody (Table 4) for 45 min at RT and membranes were thereafter washed 5 times for 5 min with TBS/T. Bound antibodies were visualized using SuperSignal chemiluminescent substrate (Pierce, Cheshire, UK) and FluorChem 8800 imaging system (Alpha Innotech Corporation, San Leandro, CA). Molecular weight markers (Table 4) were used to estimate the molecular mass of the detected band. The quantitation of the chemiluminescent signal was carried out with the use of FluorChem software version 3.1. In the case of NF-κB p65 Western blot, membranes were stripped from the bound p65 antibody and the secondary antibody before detection of the loading control laminA/C since the molecular weights of p65 and laminA/C are close to each other. In the stripping protocol, membranes were incubated for 20 min in stripping buffer (0.5 M Tris, pH 6.8, 10% SDS, 0.68% 2-mercapto-ethanol) at 55 °C and washed thereafter with TBS/T. To ensure the detachment of antibodies, the chemiluminescent signal was measured before the membrane was subjected to the treatment with laminA/C antibody as described above.
Table 4. Antibodies and molecular weight markers used in the present study

<table>
<thead>
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<th>Primary antibodies (all rabbit polyclonal antibodies):</th>
<th>MW</th>
<th>Supplied by</th>
</tr>
</thead>
<tbody>
<tr>
<td>NOS2 (M-19): sc-650</td>
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<td>Santa Cruz Biotechnology</td>
</tr>
<tr>
<td>NF-κB p65: #3034</td>
<td>65 kDa</td>
<td>Cell Signaling Technology</td>
</tr>
<tr>
<td>STAT-1α p91 (C-24): sc-345</td>
<td>91 kDa</td>
<td>Santa Cruz Biotechnology</td>
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<tr>
<td>Actin (I-19): sc-1616-R</td>
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<td>Santa Cruz Biotechnology</td>
</tr>
<tr>
<td>Lamin A/C (H-110): sc-20681</td>
<td>69/62 kDa</td>
<td>Santa Cruz Biotechnology</td>
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<table>
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<th>Secondary antibody:</th>
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<td>sc-2004: goat anti-rabbit IgG-HRP</td>
<td>Santa Cruz Biotechnology</td>
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<th>Molecular weight markers:</th>
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<tr>
<td>PageRuler Prestained Protein Ladder #SM0671</td>
<td>Fermentas Life Sciences</td>
</tr>
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6 Electrophoretic mobility shift assay (EMSA)

Electrophoretic mobility shift assay (EMSA) was used to assess the activation of NF-κB by detecting the nuclear NF-κB subunits capable of binding the nucleotides containing the consensus NF-κB-binding sequences in DNA. Nuclear proteins were extracted as described above, and the protein concentrations in the extracts were measured by the Coomassie blue method (Bradford 1976). Samples were stored at -70°C until analyzed.

Single-stranded oligonucleotides containing the consensus NF-κB binding sequences (5’-AGTTGAGGGGACTTTCCCAGGC-3’, 3’-TCAACTCCCTGGAAAGGTCCG-5’, Amersham Pharmacia Biotech, Piscataway, NJ, USA) were annealed and 5’ 32P-endabeled with DNA 5’-End Labeling Kit (Boehringer Mannheim Indianapolis, IN, USA). In the binding reactions, 10 µg of nuclear extract was incubated in 20 µl of total reaction volume containing 0.1 mg/ml (poly)dI-dC, 1 mM DTT, 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 200 mM KCl and 10 % glycerol for 20 min at RT. Thereafter 0.2 ng of 32P-labeled oligonucleotide was added and the reaction mixture was incubated for another 10 min at RT. Protein/DNA complexes were separated from the free DNA probe by electrophoresis on 4 % polyacrylamide gel. The gel was dried on filter paper and autoradiographed with an intensifying screen at -70°C. FluorChem 8800 imaging system (Alpha Innotech Corporation, San Leandro, CA) and FluorChem software version 3.1 were used to quantify the autoradiography signal.
Total RNA was extracted using RNeasy® kit (QIAGEN GmbH, Hilden, Germany) or with E.Z.N.A.® kit (Omega Bio-Tek Inc., Norcross, GA, USA). Cells were incubated with the compounds of interest and washed twice with PBS after the desired time of incubation. Thereafter cells were lysed, and total RNA was extracted. The amount of total RNA was measured spectrophotometrically and 25 ng of total RNA was reverse-transcribed to cDNA using TaqMan Reverse Transcription reagents and random hexamers (Applied Biosystems, Foster City, CA, USA). Reverse-transcriptase (RT) reaction parameters were as follows: incubation at 25°C for 10 min, RT at 48°C for 30 min, and RT inactivation at 95°C for 5 min. Total RNA extracted from the L-929 pNF-κB and L-929 pGAS cell lines was treated with DNAse I (Fermentas UAB, Vilnius, Lithuania) prior to conversion to cDNA.

cDNA formed in the RT reaction (amount corresponding to approximately 1 ng of total RNA) was subjected to PCR using TaqMan Universal PCR Master Mix, primers and probes, and ABI PRISM 7000 Sequence detection system (Applied Biosystems). The primer and probe sequences were designed by using Primer Express, version 2.0.0 (Applied Biosystems), and concentrations were optimised according to the manufacturer’s guidelines in TaqMan Universal PCR Master Mix Protocol part number 4304449 revision C. Primer and probe sequences used in this study are listed in Table 5. All probes contained 6-FAM (6-carboxy-fluorescein) as 5’-reporter dye and TAMRA (6-carboxy-tetramethyl-rhodamine) as 3’-quencher. Concentrations of primers and probes were 300 nM and 150 nM, respectively. PCR reaction parameters were as follows: incubation at 50°C for 2 min, incubation at 95°C for 10 min, and thereafter 40 cycles of denaturation at 95°C for 15 s and annealing and extension at 60°C for 1 min. Each sample was determined in duplicate.

A standard curve method was used to determine the relative mRNA levels as described in Applied Biosystems User Bulletin number 2. In short, standard curves for quantified genes were created using mRNA isolated from stimulated cells. The isolated RNA was reverse-transcribed as described. Dilution series were made from obtained cDNA ranging from 1 pg to 10 ng and were subjected to real-time PCR as described. The obtained threshold cycle values were plotted against the dilution factor to create a standard curve. Relative mRNA levels in test samples were then calculated from the standard curve. Results of iNOS and luciferase mRNA levels were normalised against GAPDH mRNA in each sample.
Table 5. Primer and probe sequences used in quantitative real-time RT-PCR

<table>
<thead>
<tr>
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<th>Sequence</th>
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<tr>
<td>Mouse iNOS forward</td>
<td>5’-CCTGGTACGGGCATTGCT-3’</td>
</tr>
<tr>
<td>Mouse iNOS reverse</td>
<td>5’-GCTCATGCGGCCTCCTT-3’</td>
</tr>
<tr>
<td>Mouse iNOS probe</td>
<td>5’-CAGCAGCGGCTCCATGACTCCC-3’</td>
</tr>
<tr>
<td>Mouse GAPDH forward</td>
<td>5’-GCATGGCCCTCCGTGTTC-3’</td>
</tr>
<tr>
<td>Mouse GAPDH reverse</td>
<td>5’-GATGTCATCATACTTTGCCAGTTT-3’</td>
</tr>
<tr>
<td>Mouse GAPDH probe</td>
<td>5’-TCGTGGATCTGACGTGCCGC-3’</td>
</tr>
<tr>
<td>Human GAPDH forward</td>
<td>5’-AAGGTCGGAGTCAACCGATTT-3’</td>
</tr>
<tr>
<td>Human GAPDH reverse</td>
<td>5’-GCAACAAATATCCACTTTACCAGGTTA-3’</td>
</tr>
<tr>
<td>Human GAPDH probe</td>
<td>5’-CGCCTGGTACCAACGGGCTGC-3’</td>
</tr>
<tr>
<td>Luciferase forward</td>
<td>5’-AAAAAGTTTCGGCAGGAGG-3’</td>
</tr>
<tr>
<td>Luciferase reverse</td>
<td>5’-TTTTCTTTGCGTGGAGTTTCCC-3’</td>
</tr>
<tr>
<td>Luciferase probe</td>
<td>5’-TGTGTTTGGACGAGTGAGTACCAGG-3’</td>
</tr>
</tbody>
</table>

Concentration of all primers was 300 nM and for all probes 150 nM. All probes contained 6-FAM (6-carboxy-fluorescein) as 5’-reporter dye and TAMRA (6-carboxy-tetramethyl-rhodamine) as 3’-quencher.

8 Actinomycin D assay

Actinomycin D assay was utilized to study the decay of iNOS mRNA. In this method, cells were incubated with the compounds of interest, and usually at or near to the time-point where the amounts of iNOS mRNA peaked (estimated from the iNOS mRNA time-curve) actinomycin D (0.1 µg/ml), which is an inhibitor of transcription, was added to the cells. Thereafter RNA was extracted at different time-points and subjected to quantitative RT-PCR to measure the remaining mRNA.

9 Transient transfection and firefly luciferase assay

To study the effects mediated by the iNOS 3’UTR, a vector containing the full length mouse iNOS 3’UTR after SV40 promoter-controlled luciferase as a reporter gene was generated and introduced to the cells by transient transfection (Soderberg et al. 2007a). Cells were grown for 24 h to 90% confluency in 24-well plates and thereafter transfected with Lipofectamine 2000 transfection reagent according to the manufacturer’s instructions (Invitrogen, Carlsbad, CA, USA). Cells were transfected either with 0.8 µg of pGL3-iNOS 3’UTR vector (Soderberg et al. 2007a) (kindly provided by Professor Matti Lang, Uppsala University, Sweden) or with 0.8 µg of pGL3-promoter vector (Promega,
Madison, WI, USA) used as a negative control (Figure 15). Four hours after transfection, fresh culture medium was changed, and the cells were further incubated and at 24 h post transfection, the culture media was changed and the cells were incubated with the compounds of interest for 24 h. Thereafter the cells were washed with PBS and lysed in passive lysis buffer (Promega, Madison, WI, USA) and centrifuged for 2 min at 12 000 rpm, +4 °C. Supernatants were collected and stored at -70 °C. Luciferase assay reagent (50 µl) (Promega, Madison, WI, USA) was added to 10 µl of a sample and luciferase activity was measured by using Victor³ multilabel counter (PerkinElmer Life and Analytical Sciences, Inc., MA, USA). Chemiluminescent signals from each sample were normalized against the protein content of the sample measured by the Coomassie blue method. The results obtained from the cells transfected with pGL3-iNOS 3’UTR were normalized against the results from the cells identically treated but transfected with pGL3-promoter vector used as control.

Figure 15. pGL3 constructs used in the study IV. A Part of a structure of the pGL3-promoter vector. B Part of a structure of the pGL-iNOS 3’UTR vector containing the full length iNOS 3’UTR (Soderberg et al. 2007a)

10 Preparation of stable L-929 pNF-κB and L-929 pGAS reporter cell lines

Professor Hartmut Kleinert at the University of Mainz, Germany kindly provided the luciferase reporter constructs to investigate NF-κB [pNFκB(luc)neo] and STAT-1 [pGAS(luc)neo] mediated transcription. pNFκB(luc)neo contained five NF-κB binding sites and pGAS(luc)neo contained four STAT-1 -binding GAS sites to drive luciferase expression. Both plasmids contained a neomycin resistance gene under the control of a TK promoter for mammalian selection. To create a stable transfection, L-929 cells were transfected with pNKxB(luc)neo or pGAS(luc)neo reporter plasmids using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. Transfected cells were selected with G418 disulfate salt (Sigma, St Louis, MO, USA) treatment (800 µg/ml). After the selection was complete, the survived clones were pooled to give rise L-929 pNF-κB and L-929 pGAS cell lines and further cultured in the presence of 400 µg/ml of G418.
11 HDAC activity assay

Nuclear extracts and the HDAC activity assay were prepared according to the manufacturer’s instructions (Histone Deacetylase Activity/Inhibitor Screening Assay, Cayman Chemicals, MI, USA) and the fluorescence was measured by using Victor\textsuperscript{3} multilabel counter (PerkinElmer Life and Analytical Sciences, Inc., MA, USA).

12 Statistics

Results are expressed as mean + standard error of mean. Statistical significance of the results was calculated by analysis of variance followed by Dunnett multiple comparisons test. Differences were considered significant when p<0.05 and further specified with ** when p<0.01 and with * when p<0.05.
Results

1 LPS-induced iNOS expression and NO production in J774 macrophages

Bacterial endotoxin lipopolysaccharide (LPS, Escherichia coli 0111:B4) was used to induce iNOS expression and NO production. iNOS mRNA accumulation was measured by quantitative real-time RT-PCR, and iNOS mRNA levels were found to be enhanced in response to LPS after a 2 h lag period reaching the maximal mRNA amount after 6 h incubation and declining thereafter. The time course of the elevation in iNOS protein at different time-points after addition of LPS was evaluated, with the maximal iNOS protein amount being detected after 24 h incubation. NO production was measured as nitrite accumulating into the culture medium and was followed up to 48 h after addition of LPS. No detectable NO production or any iNOS expression was found in untreated cells. (Figure 16)

Figure 16. LPS-induced iNOS mRNA, iNOS protein and NO production measured at different time-points after addition of LPS (10 ng/ml) in J774 macrophages. iNOS mRNA was measured by quantitative real-time RT-PCR, and results were normalized against GAPDH (n=3, mean + SEM). iNOS protein expression was measured by Western blot (n=3, mean + SEM). NO production was determined by measuring nitrite accumulation into the culture medium by Griess reaction (n=6, mean + SEM). The maximal level at each time-curve was set to represent 100%, and the other values were related to that value.
2 Effects of anti-inflammatory compounds on iNOS expression and NO production

In many inflammatory disorders, NO production through the iNOS pathway becomes enhanced, and the high amount of NO has pro-inflammatory and cytotoxic properties. The present study originated from the idea to investigate if drugs used in the treatment of inflammatory disorders such as rheumatoid arthritis and inflammatory bowel disease have any effect on iNOS expression and NO production. In addition, 36 polyphenolic compounds including dietary flavonoids were studied for their ability to inhibit iNOS expression and NO production which could be involved in their anti-inflammatory properties. Table 6 describes the compounds and their characteristics used in this study.

Cells were incubated with LPS to induce iNOS expression and NO production, and with the compound of interest for 24 h and NO production was assessed by measuring the nitrite which had accumulated into the culture media by the Griess reaction. The effects of the anti-inflammatory compounds on NO production used in this study are depicted in Table 6. The glucocorticoid, dexamethasone, and the dissociated steroid RU24858 inhibited NO production as did eight out of the 36 studied polyphenolic compounds (i.e. by more than 50%), namely flavone, daidzein, genistein, quercetin, isorhamnetin, kaempferol, naringenin and pelargonidin. Table 7 in page 72 includes all data of the effects of flavonoids used in the present study on iNOS expression and NO production. Methotrexate, sulfasalazine and 5-aminosalicylic acid were used up to relatively high concentrations (100 μM), but they were not capable of effectively inhibiting NO production. Infliximab and etanercept, used in the anticytokine therapy against TNFα, did not inhibit the LPS-induced iNOS expression and NO production. On the contrary, all three studied calcineurin inhibitors, CsA, tacrolimus and pimecrolimus inhibited LPS-induced NO production whereas another immunosuppressive agent, rapamycin, an inhibitor of mTOR, had practically no effect. 1400W, a highly iNOS selective NOS inhibitor and L-NIO, a non-selective NOS inhibitor were used to ensure that the measured nitrite originated from NO produced by the iNOS pathway. All compounds were tested for their cytotoxicity in the experimental conditions used by XTT test, and those compounds found toxic were excluded from further studies.
Table 6. Compounds used in the present study and their effects on the LPS-induced NO production in J774 macrophages

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration</th>
<th>Inhibition of LPS-induced NO production</th>
<th>Structural or pharmacological properties</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dexamethasone</td>
<td>1 µM</td>
<td>52%</td>
<td>anti-inflammatory steroid</td>
</tr>
<tr>
<td>RU24858</td>
<td>1 µM</td>
<td>44%</td>
<td>dissociated steroid</td>
</tr>
<tr>
<td>TSA</td>
<td>50 ng/ml</td>
<td>no inhibition</td>
<td>HDAC inhibitor, hydroxamate</td>
</tr>
<tr>
<td>Apicidin</td>
<td>60 nM</td>
<td>no inhibition</td>
<td>HDAC inhibitor, cyclic tetrapeptide</td>
</tr>
<tr>
<td>MC1293</td>
<td>30 µM</td>
<td>no inhibition</td>
<td>HDAC inhibitor, benzamide</td>
</tr>
<tr>
<td>Acacetin</td>
<td>100 µM</td>
<td>-</td>
<td>flavone</td>
</tr>
<tr>
<td>Apigenin</td>
<td>100 µM</td>
<td>-</td>
<td>flavone</td>
</tr>
<tr>
<td>Flavone</td>
<td>100 µM</td>
<td>93%</td>
<td>flavone</td>
</tr>
<tr>
<td>Luteolin</td>
<td>100 µM</td>
<td>-</td>
<td>flavone</td>
</tr>
<tr>
<td>Luteolin-7-glucoside</td>
<td>100 µM</td>
<td>39%</td>
<td>flavone</td>
</tr>
<tr>
<td>Vitexin</td>
<td>100 µM</td>
<td>6%</td>
<td>flavone</td>
</tr>
<tr>
<td>Daidzein</td>
<td>100 µM</td>
<td>70%</td>
<td>isoflavone</td>
</tr>
<tr>
<td>Genistein</td>
<td>100 µM</td>
<td>97%</td>
<td>isoflavone</td>
</tr>
<tr>
<td>Genistin</td>
<td>100 µM</td>
<td>31%</td>
<td>isoflavone</td>
</tr>
<tr>
<td>Isorhamnetin</td>
<td>100 µM</td>
<td>65%</td>
<td>flavanol</td>
</tr>
<tr>
<td>Kaempferol</td>
<td>100 µM</td>
<td>100%</td>
<td>flavanol</td>
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<tr>
<td>Morin</td>
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<td>Myricetin</td>
<td>100 µM</td>
<td>32%</td>
<td>flavanol</td>
</tr>
<tr>
<td>Quercetin</td>
<td>100 µM</td>
<td>90%</td>
<td>flavanol</td>
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<tr>
<td>Quercitrin</td>
<td>100 µM</td>
<td>19%</td>
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</tr>
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<td>Rutin</td>
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<td>9%</td>
<td>flavanol</td>
</tr>
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<td>flavanone</td>
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<td>+Catechin</td>
<td>100 µM</td>
<td>-</td>
<td>flavan-3-ol</td>
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<tr>
<td>-Epicatechin</td>
<td>100 µM</td>
<td>no inhibition</td>
<td>flavan-3-ol</td>
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<tr>
<td>Procyanidin B1</td>
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<td>no inhibition</td>
<td>anthocyanin</td>
</tr>
<tr>
<td>Procyanidin B2</td>
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<td>anthocyanin</td>
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<tr>
<td>Cyanidin</td>
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<td>5%</td>
<td>anthocyanin</td>
</tr>
<tr>
<td>Pelargonidin</td>
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<td>59%</td>
<td>anthocyanin</td>
</tr>
<tr>
<td>Benzoic acid</td>
<td>100 µM</td>
<td>no inhibition</td>
<td>hydroxybenzoic acid</td>
</tr>
<tr>
<td>Dodecyl gallate</td>
<td>100 µM</td>
<td>-</td>
<td>hydroxybenzoic acid</td>
</tr>
<tr>
<td>Ellagic acid</td>
<td>100 µM</td>
<td>38%</td>
<td>hydroxybenzoic acid</td>
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<tr>
<td>Gallic acid</td>
<td>100 µM</td>
<td>-</td>
<td>hydroxybenzoic acid</td>
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<tr>
<td>Methyl gallate</td>
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<td>31%</td>
<td>hydroxybenzoic acid</td>
</tr>
<tr>
<td>Octyl gallate</td>
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<td>-</td>
<td>hydroxybenzoic acid</td>
</tr>
<tr>
<td>Syringic acid</td>
<td>100 µM</td>
<td>5%</td>
<td>hydroxybenzoic acid</td>
</tr>
<tr>
<td>Chlorogenic acid</td>
<td>100 µM</td>
<td>no inhibition</td>
<td>hydroxycinnamic acid</td>
</tr>
<tr>
<td>Ferulic acid</td>
<td>100 µM</td>
<td>9%</td>
<td>hydroxycinnamic acid</td>
</tr>
<tr>
<td>Sinapic acid</td>
<td>100 µM</td>
<td>5%</td>
<td>hydroxycinnamic acid</td>
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</table>
Table 6 continues

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration</th>
<th>Inhibition of LPS-induced NO production</th>
<th>Structural or pharmacological properties</th>
</tr>
</thead>
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<tr>
<td>Methotrexate</td>
<td>100 µM</td>
<td>4%</td>
<td>DMARD</td>
</tr>
<tr>
<td>Sulfasalazine</td>
<td>100 µM</td>
<td>21%</td>
<td>DMARD&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>5-Aminosalicylic acid</td>
<td>100 µM</td>
<td>12%</td>
<td>used in the treatment of IBD</td>
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<td>Infliximab</td>
<td>10 µg/ml</td>
<td>11%</td>
<td>TNFα blocker</td>
</tr>
<tr>
<td>Etanercept</td>
<td>100 µg/ml</td>
<td>19%</td>
<td>TNFα blocker</td>
</tr>
<tr>
<td>Cyclosporin A</td>
<td>10 µM</td>
<td>80%</td>
<td>calcineurin inhibitor&lt;sup&gt;c&lt;/sup&gt;</td>
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<tr>
<td>FK-506</td>
<td>10 µM</td>
<td>26%</td>
<td>calcineurin inhibitor&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Pimecrolimus</td>
<td>10 µM</td>
<td>50%</td>
<td>calcineurin inhibitor&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Rapamycin</td>
<td>100 nM</td>
<td>no inhibition</td>
<td>mTOR inhibitor&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>1400W</td>
<td>100 µM</td>
<td>&gt;90%</td>
<td>iNOS selective NOS inhibitor</td>
</tr>
<tr>
<td>L-NIO</td>
<td>100 µM</td>
<td>&gt;90%</td>
<td>non-selective NOS inhibitor</td>
</tr>
</tbody>
</table>

<sup>a</sup>cytotoxic
<sup>b</sup>combinatory molecule of 5-aminosalicylic acid and sulfapyridine
<sup>c</sup>IMDARD = disease modifying antirheumatic drug
<sup>d</sup>IBD = inflammatory bowel disease
<sup>e</sup>immunosuppressive agent

3 Inhibition of iNOS expression and NO production by glucocorticoids. Effect of histone deacetylase inhibitors (I)

Glucocorticoids are powerful anti-inflammatory drugs used e.g. in the treatment of rheumatoid arthritis, inflammatory bowel disease, inflammatory skin disorders and asthma. In the present study, the effects of histone deacetylase (HDAC) inhibitors on iNOS expression and NO production suppressed by glucocorticoids were investigated.

