



OUTI KOSONEN

**Anti-inflammatory Effects of
NO-releasing Compounds**

*University of Tampere
Tampere 2000*

Anti-inflammatory Effects of NO-releasing Compounds

ACADEMIC DISSERTATION

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ACADEMIC DISSERTATION

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the Faculty of Medicine of the University of Tampere,
for public discussion in the small auditorium of Building B,
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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-V.

- I. Kosonen O, Kankaanranta H, Vuorinen P and Moilanen E (1997): Inhibition of human lymphocyte proliferation by nitric oxide-releasing oxatriazole derivatives. *Eur J Pharmacol* 337: 55-61.
- II. Kosonen O, Kankaanranta H, Lähde M, Vuorinen P, Ylitalo P and Moilanen E (1998): Nitric oxide-releasing oxatriazole derivatives inhibit human lymphocyte proliferation by a cyclic GMP-independent mechanism. *J Pharmacol Exp Ther* 286: 215-220.
- III. Kosonen O, Kankaanranta H, Malo-Ranta U, Ristimäki A and Moilanen E (1998): Inhibition by nitric oxide-releasing compounds of prostacyclin production in human endothelial cells. *Br J Pharmacol* 125: 247-254.
- IV. Kosonen O, Kankaanranta H, Malo-Ranta U and Moilanen E (1999): Nitric oxide-releasing compounds inhibit neutrophil adhesion to endothelial cells. *Eur J Pharmacol* 382: 111-117.
- V. Kosonen O, Kankaanranta H, Uotila J and Moilanen E: Inhibition by nitric oxide-releasing compounds of E-selectin expression in and neutrophil adhesion to human endothelial cells. *Eur J Pharmacol* (submitted).

ABBREVIATIONS

APC	antigen presenting cells
BCR	B-cell receptor
BH4	tetrahydrobiopterin
BSA	bovine serum albumin
cAMP	cyclic adenosine 3':5'-monophosphate
cGMP	cyclic guanosine 3':5'-monophosphate
ConA	concanavalin A
COX	cyclooxygenase
CTL	cytotoxic T cell
DNA	deoxyribonucleic acid
DP	PGD ₂ receptor
EDRF	endothelium-derived relaxing factor
EDTA	ethylenediamine tetraacetic acid
eNOS	endothelial nitric oxide synthase
EP	PGE ₂ receptor
E.P.R.	electron paramagnetic resonance spectrometry
ESL-1	E-selectin ligand-1
FAD	flavin adenine dinucleotide
Fas	The membrane receptor that triggers apoptosis (also known as APO-1 or CD95)
FITC	fluorescein isothiocyanate
FMLP	N-formyl-L-methionyl-L-leucyl-L-phenylalanine
FMN	flavin mononucleotide
FP	PGF _{2α} receptor
GEA 3162	1,2,3,4-oxatriazolium,5-amino-3(3,4-dichlorophenyl)-chloride
GEA 3175	1,2,3,4-oxatriazolium,3-(3-chloro-2-methylphenyl)-5[[4-methylphenyl)-sulfonyl]amino]-, hydroxide inner salt
GSH	reduced glutathione
GSNO	S-nitrosoglutathione
GTN	glyceryl trinitrate
GTP	guanosine triphosphate
HNO ₂	nitrous acid
HNO ₃	nitric acid
HTAB	hexadecyltrimethylammonium bromide
HUVEC	human umbilical vein endothelial cell
IBMX	3-isobutyl-1-methylxanthine
ICAM	intercellular adhesion molecule
IFN-γ	interferon-γ

Ig	immunoglobulin
IL	interleukin
iNOS	inducible nitric oxide synthase
IP	prostacyclin receptor
I- γ -RE	interferon- γ -responsive elements
L-NAME	N ^G -nitro-L-arginine methyl ester
L-NIL	L-N-iminoethyl lysine
L-NIO	N-iminoethyl-L-ornithine
L-NMMA	N ^G -monomethyl-L-arginine
LO \cdot	alkoxyl radical
LOO \cdot	hydroperoxyl radical
LPS	lipopolysaccharide
mAb	monoclonal antibody
MHC	major histocompatibility complex
mRNA	messenger ribonucleic acid
NADPH	reduced nicotine adenine dinucleotide phosphate
NANC	non-adrenergic, non-cholinergic
NF- κ B	nuclear factor- κ B
NK	natural killer cells
nNOS	neuronal nitric oxide synthase
NO	nitric oxide
NO ₂ ⁻	nitrite
NO ₃ ⁻	nitrate
NOS	nitric oxide synthase
NSAID	nonsteroidal anti-inflammatory drugs
\cdot O ₂ ⁻	superoxide
ODQ	1 <i>H</i> -[1,2,4]oxadiazolo[4,3,-a]quinoxalin-1-one
OH \cdot	hydroxyl radical
ONOO \cdot	peroxynitrite
ONOOH	peroxynitrous acid
PAF	platelet activating factor
PBS	phosphate-buffered saline
PDE	phosphodiesterase
PECAM-1	platelet-endothelial cellular adhesion molecule-1 (CD31)
PG	prostaglandin
PGI ₂	prostacyclin
PHA	phytohemagglutinin
PPAR	peroxisome proliferator activated receptor
PSGL-1	P-selectin glycoprotein ligand-1
PWM	pokeweed mitogen

RNOS	reactive nitrogen oxide species
RPE	R-phycoerythrin
RSNO	S-nitrosothiol
RSNO ₂	thionitrate
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SE	standard error of the mean
SIN-1	3-morpholino-sydnonimine
SNAP	S-nitroso-N-acetylpenicillamine
SNP	sodium nitroprusside
TCR	T cell receptor
TGFβ	transforming growth factor β
T _H	helper T cells
TNF	tumor necrosis factor
TNF-RE	tumor necrosis factor responsive elements
TP	thromboxane receptor
TXA ₂	thromboxane
VAP-1	vascular adhesion protein-1
VCAM-1	vascular cell adhesion molecule-1
VLA-4	very late antigen-4

INTRODUCTION

Inflammation associated with microbial infection and/or cellular injury is a localized and protective process and most importantly, a response essential to survival. However, inadvertent activation of the inflammatory cascade or lack of an appropriate “termination” signal can lead to excessive inflammatory response and destruction of the surrounding tissues. Inappropriate regulation of the inflammatory response is thought to underlie the tissue injury associated with various disorders, including inflammatory joint disease, inflammatory bowel disease and ischemia-reperfusion injury.

Nitric oxide (NO) is a unique messenger molecule involved in the regulation of diverse physiological processes including smooth muscle contractility, platelet reactivity and central and peripheral neurotransmission. In the immune system NO has been recognized as an important effector molecule for macrophages as well as a homeostatic regulator. Depending on the type and phase of the inflammatory reaction and the individual vascular or cellular responses studied, NO can exert both proinflammatory and antiinflammatory actions. Thus NO, like many other inflammatory mediators, has a dualistic regulatory function in inflammation. Initial attempts to manipulate the L-arginine:NO pathway have already demonstrated the therapeutic potential of some of these interventions. It is likely that, in the future safe and effective medicines will be developed in this field.

The present study was designed to characterize antiinflammatory properties of a group of novel NO-releasing compounds in *in vitro* set-ups. The effects of two mesoionic oxatriazole derivatives GEA 3175 and GEA 3162 were compared to earlier-known reference compounds 3-morpholino-sydnonimine (SIN-1) and S-nitroso-N-acetylpenicillamine (SNAP). The results show that NO-releasing compounds inhibit lymphocyte proliferation in a cGMP-independent fashion, inhibit prostacyclin production in endothelial cells probably by inhibiting cyclooxygenase-2 (COX-2) activity and inhibit neutrophil adhesion to and E-selectin expression in endothelial cells.

REVIEW OF LITERATURE

1. Inflammation

1.1. General aspects of inflammation

Inflammation is fundamentally a protective response the ultimate goal of which is to rid the organism of the initial cause of cell injury (e.g., microbes, toxins) as well as the consequences of such injury, i.e. the necrotic cells and tissues. Inflammatory reaction consists of innate reactions, which have no immunological basis and of specific immunological reactions. Principal effector cells of the innate immune system are neutrophils, eosinophils, basophils, mast cells, natural killer (NK) cells, and professional antigen-presenting cells (APCs). Antigen specific immune responses are mediated by T and B lymphocytes (Hale and Haynes, 1999).

The vascular and cellular responses of both acute and chronic inflammation are mediated by chemical factors derived from plasma or cells and triggered by the inflammatory stimulus. Such mediators, acting singly, in combinations, or in sequence, then amplify the inflammatory response and influence its evolution (Cotran et al., 1994). Mediators derived from plasma include the components of four enzyme cascades: the complement system, the kinin system, the coagulation system and the fibrinolytic system. When inflammatory cells are stimulated or damaged, another major mediator system is generated - the eicosanoids. Other important inflammatory mediators derived from cells are histamine, platelet-activating factor and the cytokines (Rang and Dale, 1999).

The inflammatory response is closely intertwined with the process of repair. Inflammation serves to destroy, dilute, or wall off the injurious agent, but it, in turn, induces a series of events that, as far as possible, heal and reconstitute the damaged tissue. Repair begins during the early phases of inflammation but reaches completion usually after the injurious influence has been neutralized. During repair, the injured tissue is replaced by regeneration of native parenchymal cells, or by filling of the defect with fibroblastic tissue (scarring), or most commonly by a combination of these two processes (Cotran et al., 1994).

1.1.1. Acute inflammation

Acute inflammation is the immediate and early response to an injurious agent. It is of relatively short duration, lasting for minutes, several hours, or a few days, and its main characteristics are exudation of fluid and plasma proteins (edema) and emigration of leukocytes, predominantly neutrophils. It may consist of the innate, immunologically non-specific vascular and cellular events together with a varying degree of participation of the specific immunological response.

Since the two major defensive components against microbes – antibodies and leukocytes – are normally carried in the bloodstream, it is not surprising that vascular phenomena play a major role in acute inflammation. Therefore, acute inflammation has three major components: (1) vasodilatation that leads to increased local blood flow, (2) structural changes in the microvasculature that permit the plasma proteins and leukocytes to leave the circulation (exudation), and (3) emigration of the leukocytes from the microcirculation and their accumulation in the site of injury (Cotran et al., 1994). Leukocytes ingest offending agents, kill bacteria and other microbes, degrade necrotic tissue and foreign antigens. The other side of the coin is that leukocytes may also prolong inflammation and induce tissue damage by releasing enzymes, chemical mediators, and oxygen radicals.

1.1.2. Chronic inflammation

If the pathogen or the other triggering agent persists, the condition is likely to proceed to chronic inflammation - a slow smouldering reaction which continues for months or even years and involves both tissue destruction and cell proliferation and connective tissue formation. Chronic inflammation causes some of the common and disabling human diseases, such as rheumatoid arthritis, atherosclerosis and tuberculosis. Chronic inflammation is characterized by (1) infiltration of mononuclear cells, i.e. macrophages, lymphocytes and plasma cells, (2) tissue destruction, largely induced by the inflammatory cells, and (3) attempts to repair the injury by connective tissue replacement, proliferation of small blood vessels (angiogenesis) and fibrosis (Cotran et al., 1994).

1.2. Leukocyte adhesion to endothelial cells

1.2.1. Adhesion molecules

Migration of leukocytes from the blood vessel into inflamed tissue is a central step in the process of inflammation. Binding of leukocytes to the blood vessel wall is controlled by a complex cascade of molecular interactions between the leukocyte and the endothelial cell layer, mediated by cell adhesion molecules and leukocyte-activating factors (Springer, 1995). At least three distinct families of adhesion molecules participate in leukocyte adhesion and migration. These families are selectins, integrins and certain members of an immunoglobulin superfamily [table 1].

Selectins

The selectin family is comprised of three proteins: E-(endothelial), P-(platelet), and L-(leukocyte) selectin. E-selectin and P-selectin are expressed by activated endothelial cells while L-selectin is constitutively expressed on leukocytes (Robinson et al., 1999). P-selectin exists in a preformed pool in Weibel-Palade bodies in endothelial cells and in the α -granules of platelets (McEver et al., 1989). It is rapidly (within minutes) mobilized to cell surface following

Table 1 Adhesion molecules involved in leukocyte-endothelial cell interaction

Adhesion molecule	Other names	Expression in leukocytes and endothelial cells	Ligand(s)	Activating stimuli
Selectins				
L-selectin	CD62L, LAM-1, MEL-14	All leukocytes		Constitutive, shed on activation
P-selectin	CD62P, GPI140, PADGEM	Endothelial cells, platelets	PSGL-1	Histamine, thrombin
E-selectin	CD62E, ELAM-1	Endothelial cells	ESL-1	LPS, TNF- α , IL-1
Integrins				
CD11a/CD18	LFA-1, $\alpha_4\beta_2$	All leukocytes	ICAM-1, ICAM-2, ICAM-3	PAF, LTB ₄ , C5a
CD11b/CD18	Mac-1, $\alpha_M\beta_2$	Granulocytes, monocytes, macrophages	ICAM-1, iC3b, fibrinogen	
CD11c/CD18	p150/95, $\alpha_x\beta_2$	Macrophages, monocytes, granulocytes		
VLA-4	CD49d/CD29, $\alpha_4\beta_1$	Lymphocytes, monocytes, eosinophils	VCAM-1, fibronectin	Constitutive
Immunoglobulin superfamily				
ICAM-1	CD54	Endothelial cells, leukocytes	CD11a/CD18, CD11b/CD18	Constitutive and induced by LPS, TNF- α , IL-1
ICAM-2	CD102	Endothelial cells	CD11a/CD18	Constitutive
VCAM-1	CD106	Endothelial cells	VLA-4, $\alpha_4\beta_7$	TNF- α , IL-1, interferon- γ
PECAM-1	CD31	Endothelial cells, leukocytes	PECAM-1	Constitutive
Other				
VAP-1		Endothelial cells		

ELAM = endothelial leukocyte adhesion molecule, ESL = E-selectin ligand, GMP = granule membrane protein, ICAM = intercellular adhesion molecule, IL = interleukin, LAM = leukocyte adhesion molecule, LFA = leukocyte function-associated antigen, LPS = lipopolysaccharide, LTB₄ = leukotriene B₄, PADGEM = platelet activation-dependent granule external membrane protein, PAF = platelet activating factor, PECAM = platelet endothelial cell adhesion molecule, PSGL = P-selectin glycoprotein ligand, TNF = tumor necrosis factor, VAP = vascular adhesion protein, VCAM = vascular cell adhesion molecule, VLA = very late antigen.

The lists for each of the adhesion molecules are not exclusive, but the data mentioned are those that are important in leukocyte-endothelial cell interaction. Compiled from references: Hickey and Kubes, 1997; Mojcić and Shevach, 1997; Gonzales-Amaro et al., 1998; Panes et al., 1999; Kishimoto et al., 1999; Repo and Harlan, 1999; Robinson et al., 1999.

stimulation, after which it is either recycled back inside the membrane or shed into the plasma (Tedder et al., 1995). Unlike P-selectin, the expression of E-selectin on endothelial cells is entirely under transcriptional control. E-selectin is not constitutively expressed and its synthesis is induced in endothelial cells within hours following stimulation by interleukin-1 (IL-1), tumor necrosis factor α (TNF α) or lipopolysaccharide (LPS) (Bevilacqua et al., 1987). It is then rapidly (within 16-24 hours) down-regulated (Bevilacqua et al., 1989; Vestweber and Blanks, 1999). L-selectin is constitutively expressed on virtually all circulating leukocytes but it is shed within minutes upon activation of the cells (Kishimoto et al., 1989; Vestweber and Blanks, 1999). Selectins bind to ligands containing sialylated, fucosylated carbohydrate moieties (e.g., sialyl Lewis^x) (Varki, 1997). More specific interactions with higher affinities occur between P-selectin and P-selectin glycoprotein ligand-1 (PSGL-1) (McEver and Cummings, 1997) or between E-selectin and E-selectin ligand-1 (ESL-1) (Steedmaier et al., 1995).

Integrins

Integrins are heterodimeric proteins consisting of α and β subunits. The family of integrins is divided into various subfamilies according to β subunit expressed (Springer, 1990). Integrins mediate cell-cell and cell-matrix interactions and need to be activated for binding to their counter receptors. Engagement of integrins also provides stimulatory or inhibitory signals for the cells expressing these adhesion molecules (Henricks and Nijkamp, 1998).

