RIKU KORHONEN

Regulation of Inducible Nitric Oxide Production in Activated Macrophages

ACADEMIC DISSERTATION

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LIST OF ORIGINAL COMMUNICATIONS

This thesis is based on the following original communications, referred to in the text by their roman numerals I-IV.

I. Korhonen R, Korpela R, Saxelin M, Mäki M, Kankaanranta H, and Moilanen E. (2001): Induction of nitric oxide synthesis by probiotic *Lactobacillus rhamnosus* GG in J774 macrophages and human T84 intestinal epithelial cells. *Inflammation* 25: 223-32.

II. Korhonen R, Korpela R, and Moilanen E. (2002): Signalling mechanisms involved in the induction of inducible nitric oxide synthase by *Lactobacillus rhamnosus* GG, endotoxin, and lipoteichoic acid. *Inflammation*, in press.

III. Korhonen R, Kankaanranta H, Lahti A, Lähde M, Knowles RG, and Moilanen E. (2001) Bi-directional effects of the elevation of intracellular calcium on the expression of inducible nitric oxide synthase in J774 macrophages exposed to low and to high concentrations of endotoxin. *Biochem J* 354: 351-358.

IV. Korhonen R, Lahti A, Hämäläinen M, Kankaanranta H, and Moilanen E. (2002) Dexamethasone inhibits iNOS expression and nitric oxide production by destabilizing mRNA in LPS-treated macrophages. (submitted for publication)

ABBREVIATIONS

A23187	5-(Methylamino)-2-[(2*R*,3*R*,6*S*,8*S*,9*R*)-3,9,11-trimethyl-8-[(1S)-1-methyl-2-oxo-2-(1H)-pyrrol-2-yl)-ethyl]-1,7dioxaspiro[5.5]undec-2-yl]methyl]-4-benzoxazolecarboxylic acid, calcium ionophore
ARE	adenosine-uridine-rich element
BH_4	tetrahydrobiopterin
$[Ca^{2+}]_i$	intracellular free calcium concentration
cNOS	constitutively expressed NOS (nNOS and eNOS)
CsA	cyclosporine A
DAHP	2,4-diamino-6-hydroxypyrimidine, inhibitor of BH_4 synthesis
eNOS	endothelial nitric oxide synthase
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GRE	glucocorticoid responsive element
GW273629	*S*-[2-[(1-iminoethyl)amino]ethyl]-4,4-dioxo-L-cysteine, selective iNOS inhibitor
GW274150	*S*-[2-[(1-iminoethyl)amino]ethyl]-L-homocysteine, selective iNOS inhibitor
IFNγ	gamma interferon
IL-1	interleukin-1
iNOS	inducible nitric oxide synthase
IRAK	interleukin receptor-associated kinase
IRF-1	interferon-regulatory factor 1
Jak 1, 2	members of a family of Janus kinases
L-NAME	N^G-nitro-L-arginine methyl ester, NOS inhibitor
L-NIL	L-N^G-[1-iminoethyl]lysine, NOS inhibitor
L-NIO	N-iminoethyl-L-ornithine, NOS inhibitor
L-NMMA	N^G-monomethyl-L-arginine, NOS inhibitor
LPS	lipopolysaccharide
LTA	lipoteichoic acid
MAP-kinase	mitogen- activated protein kinase
mRNA	messenger ribonucleic acid
NF-κB	nuclear factor kappa B
nNOS	neuronal nitric oxide synthase
NO	nitric oxide

NOS	nitric oxide synthase
PD98059	2-(2-Amino-3-methoxyphenyl)-4H-1-benzopyran-4-one, p42/44 MAP-kinase inhibitor
PDTC	pyrrolidinedithiocarbamate, NF-κB inhibitor
PKC	protein kinase C
Ro 31-8220	3-{1-[3-(Aminidothio)propyl]-3-indolyl}-4-(1-methyl-3-indolyl)-1H-pyrrole-2,5-dione, PCK inhibitor
RPA	ribonuclease protection assay
RT-PCR	reverse transcriptase polymerase chain reaction
SB203580	4-[5-(Fluorophenyl)-2-[4-(methylsulphonyl)phenyl]-1*H*-imidazol-4-yl]pyridine hydrochloride, p38 MAP-kinase inhibitor
STAT	signal transducers and activators of transcription
TGF-β	transforming growth factor beta
TLR	Toll-like receptor
TNFα	tumour necrosis factor alpha
3'-UTR	3´-untranslated region of mRNA
1400W	*N*-[3-(aminomethyl)benzyl]acetamidine, selective iNOS inhibitor

ABSTRACT

Inflammation is a protective response of the host to infectious/injurious factors threatening. The purpose of inflammation is to eliminate the cause of the response, and to repair and/or regenerate the injured tissue. In response to threatening factors, immune cells of the host produce inflammatory mediators such as nitric oxide (NO), cytokines and eicosanoids, which regulate the course of the inflammation.
NO is a gaseous, water- and lipid-soluble molecule that regulates various physiological responses in the cardiovascular and nervous system. In inflammation, immune and tissue cells are elicited to produce NO via inducible nitric oxide synthase (iNOS) pathway in response to infectious and injurious agents and proinflammatory cytokines secreted by the host. In immune system, NO plays a central role regulating several cellular and vascular responses of inflammation, and serves as a killing mechanism in the host-defence reactions. Experimental and clinical data suggest that increased iNOS expression and NO production are also involved in many chronic inflammatory diseases such as asthma, rheumatoid arthritis and inflammatory bowel disease.

The induction of iNOS expression and the production of NO have been found to take place in various cells along the inflammatory process, but the mechanisms involved are yet to be established. In the present study, mechanisms regulating the expression of iNOS and the production of NO in activated macrophages *in vitro*, were investigated. The detailed aims were to investigate the role of the free intracellular calcium and the effect of anti-inflammatory glucocorticoid dexamethasone on iNOS expression and NO production. In addition, the effects of probiotic bacteria *Lactobacillus rhamnosus* GG were investigated. iNOS expression and NO production were found to be induced in response to *Lactobacillus rhamnosus* GG suggesting that this probiotic has immunomodulatory effects. The main finding of the study was that dexamethasone and elevated free intracellular calcium inhibited NO production by reducing iNOS mRNA stability in macrophages exposed to bacterial endotoxin. This is a new mechanism of action by which glucocorticoids regulate NO production and the inflammatory process. The results also emphasize the importance of post-transcriptional mechanisms in the regulation of inducible NO production in activated inflammatory cells. The present study provides novel information on the regulation of NO production through iNOS pathway, and illuminates the mechanisms involved in the regulation of inflammation, which is of importance in the search of new targets for therapeutic interventions in inflammatory diseases.

INTRODUCTION

When the host is exposed to infectious/injurious factor(s), leukocytes and tissue cells secrete mediators which stimulate vascular and cellular reactions typical for the inflammatory process. The aim of the inflammatory reaction is to eliminate the chemical, physical or infectious factor(s) which have induced inflammation, and to repair damage and/or regenerate the injured tissue. Among cells activated in this process are macrophages which are able to produce various inflammatory mediators, including nitric oxide (NO). NO is a lipid- and water-soluble reactive gaseous molecule that regulates various physiological and pathophysiological processes in the body. Endogenous NO was discovered in 1987 when endothelium-derived relaxing factor was characterized to be NO (Palmer et al. 1987), and subsequently, NO was also found in neurons (Bredt and Snyder 1990). NO entered the scene of immune response in the late 1980s and early 1990s as a factor produced by a distinct enzyme, inducible nitric oxide synthase (Xie et al. 1992), and as a molecule responsible for the antitumoricidal and antimicrobial effects of activated macrophages. Since then, our conception of NO has become more diversified. iNOS expression and NO synthesis has been found to take place in various cell types, and NO has been found to be involved in several inflammatory processes, having both beneficial and detrimental effects in the host depending on the type and phase of inflammation.

In principle, induction of iNOS expression and NO formation is associated with inflammation, and iNOS is not normally present in resting cells, which underlines the importance of regulation of iNOS expression. Macrophages are important regulatory and effector cells in innate immune reactions, and have been found to produce NO via the iNOS pathway. This study was designed to investigate the mechanisms regulating iNOS expression and NO production in macrophages. The results show that regulation of iNOS expression takes place at both transcriptional and post-transcriptional levels. Since iNOS expression is related to processes both beneficial and detrimental for the host, knowledge of the mechanisms involved in the regulation of iNOS is of importance in the search for novel potential targets for pharmacological interventions in the treatment of inflammatory and other diseases.

REVIEW OF THE LITERATURE

1. Nitric Oxide

In 1987 NO was discovered to be the molecule responsible for endothelium-dependent relaxation of blood vessels following treatment with acetylcholine (Palmer et al. 1987, Ignarro et al. 1987). Soon after that, NO derived from vascular cells was shown to be synthesized from L-arginine (Palmer et al. 1988a). NO is a readily diffusible reactive gaseous molecule with half-life estimated to be up to few seconds. To date, NO has been recognized to be involved in numerous physiological and pathophysiological processes in mammals, including regulation of the circulation, neuronal functions and host defence (Wink and Mitchell 1998, Moilanen et al. 1999).

1.1. Biosynthesis of nitric oxide and isoforms of NOS

NO is formed enzymatically from L-arginine by three distinct NO synthase (NOS) enzymes. The functionally active NOS enzyme exists in a dimeric form. All three NOS monomers have a bidomain structure containing an oxygenase in N-terminal domain and a reductase in the C-terminal domain linked by a calmodulin recognition site. The reductase domain contains binding sites for co-factors NADPH, flavin adenine dinucleotide and flavin mononucleotide, whereas in the oxygenase domain there are binding sites for L-arginine and co-factors haem and tetrahydrobiopterin (BH_4) (Janssens et al. 1992, Lowenstein et al. 1992, Charles et al. 1993, Alderton et al. 2001).
The synthesis of NO from L-arginine involves two separate reactions. In the first step, termed NOS monooxygenation I reaction, L-arginine is converted to N^{ϖ}-hydroxy-L-arginine, which is an intermediate in NO synthesis, and this reaction requires NADPH and oxygen. In the second phase, termed NOS monooxygenation reaction II, N^{ϖ}-hydroxy-L-arginine is oxidized to NO and citrulline. Flavin adenine dinucleotide and flavin mononucleotide, calmodulin, and BH_4 act as co-factors in the process (Bredt and Snyder 1990, Mayer et al. 1990, Bredt et al. 1991, Stuehr et al. 1991, Alderton et al. 2001). The reactions of NO synthesis are shown in Figure 1.

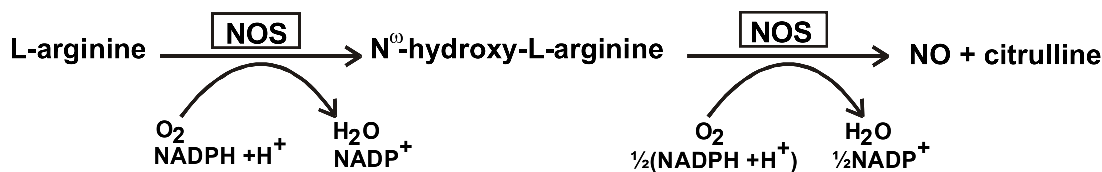


Figure 1. Biosynthesis of NO from L-arginine

All three are encoded by different genes sharing 51-57% homology in their amino acid sequence (Bredt et al. 1991, Janssens et al. 1992, Charles et al. 1993). Constitutively expressed forms of NOS (cNOS) are neuronal NOS (nNOS or NOS1) and endothelial NOS (eNOS or NOS3), the third isoform being inducible NOS (iNOS or NOS2). NO synthesis reactions are basically identical in all three NOS isoforms, but the regulation of NO production differs between them.

eNOS and nNOS were first found in the vascular endothelium and in the central and peripheral nervous system, respectively, but are now known to be expressed in various tissues and cell types. The production of NO by these constitutively expressed NO synthases is regulated mainly at the level of enzyme activity, and they produce small amounts of NO. Changes in the concentration of intracellular calcium ($[Ca^{2+}]_i$) play a central role in the regulation of NO synthesis by cNOS enzymes. Agonists such as acetylcholine in endothelial cells and glutamate in the brain induce an increase in $[Ca^{2+}]_i$ through their receptors, and activate NOS enzyme and NO production. The elevation of $[Ca^{2+}]_i$ stabilizes the calmodulin binding to its binding site in cNOS, thereby initiating NO synthesis, *i.e.* eNOS and nNOS are calcium-dependent NOS enzymes (Bredt and Snyder 1990, Abu-Soud and Stuehr 1993). In principle, both isoforms participate in the regulation of physiological processes in the cardiovascular and nervous system (Moncada and Higgs 1995, Christopherson and Bredt 1997, Alderton et al. 2001).

In addition, the expression of eNOS and nNOS is also regulated to some extent at transcriptional level. Research during recent years has revealed that nNOS expression may be regulated by e.g. neuronal activity, steroid hormones, inflammatory cytokines and bacteria-derived molecules (Liu et al. 1996, Xu et al. 1996, Kleinert et al. 2000). Similarly, eNOS expression is susceptible to expressional regulation by local environmental conditions. Shear stress of blood vessels, cellular growth, growth factors, tumour necrosis factors α (TNFα), and hypoxia have been found to modulate the expression of eNOS (Le Cras et al. 1996, Liu et al. 1996, Alonso et al. 1997, Kleinert et al. 2000).

Inducible NO production is controlled mainly via regulation of iNOS expression. iNOS is not detectable in resting cells, but its expression can be induced by several factors such as lipopolysaccharide (LPS), proinflammatory cytokines, hypoxia and foreign DNA or RNA. iNOS is expressed in various cell types, including macrophages, chondrocytes, epithelial cells, mesangial cells, smooth muscle cells and hepatocytes. Once iNOS is induced, it produces large amounts of NO for prolonged periods. Increased amounts of NO produced by the iNOS pathway are related to various pathophysiological processes, and possess both beneficial properties such as antibacterial (MacMicking et al. 1997a) and antiviral (Karupiah et al. 1993), effects and detrimental effects such as inhibition of mitochondrial respiration (Clementi et al. 1998), protein nitration and provocation of tissue injury (Boughton-Smith et al. 1993a, Kooy et al. 1997). The regulation of iNOS enzyme activity differs from that of eNOS and nNOS. Even though calmodulin binds tightly to iNOS enzyme and is needed in NO synthesis, the activity of iNOS is not changed by modulation of $[Ca^{2+}]_i$, *i.e.* iNOS is calcium-independent (Ruan et al. 1996). Once expressed, iNOS may produce NO for several hours, even days, and may induce apoptosis (programmed cell death) in the producer cells (MacMicking et al. 1997b, Moilanen et al. 1999, Alderton et al. 2001).

Recently, polymorphism of NOS enzymes has been postulated to be associated with certain clinical states. A point mutation located in –954 (G-C) bp from the transcription start site in the promoter of iNOS has been reported to be linked to protection against malaria (Kun et al. 2001), whereas variations in amounts of CCTTT pentanucleotide repeats within the iNOS promoter seem to be a risk factor for cerebral malaria (Burgner et al. 1998). Interestingly, the latter polymorphism has also been reported to be associated with protection from the development of diabetic retinopathy (Warpeha et al. 1999). Polymorphisms within eNOS and iNOS genes have been found to be implicated in cardiovascular diseases (Wang and Wang 2000, Rutherford et al. 2001). Interestingly, polymorphisms of nNOS, but not those known of iNOS, seem to also be linked to asthma (Gao et al. 2000, Wechsler et al. 2000).

1.2. Inhibitors of nitric oxide synthases (NOSs)

Inhibition of NOS activity has been proposed as a potential target for drug therapy in several diseases associated with excessive NO production. In theory, selective inhibitors for different NOS isoforms would provide targeted and efficient therapy for various diseases. Selective iNOS inhibitors are believed to be beneficial in inflammatory diseases such as bronchial asthma, inflammatory bowel diseases and rheumatoid arthritis (Hobbs et al. 1999). In addition, there are

reports of favourable effects of selective iNOS inhibitors in sepsic shock and cancer (Thomsen et al. 1997, Wray et al. 1998). Selective nNOS inhibitors are considered useful in the treatment of cerebral stroke (Christopherson and Bredt 1997, Hobbs et al. 1999, Moilanen et al. 1999). Prototypic NOS inhibitor N-monomethyl-L-arginine (L-NMMA) (Palmer et al. 1988b) is an L-arginine analog, like most NOS inhibitors, and inhibits all three NOS isoforms. In recent years, a considerable amount of research has been conducted in the search for selective inhibitors for NOS isoforms. A number of inhibitors of NOS enzymes have been identified with profiles different in respect of their capacity to inhibit NOS isoforms. In Table 1, properties of some experimentally used NOS inhibitors and three novel iNOS inhibitors are compared with respect to their potential to iNOS versus constitutively expressed NOS enzymes. (Alderton et al. 2001)

Inhibitor	*Selectivity*	
	iNOS vs. eNOS	iNOS vs. nNOS
L-NMMA	-	-
L-NAME	-	-
L-NIO	+	+
L-NIL	+	+
aminoguanine	+	+
1400W	+++	+
GW273629	++	++
GW274150	+++	++

Table 1. The selectivity of various NOS inhibitors in inhibition of inducible NOS vs. constitutively expressed NOS. - refers to non-selectivity, + refers to partial iNOS selectivity corresponding to 10-50-fold iNOS inhibition as compared to eNOS or nNOS inhibition, ++ refers to selectivity corresponding to 50-250-fold iNOS inhibition as compared to eNOS or nNOS inhibition, and +++ refers to selectivity corresponding to over 250-fold iNOS inhibition as compared to eNOS or nNOS inhibition. Selectivity is determined on the basis of IC50 values of inhibition of purified NOS isoforms. (Modified from Alderton et al., 2001)

2. Inflammation

2.1. General events in inflammation

In principle, inflammation is a protective response of the host to threatening events or agents, and the ultimate goal is to neutralize the initial cause (microbes, trauma, etc.) of the response. The vascular events in inflammation involve vasodilatation and increased permeability of the microcirculation. These events result in vascular leakage, accumulation of plasma components, and migration of leukocytes into the site of inflammation. Acute inflammation usually lasts from minutes to days and neutrophils and macrophages are the predominant infiltrating leukocytes in the acute phase. Chronic inflammation lasts several days to weeks, even months, and is characterized by the presence of lymphocytes and macrophages, and tissue destruction or necrosis. The inflammatory response is also closely linked to the activation of repair mechanisms like angiogenesis and the formation of scar tissue needed in the compensation for the destruction caused by the threatening factor(s) (Cotran et al. 1999a).