The glucocorticoid dexamethasone and RU24858 were investigated in this study. RU24858 is regarded as a dissociated steroid (Vayssiere et al. 1997) i.e. it is thought to possess GRE-independent trans-repressional properties but not GRE-mediated cis-activation/repression of GRE-regulated genes. J774 macrophages were treated with LPS or with LPS and glucocorticoid, and NO production was measured. Dexamethasone and RU24858 both inhibited LPS-induced NO production in a concentration-dependent manner (Figure 17A). Dexamethasone was more potent and probably more efficacious in inhibiting NO production. When mifepristone, which is a glucocorticoid receptor antagonist, was added to the cell culture, the inhibitory effects of dexamethasone and RU24858 were abolished (Figure 17B).
Figure 17. A Effects of different concentrations of dexamethasone and RU24858 on LPS (10 ng/ml)-induced NO production. B Effects of dexamethasone (1 μM) and RU24858 (1 μM) on LPS (10 ng/ml)-induced NO production in the absence or presence of mifepristone (3 μM). NO production in J774 cells was determined after 24 h incubation by measuring nitrite accumulation into the culture medium by Griess reaction. ** p<0.01, ns=not significant, mean ± SEM, n=6. (Reprinted with permission from: Hämäläinen et al. 2008, Pulmonary Pharmacology & Therapeutics 21: 331-339. ©Elsevier Ltd., modified)

It has been reported that histone deacetylation is involved in the mechanisms to explain why glucocorticoids inhibit IL-1β –induced GM-CSF expression (Ito et al. 2000). HDAC inhibitors were used to study if histone deacetylation has a role in the inhibitory action of glucocorticoids on iNOS expression and NO production. Trichostatin A, apicidin and MC1293 are HDAC inhibitors belonging to chemically different groups. All three HDAC inhibitors at the used drug concentrations were found to inhibit HDAC activity in the cells used in the present study (I). In the presence of HDAC inhibitors, dexamethasone and RU24858 did not inhibit LPS-induced NO production or iNOS expression (Figure 18).
Figure 18. Effects of dexamethasone (1 µM) and dissociated steroid RU24858 (1 µM) on LPS (10 ng/ml)--induced NO production (A), iNOS protein expression (B) and on iNOS mRNA accumulation (C) in the presence of HDAC inhibitors in J774 macrophages. NO production was determined after 24 h incubation by measuring nitrite accumulation into the culture medium by Griess reaction (n=6, mean + SEM). iNOS protein expression was measured by Western blot after a 24 h incubation time (n=3, mean + SEM). RNA was extracted after 12 h incubation. iNOS mRNA was measured by quantitative RT-PCR, and results were normalized against GAPDH (n=3, mean + SEM). Levels in LPS-treated cells or LPS+HDAC inhibitor treated cells represented 100% and the other values were compared to that. ** p<0.01. (Reprinted with permission from: Hämäläinen et al. 2008, Pulmonary Pharmacology & Therapeutics 21: 331-339. ©Elsevier Ltd., modified)

A549/8-Luc cells were used to study the effects of dexamethasone and TSA on iNOS promoter activity. In these cells, a construct of full length human iNOS
promoter (16 kb) (de Vera et al. 1996) in front of a luciferase gene was introduced into the cells in a pXP2 plasmid to generate cells expressing the luciferase reporter gene under the control of the iNOS promoter. Cells were stimulated with IL-1β and iNOS promoter activity was assessed by measuring luciferase mRNA levels in the cells. In the presence of TSA, the inhibitory action of dexamethasone on iNOS promoter activity was abolished (Figure 19).

Figure 19. Effects of dexamethasone on IL-1β (10 ng/ml)-induced iNOS promoter activity in the absence and in the presence of TSA in A549/8-Luc cells. Mean±SEM, n=3, *p<0.05. (Reprinted with permission from: Hämäläinen et al. 2008, Pulmonary Pharmacology & Therapeutics 21: 331-339. ©Elsevier Ltd., modified)

4 Transcriptional regulation of iNOS expression and NO production by flavonoids (II)

Flavonoids are polyphenolic compounds with a spectrum of anti-inflammatory and other biological activities. In the present study we investigated the effects of 36 naturally occurring flavonoids and related compounds on iNOS expression and NO production in macrophages exposed to LPS (see Table 6).

The polyphenolic compounds used belonged to eight chemically different groups; flavones, isoflavones, flavonols, flavanones, anthocyanins, flavan-3-ols, hydroxybenzoic acids and hydroxycinnamic acids. First, all the compounds were tested at 10 and 100 µM concentrations to evaluate their possible effects on cell
viability in the XTT test under the experimental settings in use. Further experiments were only carried out with the non-toxic compounds.

Eight of the tested 36 compounds were found to inhibit LPS-induced NO production by more than 50% and were subjected to further experiments. The effective compounds were flavone, daidzein, genistein, isorhamnetin, kaempferol, quercetin, naringenin and pelargonidin (Figure 20).

All eight effective compounds were found to inhibit NO production and iNOS protein expression in a concentration-dependent manner (Figure 21). Flavonoids were found to inhibit NO production in the following order of potency: quercetin (IC$_{50}$ ~ 25 µM) ~ kaempferol (IC$_{50}$ ~ 25 µM) > genistein (IC$_{50}$ ~ 30 µM) ~ isorhamnetin (IC$_{50}$ ~ 30 µM) > flavone (IC$_{50}$ ~ 40 µM) >
daidzein (IC$_{50}$ ~ 70 µM) > naringenin (IC$_{50}$ ~ 80 µM) > pelargonidin (IC$_{50}$ ~ 90 µM).

**Figure 21.** Effects of increasing concentrations of (A) flavone, daidzein and genistein, (B) quercetin, isorhamnetin and kaempferol, and (C) naringenin and pelargonidin on LPS-induced NO production and iNOS expression in J774 cells. NO production after 24 h incubation was determined by measuring nitrite accumulation into the culture medium by Griess reaction (n=6, mean ± SEM). iNOS protein expression was measured by Western blot after a 24 h incubation. Values in LPS-treated cells represented 100% and the other values were compared to that. Mean ± SEM, n=3, **p<0.01. (Reprinted with permission from: Hämäläinen et al. 2007, Mediators of Inflammation, doi: 10.1155/2007/45673 Hindawi Publishing Corporation, modified)

In the subsequent studies, the effects of the eight compounds (flavone, daidzein, genistein, isorhamnetin, kaempferol, quercetin, naringenin and pelargonidin) which had been found to inhibit NO production and iNOS protein expression
were investigated by quantitative real-time RT-PCR for the abilities to change iNOS mRNA expression. All the eight compounds inhibited also iNOS mRNA expression in macrophages activated by LPS (Figure 22).

\[ \text{Figure 22. Effects of flavonoids (100 \, \mu M) on LPS (100 ng/ml) -induced iNOS mRNA accumulation in J774 macrophages. RNA was extracted after 6 h incubation, and iNOS and GAPDH mRNA levels were measured by quantitative real-time RT-PCR. iNOS mRNA levels were normalized against GAPDH levels. Levels in LPS-treated cells were set to represent 100\% and the other values were related to that. Mean + SEM, n=3, **p<0.01. (Reprinted with permission from: Hämäläinen et al. 2007, Mediators of Inflammation, doi: 10.1155/2007/45673 Hindawi Publishing Corporation)} \]

To study the effects of flavonoids on the activation of transcription factors NF-κB and STAT-1 (which are critical for transcription of iNOS), the cells were incubated with the compounds of interest for 30 min (NF-κB) or for 6 h (STAT-1). Thereafter nuclear proteins were extracted and the nuclear translocation of the NF-κB p65 and STAT-1α was studied by Western blot. The nuclear translocation of p65 and STAT-1α was enhanced by LPS. All eight compounds that were found to inhibit iNOS expression (flavone, daidzein, genistein, isorhamnetin, kaempferol, quercetin, naringenin and pelargonidin) inhibited also LPS-induced NF-κB activation. In addition, daidzein, genistein, kaempferol and quercetin inhibited also the activation of STAT-1 (Figure 23).
Actinomycin D assay was performed to study the effect of flavonoids on iNOS mRNA stability. After 6 h incubation with the compounds of interest, actinomycin D was added to inhibit further transcription, and to estimate the decay of iNOS mRNA, the mRNA levels were measured before and 6 h after actinomycin D. As seen in Figure 24, daidzein and isorhamnetin had some effect...
on iNOS mRNA decay whereas the other compounds tested were virtually ineffective (Figure 24).

**Figure 24.** Effects of flavonoids on iNOS mRNA decay in LPS-treated J774 macrophages in the presence of an inhibitor of transcription (actinomycin D). Cells were treated with LPS or with LPS and flavonoid (100 μM). Actinomycin D was added to the cells 6 h after LPS. RNA was extracted before and 6 h after addition of actinomycin D. iNOS and GAPDH mRNAs were measured by quantitative real-time RT-PCR. iNOS mRNA levels were normalised against GAPDH. Normalised iNOS mRNA levels at 6 h represent 100% and other values were related to that. Mean ± SEM, n=3.

The effects on iNOS expression and NO production as well as on the activation of NF-κB and STAT-1 of all phenolic compounds used in the present study are summarized in Table 7.
<table>
<thead>
<tr>
<th>Class</th>
<th>NO production inhibition %</th>
<th>iNOS protein inhibition %</th>
<th>iNOS mRNA inhibition %</th>
<th>iNOS mRNA stability</th>
<th>NF-κB activity</th>
<th>STAT-1 activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>[10 µM]</td>
<td>[100 µM]</td>
<td>[10 µM]</td>
<td>[100 µM]</td>
<td>[100 µM]</td>
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<tr>
<td>LPS 100 ng/ml</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Flavones</td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Flavones Apigenin</td>
<td>48.2 ± 2.1</td>
<td>toxic&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
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<td></td>
<td>29.1 ± 1.6</td>
<td>toxic</td>
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<tr>
<td></td>
<td>3.1 ± 1.7</td>
<td>38.6 ± 1.1</td>
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<tr>
<td></td>
<td>35.6 ± 1.0</td>
<td>toxic</td>
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<tr>
<td>Flavones Flavone</td>
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<td>-1.9 ± 0.8</td>
<td>6.2 ± 0.7</td>
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<tr>
<td>Isoflavones</td>
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<tr>
<td>Isoflavones Daidzein</td>
<td>11.3 ± 6.3</td>
<td>70.3 ± 3.1</td>
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<td>63.3 ± 6.1</td>
<td>44.8 ± 5.3</td>
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<td>11.6 ± 2.4</td>
<td>97.4 ± 0.2</td>
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<tr>
<td>Flavonols Quercetin</td>
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<td>89.7 ± 0.3</td>
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<td>91.4 ± 0.4</td>
<td>97.2 ± 0.1</td>
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<td></td>
<td>6.0 ± 0.7</td>
<td>toxic</td>
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<td>3.5 ± 0.9</td>
<td>65.1 ± 1.6</td>
<td>6.2 ± 1.3</td>
<td>40.1 ± 9.5</td>
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<td>8.4 ± 2.2</td>
<td>99.6 ± 0.2</td>
<td>42.5 ± 4.1</td>
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<td>4.8 ± 1.3</td>
<td>40.8 ± 2.4</td>
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<td>5.1 ± 0.8</td>
<td>19.1 ± 0.8</td>
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<tr>
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<td>-2.2 ± 2.0</td>
<td>8.7 ± 1.0</td>
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<tr>
<td>Flavanones</td>
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<tr>
<td>Flavanones Taxifolin</td>
<td>3.1 ± 1.8</td>
<td>23.9 ± 1.8</td>
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<td></td>
<td>1.9 ± 2.5</td>
<td>3.9 ± 2.1</td>
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<td></td>
<td>14.7 ± 1.3</td>
<td>59.6 ± 3.3</td>
<td>-0.1 ± 10.0</td>
<td>54.9 ± 1.13</td>
<td>48.4 ± 4.7</td>
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<tr>
<td></td>
<td>5.9 ± 1.9</td>
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<td>-0.1 ± 1.0</td>
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<td>Anthocyanins</td>
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<tr>
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<tr>
<td>HBA&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>Methyl gallate</td>
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<td></td>
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<td>30.5 ± 1.0</td>
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<tr>
<td>HCA&lt;sup&gt;c&lt;/sup&gt;</td>
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<tr>
<td>Chlorogenic acid</td>
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<td>-0.9 ± 2.0</td>
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</tr>
</tbody>
</table>

<sup>a</sup> If inhibition was >50%, the compound was studied further
<sup>b</sup> If mitochondrial dehydrogenase activity was lowered by more than 20% compared to cells treated with the stimulant only, the compound was regarded as toxic
<sup>c</sup> HBA = hydroxybenzoic acid
<sup>d</sup> HCA = hydroxycinnamic acid
<sup>e</sup> no effect
5 Post-transcriptional regulation of iNOS expression by calcineurin inhibitors (III and IV)

Three calcineurin inhibitors CsA, tacrolimus and pimecrolimus, are immunosuppressive drugs used in the treatment of rheumatoid arthritis and related inflammatory diseases, inflammatory skin disorders like psoriasis and atopic dermatitis, and in the prevention of allograft rejection. To study if calcineurin inhibitors have effect on NO production, the cells were treated with LPS or LPS/IFNγ in the presence or absence of a calcineurin inhibitor. Calcineurin inhibitors were found to inhibit NO production in a concentration-dependent manner (Figure 25A and B). Expression of iNOS protein was also inhibited by calcineurin inhibitors (Figure 25C).
Figure 25. Effects of increasing concentrations of CsA, tacrolimus (FK-506) and pimecrolimus (A) on LPS-induced NO production in J774 macrophages, (B) on LPS/IFNγ-induced NO production in L-929 fibroblasts, and (C) on LPS-induced iNOS protein expression in J774 macrophages. NO production was detected after 24 h incubation by measuring nitrite accumulation into the culture medium by Griess reaction (n=6; mean ± SEM, * p<0.05, ** p<0.01). iNOS protein expression was measured by Western blot after a 24 h incubation (n=3; mean ± SEM). Levels at LPS or LPS/IFNγ stimulated cells represent 100% and the other values were related to that. (Reprinted with permission from: Hämäläinen et al., International Immunopharmacology, in press, modified. ©Elsevier Ltd)

When calcineurin inhibitors were added into the cell culture at different time-points following LPS-treatment, the accumulation of nitrite was inhibited at different levels depending on the time-point. If drugs were added 1 h prior, at the same time or up to 6-8 h post LPS treatment, then the inhibitory action was present, whereas at later time-points it declined (IV).

The nuclear translocation of the transcription factors NF-κB and STAT-1 were measured by Western blot, and NF-κB activity was measured also by the electrophoretic mobility shift assay (EMSA). Cells were incubated with the compounds of interest and then nuclear proteins were extracted. The maximal nuclear translocation of the p65 subunit of NF-κB was detected after 30 min incubation, and the translocation of the STAT-1α was increased up to 6 h incubation. Following LPS challenge, the nuclear translocations of both p65 subunit and STAT-1α were greatly enhanced as measured by Western blot. Treatment with CsA, tacrolimus or pimecrolimus (each at 10 µM) had no inhibitory effect on the nuclear translocation of p65 or STAT-1α (Figure 26A and B).

LPS-induced NF-κB activation and DNA binding activity in the absence or in the presence of calcineurin inhibitors was measured after 30 min incubation by EMSA. In unstimulated cells, a low basal NF-κB activity was detected, which was enhanced by LPS. Treatment of cells with calcineurin inhibitors had no effect of LPS-induced NF-κB activation or DNA binding as measured by EMSA (Figure 26C).
Figure 26. A Effects of CsA, tacrolimus (FK-506) and pimecrolimus on LPS-induced NF-κB activation determined as nuclear translocation of the p65 subunit of NF-κB. J774 cells were incubated for 30 min with LPS (10 ng/ml) or with LPS and calcineurin inhibitor (10 μM), and nuclear proteins were extracted. Western blot was used to detect the p65 subunit of NF-κB in the nuclear extracts. p65 levels in LPS-treated cells represent 100% and the other values were related to that (mean + SEM, n=3). B Effects of CsA, FK-506 and pimecrolimus on LPS-induced STAT-1 activation determined as nuclear translocation of STAT-1α. J774 cells were incubated for 6 h with LPS (10 ng/ml) or with LPS and calcineurin inhibitor (10 μM), and nuclear proteins were extracted. Western blot was used to detect STAT-1α in the nuclear extracts. STAT-1α levels in LPS-treated cells represent 100% and the other values were related to that (mean + SEM, n=3). C Effects of CsA, FK-506 and pimecrolimus on LPS-induced NF-κB activation and DNA binding as measured by EMSA. J774 cells were incubated for 30 min with LPS (10 ng/ml) or with LPS and calcineurin inhibitor (10 μM), and nuclear proteins were extracted. Activation of NF-κB was assessed by measuring the nuclear proteins capable of binding the nucleotide containing consensus NF-κB – binding sequences in DNA. Mean + SEM, n=3. (Reprinted with permission from: Hämäläinen et al., International Immunopharmacology, in press, modified. ©Elsevier Ltd)

The effects of calcineurin inhibitors on NF-κB and STAT-1 mediated transcription were studied by using L-929 pNF-κB and pGAS reporter cell lines
where the luciferase reporter gene is under the control of a NF-κB responsive promoter and under the control of a GAS promoter, respectively. Transcriptional activity of both NF-κB and GAS promoters was enhanced by LPS/IFNγ. Treatment with CsA or tacrolimus had no effect on NF-κB and STAT-1 mediated transcription (Figure 27).

![Image of Figure 27](image)

**Figure 27. A Effects of CsA and tacrolimus (FK-506) on NF-κB mediated transcription in LPS/IFNγ treated L-929 pNF-κB reporter cell line. B Effects of CsA and FK-506 on STAT-1 mediated transcription in LPS/IFNγ treated L-929 pGAS reporter cell line.**

Cells were treated with LPS/IFNγ or with LPS/IFNγ and calcineurin inhibitor (10 μM). RNA was extracted after 3 h incubation. LUC mRNA levels were normalized against GAPDH mRNA in each sample. LUC mRNA levels in LPS/IFNγ-treated cells represent 100% and other values were related to that. **p<0.01, mean ± SEM, n=3. (Reprinted with permission from: Hämäläinen et al., International Immunopharmacology, in press, modified. ©Elsevier Ltd)**

Accumulation of iNOS mRNA after treatment with calcineurin inhibitors was measured after shorter (6 and 8 h) and longer (24 h) incubation times. In J774 macrophages, calcineurin inhibitors had no effect on iNOS mRNA when measured after 6 h incubation but a clear reduction was found after a 24 h incubation (Figure 28A). In L-929 fibroblasts, calcineurin inhibitor had no inhibitory action when measured after 8 h incubation whereas significant inhibition was seen after 24 h incubation (Figure 28B). When iNOS mRNA levels were measured over time period of 6 to 24 hours after addition of LPS, iNOS mRNA levels were noted to decline more rapidly in the presence of calcineurin inhibitors (Figure 28C). When LPS-stimulated cells with or without calcineurin inhibitors were incubated in the presence of actinomycin D, no effects on iNOS mRNA levels were seen (Figure 28D).
Figure 28. A Effects of CsA, tacrolimus (FK-506) and pimecrolimus on LPS-induced iNOS mRNA levels in J774 macrophages. Cells were treated with LPS or with LPS and a calcineurin inhibitor (10 µM). RNA was extracted after 6 and 24 h of incubation. B Effects of CsA on LPS/IFNγ-induced iNOS mRNA levels in L-929 fibroblasts. Cells were treated with LPS and IFNγ (10 ng/ml) or with LPS/IFNγ and a calcineurin inhibitor (10 µM). RNA was extracted after 8 and 24 h of incubation. Normalised iNOS mRNA levels in LPS or LPS/IFNγ-treated cells represent 100% and other values at the same time-point were related to that. * p<0.05, ** p<0.01, mean + SEM, n=3-6. 

C Effects of CsA, FK-506 and pimecrolimus on LPS-induced iNOS mRNA decay in J774 macrophages. Cells were incubated with LPS or with LPS and a calcineurin inhibitor (10 µM) and total RNA was extracted at the time-points indicated. Normalised iNOS mRNA levels at 6 h represent 100% and other values were related to that. Mean + SEM, n=3. D Effects of CsA, FK-506 and pimecrolimus on LPS-induced iNOS mRNA decay in J774 macrophages in the presence of inhibitor of transcription (actinomycin D). Cells were treated with LPS or with LPS and a calcineurin inhibitor (10 µM). Actinomycin D was added to the cells 6 h after LPS. Thereafter RNA was extracted at the time-points indicated. Normalised iNOS mRNA levels at 6 h represent 100% and other values were related to that. Mean + SEM, n=3. (Reprinted with permission from: Härmäläinen et al., International Immunopharmacology, in press, modified. ©Elsevier Ltd)
Cells were transfected with a vector containing the 3’UTR of iNOS to study if this region is implicated in the regulation of iNOS expression by calcineurin inhibitors. At 24 h after transfection, L-929 cells were treated with LPS/IFNγ or with LPS/IFNγ and CsA, and luciferase activity in the cell lysates was measured. The luciferase activity was reduced in cells treated with LPS/IFNγ and CsA by 37% as compared to cells treated with LPS/IFNγ only, suggesting that iNOS 3’UTR is involved in the mechanisms by which calcineurin inhibitors regulate iNOS expression.

6 Summary of results (I-IV)

In the present study, several anti-inflammatory compounds were studied for their effects on NO production and iNOS expression. NO production and iNOS expression have been shown to be elevated in several inflammatory disorders and NOS inhibitors have been shown to have beneficial effects in many experimental models of inflammation (Vallance and Leiper 2002, Tinker and Wallace 2006).