The subfamily of $\beta 2$ integrins is more commonly known as leukocyte integrins since they are expressed on different types of leukocytes and are functionally related to leukocyte adhesion to endothelial cells. Members of the $\beta 2$ subfamily contain one of four different α chains designated CD11a, CD11b, CD11c and CD11d that are coupled to a common β chain, CD18. The heterodimer CD11a/CD18 is expressed on the surface of most leukocytes and interacts with intercellular adhesion molecule-1 and 2 (ICAM-1 and ICAM-2) in endothelial cells. This integrin is not stored to any appreciable degree within the leukocyte. The heterodimers CD11b/CD18, CD11c/CD18 and CD11d/CD18 are expressed predominantly in monocytes, macrophages and granulocytes. CD11b/CD18 and CD11c/CD18 are stored in the intracellular granules and are translocated to the cell surface after cell activation (Kishimoto et al., 1999). However, it has been demonstrated that upregulation of CD11b/CD18 surface expression is neither necessary nor sufficient for the stimulated leukocytes to adhere to endothelial cells (Vedder and Harlan, 1988; Philips et al., 1988; Schleiffenbaum et al., 1989). Instead, increased adhesivity may result from changes in integrin conformation, which increase receptor affinity for ligand. In addition, clustering of receptors may increase adhesivity without a change in receptor affinity (Bazzoni and Hemler, 1998; Repo and Harlan, 1999).

Ligands for CD11b/CD18 and CD11c/CD18 include ICAM-1, complement fragment iC3b and fibrinogen, whereas CD11d/CD18 mainly interacts with ICAM-3 (Gonzalez-Amaro et al., 1998; Kishimoto et al., 1999). The $\beta 1$ integrin VLA-4 (very late antigen-4) mediates the adhesion of

lymphocytes, monocytes, eosinophils and natural killer cells to activated endothelial cells, which express the counter receptor VCAM-1 (vascular cell adhesion molecule-1) (Panés et al., 1999).

Immunoglobulin superfamily

The immunoglobulin superfamily consists of proteins that are involved in antigen recognition, complement binding or cellular adhesion (Springer, 1990; Carlos and Harlan, 1994). Members of this family that are important for leukocyte adhesion to endothelial cells are ICAM-1, ICAM-2, VCAM-1 and PECAM-1 (platelet-endothelial cellular adhesion molecule-1). ICAM-1 is normally found on the surface of endothelial cells, but its expression can be significantly increased upon endothelial activation by cytokines or LPS. ICAM-1 binds to CD11a/CD18 and CD11b/CD18 on leukocytes (Carlos and Harlan, 1990; Panés et al., 1999). ICAM-2 is constitutively expressed on endothelial cells and its expression is not significantly influenced by the level of activation of the endothelial cell. ICAM-2 binds to CD11a/CD18 but with lower affinity than ICAM-1. VCAM-1, which binds to both $\beta 1$ and $\beta 7$ integrins, mediates the trafficking of monocytes and lymphocytes (Panés et al., 1999). PECAM-1 is important in transendothelial migration (Muller, 1999).

In addition to the above-mentioned “classical” adhesion molecules, novel proteins have been recently characterized which are involved in lymphocyte homing and activation (Henricks and Nijkamp, 1998). Vascular adhesion protein-1 (VAP-1) is a human endothelial sialoglycoprotein that has been shown to participate in lymphocyte recirculation and mediates the binding of lymphocytes to peripheral lymph node vascular endothelial cells (Salmi and Jalkanen, 1992; Salmi et al., 1998; Smith et al., 1998). VAP-1 expression is upregulated also in endothelial cells under inflammatory conditions in nonlymphoid tissues such as the skin and synovium, suggesting that it may mediate lymphocyte migration into inflamed tissue (Salmi et al., 1993; Salmi et al., 1997).

1.2.2. Adhesion cascade

The adhesion cascade [Figure 1] is a consequence of the release of soluble mediators that activate endothelial cells and enhance the expression of adhesion molecules in endothelial cells. In the first step some of the leukocytes entering a postcapillary venule in the site of inflammation leave the circulatory stream and adhere loosely, tentatively and reversibly to the endothelial cell surface in a process called rolling. The selectin family of adhesion molecules appears to be primarily responsible for this initial interaction (Vestweber and Blanks, 1999). Rolling leukocytes come into direct contact with endothelium, which exposes them to a variety of signals capable of promoting the next step – activating the leukocyte integrins (Travis, 1993). The leukocytes tethered by selectins are now in a position to be activated by mediators produced by activated endothelium e.g. PAF (Zimmerman et al., 1990; Lorant et al., 1991), IL-8 (Zimmerman et al., 1992) or ligands that cross-link leukocyte PECAM-1 (Berman and Muller, 1995).

Previously it was suggested that E-selectin binding induces signaling, with subsequent activation of CD11b/CD18 complex (Lo et al., 1991). Recently it has been shown that binding of neutrophils to E-selectin does not promote their activation (Repo et al., 1997). Upon activation of their integrins, leukocytes adhere tightly to the endothelial surface and start to crawl through intercellular junctions into the extravascular space. In this process, leukocytes insert pseudopods between endothelial cells and crawl through in ameboid fashion. This step is referred to as transendothelial migration, and PECAM (also known as CD31), which is expressed on the surface of leukocytes and endothelial cells, is involved in this step (Newman, 1997; Muller, 1999). After traversing the endothelial junctions, leukocytes pierce the basement membrane and emigrate in tissues toward the inflammatory focus.

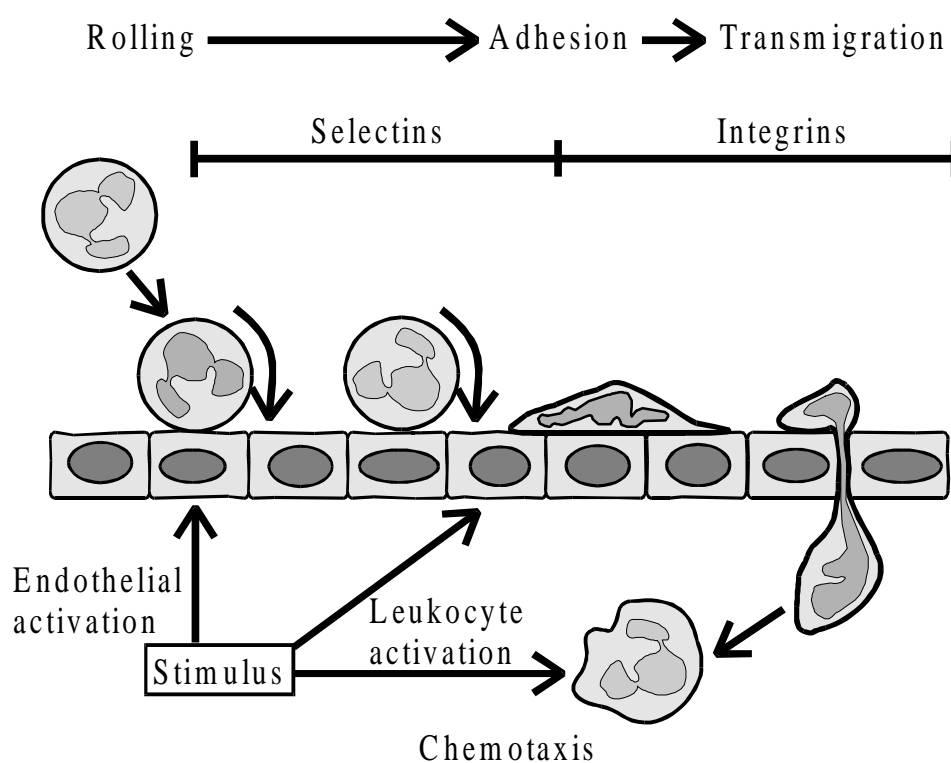


Figure 1. Schematic presentation of adhesion cascade. Modified from Travis (1993).

1.2.3. Modulation of leukocyte-endothelial cell adhesion

A large number of biological agents have been shown to promote leukocyte-endothelial cell adhesion. Some of these proadhesive agents, such as histamine, PAF and IL-8, can rapidly (within 2-3 mins) increase the level of activation and/or expression of adhesion molecules on leukocytes (e.g., CD11b/CD18)

and/or endothelial cells (e.g., P-selectin). Other proadhesive agents such as cytokines IL-1 and TNF- α , act more slowly to promote leukocyte adhesion by enhancing the transcription-dependent expression of endothelial cell adhesion molecules that act to extend and further increase the leukocyte rolling (E-selectin) and adherence/emigration (ICAM-1) responses. (Panes et al., 1999)

Although much attention has been paid on the identification and characterization of the mechanisms that promote leukocyte recruitment to sites of inflammation, very little is known about the potential role of endogenous anti-inflammatory and anti-adhesion molecules in these processes. At the same time with the transient expression of adhesion molecules, proinflammatory cytokines and cell activators, the host activates a series of anti-inflammatory systems that operate in the leukocyte-endothelium microenvironment. Some hormones and cytokines e.g., glucocorticoids, IL-10 and IL-4, are known to inhibit leukocyte adhesion in an endocrine fashion i.e. acting on a target distant from the producer cell (Perretti, 1997). Paracrine or autocrine mediators acting at the adherent leukocyte-endothelial interface include prostacyclin, adenosine and nitric oxide (Cronstein, 1994; Lefer et al., 1994; Panes et al., 1999).

1.2.4. Inhibition of adhesion: a new mechanism of action for anti-inflammatory drugs

The inappropriate recruitment of leukocytes to specific sites is a central mechanism in the pathogenesis of certain inflammatory diseases. Upregulation and/or overexpression of cell adhesion molecules is present in several diseases where inflammation and immune cells are involved (e.g., arthritis, asthma, ischemia-reperfusion injury, transplant rejection) (Henricks and Nijkamp, 1998). In view of this, therapies designed to disrupt leukocyte-endothelial interactions would be anticipated to slow the progression of inflammatory responses.

There are several potential sites at which cell adhesion can be affected (Lowe and Ward, 1997; Henricks and Nijkamp, 1998; Gonzalez-Amaro et al., 1998; Panes et al., 1999). Inhibition of adhesion molecule function can be achieved by using compounds that will bind to the adhesion molecule or its counter-receptor (e.g., soluble adhesion molecules, monoclonal antibodies directed against adhesion molecules or counter-structures and short synthetic peptides based on the structural sequences of adhesion molecules). A second potential mechanism by which upregulation of cell adhesion molecules may be suppressed is inhibition of transcription or translation of DNA or mRNA encoding critical cell adhesion molecules (e.g., by using antisense oligonucleotides). Inhibition of proinflammatory cytokines and other inflammatory mediators that upregulate cell adhesion functions is an indirect approach to antiadhesive therapy in inflammatory conditions. Furthermore, several anti-inflammatory drugs (e.g., methotrexate, glucocorticoids, cyclosporin) have been shown to affect cell adhesion molecule function and/or expression in addition to their

immunosuppressive action. The promise of NO-releasing compounds as anti-adhesive agents will be discussed below.

1.3. The function of lymphocytes in inflammation

Lymphocytes may be divided into two main groups: B cells, which are responsible for antibody production and T cells, which are responsible for cell mediated reactions. T lymphocytes recognise antigens (foreign protein or polysaccharide) as proteolytically processed peptide fragments that are bound to major histocompatibility complex (MHC) molecules on the surface of antigen presenting cells (APCs). Antigen processing is not required for recognition of antigen by B lymphocytes. On the contact with an antigen, the lymphocytes start to divide, giving rise to a large clone of cells which all have the capacity to recognise and respond to the particulate antigen. B lymphocytes differentiate into plasma cells which produce antibodies, and T cells are involved in cell-mediated immune response. In addition, some B and T lymphocytes differentiate into memory cells; a second exposure to the antigen will then result in a multiplied response (Rang and Dale, 1999; Hale and Haynes, 1999).

The ultimate function of B lymphocytes is to produce antibodies, the effector molecules of the humoral immune response. Antibodies are soluble proteins (immunoglobulins) that bind specifically to antigens or invading pathogens, inactivating them or targeting them for disposal by phagocytic cells or complement system. Membrane-bound Ig molecules also serve as the antigen-specific portion of the B-cell receptor for antigen (BCR) (Hale and Haynes, 1999).

Helper T cells ($CD4^+$, T_H) were so named for their regulatory interactions with B lymphocytes and other immune cells. Through cytokine secretion and cell surface receptor-mediated interactions, T_H cells stimulate B cells to differentiate into antibody-producing plasma cells or into memory B cells. Similar T_H interactions with macrophages result in increased macrophage production of reactive oxygen intermediates and other mediators, that enhance the cytotoxic potential of macrophages against intracellular or phagocytosed organisms. At least two general classes of T_H cells can be defined, based on their cytokine secretion profiles. T_H1 cells are characterized by production of interferon- γ (IFN- γ), IL-2 and TNF- β , cytokines that enhance macrophage reactivity and cellular immune response. T_H2 cells produce IL-4, IL-5, IL-10, and IL-13, cytokines that are important for activation of B cells. T_H1 -dominated responses are protective against most microbes and usually eliminate them. If the microbe persists despite such a response, the inflammatory reaction triggered by T_H1 cells and macrophages proceed to result in tissue injury. Large and complex parasites, which cannot be readily eliminated by T_H1 cells, are challenged by T_H2 cells, which do not kill them but interfere with their physiology. In addition, atopic diseases are related to T_H2 -dominated responses to single or multiple environmental allergens. (Romagnani, 1999; Hale and Haynes, 1999)

Cytotoxic T cells (CD8⁺,CTL) are capable of inducing lysis of target cells bearing the appropriate antigen-MHC complex. Cytotoxic T cells provide a major defense against a variety of intracellular pathogens and against tumor cells (Letvin and Schmitz, 1999).

1.3.1. Activation of T lymphocytes

T lymphocytes recognise antigen-derived peptides complexed to MHC molecules through their T cell receptors (TCR). Antigen-dependent stimulation of TCR results in a complex cascade of biochemical signalling events, with three possible outcomes: proliferation and clonal expansion, apoptosis, or anergy (unresponsiveness). The stimulus from the binding of the antigen-MHC complex to the TCR initiates the activation process but is not sufficient to induce proliferation. The avidity of TCR binding is enhanced by binding of the CD8 or CD4 accessory molecules to non-polymorphic regions of the MHC class I or class II molecules, respectively. Additional costimulatory interactions, such as between B7.1/CD80 or B7.2/CD86 on the APC and CD28 on the T cell, are also required for T-cell proliferation to occur. It is proposed that CD28 may exert its costimulatory action by facilitating the assembly of an effective scaffold of signalling elements within the TCR complex. The absence of costimulation through CD28 seems to result in the assembly of a defective scaffold that reverses slowly and may thus account for the state of unresponsiveness. The signalling pathways activated by the TCR and CD28 then engage cytosolic factors that transmit information into the nucleus to activate the genes that code for IL-2 and Fas. The IL-2 and Fas receptors employ additional signals to mediate their effects on proliferation and apoptosis, respectively (Berridge, 1997; Alberola-Ila et al., 1997; Hale and Haynes, 1999).

Lymphocytes can be stimulated *in vitro* by polyclonal activators capable of stimulating a much higher percentage of the cells than a single antigen. The most notable of the polyclonal activators are the mitogenic lectins. Phytohemagglutinin (PHA) and concanavalin A (ConA) activate T lymphocytes, leading to cell division and cytokine production. Some lectins, e.g. pokeweed mitogen (PWM), stimulate B cell proliferation and promote their differentiation into antibody-producing plasma cells (Parker, 1987). T cell mitogens presumably bind to the T-cell receptor complex and also promote a positive costimulatory signal leading to the synthesis of IL-2 (Kilpatrick, 1999). Many mitogens are effective only in the presence of “accessory cells”, particularly monocytes and macrophages, which contribute to the response in lymphocytes; some signals are cell-mediated, but others depend on soluble cytokines (Kilpatrick, 1999). ConA-stimulation is dependent on MHC II expression in accessory cells (Habu and Raff, 1977; Ahmann et al., 1978; Gallagher et al., 1986).

1.4. Prostanoids in inflammation

Prostanoids (prostaglandins, prostacyclin and thromboxane) are important mediators of a variety of physiological processes ranging from regulation of platelet aggregation and vascular tone to reproduction and inflammation. They are generated from arachidonic acid by cyclooxygenase pathway. The term prostaglandin was first introduced by von Euler in 1936. He discovered the biological activity in human semen and believed the prostate gland to be the source of the active compounds. This conception is now known to be incorrect but the name prostaglandin has remained.