The inflammatory response is divided into the first-line defence called innate immunity, and later developing defence, i.e. adaptative immunity. Innate immunity involves the epithelium as a natural barrier, phagocytic cells (neutrophils, macrophages), blood proteins (e.g. complement components), and cell-derived inflammatory mediators such as eicosanoids, reactive oxygen and nitrogen species, histamine, platelet aggregating factor and cytokines. Adaptative immunity is mediated by specific T- and B-cells, and is based on humoral and cell-mediated immunity (Abbas et al. 2000). Innate immunity provides the early response to inflammatory stimuli, for example to infection, and regulates the onset of adaptative immunity.
Innate immunity is a non-specific response activated rapidly after the introduction of inflammatory stimuli to the host. Microbes contain highly conserved molecular structures, called pathogen-associated molecular patterns (PAMP). Such PAMP–structures include such microbial cell wall components as LPS, lipoteichoic acid (LTA), peptidoglycan, mannan and glucans, as well as foreign DNA and double-stranded RNA (Astarie-Dequeker et al. 1999, Lien et al. 1999, Hemmi et al. 2001, Ozinsky et al. 2000, Alexopoulou et al. 2001). Activator cells of innate immunity, granulocytes and especially macrophages, express pattern-recognition receptors (PRR) capable of recognizing common features in PAMPs. There are three types of PRRs: secreted, endocytic and signalling. PRRs so far characterized include CD14, β2-intergrins, C-type lectins, and macrophage/scavenger receptors (Viriyakosol and Kirkland 1995, Benimetskaya et al. 1997,

Forsyth and Mathews 1996, van der Laan et al. 1999). Secreted PRRs serve as opsonins, and bind to the cell wall of a microbe and tag it for recognition by the complement system. Endocytic PRRs are expressed on the surface of phagocytes and mediate the phagocytosis and delivery of microbes to lysosomes for degradation (Medzhitov and Janeway 1997a, Medzhitov and Janeway, Jr. 2000, Zhang and Ghosh 2001).

Signalling PRRs are involved in the activation of proinflammatory reactions. The family of Toll-like receptors (TLR) has recently been found to activate innate immunity in mammals (Medzhitov et al. 1997b, Poltorak et al. 1998, Yang et al. 1998, Thoma-Uszynski et al. 2001). To date, TLR1-6 have been characterized. TLR2 recognizes widely different PAMPs, while TLR4 activation is more restricted to LPS and LTA (Lien et al. 1999, Schwandner et al. 1999, Takeuchi et al. 1999, Yoshimura et al. 1999, Dziarski et al. 2001). Co-stimulus needed for cell activation by TLR is provided by CD14 (Gegner et al. 1995, Viriyakosol and Kirkland 1995, Kirschning et al. 1998, Schroder et al. 2000). The binding of ligand (i.e. bacteria or bacterial-derived substances) to TLRs leads to activation of intracellular signalling cascades, resulting in the activation of immunological transcription factors, including nuclear factor κB (NF-κB), which regulates widely innate immunity responses (Kontgen et al. 1995, Medzhitov and Janeway, Jr. 2000, Zhang and Ghosh 2001). Activation of NF-κB has been found to induce the expression of several inflammatory mediators such as proinflammatory cytokines and adhesion proteins, and the production of eicosanoids and NO (Figure 2) (Baeuerle and Baltimore 1996, Beauparlant and Hiscott 1996). These newly synthesized factors acting singly, in sequence or in concert arouse and amplify the inflammatory response. The release of histamine and platelet aggregating factor, and induction of NO and eicosanoid production cause vasodilation and increased vascular permeability. These vascular events, together with increased adhesion protein expression, result in the accumulation of exudate and the migration of leukocytes into the inflammatory focus. As a result, signs and symptoms characteristic of inflammation develop (Cotran et al. 1999a, Abbas et al. 2000).

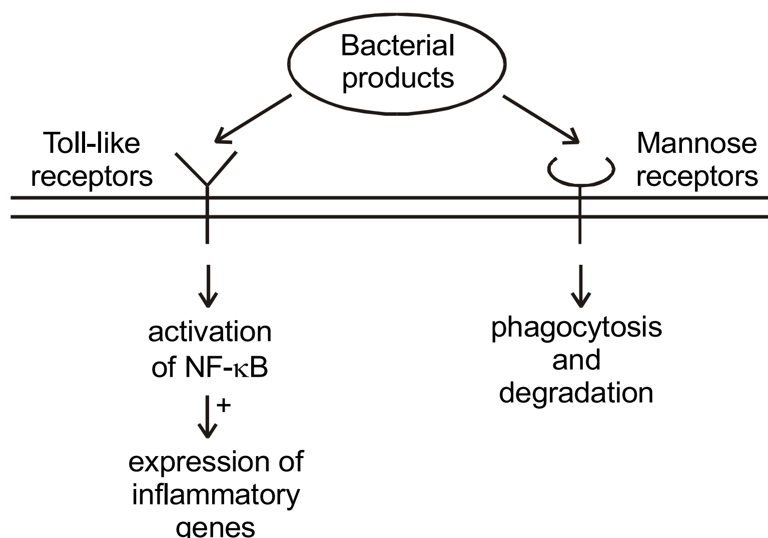


Figure 2. Simplified scheme of the cellular activation by bacterial products. Cells of innate immunity are able to recognize widely different molecular patterns found in microbes, termed as pathogen-associated molecular patterns (PAMPs), by pattern-recognition receptors (PRRs). These patterns include microbial substances such as LPS, LTA, peptidoglycan and bacterial DNA and RNA. The recognition PAMPs by PRRs, such as Toll-like receptors and mannose receptors, results in the cellular activation, and subsequently in the expression of inflammatory genes and phagocytosis of the particle recognized by PRRs. NF-κB, nuclear factor κB.

In addition to pathogenic microbes or their products, the immune response can be primed or activated by non-pathogenic microbes. Some probiotics, for example the *Lactobacillus* and *Bifidobacteria* species, have been found to induce innate immune mechanisms, including enhancement of epithelial barrier function in the gut, activation of NF-κB and cytokine production in monocytes and natural killer cells, and induction of phagocytic activity in neutrophils (Perdigon et al. 1988, Haller et al. 2000a, Haller et al. 2000b, Miettinen et al. 2000, Madsen et al. 2001). In addition, the modulation of human innate immune responses after the ingestion of some lactic acid bacteria has been reported (Schiffrin et al. 1995, Marteau et al. 1997). Probiotic bacteria regulate gut immunity by two mechanisms; limiting the growth of pathogenic bacteria and stimulating the innate immunity mechanisms in the host defence (Saxelin et al. 1991, Alander et al. 1997, Tuomola et al. 1999).

2.2. NO in inflammation and host-defence reactions

In 1985, Stuehr and Marletta showed that activated macrophages produced nitrite and nitrate in response to LPS and *Mycobacterium bovis* (Stuehr and Marletta 1985). Soon after the revolutionary

discovery that NO is an endothelium-derived relaxing factor, Hibbs and colleagues reported that the increased nitrite/nitrate production in activated macrophages was due to synthesis of NO from L-arginine, and that NO mediated cytotoxic effects of macrophages (Hibbs et al. 1988). Data accumulated thereafter to suggest that NO plays a central role in a range of inflammatory diseases. Interestingly, NO seems to have a dual role in inflammation. This effect appears to depend on the type and phase of inflammation, having both pro- and anti-inflammatory properties. Basically, NO at low concentrations, produced by constitutively expressed NOS, has anti-inflammatory effects (Kubes et al. 1991, Lefer et al. 1999), whereas NO derived from iNOS is associated with cellular/tissue dysfunction and exacerbation of inflammation e.g. through the formation of peroxynitrite (Rachmilewitz et al. 1995, Kooy et al. 1997).

In the early phase of inflammation, low amounts of NO produced by cNOS mediate many anti-inflammatory effects of NO. NO inhibits the migration of leukocytes into the inflammatory focus by reducing the expression of adhesion molecules, and by suppressing adherence and rolling of leukocytes on the endothelium (Kubes et al. 1991, Kurose et al. 1995, Ou et al. 1997, Lefer et al. 1999). Similar suppressive effects on leukocyte functions and E-selectin expression have been demonstrated with NO-donors (Kosonen et al. 1999, Kosonen et al. 2000). In intestinal inflammation models, cNOS-derived low level NO formation or administration of NO-donors has been found to be beneficial in reducing vascular leakage, protecting from tissue injury and enhancing epithelial integrity (Hutcheson et al. 1990, Payne and Kubes 1993, Szabo 2000).

Subsequently, in the advanced stage of the inflammatory process, tissue injury and worsening of the inflammation results from excess NO production by iNOS. Many of the detrimental effects of NO are due to the reaction between NO and superoxide resulting in the formation of peroxynitrite, which is a highly reactive and cytotoxic molecule. Excessive peroxynitrite formation leads to nitrated proteins, inhibition of mitochondrial respiration, depletion of cellular energetics, DNA damage, apoptosis and necrotic cell death, resulting in cellular/tissue injury (Beckman et al. 1990, Szabo et al. 1996, Kooy et al. 1997, Kim et al. 1998b, Szabo et al. 1998, Szabo 2000). The expression of iNOS and formation of peroxynitrite is involved in the pathogenesis of many chronic diseases. Marked iNOS expression and peroxynitrite formation has been found to be associated with several inflammatory states, including inflammatory bowel disease, rheumatoid arthritis, dermatitis and asthma (Moilanen et al. 1999, Szabo 2000).

In addition to its effect as a regulator of the inflammation, NO is an important effector molecule in innate immune responses (MacMicking et al. 1997b). Exposure of various inflammatory and tissue cells to bacterial products like LPS, LTA, peptidoglycan, or bacterial DNA, or to intact bacteria, induces the expression of iNOS resulting in enhanced NO production (Geller et al. 1993b, Xie et al. 1993, Hattor et al. 1997, Wheeler et al. 1997, Orman et al. 1998, Salzman et al. 1998). In these conditions, NO produced by iNOS, partly through formation of peroxynitrite, is a cytotoxic molecule serving as a killing mechanism against invading microbes (Cunha et al. 1992, Karupiah et al. 1993, MacMicking et al. 1997a, Murray and Nathan 1999, Shiloh et al. 1999, Nathan and Shiloh 2000). The importance of iNOS expression and NO formation in the control of infectious agents is well established in rodents. To date, data gathered from studies with iNOS gene-deficient mice indicate that NO has a central role in fighting viral, bacterial and protozoa infections (Bogdan 2001). The role of iNOS expression in antimicrobial reactions in humans is not clear. Human macrophages are found to induce iNOS expression and NO formation also in aseptic inflammatory conditions, e.g. rheumatoid arthritis, osteoarthritis, and aseptic loosening of joint implants (McInnes et al. 1996, Moilanen et al. 1997, Vuolteenaho et al. 2001). There are nonetheless implications that iNOS expression participates in infectious conditions in humans. Increased iNOS expression has been observed in patients with urinary tract infection, tuberculosis and malaria (Nicholson et al. 1996, Wheeler et al. 1997, Kun et al. 2001).

In addition to its role as an effector molecule in host defense, NO regulates the course of the immune response. NO has been found to affect the functions of activated T helper 1 and 2 (Th1 and Th2) cells. In rodent models, high concentrations of NO produced via iNOS pathway have been found to inhibit the proliferation of Th1 cells and affect cytokine production by T helper cells favoring the Th2 type response (Wei et al. 1995, Bauer et al. 1997, Taylor-Robinson 1997, van der Veen et al. 2000). Accordingly, NO-donors are also found to inhibit lymphocyte proliferation *in vitro* (Kosonen et al. 1998) and regulate cytokine production also in human cells *in vitro* (Bauer et al. 1997).

2.3. NOS inhibitors in experimentally induced inflammation

The role of nitric oxide in inflammation and tissue injury has been studied in several exprerimental models of inflammation using NOS inhibitors. Among the most widely investigated experimental clinical settings in relation to NO are intestinal inflammation and septic shock. Boughton-Smith and

colleagues reported in 1993 that inflammatory intestinal injury induced by intravenous administration of LPS was attenuated with a non-selective NOS inhibition L-NMMA in rats, and the time course of the action of L-NMMA suggested that NO produced by iNOS was associated with tissue injury (Boughton-Smith et al. 1993b). Another study with a selective iNOS inhibitor, aminoguanidine, showed that inhibition of iNOS reduced intestinal permeability and improved intestinal mitochondrial respiration in LPS-treated rats (Unno et al. 1997). Recent studies conducted with the selective iNOS inhibitors 1400W, L-NIL, or mercaptoethylguanidine showed that inhibition of iNOS suppressed inflammation and tissue injury in experimentally induced colitis (Zingarelli et al. 1998, Kankuri et al. 2001, Menchen et al. 2001, Yue et al. 2001).

In studies of septic shock models, mice lacking the iNOS gene have been reported to be more resistant to hypotension, vascular hyporesponsiveness and organ dysfunction, and to show better survival rates than wild-type mice (MacMicking et al. 1995, Hollenberg et al. 2000). Similar results have been obtained from studies using selective iNOS inhibitors in septic shock models. Fall in arterial pressure was attenuated with 1400W (Wray et al. 1998) and partial iNOS inhibitor S-methyl-isothiourea (Rosselet et al. 1998).

In experimental osteoarthritis, administration of the iNOS selective inhibitor L-NIL reduced cartilage and synovial inflammation and retarded the progression of joint destruction (Pelletier et al. 1998). Inhibition of iNOS by L-NIL was found to reduce or prevent joint destruction in adjuvant-induced arthritis if it was administrated prophylactically (Santos et al. 1997, Fletcher et al. 1998). In contrast, iNOS inhibition did not attenuate acute joint inflammation but exacerbated chronic inflammation and joint destruction induced by a streptococcal wall preparation in mice (McCartney-Francis et al. 2001). These opposing findings suggest that the role of iNOS expression in arthritis is related to the etiology, and possibly to the phase of disease. In addition, a highly selective iNOS inhibitor 1400W has been found beneficial in acute ischemic brain injury in rats, reducing the size of lesion and attenuating neurological dysfunction (Parmentier et al. 1999). In allergic ovalbumin-induced airway inflammation 1400W treatment reduced hyperresponsiveness and eosinophil accumulation in mice (Koarai et al. 2000).

Taken together, the induction of iNOS and NO synthesis is proinflammatory and is implicated in the development of tissue injury/dysfuntion in various inflammatory states possibly through formation of peroxynitrite. In addition, NO seems also to have anti-inflammatory effects, and the role of iNOS induction and NO synthesis may be related to the type and phase of inflammation.

3. Regulation of iNOS expression

The elevation of $[Ca^{2+}]_i$ stabilizes the binding of calmodulin to cNOS, and switches on the NO synthesis. Because iNOS contains tightly bound calmodulin, its activity is free of the modulation caused by changes in $[Ca^{2+}]_i$ concentration, and in theory, once expressed, iNOS can produce NO indefinitely until the enzyme becomes degraded. Experimental data suggest that the main controlling mechanism in inducible NO synthesis is at the level of regulation of iNOS expression. The expression of iNOS is induced in several cell types by appropriate stimulation, in both rodent and human cells. Cell types reported to express iNOS include macrophages (Lyons et al. 1992), neutrophils (Wheeler et al. 1997), vascular smooth muscle cells (MacNaul and Hutchinson 1993), fibroblasts (Kleinert et al. 1996b), chondrocytes (Charles et al. 1993), hepatocytes (Ganster et al. 2001), Kupffer cells (Kurose et al. 1997), mesangial cells (Kunz et al. 1994), glial cell (Fujimura et al. 1997) and lung and intestinal epithelial cells (Guo et al. 1995, Salzman et al. 1996). The transcriptional regulation of iNOS expression is the most thoroughly studied, and very little is known on post-transcriptional mechanisms involved. Similarly to the enhanced NO formation through increased iNOS expression, cellular down-regulation of iNOS-dependent NO formation is usually mediated by decreased iNOS expression, not iNOS enzyme modulation even though some endogenous NOS inhibitors are found (Ganster and Geller 2000, Kleinert et al. 2000).