The glucocorticoid, dexamethasone, and a dissociated steroid RU24858, flavonoids flavone, daidzein, genistein, isorhamnetin, kaempferol, quercetin, naringenin and pelargonidin, and calcineurin inhibitors CsA, tacrolimus and pimecrolimus were shown to inhibit iNOS expression and NO production in a concentration-dependent manner. More specific mechanisms of action for each subset of compounds were also investigated. Glucocorticoids were proposed to inhibit NO production and iNOS expression by a mechanism involving histone deacetylation. Flavonoids were found to inhibit the activation of transcription factor NF-κB and some of the compounds inhibited also activation of STAT-1 along with their inhibitory effect on iNOS expression and NO production. The activation of these two transcription factors is known to be a prerequisite for iNOS expression. The calcineurin inhibitors CsA, tacrolimus and pimecrolimus appeared to exert their effect on NO production and iNOS expression via destabilizing iNOS mRNA. The results are summarized in Figure 29.
Figure 29. Summary of the results obtained in the present study
1 Methodology

In these studies, immortalized cell lines were used to study NO production and iNOS expression. Cells were passaged up to 20 times, and their growing conditions were standardized and monitored. Furthermore, variations due to technical staff, cell culture medium and its supplements, cell culture plastics etc. were minimized. No infections by mycoplasma were detected.

Nitric oxide produced by cells was measured as nitrite accumulated in the cell culture medium by the Griess reaction (Green et al. 1982). Nitrite is the stable end product of NO in aqueous solution, and can be used to estimate the amount of NO which has been produced (Laurent et al. 1996). The amount of nitrite in the sample was calculated against a standard curve prepared in the same cell culture medium as used in the experiment. NOS/iNOS inhibitors were included to ensure that the measured nitrite was due to NO produced by the NOS/iNOS pathway.

Standard methods in molecular and cellular biology were used for the detection of protein and mRNA expression. In Western blot, the amount of protein loaded was controlled by measuring the protein content of each sample and by using loading controls, i.e. actin for whole cell proteins and laminA/C for nuclear extracts. Proteins were detected with specific antibodies, and visualized by a chemiluminescent reaction. Quantitative real-time RT-PCR was used to detect mRNA expression of iNOS and luciferase genes. GAPDH was used as a housekeeping gene, since its amount was not affected by different treatments. The relative amounts of iNOS and luciferase mRNA were the parameters of interest.

The actinomycin D assay was utilized to estimate the rate of mRNA degradation. However, actinomycin D may affect the stability of some mRNAs (Chen et al. 1995, Seiser et al. 1995, Dixon et al. 2000). This might be mediated by the inhibition of the transcription of a gene needed to regulate the mRNA stability or by a mechanism unrelated to the inhibition of the transcription. Taking into account these possible drawbacks of the actinomycin D assay, transient transfection of cells with a construct containing the 3’UTR of iNOS gene was also used to estimate the effects on iNOS mRNA decay in article IV.

The signaling cascade leading to the activation of transcription factor NF-κB originates from the activation of Toll/IL-1 receptor. Receptor activation leads to the activation of IKK and subsequent dissociation of IκB, which enables the
nuclear translocation and DNA binding of NF-κB subunits. IFNγ stimulation leads to the activation of JAKs, which subsequently activate the cytosolic components of the receptor, which in turn activate STAT-1, another transcription factor important for iNOS. When it forms a dimer, the activated STAT-1 can translocate to the nucleus where it binds to and activates GAS.

Different methods were used to measure the activation of NF-κB and STAT-1. Nuclear translocations of the p65 subunit of NF-κB and STAT-1α were measured by using Western blot. Nuclear translocation and DNA binding activity of NF-κB were measured by the electrophoretic mobility shift assay (EMSA). NF-κB and STAT-1 transcriptional activity was measured by using cells where the luciferase reporter gene was under the control of NF-κB and GAS promoter, respectively. EMSA has advantages over Western blot of nuclear extracts when one wishes to study nuclear translocation. EMSA measures the specific protein-DNA interaction in addition to nuclear translocation of the transcription factor. It is regarded as a highly sensitive method when radioisotope-labeled nucleic acids are used. The drawbacks of this method are that the amounts of protein/nucleic acid complex are not constant during electrophoresis, and it is difficult to estimate the appropriate exposure time for visualization in the radioautography (Hellman and Fried 2007). With the reporter constructs, it is possible to study effects on the functional activity of the transcription factors. All of the above described three methods are useful when the activation of transcription factors needs to be estimated. With respect to these three methods, reporter constructs may be regarded as the best way to estimate the actual impact on transcription even though transfection and subsequent incorporation of a vector into the cell may alter cellular behaviour to some extent.

Results (e.g. the degree of inhibition) obtained with different methods cannot be directly correlated with each other since methodologically, these techniques used are not readily comparable. For example it is not known how much inhibition at the level of a certain transcription factor is needed to achieve a particular degree of inhibition at the level of iNOS mRNA. Measurement of nitrite is linear whereas this is not the case in Western blot, with the product of the antigen-antibody reaction. The amount of PCR product becomes exponentially increased with increasing numbers of thermal cycles in the polymerase chain reaction. Furthermore, species and cell type dependent variations also exist.

2 NO production and iNOS expression by macrophages

Nitric oxide production has been shown to be induced by different stimuli in different cells, e.g. by LPS and IFNγ in murine and bovine macrophages (Lorsbach et al. 1993, Lowenstein et al. 1993, Adler et al. 1995), and by a
mixture of cytokines in human DLD-1 and A549 epithelial cells (Asano et al. 1994, Laubach et al. 1997, Linn et al. 1997). Human macrophages have been shown to express iNOS in vivo and ex vivo, but when one tries to culture these cells this capability is quickly lost (Moilanen et al. 1997). After stimulation of human iNOS, the levels of iNOS mRNA were increased by up to 20-fold though there was only 2-3 fold increase detected on promoter activity emphasizing the importance of the regulation of the iNOS mRNA stability (Linn et al. 1997). Transcriptional activation seemed to account for the most of the response to the stimulus in murine iNOS (Lowenstein et al. 1993).

In the present study, NO production and iNOS expression in murine J774 macrophages were induced by LPS. Treatment of cells with LPS evokes the activation of TLR/IL-1 signaling cascade which results in the activation of transcription factors NF-κB and STAT-1 which are both important for iNOS expression. The levels of iNOS mRNA peaked at 6 h, whereas maximal iNOS protein levels were detected 24 h after LPS. Transient induction of iNOS expression in spite of a continuous presence of stimulation has been reported also earlier, and has been shown to require de novo protein synthesis (Evans et al. 1994, Linn et al. 1997).

3 Effects of anti-inflammatory compounds on NO production

Several anti-inflammatory compounds were studied for their ability to inhibit NO production in activated macrophages. The glucocorticoid, dexamethasone, and a dissociated steroid RU24858, the calcineurin inhibitors CsA, tacrolimus and pimecrolimus, and a subset of phenolic compounds were found to inhibit NO production and iNOS expression, and will be further discussed below.

The anti-rheumatic drugs, sulfasalazine, 5-aminosalicylic acid and methotrexate, had only a minor effect on NO production even when tested at relatively high drug concentrations. High concentrations (up to 5 mM) of sulfasalazine have been reported to inhibit NF-κB activity in human colonic epithelial cells (Wahl et al. 1998). The concentrations used in the present study were lower (100 μM) but still higher than the concentrations achieved during drug treatment in vivo. In the present study, sulfasalazine was found to inhibit NO production only marginally in activated macrophages, thus probably not affecting NF-κB activity in our experimental conditions. 5-Aminosalicylic acid is a component of sulfasalazine which is used in the treatment of inflammatory bowel disease. 5-ASA (100 μM) was found to cause less than 20% inhibition of NO production. It has earlier been shown to inhibit iNOS expression in human epithelial cells, but the concentrations used in that study were in the millimolar range (Kennedy et al. 1999). Methotrexate inhibits the reduction of dihydrofolate to tetrahydrofolate, a compound needed for the synthesis of tetrahydrobiopterin (BH4), which is a cofactor for iNOS (Alderton et al. 2001). The synthesis of BH4
did not seem to be rate-limiting in our experimental setting because methotrexate had no effect on NO production. In addition, TNFα did not seem to be a critical mediator of the LPS-induced iNOS expression and NO production in murine macrophages since the TNFα blockers, infliximab and etanercept, had only a slight inhibitory effect on LPS-induced NO production.

4 Regulation of iNOS expression and NO production by glucocorticoids

Histone deacetylation has been implicated in the mechanisms of the anti-inflammatory action of glucocorticoids. In IL-1β stimulated epithelial cells, GM-CSF production was inhibited by glucocorticoids in a histone deacetylation dependent manner (Ito et al. 2000). Ligand activated glucocorticoid receptor was shown to inhibit HAT activity associated with the coactivator complex, and to recruit HDAC2 to the site of transcription. Acetylation of specific lysine residues K8 and K12 in histone H4 has been reported to be repressed (Ito et al. 2000). Glucocorticoids have also been found to reduce the TNFα-induced acetylation of lysine residues K8 and K12 in H4 in macrophages (Tsaprouni et al. 2002). The transcriptional activity of the TNFα-induced eotaxin promoter was associated with acetylation of H4 lysine residues K5 and K12, which was inhibited by glucocorticoids (Nie et al. 2005). Histone deacetylation has also been implicated in the down-regulation of IL-5 (Jee et al. 2005) and TTP (Jalonen et al. 2005) expression by glucocorticoids.

In the present study, the glucocorticoid, dexamethasone, and a dissociated steroid, RU24858, were found to inhibit iNOS expression and NO production in a concentration-dependent manner in activated macrophages. Dexamethasone and RU24858 had no effect on NO production in the presence of the glucocorticoid receptor antagonist, mifepristone, suggesting that the process is GR mediated. The inhibitory action of glucocorticoids was also abolished in the presence of histone deacetylase inhibitors TSA, apicidin and MC1293. Transcriptional activation of the iNOS promoter was measured in epithelial cells. IL-1β-induced transcriptional activation was inhibited by dexamethasone and the inhibition was absent in the presence of TSA. These results suggest that histone deacetylation is involved in the mechanisms by which glucocorticoids inhibit iNOS expression and NO production. These results do not exclude the possibility that deacetylation of other targets may play a role in the regulation of iNOS expression and NO production by anti-inflammatory steroids. For example the acetylation status of GR has been shown to affect the anti-inflammatory activity of glucocorticoids (Ito et al. 2006b).

In several cell types, NO production is sensitive to glucocorticoids (Di Rosa et al. 1990, Radomski et al. 1990, Geller et al. 1993, de Vera et al. 1996, Kleinert et al. 1996) but in others e.g. chondrocytes or in human cartilage, NO production is not inhibited by glucocorticoids (Grabowski et al. 1996b, Vuolteenaho et al.
The molecular mechanisms by which glucocorticoids have been suggested to inhibit iNOS expression and NO production are summarized in Table 8. A role for lipocortin-1 (aka annexin-1) has also been postulated but the results are somewhat contradictory (D’Acquisto et al. 1997, Bryant et al. 1998, Smyth et al. 2006).

**Table 8. Proposed mechanisms how glucocorticoids inhibit iNOS expression and NO production**

<table>
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<td><strong>Induction of arginase II</strong></td>
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<td>mouse</td>
<td>LPS</td>
<td>(Gotoh et al. 1996)</td>
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<tr>
<td><strong>Inhibition of BH₄ synthesis and L-arginine transport</strong></td>
<td>CMEC</td>
<td>rat</td>
<td>IL-1β/IFNγ</td>
<td>(Simmons et al. 1996)</td>
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<tr>
<td><strong>Inhibition of NF-κB</strong></td>
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<td>mouse</td>
<td>GM-CSF</td>
<td>(Vital et al. 2003)</td>
</tr>
<tr>
<td></td>
<td>macrophages</td>
<td>mouse</td>
<td>LPS</td>
<td>(Jeon et al. 1998)</td>
</tr>
<tr>
<td></td>
<td>hepatocytes</td>
<td>rat</td>
<td>TNFα/IL-1β/IFNγ</td>
<td>(de Vera et al. 1997)</td>
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<td>VSMC</td>
<td>rat</td>
<td>IL-1β</td>
<td>(Katsuyama et al. 1999)</td>
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<tr>
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<td>epithelial cells</td>
<td>human</td>
<td>TNFα/IL-1β/IFNγ</td>
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<td>rat</td>
<td>LPS/IFNγ</td>
<td>(Matsumura et al. 2001)</td>
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<td>mesangial cells</td>
<td>rat</td>
<td>LPS/TNFα</td>
<td>(Saura et al. 1998)</td>
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<tr>
<td><strong>Histone deacetylation</strong></td>
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<td>mouse</td>
<td>LPS</td>
<td>(Hamalainen et al. 2008)</td>
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<td><strong>Decreased iNOS mRNA stability</strong></td>
<td>macrophages</td>
<td>mouse</td>
<td>LPS</td>
<td>(Korhonen et al. 2002)</td>
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<tr>
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<td>mouse</td>
<td>LPS/IFNγ</td>
<td>(Soderberg et al. 2007b)</td>
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<tr>
<td><strong>Decreased iNOS protein stability</strong></td>
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<td>mouse</td>
<td>LPS/IFNγ</td>
<td>(Walker et al. 1997)</td>
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<tr>
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<td>mesangial cells</td>
<td>rat</td>
<td>IL-1β</td>
<td>(Kunz et al. 1996)</td>
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</table>

BH₄=tetrahydrobiopterin, CMEC=cardiac microvascular endothelial cells, VSMC=vascular smooth muscle cells

Results from the present study suggest that in addition to the mechanisms described, histone deacetylation might also be involved in mediating the inhibitory action of glucocorticoids on iNOS expression and NO production.
5 Regulation of iNOS expression and NO production by flavonoids

In the present study, the effects of 36 naturally occurring phenolic compounds on iNOS expression and NO production were studied. Eight out of the 36 compounds were found to inhibit NO production in activated macrophages. These eight flavonoids were flavone, daidzein, genistein, isorhamnetin, kaempferol, quercetin, naringenin and pelargonidin. The classes of phenolic compounds to which they belong are flavones (flavone), isoflavones (daidzein and genistein), flavonols (isorhamnetin, kaempferol and quercetin), flavanones (naringenin), and anthocyanins (pelargonidin).

Some evidence about the inhibitory action of flavonoids on NO production had been reported before the onset of the present study, and more accumulated during the course of this study. The previous literature on the effects on iNOS expression and NO production of the compounds further investigated in the present study is summarized in Table 9.

In the present study, flavone, daidzein, genistein, isorhamnetin, kaempferol, quercetin, naringenin and pelargonidin inhibited NO production and iNOS expression in a concentration-dependent manner. All of these compounds inhibited the activation of NF-κB as measured by the nuclear translocation of p65. In addition, genistein, kaempferol, quercetin and daidzein were shown to inhibit the activation of STAT-1. Daidzein and isorhamnetin were also shown to have some effects on iNOS mRNA decay which may have contributed to their anti-inflammatory properties.

Daidzein and genistein are isoflavones, and these compounds were shown to inhibit the activations of NF-κB and STAT-1, and expression of iNOS and production of NO. Both NF-κB and STAT-1 have been shown to be important transcription factors for iNOS (Xie et al. 1994, Gao et al. 1997), and two JAK inhibitors, AG-490 and WHI-P154, have been reported to inhibit iNOS expression in addition to inhibiting STAT-1 activation (Salonen et al. 2006, Sareila et al. 2006). The results from the present study confirm earlier data on the inhibitory effect of daidzein and genistein on iNOS expression and NO production, and provide further clarification of their mechanisms of action i.e. inhibition of the transcription factors NF-κB and STAT-1.

In terms of NO inhibition, flavonols were the most effective group of flavonoids in this study. Isorhamnetin, kaempferol, and quercetin effectively inhibited NO production and iNOS expression, with quercetin and kaempferol being the most potent phenolic compounds tested. Quercetin, a molecule derived from rutin after hydrolysis by glucosidases in the gastrointestinal tract, has been studied previously but only a few proposals of the mechanisms of action have been made (Table 9).
Table 9. Flavonoids shown to inhibit iNOS expression and/or NO production. A further mechanism of action is indicated if reported.

<table>
<thead>
<tr>
<th>Flavonoid</th>
<th>Cell type and stimulus</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flavone</td>
<td>macrophage, LPS</td>
<td>(Kim et al. 1999, Blonska et al. 2004)</td>
</tr>
<tr>
<td>Daidzein</td>
<td>macrophages, LPS, LPS/IFNγ</td>
<td>(Krol et al. 1995, Scuro et al. 2004)</td>
</tr>
<tr>
<td></td>
<td>inhibition of NF-κB</td>
<td>(Kim et al. 2005)</td>
</tr>
<tr>
<td></td>
<td>inhibition of NF-κB</td>
<td>(Banerjee et al. 2002)</td>
</tr>
<tr>
<td>Pelargonidin</td>
<td>macrophage, LPS</td>
<td>(Wang and Mazza 2002)</td>
</tr>
</tbody>
</table>

Quercetin has been found to inhibit IFNγ-induced STAT-1 activation in mouse BV-2 microglia (Chen et al. 2005). In the present study, quercetin was found to suppress LPS-induced STAT-1 activation in mouse macrophages, confirming the earlier finding and extending it to different cells under different stimulation. Kaempferol was found to inhibit iNOS expression and NO production, and to inhibit the activation of the transcription factors STAT-1 and NF-κB. Here it is reported for the first time that isorhamnetin inhibits NO production and iNOS expression by a mechanism likely involving inhibition of NF-κB activation.

According to earlier results, the flavanone, naringenin, was found to inhibit NO production, whereas its glycosylated counterpart naringin had no effect (Lin et al. 2005). In this study, inhibition of NF-κB is believed to mediate its
inhibitory effect on iNOS expression and NO production. The effects of anthocyanin pelargonidin on NO production have been studied in macrophages (Wang and Mazza 2002) but no further mechanistical studies were carried out. The present study confirms and extends the earlier data by revealing that pelargonidin inhibits the activation of NF-κB which is suggested to lead to the suppressed iNOS expression and NO production detected.

With respect to the structure-toxicity and structure-activity relationships, several observations were made during the present study. Glycoside moieties as substituents seemed to be important in conferring non-toxicity. Luteolin was found to be toxic when used at 100 µM concentration but the derivative luteolin-7-glucoside was not toxic. The same phenomenon was seen with apigenin and vitexin. Apigenin was toxic in our experimental conditions but vitexin, an 8-C-glucoside of apigenin, was non-toxic. Three main conclusions can be drawn from the structure-activity relationships of the compounds inhibiting NO production: a) a C-2,3 double bond is a common feature in the six most effective compounds, b) a bulky group (e.g. glycoside, rhamnoside, rutinoside or neohesperidoside) as a substituent lowered or abolished the inhibitory effect of the compound (e.g. quercetin was highly effective whereas its rhamnoside-substituted derivative quercitrin had no inhibitory effect on NO production), and c) 7 and 4’ OH-groups were found in all effective compounds except flavone, though they were present also in some ineffective compounds (Figure 30). The importance of C-2,3 double bond and the glycoside moiety effect has been earlier demonstrated in RAW264.7 macrophages and in mouse peritoneal macrophages, respectively, with different types of flavonoids (Kim et al. 1999, Matsuda et al. 2003). In primary bone marrow derived mouse macrophages, the C-2,3 double bond and B-ring position were recognized as structural features important for the inhibition of LPS-induced NO release (Comalada et al. 2006).

![Basic structure of flavonoid](image1.png)

![Quercetin](image2.png)

**Figure 30.** Basic structure of flavonoid and the chemical structure of quercetin. Features in the structures of the effective compounds in the present study are highlighted in the structure of quercetin.
Regulation of iNOS expression and NO production by calcineurin inhibitors

The calcineurin inhibitors cyclosporin A, tacrolimus and pimecrolimus were found to inhibit iNOS expression and NO production in activated macrophages and fibroblasts. No effect on the activation of transcription factors NF-κB and STAT-1 was seen after treatment with these drugs. iNOS mRNA levels were reduced at later time-points after addition of inflammatory stimuli when cells were treated with the calcineurin inhibitors. These results suggest that CsA, tacrolimus and pimecrolimus had not inhibited the early transcriptional events of iNOS expression but instead may have accelerated the degradation rate of iNOS mRNA. Studies with cells transfected with a construct containing the 3’UTR of iNOS mRNA provided further evidence that calcineurin inhibitors mediate their effect on iNOS expression and NO production by destabilizing iNOS mRNA.

CsA and/or tacrolimus have been earlier shown to inhibit NO production in different cell types e.g. macrophages (Conde et al. 1995, Hattori and Nakanishi 1995, Dusting et al. 1999), fibroblasts (Fast et al. 1993, Trajkovic et al. 1999b), hepatocytes (Muhl et al. 1993, Kunz et al. 1995, Kaibori et al. 1999), and vascular smooth muscle cells (Marumo et al. 1995). In some previously reported studies, CsA has been shown to be more potent than tacrolimus in inhibiting NO production, or tacrolimus had no inhibitory effect (Hattori and Nakanishi 1995, Marumo et al. 1995, Dusting et al. 1999, Attur et al. 2000), whereas in other studies, the situation was reversed (Kaibori et al. 1999, Strestikova et al. 2001). In the current study, CsA was found to be more potent than tacrolimus or pimecrolimus in inhibiting iNOS expression and NO production in activated macrophages and fibroblasts. Rapamycin, an immunosuppressant targeting mTOR (mammalian target of rapamycin) but not calcineurin, had no inhibitory effect on NO production indicating that calcineurin is the likely target to be mediating the effects of CsA, tacrolimus and pimecrolimus found in this study.