1.4.1. Cyclooxygenase pathway

Prostanoids are formed by the oxidative cyclization of the central 5 carbons within 20 carbon polyunsaturated fatty acids. Arachidonic acid is the most abundant precursor fatty acid for prostanoid synthesis in mammalian cells. Arachidonic acid does not occur as a free fatty acid in the cell but is esterified in membrane phospholipids, and it is released through the activation of cellular phospholipases by various stimuli. The key enzyme in the prostanoid synthesis is cyclooxygenase (COX), which catalyses the conversion of arachidonic acid to prostaglandin endoperoxides PGG₂ and PGH₂. Distinct enzymes catalyse the formation of each of the prostaglandins plus thromboxane A₂ (TXA₂) and prostacyclin (PGI₂). The cellular expression pattern of each of these synthases may profoundly influence the type of prostanoid produced by a particular cell. For example, platelets produce predominantly TXA₂, endothelial cells PGI₂ and mast cells PGD₂ (Dubois et al., 1998; Griffiths, 1999). Recently, an inducible form of human PGE₂ synthase has been characterized (Jakobsson et al., 1999). Its expression is induced by a proinflammatory cytokine IL-1 β and therefore it is likely to be significant in the enhanced PGE₂ production in inflammation.

About ten years ago, only one cyclooxygenase had been described, the isoenzyme now called as COX-1 or the constitutive enzyme. Investigators studying cell growth signalling pathways identified a unique, inducible gene product related to the known cyclooxygenase sequence (Kujubu et al., 1991; Xie et al., 1991). Concurrently, investigators looking at prostaglandin production in response to cytokines and other inflammatory factors found an increase in cyclooxygenase activity that could only be accounted for by increased expression of another cyclooxygenase (Raz et al., 1988). A second isoform, now called COX-2 or the inducible enzyme, is roughly 60% identical to the constitutive form, and COX-1 and COX-2 proteins are derived from distinct genes. COX-1 and COX-2 exhibit major differences in their regulation and expression. COX-1 is present in most tissues, performing a “housekeeping” function by synthesizing prostanoids that regulate normal cell activity. On the other hand, COX-2 is almost undetectable in most tissues, but its expression is increased during states of inflammation or in response to mitogenic stimuli and cytokines. Thus, COX-1 plays a key role in the generation of prostanoids that regulate physiologic

processes, such as gastrointestinal cytoprotection and vascular homeostasis, whereas COX-2 appears to produce prostanoids mainly at sites of inflammation [Figure 2.] (Needleman and Isakson, 1997; Dubois et al., 1998).

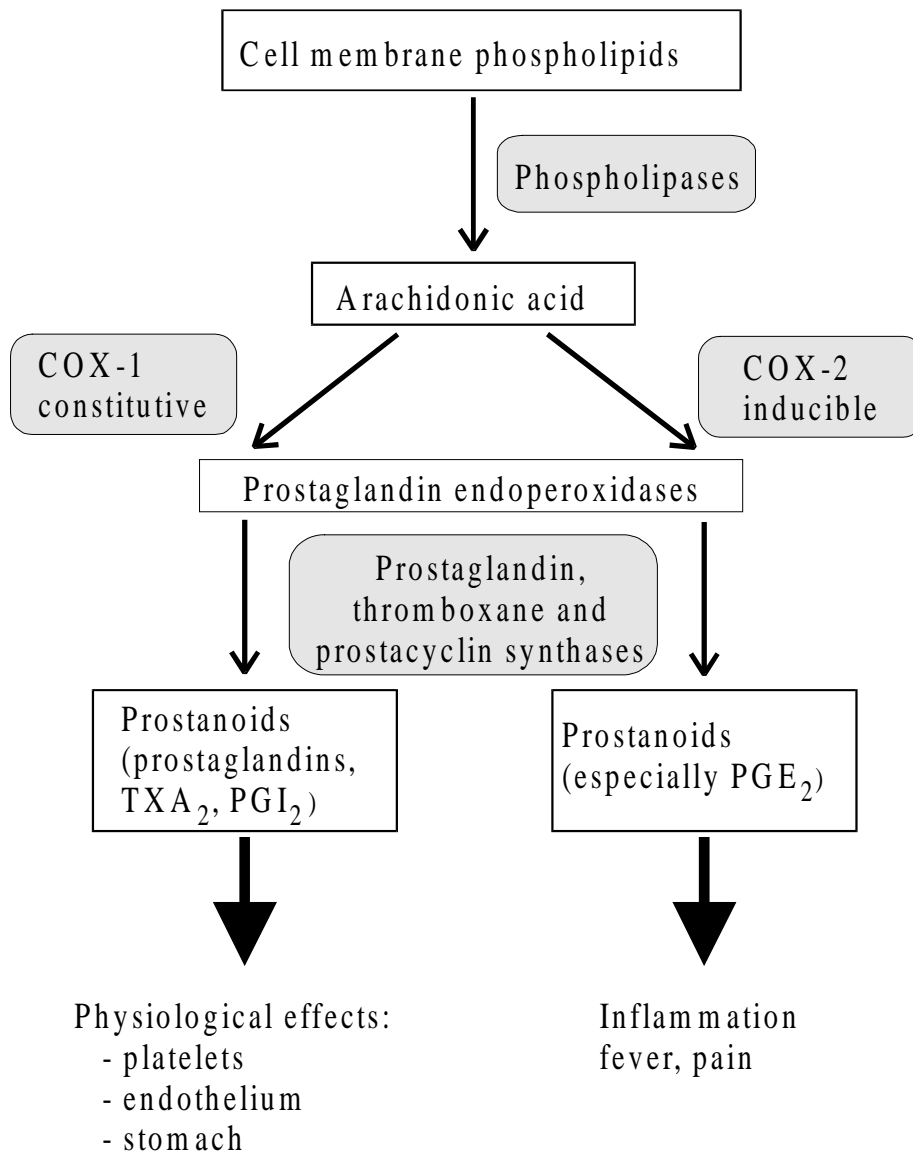


Figure 2. Cyclooxygenase pathway.
COX, cyclooxygenase; TXA₂, thromboxane A₂;
PGI₂, prostacyclin; PGE₂, prostaglandin E₂

1.4.2. Role of prostanoids in regulating the inflammatory response

Prostanoids are best thought of as autacoids, or local short-range hormones, which are formed rapidly, produce their effects locally, and then either decay

spontaneously or are destroyed enzymatically. Prostanoids exert their biologic effects by binding to a family of high-affinity cell surface receptors. Their receptors can be classified into five basic types on the basis of sensitivity to the five naturally-occurring prostanoids, PGD₂, PGE₂, PGF_{2α}, PGI₂ and TXA₂. These receptors are termed P receptors, with a preceding letter indicating the natural prostanoid to which each receptor is most sensitive. Thus the receptors are termed DP, EP, FP, IP and TP, respectively. Furthermore, EP receptors have been subdivided into four groups (EP1 through EP4). Prostaglandin receptors are coupled to G-proteins and the signal transduction pathways activated by these receptors vary. For example, the EP2, EP4 and IP receptors couple to adenylate cyclase and stimulate cAMP formation, whereas the EP1 receptor triggers increases in intracellular calcium (Coleman, 1998). In addition to these classical cell surface receptors, prostanoids have been suggested to activate the nuclear PPAR (peroxisome proliferator activated receptors) receptor family, which act directly as transcription factors upon ligand binding (Forman et al., 1997; Dubois et al., 1998).

At an inflammatory site, PGE₂ is the predominant prostanoid produced. In areas of acute inflammation mast cells release PGD₂; PGE₂ and PGI₂ are generated by the local tissues, activated leukocytes and by blood vessels. In chronic inflammation cells of the monocyte-macrophage series also release significant amounts of PGE₂. PGE₂, PGI₂ and PGD₂ are powerful vasodilators in their own right and synergise with other vasodilators such as histamine and bradykinin. It is this combined dilator action on precapillary arterioles which is responsible for the redness and increased blood flow in areas of acute inflammation. Prostanoids do not directly increase the permeability of the postcapillary venules, but they potentiate these effects of other inflammatory mediators, e.g. histamine and bradykinin and thus mediate inflammatory edema. Similarly, they do not themselves produce pain, but induce hyperalgesia and thus potentiate the effect of bradykinin and other algescic agents. Prostaglandins of the E series are also implicated in the production of fever. The increase in temperature generated by endogenous pyrogens such as IL-1 is mediated by PGE₂. (Rang and Dale, 1999; Griffiths, 1999)

In addition to the proinflammatory effects described above, prostaglandins have been shown to have a significant modulator action on inflammatory cells, decreasing their activities. PGE₂ decreases lysosomal enzyme release and the generation of toxic oxygen metabolites from neutrophils. It also inhibits macrophage and lymphocyte activation and the generation and secretion of some cytokines. (Rang and Dale, 1999; Griffiths, 1999)

1.4.3. Cyclooxygenases as a target of anti-inflammatory drugs

Since the discovery of aspirin about a century ago, this compound and other nonsteroidal anti-inflammatory drugs (NSAID) have become the drug of choice for the treatment of inflammatory and other pain and fever. Despite the wide use

of NSAIDs, their mechanism of action was not fully understood until 1971, when Vane (Vane, 1971) published his seminal observations proposing that the therapeutic action of NSAIDs rests primarily on their ability to inhibit prostaglandin synthesis. A wealth of data now suggests that the antipyretic, analgesic and anti-inflammatory actions of NSAIDs are largely the result of inhibition of COX-2, whereas many of their adverse effects (e.g., gastrointestinal toxicity) are primarily due to inhibition of COX-1. All traditional NSAIDs inhibit both COX-1 and COX-2 to varying degrees, depending on the experimental system used to measure the drug's effect (Smith et al., 1994; Laneuville et al., 1994; Gierse et al., 1995; Warner et al., 1999).

The discovery of the COX-2 isoform led to a hypothesis that selective inhibition of COX-2 would provide the potent anti-inflammatory and analgesic effects of NSAIDs without influencing COX-1 and its important physiologic functions (Needleman and Isakson, 1997). Several agents are under investigation in this new therapeutic category, and two of these, celecoxib (SC-58635) (Geis, 1999) and rofecoxib (Scott and Lamb, 1999) are now registered for clinical use in some countries. Clinical trials show that celecoxib and rofecoxib achieve analgesic efficacy in osteoarthritis and rheumatoid arthritis through specific COX-2 inhibition without showing evidence of gastrointestinal toxicity associated with other NSAIDs (Hawkey, 1999; Scott and Lamb, 1999).

2. Nitric Oxide

2.1. Introduction

In 1980, Furchgott and Zawadzki reported that the relaxation of blood vessels in response to acetylcholine required intact endothelium (Furchgott and Zawadzki, 1980). This endothelium-derived relaxing factor (EDRF) was, in 1987, determined to be nitric oxide (NO) or its derivative (Palmer et al., 1987; Ignarro et al., 1987). At the same time the importance of NO as an effector molecule in macrophage cytotoxicity was described by Hibbs et al. (Hibbs et al., 1987). It is now recognized that NO is produced by many cell types and that, in addition to regulating vessel tone, NO plays a vital role in host defense and immunity, including the modulation of inflammatory responses (Clancy et al., 1998; Moilanen et al., 1999).

NO is the best characterised member of the family of the gaseous signalling molecules in the body. The biological functions of NO can be divided into two categories. NO acts as an intercellular messenger molecule regulating physiological functions such as vascular tone, platelet aggregation, immune response and neurotransmission in the brain and in the periphery in the so-called NANC (non-adrenergic, non-cholinergic) nerves. In addition, NO synthesised in high amounts by activated macrophages possesses cytotoxic properties implicated in the ability of these cells to kill bacteria, viruses and protozoa as well as tumour cells. Although the latter function seems to be an important

mechanism in host defence, it is also a harmful and destructive action involved in the pathogenesis of autoimmune diseases and several other pathological conditions (Moncada and Higgs, 1995; Moilanen et al., 1999).

2.2. Biosynthesis of NO

NO synthesis is catalyzed by a family of NO synthase (NOS) enzymes (Knowles, 1997; Stuehr, 1999). NO is produced in the reaction of arginine with molecular oxygen to give citrulline and NO [Figure 3.]. Reduced nicotinic adenine dinucleotide phosphate NADPH acts as a co-factor and is oxidized to NADP^+ in the process. N-hydroxy-L-arginine, is formed as an intermediate in the reaction (Knowles and Moncada, 1994). Three major types of NOS have been characterized: constitutive calcium-calmodulin-dependent enzymes in endothelial cells (eNOS) and neurones (nNOS) and an inducible calcium-independent enzyme (iNOS) in macrophages and several tissue cells (Michel and Feron, 1997). All forms of the enzyme contain binding sites for flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), calmoduline, heme and tetrahydrobiopterin (BH_4) (Nathan and Xie, 1994).

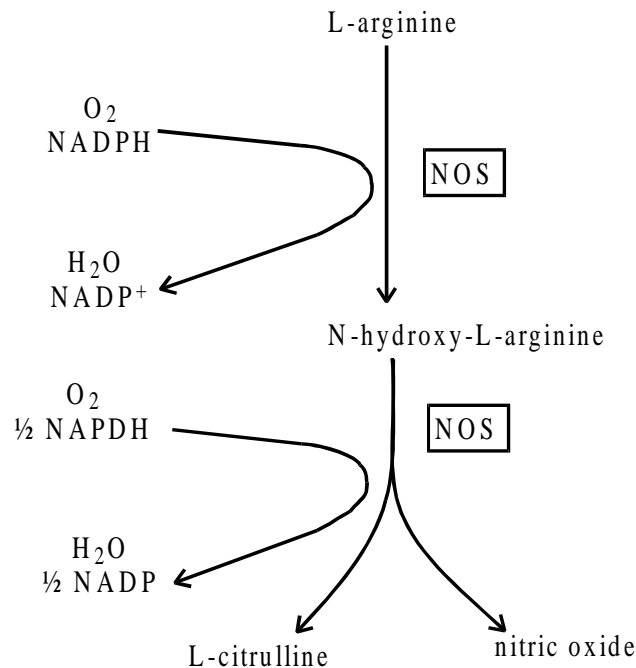


Figure 3. Nitric oxide synthase (NOS) catalyses a reaction in which nitric oxide and citrulline are formed from L-arginine. NADPH, reduced nicotinic adenine dinucleotide phosphate.

Although first described in endothelial and neuronal cells, eNOS and nNOS are present in many cell types in the body (Moncada and Higgs, 1995). Cells containing these constitutive enzymes produce physiologic amounts of NO in response to agonists such as acetylcholine in vascular endothelium, glutamate in brain, or collagen acting on platelets. Such receptor-mediated responses result in an increase in the intracellular concentrations of calcium, which is critical for activation of constitutive NOS (Knowles and Moncada, 1994; Moilanen et al., 1999).

Since iNOS contains tightly bound calmodulin, it is virtually independent of free intracellular calcium concentrations. Thus iNOS activity is regulated by protein expression rather than functional modulation (Nathan and Xie, 1994; Moilanen et al., 1999). iNOS is expressed after exposure to diverse stimuli such as inflammatory cytokines (e.g., IL-1, TNF, IFN- γ) and LPS either alone or in combination. Once expressed, the inducible enzyme generates significantly larger and sustained amounts of NO than does the constitutive isoforms (MacMicking et al., 1997; Clancy et al., 1998). iNOS gene contains consensus sequences for binding of several transcription factors, e.g. nuclear factor- κ B (NF- κ B), interferon- γ -responsive elements (I- γ -RE) and a tumor necrosis factor responsive element (TNF-RE). Several cell types including macrophages, chondrocytes, neutrophils, hepatocytes and smooth muscle cells have been reported to express iNOS in response to inflammatory stimuli but there is species and cell type variability with regard to the regulation of iNOS expression (Moilanen et al., 1999). The expression of iNOS is regulated by the balance of cytokines in microenvironment; for example, transforming growth factor β (TGF β), IL-4 and IL-10 inhibit iNOS expression in certain cell types (Clancy et al., 1998).

NO can also be generated non-enzymatically in tissues by reduction of nitrite, a reaction that takes place during acidic and reducing conditions (Weitzberg and Lundberg, 1998; Zweier et al., 1999).

2.3. NO regulating drugs

Arginine analogues such as N^G-monomethyl-L-arginine (L-NMMA), N^G-nitro-L-arginine methyl ester (L-NAME) and N-iminoethyl-L-ornithine (L-NIO) act as competitive inhibitors of NO synthases (Palmer et al., 1988; Rees et al., 1990; Moncada and Higgs, 1995). Whereas L-NMMA and L-NAME appear to be non-selective inhibitors of the various NO synthase enzymes, L-NIO has shown some selectivity toward the inducible enzyme (McCall et al., 1991; Knowles and Moncada, 1994). Recently, more selective inhibitors of iNOS have been described such as L-N-iminoethyl lysine (L-NIL) (Moore et al., 1994) and 1400W (Garvey et al., 1997). Some compounds have been reported to exhibit selectivity towards nNOS (Zhang et al., 1996; Mayer and Andrew, 1998).