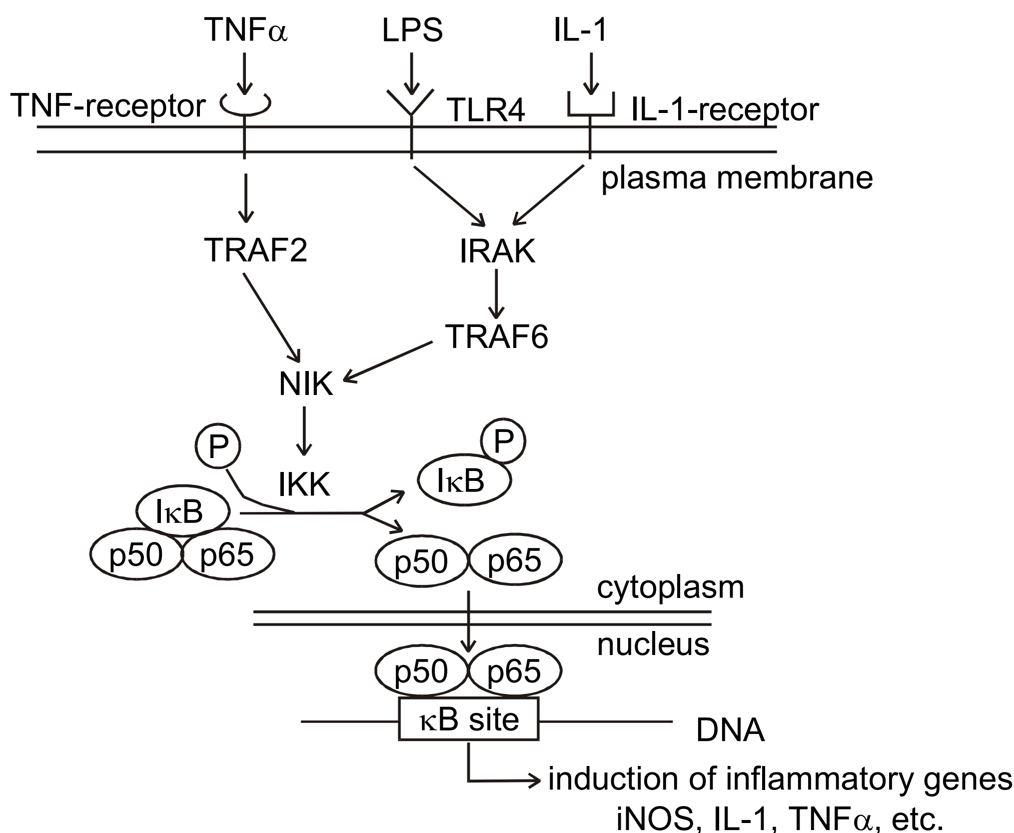
3.1. Transcriptional regulation of iNOS expression

The regulation of iNOS expression depends on the cell type and species in question, and the signal transduction pathways involved are heterogeneous. Published sequences of the mouse and human iNOS promoter region reveal binding sites for several transcription factors, including NF-κB, interferon regulatory factor 1 (IRF-1), activator protein 1, signal transducers and activators of transcription 1α (STAT1α), octamer binding factor, hypoxia-inducible factor, and nuclear factor IL-6 (Geller et al. 1993a, Xie et al. 1993, Chu et al. 1998). Several factors which are able to induce an inflammatory response also regulate iNOS expression. Such factors include pathogenic Gram-positive and Gram-negative bacteria, bacterial products (LPS, LTA, peptidoglycan), foreign DNA and RNA, cytokines, and hypoxia. LPS has been found to induce iNOS expression both *in vitro* and *in vivo*, and LPS-mediated iNOS expression and NO formation has been shown to be involved in the development of hypotension, multi-organ failure and death in sepsic shock (MacMicking et al. 1995, Wei et al. 1995, Gunnett et al. 1998, Kristof et al. 1998). Accordingly, enhanced iNOS

expression has been found to take place in response to Gram-positive bacteria, and in these conditions the most important component in the induction of iNOS expression is considered to be LTA. Peptidoglycan has also been found to induce iNOS expression in vitro in concert with LTA (De Kimpe et al. 1995, Kengatharan et al. 1996, Kengatharan et al. 1998). In addition, gamma interferon (IFNγ), a prominent cytokine in innate immune responses, is along with LPS a major inducer of iNOS expression, acting solely or in concert with LPS or other cytokines. Together with these factors, several other cytokines are also found to regulate induction of iNOS expression and NO formation (Ganster and Geller 2000). The regulation of iNOS induction differs between rodent and human cells. Rodent cells express iNOS readily in response to LPS and proinflammatory cytokines such as IFNγ, TNFα and interleukin-1β (IL-1β), whereas human cells seem to require a combination of cytokines or more complex treatments to induce iNOS expression.

3.2. NF-κB in iNOS expression

The NF-κB/Rel family of transcription factors widely regulate inflammatory and immune responses, the cell cycle and cell differentiation. NF-κB is activated by various signals and intracellular signalling cascades converge in NF-κB activation. LPS as well as the proinflammatory cytokines TNFα and IL-1 are among the most potent inducers of this event. NF-κB dimers are present in cytosol bound to inhibitory κB (IκB) proteins, which retain it in an inactive state. The activation of TNFα receptor leads to the subsequent activation of tumour necrosis factor receptor-associated factor 2 (TRAF2), and the activation of IL-1 receptor activates tumour necrosis factor receptor-associated factor 6 (TRAF6) through interleukin 1-associated kinase (IRAK) (Cao et al. 1996, Kirschning et al. 1998, Pomerantz and Baltimore 1999). TLR activation by LPS results in the activation of IRAK and TRAF6. Activated TRAF6 and 2 induce signalling cascades which result in the activation of NF-κB-inducing kinase (NIK) which phosphorylates and activates IκB kinase (IKK) (Malinin et al. 1997, Kopp and Medzhitov 1999, Silverman and Maniatis 2001). IKK phosphorylates IκB, resulting in the ubiquitination and degradation of IκB in the proteosome. Translocation signals in the protein structure of NF-κB are revealed, allowing NF-κB to be carried into the nucleus, where it activates the expression of target genes (Figure 3). (Guha and Mackman 2001, Joyce et al. 2001).



<u>Figure 3. A simplified scheme of the pathways involved in the activation of NF-kB.</u> The binding of TNFα to its receptor leads to the activation of TNF-receptor associated factor 2 (TRAF2), which in turn activates NF-κB-inducing kinase (NIK). The activation of NIK leads to subsequent activation of IκB kinase (IKK). IKK phosphorylates IκB, resulting in the dissociation of IκB from NF-κB, revealing the translocation signals of NF-κB, and then NF-κB is carried into the nucleus, where it binds to its specific binding site in the promoter region of the target gene. The activation of IL-1 receptor and Toll-like receptor 4 (TLR4) by IL-1 and LPS respectively, activates interleukin-1-receptor associated kinase (IRAK), which subsequently leads to the activation of TRAF6 and NIK, resulting in the activation of NF-κB. p50 and p56, members of NF-κB/Rel family of transcription factors; κB site, a specific nucleotide sequence recognized by NF-κB in the promoter of target gene; p, phosphate residue.

NF-κB is a key transcription factor involved in the expression of iNOS in both rodent and human cells. Several lines of evidence unquestionably emphasize the prominent role of NF-κB. Enhanced iNOS expression in response to LPS challenge has been shown to be mediated through activation of NF-κB in several mouse cell types, and the important binding sites for NF-κB in the promoter region of iNOS are located between –76 and –85 bp and –962 and–971 bp from the transcription start site (Xie et al. 1994, Weisz et al. 1996). Another major inducer of iNOS expression, IFNγ, has also been found to activate NF-κB, and the binding site involved in IFNγ-induced activation of NF-

κB has been located between –962 and –971 bp. This binding site is necessary for synergistic activation of iNOS transcription by LPS and IFNγ (Xie et al. 1993, Weisz et al. 1996).

Transcription of the human iNOS gene is also tightly regulated by NF-κB, although the promoter region is distinct from that of mouse iNOS. There are several binding sites for NF-κB in the promoter region of human iNOS, but the locations of NF-κB binding sites relevant for induction of human iNOS differ from those of the mouse iNOS promoter. After stimulation with a combination of cytokines TNFα, IL-1β, and IFNγ, a fundamentally important NF-κB binding site has been located at site –5.8 kb upstream of the transcription starting site, and enhancer NF-κB sites located at positions –5.2, -5.5, and –6.1 kb (de Vera et al. 1996, Taylor et al. 1998). Further evidence showing the prominent role of NF-κB in iNOS transcription is emphasized by the fact that pyrrolidinedithiocarbamate (PDTC), an inhibitor of NF-κB activation (Schreck et al. 1992, Liu et al. 1999), blocks the induction of iNOS expression and NO formation induced by LPS in murine cells and combinations of cytokines in human cells (Xie et al. 1994, Kleinert et al. 1996b, Nunokawa et al. 1996, Taylor et al. 1998, Lahti et al. 2000).

3.3. Role of IRF-1 in iNOS expression

In addition to NF-κB, IRF-1 has been reported to a play role in the regulation of iNOS transcription. Binding of IFNγ to its receptor leads to the activation of Janus family protein tyrosine kinases (Jak 1 and Jak 2), which in turn activate transcription factor STAT1α by phosphorylation in cytosol. The activated of STAT1α translocates to the nucleus, binds to the gamma-activated site in the promoter of the primary response gene for IFNγ response, and activates transcription. One group of primary response genes to IFNγ is a family of transcription factors termed interferon regulatory factors (IRFs), including IRF-1, IRF-2, and proteins known as p48 and ICSPB (Decker et al. 1991, Shuai et al. 1992, Silvennoinen et al. 1993, Boehm et al. 1997). Both mouse and human iNOS promoters contain binding sites for IRF-1. The role of IRF-1 in iNOS transcription has been studied mostly in mouse macrophages. Studies conducted with IRF-1 gene knock-out mice show that, along with NF-κB, IRF-1 has a central role in iNOS transcription, since macrophages derived from mice lacking the gene for IRF-1 do not express iNOS in response to LPS or cytokines (Martin et al. 1994, Kamijo et al. 1994). The binding site of IRF-1 important in iNOS expression is located at position –913 to –923 bp from the transcription start site. Although macrophages derived from IRF-1-deficient mice failed to express iNOS in response to LPS and IFNγ, IRF-1 has usually been suggested to act in

synergy with NF-κB to enhance iNOS expression possibly by exerting an effect in the assembly of NF-κB (Martin et al. 1994, Weisz et al. 1996, Kim et al. 1997). Additionally, in glial cells from mice lacking the IRF-1 gene, expression of iNOS in response to LPS and IFNγ is markedly reduced, but not totally blocked. In the study in question, the inhibition of NF-κB by diethyldithiocarbamate abrogated the expression of iNOS in response to LPS and IFNγ, and the authors suggested that NF-κB is essential and IRF-1 provides an additional signal in iNOS induction (Fujimura et al. 1997). However, in respect of iNOS expression, chondrocytes derived from IRF-1-deficient mice have shown intact responses to IFNγ and LPS or IL-1β, suggesting that the role of IFNγ in iNOS expression is a cell type-dependent phenomenon (Shiraishi et al. 1997). In addition to the role involved in transcription, IFNγ has been thought to exert other effects on iNOS expression, possibly through stabilization of iNOS mRNA and/or protein (Weisz et al. 1994, Salkowski et al. 1997).

Together with binding sites for IRF-1, the iNOS promoter also contains gamma-activated site sequences. In mouse macrophages stimulated with LPS and IFNγ, STAT1α has been found to bind to gamma-activated site in the iNOS promoter, and to increase iNOS expression to some extent, which would imply that STAT1α has a direct enhancer role in NF-κB-mediated iNOS transcription in mouse macrophages (Gao et al. 1997). Additionally, enhanced iNOS expression following LPS stimulation has been found to be partially mediated by increased IFN-αβ expression, leading to STAT1α activation in mouse macrophages (Gao et al. 1998). In human cells, the role of STAT1α may be more important. In human intestinal epithelial cells, expression of iNOS in response to stimulation with a mixture of the cytokines IFNγ, TNFα and IL-1β was reduced by the Jak2 inhibitors tyrphostin B42 and B25, suggesting a significant role of STAT1α activation in iNOS expression in these cells (Kleinert et al. 1998).

3.4. Other transcription factors associated with iNOS expression

Recent data suggest that human iNOS transcription is also affected by activator protein 1, whose role, however, is somewhat controversial. Activator protein-1 is a heterodimer of the proto-oncogenes *fos* and *jun*, and it can bind to DNA and activate transcription. Human iNOS transcription has been shown to be regulated by activator protein-1 along with NF-κB in lung epithelial cells by a MAP-kinase-dependent mechanism (Marks-Konczalik et al. 1998, Kristof et al. 2001). In contrast, in intestinal epithelial cells, stimulation with cytokines has led to reduced

activator protein-1 DNA binding activity together with enhanced iNOS expression (Kleinert et al. 1998). This suggests that the regulation of human iNOS transcription by activator protein-1 may be cell type-dependent, and reinforces the assumption of heterogeneity in the regulation of human iNOS expression. In LPS-stimulated mouse macrophages, activator protein-1 has been found to be activated in response to increased $[Ca^{2+}]_i$ concomitantly with increased iNOS expression and NO formation. However, activator protein-1-dependent iNOS transcription was not studied (Chen et al. 1998). In addition, some other transcription factors, e.g. octamer binding factor-1, nuclear factor-IL6, hypoxia-inducible factor, and high mobility group-I(Y), cyclic AMP-responsive element-binding protein (known as CREB), and CCAAT/enhancer-binding protein (known as C/EBP), have been held to participate in the regulation of iNOS expression in some cell types (Kinugawa et al. 1997, Xie 1997, Eberhardt et al. 1998, Perrella et al. 1999, Ganster and Geller 2000).

3.5. Second messenger pathways involved in the expression of iNOS

3.5.1. Tyrosine kinases

There are studies on single intracellular signalling molecules involved in the regulation of iNOS expression, but second messenger pathways regulating iNOS expression remain to be established. Several intracellular factors are found to participate in the signal transduction resulting in the induction of iNOS expression. Among the early regulators of iNOS expression are tyrosine kinases. Activation of these kinases has been reported to regulate the induction of iNOS and NO production through NF-κB, and the inhibition of tyrosine kinase activation by pharmacological inhibitors abrogates iNOS expression (Geng et al. 1995b, Kleinert et al. 1996b, Chen et al. 1998b). Studies characterizing the role of tyrosine kinases have often been conducted with the pharmacological inhibitor genistein, and it has been suggested that they act at the level of activation of NF-κB (Chen et al. 2000, Chen et al. 2001).

3.5.2. MAP-kinases

Recently, another family of protein kinases, namely mitogen-activated protein kinases (MAP-kinases), has been found to participate in iNOS expression. Both p42/44 (also known as Erk 1/2) and p38 have been reported to be involved in the regulation of iNOS expression in different cell types, including dendritic cells, cardiac myocytes, glia cells and chondrocytes (Bhat et al. 1998,

Cruz et al. 1999, Kan et al. 1999, Martel-Pelletier et al. 1999, Patel et al. 1999). MAP-kinase subgroups p42/44 and p38 participate in the regulation of iNOS expression following cytokine or LPS stimulation in several cells types, but results are somewhat controversial, and both enhancing and inhibiting effects have been reported. In earlier studies, inhibitors of MAP-kinases p42/44 and p38 were found to inhibit iNOS expression in LPS-treated macrophages, but concentrations of inhibitors used in those studies were relatively high and non-specific effects cannot be ruled out (Ajizian et al. 1999, Chen and Wang 1999). More recent studies with MAP-kinase inhibitors at concentrations which specifically block p42/44 and p38 pathways have shown that p42/44 MAP-kinase up-regulates but is not essential for iNOS induction in mouse macrophages and human intestinal epithelial cells (Lahti et al. 2000, Chan and Riches 2001). Inhibition of p38 has resulted in enhanced NO production and iNOS expression in macrophages and intestinal epithelial cells without affecting the activation of NF-κB (Patel et al. 1999). These results suggest differential roles of p42/44 and p38 in iNOS expression. Thus, the precise role of MAP-kinases in the induction of iNOS expression remains to be established. (Ganster and Geller 2000).

3.5.3. Phospholipase C and protein kinase C

Phospholipase C and protein kinase C (PKC) pathways have also been found to participate in the regulation of iNOS expression. LPS stimulation of macrophages leads to activation of phospholipase C and subsequent formation of diacylglyserol and inositol 1,4,5-trisphosphate. Diacylglycerol is involved in the activation of PKC, while inositol 1,4,5-trisphosphate participates in the regulation of $[Ca^{2+}]_i$ levels. Results from studies with PKC inhibitors suggest that PKC activation is involved in iNOS expression, but different PKC-isoforms have either positive or negative effects on iNOS expression (Fujihara et al. 1994, Jun et al. 1994, Paul et al. 1997). More recent studies show that phosphoinositide-specific-phospholipase C and phosphatidylcholine-specific-phospholipase C and PKC-α, -βI, and –δ isoforms are of importance in iNOS expression via an NF-κB-dependent mechanism in mouse macrophages, whereas in astrocytes, phosphatidylcholine-specific-phospholipase C and PKC-η are of importance following LPS stimulation (Chen et al. 1998a, Chen et al. 1998b).

3.5.4. Intracellular Ca^{2+}

Numerous signals leading to leukocyte activation mediate their effects through the elevation of $[Ca^{2+}]_i$ (Kankaanranta and Moilanen 1995, Kankaanranta et al. 1996, Cotran et al. 1999a). Most commonly, activation of non-excitable cells by elevated $[Ca^{2+}]_i$ is mediated by a process called *capacitative Ca^{2+} entry* (also known as *store-operated Ca^{2+} entry*). In this signalling, ligand binding to its G-protein-linked receptor leads to the activation of phospholipase C, and the formation of diacylglycerol and inositol 1,4,5-trisphosphate. Inositol 1,4,5-trisphosphate binds to its receptor located on the surface of the endoplasmic reticulum, resulting in the release of Ca^{2+} into the cytocol. This Ca^{2+} release activates TRP3 calcium channels in plasma membrane, and thereby allows Ca^{2+} influx into the cell, causing a *calcium release-activated calcium current* (I_{CRAC}), which serves as a Ca^{2+} signal (Broad et al. 2001, Putney et al. 2001). Capacitative calcium entry can be mimicked by pharmacological tools such as calcium ionophores A23187 and ionomycin, and thapsigargin, which is an inhibitor of microsomal Ca^{2+}-ATPase providing an inositol 1,4,5-trisphosphate –like Ca^{2+} signal (Thastrup et al. 1990).

Unlike cNOS activity, iNOS-dependent NO synthesis is considered to be independent of rapid changes in $[Ca^{2+}]_i$. Although $[Ca^{2+}]_i$ does not enhance NO production by modulating the activity of iNOS enzyme, there are reports suggesting that it may regulate iNOS expression in some cell types (Denlinger et al. 1996, Chen et al. 1998).

The increase in $[Ca^{2+}]_i$ may also inhibit NO formation. In microvascular endothelial cells, $[Ca^{2+}]_i$ elevating agents have been shown to inhibit iNOS expression and NO formation induced by TNFα and IL-1β (Bereta et al. 1994). In IL-1β-stimulated chondrocytes, Ca^{2+} ionophore was reported to suppress iNOS expression and NO synthesis (Geng and Lotz 1995a). Thus, the role Ca^{2+} in the induction of iNOS expression and NO production is not thoroughly understood and further studies are called for to clarify the mechanisms involved.