The immunosuppressive effect of calcineurin inhibitors in T-cells is mediated by inhibition of the activation of the transcription factor NF-AT. There is no evidence that the iNOS promoter would contain a binding site for NF-AT (Kleinert et al. 2004, Korhonen et al. 2005), and therefore other mechanisms were explored. It has been reported that NOS activity is inhibited by CsA and tacrolimus in peritoneal macrophages (Conde et al. 1995), but in our study the inhibitory effect of the calcineurin inhibitors declined when the drugs were added 6-12 h after LPS. This suggests that the inhibitory effect is not mediated by inhibition of activity of iNOS. Inhibition of iNOS expression by calcineurin inhibitors has been reported, and NF-κB signaling has been suggested to be the target of these drugs (Kim et al. 2004b). NF-κB DNA binding and luciferase κB reporter were inhibited by CsA in both peritoneal and RAW264.7 macrophages stimulated with LPS (Kim et al. 2004b). After 16 h of incubation, NF-κB binding was inhibited by CsA in IL-1β stimulated rat mesangial cells (Kunz et al. 1995), and tacrolimus inhibited the activation of NF-κB in rat hepatocytes.
(Kaibori et al. 1999, Tunon et al. 2003). In human intestinal microvascular cells stimulated by LPS/TNFα, no inhibition of NF-κB was found (Rafiee et al. 2002). These different results may reflect species, cell and stimulus dependent regulation, but also the methods used to measure the activation of NF-κB have varied between studies. Since NF-κB and STAT-1 are important transcription factors for iNOS, the effects of the calcineurin inhibitors on their activation were evaluated. In the present study, calcineurin inhibitors were not found to inhibit the nuclear translocation of either NF-κB or STAT-1 (measured by Western blot), the nuclear translocation and DNA binding activity of NF-κB (measured by EMSA), or the NF-κB or STAT-1 mediated transcription (measured with luciferase constructs).

All three calcineurin inhibitors, CsA, tacrolimus and pimecrolimus, were found to reduce iNOS mRNA levels at later time-point after LPS stimulation whereas no reduction was seen at an early time-point. This suggests that the drugs most probably affect the degradation of iNOS mRNA. The hypothesis was supported by the data from the time-curve of iNOS mRNA where LPS-induced iNOS mRNA degradation seemed to be accelerated in cells treated with calcineurin inhibitors as compared to cells treated with LPS alone. However, the phenomenon was not seen in the presence of actinomycin D, an inhibitor of transcription, when this compound was added after a 6 h incubation. This finding indicates that the synthesis of a factor necessary for the effects of the calcineurin inhibitors might be blocked by the addition of actinomycin D, and therefore no acceleration of degradation was observed. This is supported by findings that CsA and tacrolimus induced deadenylation and subsequent degradation of IL-3 mRNA (Nair et al. 1999), and the destabilization of IL-3 mRNA was dependent on ongoing transcription (Nair et al. 1994). Another possibility is that the calcineurin inhibitors suppress events needed for prolonged transcription of iNOS following LPS-stimulation, and those transcriptional events are inhibited by actinomycin D. The stability of some mRNAs has been shown to be directly affected by actinomycin D (Chen et al. 1995, Seiser et al. 1995, Dixon et al. 2000), and this might also explain the present results from the actinomycin D experiment. In the present study, cells transfected with a reporter construct containing the 3’UTR of iNOS mRNA were utilized to study the effect of the calcineurin inhibitors on the iNOS 3’UTR, a region known to be important for the regulation of the stability of iNOS mRNA. In activated cells treated with CsA, the amount of reporter was significantly lower than in cells treated with the stimulant alone. These results suggest that 3’UTR-dependent destabilization of iNOS mRNA is involved in the mechanisms by which calcineurin inhibitors down-regulate iNOS expression and NO production. Other potential mechanisms for the calcineurin inhibitors which were not investigated in the present study include altering the stability of iNOS protein, effect on transcription factor IRF-1, effect on MKP-1 and the role of TGFβ. The effects on mitogen activated protein kinases p38, Erk1/2 and JNK were studied, but calcineurin inhibitors were not observed to alter their activation.
7 Down-regulation of NO production and iNOS expression by anti-inflammatory compounds

In the present study, anti-inflammatory compounds were studied for their effects on iNOS expression and NO production in activated macrophages and fibroblasts. Glucocorticoids, flavonoids and calcineurin inhibitors were all found to inhibit NO production and iNOS expression, and different mechanisms of action were suggested.

The inhibitory effect of glucocorticoids was abolished in the presence of histone deacetylase inhibitors suggesting that histone deacetylation is involved in the mechanisms by which glucocorticoids inhibit NO production and iNOS expression. This route of inhibition targets the early transcriptional events involved in iNOS expression.

The present study suggests that inhibition of the activation of transcription factors NF-κB and/or STAT-1 mediates the inhibitory effects of the flavonoids flavone, daidzein, genistein, isorhamnetin, kaempferol, quercetin, naringenin and pelargonidin on both iNOS expression and NO production.

In contrast to the glucocorticoids and flavonoids, which were postulated to target early transcriptional events of iNOS expression, the calcineurin inhibitors were found to possess post-transcriptional effects. Down-regulation of iNOS expression and NO production by calcineurin inhibitors is believed to be mediated by enhanced iNOS mRNA decay.

In several inflammatory diseases, iNOS expression and NO production are increased, and NO can act as a proinflammatory and cytotoxic mediator. In experimental inflammatory models, NOS inhibitors have proven to be effective in attenuating the inflammation. The results obtained in this study have extended our knowledge of the anti-inflammatory mechanisms of glucocorticoids, flavonoids and calcineurin inhibitors. Regulation of iNOS expression was found to occur both at the transcriptional and post-transcriptional levels. These findings may be fundamental in the development of novel anti-inflammatory drugs with more precise target specificity. Understanding the transcription of proinflammatory genes, and more importantly, their regulation at the level of mRNA and protein stability is important when the aim is to inhibit ongoing expression of inflammatory factors.
Summary and conclusions

The aim of the present study was to investigate the effects of anti-inflammatory compounds on iNOS expression and NO production in activated macrophages. Studies were carried out to define in more detail the mechanisms of action of the identified effective compounds. The findings and conclusions are as follows:

1. Glucocorticoids are widely used in the treatment of inflammatory diseases such as rheumatoid arthritis, IBD and asthma. Histone deacetylation is a novel mechanism proposed to mediate some of the anti-inflammatory effects of glucocorticoids. In the present study, the glucocorticoid dexamethasone and a dissociated steroid, RU24858, were found to inhibit iNOS expression and NO production by a mechanism related to histone deacetylation.

2. Polyphenolic compounds possess anti-inflammatory properties and were studied for their effects on iNOS expression and NO production. The flavonoids daidzein, genistein, kaempferol and quercetin inhibited the activation of NF-κB and STAT-1 along with their inhibitory effect on iNOS expression and NO production. Flavone, isorhamnetin, naringenin and pelargonidin inhibited the activation of NF-κB as well as iNOS expression and NO production. In addition, daidzein and isorhamnetin were found to increase the decay of iNOS mRNA.

3. Calcineurin inhibitors are immunosuppressive drugs used in the prevention of allograft rejection and in the treatment of inflammatory diseases. In the present study, calcineurin inhibitors CsA, tacrolimus and pimecrolimus were found to down-regulate iNOS expression and NO production, probably by enhancing the degradation of iNOS mRNA.

Inhibition of iNOS expression and NO production are likely to be involved in the anti-inflammatory mechanisms of glucocorticoids, calcineurin inhibitors, and a subgroup of flavonoids. The present findings of the molecular mechanisms involved in those effects may be utilized in the development of novel anti-inflammatory drugs with more specific target selectivities.
Kiitokset (Acknowledgements)

This study was carried out in the Immunopharmacology Research Group, Medical School, University of Tampere, Finland.

Väitöskirjatyön ohjaajalle, professori Eeva Moilaselle tahdon osoittaa suuret kiitokset mahdollisuudesta suorittaa jatko-opinnot Immunofarmakologian tutkimusryhmässä. Olen vuosien varrella saanut olla mukana monissa erilaisissa projekteissa ja tehtävissä, joista olen oppinut paljon. Kiitos korvaamattomasta opastuksestasi ja tuestasi tieteen poluilla.

Tahdon kiittää väitöskirjan esitarkastajaa sekä seurantaryhmäni jäsentä dosentti Häkan Granlundia hänenä samamattani rakentavista kommenteista ja parannusehdotuksista koskien väitöskirjatyötäni matkan varrella ja käskirjoituskumppaneissa. Professor Brendan Whittle is gratefully acknowledged for his constructive criticism and advice to improve this thesis.


Immunofarmakologian tutkimusryhmä on ollut hyvä paikka tehdä työtä, ja kiitos siitä kuuluu kaikille ryhmän jäsenille – niin nykyisille kuin jo muuhin tehtävänä siirtynyille. Ryhmässämme on hyvä henki, mistä kuuluu kiitos teille kaikille.

Ystäville ja perheelle kiitos siitä, että elämää on myös työn ja tieteen ulkopuolella. Kiitos appivanhemmille Liisalle ja Ekille avustanne arjen pyörityksessä. Veljille Ristolle ja Jyrkille perheineen kiitos ystävyydestänne ja tuestanne.


Juhalle kiitos rakkaudestasi, tuestasi, käräsivällisyystäsi ja siitä kuinka mahtava isä olet tytöllemme Tuulille ja Merille, elämämme tärkeimmile.

This study was supported by the Finnish Cultural Foundation, the Finnish Cultural Foundation Pirkanmaa Regional Fund, the Medical Research Fund of Tampere University Hospital, Drug 2000 program by the Finnish Funding Agency for Technology and Innovation (TEKES), the Academy of Finland, and Tampere Graduate School in Biomedicine and Biotechnology.

Tampere 1.8.2008

Mari Hämäläinen
References


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Inhibition of iNOS expression and NO production by anti-inflammatory steroids. Reversal by histone deacetylase inhibitors

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PII: S1094-5539(07)00074-0
DOI: doi:10.1016/j.pupt.2007.08.003
Reference: YPUPT 791

To appear in: Pulmonary Pharmacology & Therapeutics

Received date: 24 April 2007
Revised date: 4 July 2007
Accepted date: 8 August 2007

Cite this article as: Mari Hääläinen, Riikka Lilja, Hannu Kankaanranta and Eeva Moilanen, Inhibition of iNOS expression and NO production by anti-inflammatory steroids. Reversal by histone deacetylase inhibitors, Pulmonary Pharmacology & Therapeutics (2007), doi:10.1016/j.pupt.2007.08.003

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Inhibition of iNOS expression and NO production by anti-inflammatory steroids. Reversal by histone deacetylase inhibitors.

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Type of contribution: Regular paper
Date of preparation: 18.4.2007, revised 4.7.2007
Number of text pages: 18
Number of figures: 6
Abstract

In inflammation, nitric oxide (NO) is produced by inducible nitric oxide synthase (iNOS) induced by bacterial products and cytokines, and NO acts as a regulatory and proinflammatory mediator. Glucocorticoids are powerful anti-inflammatory agents that inhibit the expression of iNOS and various other inflammatory factors. Histone deacetylation has been recently described as a novel mechanism how glucocorticoids down-regulate transcriptional activation of some inflammatory genes. The aim of the present study was to investigate the effects of inhibitors of histone deacetylation on the suppressive effects of glucocorticoids on NO production and iNOS expression.

Dexamethasone and a dissociated glucocorticoid RU24858 inhibited NO production, and iNOS protein and mRNA expression in macrophages exposed to bacterial lipopolysaccharide (LPS). In the presence of a glucocorticoid receptor (GR) antagonist mifepristone, dexamethasone and RU24858 had no effect on NO production. The role of histone deacetylation in the glucocorticoid effect was studied by using three structurally different inhibitors of histone deacetylases (HDACs); trichostatin A, apicidin and MC1293. HDAC inhibitors reversed the effects of dexamethasone and RU24858 on iNOS expression and NO production. Stably transfected A549/8 cells containing luciferase gene under the control of human iNOS promoter were used in promoter-activity studies. iNOS promoter activity induced by IL-1β was inhibited by dexamethasone and the inhibitory effect was reversed by HDAC inhibitor trichostatin A.

The results suggest that glucocorticoids inhibit iNOS expression and NO production by a GR-mediated and GRE-independent manner through histone deacetylation and transcriptional silencing.

Keywords: nitric oxide, inducible nitric oxide synthase, inflammation, glucocorticoids, histone deacetylation, macrophages
1. Introduction

Nitric oxide (NO) is a gaseous short-living signalling molecule with several physiological and pathophysiological effects. NO is produced by three nitric oxide synthases (NOS); endothelial NOS (eNOS), neuronal NOS (nNOS) and inducible NOS (iNOS). eNOS and nNOS are calcium-dependent constitutively expressed enzymes responsible for the low physiological production of NO, whereas inducible NOS is calcium-independent enzyme producing higher amounts of NO [1]. Many cell types express iNOS when exposed to bacterial products or proinflammatory cytokines, and in inflammation high and prolonged production of NO may lead to cytotoxic and proinflammatory effects [2,3]. NO production through iNOS pathway is mainly regulated at transcriptional level but also post-transcriptional regulatory mechanisms have been reported [4].

Glucocorticoids have been reported to inhibit NO production and iNOS expression in several cell types including vascular endothelial cells [5], epithelial cells [6] and macrophages [7], yet in some cell types like in chondrocytes, glucocorticoids have failed to inhibit NO production [8,9]. Glucocorticoids have been shown to exert their inhibitory effect on NO production and iNOS expression through inhibition of the transcription factor nuclear factor (NF)-κB. The inhibition of nuclear translocation of NF-κB or its DNA binding as well as increase of inhibitory IκB by glucocorticoids have been reported [6,10,11]. Interestingly, in epithelial cells dexamethasone was not found to affect the expression of p65, p50 or IκBα nor NF-κB activation or its DNA-binding but still it repressed IL-1β-induced iNOS mRNA expression by inhibiting NF-κB-dependent transcription [12]. Also other mechanisms of action for glucocorticoids in repressing NO production have been suggested [13-16].

Most of the anti-inflammatory actions of glucocorticoids are exerted by activation of transcription of anti-inflammatory genes or by inhibition of pro-inflammatory transcription factors or mechanisms leading to reduced transcription of pro-inflammatory genes. Histone deacetylation by glucocorticoids has been suggested as a novel mechanism for transcriptional silencing of pro-inflammatory genes. [17,18]
In regulating gene expression, histones have a significant role. DNA in chromosomes is packaged into chromatin, where it is wound around an octamer of core histones H2A, H2B, H3 and H4 forming a nucleosome. The N-terminal tails of core histones are highly conserved and susceptible to modifications such as acetylation, methylation, phosphorylation and ubiquitination. Acetylation of the N-terminal tails of core histones is in relation to transcriptional activity, whereas hypoacetylation is correlated with transcriptional silencing [19,20]. Transcriptional coactivator complexes like CBP (cAMP-response-element-binding protein-binding protein)/p300 have intrinsic histone acetyltransferase (HAT) activity. Complexes are not directly bound to DNA but they are recruited to particular promoters through interactions with DNA-bound transcription factors to activate transcription [21]. Histone deacetylases (HDACs) interact with co-repressor molecules to silence gene expression [22]. Also other proteins than histones have been identified as substrates for HDACs, e.g. glucocorticoid receptor (GR) has been shown to be deacetylated by HDAC2 [23,24].

In IL-1β-treated epithelial cells dexamethasone has been shown to down-regulate GM-CSF production by a mechanism related to deacetylation of lysine residues 8 and 12 in histone H4 [25]. In macrophages TNF-α-mediated acetylation of lysine residues 8 and 12 in histone H4 was reported to be reduced by dexamethasone [26]. The aim of the present study was to investigate if histone deacetylation might be involved in the mechanisms how glucocorticoids down-regulate NO production through iNOS pathway by using HDAC-inhibitors as pharmacological tools.

2. Materials and methods

2.1 Materials

Dulbecco’s Modified Eagle’s Medium and foetal bovine serum were from Cambrex Bioproducts Europe (Verviers, Belgium). All medium supplements were obtained from Invitrogen (Carlsbad, CA, USA). M1293 and apicidin were from Alexis Corporation (San Diego, CA, USA), dexamethasone from Orion Corporation (Espoo, Finland) and RU24858 was kindly provided by Aventis Pharma (Romainville Cedex, France). All other reagents were from Sigma (St. Louis, MO, USA).
2.2 Cell culture

Murine J774 macrophages were obtained from American Type Culture Collection (Rockville, MD, USA). Macrophages were cultured at 37°C (in 5% carbon dioxide) in Dulbecco's Modified Eagle's Medium (DMEM) with glutamax-I containing 10% heat-inactivated foetal bovine serum, penicillin (100 units ml\(^{-1}\)), streptomycin (100 µg ml\(^{-1}\)) and amphotericin B (250 ng ml\(^{-1}\)). A549/8-Luc cells stably transfected with plasmid pXP2-16kb containing full length human iNOS promoter (16 kb) in front of a luciferase gene [27] were used in the experiments on the regulation of human iNOS promoter. The A549/8-Luc cells were kindly provided by Dr. Hartmut Kleinert, University of Mainz, Germany. A549/8-Luc cells were cultured at 37°C in 5% CO\(_2\) atmosphere in DMEM with 1 mM sodium pyruvate containing 5% heat-inactivated foetal bovine serum, 2.5 µg ml\(^{-1}\) Polymyxin B and 0.5 mg ml\(^{-1}\) G418 for selection. Both cell lines were harvested with trypsin-EDTA, and cells were seeded in 96-well plates for XTT-test, in 24-well plates for nitrite measurements and RNA extraction, in 6-well plates for Western blot analysis and in 10-cm dishes for HDAC activity assay and grown to confluence before the commencement of the experiments. Confluent cell cultures were exposed to fresh culture medium containing the compounds of interest and cultured for indicated times.

2.3 XTT-test

Cell viability was tested using the Cell Proliferation Kit II that measures the cells’ ability to metabolise XTT to formazan by mitochondrial dehydrogenase activity, a function that only occurs in viable cells (Roche Diagnostics GmbH, Mannheim, Germany). Cells were incubated with the tested compounds and LPS/IL-1β for 20h before addition of sodium 3’-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis (4-methoxy-6-nitro) benzene sulphonic acid hydrate (XTT) (final concentration 0.3 mg ml\(^{-1}\)) and N-methyl dibenzopyrazine methyl sulphate (8.2 µM). Cells were incubated for another 4h and the amount of formazan accumulated in growth medium was assessed spectrophotometrically. Triton-X treated cells were used as a positive control. Conditions were considered toxic if the cells’ ability to metabolise XTT to
formazan was lowered by more than 20% as compared to cells exposed to LPS/IL-1β only.

2.4 Nitrite determinations

Measurement of nitrite accumulation into the culture medium was used to determine NO production. At indicated time points the culture medium was collected and nitrite was measured by Griess reaction [28]. A NOS inhibitor L-NIO was used to ensure that the measured nitrite was due to NO produced by NOS pathway in the cell culture.

2.5 Western blot analysis of iNOS protein

After desired time of incubation cell pellets were lysed in ice-cold extraction buffer (10 mM Tris-base, pH 7.4, 5 mM EDTA, 50 mM NaCl, 1% Triton X-100, 0.5 mM phenylmethyl sulphonyl fluoride, 1 mM sodiumorthovanadate, 20 µg ml⁻¹ leupeptin, 50 µg ml⁻¹ aprotinin, 5 mM NaF, 2 mM sodiumpyrophosphate and 10 µM n-octyl-beta-D-glucopyranoside). Samples were incubated on ice for 15 min, centrifuged, and the resulting supernatant was diluted 1:4, boiled for 5 min in sample buffer (6.25 mM Tris-HCl, 10% glycerol, 2% SDS and 0.025% 2-mercapto-ethanol) and stored at -20°C until analysed. The Coomassie blue method was used to measure the protein content of the samples [29]. Protein samples (20 µg) were separated by SDS-PAGE on 8% polyacrylamide gel and transferred to nitrocellulose membrane. The bound antibody (rabbit polyclonal anti-iNOS antibody M-19, Santa Cruz Biotechnology, Santa Cruz, CA, USA) was detected using goat anti-rabbit polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and visualised by SuperSignal West Pico chemiluminescent substrate (Pierce, Cheshire, UK) and FluorChem 8800 imaging system (Alpha Innotech Corporation, San Leandro, CA, USA). The quantitation of the chemiluminescent signal was carried out with the use of FluorChem software version 3.1.
2.6 RNA extraction and real-time RT-PCR of iNOS, luciferase and GAPDH mRNAs

J774 and A549/8-Luc cells stimulated with the compounds of interest were trypsinised after the desired time of incubation. The cell pellets were washed twice with PBS, lysed and purified using QIAshredder™ (QIAGEN, Valencia, CA). Thereafter RNA was extracted using RNeasy® kit for isolation of total RNA (QIAGEN Inc.). Total RNA (25 ng) was reverse-transcribed to cDNA using TaqMan Reverse Transcription reagents and random hexamers (Applied Biosystems, Foster City, CA, USA). Reverse-transcriptase (RT) reaction parameters were as follows: incubation at 25ºC for 10 min, RT at 48ºC for 30 min, and RT inactivation at 95ºC for 5 min. cDNA obtained from the RT reaction (amount corresponding to approximately 1 ng of total RNA) was subjected to PCR using TaqMan Universal PCR Master Mix and ABI PRISM 7000 Sequence detection system (Applied Biosystems). The primer and probe sequences and concentrations were optimised according to the manufacturer’s guidelines in TaqMan Universal PCR Master Mix Protocol part number 4304449 revision C and were as follows: 5'-CCTGGTACGCGCCATTGCT-3’ (mouse iNOS forward), 5'-GCTCATGCGCCTCCTT-3’ (mouse iNOS reverse), 5’-CAGCAGCGGCTCCATGACTCCC-3’ (mouse iNOS probe), 5’-GCATGGCCTTCCGTTCTC-3’ (mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) forward), 5’-GATGTCATCATACTTGGCAGGTTT-3’ (mouse GAPDH reverse), 5’-TGTTGATCTGACGTGCCGCC-3’ (mouse GAPDH probe), 5’-AAAAAGTTGCGCGGAGGAG-3’ (luciferase forward), 5’-TTTTTCTTTCGCTGAGTTC-3’ (luciferase reverse), 5’-TGTTGATCTGACGTGCCGCC-3’ (human GAPDH forward), 5’-GCAACAAATATCCACTTACCAGGTTA-3’ (human GAPDH reverse), 5’-CGCCTGGTACGCGCCATTGCT-3’ (human GAPDH probe). Concentration for all primers was 300 nM and for probes 150 nM. All probes contained 6-FAM (6-carboxy-fluorescein) as 5’-reporter dye and TAMRA (6-carboxy-tetramethylrhodamine) as 3’-quencher. Each sample was determined in duplicate. Results of iNOS and LUC mRNA levels were normalised against GAPDH mRNA in each sample.
2.7 Preparation of nuclear extracts for HDAC activity assay

Nuclear extracts from J774 cells were prepared, and the HDAC activity assay was performed according to manufactures’ instructions (Histone Deacetylase Activity/Inhibitor Screening Assay, Cayman Chemicals, MI, USA).