Anti-inflammatory corticosteroids inhibit iNOS expression in some (Radomski et al., 1990; Di Rosa et al., 1990; O'Connor and Moncada, 1991) but not in all cell types and have been reported to do so by down-regulating transcription factor NF- κ B (Kleinert et al., 1996). In addition, posttranscriptional regulation has been suggested (Kunz et al., 1996). Glucocorticoids inhibit NO production also by limiting tetrahydrobiopterin (BH₄) and L-arginine availability (Simmons et al., 1996; Gotoh et al., 1996). In addition, anti-inflammatory and immunosuppressive drug cyclosporin A has been reported to inhibit induction of iNOS (Muhl et al., 1993; Hattori and Nakanishi, 1995). Methotrexate inhibits the formation of BH₄, an essential co-factor of NOS (Gross and Levi, 1992; Saura et al., 1996).

2.4. Biochemical actions of NO

The physiological chemistry of NO is a complex process encompassing numerous potential reactions. Consideration of these chemical reactions provides a blueprint by which one may distinguish the regulatory processes and/or anti-inflammatory effects of NO from its potential toxic and/or proinflammatory effects (Grisham et al., 1999). NO chemistry can be separated into direct and indirect effects (Wink and Mitchell, 1998). Direct effects are those reactions in which NO interacts directly with biological molecules. In contrast, indirect effects are derived from the reaction of NO with either superoxide or oxygen, which yields reactive nitrogen oxide species (RNOS). Under normal physiological conditions, cells produce small but significant amounts of NO and only trace amounts of reactive oxygen species dictating that direct NO chemistry will predominate in normal tissues. Direct effects of NO are involved primarily in regulatory, protective and anti-inflammatory processes *in vivo*. However, in tissues where iNOS has been upregulated indirect effects such as nitrosation, nitration, and oxidation will prevail. Reactive nitrogen oxide species have been suggested as mediators of the pathophysiology associated with inflammation (Grisham et al., 1999).

2.4.1. Direct effects of NO

Reaction between NO and metal complexes

The reaction of NO with certain metals to form nitrosyl complexes occurs *in vivo* primarily with iron-containing proteins (Wink and Mitchell, 1998). One of the most facile NO reactions with metalloproteins is that with heme-proteins. The most notable of these is the reaction of NO and guanylate cyclase, which stimulates the formation of cGMP from GTP (Ignarro, 1990). Synthesis of cGMP mediates many physiological effects of NO such as regulation of vascular tone, inhibition of platelet aggregation, and regulation of neurotransmission (Moncada et al., 1991). This same type of chemistry can also inhibit other metalloproteins such as cytochrome P-450, NOS, cytochrome oxidase, and catalase (Grisham et al., 1999).

Various metal-oxygen and metallo-oxo complexes rapidly react with NO. An important direct effect of NO is the reaction between NO and oxyhemoglobin

to form methemoglobin and nitrate (Murphy and Noack, 1994). Due to the high concentration of oxyhemoglobin and its rapid reaction with NO, this is one of the primary metabolic fates, as well as a primary detoxification mechanism for NO *in vivo* (Wink and Mitchell, 1998). Another physiologically relevant reaction is the reaction of NO with high valent metal complexes. Metallo-oxo species are formed from oxidation of metal species or metal-oxygen complexes (hemoglobin, myoglobin, cytochromes) by agents such as hydrogen peroxide. Such hypervalent complexes are powerful oxidants that can lead to cellular damage such as lipid peroxidation. NO rapidly reacts with these hypervalent complexes and reduces them to their original oxidation state protecting tissue from peroxide-mediated damage (Gorbunov et al., 1995; Wink and Mitchell, 1998).

It has become apparent that iron or hemoprotein-catalyzed oxidative reactions may mediate some of the pathophysiology associated with acute and chronic inflammation (Jour'dHeuil et al., 1998). NO may modulate iron-catalyzed oxidation reactions such as the superoxide (O_2^-) driven Fenton reaction, which produces powerful oxidants like the hydroxyl radical ($OH\cdot$) and metalloxo complexes. These data demonstrate that NO may have remarkable antioxidant capabilities (Grisham et al., 1999).

Reaction of NO with radical species

Another direct effect of NO is its reaction with other radical species. Because NO has an unpaired electron, it will rapidly react with lipid alkoxy ($LO\cdot$) and alkyl hydroperoxyl ($LOO\cdot$) radicals. NO terminates propagation of peroxidation reactions thus limiting the formation of lipid peroxides (Rubbo et al., 1994; Grisham et al., 1999).

2.4.2. Indirect effects of NO

NO/O₂ reaction

NO reacts with O_2 to yield reactive intermediates, which in turn may mediate additional nitrosative and/or oxidative reactions (Grisham et al., 1999). Autoxidation of NO in an aqueous environment leads to the formation of N_2O_3 , which is a potent nitrosating agent (Wink and Mitchell, 1998). N_2O_3 has been shown to *N*- and *S*-nitrosate a variety of biological targets. Interaction of N_2O_3 with amino compounds yields *N*-nitrosamines (Tamir and Tannenbaum, 1996). *S*-nitrosothiols (RSNOs) are formed via the interaction between N_2O_3 and certain thiols (Wink and Mitchell, 1998). Nitrosation of thiols in proteins has variety of different effects from inhibiting enzyme activity to forming *S*-nitrosothiol adducts that have been suggested to play an important role in NO transport and signal transduction pathways. Because many RSNOs release NO either spontaneously or after metabolism, they are capable of mediating many of the biological functions of NO (Grisham et al., 1999). *S*-nitroso-albumin is thought to be the most abundant nitrosothiol in human plasma. In addition, because reduced glutathione (GSH) has high affinity for N_2O_3 , it is likely to play

a critical role in protecting cells from the damaging effects of NO (Luperchio et al., 1996).

NO/O₂⁻ reaction

NO and O₂⁻ rapidly interact to produce the potent cytotoxic oxidant peroxynitrite (ONOO⁻) and its conjugate acid peroxynitrous acid (ONOOH). In neutral solution peroxynitrite is a powerful oxidant, which oxidizes thiols or thioethers, nitrates tyrosine residues, nitrates and oxidizes guanosine, degrades carbohydrate, initiates lipid peroxidation and cleaves DNA (Grisham et al., 1999). The formation of ONOO⁻ is limited by the competing reaction of O₂⁻ with superoxide dismutase (Wink and Mitchell, 1998). Another factor that limits harmful effects of peroxynitrite is the reaction between peroxynitrite and either NO or O₂⁻. Maximum oxidation chemistry mediated by peroxynitrite occurs when the fluxes of NO and superoxide are equivalent. In the presence of excess NO or superoxide, peroxynitrite is proposed to be converted to nitrosating species N₂O₃ (Miles et al., 1996; Wink et al., 1997). There exists a balance between the chemistry of RNOS associated with oxidative stress and that of nitrosative chemistry. The chemistry of peroxynitrite is limited to sites close to the O₂⁻ source (Wink and Mitchell, 1998).

3. NO-releasing compounds

3.1. Biochemical pathways of NO formation from different NO donors

Nitrovasodilators have been in clinical use for more than a century for the treatment of angina and are still widely used in numerous conditions (Moncada and Higgs, 1995). In addition, NO-releasing compounds are used as experimental tools to either mimic an endogenous NO-related response or substitute for an endogenous NO deficiency. These compounds can be divided into different groups that include the organic nitrates and nitrites, inorganic nitroso compounds (e.g., sodium nitroprusside, SNP), S-nitrosothiols, sydnonimines, furoxans, mesoionic oxatriazole derivatives and NONOates (Feelisch, 1998) [Figure 4.]. All these compounds are prodrugs that exert their pharmacological actions via a common pathway, i.e. the release of NO.

3.1.1. Organic nitrates

Organic nitrates chemically represent esters of nitric acid (HNO₃). Apart from their therapeutic application as antianginal drugs, some of these compounds are highly flammable solvents, propellants, and explosives (Feelisch, 1991). Clinically used representatives include glyceryl trinitrate (GTN), isosorbide dinitrate, and isosorbide 5-mononitrate. Ferid Murad analysed the mechanisms of action of glyceryl trinitrate and other related vasodilators, and he suggested in 1977 that these compounds release NO, which enhances cGMP production and relaxes smooth muscle (Katsuki et al., 1977a; Katsuki et al., 1977b; Arnold et al., 1977). For NO release to occur, organic nitrates require either enzymatic or non-

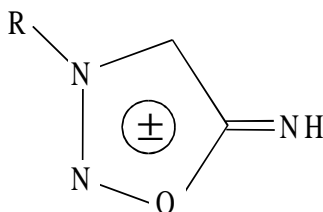
enzymatic bioactivation. It is likely that multiple intracellular and extracellular pathways contribute to NO formation from these compounds, but the relative importance of individual metabolic systems is poorly understood (Feelisch, 1998). The activity of glutathione-S-transferase and cytochrome P₄₅₀ related enzymes is thought to be involved in the bioactivation of organic nitrates (Feelisch, 1993). Thiols present in the cytosol are likely to account for non-enzymatic nitrate metabolism, and in both cases an unstable thionitrate (RSNO₂) may be the common intermediate. Certain structural prerequisites of the thiol are thought to account for the finding that under physiological conditions only a limited number of sulphhydryl-containing compounds (e.g., cysteine) react with organic nitrates to form NO, whereas virtually all thiols decompose (inactivate) organic nitrates to nitrite (NO₂⁻) (Feelisch, 1991).

Organic nitrates R - O - NO₂

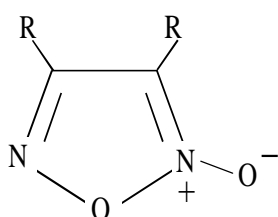
Organic nitrites R - O - NO

S-nitrosothiols R - S - NO

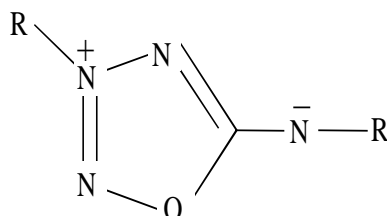
Sydnones



Furoxans



Mesoionic oxatriazole derivatives



NONOates R-[N(O)NO]⁻

Figure 4. The chemical structure of NO-releasing compounds

3.1.2. Organic nitrites and S-nitrosothiols

Organic nitrites, such as amyl nitrite, are chemically esters of nitrous acid (HNO_2). They react with thiol groups to form unstable S-nitrosothiols, which decompose to NO (Feelisch, 1991). S-nitrosothiol compounds are sulfur analogues of organic nitrites. At least two S-nitrosothiols have been prepared as stable solids and have been characterized. They are S-nitroso-N-acetylpenicillamine (SNAP) and S-nitrosoglutathione (GSNO) (Butler and Rhodes, 1997). In physiological buffers, S-nitrosothiols decompose to yield the corresponding disulfide (RSSR) and NO by cleavage of S-N bond. This process is enhanced by heat, light and metal ions (Singh et al., 1996). Another important reaction of S-nitrosothiols, which has been implicated in their biological activity, is transnitrosation (Feelisch, 1998). This reaction consists of the transfer of bound NO from one thiol group to another.

3.1.3. Sodium nitroprusside

Sodium nitroprusside (SNP) liberates NO by an as yet unknown mechanism. Relatively small amounts of NO released from SNP *in vitro* are not sufficient to account for its marked dilatory potency and it is likely to have additional effects unrelated to the generation of NO (Feelisch, 1993). In biological systems, both non-enzymatic and enzymatic NO release from SNP may occur. SNP decomposition is accompanied by the formation of cyanide (Feelisch, 1998).

3.1.4. Sydnonimines

The best known compound of sydnonimines is SIN-1 (3-morpholino-sydnonimine), which arises from hepatic cleavage of the prodrug molsidomine. Sydnonimines rapidly decompose to release NO without the need for any cofactor such as thiols. The first step in the transformation of SIN-1 is the hydroxyl ion-dependent conversion to the open-ring form SIN-1A. This compound releases NO via a radical process following reaction with molecular oxygen. In the course of this reaction superoxide (O_2^-) is formed, which together with NO can form peroxynitrite (Bohn and Schonafinger, 1989; Feelisch et al., 1989; Hogg et al., 1992). The reaction between NO and superoxide to form peroxynitrite may be regarded as an inactivation route of these two reactive molecules (Gryglewski et al., 1986). On the other hand, peroxynitrite is an active oxidant and nitrating agent, which may be responsible for some of the effects of SIN-1 (Crow and Beckman, 1995). However, oxidants other than O_2 and certain enzymes can promote oxidation of and NO release from SIN-1 in biological systems and no peroxynitrite is formed (Feelisch, 1998; Singh et al., 1999). Furthermore, not all the biological effects of sydnonimines can be attributed to NO. SIN-1-induced suppression of neutrophil degranulation was shown not to be mediated by cGMP, NO or ONOO^- , but probably by the NO-lacking metabolite, SIN-1C (Kankaanranta et al., 1997).

3.1.5. Furoxans

Furoxans are another group of heterocyclic compounds, which have been shown to possess NO-mimetic pharmacological activities (Feelisch et al., 1992; Ferioli et al., 1995; Civelli et al., 1996). Furoxans liberate NO after reacting with sulphhydryl groups of low molecular weight thiols and proteins (Feelisch et al., 1992; Medana et al., 1994). New furoxan derivatives have been reported to release NO spontaneously independently of thiols and without requiring enzymatic metabolism (Hecker et al., 1995).

3.1.6. Mesoionic oxatriazole derivatives

A series of new NO-releasing mesoionic oxatriazole derivatives has been synthesized in the GEA's Chemical Laboratories. Compounds are structurally related to sydnonimines and furoxans and the structures of GEA 3162 and GEA 3175 are presented in figure 5. GEA compounds have been shown to have NO-dependent pharmacological effects: they have been reported to have vasodilator, antiplatelet, fibrinolytic (Corell et al., 1994) and antibacterial (Virta et al., 1994) activities as well as to inhibit neutrophil functions (Moilanen et al., 1993; Wanikiat et al., 1997), suppress tumour cell growth (Vilpo et al., 1997), regulate glycosaminoglycan synthesis in articular cartilage (Järvinen et al., 1995), inhibit oxidation of low density lipoprotein (Malo-Ranta et al., 1994) and induce relaxation of bronchioles (Vaali et al., 1996; Hernandez et al., 1998) and trachea (Vaali et al., 1998).

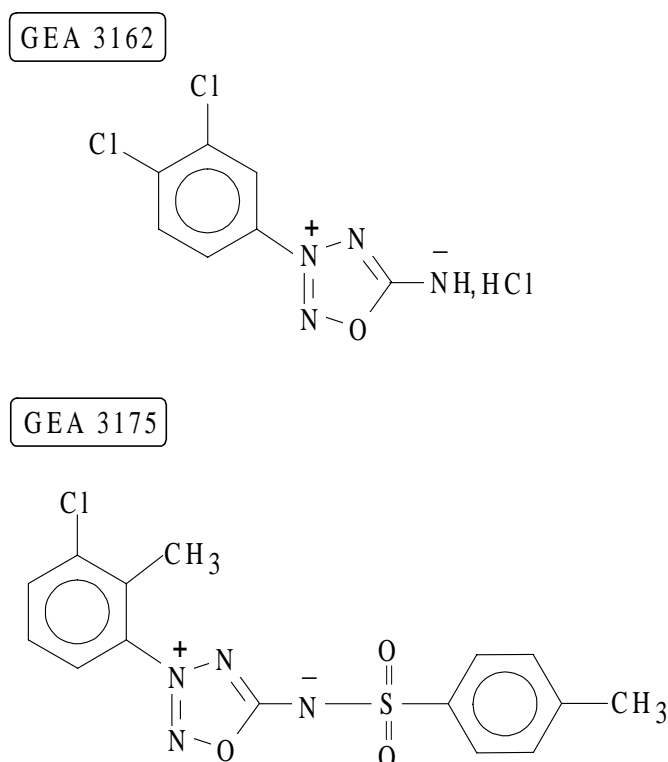


Figure 5. The chemical structures of GEA compounds.