3.5.5. Cyclic nucleotides

Cyclic nucleotides are found to regulate iNOS expression to some extent. Cyclic AMP has both activating and inhibiting effects on iNOS expression and NO synthesis depending on the cell type in question. The elevation of cyclic AMP concentration has been reported to enhance iNOS expression by mechanism involving the activation of NF-κB in fibroblasts (Kleinert et al. 1996b). In another study conducted with rat mesangial cells treated with IL-1β, iNOS expression was increased by a

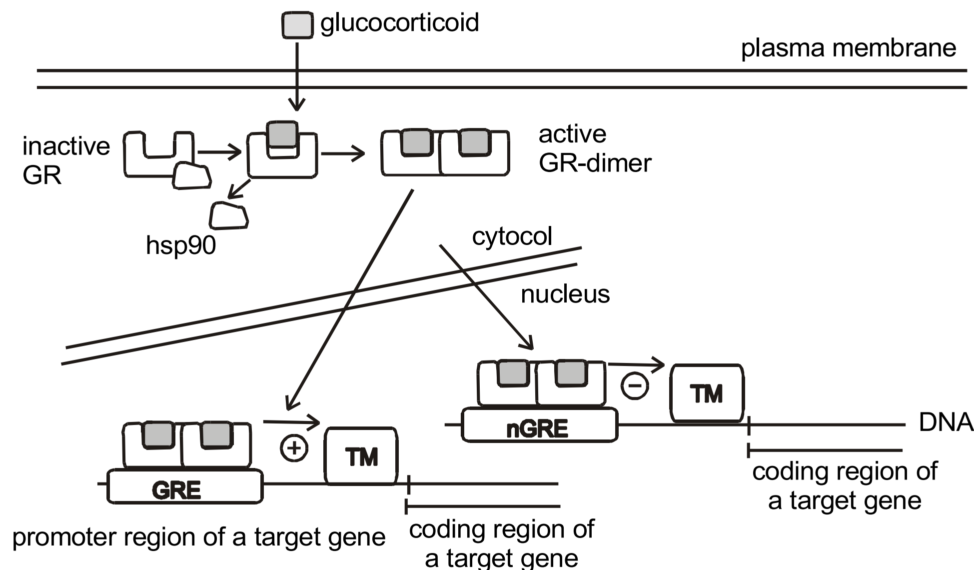
cAMP-dependent mechanism, and the authors concluded that iNOS expression was due to activation of NF-κB, cyclic AMP-responsive element-binding protein (known as CREB) and CCAAT/enhancer-binding protein (known as C/EBP) (Eberhardt et al. 1998). In hepatocytes, cAMP has been found to inhibit iNOS expression by a mechanism involving the inactivation of NF-κB (Mustafa and Olson 1998). On the other hand, cyclic GMP, which is normally related to the regulation of physiological events of NO, has been found to inhibit iNOS expression by a mechanism related to destabilization of iNOS mRNA in mesangial cells (Perez-Sala et al. 2001).

3.6. Factors involved in down-regulation of iNOS expression

In physiological and pathophysiological conditions, the termination of inducible NO synthesis is usually dependent on the down-regulation of the expression of iNOS, and not on the modulation of enzyme activity. Factors involved in the suppression of iNOS expression and subsequent NO synthesis have been to some extent defined; they include endogenous and exogenous glucocorticoids, transforming growth factor β (TGF-β), interleukin-10, and interleukin-4. In addition, some pharmaceutical compounds are also found to inhibit iNOS expression.
Glucocorticoids are widely used as anti-inflammatory drugs in various diseases. Glucocorticoids are known to up-regulate or down-regulate the expression of a number of genes. Traditionally, glucocorticoids are thought to regulate gene expression at genomic level by activating or suppressing gene transcription through a glucocorticoid-responsive element (GRE) and a negative glucocorticoid-responsive element (nGRE), respectively (Figure 4) (Adcock 2000, Newton 2000).

Although glucocorticoids inhibit the expression of several genes, especially inflammatory genes, suppression of transcription through nGRE is relatively uncommon. Other, non-genomic mechanisms of glucocorticoid action have been proposed (Adcock 2000, Newton 2000). Glucocorticoids have been found to inhibit inflammatory transcription factors by direct protein-protein interaction. Initially, this mechanism was found to take place between activator protein-1 and GR, resulting in inhibition of gene expression (Schule et al. 1990, Yang-Yen et al. 1990, Konig et al. 1992). Since then, GR has also been found to interact with an NF-κB subunit p65 and thereby to antagonize the interaction of NF-κB and transcription machinery, leading to decreased transcription and gene expression (Ray and Prefontaine 1994, De Bosscher et al. 2000). Other mechanisms such as competition of transcription co-activators with other transcription factors (Lee

et al. 1998, Sheppard et al. 1998), or induction of inhibitory protein I-κB leading to inactivation of NF-κB (Auphan et al. 1995), have also been proposed.



<u>Figure 4. Classical mechanisms of action of glucocorticoids.</u> The glucocorticois action is mediated through glucocorticoid receptor. In inactive form, glucocorticoid receptor (GR) is located in cytocol and bound to inhibitory proteins, such as 90 kDa heat shock proteins (hsp90). Binding of glucocorticoid to GR leads to dissociation of hsp90 from GR allowing subsequent dimerisation of GR-monomers. GR-dimer translocates to nucleus, and binds to glucocorticoid-responsive element (GRE) of a target gene promoting gene expression. Alternatively, GR-dimer may bind to negative GRE (nGRE) leading in the inhibition of gene expression. TM, transcription machinery.

In principal, glucocorticoids are thought to suppress iNOS expression and NO synthesis. Early findings with mouse macrophages and vascular endothelial muscle cells suggested that glucocorticoids decrease iNOS expression and NO formation following stimulation with LPS and IFNγ (Radomski et al. 1990, Di Rosa et al. 1990). This conception has since been confirmed in a number of studies with other cell types. Glucocorticoids have been shown to reduce iNOS expression and NO formation also in *in vivo* conditions (Tepperman et al. 1993, Guo et al. 1995). The mechanism by which glucocorticoids suppress iNOS expression is not clear. In rat mesangial cells, dexamethasone has been found to suppress iNOS expression and NO formation by reducing the iNOS transcription rate, mRNA translation and protein stability (Kunz et al. 1996). In rat hepatocytes, dexamethasone suppresses iNOS expression by a mechanism involving the reduction in NF-κB activity through increased IκB expression (de Vera et al. 1997).

In human cells, the inhibition of iNOS expression by glucocorticoids seems to be cell type-dependent, as well as the proposed mechanism of action. Dexamethasone was reported to inhibit cytokine-induced iNOS expression by reducing DNA-binding activity of NF-κB and subsequent iNOS transcription in lung epithelial cells (Kleinert et al. 1996a), whereas in cytokine-treated intestinal epithelial cells, dexamethasone has proved ineffective in reducing the transcription and expression of iNOS (Kleinert et al. 1998, Salzman et al. 1996). Inducible NO synthesis in isolated human chondrocytes, as well as in articular cartilage from patients suffering from osteoarthritis, is markedly resistant to glucocorticoids, and NO accumulation is not significantly affected by glucocorticoids (Palmer et al. 1993, Grabowski et al. 1996, Vuolteenaho et al. 2001).
Another immunosuppressive drug, cyclosporine A (CsA), has also been found to inhibit iNOS expression and NO formation in macrophages, fibroblasts and glial cells, but the mechanism involved is unknown (Dusting et al. 1999, Trajkovic et al. 1999a, Trajkovic et al. 1999b).

TGF-β is a family of cytokines consisting of three isoforms (TGF-β1-3), and these are potent modulators of immune responses, affecting the function and proliferation of several types of immune cells. All isoforms of TGF-β have been reported to participate in the regulation of iNOS expression, and it has been suggested that increased expression of iNOS due to decreased TGF-β expression is implicated in pathophysiological processes (Vodovotz 1997). A role of TGF-β in the control of iNOS expression and NO production *in vivo* has been demonstrated in animal studies. TGF-β reduces iNOS expression in several organs and decreases mortality in rats with bacterial sepsis induced by LPS (Perrella et al. 1996a). TGF-β knock-out mice have been found to suffer from multifocal inflammation which correlated with increased iNOS expression and elevated serum nitrite/nitrate levels (Vodovotz et al. 1996). TGF-β may suppress the expression of iNOS by several mechanisms. Reduction of iNOS transcription by TGF-β is considered to be independent of modulation of NF-κB activity, and it has been suggested that reduced expression of transcription factor high mobility group I(Y) by TGF-β is responsible for decreased iNOS transcription (Perrella et al. 1994, Perrella et al. 1996b, Pellacani et al. 2001). In addition, post-transcriptional effects contribute to the suppression of iNOS expression by TGF-β (Vodovotz et al. 1993, Perrella et al. 1994).

Interleukin-4 and interleukin–10 are also down-regulators of iNOS expression and NO production in certain conditions. Interleukin-4 has been found to inhibit iNOS expression and NO formation by inhibiting the induction of iNOS mRNA and subsequent iNOS protein expression and NO synthesis

(Paludan et al. 1997, Nemoto et al. 1999, Patton et al. 2002). Correspondingly, interleukin-10 has been found to inhibit iNOS mRNA expression and NO production, possibly counteracting the effect of IFNγ (Cunha et al. 1992, Chomarat et al. 1993, Dugas et al. 1998, Nemoto et al. 1999). This is of interest, since interleukin-10 suppresses expression of certain cytokines by destabilizing their mRNAs (Bogdan et al. 1992, Kishore et al. 1999).

4. Regulation of iNOS messenger RNA

4.1. General aspects of mRNA

Genetic information stored as a specific nucleotide sequence in DNA needs to be transcribed into a specific nucleotide sequence of a messenger RNA (mRNA) before it can be translated into a functional protein. This phenomenon is termed gene expression. The mRNA molecule of a given gene contains the coding sequence of the gene, the promoter and enhancer regions, cap structure in the 5' untranslated region (UTR), and poly(A)tail in the 3'-UTR. The functions of these untranslated structures in mRNAs are involved in mRNA splicing, initiation of transcription, and protection of mRNA from degradation by exonucleases (Granner 1993). The time of decay of mRNAs varies depending on the gene in question. Some mRNAs are particularly stable, while certain mRNAs are more susceptible to degradation. There are mRNAs which contain adenosine-uridine-rich elements (ARE), e.g. AUUUA located in the 3'-UTR upstream of the poly(A)tail, and three classes of AREs have been identified (Chen and Shyu 1995). mRNAs containing AREs include cytokine, proto-oncogene and growth factor mRNAs. These ARE sequences are considered to mediate a rapid degradation of mRNA (Shaw and Kamen 1986, Lagnado et al. 1994, Zubiaga et al. 1995, Malter 1998).

4.2. Regulation of mRNAs of transiently expressed factors involved in inflammation

In most cases, gene expression of factors involved in inflammation is controlled at transcriptional level. Basically, most of the genes involved in regulation of the inflammatory process are silent in resting cells, their expression being induced by inflammatory stimuli such as LPS, peptidoglycan, LTA, double-stranded DNA and RNA, and many proinflammatory cytokines (Abbas et al. 2000). These agents induce the activation of NF-κB and other immunological transcription factors

(Beauparlant and Hiscott 1996), which in turn induce expression of the mRNA of cytokines, growth factors and adhesion proteins, and mRNA is further translated into functionally active proteins.

Gene expression can also be regulated at post-transcriptional level. One mechanism here is regulation of the stability of mRNA. In fact, regulation of the stability of mRNA is a common means of immunological cells to adjust gene expression and thereby respond to environmental changes in mammalian cells (Lindstein et al. 1989, Koeffler et al. 1988, Akashi et al. 1990). ARE-containing mRNAs are susceptible to regulation of mRNA expression by mechanisms involving changes in the stability of the given mRNA. The degradation of granulocyte/macrophage-colony stimulating factor mRNA was first shown to be mediated by a mechanism dependent on ARE-sequences in the 3'-UTR (Shaw and Kamen 1986). Thereafter, several cytokine mRNAs as well as growth factor and proto-oncogene mRNAs have been shown to be regulated by stabilization/destabilization (Lindstein et al. 1989, Zubiaga et al. 1995, Stoecklin et al. 1994, Carballo et al. 1998). Such mRNAs include *c-fos*, interleukin-2, IFNγ, TNFα, and interleukin-3. In addition, the destabilization of these mRNA has been found to be protein synthesis-dependent (Koeffler et al. 1988, Tobler et al. 1988, Akashi et al. 1990). A number of agents have been found to regulate gene expression by affecting mRNA stability. Glucocorticoids are known to destabilize the mRNA of interleukin-2 (Fessler et al. 1996), and through the ARE region the mRNAs of cyclooxygenase-2 (Ristimäki et al. 1996), IFNγ (Peppel et al. 1991), and cyclin-D3 (Garcia-Gras et al. 2000). In addition, cyclosporine inhibits cell growth by destabilizing interleukin-3 mRNA (Nair et al. 1994). Interleukin-10 is an anti-inflammatory cytokine, and it has been shown to destabilize the mRNAs of cytokines and chemokines (Kim et al. 1998a, Kishore et al. 1999).

The precise mechanisms by which the ARE regions are involved in the regulation of those mRNAs remain unclarified. To date, a few proteins have been proposed as candidates mediating the regulation of mRNA stability. Such factors include heterogeneous ribonucleoprotein D (hnRNP D or AUF1), HuR (a member of embryonic lethal abnormal vision (ELAV) RNA-binding protein family), and the zinc finger protein tristetraprolin (TTP) (Wilson and Brewer 1999, Brennan and Steitz 2001, Mitchell and Tollervey 2000). TTP has been identified as a factor binding to ARE sequences and mediating the destabilization of the mRNAs of TNFα (Carballo et al. 1998, Lai et al. 1999), granulocyte/macrophage-colony stimulating factor (Carballo et al. 2000), and interleukin-3 (Stoecklin et al. 2000). Likewise, AUF1 is also able to bind to ARE regions and mediate rapid mRNA decay of certain genes (DeMaria and Brewer 1996), including c-*myc*, parathyroid hormone,

granulocyte/macrophage-colony stimulating factor, c-*fos*, and c-*jun* (Brewer 1991, Buzby et al. 1999, Loflin et al. 1999, Sela-Brown et al. 2000, Xu et al. 2001). Recently, another protein capable of binding to mRNA and mediating its rapid decay has been identified. KSRP, a protein originally found to be involved in neuronal mRNA splicing (Min et al. 1997), has been shown to mediate ARE-containing mRNA degradation (Chen et al. 2001). The mechanisms which mediate mRNA degradation by these agents remained unknown until very recently. TTP and KSRP were shown to present ARE-containing mRNA to exosomes, which are multiprotein complexes having 3'-5' exoribonuclease activity (Mitchell et al. 1997), and thus enabling the interaction between exosome and mRNA (Chen et al. 2001). (Figure 5)

In contrast to TTP, AUF1, and KSRP which mediate the increased mRNA decay, HuR has been identified as a protein involved in the stabilization of ARE-containing mRNAs (Brennan and Steitz 2001). HuR has been found to bind to the ARE regions of many cytokine and angiogene mRNAs, e.g. interleukin-8, interleukin-6, TNFα, TGF-β, cyclooxygenase 2, vascular endothelial growth factor, and to protect these mRNAs from rapid degradation *in vitro* (Levy et al. 1998, Ford et al. 1999, Nabors et al. 2001). The mRNA-stabilizing function of HuR has also been confirmed in *in vivo* conditions, and the mechanism whereby HuR protects mRNA is related to its ability to prevent the interaction of mRNA and the exosome responsible for 3'-5' degradation (Fan and Steitz 1998, Peng et al. 1998, Chen et al. 2001).

4.3. Regulation of iNOS mRNA stability

The induction of iNOS is tightly regulated at transcriptional level, but there is some evidence of the significance of post-transcriptional regulation in iNOS expression. iNOS expression has been found to be a transient phenomenon: iNOS mRNA expression is induced by proinflammatory stimuli such as LPS and cytokines, peaking in 3-6 hours after introduction of the stimuli, and gradually declining thereafter in a protein synthesis-dependent manner (Evans et al. 1994, Lähde et al. 2000). In addition, there are further observations emphasizing the importance of post-transcriptional mechanisms in iNOS mRNA expression. iNOS mRNA expression has been found to be increased in macrophages treated with a combination of LPS and IFNγ as compared to cells stimulated solely with LPS, without a corresponding change in the iNOS transcription rate (Weisz et al. 1994). In resting human intestinal epithelial cells, some basal iNOS mRNA transcription has been detected,

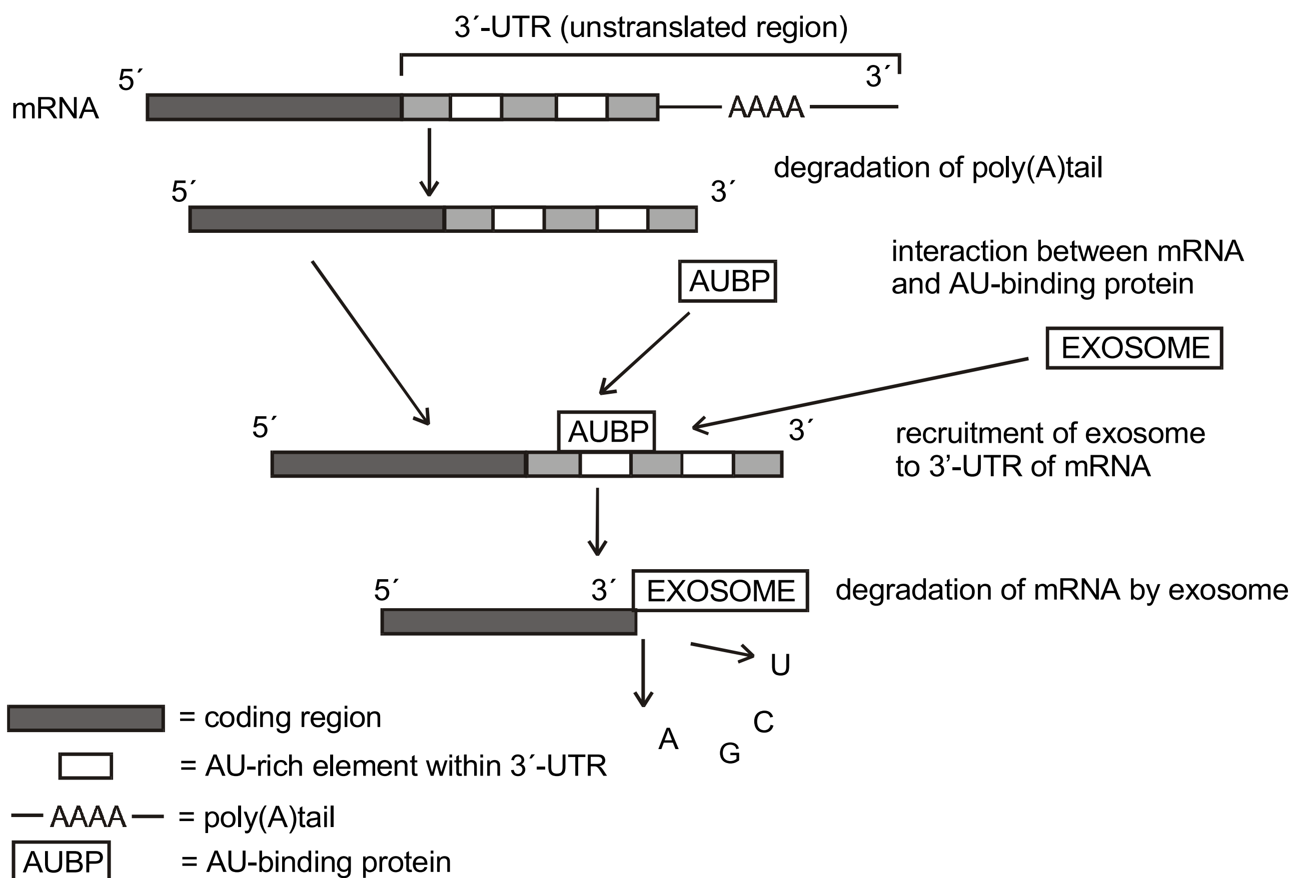


Figure 5. The mRNA degradation pathway. The 3'-end of mRNAs contains a poly(A)tail which prevents inappropriate degradation. In eukaryotic cells, mRNA degradation is thought to take place in the 3'-5' direction. First, poly(A)tail is removed by deadenylating nucleases. AU-binding protein(s) bind to ARE motifs tagging mRNA for exosome. Exosome is a protein complex containing at least ten subunits and responsible for exonucleolytic degradation of the body of mRNA. AU-binding proteins involved in the degradation of mRNA include tristetraprolin, AUF1 and KSRP. Another AU-binding protein, HuR, is involved in the stabilization of mRNA, possibly by preventing the interaction between mRNA and exosome.

and iNOS mRNA expression is markedly increased by cytokine mixture with only a minor change in transcription rate (Linn et al. 1997).