2.8 Statistics

Results are expressed as mean + standard error of mean (SEM). When appropriate, statistical significance of the results were calculated by analysis of variance followed by Dunnett multiple comparison’s test. Differences were considered significant when p<0.05

3. Results

3.1 The effects of glucocorticoids on LPS-induced NO production

Lipopolysaccharide (LPS) was used to induce NO production in J774 macrophages. NO production was not detectable in untreated cells. Dexamethasone and a dissociated steroid RU24858 [30] both inhibited NO production in a concentration-dependent manner, dexamethasone being somewhat more potent than RU24858 on molar basis (Figures 1A and B). Dexamethasone and RU24858 had no inhibitory effect on NO production in the presence of a glucocorticoid receptor antagonist mifepristone (Figure 1C). Cytotoxicity was measured by XTT-test and none of the experimental treatments were found to be toxic.

3.2 The effects of glucocorticoids on LPS-induced NO production in the presence of HDAC inhibitors trichostatin A, apicidin and MC1293

Trichostatin A (TSA) was used as a non-selective HDAC inhibitor. Dexamethasone (1 µM) and RU24858 (1 µM) inhibited LPS-induced NO production by 52 and 44%, respectively. In the presence of TSA the inhibitory actions of dexamethasone and RU24858 on NO production in J774 macrophages were abolished (Figure 2A). In
addition to TSA, two other HDAC inhibitors (apicidin and MC1293) were used to study the effect of HDAC inhibition on the inhibitory action of dexamethasone on NO production. As seen in figure 2B, dexamethasone had no inhibitory effect on LPS-induced NO production in J774 macrophages in the presence of apicidin or MC1293.

To confirm that used HDAC inhibitors TSA, apicidin and MC1293 inhibit HDAC activity in our experimental conditions, an enzyme assay was performed. TSA (50 ng ml⁻¹ (165 nM)), apicidin (60 nM) and MC1293 (30 µM) inhibited HDAC activity in J774 nuclear extracts by 92, 68 and 49%, respectively (Figure 3).

3.3 The effects of glucocorticoids on iNOS protein and mRNA expression in the absence or presence of trichostatin A

The effects of dexamethasone, RU24858 and TSA on iNOS protein expression in J774 macrophages were studied by Western blot analysis. Dexamethasone and RU24858 were found to suppress LPS-induced iNOS protein expression, and this inhibitory action was reversed by TSA (Figure 4).

The effects of dexamethasone and RU24858 were investigated also on iNOS mRNA expression by quantitative reverse-transcriptase real-time PCR. After incubation for 12h untreated cells and cells treated with LPS and dexamethasone or with LPS and RU24858 in the absence or presence of TSA were lysed and RNA was extracted. Untreated cells did not produce detectable amounts of iNOS mRNA. As seen in figure 5A, both dexamethasone (1 µM) and RU24858 (1 µM) significantly (>60%) inhibited the accumulation of iNOS mRNA (Figure 5A). In the presence of TSA the inhibitory effects of dexamethasone and RU24858 were abolished (Figure 5B).

3.4 The effects of dexamethasone and trichostatin A on iNOS promoter activity in A549/8-Luc cells

To study the effects of dexamethasone and TSA on iNOS promoter activity we used A549/8-Luc cells, which had been stably transfected with plasmid pXP2-16kb
containing full length human iNOS promoter (16 kb) in front of a luciferase gene [27] to generate cells expressing luciferase reporter gene under control of iNOS promoter.

iNOS promoter activity was enhanced by interleukin (IL)-1β (10 ng ml⁻¹) as measured by luciferase mRNA levels. Addition of dexamethasone (1 µM) diminished iNOS promoter activity to the level in untreated control cells. In the presence of TSA dexamethasone had no effect on iNOS promoter activity (Figure 6).

4. Discussion

In the present study, we studied the effects of dexamethasone and RU24858, which is a dissociated glucocorticoid [30], on NO production and iNOS expression in macrophages exposed to LPS. Dexamethasone and RU24858 both inhibited LPS-induced NO production in a concentration-dependent manner, and the inhibitory action was reversed in the presence of HDAC inhibitors trichostatin A, apicidin and MC1293.

Eleven histone deacetylases (HDACs) belonging to three different groups have been described [31]. HDAC inhibitors are of natural origin or synthesised, and they have promise as antitumor and anti-inflammatory agents [32,33]. In the present study, three HDAC inhibitors were used. Trichostatin A (TSA) and apicidin have been reported to act at nanomolar concentrations, and they belong to hydroxamate and cyclic tetrapeptide groups, respectively. MC1293 is a benzamide derivative which is effective at micromolar concentrations [34]. In the present study, TSA abrogated the inhibitory effects of dexamethasone and RU24858 on NO production, and iNOS protein and mRNA expression. Dexamethasone’s effect on NO production was also reversed by apicidin and MC1293. Using an iNOS promoter construct in A549/8 cells, we studied the effects of dexamethasone and TSA on iNOS promoter activity. We found dexamethasone to inhibit IL-1β-induced iNOS promoter activity, and this inhibitory effect was reversed by TSA.
There are $\alpha$- and $\beta$-variants of glucocorticoid receptor (GR) and it is also subjected to posttranslational modifications like phosphorylation which affect the activity of the receptor [35]. Binding of a glucocorticoid to a glucocorticoid receptor in cytoplasm leads to dissociation of molecular chaperones from GR and induction of its nuclear translocation. Ligand activated GRs translocate to nucleus, and bind as a dimer to glucocorticoid responsive element (GRE) in DNA. In transcriptional activation interaction of GR with positive GRE leads to enhanced transcription whereas some genes are down-regulated through negative GRE [36]. Glucocorticoids have also been shown to have effect on the stability of some mRNAs or proteins [37,38]. Anti-inflammatory effects of glucocorticoids can be partly explained by activation of anti-inflammatory genes, but most of the anti-inflammatory actions of glucocorticoids are believed to be mediated by suppressing transcription of pro-inflammatory genes by interacting with transcription factors or transcriptional mechanisms [17].

Deacetylation of specific lysine residues in histone H4 by glucocorticoids leading to transcriptional silencing has recently been proposed as a mechanism for the transrepressional anti-inflammatory effects of glucocorticoids [18]. In IL-1$\beta$ stimulated A549 epithelial cells dexamethasone inhibited GM-CSF release by a mechanism related to repression of the acetylation of lysines 8 and 12 in histone 4. Inhibition of acetylation was associated with inhibition of HAT activity and recruitment of HDAC2 to the site of transcription [25]. In accordance, dexamethasone was found to suppress TNF-$\alpha$ -induced acetylation of lysine residues 8 and 12 in histone H4 in macrophages [26]. Histone deacetylation has been reported to be involved also in the inhibitory effect of dexamethasone on the expression of IL-5 [39] and tristetraprolin [40]. Our results are in concordance with those earlier results and extend the previous data by showing that HDAC inhibitors reverse also the inhibitory action of dexamethasone on NO production, iNOS expression and iNOS promoter activity in cells exposed to inflammatory stimuli.

NO production and iNOS expression is inhibited by glucocorticoids in many cell types including vascular endothelial cells [5], epithelial cells [6], hepatocytes [11] and macrophages [7]. Glucocorticoids may inhibit NO production and iNOS expression by several distinct but simultaneous mechanisms and the principal mechanisms may vary depending on the cell-type and the inflammatory stimuli. Following mechanisms
have been suggested: inhibition of the synthesis of a cofactor tetrahydrobiopterin and transport of the substrate L-arginine into the cell [15], induction of arginase II (which consumes the substrate L-arginine) [13], inhibition of transcription factor NF-κB [6,11], and destabilisation of iNOS mRNA [16]. Findings in the present study propose a novel mechanism that is likely involved in the inhibitory action of dexamethasone on NO production, i.e. transcriptional silencing through histone deacetylation. Dexamethasone has earlier been shown to inhibit IL-1β induced GM-CSF release in epithelial cells [25], transcription of IL-5 in T cells [39] and production of tristetraprolin in macrophages [40] by a mechanism involving histone deacetylation, and our results suggest that iNOS expression is regulated by a similar mechanism.

In the present study, dexamethasone and a dissociated steroid RU24858 were shown to inhibit NO production and iNOS expression in LPS-treated macrophages in a concentration-dependent manner. HDAC inhibitors TSA, apicidin and MC1293 reversed the inhibitory effects of dexamethasone and RU24858 in NO production. In A549/8-Luc cells the inhibitory effect of dexamethasone on iNOS promoter activity was reversed by TSA. These results suggest that histone deacetylation is involved in the mechanisms how glucocorticoids inhibit NO production and iNOS expression in response to inflammatory stimuli.

Acknowledgements

We wish to thank Dr. Hartmut Kleinert for providing us with A549/8-Luc cell line, Mrs Niina Ikonen for skilful technical assistance and Mrs Heli Määttä for secretarial help. The study was supported by grants from the Medical Research Fund of Tampere University Hospital, Finland.

References


Figure captions

Figure 1
A Effects of increasing concentrations of dexamethasone on LPS (10 ng ml\(^{-1}\)) – induced NO production.
B Effects of increasing concentrations of RU24858 on LPS (10 ng ml\(^{-1}\)) – induced NO production.
C Effect of mifepristone (3 µM) on the inhibitory action of dexamethasone (1 µM) and RU24858 (1 µM) on LPS (10 ng ml\(^{-1}\)) – induced NO production.
NO production in J774 macrophages during a 24h incubation time was determined by measuring nitrite accumulation into the culture medium by Griess reaction. Mean + SEM, n=4-6, **p<0.01 as compared to cells treated with LPS alone.

Figure 2
A Effects of dexamethasone (1 µM) and RU24858 (1 µM) on LPS (10 ng ml\(^{-1}\)) – induced NO production in the absence or presence of TSA (50 ng ml\(^{-1}\)).
B Effect of dexamethasone (1 µM) on LPS (5 ng ml\(^{-1}\)) – induced NO production in the absence or presence of apicidin (60 nM) or MC1293 (30 µM).
NO production in J774 macrophages was determined after 24h incubation by measuring nitrite accumulation into the culture medium by Griess reaction. LPS or LPS+TSA/apicidin/MC1293 - induced NO production was set as 100% and other values were related to that. Mean + SEM, n=6, ** p<0.01 as compared to cells treated with LPS alone.

Figure 3 Effects of TSA (50 ng ml\(^{-1}\)), apicidin (60 nM) and MC1293 (30 µM) on total HDAC activity in J774 nuclear extracts. Mean + SEM, n=6.

Figure 4 Effects of dexamethasone (1 µM) and RU24858 (1 µM) on LPS (10 ng ml\(^{-1}\)) – induced iNOS protein expression A in the absence or B in the presence of TSA (50 ng ml\(^{-1}\)) during a 24h incubation time in J774 macrophages. Equal amounts of protein (20 µg/lane) were loaded into SDS-PAGE gel, and iNOS protein expression was detected by Western blot analysis. The quantitation of the chemiluminescent signal was carried out using the FluorChem software version 3.1. LPS-induced or LPS and
TSA-induced iNOS protein expression was set as 100% and other values were related to that. Mean + SEM, n=3.

**Figure 5** Effects of dexamethasone (1 µM) and RU 24858 (1 µM) on LPS (10 ng ml$^{-1}$)–induced iNOS mRNA accumulation in the absence or B in the presence of TSA (50 ng ml$^{-1}$) in J774 macrophages. RNA was extracted after 12h incubation, and iNOS and GAPDH mRNAs were measured by real-time RT-PCR. iNOS mRNA levels were normalised against GAPDH. iNOS mRNA levels at LPS or LPS+TSA–treated cells were set as 100% and other values were related to that. Mean + SEM, n=3.

**Figure 6** Effects of dexamethasone (1 µM) in the absence or presence of TSA (50 ng ml$^{-1}$) on IL-1β (10 ng ml$^{-1}$)–induced iNOS promoter activity in A549/Luc cells. RNA was extracted after 6h incubation, and LUC and GAPDH mRNAs were measured by real-time RT-PCR. LUC mRNA levels were normalised against GAPDH. Mean + SEM, n=3.
Figure 1 Hämäläinen et al.
Figure 2 Hämäläinen et al.
Figure 3 Hämäläinen et al.
Figure 4 Hämäläinen et al.
Figure 5 Hämäläinen et al.
Figure 6 Hämäläinen et al.
Research Article

Anti-Inflammatory Effects of Flavonoids: Genistein, Kaempferol, Quercetin, and Daidzein Inhibit STAT-1 and NF-κB Activations, Whereas Flavone, Isorhamnetin, Naringenin, and Pelargonidin Inhibit only NF-κB Activation along with Their Inhibitory Effect on iNOS Expression and NO Production in Activated Macrophages

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Received 18 December 2006; Revised 16 March 2007; Accepted 26 April 2007

Inflammation, bacterial products and proinflammatory cytokines induce the formation of large amounts of nitric oxide (NO) by inducible nitric oxide synthase (iNOS), and compounds that inhibit NO production have anti-inflammatory effects. In the present study, we systematically investigated the effects of 36 naturally occurring flavonoids and related compounds on NO production in macrophages exposed to an inflammatory stimulus (lipopolysaccharide, LPS), and evaluated the mechanisms of action of the effective compounds. Flavone, the isoflavones daidzein and genistein, the flavonols isorhamnetin, kaempferol and quercetin, the flavanone naringenin, and the anthocyanin pelargonidin inhibited iNOS protein and mRNA expression and also NO production in a dose-dependent manner. All eight active compounds inhibited the activation of nuclear factor-κB (NF-κB), which is a significant transcription factor for iNOS. Genistein, kaempferol, quercetin, and daidzein also inhibited the activation of the signal transducer and activator of transcription 1 (STAT-1), another important transcription factor for iNOS. The present study characterizes the effects and mechanisms of naturally occurring phenolic compounds on iNOS expression and NO production in activated macrophages. The results partially explain the pharmacological efficacy of flavonoids as anti-inflammatory compounds.

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1. INTRODUCTION

Nitric oxide (NO) is produced from L-arginine by three nitric oxide synthase (NOS) enzymes; endothelial NOS (eNOS), neuronal NOS (nNOS), and inducible NOS (iNOS). Low physiological levels of NO are produced by constitutively expressed eNOS and nNOS, whereas iNOS is responsible for prolonged production of larger amounts of NO. iNOS is induced by bacterial products and inflammatory cytokines in macrophages and some other cells [1–3]. NO production is increased in inflammation and has proinflammatory and regulatory effects [4, 5]. In addition, peroxynitrite formation in a reaction of NO and superoxide may lead to increased cytotoxicity. The experimental data support the idea that compounds inhibiting expression or activity of iNOS are potential anti-inflammatory agents [6–9].

Flavonoids are naturally occurring polyphenolic compounds containing two benzene rings linked together with a heterocyclic pyran or pyrone ring. Flavonoids are normal constituents of the human diet and are known for a variety of biological activities. Some of these act as enzyme inhibitors and antioxidants, and have been reported to have anti-inflammatory properties. However, the molecular mechanisms explaining how flavonoids suppress the inflammatory response are not known in detail [10, 11]. There are studies showing that certain flavonoids down-regulate NO
production in response to inflammatory stimuli [12–14], but no more precise mechanisms of action are known.

In the present study, we investigated the effects of 36 naturally occurring compounds representing different groups of flavonoids and related compounds on iNOS expression and NO production in activated macrophages systematically, and evaluated the mechanisms of action of the effective compounds.

2. MATERIALS AND METHODS

2.1. Materials

Luteolin, luteolin-7-glucoside, vitexin, daidzein, genistein, genistin, rhamnetin, isorhamnetin, kaempferol, myricetin, taxifolin, naringin, ferulic acid, pelargonidin, procyanidin B1, and procyanidin B2 were obtained from Extrasynthese (Lyon, France). Acacetin, cyanidin, flavone, morin, and quercitrin were obtained from Carl Roth GmbH (Karlruhe, Germany). Quercetin, rutin, and benzoic acid were obtained from Merck (Darmstadt, Germany). Naringenin, hesperidin, chlorogenic acid, dodecyl gallate, methyl gallate, octyl gallate, and sinapic acid were obtained from Fluka (Buchs SG, Switzerland).

Dulbecco's modified eagle medium and its supplements were obtained from Gibco BRL (Paisley, UK). All other reagents were obtained from Sigma (St. Louis, MO, USA) unless otherwise stated.

2.2. Cell culture

Murine J774 macrophages were obtained from American Type Culture Collection (Rockville, MD, U.S.A.). Cells were cultured at 37°C (in 5% carbon dioxide) in Dulbecco's modified eagle's medium (DMEM) with glutamax-I containing 10% heat-inactivated foetal bovine serum, penicillin (100 units/ml), streptomycin (100 μg/ml), and amphotericin B (250 ng/ml). Cells were harvested with trypsin-EDTA. Cells were seeded in 96-well plates for XTT-test, in 24-well plates for nitrite measurements, in 6-well plates for iNOS Western blot and RNA extraction, and in 10-cm dishes for p65 and STAT-1 Western blot. Confluent cells were exposed to fresh culture medium containing the compounds of interest and cultured for the times indicated.

2.3. XTT-test

Cell viability was tested using cell proliferation kit II that measures the cells' ability to metabolize XTT to formazan by mitochondrial dehydrogenase activity, a function that only occurs in viable cells (Roche Diagnostics GmbH, Mannhein, Germany). Cells were incubated with the tested compounds and LPS for 20 hours before addition of sodium 3'-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis (4-methoxy-6-nitro) benzene sulphonic acid hydrate (XTT) (final concentration 0.3 mg/ml) and N-methyl benzopyrazine methyl sulphate (8.2 μM). Cells were incubated for another 4 hours and the amount of formazan accumulating in growth medium was assessed spectrophotometrically. Triton-X treated cells were used as a positive control. Conditions were considered nontoxic if the cells’ ability to metabolize XTT to formazan was more than 80% of that of cells exposed to LPS only.

2.4. Nitrite determinations

Measurement of nitrite accumulation into the culture medium was used to determine NO production. At the indicated time points, the culture medium was collected and nitrite was measured by the Griess reaction [15]. A NOS inhibitor L-NIO (1 mM) and a highly selective iNOS inhibitor 1400 W (1 mM) were added at the beginning of the incubation to cells that were stimulated with LPS (100 ng/ml) to ensure that the measured nitrite was due to NO produced by the iNOS pathway in the cell culture.

2.5. Preparation of cell lysates for iNOS Western blot

After the desired time of incubation cell lysates were prepared as described earlier [16]. The Coomassie blue method was used to measure the protein content of the samples [17].

2.6. Preparation of nuclear extracts for p65 and STAT-1 Western blot

Cells were seeded on 10-cm dishes and were grown to confluence. Cells were incubated with the compounds of interest for 30 minutes (p65) or for 6 hours (STAT-1α). After incubation, samples were prepared as described earlier [18]. The Coomassie blue method was used to measure the protein content of the samples [17].

2.7. Western blot analysis of iNOS, p65, and STAT-1α proteins

Protein samples (20 μg) were separated by SDS-PAGE on 8% polyacrylamide gel and transferred to nitrocellulose membrane. Bound antibody (rabbit polyclonal antibodies for iNOS, STAT-1α (Santa Cruz Biotechnology, Santa Cruz, CA, USA), or for p65 subunit of NF-κB (Cell Signaling Danvers, MA, USA)) was detected using goat anti-rabbit polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), or for p65 subunit of NF-κB (Cell Signaling Danvers, MA, USA), and visualised by SuperSignal chemiluminescent substrate (Pierce, Cheshire, UK) and FluorChem 8800 imaging system (Alpha Innotech Corporation, San Leandro, CA). The quantitation of the chemiluminescent signal was carried out using FluorChem software version 3.1.

2.8. RNA extraction and real-time RT-PCR of iNOS and GAPDH mRNAs

J774 cells stimulated with the compounds of interest were trypsinised after the desired time of incubation. Cell homogenization, RNA extraction, reverse transcription, and quantitative PCR were performed as described earlier [16]. The primer and probe sequences and concentrations were
optimized according to the manufacturer’s instructions in TaqMan Universal PCR Master Mix Protocol part number 4304449 revision C and were as follows: 5'-CCTGGTACGGGCTTGT-3', 5'-GCTCATGCGGCCTCCCTTAA-3' (forward and reverse mouse iNOS primers, resp., both 300 nM), 5'-CAGCAGCGCTCCATGACTCC-3' (mouse iNOS probe containing 6-FAM as 5'-reporter dye and TAMRA as 3'-quencher, 150 nM), 5'-GCATGGCCTTCCATGACCTCC-3' (mouse GAPDH primers, resp., both 300 nM), 5'-TGGATCTGACGTGCCGCC-3' (mouse GAPDH probe containing 6-FAM as 5'-reporter dye and TAMRA as 3'-quencher, 150 nM). Results of iNOS mRNA levels were normalized against GAPDH mRNA in each sample.

2.9. Statistics

Results are expressed as mean ± standard error of mean (SEM). The statistical significance of the detected differences was calculated by analysis of variance followed by Dunnett multiple comparison’s test. Differences were considered significant when \( P < .05 \).

3. RESULTS

The tested compounds \( (n = 36) \) represented eight groups of flavonoids and related compounds: flavones, isoflavones, flavonols, flavanones, flavan-3-ols, anthocyanins, hydroxybenzoic acid (HBA) group, and hydroxycinnamic acid (HCA) group. The tested compounds are listed and their structures are shown in Figure 1 and Table 1. Possible cytotoxic effects were tested by XTT-test. Compounds that were toxic at 100 \( \mu \text{M} \) (see Table 2) were excluded from further studies.

3.1. Effects of flavonoids on LPS-induced NO production in J774 cells

Untreated J774 macrophages did not produce detectable amounts of NO during 24-hour incubation, but LPS (100 ng/ml) enhanced NO production significantly. In the first experiments, flavonoids were used at 10 \( \mu \text{M} \) and 100 \( \mu \text{M} \) concentrations. The compounds inhibiting NO production by more than 50% at 100 \( \mu \text{M} \) concentration compared to LPS-treated control were flavone, daidzein, genistein, isorhamnetin, kaempferol, quercetin, naringenin, and pelargonidin (see Table 2). NOS inhibitor L-NIO (1 mM) and a selective iNOS inhibitor 1400 W (1 mM) were used as control compounds, and they inhibited LPS-induced NO production by more than 90%.