The NO-releasing properties of mesoionic oxatriazole compounds have been characterized by documenting their ability to inhibit platelet aggregation, induce cGMP synthesis in platelets, convert oxyhemoglobin to methemoglobin, generate nitrite and nitrate in aqueous solutions and to form nitrosyl-hemoglobin complex (Karup et al., 1994; Kankaanranta et al., 1996). After addition of water compounds are likely to undergo an acid catalyzed ring opening (Karup et al., 1994). The next step, in which NO is released, is speculative and *in vitro* and *in vivo* pathways may differ. GEA compounds consume oxygen only when used at high concentrations and thereby an oxygen-dependent mechanism of NO release, similar to that found for SIN-1, is unlikely in the case of mesoionic oxatriazole derivatives (Karup et al., 1994; Holm et al., 1998). GEA 3162 has been reported to release only negligible amounts of superoxide and peroxyxynitrite, which suggest that GEA compounds differ from SIN-1 also in this matter (Holm et al., 1998). GEA 3162 has been shown to release NO spontaneously in buffer solutions (Kankaanranta et al., 1996; Holm et al., 1998). GEA 3175, which is the sulphonamide derivative of the parent compound GEA 3162, is only a weak NO-donor in phosphate buffer. However, in the presence of living cells or plasma GEA 3175 releases comparable amounts of NO (Kankaanranta et al., 1996). This may indicate a need for enzymatic degradation or the presence of thiols in order to release NO. This idea is further supported by the finding that L-cysteine enhances the release of NO from GEA 3175 (Karup et al., 1994; Cohen et al., 1995).

3.1.7. NONOates

NONOates (NO/nucleophile complexes) are compounds that are capable of spontaneously generating NO (Morley and Keefer, 1993a). NONOates can be formed by exposing different nucleophilic compounds (usually an amine, e.g. diethylamine or spermine) to NO (Maragos et al., 1991; Morley et al., 1993b). It is thought that NONOates generate NO by acid-catalyzed dissociation with regeneration of free nucleophile and NO, although enzymatic metabolism *in vivo* cannot be excluded (Feelisch, 1998).

3.2. Pharmacological effects

Although many groups of NO-donors are under development, the organic nitrates are still practically the only NO-donors in clinical use in addition to sodium nitropruside in some indications and molsidomine in some countries. The most important indications for nitrates are stable and unstable angina pectoris, acute myocardial infarction and congestive heart failure. The beneficial effects of these drugs in angina result from their dilator action on arterial and venous smooth muscle and from their ability to dilate large coronary vessels and thus increase coronary flow (Moncada and Higgs, 1995).

3.3. Tolerance

Repeated administration of nitrates to smooth muscle preparation *in vitro* results in a diminishing relaxant effect. Prolonged exposure to organic nitrates has long been known to induce tolerance to the cardiovascular effects of these drugs in humans and experimental animals, but the mechanism underlying this phenomenon is not completely understood. Nitrate tolerance is a complex process and the mechanisms likely involve several independent factors (Glasser, 1999). Traditionally tolerance has been explained by the tissue depletion of reduced sulphhydryl groups, reduced nitrate biotransformation and desensitization of the target enzyme guanylate cyclase (Munzel and Harrison, 1997). As vasodilators, nitrates activate compensatory neurohumoral mechanisms such as the renin-angiotensin system and increase catecholamine and vasopression levels, all of which may attenuate their vasodilator potency (Munzel and Harrison, 1997). Recently, several new mechanisms of nitrate tolerance have been suggested. Nitrates have been found to increase endothelin production within the vascular smooth muscle (Glasser, 1999). Also enhanced formation of superoxide radicals has been proposed to play a role in the development of nitrate tolerance (Dikalov et al., 1998).

An important therapeutic goal is the development of NO-releasing drugs that exert the positive effects of organic nitrates, but lack significant tolerance development. SIN-1, furoxans and NONOates have been reported to be free of tolerance in *in vitro* experiments (Hecker et al., 1995; Hinz and Schroder, 1998; Hinz and Schroder, 1999). In addition, treatment with SIN-1 *in vivo* did not produce tolerance and could overcome the loss of vascular relaxation associated with long-term glyceryl nitrate therapy (Govantes et al., 1996; Sutsch et al., 1997).

4. NO in inflammation

Evidence from studies using a number of experimental models has suggested a pathophysiologic role of NO in a range of inflammatory diseases. Like several other earlier known inflammatory mediators such as prostaglandins or IL-1, NO seems to have a dual regulatory function in inflammation. Depending on the type and phase of the inflammatory reaction and the individual vascular or cellular responses studied, NO can exert both proinflammatory and antiinflammatory properties. Constitutively produced NO appears to offer protection against acute inflammatory insults, whereas expression of iNOS is associated with widespread tissue injury and inflammation (Moilanen et al., 1999).

In inflammation NO can act both as a direct effector and as a regulator of other effectors (Nathan, 1997). One effector function includes direct cytotoxicity toward tumor cells, microorganisms, and host cells. There is considerable evidence that NO contributes to tissue destruction in inflammatory diseases. iNOS is expressed in inflamed tissue and a correlation of iNOS expression with

disease activity has been seen (Kolb and Kolb-Bachofen, 1998). For example, increased production of NO (and/or increased iNOS expression) has been implicated in sepsis, inflammatory bowel diseases, psoriasis, rheumatoid arthritis and osteoarthritis, multiple sclerosis, asthma and diabetes mellitus (Clancy et al., 1998). High doses of NO can trigger both necrotic and apoptotic pathways of cell death and several signs of inflammation are reversed by NOS inhibitors. However, data are accumulating that NO generated by iNOS is more than just a cytotoxic molecule in immune defence. In addition, it appears to exert substantial immunoregulatory activity. In some cases, both pharmacological inhibition and genetic inactivation of iNOS have led to increased pathology of inflammatory diseases. Therefore, the impact of NO on immune cell function is not merely damaging. Rather, immune cells appear to exhibit a specific response when exposed to NO (Kolb and Kolb-Bachofen, 1998).

NO serves as a bifunctional regulator of apoptosis: physiologically relevant levels of NO suppress apoptotic pathways by several mechanisms (Kim et al., 1999). Higher rates of NO production can overwhelm cellular protective mechanisms and shift the balance toward apoptotic death in some cell types. The presence of superoxide may also divert NO to a toxic pathway by leading to the formation of peroxynitrite.

4.1. NO in the vascular events of inflammation

NO is a potent vasodilator which modulates the early vascular responses of the acute inflammation as well as mediates the profound vasodilation associated with the septic shock syndrome. The vascular leakage associated with an inflammatory reaction seems to be regulated by NO in a biphasic manner as described in endotoxin-induced intestinal inflammation (Laszlo et al., 1994; Laszlo et al., 1995). In the early stages of the response low levels of NO produced by the constitutive NOS are protective and anti-inflammatory. After a few hours when iNOS is induced inhibitors of NOS suppress extravasation and the inflammatory response suggesting that high concentrations of NO are proinflammatory and augment the vascular leakage. The studies with selective inhibitors of iNOS support the detrimental role of iNOS in endotoxin-induced vascular injury (Garvey et al., 1997; Laszlo and Whittle, 1997).

4.1.1. NO and leukocyte adhesion

The importance of NO as an endogenous inhibitor of neutrophil adhesion to vascular endothelium was first described by Kubes et al. (Kubes et al., 1991). Inhibition of NOS augments leukocyte adhesion, whereas NO donors have been shown to inhibit leukocyte adhesion to endothelial cells (Hickey and Kubes, 1997).

The detailed mechanisms underlying the inhibitory action of NO on leukocyte adhesion are not known. The significance of the inactivation of superoxide anion

by NO and suppression of mast cell functions has been suggested (Kubes et al., 1993; Gaboury et al., 1993). More recently, the data has been extended to suggest that NO may inhibit several of the multistep cascade events that are involved in leukocyte recruitment including (a) rapid P-selectin-dependent leukocyte rolling, (b) CD18-dependent leukocyte adhesion, and (c) endothelial cell adhesion molecule expression. First, NO attenuates leukocyte rolling along the endothelium by inhibiting the expression of P-selectin on the vascular endothelium (Davenpeck et al., 1994; Gauthier et al., 1994; Armstead et al., 1997). Second, NO inhibits the firm adhesion of leukocytes to endothelium (Lefer, 1997). Antibodies directed against β 2-integrin CD18 prevented the leukocyte adhesion enhanced by NOS-inhibitors (Kubes et al., 1991; Niu et al., 1994; Mitchell et al., 1998). This suggests that NO mediates its antiadhesive effect through the leukocyte adhesion molecule CD11/CD18. NO inhibit the firm adhesion of leukocytes to endothelial cells partially by inhibiting ICAM-1 and VCAM-1 expression (De Caterina et al., 1995; Khan et al., 1996). NO is believed to repress VCAM-1 gene transcription, in part, by inhibiting NF- κ B (De Caterina et al., 1995; Khan et al., 1996).

The inhibition of leukocyte adhesion to endothelium is of major importance not only as a protective mechanism in inflammation but also in the ischemia-reperfusion injury and in tumour metastatic mechanism. Restitution of blood flow to ischemic tissues is associated with neutrophil-mediated ischemia-reperfusion injury (Granger and Korthuis, 1995). Ischemia-reperfusion injury exhibits many of the same characteristics as acute inflammation, including fluid accumulation, hyperemia and inflammatory cell infiltration (Grisham et al., 1998). Accumulation of neutrophils to postischemic tissues is mediated by various coordinately regulated adhesion molecules on the surface of leukocytes and endothelial cells (Lefer and Lefer, 1996). Reduced myocardial necrosis and neutrophil accumulation has been reported by NO-donors in an acute myocardial ischemia and reperfusion in animal models suggesting a novel indication for the therapeutic use of NO-releasing compounds (Lefer et al., 1993; Fukuda et al., 1995; Liu et al., 1998).

4.2. NO in the cellular events of inflammation

NO is an important effector molecule in murine macrophage-mediated host defence. In response to endotoxin (LPS) and IFN- γ these cells produce high amounts of NO which contributes to their cytostatic/cytotoxic action toward pathogens and tumor cells (MacMicking et al., 1997). The presence of iNOS and the role of NO in the function of human mononuclear phagocytes has, however, been more difficult to demonstrate. Human monocyte-macrophages do not express iNOS in response to the cytokine combinations known to induce NO synthesis in mouse macrophages. Other pathways leading to induction of iNOS in human monocyte-macrophages have been recently identified and data are also accumulating to show that NO has a role in the antimicrobial mechanisms of

human macrophages (Vouldoukis et al., 1995; Moilanen et al., 1997; Moilanen et al., 1999).

There are also interspecies differences in the synthesis of NO by neutrophils (Moilanen et al., 1999). Rat neutrophils are known to produce NO in response to inflammatory stimuli, but in human neutrophils no detectable NO-synthesis has been found (Holm et al., 1999). NO-releasing compounds are potent inhibitors of neutrophil functions including degranulation, chemotaxis, leukotriene B₄ release, and superoxide anion production *in vitro* (Moilanen et al., 1993). In addition, NO can exert also stimulatory effects on neutrophils reflecting a concentration-dependent biphasic action (Moilanen et al., 1999).

4.2.1. NO and lymphocytes

NO suppresses T lymphocyte responses. In murine models, production of large amounts of NO by activated macrophages accounts for their ability to suppress lymphocyte proliferation (Albina et al., 1991; Mills, 1991). Proliferative response of activated T lymphocytes is susceptible to NO whereas mediators like prostaglandins or hydrogen peroxide rather than NO are responsible for the macrophage-induced inhibition of B lymphocyte proliferation (Albina et al., 1991). In addition, NO produced by macrophages inhibits T cell proliferation in experimentally induced infections such as murine trypanosomiasis and mycobacterial infections (Sternberg and McGuigan, 1992; Schleifer and Mansfield, 1993; Maw et al., 1997). There are also data suggesting the mediator role of NO in the tumour-induced suppression of tumour-infiltrating lymphocytes (Lejeune et al., 1994). Using murine T-cell lines Taylor-Robinson et al. found that T-helper type 1 (T_H1) but not T-helper type 2 (T_H2) cells produced large amounts of NO which serves as a regulatory mechanism leading to suppressed function of T_H1 lymphocytes. Specific impairment of T_H1 cells was observed while T_H2 cell function appeared largely unaffected (Taylor-Robinson et al., 1993; Taylor-Robinson et al., 1994). A key target for NO action in murine lymphocytes is the IL-2 gene. Exposure to NO suppresses IL-2 gene expression and consequently, NO modulates the T_H1/T_H2 balance by favouring T_H2 responses (Taylor-Robinson, 1997). There are also data suggesting that NO induces a similar bias in the human immune system (Chang et al., 1997; Kolb and Kolb-Bachofen, 1998; Roozendaal et al., 1999). This proposed role of NO fits in with the observation that mice with a disrupted iNOS gene exhibit enhanced T_H1 reactivity (Wei et al., 1995).

In addition to the inhibitory action of higher concentrations of NO on T-lymphocyte proliferation, NO gas at nanomolar concentrations has been shown to stimulate resting human peripheral blood mononuclear cells (Lander et al., 1993a; Lander et al., 1993b). NO enhanced NF- κ B binding activity and TNF- α secretion in human peripheral blood mononuclear cells. A recent study shows that a monomeric G protein p21ras is a target of NO in T lymphocytes and the

activation of p21ras could explain these immunostimulatory properties of low concentrations of NO (Lander et al., 1995).

4.3. NO and cyclooxygenase

Stimulation of various cells by cytokines and other inflammatory mediators results in the induction of both iNOS and COX-2. Depending on the cell type and experimental condition used, NO has been found either to stimulate, to inhibit or not to influence prostanoid synthesis.

Some investigators have demonstrated that NO potentiates the formation of prostaglandins. Salvemini et al. (Salvemini et al., 1993) have shown that addition of NOS inhibitors to LPS-stimulated mouse macrophages results not only in a decrease in nitrite formation, but also a four- to fivefold decrease in prostaglandin E₂ biosynthesis. In addition, they suggested that the effect of NO on prostaglandin synthesis is cGMP independent. The ability of NO to enhance prostaglandin biosynthesis has been observed in other cell culture and *in vivo* models. These include various rat tissues (Corbett et al., 1993; Franchi et al., 1994; Salvemini et al., 1995b; Sautebin et al., 1995; Lianos et al., 1998; Nogawa et al., 1998), bovine endothelial cells (Davidge et al., 1995; Salvemini et al., 1996), human airway epithelial cells (Watkins et al., 1997), and a rabbit renal inflammation model (Salvemini et al., 1994). Although many studies indicate that NO directly interacts with cyclooxygenase to stimulate prostaglandin production (Salvemini et al., 1995a), some have suggested that this phenomenon may result (at least in part) from an effect of NO on transcription of the cyclooxygenase gene (Tetsuka et al., 1996; Hughes et al., 1999). Hughes et al. (Hughes et al., 1999) noted that the effects of NO on cyclooxygenase expression at transcriptional and post-transcriptional levels are highly dependent on the cytokines involved in cellular stimulation. Tetsuka et al. (Tetsuka et al., 1996) and Watkins et al. (Watkins et al., 1997) suggest that the stimulatory effect of NO is mediated through a cGMP-dependent mechanism in rat mesangial cells and human lung epithelial cells, respectively.

The inhibition of COX-2 expression by NO has been observed in mouse and rat macrophages. In these studies, NOS inhibitors act to increase COX-2 expression and prostaglandin biosynthesis in response to cytokine stimulation and the addition of NO donors reverse this effect (Swierkosz et al., 1995; Habib et al., 1997). The ability of NO to inhibit prostaglandin synthesis has also been observed in cartilage from osteoarthritis patients (Amin et al., 1997). In addition to inhibition of cyclooxygenase expression by NO, Swierkosz et al. (Swierkosz et al., 1995) and Stadler et al. (Stadler et al., 1993) suggest that NO also directly inhibits cyclooxygenase activity.

Several *in vitro* studies have been undertaken to determine the nature of the interaction between NO and COX proteins; however, the results of these investigations have also varied widely. Some investigators note that NO

stimulates prostaglandin synthesis by COX (Hajjar et al., 1995; Maccarrone et al., 1997). Others observe either no stimulation or inhibition of the enzyme by NO (Kanner et al., 1992; Tsai et al., 1994; Curtis et al., 1996). It is well established that NO binds efficiently to many iron-containing proteins. Cyclooxygenase is a heme-containing enzyme and the chemical interaction between NO and the heme moiety of COX has been demonstrated (Karthien et al., 1987). However, it has been demonstrated, that NO does not stimulate COX activity through an interaction with the heme prosthetic group (Tsai et al., 1994; Hajjar et al., 1995; Goodwin et al., 1999). Although in numerous *in vivo* and cellular systems NO has a stimulatory effect on prostaglandin synthesis, no clear mechanism by which NO may interact directly with the COX protein to stimulate activity has been found. Most possible interactions between NO and COX are more likely to result in inhibition rather than activation (Goodwin et al., 1999). One possible explanation is that the enhancement of prostaglandin synthesis by NO may be due to scavenging of superoxide by the NO/O₂⁻ reaction. Superoxide inhibits cyclooxygenase by reducing the heme iron to its inactive ferrous (Fe²⁺) state. The presence of small amounts of NO would prevent this reduction by interacting with and scavenging O₂⁻. This would maintain the COX-associated heme iron in the ferric (Fe³⁺) form, resulting in the formation of prostaglandins. On the other hand, overproduction of large amounts of NO would inhibit COX activity (Wink and Mitchell, 1998; Grisham et al., 1999). In addition, it has been shown that peroxynitrite, the coupling product of nitric oxide and superoxide, activates cyclooxygenase (Landino et al., 1996).