A few factors have been found to affect the iNOS mRNA degradation rate. TGF-β diminishes iNOS expression by reducing iNOS mRNA stability and translation in mouse macrophages (Vodovotz et al. 1993), and TGF-β-deficient mice show enhanced iNOS mRNA and NO production (Vodovotz et al. 1996). Another anti-inflammatory cytokine, interleukin-4, potentiates IFNγ -induced iNOS expression by stabilizing iNOS mRNA in human airway epithelial cells. The stabilization was due to factor(s) released by the cells shortly after the introduction of cytokines (Guo et al. 1997). In

addition, the elevation of $[Ca^{2+}]_i$ has been reported to reduce NO production through enhanced iNOS mRNA degradation in chondrocytes (Geng and Lotz 1995a). Recently, cyclic GMP was found to destabilize iNOS mRNA in human mesangial cells stimulated with a combination of IL-1β and TNFα (Perez-Sala et al. 2001), suggesting a mechanism for feed-back down-regulation of NO formation through the iNOS pathway by NO itself.

Murine iNOS cDNA contains two copies of AUUUA motifs, and human iNOS cDNA evincecs four AUUUA motifs in the 3'-UTR (Lyons et al. 1992, Geller et al. 1993a). However, the role of ARE sequences in the destabilization of iNOS mRNA has not been confirmed. Instead, there are data suggesting that the 3'-UTR of iNOS mRNA participates in the regulation of iNOS expression in cooperation with the promoter region (Nunokawa et al. 1997). A recent finding indicates that HuR, an ARE binding protein involved in the stabilization of transiently expressed mRNAs, binds to ARE sequences found in the 3'-UTR of iNOS mRNA and stabilizes it (Rodriguez-Pascual et al. 2000).

4.4. mRNA as a target for therapy

There is a growing body of evidence suggesting that alterations in the stability of the mRNAs of transiently expressed gene play a role in the pathogenesis of a number of diseases. Such diseases include Alzheimer´s disease and tumours. In Alzheimer's disease, the hallmark is the deposition of β-amyloid, formed out of amyloid precursor protein, in the brain, especially around the blood vessels. A local inflammatory process has been suggested to take place in the pathogenesis of in the disorder (Lue et al. 2001). Interestingly, amyloid precursor protein mRNA is stabilized by growth factors (Rajagopalan and Malter 2000), and an RNA binding protein hnRNP C, which can bind to amyloid precursor protein mRNA, stabilizes it, and results in an increased synthesis of amyloid precursor protein (Rajagopalan et al. 1998). In certain brain tumours, there are observations that the mRNA-stabilizing protein HuR is expressed, and results in the enhanced expression of cytokines and angiogenic factors by a mechanism related to the inhibition of decay of the mRNAs of the genes (Nabors et al. 2001). In addition, the mRNA of vascular endothelial growth factor is stabilized by HuR in hypoxic conditions (Levy et al. 1998).

TNFα has a prominent role in a number of inflammatory diseases. Mice lacking the gene for TTP have elevated levels of TNFα and the animals develop an inflammatory syndrome characterized by cacexia, arthritis, conjunctivitis, glomerular mesangial thickening and increased concentrations of anti-DNA and antinuclear antibodies. Enhanced TNFα expression was interpreted to be largely due to the impairment of normal degradation of TNFα mRNA (Taylor et al. 1996, Carballo et al. 1998). In alcohol liver disease, increased TNFα expression is related to liver injury, and, according to a recent finding, ethanol results in stabilization of TNFα mRNA in hepatocytes, which in turn enhances TNFα expression (Tilg and Diehl 2000, Kishore et al. 2001). Asthmatic inflammation is characterized by pulmonary eosinophilia. In the pathogenesis of asthma, prolonged eosinophilic survival is considered to play a prominent role (Giembycz and Lindsay 1999, Kankaanranta et al. 2000). A recent finding suggests that the stabilization of granulocyte/macrophage-colony stimulating factor mRNA by TNFα through ARE motifs leads to its increased expression contributing to the prolonged survival of pulmonary eosinophils in *in vivo* conditions (Esnault and Malter 2001, Capowski et al. 2001).

eNOS is expressed constitutively and is responsible for the production of NO, participating in the physiological regulation of endothelial function. In blood vessels with atherosclerotic plaques, eNOS derived NO formation is impaired, and one proposed mechanism is decreased eNOS expression caused by TNFα produced in the affected endothelium (De Meyer and Herman 2000). It has been shown that impaired eNOS expression is due to accelerated eNOS mRNA degradation by TNFα. The mechanism of eNOS mRNA degradation by TNFα involves binding of a cytosolic protein to the 3'-UTR of eNOS mRNA (Alonso et al. 1997). Another interesting finding shows that cerivastatin, an HMG-CoA reductase inhibitor, prevents the down-regulation of eNOS and stabilized eNOS mRNA by inhibiting the binding of cytosolic proteins to the 3'-UTR of eNOS mRNA (Gonzalez-Fernandez et al. 2001).

As noted above, several lines of evidence suggest that the alteration of mRNA stability plays a role in the pathophysiological processes involved in a number of diseases. This furthers our understanding of the course of diseases and the mechanisms underlying pathological changes in the host, and may have implications regarding the etiology of a given disease. The changes in the regulation of mRNA stability of transiently expressed genes seem to be related to malignancies and diseases which show changes in factors regulating inflammatory processes and the cell cycle. This may hold prospects of new approaches in the treatment of cancers and various inflammatory

diseases. However, basic research is needed to clarify the mechanisms and factors regulating the targeted mRNA turnover before any therapeutic expectations can be evaluated.

Increased iNOS expression and NO synthesis are induced in inflammation, but the underlying mechanisms are unclear. The induction of transcription is of crucial importance in iNOS expression, but it is likely that post-transcriptional mechanisms are of significance, and here regulation of mRNA stability has a central role. At present, our understanding of the transcriptional regulation of iNOS is rather limited, and even less is known regarding the regulation of iNOS mRNA stability. Research focusing on events regulating iNOS expression, and especially iNOS mRNA stability, is of importance in deepening our understanding of the mechanisms regulating NO production, and in the search for targets for novel pharmacological interventions.

AIM OF THE STUDY

Increased NO synthesis by the iNOS pathway is involved in the pathogenesis of various inflammatory diseases. The aim of the present study was to investigate the mechanisms involved in the regulation of iNOS expression in activated macrophages.

The detailed aims were:

1. to investigate the effect of probiotic *Lactobacillus* GG on iNOS expression and NO production, and to explore the intracellular pathways participating in the process,
2. to assess the role of free cytosolic Ca^{2+} in the expression of iNOS and NO production, and
3. to investigate the effect and mechanism of action of dexamethasone in iNOS expression and NO formation

MATERIALS AND METHODS

1. Cell cultures (I-IV)

iNOS expression and NO production were studied in murine J774 macrophages (American Type Culture Collection No: TIB-67) and T84 human colon epithelial cells (American Type Culture Collection No: CCL 248). J774 murine macrophages and human T84 colon epithelial cells were cultured at 37°C in humidified 5% carbon dioxide atmosphere in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum, penicillin (100 U/ml), streptomycin (100 μg/ml) and amphotericin B (250 ng/ml) and harvested with trypsin-EDTA. Cells were grown to confluence in 24 well plates, and confluent cell monolayers were exposed to culture medium containing the bacteria and/or compounds of interest. For other subsequent analyses, culture medium was collected and cells were harvested after the time indicated.

2. Determination of cell viability and apoptosis (III)

The number of viable cells was estimated by colorimetric assay using Cell Proliferation Kit II (Boeringer Mannheim GmbH, Mannheim, Germany). Cells were seeded on 96-well plates, and incubated at 37°C for 24 hours in the presence of compounds of interest. A tetrazolium salt XTT (sodium 3′- [1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis (4-methoxy-6-nitro) benzene sulfonic acid hydrate), which is metabolized to formazan by intact mitochondrial dehydrogenases, and an electron coupling reagent (1,25 mM N-methyl dibenzopyrazine methyl sulfate) were added and the cells were incubated for another 4 hours. The viability of cells was estimated on the basis of formazan formed, this being detected spectrophotometrically.

Macrophage apoptosis was determined by propidium iodide (PI) staining of DNA fragmentation and flow cytometry (FACScan, Becton Dickinson, San Jose, CA). Briefly, the 200xg cell pellet was gently resuspended in hypotonic fluorochrome solution (PI 25 μg/ml in 0.1% sodium citrate and 0.1% Triton X-100). The tubes were left in the dark overnight at 4°C before flow cytometric analysis. Cells showing reduced relative DNA content were considered apoptotic.

3. Nitrite and nitrate assays (I-IV)

In aqueous solutions, NO is metabolized rapidly into nitrite. NO production was measured as nitrite (NO_2^-), accumulated in the culture medium, by Griess reagent (1% sulfanilamine, 0,1% naphtyletylene diamine dihydrochoride and 2,5% phosphoric acid). Nitrite reacts with Griess and forms purple azo dye, which can be detected spectrophotometrically at a wave-length of 546 nm. Sodium nitrite was used as standard (Green et al. 1982). The detection limit for the assay was 1 μM.

In some experiments nitrite or nitrite + nitrate was measured by ozone-chemiluminescence. When the nitrite + nitrate accumulation into the culture medium was measured, vanadium (III) chloride in hydrochloric acid was added to convert nitrite and nitrate to NO, which was then quantified by the ozone-chemiluminescence method (Braman and Hendrix 1989). Samples were first treated with ethanol at – 20 °C for two hours to precipitate proteins. An aliquot of the supernatant was injected into a cylinder containing a saturated solution of VCL_3 in 1 M HCl (0,8 g VCl_3 per 100 ml of 1 M HCl) at 95 °C and NO formed in these reducing conditions was measured by the NO analyser NOA 280 (Sievers Instruments Inc., Boulder, CO, USA) using sodium nitrate as standard. When nitrite concentrations were measured, deproteinized samples were injected into a cylinder containing sodium iodide (1% wt/vol) in acetic acid at room temperature to convert nitrite to NO, which was measured by NO analyser NOA 280 using sodium nitrite as a standard (Holm 2000)

4. Western blot analysis (I-IV)

Cell pellets were lysed in ice cold extraction buffer (10 mM Tris-base, 5 mM EDTA, 50mM NaCl, 1% Triton-X-100, 0.5 mM phenylmethyl sulfonyl fluoride, 2 mM Na-orthovanadate, 10 μg/ml leupeptin, 25 μg/ml aprotinin, 1.25 mM NaF, 1 mM Na-pyrophosphate, 10 mM n-octyl-β-D-glucopyranoside). An aliquot of the supernatant was used to determine protein by the Coomassie blue method (Bradford 1976). Following incubation on ice for 15 min, samples were centrifuged and the resulting supernatant boiled for 5 min in sample buffer (62.5 mM Tris-HCl, 20% glycerol, 2% SDS and 10mM 2-mercapto-ethanol). Protein samples were separated by SDS-PAGE on 10% polyacrylamide gels and transferred to nitrocellulose (Laemmli 1970). iNOS protein was identified by Western blot using rabbit polyclonal iNOS antibody (Santa Cruz Biotechnology, Santa Cruz,

CA, USA). The membrane was then washed and incubated with a horseradish peroxidase-linked anti-rabbit IgG. The nitrocellulose was developed by the ECL™ Western blot detection system. Densitometric analysis was carried out on SigmaGel™ software.

5. Reverse-transcriptase polymerase chain reaction (RT-PCR) (I, III, IV)

Cells were grown on 6-well plates to confluency, and then stimulated for the time indicated. Cell monolayers were washed twice with ice-cold PBS. Subsequently, the cells were lysed and cell lysate homogenized using a QIAshredder™ (QIAGEN Inc, Santa Clarita, CA, U.S.A.) and extraction of total RNA was carried out with the RNeasy® kit for isolation of total RNA (QIAGEN Inc). Synthesis of cDNA from mRNA and subsequent amplification of cDNA by PCR was performed with a GeneAmp® Thermostable r*Tth* Reverse Transcriptase RNA PCR Kit (Perkin-Elmer; Roche Molecular Systems Inc., Branchburg, NJ, U.S.A.) or by RobusT RT-PCR Kit (Finnzymes Oy, Espoo, Finland) according to manufacturer´s instructions. Primers for murine iNOS mRNA were the following: the left primer was 5'-ATGCCCGATGGCACCATCAGA-3' and the right primer 5'-CACTTCCTCCAGGATGTTGTA-3', resulting in a 372 bp product (Lyons et al. 1992, Moilanen et al. 1997). Primers for TTP (left primer 5’-TACGAGAGCCTCCAGTCGAT-3’ and right 5’-GCGAAGTAGGTGAGGGTGAC-3’, resulting in a 238 bp product) were designed using Primer3 software (code available at Steve Rozen, Helen J. Skaletsky (1996,1997) http://www-genome.wi.mit.edu/genome_software/other/primer3.html) from a mouse TTP sequence (Genbank accession NM_011756). For murine GAPDH, the left primer was 5’-CGGAGTCAACGGCTTTGGTCGTAT-3’ and right 5’-AGCCTTCTCCATGGTGGTGAAGAC-3’ resulting in a 306 bp product (Wong et al. 1994). Products were analysed on 2% agarose gel containing ethidium bromide and visualized with UV light. TTP RT-PCR products were analysed using FluorChem (Alpha Innotech Corporation, San Leandro, CA, USA). Primers were obtained from Pharmacia Biotech; Genosys Biotechnologies Inc., Cambridge, UK. PCR products were sequenced.

6. Ribonuclease protection assay (RPA) (IV)

Cells were grown to confluency in 6-well plates, and compounds of interest were supplied in a fresh culture medium at the beginning of the experiment. Cells were washed twice with PBS, and extraction of total RNA carried out as described above. Murine iNOS and GAPDH mRNAs were detected by ribonuclease protection assay using murine iNOS (Cayman Chemical, Ann Arbor, MI, U.S.A.) and GAPDH probe template (PharMingen, San Diego, CA, U.S.A.), respectively. In brief, 3 µg of total RNA was hybridized overnight with the ^{32}P-labeled RNA probes, which had been synthesized from the templates using T7 RNA polymerase (RiboQuant™ In Vitro Transcription Kit; PharMingen, San Diego, CA, U.S.A.). The RNase protection assay was carried out using RiboQuant™ Ribonuclease Protection Assay Kit (PharMingen, San Diego, CA, U.S.A.). Single-stranded RNA and free probe were digested by RNase A and T1. Subsequently, protected mRNAs (371 and 97 base pairs for iNOS and GAPDH, respectively) were separated on a 6 % denaturing polyacrylamide gel and the gel exposed on film. The levels of mRNA expression were quantified by densitometric analysis using SigmaGel™ software. The value of iNOS expression was normalized against GAPDH (Melton et al. 1984).

7. Electrophoretic mobility shift assay (EMSA) (III)

J774 macrophges were incubated with the tested compounds for 30 min. The cells were then rapidly washed with ice cold PBS and solubilized in hypotonic buffer A (10 mM HEPES-KOH, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.2 mM PMSF, 10 µg/ml leupeptin, 25 µg/ml aprotinin, 0.1mM EGTA, 1 mM Na_2VO_4, 1mM NaF). After incubation for 10 min on ice cells were vortexed for 30 s and the nuclei separated by centrifugation at 4 °C, 15000 rpm for 10 s. The nuclei were resuspended in buffer C (20 mM HEPES-KOH, pH 7.9, 25 % glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 0.2 mM PMSF, 10 µg/ml leupeptin, 25 µg/ml aprotinin, 0.1mM EGTA, 1 mM Na_3VO_4, 1mM NaF), incubated for 20 min on ice, and vortexed for 30 s, and nuclear extracts were obtained by centrifugation at 4 °C, 15000 rpm for 2 min. The protein content of the nuclear extracts was measured by the Coomassie blue method (Bradford 1976).