If the compound inhibited NO production by more than 50% at 100 \( \mu \text{M} \) concentration, a dose-response effect was studied. All eight active compounds (flavone, daidzein, genistein, isorhamnetin, kaempferol, quercetin, naringenin,
<table>
<thead>
<tr>
<th>Class</th>
<th>Derivatives</th>
<th>Substituents</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1&lt;sup&gt;+&lt;/sup&gt;</td>
<td>3</td>
</tr>
</tbody>
</table>

### Flavones

- **Acacetin**: H OH OH H OCH<sub>3</sub> H
- **Apigenin**: H OH OH H OH H
- **Flavone**: H H H H H H
- **Luteolin**: H OH OH OH OH OH H
- **Lut-7-glucoside**: H OH OGl Glc OH OH H
- **Vitexin**: H OH OH OH OH H

### Isoflavones

- **Daidzein**: H H OH OH H OH H
- **Genistein**: H OH OH H OH H
- **Genistin**: H OH OGl Glc H OH H

### Flavonols

- **Isorhamnetin**: OH OH OH OCH<sub>3</sub> OH H
- **Kaempferol**: OH OH OH OH H OH H
- **Morin**: OH OH OH OH H OH H
- **Myricetin**: OH OH OH OH OH OH OH
- **Quercetin**: OH OH OH OH OH H
- **Quercitrin**: ORha OH OH OH OH H
- **Rhamnetin**: OH OH OCH<sub>3</sub> OH OH H
- **Rutin**: ORu OH OH OH OH H

### Flavanones

- **Naringenin**: H OH OH H OH H
- **Naringin**: H OH ONeo H OH H
- **Taxifolin**: OH OH OH OH OH H

### Flavan-3-ols

- **+Catechin**: OH OH OH OH OH H
- **−Epicatechin**: OH OH OH OH OH H

### Procyanidin B1

- Dimer of epicatechin and catechin linked via their carbons 4 and 8, respectively.

### Procyanidin B2

- Dimer of two epicatechin molecules linked via carbons 4 and 8.

### Anthocyanins

- **Cyanidin**: OH OH OH OH OH H
- **Pelargonidin**: OH OH OH OH OH H

### HBA

- **Benzoic acid**: H H
- **Dodecyl gallate**: COO(CH<sub>2</sub>)<sub>11</sub>CH<sub>3</sub> OH OH
- **Ellagic acid**: see below
- **Gallic acid**: OH OH
- **Methyl gallate**: COOCH<sub>3</sub> OH OH
- **Octyl gallate**: COOCH<sub>2</sub>(CH<sub>2</sub>)<sub>6</sub>CH<sub>3</sub> OH OH
- **Syringic acid**: OCH<sub>3</sub> OCH<sub>3</sub>

### HCA

- **Chlorogenic acid**: see below
- **Ferulic acid**: OCH<sub>3</sub>
- **Sinapic acid**: OCH<sub>3</sub> OCH<sub>3</sub>

<sup>1</sup>if other than in basic structure

Gl=glucoside, Rha=rhamnoside, Ru=rutinoside, Neo=neohesperidoside

<sup>2</sup>OH group is in front of plane of paper, <sup>3</sup>OH group is behind plane of paper
Table 2: Effects of phenolic compounds on cell viability and on LPS-induced (100 ng/ml) NO production in J774 macrophages. No detectable NO production was found in untreated cells. Mean ± standard error of mean (SEM).

<table>
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<th>Class</th>
<th>Toxicity[a]</th>
<th>NO production (inhibition%)</th>
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<tr>
<td></td>
<td>[100 µM]</td>
<td>[10 µM]</td>
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<tr>
<td><strong>LPS 100 ng/ml</strong></td>
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<tr>
<td><strong>Flavonoids</strong></td>
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<td></td>
</tr>
<tr>
<td>Acacetin</td>
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<tr>
<td>Apigenin</td>
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<td>Flavone</td>
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<td>Taxifolin</td>
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<td>+ Catechin</td>
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<tr>
<td>− Epicatechin</td>
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<td></td>
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<tr>
<td>Syringic acid</td>
<td>–</td>
<td>6.7 ± 1.5</td>
</tr>
<tr>
<td><strong>HCA</strong>[c]</td>
<td></td>
<td></td>
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<tr>
<td>Chlorogenic acid</td>
<td>–</td>
<td>−4.8 ± 3.5</td>
</tr>
<tr>
<td>Ferulic acid</td>
<td>–</td>
<td>6.6 ± 1.1</td>
</tr>
<tr>
<td>Sinapic acid</td>
<td>–</td>
<td>8.9 ± 1.9</td>
</tr>
</tbody>
</table>

[a] Cytotoxicity was tested by XTT-test and compounds that showed cytotoxicity at 100 µM concentrations were excluded from further study. [b] HBA = hydroxybenzoic acid. [c] HCA = hydroxycinnamic acid, n = number of replicates.
and pelargonidin) inhibited NO production in a dose-dependent manner in the following order: quercetin (IC$_{50}$ ∼ 25 μM) > kaempferol (IC$_{50}$ ∼ 25 μM) > genistein (IC$_{50}$ ∼ 30 μM) > isorhamnetin (IC$_{50}$ ∼ 30 μM) > flavone (IC$_{50}$ ∼ 40 μM) > daidzein (IC$_{50}$ ∼ 70 μM) > naringenin (IC$_{50}$ ∼ 80 μM) > pelargonidin (IC$_{50}$ ∼ 90 μM) (see Figure 2).

3.2. Effects of flavonoids on LPS-induced iNOS protein expression

The effects of those eight flavonoids inhibiting NO production by more than 50% at 100 μM concentrations were tested on iNOS protein expression by Western blot analysis. Unstimulated cells did not express detectable amounts of iNOS protein and LPS enhanced iNOS protein expression considerably. All eight active compounds (flavone, daidzein, genistein, isorhamnetin, kaempferol, quercetin, naringenin, and pelargonidin) inhibited LPS-induced iNOS protein expression (see Figure 3).

3.3. Effects of flavonoids on LPS-induced iNOS mRNA levels

iNOS mRNA was measured by quantitative real-time RT-PCR. Cells were incubated with LPS (100 ng/ml) or with LPS and the tested flavonoid (flavone, daidzein, genistein, isorhamnetin, kaempferol, quercetin, naringenin, or pelargonidin (100 μM)) for 6 hours. This incubation time was chosen according to the time curve of iNOS mRNA, where the maximal iNOS mRNA levels were between 6 and 8 hours after addition of LPS. Untreated cells expressed very low levels of iNOS mRNA and LPS enhanced iNOS mRNA expression considerably. All eight tested flavonoids significantly lowered iNOS mRNA levels when measured after 6-hour incubation in the following order of potency: quercetin > kaempferol > genistein > isorhamnetin > flavone > naringenin > daidzein > pelargonidin (see Figure 4).

3.4. Effects of flavonoids on LPS-induced activation of transcription factors NF-κB and STAT-1

NF-κB and STAT-1 are important transcription factors for iNOS [19, 20]. Therefore we measured the effects of the eight effective flavonoids on NF-κB and STAT-1 activations by measuring the nuclear translocation of the factors by Western blot.

In unstimulated cells, low basal activity of NF-κB was detected and was significantly enhanced after LPS challenge. The maximal activation was found 30 minutes after LPS addition, and that incubation time was used in the subsequent studies. Quercetin, naringenin and pelargonidin inhibited the LPS-induced activation of NF-κB by more than 80%. Flavone, genistein, isorhamnetin, kaempferol, and daidzein had a moderate (57%–72% inhibition) inhibitory effect (see Figure 5).

Nuclear STAT-1 levels were significantly enhanced after LPS challenge. The maximal effect was found 6 hours after LPS addition, and that time point was chosen for subsequent studies. The LPS-induced activity of STAT-1 was nearly

![Figure 2](image-url)
Figure 3: (a) Effects of flavone, daidzein, and genistein on LPS (100 ng/ml)-induced iNOS protein expression in J774 cells during a 24-hour incubation time. (b) Effects of quercetin, isorhamnetin, and kaempferol on LPS (100 ng/ml)-induced iNOS protein expression in J774 cells during a 24-hour incubation time. (c) Effects of naringenin and pelargonidin on LPS (100 ng/ml)-induced iNOS protein expression in J774 cells during a 24-hour incubation time. iNOS protein expression was detected by Western blot. Mean ± SEM, n = 3, **P < .01.

Figure 4: Effects of flavonoids on iNOS mRNA expression. Cells were treated with LPS (100 ng/ml) or with LPS and the tested compound (100 μM) and RNA was extracted after 6 hours. iNOS and GAPDH mRNA were measured by real-time RT-PCR and iNOS mRNA levels were normalised against GAPDH. Mean ± SEM, n = 3, **P < .01.

Figure 5: Effects of flavonoids on LPS-induced NF-κB activation determined as nuclear translocation of NF-κB. J774 cells were incubated for 30 minutes with LPS (100 ng/ml) or with LPS and the flavonoid (100 μM), and nuclear proteins were extracted. Western blot was used to detect the p65 subunits of NF-κB in the nuclear extracts. p65 levels in LPS-treated cells were set at 100% and the other values were related to that. The dotted line represents the nuclear p65 levels in untreated control cells. Mean ± SEM, n = 4–6, **P < .01 as compared to LPS-induced level.

totally (91% inhibition) inhibited by quercetin. Daidzein, genistein, and kaempferol had a moderate (32%–41% inhibition) inhibitory effect whereas flavone, isorhamnetin, naringenin, and pelargonidin showed no inhibitory effect on the activation of STAT-1 (see Figure 6).
4. DISCUSSION

Flavonoids are nonessential dietary factors, and humans consume about 1-2 g of flavonoids daily. Flavonoids are abundantly present in fruits, vegetables, seeds, nuts, tea, and red wine, and the flavonoid mostly consumed is quercetin. Flavonoids are believed to act as health-promoting substances, and some of them have antioxidant and anti-inflammatory properties [10, 11]. Anti-inflammatory effects have also been found in vivo. For instance, genistein was reported to inhibit LPS-induced septic response in rat [21] and quercetin suppressed experimentally induced arthritis in rat [22].

In the present study, we investigated the effects of flavonoids and related compounds belonging to eight classes (flavones, isoflavones, flavonols, flavanones, flavan-3-ols, anthocyanins, HBA, and HCA) on iNOS expression and NO production in activated macrophages. Eight effective compounds were found. Four compounds (genistein, kaempferol, quercetin, and daidzein) inhibited LPS-induced STAT-1 and NF-κB activations, and iNOS expression. In addition, four compounds (flavone, isorhamnetin, naringenin, and pelargonidin) inhibited NF-κB activation and iNOS expression but had no effect on STAT-1.

Isoflavones daidzein and genistein inhibited LPS-induced iNOS expression and NO production in a dose-dependent manner, whereas genistin was less effective. Daidzein and genistein also inhibited activations of STAT-1 and NF-κB, which are important transcription factors for iNOS [19, 20]. To our knowledge, their effects on STAT-1 activation have not been reported previously, whereas suppression of DNA-binding of NF-κB by genistein has been reported [23]. Our results confirm earlier observations on the inhibitory effects of daidzein and genistein on iNOS expression and NO production [13, 24–27], and provide a mechanism for the effect through suppression of STAT-1 and NF-κB activations.

In the flavonol group, isorhamnetin, kaempferol, and quercetin inhibited NO production and iNOS protein and mRNA expression, quercetin and kaempferol being the most potent of the phenolic compounds tested. Isorhamnetin, kaempferol, and quercetin all inhibited NF-κB activation, and quercetin and kaempferol also had an effect on STAT-1 activation. This is the first study to show that isorhamnetin reduces iNOS expression, and that the effect may well be mediated by inhibition of NF-κB activation. Kaempferol has previously been shown to inhibit iNOS expression and NO production [13, 28]. Here we confirm those findings and show that kaempferol inhibits STAT-1 and NF-κB activations, which are implicated in their effects on iNOS expression. Chen et al. [29] have reported that quercetin inhibits IFNγ-induced STAT-1 activation in mouse BV-2 microglia. In the present study we found that quercetin also suppressed LPS-induced activation of STAT-1 in macrophages supporting the idea that its effects on STAT-1 are stimulus and cell-type independent. Quercetin inhibited LPS-induced STAT-1 activation along with its inhibitory effect on iNOS expression and NF-κB activation. Inhibition of STAT-1 activation by quercetin is likely involved in the mechanisms by which it inhibits iNOS expression because JAK inhibitors AG-490

**Figure 6:** (a)–(c) Effects of flavonoids on LPS-induced STAT-1 activation determined as nuclear translocation of STAT-1α. J774 cells were incubated for 6 hours with LPS (100 ng/ml) or with LPS and the flavonoid (100 μM), and nuclear proteins were extracted. Western blot was used to detect STAT-1α in the nuclear extracts. STAT-1α levels in LPS-treated cells were set at 100% and the other values were related to that. The dotted line represents the nuclear STAT-1α levels in untreated control cells. Mean + SEM, n = 3, **P < .01, *P < .05 as compared to LPS-induced level.
and WHI-P154 have been shown to inhibit iNOS expression along with their suppressive actions on STAT-1 activation \[18, 30\].

Concurring with earlier results, we found flavanone naringenin to inhibit LPS-induced NO production while its glycosylated counterpart naringin had no effect \[31\]. Our results suggest that the inhibitory effect of naringenin is likely to be at transcriptional level through inhibition of the activation of NF-κB. Pelargonidin has been reported to inhibit NO production in macrophages \[26\]. Here we extend the data by showing that pelargonidin suppresses NO production by reducing iNOS expression through inhibiting the activation of transcription factor NF-κB.

Regarding the structural requirements of flavonoids for the inhibition of NO production, three main features could be found:  
(a) a C-2,3 double bond is a common feature in the six most effective compounds,

(b) a bulky group (e.g., glycoside, rhamnose, rutinoside, or neohesperidoside) as a substituent lowered or abolished the compound’s inhibitory effect (e.g., quercetin was highly effective whereas its rhamnose-substituted derivative quercitrin was ineffective),

(c) 7 and 4’ OH-groups were found in all effective compounds but this alone did not differentiate active from ineffective compounds.

Related structure-activity relationships regarding PGE\(_2\) inhibition have been reported in rat peritoneal macrophages \[32\].

Earlier studies have shown that some flavonoids inhibit NO production in response to inflammatory stimuli \[13, 26, 28, 33, 34\]. The present study extends the previous knowledge by systematically comparing the effects of a large series of compounds in standardized experimental conditions. Moreover, we investigated the effects of the eight effective compounds not only on NO production but also on iNOS mRNA and protein expression, and on the activation of inflammatory transcription factors NF-κB and STAT-1. Some anti-inflammatory flavonoids have been shown to inhibit activation of NF-κB and the effect has been linked to their antioxidant properties \[29, 35\]. Here we found that all the effective compounds (flavone, daidzein, genistein, isorhamnetin, kaempferol, quercetin, naringenin, and pelargonidin) inhibited LPS-induced NF-κB activation. In addition, genistein, kaempferol, quercetin, and daidzein also inhibited STAT-1 activation. To our knowledge, the inhibitory effects of genistein, kaempferol, and daidzein on STAT-1 activation have not been reported previously, whereas quercetin was found to inhibit IFNγ-induced activation of STAT-1 in mouse BV-2 microglia \[29\]. The mechanisms by which genistein, kaempferol, quercetin, and daidzein inhibit STAT-1 activation are not known, but may be associated with inhibition of phosphorylation of STAT-1 or its up-stream kinase JAK2 \[36\]. Interestingly, the three most potent inhibitors of iNOS expression and NO production, that is, genistein, kaempferol, and quercetin, inhibited both NF-κB and STAT-1 activations, whereas those flavonoids inhibiting only NF-κB had a smaller effect on iNOS expression. Because NF-κB and STAT-1 are involved in the activation of several inflammatory genes, flavonoids that inhibit activation of NF-κB and/or STAT-1 are likely to down-regulate production of an array of inflammatory mediators in addition to iNOS. Therefore the present results offer an additional molecular mechanism for the anti-inflammatory action of flavonoids.

In conclusion, we compared the effects of 36 naturally occurring flavonoids and related polyphenolic compounds on LPS-induced NO production and iNOS expression in activated macrophages. The flavonoid classes containing the most effective compounds were isoflavones and flavonols. We identified eight compounds as being able to inhibit LPS-induced NO production and iNOS expression. Four compounds (genistein, kaempferol, quercetin, and daidzein) inhibited activation of both of the important transcription factors for iNOS, that is, STAT-1 and NF-κB, whereas four compounds (flavone, isorhamnetin, naringenin, and pelargonidin) inhibited only NF-κB. The results partly explain the anti-inflammatory effects of flavonoids.

**ACKNOWLEDGMENTS**

The authors wish to thank Mrs Niina Ikonen and Mrs Jaana Tägström for skilful technical assistance and Mrs Heli Määttä for secretarial help. The study was supported by grants from the Finnish Funding Agency for Technology and Innovation (TEKES), the Medical Research Fund of Tampere University Hospital, Finland, and the Academy of Finland.

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Calcineurin inhibitors, cyclosporin A and tacrolimus inhibit expression of inducible nitric oxide synthase in colon epithelial and macrophage cell lines

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Abstract
Nitric oxide (NO) production is increased in inflammatory bowel disease (IBD) and selective iNOS inhibitors have proved to be anti-inflammatory in experimentally induced colitis. The aim of the present study was to test if drugs used in the treatment of IBD affect NO production in colon epithelial and macrophage cell lines. We tested the effects of cyclosporin A, tacrolimus (FK-506), methotrexate, sulfasalazine, 5-ASA and two novel TNF-α antagonists etanercept and infliximab on lipopolysaccharide (LPS) -induced NO production in human T84 colon epithelial cells and in murine J774 macrophages. Cyclosporin A and FK-506 inhibited iNOS expression, and subsequent NO production, in a dose-dependent manner at therapeutically achievable drug concentrations in both cell lines. The effect was most pronounced when cyclosporin A was given 1h prior to 4h after LPS, and declined thereafter, indicating that cyclosporin A does not inhibit iNOS activity. Neither cyclosporin A nor FK-506 altered the activation of nuclear factor-κB (NF-κB) that is a critical transcription factor for iNOS. Sulfasalazine inhibited NO production slightly only when given at high (100 µM) drug concentrations. Methotrexate, 5-ASA and TNFα antagonists infliximab and etanercept were practically ineffective. Two inhibitors of phosphatase calcineurin, cyclosporin A and FK-506, inhibited iNOS expression and NO production in human T84 colon epithelial cells and in murine J774 macrophages by a NF-κB independent manner. These findings are implicated in the anti-inflammatory action of these compounds.

Keywords: nitric oxide, iNOS, cyclosporin A, tacrolimus, sulfasalazine, colitis

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1. Introduction

Nitric oxide (NO) is produced in increased amounts and acts as a pro-inflammatory and cytotoxic mediator in various inflammatory diseases (Moilanen et al., 1999). NO is synthesised from L-arginine by nitric oxide synthase (NOS) enzymes. Two types of NOS enzymes have been identified: constitutively expressed calcium-dependent cNOS and inducible calcium-independent iNOS (Knowles and Moncada, 1994; Alderton et al., 2001). iNOS is induced by inflammatory cytokines and bacterial products in various cell types (Moilanen et al., 1999). When iNOS is induced it produces high amounts of NO for prolonged periods which is related to immunoregulatory, antimicrobial and cytotoxic actions of NO.

Nitric oxide production is increased in inflammatory bowel disease (IBD) where NO is mainly produced by macrophages, neutrophils and epithelial cells. In experimentally induced intestinal inflammation NO seems to have a biphasic effect. In the early stages of inflammation low levels of NO produced by cNOS are protective but later on, when NO is produced in high amounts by iNOS, it is associated with mucosal lesions, ulcerations, intraluminal bleeding, bowel dilatation and dysfunction, and has pro-inflammatory and destructive effects (Laszlo et al., 1994; Guslandi, 1998; Miller and Sandoval, 1999). NO production and iNOS activity have been shown to be increased in active ulcerative colitis (Kimura et al., 1997; Boughton-Smith et al., 1993; Lundberg et al., 1994; Kimura et al., 1998). NOS inhibitors with relative selectivity towards iNOS have been reported to have anti-inflammatory effects in endotoxin-induced intestinal barrier dysfunction in rats (Unno et al., 1997), in TNBS-induced colitis in rats (Zingarelli et al., 1998) and in spontaneous colonic inflammation occurring in HLA-B27 transgenic rats (Aiko et al., 1998). In recent studies, a highly selective iNOS inhibitor 1400W has proved to have a significant anti-inflammatory action in severe TNBS-induced colitis (Kankuri et al., 2001; Menchen et al., 2001) and in LPS-provoked injury in rat gastroduodenal microvasculature (Kiss et al., 2001). These results suggest that inhibition of iNOS in gut inflammation is of therapeutic value. Therefore, we decided to investigate the effects of drugs used in the treatment of IBD (cyclosporin A, FK-506, sulfasalazine, methotrexate, 5-ASA, infliximab and etanercept) on iNOS expression and NO production in two different cell lines, in human intestinal T84 epithelial cells and in J774 murine macrophages. The latter cell line was used because iNOS pathway is well characterised in that cell line. We used also human intestinal T84 epithelial cells, which have recently been shown to express iNOS and produce NO in response to inflammatory stimulus (Lahde et al., 2000). In these conditions, we investigated the effects of two calcineurin inhibitors cyclosporin A and tacrolimus (FK-506), two TNF-α antagonists infliximab and etanercept and three other drugs for inflammatory bowel disease sulfasalazine, methotrexate and 5-aminosalicylic acid, on cellular iNOS expression and NO production in response to inflammatory stimuli.

2. Materials and methods

2.1 Materials

Dulbecco’s modified Eagle medium and its supplements were from Gibco BRL (Paisley, Scotland, UK). Cyclosporin A was supplied by Calbiochem (La Jolla, CA, USA). Infliximab was from Centocor B.V (Leiden, The Netherlands), etanercept was from Wyeth Lederle (Espoo, Finland)
and methotrexate from Orion Corporation (Espoo, Finland). All other reagents were from Sigma (St. Louis, MO, USA).