4.4. Therapeutic potential of NO-releasing drugs

Reduced generation of NO has been implicated in a number of clinical conditions (Moncada and Higgs, 1995). In these, or even in some situations in which NO production is unimpaired, it may be desirable to mimic or enhance the physiological generation of NO.

It is likely that NO from constitutive eNOS plays a role in the early stages of inflammation as a mechanism to decrease and limit the process by inhibiting white cell activation and platelet aggregation and by inducing vasodilatation. Compounds that release nitric oxide in small amounts over a prolonged period of time may be useful in preventing tissue injury in early stages of inflammation (Muscara and Wallace, 1999). In contrast, NO from iNOS contributes to many aspects of chronic inflammation. The use of selective iNOS inhibitors may be of benefit in the management of chronic inflammatory processes (Hobbs et al., 1999).

Traditional uses of NO donors have been based on their vasodilator properties. The anti-inflammatory effects should be achieved without causing significant hypotension. One interesting possibility lies in the development of NO donors that will selectively target the tissue for which the NO replacement therapy is intended. NO-releasing [N(O)NO]- group has been conjugated to a wide variety

of other functional molecules, including amino acids, proteins, polyamines, drugs, nucleosides, synthetic polymers, polysaccharides and phospholipids. By designing the molecule such that the protecting group can be removed only by enzymes concentrated in a given target organ or cell type, prodrugs can be prepared that circulate freely but generate NO preferentially at the site of metabolism. Such agents have proven useful for protecting the liver from TNF- α -induced apoptotic cell death, localizing anticoagulant activity, promoting healing of coronary arteries after balloon angioplasty, inducing penile erections and selectively dilating a given blood vessel or vascular bed (Saavedra et al., 1996; Smith et al., 1996; Keefer et al., 1996; Saavedra et al., 1997; Keefer, 1999; Champion et al., 1999).

In gastrointestinal tract NO donors accelerate healing of preexisting ulcers. Gastric ulcers in rats were shown to be healed at a greatly accelerated rate when the rats were treated daily with NO-donors (Elliott et al., 1995; Asmawi et al., 1999). An NO-donor (glyceryltrinitrate) has also been successfully employed in a clinical setting to accelerate the healing of anal fissures associated with Crohn's disease (Lund and Scholefield, 1997). Nonsteroidal anti-inflammatory drugs (NSAIDs) represent one of the most widely used classes of therapeutic agents, but their tendency to cause ulceration and bleeding in the gastrointestinal tract is a major problem. It has been proposed that addition of an NO-releasing moiety to standard NSAIDs would result in the formation of anti-inflammatory drugs with markedly reduced ulcerogenic properties (Muscara and Wallace, 1999). These so-called "NO-NSAIDs" have been characterized in several species and have been shown to have comparable or superior anti-inflammatory and analgesic activity to standard NSAIDs while sparing the gastrointestinal tract and kidney of injury. Unlike the parent drugs from which they are derived, NO-NSAIDs do not cause a reduction in gastric mucosal blood flow and do not evoke leukocyte adhesion to vascular endothelium in mesenteric venules (Wallace et al., 1994; Somasundaram et al., 1997; Fiorucci et al., 1999). The success of the NO-NSAID approach has led to this strategy being applied to other classes of drugs that could benefit from addition of an NO-releasing moiety, such as glucocorticoids and 5-aminosalicylic acid (Muscara and Wallace, 1999).

Since one of the main features of inflammation is the infiltration of leukocytes, it has been proposed that NO donors can reduce damaging effects of an inappropriate inflammatory response through inhibition of leukocyte infiltration (Hickey and Kubes, 1997). This approach is already being assessed in ischemia-reperfusion and in atherosclerosis (Kurose et al., 1994; Gauthier et al., 1995). NO levels are decreased in a variety of circulatory disorders, including myocardial ischemia-reperfusion, hypercholesterolemia and atherosclerosis. The reduction in NO release leads to an increased leukocyte adherence to coronary vascular endothelium and it is therefore not surprising that replacement therapy to restore the NO deficit has been considered in these disease states (Lefer, 1997).

Initial attempts to target NO-releasing therapy to the sites of inflammation are already demonstrating the therapeutic potential of some of these interventions. The challenge will be to distinguish between the physiological and protective actions of NO that are to be maintained or enhanced and the pathological roles that need to be reduced or abolished.

AIMS OF THE STUDY

The aim of the study was to investigate the effects of NO-releasing compounds on inflammatory cells *in vitro* and the mechanisms of their anti-inflammatory actions. Two novel NO-releasing mesoionic oxatriazole derivatives (GEA 3162 and GEA 3175) were compared to earlier known NO-donors SIN-1 and SNAP.

The detailed aims were:

1. To examine the effects of NO-releasing compounds on lymphocyte proliferation, and the role of cGMP in this response.
2. To study the effects of NO-releasing compounds on prostacyclin production in inflammatory activated endothelial cells.
3. To study the effects of NO-releasing compounds on neutrophil adhesion to endothelial cells.

MATERIALS AND METHODS

1. Lymphocyte proliferation (I,II)

1.1. Cell isolation and proliferation assay

Human peripheral blood mononuclear cells were isolated by Ficoll-Paque gradient centrifugation from venous blood obtained from healthy volunteers who had abstained from any drugs for at least one week before sampling. The cell suspension consisted of lymphocytes ($90.0 \pm 1.7\%$), monocytes ($7.4 \pm 1.5\%$) and polymorphonuclear leukocytes ($2.6 \pm 0.5\%$) (mean \pm SE, $n = 7$). The cells were suspended in RPMI 1640 Glutamax supplemented with 10% heat inactivated fetal bovine serum, penicillin (100 U/ml), streptomycin (100 μ g/ml) and amphotericin B (250 ng/ml). Lymphocyte proliferation was induced by ConA. The cells were cultured in 96-well plates (2×10^5 cells / 200 μ l). NO-donors and other compounds tested were added into the culture just before ConA. The cells were incubated for 2 days at 37°C (in 5% CO₂) and then pulsed for 20 h with 0.1 μ Ci [³H]thymidine. The cells were harvested and the incorporated radioactivity was measured by β -counter.

1.2. cGMP production in human peripheral blood mononuclear cells

Peripheral blood mononuclear cells (5×10^6 cells in 500 μ l of RPMI) were incubated with ConA (1 μ g/ml) and the NO-donors either in the presence or absence of a phosphodiesterase inhibitor (type V PDE-inhibitor zaprinast 25 μ M or nonspecific PDE-inhibitor 3-isobutyl-1-methylxanthine IBMX 100 μ M) or guanylate cyclase inhibitor (1*H*-[1,2,4]oxadiazolo[4,3,-a]quinoxalin-1-one; ODQ 10 μ M) for the indicated time at 37°C. The incubations were terminated by addition of ice cold trichloroacetic acid (final concentration 6%) and the samples were centrifuged (10 000 \times g for 5 min). The supernatants were washed four times with water-saturated ether, diluted with an equal volume of 100 mM sodium acetate buffer (pH 6.2) and stored at -20°C until assayed for cGMP. The cGMP samples were acetylated and measured by radioimmunoassay as described earlier (Axelsson et al., 1988; Moilanen et al., 1993).

2. Prostacyclin production (III)

2.1. Human umbilical vein endothelial cell culture

Human umbilical vein endothelial cells (HUVECs) were isolated by treatment of human umbilical veins with 0.01% collagenase (Zimmerman et al., 1990). The cells were suspended in RPMI 1640 Glutamax-medium supplemented with 20% heat-inactivated fetal bovine serum, endothelial cell growth supplement (10 μ g/ml), penicillin (100 units/ml), streptomycin (100 μ g/ml) and amphotericin B (250 ng/ml). Cells were grown to confluence in plastic dishes, then removed by

treatment with trypsin-EDTA (ethylenediamine tetraacetic acid), and seeded to gelatin-coated 24 well plates.

2.2. Prostacyclin production

Confluent cultures of HUVECs were stimulated by LPS for 6 h. NO-donors and other compounds tested were added into the culture at the beginning of the incubations or at the time point indicated. The medium was collected and the stable metabolite of prostacyclin (6-keto-PGF_{1α}) was measured by radioimmunoassay after Amprep C2 minicolumn purification.

2.3. Western blot analysis

Cell pellets from HUVECs were lysed in extraction buffer (pH 7.4) at 4°C (10 mM Tris-base, 5 mM EDTA, 50 mM NaCl, 1% Triton-X-100, 0.5 mM phenylmethylsulfonyl 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). After centrifugation the supernatant was collected and an aliquot of the supernatant was used to determine protein by the Coomassie blue method (Bradford, 1976). Supernatants were then mixed 1:4 with sample buffer (62.5 mM Tris, pH 6.8, 10% glycerol, 2% SDS, 0.025% bromophenol blue and 5% β-mercaptoethanol) and heated at 100°C for 5 min. Proteins were separated by SDS-PAGE on 10% polyacrylamide gels and then transferred to nitrocellulose. The nitrocellulose was then blocked with 5% non-fat milk, followed by incubation with goat polyclonal antibody (1:500 dilution) raised against a peptide corresponding to amino acids 27-46 mapping at the amino terminus of the COX-2 precursor for 45 min at room temperature. The membrane was then washed and incubated with a horseradish peroxidase linked anti-goat IgG. The nitrocellulose was developed using the ECLTM Western blot detection system and densitometric analysis was carried out using SigmaGelTM software.

2.4. Nitrite assay

Nitrite (NO₂⁻), a stable product of NO in aqueous solutions was measured from the incubation medium with Griess reagent (1% sulfanilamide, 0.1% naphthylethylene diamine dihydrochloride and 2.5% phosphoric acid). Nitrite reacts with Griess reagent to form a purple azo dye. Its absorbance at 546 nm was detected by a spectrophotometer (Green et al., 1982). The detection limit of the assay was 1 µM.

3. Neutrophil adhesion to endothelial cells (IV)

3.1. Co-cultures of endothelial cells and polymorphonuclear leukocytes

Rabbit B4 aortic endothelial cells (Buonassisi and Venter, 1976) were cultured on 24-multiwell plates to confluence. Human polymorphonuclear leukocytes were isolated from citrated blood of healthy donors by gradient centrifugation on Ficoll-Paque as described earlier (Moilanen et al., 1988). Polymorphonuclear leukocytes (1×10^6 cells in 1 ml RPMI medium supplemented with 5 % heat-inactivated fetal bovine serum, 100 U/ml penicillin, 100 μ g/ml streptomycin and 250 ng/ml amphotericin B) were added to the endothelial cell cultures supplemented with or without the NO donors or other compounds tested. After 30 min incubation at 37°C (in 5 % CO₂), TNF- α (10 U/ml) was added to induce adhesion. The co-cultures were washed twice with phosphate-buffered saline (PBS) after 30 min to remove non-adherent leukocytes. Hexadecyltrimethylammonium bromide (HTAB 0.5%; 1 ml/well) was added to lyse cells. The number of adherent leukocytes was quantitated by myeloperoxidase assay (Bailey and Fletcher, 1988).

3.2. cGMP production in polymorphonuclear leukocytes.

Polymorphonuclear leukocytes (5×10^6 cells in 500 μ l of Dulbecco's PBS) were incubated with the NO donor for 30 min at 37°C. The incubations were terminated by addition of ice cold trichloroacetic acid (final concentration 6%), and the cGMP was measured as described above.

3.3. CD11/CD18 expression in polymorphonuclear leukocytes

Polymorphonuclear leukocytes (2×10^6 cells in 1000 μ l of Dulbecco's PBS + 0.25 % BSA) were incubated with the NO donor at 37°C. After 10 min incubation TNF- α (10 U/ml) was added for 30 min. The incubations were stopped by addition of cold PBS (2 ml) and then the cells were washed with cold PBS. Cells were then stained (30 min; +4°C) with fluorescein isothiocyanate (FITC) labelled CD18 monoclonal antibody (MHM23) and after two washes with PBS + 2% BSA they were analysed by flow cytometry (FACScan; Becton Dickinson). A purified mouse monoclonal IgG1 antibody (DAK-G01) conjugated with FITC was used as a negative control. The antibody specificity was directed towards *Aspergillus niger* glucose oxidase, an enzyme which is neither present nor inducible in mammalian tissue.

4. Adhesion molecule expression in and neutrophil adhesion to human endothelial cells (V)

4.1. Adhesion assay

The confluent monolayers of HUVECs were incubated with LPS for 6 hours at 37°C in 5 % CO₂ either in the presence or absence of NO donors or a NOS inhibitor. Thereafter the cells were washed and human polymorphonuclear leukocytes (5 x 10⁵ cells / 500 µl) were added into the culture. After 20 min incubation, the co-cultures were washed twice with PBS to remove non-adherent leukocytes. 0.5% HTAB (1 ml/well) was added to lyse cells. The number of adherent leukocytes was quantitated by myeloperoxidase assay (Bailey and Fletcher, 1988).

4.2. E-selectin and ICAM-1 expression

Endothelial cells were incubated with the culture medium (unstimulated cells) or LPS (10 ng/ml) for 6 hours either in the presence or absence of NO donors. The cells were detached from flasks using 0.02% EDTA in PBS, washed and suspended in PBS. Then the cells were incubated (30 min; 4°C) with FITC-labelled monoclonal anti-CD62E (E-selectin) antibody (1.2B6), R-phycoerythrin (RPE)-labelled monoclonal anti-CD54 (ICAM-1) antibody (HA58) or with similarly labelled negative control (DAK-G01; see above). After two washes with PBS containing 2% BSA, cells were fixed in 1% paraformaldehyde in 0.15M NaCl. Expression of surface antigens was analysed by flow cytometry (FACSscan; Becton Dickinson). Fluorescence intensities of the samples were reported after subtracting the mean fluorescence intensity of the cells exposed to the negative control antibody.

5. Cell viability

To evaluate a direct cytotoxicity of the NO donors, Trypan blue staining and the measurements of released lactate dehydrogenase (The committee on enzymes of the Scandinavian society for clinical chemistry and clinical physiology, 1974) were included in the protocols. Triton-X (1%) treatment was used as a positive control. None of the NO donors tested decreased leukocyte viability as measured by these tests in the incubation conditions described above. The NO donors did neither alter the morphology of endothelial cell monolayers as examined under a phase-contrast microscope.

6. Drugs and chemicals

Two mesoionic oxatriazole derivatives GEA 3162 (1,2,3,4-oxatriazolium,5-amino-3(3,4-dichlorophenyl)-chloride) and GEA 3175 (1,2,3,4-oxatriazolium,-3-(3-chloro-2-methylphenyl)-5-[[4-methylphenyl)sulfonyl]amino]-, hydroxide

inner salt) as well as SIN-1 (3-morpholino-sydnonimine), SNAP (S-nitroso-*N*-acetylpenicillamine) and an analogue of cGMP, 8-*p*-chlorophenylthio-cGMP, were kindly provided by GEA Ltd. (Copenhagen, Denmark). To prevent the decomposition of NO-donors before experiment, the stock solutions of NO-donors were dissolved in dimethylsulfoxide and protected from light. Aqueous solutions were prepared just before use (Feelisch, 1991; Feelisch, 1998).