Single-stranded oligonucleotides (5'-AGTTGAGGGGACTTTCCCAGGC-3', 3'-TCAACTCCCCTGAAAGGGTCCG-5') (Amersham Pharmacia Biotech) containing the NF-κB-

binding sequences were annealed and the 5'[^{32}P]-end labeled using the DNA 5'-End Labeling Kit (Boehringer Mannheim GmbH, Mannheim, Germany). For binding reactions, 10 μg of nuclear extract was incubated in 20 μl of the 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, 10 % glycerol 20 min at room temperature; 0,2 ng ^{32}P-labeled oligonucleotide probe was added to the reaction mixture and incubated for 10 minutes. Thereafter, protein/DNA complexes were separated from free DNA probe by electrophoresis on a native 4 % polyacrylamide gel, and the dried gel exposed on film using an intensifying screen at –70 °C (Fried and Crothers 1981).

8. Measurement of $[Ca^{2+}]_i$ *and* Ca^{2+} *influx (III)*

Cells (50×10^6/ml) were loaded with the acetoxymethyl ester form of the fluorescent probe Fura-2 (5 μM) for 30 min at 37°C in a shaking water-bath. Macrophages were then suspended in two volumes of HEPES buffer (containing 145 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$ and 1.5 mM Ca^{2+}, pH 7.40 at 37°) and kept at room temperature for 10 min to allow reequilibration. The cells were washed twice and suspended in HEPES buffer to yield a cell suspension containing 5×10^6 macrophages /ml of buffer. Changes in fluorescence were recorded with a Shimadzu RF-5000 spectrofluorometer (Shimadzu Corp., Kyoto, Japan) in thermostated (37°) quartz cuvettes, with continuous stirring. The excitation wavelengths were set at 340 nm and 380 nm, and the emission wavelength at 500 nm, when changes in $[Ca^{2+}]_i$ were measured.

Cells were incubated with the drug studied for 3 min before stimulation. The increases in $[Ca^{2+}]_i$ were induced by addition of the inhibitor of Ca^{2+}-ATPase thapsigargin (100 nM) or the calcium ionophore A23187 (1 μM). Calibration of the signal was performed as previously described (Grynkiewicz et al. 1985). The maximal fluorescence (F_{max}) was measured after addition of 2 μM ionomycin, and the minimum fluorescence (F_{min}) in the presence of 25 mM EGTA, pH 8.6, and 0.1% Triton 100. The $[Ca^{2+}]_i$ values were calculated from the equation $[Ca^{2+}]_i$ (nM) = $R \cdot 224 \cdot (F-F_{min})/(F_{max}-F)$, where 224 represents the dissociation constant for Fura-2, F is the fluorescence of the intact cell suspension, and R is the ratio F_{min}/F_{max} at 380 nm.
The resting $[Ca^{2+}]_i$ of macrophages suspended in HEPES was 114 ± 16 nM (n=10). After stimulation with 100 nM thapsigargin and 1 μM A23187, the maximal $[Ca^{2+}]_i$ values were 303 ± 61 nM (n=5) and 261 ± 89 nM (n=5), respectively.

9. 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 $P < 0.05$.

10. Materials

Table 2. List of the pharmacological compounds used in the study.

Compound	*Function*	*Reference*
A23187	Ca^{2+} ionophore	Ishida and Shibata 1980
actinomycin D	inhibitor of RNA synthesis	Aktipis and Panayotatos 1981
cycloheximide	inhibitor of eukaryotic protein synthesis	Obrig et al. 1971
cyclosporine	inhibitor calcineurin-dependent phosphatase	Fruman et al. 1992
DAHP	inhibitor of BH_4 synthesis	Gross and Levi 1992
dexamethasone	glucocorticoid	Radomski et al. 1990
genistein	inhibitor of tyrosine kinases	Akiyama et al. 1987
L-NAME	NOS inhibitor	Janssens et al. 1992
L-NMMA	NOS inhibitor	Palmer et al. 1988
L-NIO	NOS inhibitor	Charles et al. 1993
PD98059	inhibitor of p42/44 MAP-kinase	Alessi et al. 1995
PDTC	inhibitor of NF-κB activation	Schreck et al. 1992
Ro 31-8220	inhibitor of protein kinase C	Davis et al. 1989
RU24858	dissociated glucocorticoid	Vanden Berghe 1999
SB203580	inhibitor of p38 MAP-kinase	Cuenda et al. 1995
sepiapterin	pre-cursor of BH_4	Werner-Felmayer et al. 1993
thapsigargin	inhibitor of microsomal Ca^{2+}-ATPase	Thastrup et al. 1990
1400W	selective iNOS inhibitor	Garvey et al. 1997

Culture media, fetal bovine serum, antibiotics and trypsin-EDTA were purchased from Gibco BRL, Paisley, Scotland, UK. Recombinant murine interferon γ (IFNγ), human IFNγ and human tumour necrosis factor α (TNFα) were provided by Immugenex Corp., Los Angeles, CA, USA. Recombinant human interleukin-1β (IL-1β) was supplied by Genzyme, Cambridge, MA, USA.

Lipopolysaccharide from *Escherichia coli* 0111:B4, lipoteichoic acid from *Staphylococcus aureus*, and N^G-nitro-L-arginine methyl ester (L-NAME), thapsigargin, A233187, Fura-2/AM, cycloheximide, and actinomycin D were from Sigma, St. Louis, MO, USA. Freeze-dried, viable and heat-inactivated *Lactobacillus rhamnosus* GG products, *Lactobacillus rhamnosus LC* 705, *Escherichia coli* B44, and 2-pyrrolidone-5-carboxylic acid (PCA) were provided by Valio Ltd, Helsinki, Finland. N-monomethyl-L-arginine (L-NMMA, Clinalfa, Läufelfingen, Switzerland); L-N-iminoethyl-ornithine (L-NIO), sepiapterin, 2,4-diamino-6-hydroxypyrimidine (DAHP) (Alexis Corp., Läufelfingen, Switzerland); pyrrolidinedithiocarbamate (PDTC), genistein (Tocris, Langford, Bristol, UK); Ro 31-8220 (Roche Products Ltd, Hertfordshire, UK); PD98059, SB203580, (Calbiochem, La Jolla, CA, USA); cyclosporine, (Sandoz Pharma Ltd., Basel, Switzerland); dexamethasone (Orion Corp., Espoo, Finland), RU24858 (Aventis Pharma, Romainville Cedex, France) were obtained as indicated. [α-^{32}P]UTP was obtained from Amersham Pharmacia Biotech, Buckinghamshire, UK. 1400W was a kind gift from Dr. Richard G. Knowles (Glaxo-Wellcome Research and Development, Stevenage, UK). All other reagents were obtained from Sigma Ltd. St. Louis, MO, USA.

RESULTS

The present study investigated the induction of iNOS mRNA and protein and production of NO in response to inflammatory stimuli, mainly LPS, IFNγ and LTA, and the intracellular mechanisms involved in the process, especially the role of $[Ca^{2+}]_i$. Further, the mechanism of action of dexamethasone, a glucocorticoid analog used as an anti-inflammatory agent, in reduced NO formation was studied. In addition, the effect of the Gram-positive probiotic *Lactobacillus rhamnosus* GG (*Lactobacillus* GG) (American Type Culture Collection No: 53103) on NO formation was investigated. Two other bacterial strains, Gram-negative *Escherichia coli* B44, and another Gram-positive *Lactobacillus* strain *rhamnosus* LC 705 were also tested for reference.

1. The effect of LPS and IFNγ on the induction of NO synthesis

Resting J774 macrophages did not produce NO, and iNOS protein and mRNA were not detectable. Treatment of cells with IFNγ (5 ng/ml) also failed to induce detectable NO synthesis and iNOS protein expression (I-IV). LPS alone or in combination with IFNγ was able to induce marked NO synthesis, as well as the expression of iNOS mRNA, detected by RT-PCR and RPA, and iNOS protein, detected by Western blot (II-IV). In addition, a Gram-negative bacteria *Escherichia coli* B44 induced marked NO synthesis (I). iNOS expression and NO synthesis were both abrogated by the protein synthesis inhibitor cycloheximide (1 μg/ml) and transcription factor NF-κB inhibitor PDTC (100 μM) indicating that LPS- or LPS+IFNγ-induced iNOS expression and NO production require the activation of NF-κB and *de novo* protein synthesis. NO production was totally inhibited by the non-selective NOS inhibitor L-NMMA, as well as by the selective and highly selective iNOS inhibitors L-NIO and 1400W, respectively (I-IV). These data indicate that the nitrite/NO detected was produced by iNOS (II). After stimulation of cells with a low LPS concentration in the presence of IFNγ (II), NO formation was attenuated by genistein (protein tyrosine kinase inhibitor). Partial inhibition was also achieved by Ro 31-8220 (PKC inhibitor), PD98059 (inhibitor of p42/44 MAP-kinase), and CsA. SB203580 (inhibitor of p38 MAP-kinase) did not regulate NO synthesis in these conditions. (II)

2. Induction of iNOS expression and NO production by Lactobacillus GG and its pharmacological regulation (I and II)

Freeze-dried *Lactobacillus* GG induced NO synthesis and iNOS protein expression in J774 macrophages and the effect was enhanced by IFNγ. In the presence of IFNγ, *Lactobacillus* GG induced detectable mRNA expression at a time point of 2 h after the stimulation, and iNOS mRNA peaked at 4 to 12 h and gradually declined between 12-48 h after stimulation. NO synthesis and iNOS protein expression were abrogated by cycloheximide and PDTC. iNOS mRNA expression was inhibited by PDTC, but not by cycloheximide. In the presence of IFNγ, cultured and heat-inactivated *Lactobacillus* GG as well as *Lactobacillus rhamnosus* LC 705 induced NO synthesis comparable to that induced by freeze-dried *Lactobacillus* GG. *Lactobacillus* GG-induced NO formation was inhibited by the non-selective NOS inhibitors L-NMMA and L-NAME, by partially the selective iNOS inhibitor L-NIO, and the highly selective iNOS inhibitor 1400W in a dose-dependent manner (see Figure 6 and Table 3). These data indicate that NO formation induced by *Lactobacillus* GG was due to induction of iNOS. In addition, *Lactobacillus* GG induced NO synthesis in T84 human epithelial cells primed with IFNγ, TNFα and IL-1β.

Signalling mechanisms involved in the induction of iNOS expression and NO production by *Lactobacillus* GG were studied by pharmacological means, and compared to those involved in the LTA- and LPS-induced signalling. LTA and LPS are cell wall components of Gram-positive and Gram-negative bacteria, respectively. *Lactobacillus* GG-induced iNOS expression and NO production were inhibited by inhibitors of protein tyrosine kinase and NF-κB, indicating the crucial role of these factors in induction, as was also the case in LTA- and LPS-treated cells. Inhibitors of p42/44 and p38 MAP kinases (PD98059 and SB203580, respectively) and cyclosporine partially inhibited NO production and iNOS protein expression in cells stimulated with *Lactobacillus* GG, LTA and LPS. This suggests that the effects of these three stimuli on iNOS expression and NO synthesis are mediated through a common pathway (see Figure 6).

	L-NMMA	L-NAME	L-NIO	1400W
no drug	100	100	100	100
10 μM	78 ± 9.5	89 ± 8.6	56 ± 7.4	80 ± 6.4
100 μM	43 ± 4.6	48 ± 7.2	13 ± 2.3	19 ± 1.9
1000 μM	11 ± 4	29 ± 16	10 ± 2.7	4.7 ± 2.5

Table 3. The effect of NOS inhibitors on NO formation in J774 macrophages treated with a combination of Lactobacillus rhamnosus GG and IFNγ. Results are expressed as % of control, mean ± SEM. (Reproduced, with permission, from Korhonen et al., 2001, Inflammation. Vol 25, pp 223-232. © Kluwer Academic/Plenum Publisher.)

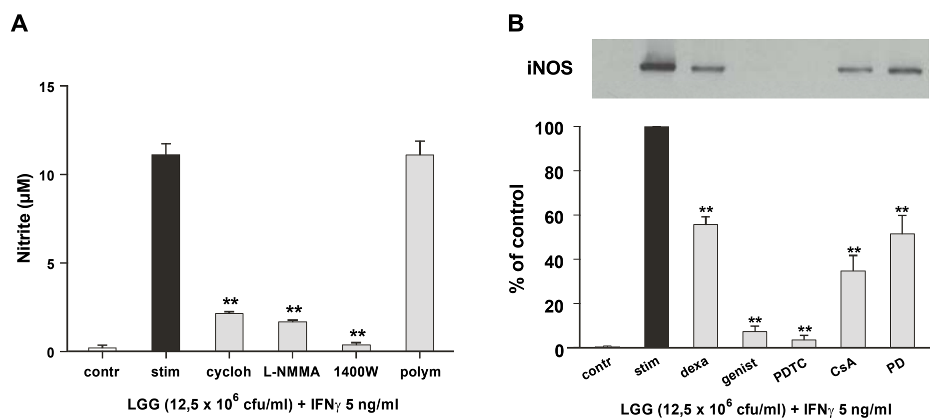


Figure 6. The effect of Lactobacillus GG on NO formation and expression of iNOS protein in J774 macrophages primed with IFNγ. (A) Cells were incubated for 24 h and NO production then measured as nitrite accumulated in the culture medium. (B) Cells were incubated for 12 h, and then harvested for protein extraction. iNOS protein was detected by Western blot. Concentrations of the pharmaceutical compounds: cycloheximide (cycloh; 1 μg/ml), L-NMMA (1 mM), 1400W (1 mM), polymyxin B (Polym; 1 μg/ml), dexamethasone (dexa; 10 μM), genistein (genist; 100 μM), PDTC (100 μM), cyclosporine (CsA; 10 μM), and PD98059 (PD; 10 μM) were as indicated. Results are expressed as mean ± SEM, n=6 and n=4 for nitrite determination and Western blot, respectively. (Reproduced, with permission, from Korhonen et al., 2002, Inflammation, in press. © Kluwer Academic/Plenum Publisher.)

BH_4 is an essential co-factor in NO synthesis, and its expression is induced by LPS. The inhibition of BH_4 synthesis by DAHP reduced NO formation by 40% in cells stimulated with the combination of *Lactobacillus* GG and IFNγ, and the effect of DAHP was reversed by supplementation of sepiapterin, a precursor of BH_4.

3. Regulation of iNOS expression and NO production by $[Ca^{2+}]_i$ (III)

The role of the elevated concentration of cytosolic calcium $[Ca^{2+}]_i$ in iNOS expression and NO production in LPS-stimulated cells was investigated using thapsigargin and A23187 as agents to induce elevated $[Ca^{2+}]_i$. Thapsigargin alone did not induce detectable NO synthesis or iNOS protein expression. Combining thapsigargin with a low concentration of LPS (1 ng/ml) resulted in increased iNOS mRNA and protein expression, as well as NO production, as compared to LPS alone. Thapsigargin-induced potentiation of NO production was most potent when thapsigargin was added to the cells 1 h prior or along with LPS, and it gradually decreased if thapsigargin was administered 1 – 4 h after LPS stimulation. Similarly to thapsigargin, A23187 induced NO production in cells primed with LPS (1 ng/ml).

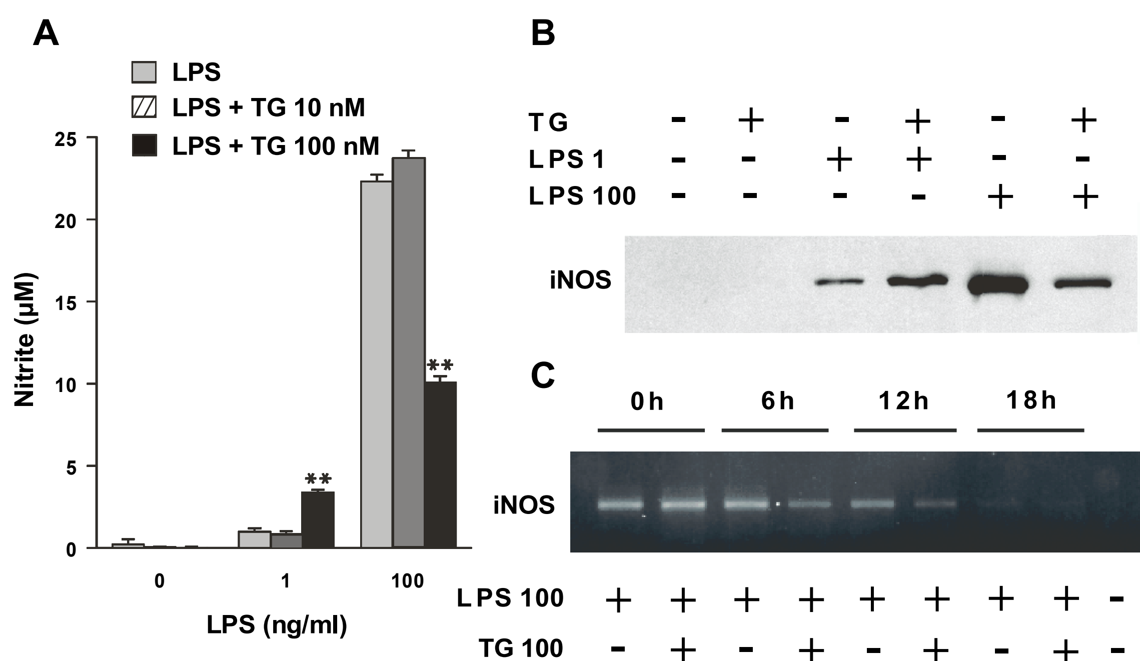


Figure 7. The effect of thapsigargin on NO production, iNOS protein expression and iNOS mRNA degradation in J774 macrophages stimulated with LPS (1 or 100 ng/ml). (A) NO was measured as nitrite in the culture medium after 24 h incubation; results are expressed as mean + SEM, n=6. (B) Cells were incubated for 12 h and then harvested for protein extraction. iNOS protein was detected by Western blot. (C) Cells were stimulated with LPS for 6 h, and actinomycin then added. Subsequently, cells were incubated for the time indicated, and then harvested for RNA extraction. iNOS mRNA was detected by RT-PCR. Concentrations of thapsigargin (TG) were 10 and 100 nM in nitrite experiments 100 nM in Western blot and RT-PCR experiments. (Reproduced, with permission, from Korhonen et al., 2001, Biochemical Journal, Vol. 354, pp 351-358. © the Biochemical Society.)