2.2 Cell culture

Human T84 colon epithelial cells and murine J774 macrophages were obtained from American Type Culture Collection (Rockville, MD, U.S.A). Cells were cultured at 37 °C (in 5% carbon dioxide) in Dulbecco’s Modified Eagle’s Medium (DMEM) with glutamax-I containing 10 % (5% in T84 cells) heat-inactivated foetal bovine serum, penicillin (100 units/ml), streptomycin (100 µg/ml) and amphotericin B (250 ng/ml). Cells were harvested with trypsin-EDTA. Cells were seeded in 24 well plates for nitrite measurements, in 6 well plates for Western blot analysis and in 10-cm dishes for electrophoretic mobility shift assay. Confluent cells were exposed to fresh culture medium containing the compounds of interest.

2.3 XTT-test

Cell viability was tested using Cell Proliferation Kit II (Boehringer Mannheim, Indianapolis, IN, USA). Cells were incubated with the tested compounds for 20 h before addition of sodium 3’-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis (4-methoxy-6-nitro) benzene sulfonic acid hydrate (XTT) (final concentration 0.3 mg/ml) and N-methyl dibenzopyrazine methyl sulfate (1.25 mM). Then cells were further incubated for 4 h and the amount of formazan accumulated in growth medium was assessed spectrophotometrically. Triton-X treated cells were used as a positive control.

2.4 Nitrite determinations

Measurement of nitrite accumulation into the culture medium was used to determine NO production. At indicated time points the culture medium was collected and nitrite was measured by Griess reaction (Green et al., 1982).

2.5 Western blot analysis

After desired time of incubation cell pellets were lysed in ice-cold extraction buffer (10 mM Tris-base, 5 mM EDTA, 50 mM NaCl, 1 % Triton-X, 0.5 mM phenylmethyl sulfonyle fluoride, 2 mM sodiumorthovanadate, 10 µg/ml leupeptin, 25 µg/ml aprotinin, 1.25 mM NaF, 1 mM sodiumpyrophosphate and 10 mM n-octyl-beta-D-glucopyranoside). After extraction by incubation on ice for 15 min samples were centrifuged and the resulting supernatant was diluted 1:4 and boiled for 5 min in sample buffer (6.25 mM Tris-HCl, 10 % glycerol, 2 % SDS and 0.025% 2-mercapto-ethanol) and stored at −20 °C until analysed. Coomassie blue method was used to measure the protein content of the samples (Bradford, 1976). Protein samples (20 µg) were separated by SDS-PAGE on 8% polyacrylamide gel and transferred to nitrocellulose membrane. iNOS protein was detected and identified by Western blotting using rabbit polyclonal antibody (M-19 for murine J774 macrophages and N-20 for human T84 epithelial cells) obtained from Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.

2.6 Preparation of nuclear extracts

Cells were seeded on 10-cm dishes and were grown to confluency. Cells were incubated with the compounds of interest for 30 min. After incubation, cells were washed with ice-cold PBS and solubilised in hypotonic buffer A (10 mM HEPES-KOH, pH 7.9, 1.5 mM MgCl$_2$, 10 mM KCl, 0.5 mM dithiotreitol (DTT), 0.2 mM PMSF, 10 µg/ml leupeptin, 25 µg/ml aprotinin, 0.1 mM EGTA, 1mM Na$_2$VO$_3$, 1 mM NaF) and incubated for 10 min on ice. Thereafter, cells were vortexed for 30 s
and the nuclei were separated by centrifugation at 4 °C, 15,000 rpm, 10 s. Nuclei were suspended in buffer C (20 mM HEPES-KOH, pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM DTT, 0.2 mM PMSF, 10 µg/ml leupeptin, 25 µg/ml aprotinin, 0.1 mM EGTA, 1 mM Na₃VO₄, 1 mM NaF) and incubated on ice for 20 min. Nuclei were vortexed 30 s and nuclear extracts were obtained by centrifugation at 4 °C and 15,000 rpm for 2 min. Coomassie blue method was used to measure the protein content of the samples (Bradford, 1976).

2.7 Electrophoretic mobility shift assay

Single-stranded oligonucleotides (5' -AGTTGAGGGGACTTTCCCAGGC-3' and 3' -TCAACTCCCCTGAAAGGGTCCG-5'; Amersham Pharmacia Biotech (Piscataway, NJ, USA)) containing the nuclear factor-κB (NF-κB) -binding sequences were annealed and 5' ³²P-end labeled with DNA 5'-End Labeling Kit (Boehringer Mannheim (Indianapolis, IN, USA)). For binding reactions 10 µg of nuclear extract was incubated in 20 µl of total reaction volume containing 0.1 mg/ml (poly) dI- dC, 1 mM DTT, 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 200 mM KCl and 10 % glycerol for 20 min at room temperature. Thereafter 0.2 ng of ³²P-labeled oligonucleotide was added and reaction mixture was incubated for 10 min at room temperature. Protein/DNA complexes were separated from DNA probe by electrophoresis on 4 % polyacrylamide gel. Gel was dried on filter paper and autoradiographed with intensifying screen at –70 °C.

2.8 Statistics

Results are expressed as mean ± standard error of mean (SEM). Statistical significance was calculated by analysis of variance supported by Dunnett adjusted significance levels. Differences were considered significant when P<0.05.

3. Results

3.1. Effects of cyclosporin A and FK-506 on NO production

Bacterial endotoxin (lipopolysaccharide, LPS) induced iNOS expression and NO production in human T84 epithelial cells and in murine J774 macrophages. Cyclosporin A inhibited NO production (measured as nitrite accumulation into the culture medium) in a dose-dependent manner in both cell lines. Exposure to increasing concentration of CsA resulted in a 34% and 10% (1 µM), 59% and 30% (3 µM) and 80% and 52% (10 µM) inhibition of NO production during a 24h incubation in T84 and J774 cells, respectively (Fig.1A).

![Figure 1](image-url)
production in T84 and in J774 cells during a 24h incubation time. NO production was determined by measuring nitrite accumulation into the culture medium by Griess reaction. Mean ± SEM, n=6. ** indicates P< 0.01 as compared to cells incubated without CsA or FK-506.

FK-506 (tacrolimus) is another inhibitor of Ca\(^{2+}\)/calmodulin dependent calcineurin. Mimicking the action of CsA, FK-506 had also a dose-dependent inhibitory action on LPS-induced NO production (Fig. 1B). Cytotoxic action of CsA or FK-506 as a contributing factor was ruled out by XTT-test.

The time-course of the inhibitory action of CsA on NO production is shown in Figure 2. The inhibition was most pronounced when CsA was given from 1h prior to 4h after LPS. The suppressive action declined, and in the case of T84 cells was totally reversed, if CsA was given at 8h or later following LPS. These data suggest that CsA inhibits iNOS expression rather than iNOS activity (Fig.2).

**Figure 2.** Time-course of the inhibitory action of CsA. T84 and J774 cells were incubated for 24h with LPS and CsA was added at indicated time points. NO production was determined by measuring nitrite accumulation into the culture medium by Griess reaction. Mean ± SEM, n=6. ** indicates P< 0.01 as compared to cells incubated without CsA.

3.2. Effects of cyclosporin A and FK-506 on iNOS protein expression

In the further studies, we investigated the effect of CsA and FK-506 on iNOS expression by Western blot (Fig.3). Cells cultured in the absence of LPS did not contain detectable amounts of iNOS protein. iNOS expression was significantly enhanced in both cell types following exposure to LPS. CsA (1-10 µM) and FK-506 (1-30 µM) suppressed this LPS-induced iNOS expression in a dose-dependent manner in both cell types.

**Figure 3.** The effects of CsA and FK-506 on LPS-induced iNOS protein expression in T84 and J774 cells as detected by Western blot analysis. Cells were incubated for 24h with compounds of interest and iNOS was detected by immunoblot with specific antibody against iNOS. The figure shows a representative of four separate experiments with similar results.

3.3. Effects of cyclosporin A and FK-506 on transcription factor NF-κB activation

NF-κB is an important transcription factor of iNOS (Xie et al., 1994). Therefore, we measured the effects of CsA on NF-κB activation by EMSA (Fig. 4). In the absence of LPS there was a low basal activity of NF-κB that was significantly enhanced following LPS. CsA or FK-506 had no effect on LPS-induced NF-κB activation.

3.4. Effects of methotrexate, sulfasalazine, 5-aminosalicylic acid, infliximab and etanercept on NO production

Out of the five other drugs tested, sulfasalazine (100 µM) inhibited LPS-induced NO production by 26% in T84 cells and by 21% in J774 cells (Table 1). Methotrexate, sulfasalazine and 5-ASA inhibited NO production by less than 20% at 100 µM concentrations and had no effect at concentrations of 30 µM or lower.
Figure 4. The effects of CsA and FK-506 on LPS-induced NF-kB activation. Human T84 colon epithelial cells were incubated for 30 minutes with LPS or with LPS and CsA or with LPS and FK-506. NF-kB binding activity was determined by electrophoretic mobility shift assay. The figure shows a representative of four separate experiments with similar results.

TNFα antagonists infliximab (10 µg/ml) and etanercept (100 µg/ml) did not significantly alter LPS-induced NO production.

Table 1.
Effects of methotrexate, sulfasalazine, 5-ASA, infliximab and etanercept on endotoxin (1 µg/ml)-induced NO production in T84 cells and in J774 cells.

<table>
<thead>
<tr>
<th></th>
<th>T84 nitrite (% of control)</th>
<th>J774 nitrite (% of control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>methotrexate (100 µM)</td>
<td>83 ± 0.72**</td>
<td>96 ± 1.5</td>
</tr>
<tr>
<td>sulfasalazine (100 µM)</td>
<td>74 ± 4.9**</td>
<td>79 ± 0.64**</td>
</tr>
<tr>
<td>5-ASA (100µM)</td>
<td>90 ± 0.79**</td>
<td>88 ± 1.5**</td>
</tr>
<tr>
<td>infliximab (10 µg/ml)</td>
<td>110 ± 2.5**</td>
<td>89 ± 1.7**</td>
</tr>
<tr>
<td>etanercept (100 µg/ml)</td>
<td>105 ± 2.4</td>
<td>81 ± 1.3**</td>
</tr>
</tbody>
</table>

Endotoxin and drugs tested were added to the cells at the beginning of the incubation; cell were incubated for 24 h and, thereafter, nitrite was measured from the culture as a marker of NO production. ** indicates P<0.01. Mean ± standard error of mean (SEM), n=6.

4. Discussion

Cyclosporin A (CsA) is a potent immunosuppressive agent. It acts primarily as an inhibitor of T-cell activation, but it has been reported to affect also other cell types (Schreiber and Crabtree, 1992). In T-cells CsA acts by suppressing T-cell receptor-activated signal transduction pathway. CsA inhibits the action of Ca²⁺/calmodulin-dependent phosphatase calcineurin resulting in a suppressed activation of transcription factor of activated T cells (NF-AT). This action leads to reduced production of IL-2 and other cytokines in activated T cells. The most important clinical use of CsA is in the prevention and treatment of allograft rejection. It is also used in autoimmune disorders such as rheumatoid arthritis and IBD (Faulds et al., 1993).

CsA was reported to inhibit LPS-induced NO production in macrophages and it was proposed to do this by suppressing iNOS induction rather than by inhibiting NOS activity (Hattori and Nakanishi, 1995). In another study CsA was found to inhibit NOS activity in a dose-dependent manner in casein-elicited murine peritoneal macrophages (Conde et al., 1995). In murine macrophage cell line RAW 264.7 LPS-induced NO production was found to be inhibited at iNOS mRNA level by CsA (Attur et al., 2000). In the present study we found that CsA inhibits LPS-induced NO production in a dose-dependent manner in human T84 cells and in murine J774 macrophages. Time-course of this inhibitory action suggests that CsA inhibits iNOS expression rather than NOS activity since 1h before to 4h after the LPS-stimulation the suppressive action of CsA on NO production was most efficient declining thereafter. The Western blot analysis confirmed the inhibitory action of CsA on iNOS protein expression after LPS challenge. NF-κB is an important transcription factor in the induction of iNOS gene (Xie et al., 1994). Therefore, we tested if CsA has any effect on the nuclear translocation and DNA-binding of NF-κB on LPS-induced T84 cells. Low basal activity of NF-κB was significantly increased after LPS challenge but CsA had
no effect on NF-κB activity. This result suggests that CsA mediates its effect on LPS-induced NO production by an NF-κB-independent manner.

Tacrolimus (FK-506) is another immunosuppressive compound that has a very similar mechanism of action as CsA. In activated T-cells CsA and FK-506 exert their effect on the transcription factor NF-AT (Schreiber and Crabtree, 1992). When bound to their cytosolic immunophilin receptors, CsA and FK-506 inhibit the action of a protein phosphatase, calcineurin, resulting in reduced dephosphorylation of the cytosolic component of transcription factor NF-AT. As a consequence, nuclear translocation of NF-AT is inhibited. In our experiments, FK-506 had a very similar inhibitory action as CsA on NO production and iNOS expression both in human T84 colon epithelial cells and in mouse J774 macrophages. These data suggest that the effect of CsA and FK-506 is mediated through inhibition of calcineurin either by NF-AT dependent or independent manner. The role of NF-AT in the regulation of iNOS expression is not known. We did a computer survey (MatInspector V2.2, Quandt et al., 1995) and found that there are several potential binding sites for NF-AT in human iNOS promoter region but we did not find in the literature convincing data on any significant role of NF-AT in the regulation of iNOS transcription. On the other hand, our time-response curve of the effect of CsA on inducible NO production suggest that the effect is not a clear transcriptional one but more likely takes place at post-transcriptional level. Further studies are needed to specify the target of CsA and FK-506 in this process.

We also tested the effects of methotrexate, sulfasalazine, 5-ASA, infliximab and etanercept on LPS-induced NO production in T84 and in J774 cells. Methotrexate inhibits the reduction of dihydrofolate to tetrahydrofolate, which is needed in the synthesis of tetrahydrobiopterin, a cofactor of iNOS (Alderton et al., 2001). In T84 cells a slight inhibition on LPS-induced NO production by high concentrations (100 µM) of methotrexate was detected but not in J774 cells. These results suggest that in our experimental conditions the synthesis of tetrahydrobiopterin is not a rate-limiting factor for inducible NO production. In macrophages from rats with adjuvant-induced arthritis methotrexate has been shown to suppress NO production (Omata et al., 1997). 5-aminosalicylic acid, when used at mill molar concentrations, has been shown to inhibit iNOS transcription in human intestinal epithelial cell lines DLD-1 and Caco-2BBe (Kennedy et al., 1999). We used lower concentrations (up to 100 µM) of 5-ASA and found a slight effect on LPS-induced NO production with the highest concentration used. Wahl et al. have reported that sulfasalazine, a combinatorial molecule of 5-ASA and sulfapyridine, is a specific inhibitor of NF-κB activation when used at 2-5-mM concentrations (Wahl et al., 1998). We used 100 µM of sulfasalazine and a 26% and 21% inhibition was found in LPS-induced NO production in T84 and in J774 cells, respectively. These data suggest that with concentrations we used sulfasalazine does not significantly inhibit the activation of NF-κB since NF-κB is a critical transcription factor for iNOS (Xie et al., 1994). TNFα antagonists infliximab and etanercept had no effect on NO production in T84 or in J774 cells indicating that TNFα is not mediating or enhancing LPS-induced NO production in these cell types.

In summary, two calcineurin inhibitors, CsA and FK-506, inhibited LPS-induced NO production and iNOS expression in human intestinal T84 cells and in murine J774 macrophages by a NF-κB-independent manner. These results are
implicated in the anti-inflammatory action of cyclosporine and tacrolimus.

Acknowledgements

We wish to thank Mrs Niina Ikonen and Mrs Heli Määttä for their skilful technical assistance. The study was supported by the Academy of Finland and the Medical Research Fund of Tampere University Hospital.

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Calcineurin inhibitors down-regulate iNOS expression by destabilising mRNA

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Abstract

In inflammation, bacterial products and pro-inflammatory cytokines induce the expression of inducible nitric oxide synthase (iNOS) and formation of high amounts of nitric oxide (NO). In a number of inflammatory diseases NO has pro-inflammatory and cytotoxic effects. The aim of the present study was to investigate the effects of immunosuppressive drugs cyclosporin A (CsA), tacrolimus (FK-506) and pimecrolimus on NO production through iNOS pathway in activated macrophages and fibroblasts. Calcineurin inhibitors (CsA, FK-506 and pimecrolimus) inhibited NO production and iNOS expression in a concentration-dependent manner, CsA being more potent than FK-506 and pimecrolimus. No effect on the activation or activity of the transcription factors nuclear factor-κB (NF-κB) and signal transducer and activator of transcription 1 (STAT-1) was found. CsA, FK-506 and pimecrolimus did not reduce iNOS mRNA levels when measured 6-8h after the inflammatory stimulus, but significantly lower levels of iNOS mRNA were found after 24h incubation. Also, in cells transfected with a luciferase gene under the control of 3’ untranslated region (3’ UTR) of iNOS, CsA reduced luciferase activity.

In conclusion, the results suggest that calcineurin inhibitors cyclosporin A, tacrolimus (FK-506) and pimecrolimus inhibit iNOS expression and NO production in response to inflammatory stimuli by enhancing the decay of iNOS mRNA by a 3’UTR-dependent manner. The findings add our knowledge on the anti-inflammatory effects of CsA, FK-506 and pimecrolimus, and suggest that calcineurin may have a role in the regulation of iNOS expression.

Keywords: nitric oxide, nitric oxide synthase, inflammation, calcineurin inhibitors, mRNA stability, macrophages

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Introduction

Nitric oxide (NO) is a gaseous signaling molecule with various regulatory functions in human physiology and pathophysiology. It is produced in increased amounts in inflammation and is known to act as a regulatory, pro-inflammatory and cytotoxic modulator in various inflammatory diseases [1,2]. NO is produced by three nitric oxide synthase (NOS) enzymes; endothelial NOS (eNOS), neuronal NOS (nNOS) and inducible NOS (iNOS). eNOS and nNOS are calcium-dependent constitutive enzymes and are responsible for the low physiological production of NO. iNOS is increased in response to bacterial products and pro-inflammatory cytokines, and once expressed it produces large amounts of NO for prolonged periods [2-4]. High levels of NO and peroxynitrite (which is formed in a reaction of nitric oxide and superoxide) can lead to increased cytotoxicity in the inflammatory focus. Data from experimental inflammatory models show that compounds that inhibit iNOS expression or the enzymatic activity of iNOS have anti-inflammatory effects [5-7].

Calcineurin inhibitors cyclosporin A (CsA) and tacrolimus (FK-506) are used as immunosuppressants in the prevention of allograft rejection and as anti-inflammatory drugs. Once bound to their intracellular receptor immunophilins in lymphocytes, these drugs inhibit Ca²⁺/calmodulin-dependent protein phosphatase calcineurin, which leads to the inhibition of the activation and nuclear translocation of the transcription factor NFAT (nuclear factor of activated T cells). NFAT is important in the transcriptional regulation of various cytokines in activated T-cells. CsA and FK-506 have been shown to also affect other cells by NFAT-dependent and -independent mechanisms [16-20]. We and others have shown that calcineurin inhibitors reduce NO production in macrophages exposed to inflammatory stimuli [21-26]. The aim of the present study was to investigate in more detail the mechanisms mediating the inhibitory effects of CsA, FK-506 and pimecrolimus on NO production in response to inflammatory stimuli.

Methods

Materials

Dulbecco’s Modified Eagle’s Medium (DMEM) and Eagle’s Minimal Essential Medium (EMEM) were from Cambrex Bioproducts Europe (Verviers, Belgium). All medium supplements were obtained from Invitrogen (Carlsbad, CA, USA) except that non-essential amino acids, sodium pyruvate and sodium bicarbonate were from Cambrex Bioproducts Europe (Verviers, Belgium). Cyclosporin A and FK-506 were supplied by Calbiochem (La Jolla, CA, USA) and pimecrolimus by Novartis Pharma AG (Basel, Switzerland). pGL3-promoter vector, passive lysis buffer and luciferase assay reagents were from Promega (Madison, WI, USA). All other reagents were from Sigma (St. Louis, MO, USA).
Cell culture
Murine J774 macrophages and murine L-929 fibroblasts were obtained from American Type Culture Collection (Rockville, MD, USA). Macrophages were cultured at 37°C (in 5% carbon dioxide) in DMEM with glutamax-I containing 10% heat-inactivated foetal bovine serum, penicillin (100 units/ml), streptomycin (100 µg/ml) and amphotericin B (250 ng/ml). Fibroblasts were cultured at 37°C (in 5% carbon dioxide) in EMEM containing 10% heat-inactivated foetal bovine serum, penicillin (100 units/ml), streptomycin (100 µg/ml) and amphotericin B (250 ng/ml) and supplemented with sodium bicarbonate (1 mM), non-essential amino acids (0.1 mM) and sodium pyruvate (0.15%). Cells were passaged up to 20 times and harvested with trypsin-EDTA. Cells were seeded in 96-well plates for XTT-test, in 24-well plates for nitrite measurements, RNA extraction and transfection experiments, in 6-well plates for iNOS Western blot analysis and in 10-cm dishes for NF-κB and STAT-1 Western blot, and grown to confluence (approximately 90% confluency in transfection experiments) before the commencement of the experiments. Confluent cell cultures were exposed to fresh culture medium containing the compounds of interest and cultured for the indicated times.

Preparation of stable L-929 pNF-κB and L-929 pGAS reporter cell lines
Luciferase reporter constructs for NF-κB [pNFκB(luc)neo] and STAT-1 [pGAS(luc)neo] were kindly provided by Professor Hartmut Kleinert at the University of Mainz, Germany. pNFκB(luc)neo contained five NF-κB binding sites and pGAS(luc)neo four GAS sites to drive luciferase expression. Both plasmids had a neomycin resistance gene under control of TK promoter for mammalian selection. To create a stable transfection, L-929 cells were transfected with pNKκB(luc)neo or pGAS(luc)neo reporter plasmids using Lipopectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. Transfected cells were selected with G418 disulfate salt (800 µg/ml) (Sigma-Aldrich Co., St Louis, MO, USA). After selection, the survived clones were pooled to give rise to L-929 pNF-κB and L-929 pGAS cell lines and further cultured in the presence of 400 µg/ml of G 418.