8-Bromo-cGMP, 8-bromo-cAMP, zaprinast, endothelial cell growth supplement, HTAB, LPS (from *E. coli* 026:B6), ketoprofen, cycloheximide, arachidonic acid, Tris-base, Triton-X-100, phenylmethylsulfonyl fluoride, Na-orthovanadate, leupeptin, aprotinin, Na-pyrophosphate, n-octyl- β -D-glucopyranoside, Tris, glycerol, SDS, bromophenol blue, β -mercaptoethanol, sulfanilamide and naphthylethylene diamine dihydrochloride were obtained from Sigma (St. Louis, MO, USA). Culture media, fetal bovine serum, antibiotics and trypsin-EDTA (Gibco, Paisley, UK), ConA and Ficoll-Paque (Pharmacia, Uppsala, Sweden), recombinant human TNF- α (Genzyme, Cambridge, MA, USA), 125 I-labelled cGMP (DuPont, Boston, MA, USA), FITC-conjugated anti-human CD18 mAb, FITC- and RPE-conjugated negative controls for flow cytometry (Dako, Glostrup, Denmark), RPE- conjugated anti-human CD54 mAb (Pharmingen, San Diego, CA, USA), FITC-conjugated anti-human CD62E mAb (Calbiochem, La Jolla, CA, USA), [methyl- 3 H]thymidine (Amersham International, Buckinghamshire, UK), collagenase A (Boehringer Mannheim, Germany), L-NMMA (Clinalfa, Läufelfingen, Switzerland), IBMX (EGA-Chemie, Steinheim, Germany), ODQ (Tocris Cookson, Bristol, UK), L-NIO and NS-398 (Alexis, Läufelfingen, Switzerland) were obtained as indicated. Radioimmunoassay kits for 6-keto-PGF $_{1\alpha}$ were from the Institute of Isotopes of Hungarian Academy of Sciences (Budapest, Hungary). Amprep C2 minicolumns and ECLTM Western blot detection system were from Amersham International (Buckinghamshire, UK). COX-2 antibody and horseradish peroxidase linked anti-goat IgG were from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

7. Statistics

Results are expressed as mean \pm SE. Statistical significance was calculated by analysis of variance supported by Dunnett's or Bonferroni's multiple comparisons test and by Friedman nonparametric repeated measures test followed by Dunn's multiple comparisons test. The data were analysed using BMDP Statistical Software (Los Angeles, CA, USA) or GraphPad InStat (San Diego, CA, USA). Differences were considered significant when $P < 0.05$.

RESULTS

1. Effects of NO donors on human lymphocyte proliferation (I, II)

The three NO-donors, GEA 3162, GEA 3175 and SNAP, inhibited in a dose-dependent manner the proliferative responses of human mononuclear cells stimulated with submaximal concentrations (1 µg/ml) of ConA (I, II). On a molar basis the two new mesoionic oxatriazole derivatives (GEA 3162 and GEA 3175) were more potent than the earlier known NO-releasing compound SNAP. The antiproliferative action of NO-donors was attenuated when red blood cells were added into the culture suggesting that NO is involved in the action of these compounds (I). Hemoglobin inhibits the action of NO by binding it to form nitrosylhemoglobin or by metabolising it to inactive nitrate (Murphy and Noack, 1994).

The antiproliferative effect of NO-donors was most pronounced when the cells were stimulated with submaximal concentrations of ConA (0.1 and 1 µg/ml). When ConA concentrations were increased, the antiproliferative action of NO-donors was reversed (I). These data together with the finding that trypan blue staining and lactate dehydrogenase release was not affected by treatment with NO-donors suggest that the NO-donors in these concentrations have antagonisable and regulatory rather than cytotoxic effect on ConA-activated mononuclear cells.

Lander et al. (Lander et al., 1995; Lander et al., 1993a; Lander et al., 1993b) have reported that NO at low concentrations stimulates lymphocytes. Therefore we tested the effects of a wide concentration range of the NO-donors and only antiproliferative action was found (I).

At physiological concentrations of NO the enzyme guanylate cyclase is the principal target of NO (Ignarro, 1991). In the second set of experiments, the possible mediator role of cGMP in the antiproliferative action of NO donors in human lymphocytes was tested (II). First, NO donors caused a rapid and transient increase in cGMP production in mononuclear cells. After 30-min or 2-hr incubation with the NO donors, cGMP levels in mononuclear cells were substantially increased. However, there was no clear correlation between the degree of the antiproliferative action of NO-donors and their ability to raise cGMP. Second, an inhibitor of guanylate cyclase, ODQ (Garthwaite et al., 1995) inhibited NO donor-induced cGMP production, whereas the antiproliferative action of NO donors remained unaltered. Third, phosphodiesterase (PDE) inhibitors zaprinast and IBMX (Beavo, 1995) potentiated and prolonged NO donor-induced increase in the concentrations of cGMP but did not enhance the antiproliferative action of NO donors. Fourth, two analogs of cGMP, 8-bromo-cGMP and a more cell-permeable compound, 8-*p*-chlorophenylthio-cGMP (Miller et al., 1973; Butt et al., 1992), did not inhibit ConA-stimulated lymphocyte proliferation when used in concentrations of up to 300 µM. At

millimolar concentrations, 8-bromo-cGMP had a moderate inhibitory action. At these higher concentrations, unspecific effects like increased cAMP cannot be ruled out. Taken together, these data suggest that NO donors inhibit lymphocyte proliferation in a cGMP-independent manner.

2. Effects of NO donors on prostacyclin production in human endothelial cells (III)

In this set of experiments LPS-treated (6 h) human endothelial cells were used to study the effects of NO-releasing compounds on prostacyclin formation. GEA 3175 (1-30 μ M) inhibited LPS-induced production of 6-keto-PGF_{1 α} (a stable metabolite of prostacyclin) in HUVECs in a concentration-dependent manner being more potent than SNAP. These experimental conditions measure prostacyclin production principally by COX-2 as evidenced by the effect of a specific inhibitor of COX-2, NS-398 (Futaki et al., 1994), which inhibited the accumulation of prostacyclin into the culture medium by >90%. Western blot analysis confirmed the presence of COX-2 protein in unstimulated and to a higher extent in LPS-stimulated cells. This is consistent with the earlier data indicating that serum added into the culture medium contains factor(s) able to induce COX-2 in cell culture conditions (O'Banion et al., 1992). The effect of NO-donors on COX-1 was tested in the presence of a selective inhibitor of COX-2 and no effect was found. To study the possible effect of NO donors on phospholipases, exogenous arachidonic acid was added into the culture. The inhibition of prostacyclin synthesis by GEA 3175 was similar in the presence and in the absence of exogenous arachidonic acid suggesting that the action of NO donors is against cyclooxygenases rather than phospholipases. The addition of red blood cells into the culture reversed the inhibitory action of NO donors indicating that the effect was due to NO released from the compounds.

NO could regulate prostacyclin production by COX-2 at the level of enzyme activity or through the synthesis of COX-2 protein. In the present experiments, NO donors inhibited prostacyclin production regardless of whether the compounds were added into the cultures together with LPS or 6 h afterwards at the time when a high-level induction of COX-2 was evident. These findings suggest that NO-donors inhibit the activity of COX-2 in cultured HUVECs. In further experiments NO-releasing compounds did not alter the amount of COX-2 protein in LPS-treated HUVECs implying that only the activity but not expression of COX-2 was affected.

Inhibitors of NO synthase, L-NMMA (up to 1 mM) or L-NIO (up to 2 mM) did not alter LPS-induced 6-keto-PGF_{1 α} production in HUVECs. In addition, LPS (10 ng/ml) failed to induce detectable nitrite production in HUVECs.

3. Effects of NO donors on neutrophil adhesion to endothelial cells (IV,V)

The effects of NO donors on neutrophil adhesion to endothelial cells were studied in two models. In the first set of experiments the effects of NO-donors on the adhesion of TNF- α -stimulated polymorphonuclear leukocytes was studied in a co-culture of human polymorphonuclear leukocytes and B4 endothelial cells. In the subsequent experiments human umbilical vein endothelial cells were stimulated by LPS to induce adhesion molecule expression and the effects of NO donors on adhesion molecule expression in and leukocyte adhesion to LPS-stimulated endothelial cells were studied.

First, polymorphonuclear leukocyte adhesion to endothelial cells was induced by addition of TNF- α (10 U/ml; 30 min) into the co-cultures of human polymorphonuclear leukocytes and B4 endothelial cells (IV). GEA 3162 (3-10 μ M) and GEA 3175 (10-30 μ M) inhibited the adhesion process in a concentration-dependent manner being more potent than SIN-1. When separately exposing endothelial cells and neutrophils to NO donors, neutrophils rather than endothelial cells seemed to be the target of the effect of NO. Flow cytometric analysis showed that NO donors did not alter TNF- α induced CD11/CD18 surface expression in polymorphonuclear leukocytes. The inhibitory action of NO donors on adhesion paralleled with the increased synthesis of cGMP in polymorphonuclear leukocytes. Analogues of cGMP (8-bromo-cGMP and 8-*p*-chlorophenylthio-cGMP) inhibited neutrophil adhesion suggesting a role for cGMP in the action of NO donors.

In the second set of experiments incubation of confluent monolayers of HUVECs with LPS stimulated the adhesion of polymorphonuclear leukocytes to HUVECs (V). Flow cytometric analysis showed that LPS treatment upregulated the expression of adhesion molecules E-selectin and ICAM-1 in HUVECs. GEA 3175 (10-30 μ M) inhibited LPS-induced adhesion being more potent than SNAP. The increased E-selectin expression induced by LPS was significantly attenuated by both NO donors whereas ICAM-1 expression remained unaltered.

These data show that NO-donors inhibit neutrophil adhesion to endothelial cells by suppressing both leukocyte and endothelial cell-related events involved in the adhesion process.

DISCUSSION

1. NO-releasing compounds

In the present study the effects of chemically different NO-releasing compounds were tested on inflammatory cells *in vitro*. Two recently characterized mesoionic oxatriazole derivatives GEA 3162 and GEA 3175 were compared to earlier known compounds SIN-1 and SNAP. Mesoionic oxatriazole derivatives have been shown to release NO spontaneously in buffer solutions (GEA 3162) and in the presence of living cells (GEA 3175) (Kankaanranta et al., 1996; Holm et al., 1998). As a sign of NO-production in our experimental conditions mesoionic oxatriazole derivatives as well as reference compounds rapidly increased the levels of cGMP in neutrophils and lymphocytes. Addition of red blood cells into the cell culture reversed the effects of NO-donors on cGMP production, lymphocyte proliferation and prostacyclin production confirming that the effects were due to NO released from the compounds. Hemoglobin inhibits the action of NO by binding it to form nitrosylhemoglobin or by metabolising it to inactive nitrate (Murphy and Noack, 1994). Therefore we suggest that the present effects of NO-donors on inflammatory cells *in vitro* are NO-dependent.

The kinetics of NO-release from GEA compounds has been previously studied by following the formation of nitrite and nitrate in buffer solutions and by measuring E.P.R. signals of nitrosylhemoglobin of venous blood treated with these NO-donors (Kankaanranta et al., 1996). In venous blood, GEA 3162 and GEA 3175 gave rise to a nitrosylhemoglobin-like E.P.R. signal in minutes. During a 2 h follow-up time the amplitude of this signal was not markedly increased suggesting a rapid release of NO. When GEA 3162 was incubated in buffer solution, the concentration of nitrite + nitrate detected after 40 min incubation was 50 % of the original concentration of the NO-donor (Kankaanranta et al., 1996). In the present study the kinetics of the increased production of cGMP in human mononuclear cells was studied (I, II). In human mononuclear cells cGMP levels induced by GEA 3162 were higher after 30 min than after 2 h incubation, whereas cGMP levels after GEA 3175 and SNAP continued to increase, when the incubation time was prolonged from 30 min to 2 h. When the NO-donors were incubated with mononuclear cells for 24 h, cGMP levels comparable to the pre-treatment values were found. These results are in agreement with the previous results where the kinetics of the increased production of cGMP was measured in human neutrophils (Moilanen et al., 1993). When the neutrophils were incubated with GEA 3162, cGMP levels peaked during 10 min incubations and reduced thereafter whereas cGMP levels in cells treated with SNAP continued to increase during the 90 min follow-up. These findings support the idea that the release of NO from these two GEA compounds is rapid in onset and peaks in the early phases of the incubation, GEA 3162 being more rapid NO-releaser than GEA 3175.

In the present study SIN-1 and SNAP were used as reference compounds. SIN-1 is known to produce both NO and superoxide anion, which may result qualitatively different results with SIN-1 and GEA compounds (discussed below). In our previous studies SNAP has been shown to be a potent NO donor without concomitant superoxide release (Holm et al., 1998). The kinetics of NO production from SNAP appeared to be somewhat different in buffer solution and in the presence of cells. In buffer solution NO release from SNAP was rapid, it peaked at 15 min and decreased thereafter (Kankaanranta et al., 1996; Holm et al., 1998). As seen in the present study cGMP measurements in lymphocytes and neutrophils suggest slower NO release (Moilanen et al., 1993; Kosonen et al., 1997). These results indicate somewhat different mechanisms of NO release in buffer solution and in the presence of living cells, both in the case of GEA compounds and SNAP.

As measured by Trypan blue staining and lactate dehydrogenase release none of the NO-donors decreased cell viability when used in the indicated drug concentrations. NO donors did neither alter the morphology of endothelial cells when studied under microscope. Furthermore, the inhibition of lymphocyte proliferation was antagonisable: when mitogen concentrations were increased, the antiproliferative action of NO-donors was reversed. Increased production of cGMP in lymphocytes and neutrophils also suggest that NO-donors did not decrease cell viability. In addition, further studies in our laboratory have shown that NO-donors in concentrations used in the present experiments do not induce apoptosis in lymphocytes (Kankaanranta H, unpublished results). In conclusion, the effects of NO-donors were regulatory rather than cytotoxic and the compounds proved to be useful tools in *in vitro* studies.

2. Antiproliferative effects of NO donors in human lymphocytes

NO-releasing compounds inhibited lymphocyte proliferation in NO-dependent and antagonisable manner (I). These data are consistent with the earlier findings that NO produced by activated macrophages inhibit T-lymphocyte proliferation *in vitro* (Hoffman et al., 1990; Albina et al., 1991) and in some experimentally induced infections *in vivo* (Sternberg and McGuigan, 1992; Schleifer and Mansfield, 1993). Increased synthesis of cGMP mediates the vasodilatory, antiaggregatory and various other actions of NO. In vascular smooth muscle cells NO has been reported to inhibit cell proliferation by a cGMP-dependent (Etienne et al., 1996; Yu et al., 1997) and independent (Lahteenmaki et al., 1998) mechanisms. In the present study, we tested the mediator role of cGMP in the antiproliferative action of NO donors in ConA-stimulated lymphocytes and found that the characteristics originally presented by Sutherland et al. (Sutherland et al., 1968) for a second messenger could not be demonstrated (II). Our data suggest that NO donors inhibit lymphocyte proliferation in a cGMP-independent manner. In addition to guanylate cyclase NO has direct effects on various other enzymes. NO inhibits iron containing enzymes including aconitase in Krebs cycle, complexes I and II of the mitochondrial respiratory chain (Drapier and Hibbs,

1988; Stadler et al., 1991) and ribonucleotide reductase, the rate-limiting enzyme in DNA synthesis (Lepoivre et al., 1990; Kwon et al., 1991). These effects might well be involved in the antiproliferative action of NO.

Induction of apoptosis appears to be an important mechanism in the regulation of clonal expansion of T-cells (Kolb and Kolb-Bachofen, 1998). At low concentrations, NO has been reported to protect cells from apoptosis. Higher rates of NO production can overwhelm cellular protective mechanisms and shift the balance toward apoptotic death in some cell types (Kim et al., 1999). Thus, it is possible that NO regulates the T_H1/T_H2 balance by promoting or suppressing apoptosis at high/low doses (Kolb and Kolb-Bachofen, 1998). However, recent yet unpublished studies in our laboratory showed that the inhibition of lymphocyte proliferation by NO-releasing compounds could not be explained by enhanced rate of apoptosis (Kankaanranta H, unpublished results).

In murine lymphocytes, exposure to NO suppresses IL-2 gene expression and consequently, NO modulates the T_H1/T_H2 balance by favouring T_H2 responses (Taylor-Robinson, 1997). Our preliminary results show, that NO-releasing compounds inhibit IL-2 production in human lymphocytes which is consistent with the data in communications I and II on NO-induced antiproliferative action on mitogen-stimulated human lymphocytes (Kosonen O, unpublished results).

3. NO donors and cyclooxygenase

In the present study NO-releasing compounds inhibited prostacyclin synthesis in human endothelial cells exposed to a proinflammatory agent LPS (III). Our results on the inhibitory action of NO on inducible eicosanoid synthesis are supported by the findings of Stadler et al. (1993), Swierkosz et al. (1995) and Amin et al. (1997). These articles report that NO inhibits prostanoid production by macrophages (Swierkosz et al 1995), Kupffer cells (Stadler et al., 1993) or chondrocytes (Amin et al., 1997) activated by inflammatory stimuli to express COX-2 activity.