In contrast, thapsigargin clearly inhibited NO formation and iNOS protein expression is cells treated with a high concentration of LPS (100 ng/ml). The suppressed iNOS protein expression and NO formation was associated with the destabilization of iNOS mRNA by thapsigargin. Thapsigargin did not alter activation of NF-κB, which is a critical transcription factor for iNOS, with respect to LPS treatment. Thus, the elevation of $[Ca^{2+}]_i$ has bi-directional effects on iNOS expression and NO formation in LPS-treated cells depending on the LPS concentration. In cells primed with a low LPS concentration, elevated $[Ca^{2+}]_i$ enhances iNOS expression and NO production, whereas the elevation of $[Ca^{2+}]_i$ inhibits iNOS expression and NO synthesis in cells treated with a high LPS concentration (Figure 7).

4. The effect of dexamethasone on iNOS expression and NO production in cells treated with LPS and a combination of LPS and IFNγ

The effect of dexamethasone on iNOS expression and NO production in cells treated with LPS (10 ng/ml) and a combination of LPS (10 ng/ml) and IFNγ (5 ng/ml) was investigated. Dexamethasone reduced NO production and iNOS expression in a dose-dependent manner in cells treated with LPS. Decreased NO formation was not reversed by supplementation of L-arginine. In the presence of mifepristone, a competitive glucocorticoid receptor antagonist, dexamethasone had no effect on iNOS expression or NO production. This indicates that the suppressive effect of dexamethanone is mediated through activation of the glucocorticoid receptor. Interestingly, dexamethasone failed to inhibit iNOS expression and NO formation in cells stimulated with a combination of LPS and IFNγ.

Dissociated glucocorticoids are synthetic steroid analogs having an inhibitory effect on NF-κB and activator protein-1 activation comparable to that of glucocorticoids, but they do not regulate gene expression by a GRE-mediated mechanism (Vanden Berghe et al. 1999). Here, the effect of one such compound, RU24858, was tested. RU24858 inhibited NO synthesis and iNOS protein expression in cells treated with LPS; thus, the effect was comparable to that of dexamethasone. In contrast, cells treated with a combination of LPS and IFNγ were resistant to RU24858 with respect to iNOS protein expression and NO production.

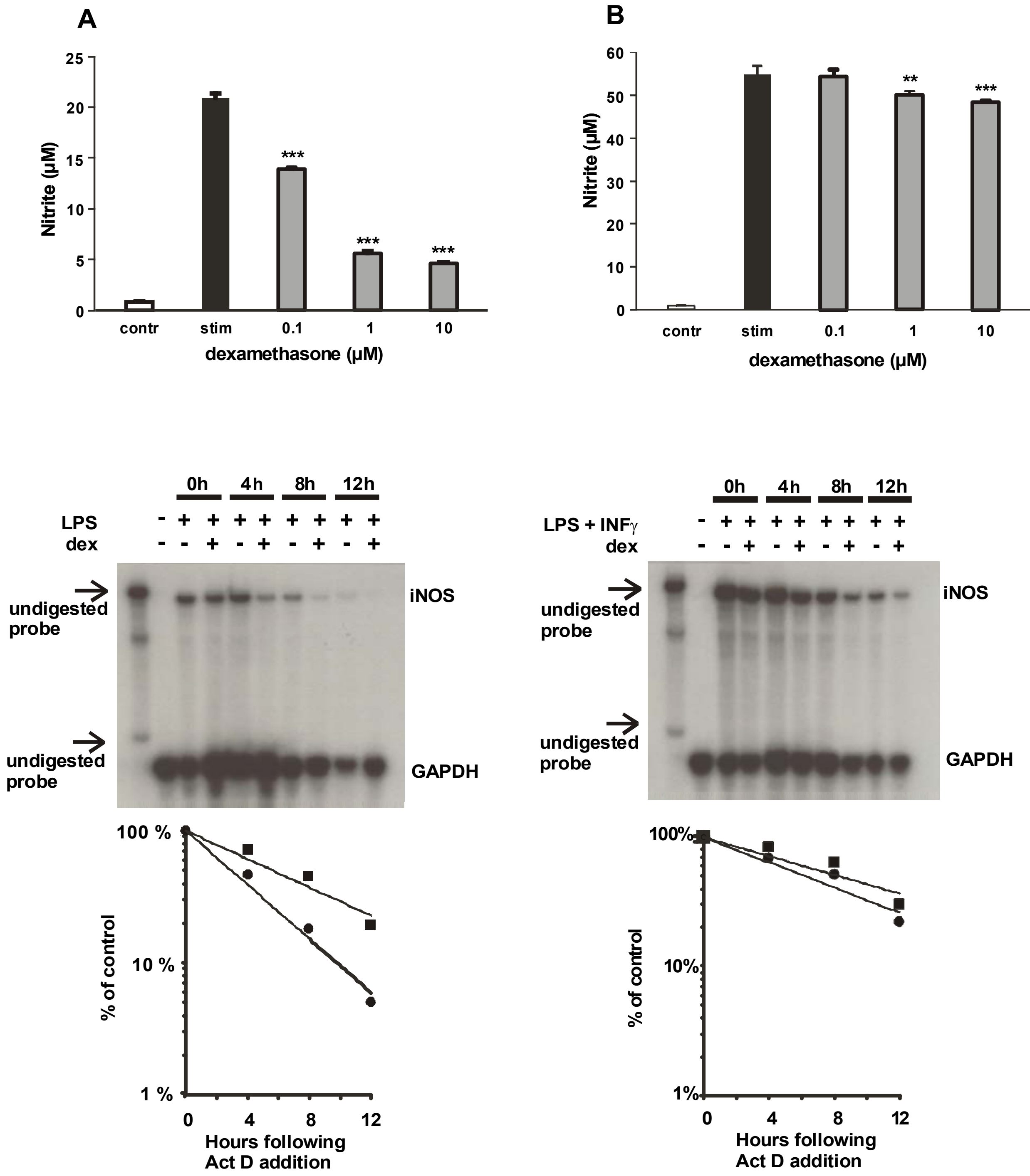


Figure 8. The effect of dexamethasone on NO formation and iNOS mRNA expression and degradation in macrophages treated with (A) LPS and (B) LPS + IFNγ. In nitrite experiments, cells were incubated for 24 h with compounds of interest, and then harvested for nitrite determination. Results are expressed as mean + SEM, n=6. In mRNA experiments, cells were stimulated for 6 h before addition of actinomycin D. Thereafter, cells were incubated for the time indicated, and then harvested for RNA extraction. iNOS mRNA was detected by RPA. Results are given as mean, n=3.

The effect of dexamethasone on iNOS mRNA levels and stability was also investigated. In cells treated with LPS, dexamethasone exerted no significant inhibitory effect on steady-state iNOS mRNA levels after 6 h incubation, but was found to destabilize iNOS mRNA. The effect of dexamethasone on iNOS mRNA expression proved to be protein synthesis-dependent in cells treated with LPS, suggesting that dexamethasone induces synthesis of an mRNA-destabilizing protein. In addition, dexamethasone-induced destabilization of iNOS mRNA was clearly, but not totally, suppressed in cells treated with a combination of LPS and IFNγ (Figure 8).

Thus, dexamethasone was found to inhibit LPS-induced NO synthesis and iNOS protein expression, and was associated with destabilization of iNOS mRNA. The effect of dexamethasone on decreased iNOS mRNA expression was counteracted by inhibition of protein synthesis. In contrast, dexamethasone did not inhibit NO production, iNOS expression, or iNOS mRNA steady-state levels in cells treated with a combination of LPS and IFNγ.

5. Regulation of iNOS mRNA stability

The role of iNOS mRNA stability in the regulation of iNOS expression and NO production with thapsigargin (III) and dexamethasone (IV) was assessed. In these studies, dexamethasone was found to destabilize iNOS mRNA.

The iNOS mRNA half-life was reduced from 5 h 40 min to 3 h (by 46%) by dexamethasone in LPS treated cells. In cells treated with a combination of LPS and IFNγ, the half-life of iNOS mRNA was increased to 8 h 20 min and dexamethasone reduced the half-life by 24% (IV). In addition, elevation of $[Ca^{2+}]_i$ was found to increase the degradation of iNOS mRNA in cells treated with a higher concentration of LPS (III).

TTP is an LPS- and growth factor-inducible protein involved in the destabilization of cytokine mRNAs; it was thus hypothetisized that the effect of dexamethasone on iNOS mRNA destabilization would be mediated by increased production/activity of TTP. The induction of TTP by LPS was therefore measured. J774 macrophages showed some basal expression of TTP mRNA. TTP mRNA expression was increased by LPS. Dexamethasone reduced LPS-induced TTP mRNA expression. It is thus unlikely that TTP is the factor responsible for the destabilization of iNOS mRNA by dexamethasone.

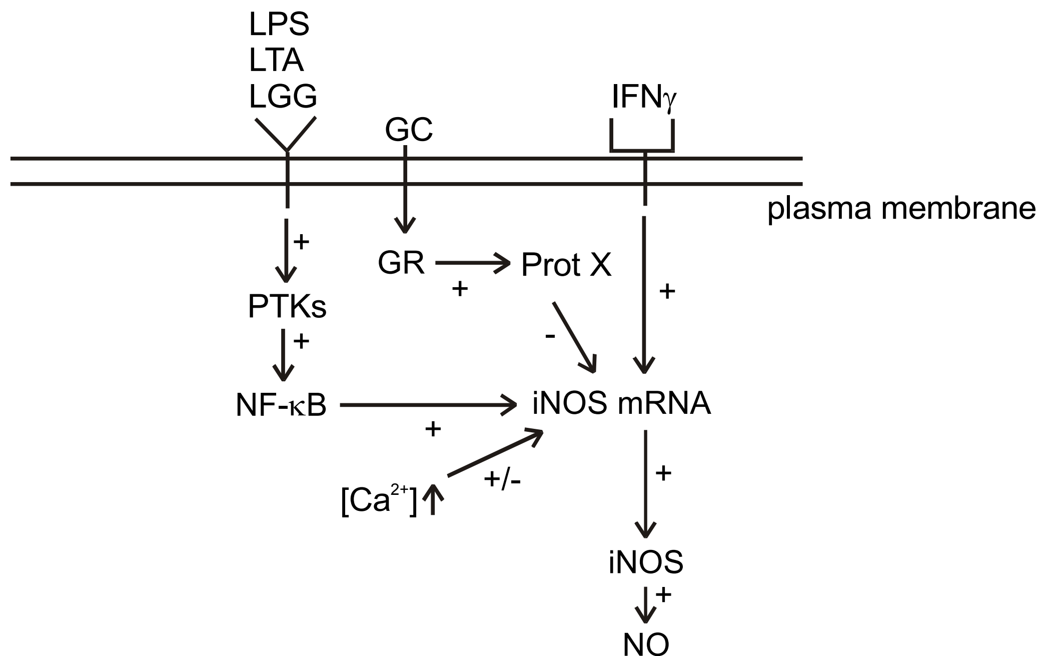


Figure 9. Summary of the main results on the regulation of iNOS expression in J774 macrophages. *LPS (and LTA and Lactobacillus GG) induces iNOS expression through activation of tyrosine kinases and NF-κB. IFNγ enhanced iNOS expression by stabilizing iNOS mRNA. According to the literature, IFNγ may also increase iNOS transcription by activating NF-κB synergistically with IRF-1 and/or STAT1. Increased intracellularCa^{2+} increases iNOS expression in cells treated with low LPS concentration by mechanism independent of NF-κB. In cells treated with higher LPS concentration, Ca^{2+} suppresses iNOS expression by destabilizing iNOS mRNA. Dexamethasone decreases iNOS expresion by destabilizing iNOS mRNA in LPS-treated cells, and the effect of dexamethasone requires protein synthesis. The effect of dexamethasone is over-run by IFNγ. LGG, Lactobacillus rhamnosus GG; GC, glucocorticoids; GR, glucocorticoid receptor; Prot X, unknown protein; NF-κB, nuclear factor κB;*

DISCUSSION

1. Methodology

NO is a reactive molecule in aqueous solution with half-life estimated to be up few seconds, and cannot therefore itself be detected in culture medium. However, metabolites of NO, nitrite (NO_2^-) and nitrate (NO_3^-) can be measured. Nitrite is the main metabolite of NO in many *in vitro* conditions, whereas nitrate is formed in the blood. The most common means to detect nitrite is the Griess reaction. This is a convenient and simple method to determine NO formation, and can easily be applied to a large number of samples. (Green et al. 1982, Moshage et al. 1995). Low concentrations of NO can be determined by the ozone-chemiluminescence method. This approach can be also used to measure nitrite and nitrate if these compounds are first reduced to NO. This is the most accurate measn of measuring NO, and a more convenient way to determine nitrite + nitrate production than traditional methods based on the enzymatic or chemical conversion of nitrate to nitrite (Hampl et al. 1996). Using NOS inhibitors, it is possible to differentiate the nitrite/nitrate derived from NO and possibly other pathways of cellular metabolism.

Macrophages have a pivotal role in innate immune responses, and NO produced by these cells is an important effector molecule in infections (MacMicking et al. 1997, Bogdan 2001). Macrophage cell lines provide a convenient model for studies of the regulation of iNOS expression. Murine macrophage cell line J774 has previously been found to produce NO in response to LPS and the combination of LPS and IFNγ through iNOS pathway (Stuehr and Marletta 1987). Methods used to detect the expression of iNOS (Western blot, RT-PCR, RPA) and transcription factors (EMSA), as well as $[Ca^{2+}]_i$ (flow cytometry) are well established in research into cellular and molecular biology.

2. Lactobacillus GG and NO production

Lactobacillus GG has been found to have beneficiacl effects in gastro-intestinal disorders and atopic diseases (Alvarez-Olmos and Oberhelman 2001, Kalliomäki et al. 2001), but its mechanisms of action have remained largely unknown. There are reports showing that *Lactobacillus* GG has immunostimulatory effects in the host, e.g. it has been shown to promote local antibody production in the intestine and to induce cytokine expression (Kaila et al. 1992, Miettinen et al. 1998).

In the present study, J774 macrophages were found to express iNOS and to produce NO in response to *Lactobacillus* GG and IFNγ. iNOS expression and NO formation were dependent on protein tyrosine kinase and NF-κB activation, since genistein and PDTC abrogated the responses. To a lesser extent, activation of p42/44 and p38 MAP-kinases was also involved. Our results on *Lactobacillus* GG-induced NO production are in line with previous findings showing that induction of iNOS expression by LPS is dependent on the activation of protein tyrosine kinase down-stream of CD14, but upstream of phospholipase C (Chen et al. 1998b). Similarly, iNOS expression induced by *Lactobacillus* GG or LTA was inhibited by genistein and PDTC, indicating the crucial role of protein tyrosine phosphorylation and activation of NF-κB in the process. Indeed, direct activation of NF-κB by non-pathogenic bacteria, including *Lactobacillus* GG, has been shown to take place, leading to expression of IFNγ, interleukin-12, and interleukin-18 in NK cells and monocytes (Miettinen et al. 1998, Miettinen et al. 2000).

In the present experiments, PKC activation participated in the induction of iNOS by *Lactobacillus* GG, LPS, and LTA, because inhibition PKC by Ro 31-8220 at a concentration specific for PKC (Murphy and Westwick 1992), caused partial inhibition on NO formation. This is in line with previous findings suggesting that PCK activation participates in LPS-induced iNOS expression (Paul et al. 1997, Chen et al. 1998b).

There are fragmentary data concerning MAP-kinase activation following LTA stimulation. In monocytes, LTA derived from *Treponema* species has been shown to activate p42/44, p38, and c-Jun N-terminal kinases 1 and 2 (Schroder et al. 2001). In the present study, iNOS expression and NO production were partially inhibited by the MAP-kinase inhibitors PD98059 and SB203580 in cells treated with *Lactobacillus* GG and IFNγ. NO synthesis induced by LTA + IFNγ was also partially inhibited by the p42/44 inhibitor PD98059, but did not respond to the p38 inhibitor SB203580. This suggests that activation of p42/44 and p38 MAP-kinases enhances but is not of major importance in the induction of NO production in response to *Lactobacillus* GG.

Cyclosporine markedly inhibited *Lactobacillus* GG-induced iNOS protein expression and NO formation in IFNγ-primed J774 macrophages. Cyclosporine is an inhibitor of calcium/calmodulin-dependent phosphatase subsequently inhibiting the activation of nuclear factor of activated T cells (also known as NF-AT). Although nuclear factor of activated T cells is not considered to be involved in the induction of iNOS transcription, it may regulate unknown factor(s) which play role

in iNOS expression. BH_4 is an important co-factor in NO synthesis (Alderton et al. 2001), and its production is induced by inflammatory signals activating NF-κB (Hattori and Gross 1993, Hattori et al. 1997). Inhibition of BH_4 synthesis by DAHP inhibited NO production, and the effect was attenuated by supplementation of the precursor of BH_4 in J774 macrophages stimulated with *Lactobacillus* GG and IFNγ. These findings suggest that *Lactobacillus* GG induces the availability of co-factors needed for NO synthesis along with iNOS induction.

Toll receptor (TLR) activation has a prominent role in the activation of innate immunity. LTA, along with LPS, activate TLR4, while TLR2 is activated by a wider range of factors. Activation of both receptor types subsequently activates serine/threonine kinase IRAK, resulting in the activation of NF-κB. Intracellular signal transduction pathway(s) in this step of activation are yet to be established, although mechanisms involving tyrosine phosphorylation of TLR2, and activation of serine/threonine protein kinase IRAK have been proposed (Schwandner et al. 1999, Takeuchi et al. 1999, Yoshimura et al. 1999, Arbibe et al. 2000, Opitz et al. 2001). Although not investigated in the present study, it is likely that induction of iNOS and NO production by *Lactobacillus* GG may be mediated through TLR2 or/and TLR4, and that LTA is the structure responsible for the activation.