XTT-test
Cell viability was tested using Cell Proliferation Kit II that measures the cells’ ability to metabolise XTT to formazan by mitochondrial dehydrogenase activity, a function that only occurs in viable cells (Roche Diagnostics GmbH, Mannheim, Germany). Cells were incubated with the tested compounds and LPS or LPS+IFNγ for 20h before the addition of sodium 3'-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis-(4-methoxy-6-nitro) benzene sulfonic acid hydrate (XTT) (final concentration 300 µg/ml) and N-methyl dibenzopyrazine methyl sulphate (8.2 µM). Cells were incubated for another 4h and the amount of formazan accumulating in growth medium was assessed spectrophotometrically. Triton-X treated cells were used as a positive control. Conditions were considered toxic if the cells’ ability to metabolise XTT to formazan was lowered by more than 20% as compared to cells exposed to LPS or LPS+IFNγ only.

Nitrite determinations
Measurement of nitrite accumulation into the culture medium was used to determine NO production. At the indicated time points, the culture medium was collected and nitrite was measured by the Griess reaction. A NOS inhibitor L-NIO was used to ensure that the measured nitrite was due to NO produced by the NOS pathway in the cell culture.

Preparation of cell lysates for iNOS Western blot
After the desired time of incubation the cell pellets were lysed in ice-cold extraction buffer (10 mM Tris-base, pH 7.4, 5 mM EDTA, 50 mM NaCl, 1% Triton X-100, 0.5 mM phenylmethyl sulphonyl fluoride, 1 mM sodiumorthovanadate, 20 µg/ml leupeptin, 50 µg/ml aprotinin, 5 mM NaF, 2 mM sodiumpyrophosphate and 10 µM n-octyl-beta-D-glucopyranoside). Samples were...
incubated on ice for 15 min, centrifuged, and the resulting supernatant was diluted 1:4, boiled for 5 min in sample buffer (6.25 mM Tris-HCl, 10% glycerol, 2% SDS and 0.025% 2-mercapto-ethanol) and stored at -20°C until analysed. The Coomassie blue method was used to measure the protein content of the samples.

**Preparation of nuclear extracts for p65 and STAT-1α Western blot**

The cells were seeded on 10-cm dishes and grown to confluence. The cells were incubated with the compounds of interest for 30 min (p65) or for 6h (STAT-1α). After incubation, the cells were washed with ice-cold PBS and solubilised in hypotonic buffer A (10 mM HEPES-KOH, pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM dithiotreitol (DTT), 0.2 mM PMSF, 10 µg/ml leupeptin, 25 µg/ml aprotinin, 0.1 mM EGTA, 1 mM Na₂VO₄, 2 mM NaF) and incubated for 10 min on ice. Thereafter, the cells were vortexed for 30 s and the nuclei were separated by centrifugation at 4°C, 15 000 rpm, 10 s. The samples were suspended in buffer C (20 mM HEPES-KOH, pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM DTT, 0.2 mM PMSF, 10 µg/ml leupeptin, 25 µg/ml aprotinin, 0.1 mM EGTA, 1 mM Na₂VO₄, 1 mM NaF) and incubated on ice for 20 min. The samples were vortexed for 30 s and nuclear extracts were obtained by centrifugation at 4°C and 15 000 rpm for 2 min. The Coomassie blue method was used to measure the protein content of the samples.

**Western blot analysis of iNOS, p65 and STAT-1α proteins**

Protein samples (20 µg) were separated by SDS-PAGE on 8% polyacrylamide gel and transferred to nitrocellulose membrane. Bound antibody [rabbit polyclonal antibodies for iNOS and STAT-1α (Santa Cruz Biotechnology, Santa Cruz, CA, USA), or for p65 subunit of NF-κB (Cell Signaling, Danvers, MA, USA)] was detected using goat anti-rabbit polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and visualised by SuperSignal® chemiluminescent substrate (Pierce, Cheshire, UK) and FluorChem 8800 imaging system (Alpha Innotech Corporation, San Leandro, CA). The quantitation of the chemiluminescent signal was carried out using FluorChem software version 3.1. Actin and laminA/C were used as loading controls for iNOS in whole cell extracts, and for p65 and STAT-1α in nuclear extracts, respectively. Antibodies for actin and laminA/C were from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

**RNA extraction and real-time RT-PCR of iNOS, LUC and GAPDH mRNAs**

Cells stimulated with the compounds of interest were trypsinised after the desired time of incubation. The cells were washed twice with PBS, lysed and purified using QIAshredder™ (QIAGEN, Valencia, CA, USA). Thereafter RNA was extracted using RNeasy® kit for isolation of total RNA (QIAGEN Inc.). Total RNA (25 ng) was reverse-transcribed to cDNA using TaqMan Reverse Transcription reagents and random hexamers (Applied Biosystems, Foster City, CA, USA). Reverse-transcriptase (RT) reaction parameters were as follows: incubation at 25°C for 10 min, RT at 48°C for 30 min, and RT inactivation at 95°C for 5 min. Total RNA extracted from the L-929 pNF-kB and L-929 pGAS cell lines was treated with DNase I (Fermentas UAB, Vilnius, Lithuania) prior conversion to cDNA. cDNA obtained from the RT reaction (amount corresponding to approximately 1 ng of total RNA) was subjected to PCR using TaqMan Universal PCR Master Mix and ABI PRISM 7000 Sequence detection system (Applied Biosystems). The primer and probe sequences and concentrations were optimised according to the manufacturer’s instructions in TaqMan Universal PCR Master Mix Protocol part number 4304449 revision C and were as follows: 5’-CCTGGTACGGGCATTGCT-3’, 5’-GCTCATGCGGCCTCCTT-3’, (forward and reverse mouse iNOS primers, respectively), 5’-CAGCAGGGCTCCATGACTCCC-3’ (mouse iNOS probe), 5’-AAAAAGTTG CGCGAGGAG-3’, 5’-TTTTTCATTGC
GTCGAGTTTTCC-3’ (forward and reverse luciferase primers, respectively), 5’-TGTGTGTGGACGAAAGTACCGAAGTGCTTTAC3’ (luciferase probe), 5’-GCATGGCCTTCCGTGTTC-3’, 5’-GATGTCATCATACTTGGCAAGTTT-3’ (forward and reverse mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers, respectively), 5’-TCGTGGATCTGGTACGCC-3’ (mouse GAPDH probe).

Concentrations for all primers were 300 nM and for probes 150 nM. All probes contained 6-FAM (6-carboxy-fluorescein) as 5’-reporter dye and TAMRA (6-carboxy-tetramethyl-rhodamine) as 3’-quencher.

The PCR reaction parameters were as follows: incubation at 50ºC for 2 min, incubation at 95ºC for 10 min, and thereafter 40 cycles of denaturation at 95ºC for 15 s and annealing and extension at 60ºC for 1 min. Each sample was determined in duplicate. The relative mRNA levels in the test samples were then calculated from the standard curve. The results of iNOS mRNA levels were normalised against GAPDH mRNA in each sample.

**Actinomycin D assay**

Actinomycin D assay was utilized to study the decay of iNOS mRNA. In this method, cells were incubated with the compounds of interest for 6 h and actinomycin D (0.1 µg/ml), which is an inhibitor of transcription, was then added to the cells. RNA was extracted at different time points following addition of actinomycin D, and subjected for quantitative RT-PCR to measure the remaining iNOS mRNA.

**Transient transfection and firefly luciferase assay**

Luciferase reporter gene construct pGL3-iNOS 3’UTR (kindly provided by Professor Matti Lang, Uppsala University, Sweden) contains the full length mouse iNOS 3’UTR cloned behind luciferase reporter gene in pGL3-promoter vector (Promega, Madison, WI, USA) [12]. L-929 cells were grown on 24-well plates to approximately 90% confluency for 24h. The cells were then transfected with 0.1 µg/ml, which is an inhibitor of transcription, was then added to the cells. RNA was extracted at different time points following addition of actinomycin D, and subjected for quantitative RT-PCR to measure the remaining iNOS mRNA.

**Figure 1**

A Effects of increasing concentrations of CsA, FK-506 and pimecrolimus on LPS (10 ng/ml) – induced NO production in J774 macrophages. B Effects of increasing concentrations of CsA, FK-506 and pimecrolimus on LPS (100 ng/ml) and IFNγ (10 ng/ml) – induced NO production in L-929 fibroblasts. C Effects of CsA, FK-506 and pimecrolimus (10 µM) on LPS (10 ng/ml) – induced NO production in J774 cells. Drugs were added into the cell culture at different time points in relation to LPS. NO production was determined after 24h incubation time by measuring nitrite accumulation into the culture medium by Griess reaction. NO production was not detectable in untreated cells. NO levels in LPS-treated cells were set as 100% and the other values were related to that. **p<0.01, *p<0.05, mean ± SEM, n=6.**
Effects of calcineurin inhibitors CsA, FK-506 and pimecrolimus on NO production

Bacterial lipopolysaccharide (LPS) induced iNOS expression and NO production in J774 macrophages. NO production was not detectable in untreated cells. When CsA, FK-506 or pimecrolimus was added into the cell culture with LPS, the drugs inhibited NO production in a concentration-dependent manner. CsA was more potent than FK-506 and pimecrolimus (Figure 1A). The effects of calcineurin inhibitors on NO production were also tested in L-929 mouse fibroblast cells. In untreated cells no NO production was detected. All three inhibitors reduced NO production in a concentration-dependent manner in cells stimulated with the combination of LPS and interferon γ (IFNγ) (Figure 1B).

In the subsequent studies we added the calcineurin inhibitors into the macrophage culture 1h prior, at the same time, or 1-12h after LPS, and measured their effects on NO production. The inhibitory action of CsA, FK-506 and pimecrolimus was most pronounced when the drug was added 1h prior, at the same time, or 1-6h after LPS (Figure 1C). If the drug was added at later time-points (8 and 12h after LPS) the inhibitory action declined. Cytotoxicity was assessed by XTT-test, and the combinations of compounds used were not toxic for the cells.

Results

Effects of calcineurin inhibitors CsA, FK-506 and pimecrolimus on LPS, or LPS and IFNγ-induced iNOS expression and NO production

Bacterial lipopolysaccharide (LPS) induced iNOS expression and NO production in J774 macrophages. NO production was not detectable in untreated cells. When CsA, FK-506 or pimecrolimus was added into the cell culture with LPS, the drugs inhibited NO production in a concentration-dependent manner. CsA was more potent than FK-506 and pimecrolimus (Figure 1A). The effects of calcineurin inhibitors on NO production were also tested in L-929 mouse fibroblast cells. In untreated cells no NO production was detected. All three inhibitors reduced NO production in a concentration-dependent manner in cells stimulated with the combination of LPS and interferon γ (IFNγ) (Figure 1B).

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The effects of CsA, FK-506 and pimecrolimus were studied on iNOS protein expression in J774 macrophages by Western blot. Untreated cells did not express detectable amounts of iNOS protein but its expression was significantly increased by LPS. All three calcineurin inhibitors suppressed iNOS protein expression (Figure 2).

Effects of calcineurin inhibitors on transcription factors NF-κB and STAT-1

Nuclear factor - κB (NF-κB) and signal transducer and activator of transcription 1 (STAT-1) are important transcription factors for iNOS [27,28]. Therefore we studied whether calcineurin inhibitors CsA, FK-506 or pimecrolimus modulate LPS-induced
NF-κB and STAT-1 activation in J774 cells by measuring the nuclear translocation of the factors by Western blot, and by measuring NF-κB and STAT-1 mediated transcription by using reporter cell lines. LPS enhanced NF-κB levels in nuclear extracts, and maximal effect was found after 30 min incubation. CsA, FK-506 or pimecrolimus (10 μM) did not inhibit the LPS-induced nuclear translocation of NF-κB (Figure 3A).

Transcriptional activity of NF-κB responsive promoter in L-929 pNF-κB cells was enhanced by LPS+IFNγ, and no inhibition on the NF-κB mediated transcription was seen in the presence of calcineurin inhibitors (Figure 3B). LPS also increased nuclear STAT-1 levels, which were increased up to 6h incubation. CsA, FK-506 or pimecrolimus (10 μM) had no effect on the LPS-induced nuclear translocation of STAT-1 (Figure 3C). Gamma-activated site (GAS) is a STAT-1 binding site in target genes, e.g. in iNOS. Therefore, we used L-929 pGAS cell line to investigate STAT-1 mediated transcription. Transcriptional activity of GAS promoter was enhanced after treatment with LPS+IFNγ. In the presence of calcineurin inhibitors, GAS-mediated transcription remained unaltered (Figure 3D).

Effects of calcineurin inhibitors on iNOS mRNA expression and stability
To investigate the effects of calcineurin inhibitors on iNOS mRNA expression, LPS-treated macrophages were incubated in the presence or in the absence of CsA, FK-506 or pimecrolimus (10 μM), and iNOS mRNA levels were measured by quantitative real-time RT-PCR after 6 and 24h incubations. Calcineurin inhibitors had practically no effect on LPS-induced iNOS mRNA levels during 6h incubation. However, all three calcineurin inhibitors significantly reduced iNOS mRNA levels when measured after 24h incubation (Figure 4A). Similarly to the results from the experiments with J774 macrophages, CsA reduced iNOS mRNA levels in L-929
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![Graph A]

**Figure 4 A** Effects of CsA, FK-506 and pimecrolimus on LPS-induced iNOS mRNA accumulation in J774 macrophages. Cells were treated with LPS (10 ng/ml) or with LPS and calcineurin inhibitor (10 µM). RNA was extracted after 6 and 24h incubation.

![Graph B]

**Figure 4 B** Effects of CsA, FK-506 and pimecrolimus on LPS-induced iNOS mRNA accumulation in L-929 fibroblasts. Cells were treated with LPS (100 ng/ml) and IFN-γ (10 ng/ml) or with LPS+IFN-γ and calcineurin inhibitor (10 and 30 µM). RNA was extracted after 8 and 24h incubation.

iNOS and GAPDH mRNAs were measured by real-time RT-PCR. iNOS mRNA levels were normalised against GAPDH. Untreated cells did not produce detectable amounts of iNOS mRNA in either cell line. iNOS mRNA levels in LPS or LPS+IFN-γ treated cells were set at 100% and other values were related to that. * p<0.05, ** p<0.01, mean ± SEM, n=3-6.

To further investigate the kinetics of the reduction of iNOS mRNA accumulation, J774 cells were treated with LPS in the presence or in the absence of calcineurin inhibitor (10 µM), and iNOS mRNA levels were measured after different incubation times. iNOS mRNA levels declined faster in cells treated with LPS and calcineurin inhibitors compared to cells treated with LPS alone (Fig. 5A). This suggests that calcineurin inhibitors may regulate iNOS mRNA expression by affecting mRNA stability.

In the further studies, actinomycin D assay was carried out. The cells were first cultured with LPS and CsA, FK-506 or pimecrolimus (10 µM) for 6h and thereafter actinomycin D was added to stop mRNA synthesis. iNOS mRNA levels were then measured 0, 2, 4, 6 and 18h after addition of actinomycin D. Surprisingly, in these experiments we did not see any differences in the mRNA decay rates between cells treated with or without calcineurin inhibitors (Figure 5B). Because, it is known that actinomycin D may itself affect the stability of mRNAs [29,30] we wanted to have another measure of iNOS mRNA stability. To obtain that, L-929 cells were transiently transfected with a luciferase reporter construct containing full length iNOS 3’ untranslated region (UTR) cloned behind luciferase gene [12]. When the cells were stimulated with LPS and IFNγ, luciferase activity was reduced in those cells treated with CsA compared to cells treated with LPS+IFNγ only (Figure 5C). This suggests that calcineurin inhibitors regulate iNOS stability by a 3’UTR-dependent mechanism.

**Discussion**

Calcineurin inhibitors cyclosporin A and FK-506 (tacrolimus) are potent immunosuppressive and anti-inflammatory agents proved to be beneficial in the treatment of allograft rejection and inflammatory diseases such as rheumatoid arthritis [18,31,32]. In the present study, we found that CsA, FK-506 and pimecrolimus down-regulated NO production by destabilising iNOS mRNA. The results obtained from reporter gene experiments suggest that CsA exerts its effect by affecting the 3’ UTR of iNOS mRNA.

Calcineurin inhibitors CsA, FK-506 and pimecrolimus inhibited LPS-induced NO production in a concentration-dependent manner in macrophage and fibroblast cell lines. In T-cells, CsA and FK-506 act by suppressing the T-cell receptor activated transduction pathway. The drugs inhibit
calcineurin, a Ca\(^{2+}\)/calmodulin-dependent phosphatase, resulting in the inhibition of the activation of transcription factor NFAT [17]. In addition, CsA and FK-506 have been reported to have NFAT-independent effects in some cell types [19,20]. iNOS promoter has not been reported to contain a binding site for NFAT [2,3]. Therefore it is likely that inhibition of NFAT by CsA and FK-506 is not the mechanism by which these drugs regulate iNOS expression and we sought other mechanisms of action.

NF-κB and STAT-1 are important transcription factors for the induction of iNOS expression [27,28]. Therefore we tested the effects of CsA, FK-506 and pimecrolimus on LPS-induced activation of NF-κB and STAT-1. NF-κB and STAT-1 were activated by LPS, but the nuclear translocation of those transcription factors was not suppressed by calcineurin inhibitors measured by Western blot. Thereafter effects of calcineurin inhibitors on NF-κB and STAT-1 mediated transcription was measured by using reporter cell lines. Calcineurin inhibitors were not found to inhibit transcription driven by NF-κB or GAS promoters. CsA has been reported to inhibit NF-κB activation in rat mesangial cells [33] but the present results do not support suppressed NF-κB activation as the mechanism of the inhibitory effect of calcineurin inhibitors on iNOS expression in activated macrophages.

We investigated the effects of calcineurin inhibitors when given at different time-points in relation to the stimulus LPS. If the drugs were added 4-6h after LPS, they still had a clear inhibitory effect on NO production, but no inhibition was seen if the drugs were given 12h after LPS. These results suggest that the calcineurin inhibitors do not inhibit the activity of iNOS enzyme or the early transcriptional events of iNOS gene.

The post-transcriptional regulation of transiently expressed inflammatory genes is potentially important therapeutically. The control of iNOS expression at the level of the regulation of iNOS mRNA stability has gained increased attention. iNOS mRNA contains AU-rich elements in its
3’ untranslated region [34,35] which are known to destabilise mRNA. TGF-β and 8-Br-cGMP have been reported to destabilise iNOS mRNA in mouse peritoneal macrophages and in human mesangial cells respectively [36,37]. Dexamethasone and a JNK inhibitor SP600125 have been shown to destabilise iNOS mRNA in murine J774 macrophages [38,39], and using siRNA approach, JNK was recently shown to stabilise iNOS mRNA in human epithelial cells [40].

Proteins known to bind to and regulate iNOS mRNA stability include HuR (a member of the embryonic lethal abnormal vision (ELAV) protein family), KH-type splicing regulatory protein (KSRP) and polypyrimidine tract binding protein (PTB) [9,11,13,15]. In addition, a cytokine inducible protein tristetraprolin (TTP) has been shown to regulate iNOS mRNA stability by governing the interaction of destabilising protein KSRP and iNOS mRNA [14,15]. Also, heterogeneous nuclear ribonucleoprotein (hnRNP) L has been shown to interact with the 3’UTR of the iNOS mRNA and may have role in the regulation of iNOS expression [11]. In the present study, we tested the hypothesis that calcineurin inhibitors enhance iNOS mRNA decay. During 6 or 8h incubation in stimulated macrophages or fibroblasts respectively, calcineurin inhibitors had no effects on iNOS mRNA levels whereas when measured after 24h incubation iNOS mRNA levels were significantly lower in cells treated with calcineurin inhibitors. This suggests that post-transcriptional events are important in the inhibitory action of calcineurin inhibitors. However, calcineurin inhibitors did not alter iNOS mRNA levels when inhibitor of transcription actinomycin D was added into the culture 6h after LPS and calcineurin inhibitors. This suggests that calcineurin inhibitors may be involved in the regulation of the synthesis of factors that regulate iNOS mRNA stability and that the production of these factors is inhibited by actinomycin D. This is supported by the earlier finding by Nair et al. (1994) who demonstrated that CsA destabilised IL-3 mRNA. This required ongoing transcription and the effect was not seen in the presence of actinomycin D [41]. Since actinomycin D may modulate the effect of calcineurin inhibitors, we used one calcineurin inhibitor, CsA, to investigate mRNA stability in a reporter gene setup. iNOS mRNA stability is known to be regulated by 3’UTR of the mRNA [2,3]. Therefore, we used a luciferase reporter construct containing the full length 3’UTR of murine iNOS mRNA cloned behind the luciferase gene. CsA reduced luciferase activity compared to the control cells stimulated with LPS and IFNγ in the absence of CsA. This suggests that CsA targets 3’UTR of iNOS mRNA and regulates iNOS expression through mRNA stability.

Kim et al. [25] demonstrated that calcineurin is required for full iNOS expression in macrophages, and other studies have shown that calcineurin inhibitors CsA and/or FK-506 inhibit NO production in macrophages [21-24,26]. In the present study, CsA inhibited NO production more potently than FK-506, which has also previously been shown in macrophages [22,24]. We found that another calcineurin inhibitor, pimecrolimus, also inhibited iNOS expression in activated macrophages and fibroblasts. The present study extends the previous data by providing evidence to support that calcineurin inhibitors CsA, FK-506 and pimecrolimus inhibit LPS-induced NO production and iNOS expression in response to inflammatory stimuli by destabilising iNOS mRNA by a 3’UTR-dependent mechanism.

Acknowledgements
We wish to thank Professor Hartmut Kleinert at the University of Mainz, Germany for kindly providing us the luciferase reporter constructs for NF-κB [pNFxB(luc)neo] and STAT-1 [pGAS(luc)neo], Professor Matti Lang for providing the pGL3-iNOS 3’UTR reported gene construct, and Mrs Niina Ikonen, Mrs Jaana Tägtström and Mrs Heli Määttä for their skilful technical assistance. The study was supported by grants from the Finnish Cultural Foundation, the Finnish Cultural Foundation Pirkannaa Regional
Fund, the Medical Research Fund of Tampere University Hospital and Drug 2000 program by the Finnish Funding Agency for Technology and Innovation (TEKES).

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