To study the possible effect of NO donors on phospholipases, exogenous arachidonic acid was added into the culture. Phospholipases proved to be an unlike target of the inhibitory action of NO-donors because addition of exogenous arachidonic acid did not reverse the response.

The experimental conditions in this study measured prostacyclin production principally by COX-2 as evidenced by Western blot analysis and the effect of a COX-2 specific inhibitor. Our results suggest that NO donors inhibit COX-2 activity but not COX-2 expression in LPS stimulated endothelial cells. In a recent report, NO was found to inhibit also COX-2 protein expression in murine macrophages (Habib et al., 1997). We studied the effects of NO donors on COX-2 expression in HUVECs by Western blot analysis and did not find any effect on COX-2 protein levels although PGI₂ synthesis was suppressed significantly.

These differences may be explained by different mechanisms regulating induction of COX-2 gene or stability of COX-2 mRNA between various cells and species.

In contrast to our data, some studies in the literature show that NO enhances cyclooxygenase activity in certain cell types (Salvemini et al., 1993; Corbett et al., 1993; Hajjar et al., 1995). This controversy may be due to tissue-specific expression and regulation of NOS and COX in various cell types, as well as the influence of other auto- or paracrine mediators in the microenvironment. An example of cell-cell variation has been illustrated by studies of Janabi et al. (Janabi et al., 1996) in which endogenous NO activated PGF_{2α} production in human microglial cells but not in astrocytes. Also the concentration of NO has a complex (direct or indirect) biphasic effect on prostaglandin production (Goodwin et al., 1999; Patel et al., 1999). Based on a careful study on the experimental conditions used in these studies it is tempting to speculate that the contradictory results on the effects of NO on eicosanoid synthesis could be explained by a different action of NO on COX-1 and COX-2 isoenzymes either directly or indirectly. Davidge et al. (Davidge et al., 1995) reported that endogenous NO increased eicosanoid production in bovine endothelial cells in response to calcium ionophore A23187 in culture conditions where Western blot analysis did not show any sign of induction of COX-2. In the study of Salvemini et al. (Salvemini et al., 1996) nitrovasodilators caused increased prostacyclin production in bovine aortic endothelial cells when incubated in arachidonic acid-supplemented Hank's balanced salt solution in the absence of serum and other factors known to induce COX-2. A direct NO-mediated activation of COX-1 has been shown after S-nitrosation of cysteine residues in the catalytic domain of the enzyme (Hajjar et al., 1995). Based on these studies NO seems to activate COX-1 isoenzyme and the inhibitory effects of NO are related to COX-2 isoenzyme.

6-keto-PGF_{1α} is often used as an indicator of cyclooxygenase activity. This prostaglandin is a breakdown product of prostacyclin, which in turn is synthesized from arachidonic acid by a sequence action of cyclooxygenase and prostacyclin synthase. Based on the present experiments it is not possible to rule out the effect of NO on prostacyclin synthase. Indeed, it has been reported that dependent on a local NO concentration, this mediator may either activate or inhibit the enzyme prostacyclin synthase (Wade and Fitzpatrick, 1997). Recently, peroxynitrate was shown to nitrate and inactivate prostacyclin synthase (Zou and Bachschmid, 1999).

LPS induces expression of iNOS and production of high amounts of NO in several rodent cell types whereas in human cells additional stimuli are usually needed (Mossalayi et al., 1994; Vouldoukis et al., 1995; Moilanen et al., 1999). Expression of iNOS in freshly isolated human umbilical vein endothelial cells treated with a mixture of cytokines TNF-α, IL-1β and LPS was recently demonstrated (Orpana et al., 1997; Ranta et al., 1998). However, cultured human umbilical vein endothelial cells do not express iNOS when exposed to

proinflammatory stimuli (Rosenkranz-Weiss et al., 1994). This may imply that endothelial cells that are conventionally cultured for several passages have lost their ability to express iNOS, whereas freshly isolated cells maintain this capacity. In the present study, no measurable nitrite production as a marker of NO synthesis was found. L-NMMA and L-NIO, inhibitors of NOS, failed to alter prostacyclin synthesis in endothelial cells. These results suggest that in human umbilical vein endothelial cells cultured for 2 passages LPS induces low, if any, NO production and that endogenous NO does not regulate prostacyclin production. This does not exclude the possibility that endogenous NO might regulate neutrophil adhesion in human endothelium *in vivo*. A link between NO and the regulation of eicosanoid synthesis could represent an important mechanism in controlling vascular and inflammatory responses in pathophysiological states and during treatment with nitrovasodilators.

4. Antiadhesive effects of NO donors

In the present studies (IV, V) we found that chemically unrelated NO donors inhibited neutrophil adhesion to endothelial cells. This is consistent with the earlier reports showing that inhibition of NO synthesis *in vivo* with analogues of L-arginine enhances cell adhesion in the microcirculation (Kubes et al., 1991; Arndt et al., 1993; Kurose et al., 1993) and that NO-releasing compounds have therapeutic potential in preventing neutrophil infiltration in e.g. ischemia-reperfusion injury (Fukuda et al., 1995; Liu et al., 1998). The detailed mechanisms involved in the inhibitory action of NO on the leukocyte adhesion are not known. The significance of the inactivation of superoxide anion by NO and suppression of mast cell functions has been suggested (Kubes et al., 1993; Gaboury et al., 1993). More recently, inhibition of certain adhesion molecules in endothelial cells has been described. In the present study, we tested the effects of novel NO donors on neutrophil adhesion to endothelial cells *in vitro* in two different models to find out the mechanisms involved in the antiadhesion effects of NO-donors. In the first model the effects of NO-donors on the adhesion of TNF- α -stimulated polymorphonuclear leukocytes were studied (IV). In the second model human umbilical vein endothelial cells were stimulated by LPS to induce adhesion molecule expression and the effects of NO donors on adhesion molecule expression in and leukocyte adhesion to LPS-stimulated endothelial cells were investigated (V).

In the first model neutrophils rather than endothelial cells seemed to be the target of the effect of NO. Our results demonstrated that NO-releasing compounds inhibited TNF- α -induced polymorphonuclear leukocyte adhesion to endothelial cells (IV). Leukocyte adhesion induced by proinflammatory mediators such as cytokines and chemoattractants is primarily mediated via leukocyte β 2-integrins (Carlos and Harlan, 1994). β 2-integrin expression in neutrophils is increased by a variety of agonists: phorbol esters, FMLP, leukotriene B₄ and TNF- α (Carlos and Harlan, 1994). Kubes et al. (1991), Niu et al. (1994) and Mitchell et al. (1998) reported that antibodies directed against β 2-integrin CD18 prevented the

leukocyte adhesion enhanced by nitric oxide synthase (NOS)-inhibitors. This suggests that NO mediates its antiadhesive effect through the leukocyte adhesion molecule CD11/CD18. In our study stimulation of neutrophils with TNF- α resulted in an increase in surface expression of CD11/CD18 confirming the earlier findings (Gamble et al., 1985; Lo et al., 1989). NO donors did not suppress TNF- α -induced surface expression of CD11/CD18. Both quantitative and qualitative changes occur in β 2-integrins after cell activation (Arnaout, 1990). However, it has been demonstrated that upregulation of CD11/CD18 surface expression is neither necessary nor sufficient for the stimulated neutrophils to adhere to cultured endothelial cells (Vedder and Harlan, 1988; Philips et al., 1988; Schleiffenbaum et al., 1989) and that qualitative changes in adhesion molecule avidity play a more critical role in regulation of β 2-integrin function (Carlos and Harlan, 1994). The present results show that NO donors do not alter CD11/CD18 surface expression in TNF- α -stimulated polymorphonuclear leukocytes but they may cause changes in the β 2-integrin avidity. In addition, S-nitrosation of the critical SH groups on the surface of polymorphonuclear leukocytes could decrease adhesion and leukocyte infiltration (Grisham et al., 1999).

The data presented in our study also indicate a role for cGMP in the modulation of neutrophil adhesion by NO donors. The concentration-response curves of the cGMP-enhancing effect by various NO donors correlated with their inhibitory action of neutrophil adhesion. Two analogues of cGMP, 8-p-chlorophenylthio-cGMP and 8-bromo-cGMP, inhibited neutrophil adhesion. 8-p-chlorophenylthio-cGMP caused the effect at lower concentrations than 8-bromo-cGMP probably because it penetrates well into the cells and is a poor substrate for cGMP-degrading phosphodiesterases (Butt et al., 1992). These results suggest that the inhibitory action of NO donors on neutrophil adhesion might be a cGMP-mediated process. That assumption is consistent with the studies of Kurose et al. (Kurose et al., 1993) and Davenpeck et al. (Davenpeck et al., 1994) who found that analogues of cGMP prevented leukocyte influx elicited by NOS-inhibitor L-NAME.

In this first adhesion model (IV) SIN-1 was used as a reference compound. Unlike GEA 3162 and SNAP (Holm et al., 1998) SIN-1 is known to produce both NO and superoxide anion, and the reaction between these two molecules results in the formation of peroxynitrite (Feelisch, 1991; Hogg et al., 1992). The reaction between NO and superoxide anion may be regarded as an inactivation route of these two reactive molecules (Gryglewski et al., 1986) and could explain the lower potency of SIN-1 found in the present experiment. On the other hand, the product formed in this reaction, i.e., peroxynitrite, is an active oxidant and nitrating agent, which may be responsible for some of the effects of SIN-1 (Crow and Beckman, 1995). Activated neutrophils produce superoxide anion, and studies in our laboratory have been recently shown that when activated but not resting neutrophils are exposed to a NO donor, detectable amounts of peroxynitrite are formed (Holm et al., 1999). TNF- α has been shown to activate

neutrophils to produce superoxide anion (Tsujimoto et al., 1986; Menegazzi et al., 1994). Therefore, peroxynitrite may be formed in these culture conditions after addition of either SIN-1 or GEA compounds and peroxynitrite may be implicated in the actions of NO donors.

In the second model of leukocyte/endothelial cell adhesion (V), by selectively exposing cultured endothelial cells to NO donors, we identified adhesion molecule expression in endothelial cells as a potential target of NO. NO-releasing compounds inhibited E-selectin expression in and neutrophil adhesion to LPS-stimulated endothelial cells (V).

In human umbilical vein endothelial cells LPS induces the expression of adhesion molecules E-selectin and ICAM-1, which mediate neutrophil adhesion. E-selectin is induced on the transcriptional level, and maximal levels of E-selectin protein are expressed at the cell surface within 4 h after stimulation (Bevilacqua et al., 1987; Vestweber and Blanks, 1999). ICAM-1 is constitutively expressed in human endothelial cells and the expression is increased by LPS (Dustin and Springer, 1988; Lee et al., 1995). In our study stimulation of human umbilical vein endothelial cells for 6 h with LPS increased surface expression of both E-selectin and ICAM-1. We found that GEA 3175 and SNAP suppressed LPS-induced expression of E-selectin in endothelial cells. Others (De Caterina et al., 1995; Spiecker et al., 1998) have reported that NO donors inhibit also IL-1 α - and TNF- α -induced E-selectin expression in human saphenous vein endothelial cells. These results suggest that NO reduces E-selectin-mediated adhesion process in a stimulus-independent manner. In the present study NO-releasing compounds did not alter LPS-induced ICAM-1 expression confirming the studies of Biffl *et al.* (Biffl et al., 1996) and Tsao *et al.* (Tsao et al., 1996) who studied LPS-stimulated human umbilical vein endothelial cells and human aortic endothelial cells. In addition, NO donors have been reported to inhibit ICAM-1 expression in human endothelial cells after stimulation with cytokines IL-1 β and TNF- α (Takahashi et al., 1996; Spiecker et al., 1998). The present data show that LPS-induced ICAM-1 expression in human vascular endothelial cells is resistant to NO suggesting that NO regulates ICAM-1 expression in a stimulus dependent manner. This may well be the case as LPS-induced ICAM-1 expression was not inhibited by IL-1 or TNF- α antibodies indicating that it was not an autocrine effect mediated by the LPS-induced IL-1 or TNF- α (Lee et al., 1995). Accordingly, dexamethasone has been shown to regulate ICAM-1 expression in a stimulus-dependent manner (Burke-Gaffney and Hellewell, 1996).

Expression of E-selectin on coronary endothelium after myocardial ischemia reperfusion has been described (Shen and Verrier, 1994). Therefore the present findings on the inhibitory action of NO donors on E-selectin expression in endothelial cells may be implicated not only in inflammation but also in the mechanisms of the protective action of NO donors in ischemia-reperfusion injury. In conclusion, these data show that NO-donors inhibit neutrophil adhesion

to endothelial cells by suppressing both leukocyte and endothelial cell-related events involved in the adhesion process.

5. Conclusion

The present study demonstrates that NO-releasing compounds have NO-dependent anti-inflammatory effects on inflammatory cells *in vitro*. The results show that NO-releasing compounds (1) inhibit human lymphocyte proliferation by a cGMP-independent mechanism, (2) inhibit LPS-induced production of prostacyclin in human endothelial cells probably by inhibiting COX-2 activity and (3) inhibit neutrophil adhesion to endothelial cells by suppressing both leukocyte and endothelial cell-related events involved in the adhesion cascade. These results indicate that NO serves as a regulatory molecule in inflammation and has anti-inflammatory actions. This hypothesis is supported by data obtained in *in vivo* experiments. Animal studies have shown that inhibition of NO synthesis *in vivo* with analogues of L-arginine enhances cell adhesion in the microcirculation (Kubes et al., 1991; Arndt et al., 1993; Kurose et al., 1993) whereas NO-releasing compounds have therapeutic potential in preventing neutrophil infiltration in e.g. ischemia-reperfusion injury (Fukuda et al., 1995; Liu et al., 1998). NO produced by activated macrophages inhibit T-lymphocyte proliferation in some experimentally induced infections *in vivo* (Sternberg and McGuigan, 1992; Schleifer and Mansfield, 1993). In iNOS deficient mice LPS-induced leukocyte adhesion to endothelium is increased and wound healing is impaired. Chronic rejection of allografts is more severe in iNOS deficient mice than in wild types, apparently by inhibition of inflammatory cell accumulation and neointimal smooth muscle cell proliferation and the development of graft arteriosclerosis (Nathan, 1997).

NO, like many other inflammatory mediators, seems to have a dual regulatory function in inflammation. Expression of iNOS is associated with widespread tissue injury and inflammation and the use of selective iNOS inhibitors has a promise in the treatment of certain inflammatory diseases (Hobbs et al., 1999) whereas in some situations it is desirable to mimic or enhance physiological generation of NO. When used in ischemic heart disease anti-inflammatory actions may mediate some of the beneficial effects of NO donors. On the other hand, anti-inflammatory and immunosuppressive actions may appear as side effects of NO-releasing compounds. Thus, the immunosuppressive actions of NO donors offer therapeutic possibilities and may lead to adverse effects when these drugs are used in other indications.

SUMMARY AND CONCLUSIONS

The present studies were designed to investigate the effects of NO-releasing compounds on leukocyte and endothelial cell functions related to inflammatory response *in vitro* and to elucidate the mechanisms of their anti-inflammatory actions. The two recently characterized NO-releasing mesoionic oxatriazole derivatives GEA 3162 and GEA 3175 were compared to earlier known NO donors 3-morpholino-sydnominine (SIN-1) and S-nitroso-*N*-acetylpenicillamine (SNAP).

The major findings were:

1. NO-releasing compounds inhibited mitogen-induced proliferation of human lymphocytes by a cGMP-independent mechanism.
2. NO-releasing compounds inhibited LPS-induced production of prostacyclin in human endothelial cells. The data suggest that NO-releasing compounds inhibit cyclooxygenase-2 (COX-2) enzyme activity but not COX-2 protein expression.
3. NO-releasing compounds inhibited the adhesion of TNF- α -stimulated neutrophils to endothelial cells. NO donors did not alter CD11/C18 surface expression in TNF- α -stimulated neutrophils. The data indicate a role for cGMP in the modulation of neutrophil adhesion by NO donors.
4. By selectively exposing cultured endothelial cells to NO donors, we have identified adhesion molecule expression in endothelial cells as a potential target of NO. NO-releasing compounds inhibited E-selectin expression in and neutrophil adhesion to LPS-stimulated endothelial cells.

In conclusion, chemically different NO-releasing compounds were shown to have NO-dependent anti-inflammatory effects in various models *in vitro* indicating that NO serves as a regulatory molecule in inflammation. The immunosuppressive action of NO-releasing compounds offers therapeutic possibilities and may lead to adverse effects when NO-releasing compounds are used in other indications.

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