The promotion of local antibody production by *Lactobacillus* GG has been found to take place in the intestine of subjects suffering from rotavirus diarrhea (Kaila et al. 1992), and *Lactobacillus* GG has been shown to induce cytokine production by a mechanism dependent on NF-κB activation, and to regulate T-cell functions (Miettinen et al. 1998, Pelto et al. 1998, Miettinen et al. 2000). Results in the present study show that non-pathogenic *Lactobacillus* GG induced iNOS expression and NO production in macrophages. In addition, *Lactobacillus* GG was also found to induce NO production in T84 colon epithelial cells. These findings are in line with earlier reports suggesting that *Lactobacillus* GG has immunostimulatory effects and is able to activate innate immunity and modulate immunological responses. Taken together, these data support the conception that probiotic bacteria modulate immune reactions in the host apart from their ability to transiently colonize the intestine and thereby limit the growth of pathogens (Saxelin et al. 1991, Alander et al. 1997, Tuomola et al. 1999, Mack et al. 1999). Induction of low-level production of NO by *Lactobacillus* GG may be of importance in the healing processes in the gut. These may include modulation of leukocyte migration into the intestinal tissue, augmentation of wound healing, and maintainance of appropriate blood flow in the intestinal wall. In fact, during recent years, the earlier view that the induction of iNOS in gut inflammation inevitably leads to cytotoxic effects has been challenged. In

intestinal inflammation models, LPS- or TNFα-induced leukocyte rolling was shown to be enhanced in the mesenteric microvasculature or in liver sinusoids by iNOS inhibitors and in iNOS-deficient mice (Kubes et al. 1997, Hickey et al. 1997). Acetic acid-induced mild intestinal inflammation was more severe in iNOS-deficient mice as estimated by leukocyte migration and macroscopic damage (McCafferty et al. 1997). These findings would imply that the effects of iNOS-derived NO may depend on the level of iNOS expression and NO production, and/or pathophysiological condition.

3. The role of $[Ca^{2+}]_i$ in iNOS expression

In the present study, the effect of elevation of $[Ca^{2+}]_i$ on the induction of iNOS expression and NO production was investigated. In order to mimic capacitative, i.e. transient receptor-mediated Ca^{2+} entry (Putney et al. 2001), thapsigargin and calcium ionophore A23187 were used as agents to induce elevation of $[Ca^{2+}]_i$. Elevated $[Ca^{2+}]_i$ was found to have a bi-directional effect on NO production. In cells treated with a low LPS concentration, the elevation of $[Ca^{2+}]_i$ increased iNOS mRNA and protein expression and NO production. In contrast, NO production as well as iNOS protein expression were reduced by elevation of $[Ca^{2+}]_i$ in macrophages treated with a higher LPS concentration. The reduced iNOS protein expression was associated with increased iNOS mRNA degradation.

In earlier studies, $[Ca^{2+}]_i$ increased by thapsigargin or calcium ionophores has been found to enhance NO production in peritoneal macrophages primed with IFNγ or LPS (Raddassi et al. 1994, Park et al. 1996) and in an LPS-treated mouse macrophage cell line (Denlinger et al. 1996, Chen et al. 1998). Our results are concomitant with these findings. NO production was also increased by the combination of thapsigargin and phorbol 12-myristate 13-acetate (known as PMA), a PKC activator in peritoneal macrophages (Park et al. 1996), and down-regulation of PKC did not affect UTP-induced, Ca^{2+}-mediated NO production (Chen et al. 1998), suggesting that Ca^{2+} induces NO formation by a mechanism independent of PKC, as was the case in our experiments in which Ro 31-8220 failed to inhibit thapsigargin-induced NO formation.

In T-cells, NF-κB has been reported to be activated by a synergistic mechanism of PKC and elevated $[Ca^{2+}]_i$ (Frantz et al. 1994, Steffan et al. 1995). Although NF-κB is a critical transcription

factor in the induction of iNOS expression, experimental data do not support the hypothesis that increased iNOS expression by thapsigargin is mediated through enhanced NF-κB activation. Another Ca^{2+}-regulated transcription factor, activator protein-1, may be involved, since it has been thought to contribute to increased iNOS expression by the Ca^{2+} signal (Chen et al. 1998). Unfortunately, the effect of $[Ca^{2+}]_i$ on the activation of activator protein-1 was not measured in this study.

There are only fragmentary data concerning the down-regulation of NO production by $[Ca^{2+}]_i$. iNOS expression and NO synthesis were inhibited by thapsigargin and calcium ionophore ionomycin in mouse endothelial cells (Bereta et al. 1994). In another study, agonist-induced activation of purinoreceptors, which cause a transient Ca^{2+} influx into cytocol, was reported to inhibit iNOS expression and NO formation by an unknown mechanism in mouse macrophages treated with LPS (Denlinger et al. 1996). In the present study, thapsigargin was found to inhibit iNOS expression by a mechanism dependent on the destabilization of mRNA in activated macrophages. This is in line with an earlier finding in IL-1β-stimulated chondrocytes, where the suppression of iNOS expression and NO production by thapsigargin and A23187 was associated with the destabilization of iNOS mRNA (Geng and Lotz 1995a).

A number of factors induced in the inflammatory context, as well as pathogenic bacteria, are able to induce a receptor-mediated transient elevation in $[Ca^{2+}]_i$ (Cotran et al. 1999b). Controlling iNOS-derived NO production in different states of inflammation is of importance. Ca^{2+} may provide an additional signal resulting in the induction of iNOS and NO in the early stage of infection/inflammation, thereby promoting an inflammatory response in order to eliminate the cause. Interestingly, pathogenic *E. coli* has been reported to induce Ca^{2+} oscillations which activate inflammatory transcription factors in renal epithelial cells (Uhlén et al. 2000). In an advanced state of inflammation, when excess NO synthesis is involved in detrimental effects, i.e. inhibition of mitochondrial respiration, DNA damage and tissue injury against the host, inappropriate iNOS expression would be down-regulated by receptor-mediated Ca^{2+} signal, thereby attenuating the inflammatory response.

4. Regulation of iNOS expression and NO production by dexamethasone

Glucocorticoids are among the most potent anti-inflammatory drugs, and are capable of suppressing the expression of various genes regulating inflammatory responses. In early studies, glucocorticoids were found to inhibit inducible NO production in endothelial cells and macrophages (Radomski et al. 1990, Di Rosa et al. 1990). This effect has since been confirmed in different cell types in several reports, including the present study, but not in all cell types, for example chondrocytes (Grabowski et al. 1996) and intestinal epithelial cells (Kleinert et al. 1998, Salzman et al. 1996). In the present study, iNOS protein expression and NO production were inhibited by dexamethasone in a dose-dependent manner LPS-stimulated in J774 macrophages. Interestingly, when LPS was combined with IFNγ, dexamethasone did not inhibit iNOS expression and NO synthesis.

The mechanism by which glucocorticoids inhibit iNOS expression and NO formation is not clear, and both transcriptional and post-transcriptional effects have been proposed. Glucocorticoids are known to activate gene expression, and this is mediated through binding of the glucocorticoid-receptor complex to GRE motifs in the promoter of the target gene. Effects involved in the inhibition of gene expression are related to the anti-inflammatory effects of glucocorticoids, and are considered to be mediated via inhibition of transcription factors, including NF-κB and activator protein-1. The attenuation of iNOS expression by glucocorticoids has been held to be related to the inhibition of NF-κB. Two distinct mechanisms have been proposed. Glucocorticoids induce the expression of IκB, which in turn binds to and thereby inactivates NF-κB (Auphan et al. 1995, Scheinman et al. 1995a). This has been found to take place in rat hepatocytes stimulated with cytokines, leading to suppression of iNOS mRNA expression (de Vera et al. 1997). Another mechanism, independent of the expression of IκB, whereby NF-κB activation is inhibited by glucocorticoids, involves protein-protein interaction between NF-κB subunits and the glucocorticoid receptor, resulting in the reduced DNA binding of NF-κB (Ray and Prefontaine 1994, Scheinman et al. 1995b, Heck et al. 1997) and suppression of iNOS mRNA, and subsequent protein expression in lung epithelial cells (Kleinert et al. 1996a). Similar interactions between glucocorticoid receptor and other transcription factors, e.g. activator protein-1 and nuclear factor of activated T cells, have also been suggested (Smith et al. 2001).

In the present study, although NO production was markedly reduced, steady-state levels of iNOS mRNA were not significantly decreased by dexamehasone up to 6 h after stimulation with LPS.

Although the rate of iNOS transcription was not measured, this implies that the effect of dexamethasone on iNOS expression induced by LPS is not mainly mediated by inhibition of transcription. This suggests a post-transcriptional mechanism. Glucocorticoids are found to down-regulate certain inflammatory genes by destabilizing their mRNA. In the present study, iNOS mRNA was found to be destabilized by dexamethasone in cells treated with LPS, contributing to the reduction of iNOS protein expression and NO formation. In addition, iNOS mRNA expression was attenuated by an inhibitor of protein synthesis, suggesting that the effect of dexamethasone was *de novo* protein synthesis-dependent. In the literature, one earlier report concerning the destabilizing effect of dexamethasone on iNOS mRNA is to be found (Walker et al. 1997). Taken together, these findings highlight the importance of a post-transcriptional mechanism, in this case the increased degradation of iNOS mRNA, in the down-regulation of iNOS expression and NO production by glucocorticoids.

IFNγ has been shown to enhance NO production and iNOS expression. Transcriptional and post-transcriptional effects have been suggested. In the present study, in cells stimulated with a combination of LPS and IFNγ, the expression of iNOS mRNA and protein as well as NO formation were enhanced as compared to LPS-treated cells, and this was concomitant with stabilization of iNOS mRNA. These data are supported by earlier findings showing that IFNγ stabilizes iNOS mRNA in LPS-treated macrophages (Weisz et al. 1994) and in IL-1β-stimulated Langerhans islet cells (Heitmeier et al. 1997). In addition, destabilization of iNOS mRNA by dexamethasone was clearly counteracted by IFNγ. This suggests that the effect of dexamethasone on iNOS mRNA stability may be, at least partially, stimulus-dependent, which is a new finding.

5. The regulation of iNOS mRNA stability by dexamethasone and $[Ca^{2+}]_i$

Transcriptional regulation of expression of both rodent and human iNOS has been an object of intense research during the last few years, and the importance of regulation of iNOS expression at the level of transcription is beyond dispute. However, the role of post-transcriptional mechanisms in the regulation of iNOS expression and NO formation has been far less thoroughly explored. In rat smooth muscle cells and mouse macrophages, following stimulation with IFNγ, IL-1β and TNFα or LPS and IFNγ, respectively, degradation of iNOS mRNA has been found to be mediated by a newly synthesized, yet unknown protein (Evans et al. 1994). In the present study, iNOS expression

induced either by LPS or a combination of LPS and IFNγ was not attenuated by the protein synthesis inhibitor cycloheximide. iNOS mRNA contains ARE motifs in 3´-UTR, which are generally related to rapid degradation of mRNA (Malter 1998). Recently, ARE motifs have been found to have a boarder function in the regulation of mRNA stability. The mRNA-stabilizing protein HuR has been shown to stabilize mRNAs through ARE motifs (Brennan and Steitz 2001). ARE-mediated stabilization of iNOS mRNA by HuR has been reported (Rodriguez-Pascual et al. 2000), degradation not. According to a preliminary finding in this study, the mRNA of TTP is present, and is inducible by LPS in J774 macrophages, and may be involved in the regulation of iNOS expression.

Besides dexamethasone, IFNγ and $[Ca^{2+}]_i$, only a few factors have been found to regulate iNOS mRNA stability. TGF-β, a potent endogenous suppressor of iNOS expression, destabilizes iNOS mRNA in addition to its inhibitory effect on transcription, which both contribute to the reduction of NO formation (Vodovotz et al. 1993). NO-donors have also been found to down-regulate iNOS expression through cyclic GMP partly by destabilizing mRNA in mesangial cells (Perez-Sala et al. 2001). In human ventricular myocytes stimulated with TNFα, IL-1β, LPS and IFNγ, marked iNOS mRNA levels were detected in the total absence of iNOS protein or NO synthesis, suggesting a prominent role of post-transcriptional regulation in iNOS expression (Luss et al. 1997). In pancreatic islet β-cells, activation of PKCδ has been found to stabilize iNOS mRNA following IL-1β-stimulation (Carpenter et al. 2001). In rat cardial fibroblasts stimulated with IL-1β, iNOS mRNA is stabilized and NO production enhanced by an β-adrenergic agonist isoproterenol in the absence of increased transcription. The human lung epithelium has been shown to express iNOS continuously (Guo et al. 1995) and this is due to the presence of IFNγ and interleukin-4 in lung tissue which stabilizes iNOS mRNA (Guo et al. 1997). Although there are no data on the detailed mechanisms whereby iNOS mRNA stability is regulated, these reports show that mRNA stability is of importance in the regulation of iNOS expression. Exploring the mechanisms regulating the stability of the mRNAs of iNOS and other inflammatory genes may reveal potential targets for novel drug therapy, especially in the treatment of inflammatory diseases.

SUMMARY AND CONCLUSIONS

The purpose of the present study was to investigate the mechanisms involved in the regulation of iNOS expression and NO production in macrophages activated by *Lactobacillus* GG, LPS and IFNγ. Results show that the expression of iNOS is regulated not only at transcriptional but also at post-transcriptional level.

The major findings and conclusions were:

1. *Lactobacillus* GG induced the expression of iNOS and NO production in J774 macrophages in the presence of IFNγ. iNOS induction by *Lactobacillus* GG was dependent on the activation of protein tyrosine kinase(s) and NF-κB, and to lesser extent, on the activation of protein kinase C and p42/44 and p38 mitogen-activated protein kinases. These data support the conception that *Lactobacillus* GG has immunomodulatory effects in immune/inflammatory cells.

2. Elevated cytosolic Ca^{2+} induced iNOS expression and NO formation in macrophages stimulated with a low concentration of LPS by a mechanism independent of the activation of NF-κB, although NF-κB activation was crucial for the LPS-effect. A corresponding Ca^{2+} signal attenuated iNOS protein expression and NO synthesis by destabilizing iNOS mRNA in cells exposed to a higher LPS concentration. These findings show that, even though the activity of iNOS enzyme is not modulated by Ca^{2+}, iNOS expression is regulated by Ca^{2+} in a bi-directional manner, and this may be of importance in the regulation of the inflammatory process.

3. Dexamethasone inhibited iNOS expression and NO production in macrophages stimulated with LPS, and this was associated with destabilization of iNOS mRNA. In cells stimulated with LPS and IFNγ, dexamethasone did not attenuate iNOS protein expression and NO synthesis. In addition, destabilization of iNOS mRNA by dexamethasone was partially counteracted by IFNγ. These data suggest that dexamethasone decreases inducible NO formation by destabilizing iNOS mRNA, and the effect of dexamethasone is dependent on the stimulus.

4. Degradation of iNOS mRNA was enhanced by dexamethasone and elevated cytosolic Ca^{2+}, contributing to reduced iNOS expression and NO production. These findings emphasize the role of post-transcriptional mechanisms in the regulation of iNOS expression along with transcriptional regulation. The mechanisms involved in the regulation of iNOS mRNA are potential targets for the development of novel anti-inflammatory treatments.

YHTEENVETO

Tulehdus on elimistön suojareaktio taudinaiheuttajia tai muita vahingollisia tekijöitä vastaan. Tällaisia tekijöitä ovat muun muassa mikrobit, erilaiset haitalliset kemialliset tai fysikaaliset tekijät, sekä mekaaniset vauriot. Tulehdusreaktion tarkoitus on poistaa elimistön hyvinvointia uhkaava tekijä, ja korjata syntyneet kudosvauriot. Tulehdusta säätelevät elimistön tuottamat välittäjäaineet, joita ovat muun muassa typpioksidi, eikosanoidit sekä sytokiinit.

Typpioksidi on vesiliukoinen, kaasumainen molekyyli, joka säätelee muun muassa monia sydän- ja verisuoniston fysiologisia toimintoja sekä toimii hermovälittäjäaineena. Immuunijärjestelmässä tulehduksen välittäjäaineet saavat valkosolut ja monet elimistön muut solut ilmentämään indusoituvaa typpioksidisyntaasi-entsyymiä, joka tuottaa suuria määriä typpioksidia. Tällöin typpioksidi toimii sekä elimistön puolustusvasteita säätelevänä välittäjäaineena että antimikrobisena ja sytotoksisena tekijänä. Tiedetään, että typpioksidi liittyy moniin kroonisiin tulehdussairauksiin kuten astmaan, nivelreumaan ja tulehduksellisiin suolistosairauksiin.

Monet elimistön solut tuottavat typpioksidia vasteena tulehduksellisille signaaleille, mutta solunsisäiset mekanismit, jotka säätelevät typpioksidin tuottoa, ovat vielä epäselviä. Väitöskirjatyössä tutkittiin makrofagien typpioksidituotantoon liittyviä mekanismeja. Tutkimuksessa havaittiin probioottina käytettävän *Lactobacillus rhamnosus* GG:n saavan aikaan typpioksidin tuotannon makrofageissa, eli *Lactobacillus rhamnosus* GG:llä näyttää olevan elimistön immuunivastetta sääteleviä vaikutuksia. Tutkimuksen päälöydös oli, että tulehduslääkkeenä käytettävä glukokortikoidi deksametasoni ja vapaa solunsisäinen kalsium vähensivät typpioksidin tuotantoa post-transkriptionaalisella tasolla, ja vaikutuksen havaittiin liittyvän typpioksidia tuottavan entsyymin, indusoituvan typpioksidisyntaasin, lähetti-RNA:n nopeutuneeseen hajoamiseen. Tulos korostaa post-transkriptionaalisten mekanismien merkitystä indusoituvan typpioksidituotannon säätelyssä. Kyseessä on myös uusi glukokortikoidien vaikutusmekanismi, joka osaltaan selittää ”kortisonin” tulehdusta lievittävää vaikutusta. Tutkimus tuo uutta tietoa typpioksidituottoa säätelevistä mekanismeista, ja valaisee tulehduksen solutason mekanismeja, mikä on tärkeää tulehduksellisten sairauksien täsmälääkkeiden ja muiden kohdennettujen hoitojen kehitystyössä.

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