PÄIVI HOLM

# Nitric Oxide and Peroxynitrite Production by NO-donors and Inflammatory Cells

*University of Tampere*
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ACADEMIC DISSERTATION
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ACADEMIC DISSERTATION

To be presented, with the permission of
the Faculty of Medicine of the University of Tampere,
for public discussion in the small auditorium of Building B,
Medical School of the University of Tampere,
Medisiinarinkatu 3, Tampere, on October 21st, 2000, at 12 o'clock.

*University of Tampere*
*Tampere 2000*

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## LIST OF ORIGINAL COMMUNICATIONS

This thesis is based on the following original communictions.

I. H. Kankaanranta, E. Rydell, A.-S. Petersson, P. Holm, E. Moilanen, T. Corell, G. Karup, P. Vuorinen, S.B. Pedersen, Å. Wennmalm, T. Metsä-Ketelä: Nitric oxide-donating properties of mesoionic 3-aryl substituted oxatriazole-5-imine derivatives. Br J Pharmacol 117:401-406, 1996.

II. P. Holm, H. Kankaanranta, T. Metsä-Ketelä, E. Moilanen: Radical releasing properties of nitric oxide-donors GEA 3162, SIN-1 and SNAP. Eur J Pharm 346: 97-102, 1998.

III. P. Holm, H. Kankaanranta, S.S. Oja, R.G. Knowles, E. Moilanen: No detectable NO synthesis from L-arginine or $N^G$-hydroxy-L-arginine in fMLP-stimulated human blood neutrophils despite production of nitrite, nitrate and citrulline from $N^G$-hydroxy-L-arginine. J Leuk Biol 66:127-134, 1999.

IV. P. Holm, M. Lähde, H. Kankaanranta, T. Moilanen, N. Al-Saffar, S.S. Oja, T. Tuomiranta, R. Peltomaa, M. Leirisalo-Repo, E. Moilanen: Enhanced nitric oxide production in synovitis. Cellular sources and relation to synovial fluid cytokine concentration (submitted).

## ABBREVIATIONS

| | |
|---|---|
| ADP | adenosine 5’ -diphosphate |
| cGMP | cyclic guanosine 3’:5’ -monophosphate |
| COX | cyclooxygenase |
| EDRF | endothelium –derived relaxing factor |
| ELISA | enzyme linked immunosorbent assay |
| eNOS | endothelial nitric oxide synthase |
| ESR | electron spin resonance |
| FMLP | n-formyl-L-methionyl-L-leucyl-L-phenylalanine |
| 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 |
| $H_2O_2$ | hydrogen peroxide |
| HPLC | high pressure liquid chromatography |
| IBMX | 3-isobuthyl-1-methylxanthine |
| IFN-γ | interferon-γ |
| IL | interleukin |
| iNOS | inducible nitric oxide synthase |
| LDL | low density lipoprotein |
| LPS | lipopolysaccaride |
| metHb | methemoglobin |
| NFκB | nuclear factor κB |
| nNOS | neuronal nitric oxide synthase |
| L-NAME | $N^G$-nitro-L-arginine methyl ester |
| L-NIO | N-iminoethyl-L-ornithine |
| L-NMMA | $N^G$-monomethyl-L-arginine |
| NO | nitric oxide |
| NOS | nitric oxide synthase |
| $NO_x$ | nitrogen oxides |
| $O_2^{\cdot -}$ | superoxide anion |
| $O_3$ | ozone |
| OH-L-Arg | $N^G$-hydroxy-L-arginine |
| $ONOO^-$ | peroxynitrite |
| oxyHb | oxyhemoglobin |
| PRP | platelet rich plasma |
| RA | rheumatoid arthritis |
| RIA | radioimmunoassay |
| RT-PCR | reverse transcriptase polymerace chain reaction |
| SF | synovial fluid |
| SIN-1 | 3-morpholino-sydnonimine |
| SNAP | S-nitroso-*N*-acetylpenicillamine |
| SOD | superoxide dismutase |
| TNF-α | tumor necrosis factor-α |

## 1. INTRODUCTION

In 1980 Furchgott and Zawadzki described the endothelium-derived relaxing factor (EDRF) (Furchgott and Zawadzki, 1980). The term EDRF was coined to account for the finding that upon stimulation with acetylcholine the endothelium releases a relaxing factor. In 1987 pharmacological and chemical evidence indicated that EDRF is identical with or closely related to nitric oxide (NO), a free radical gas, synthesized from L-arginine (Ignarro et al., 1987; Palmer et al., 1987). At the same time it was found that the EDRF/NO released by endothelial cells is a potent inhibitor of platelet aggregation and adhesion to the vascular wall. This effect was found to be mediated by stimulation of soluble guanylate cyclase in the platelets (Busse et al., 1987; Radomski et al., 1987; Alheid et al., 1987). In parellel with the discovery of endothelial NO production, it was observed that murine macrophages stimulated with lipopolysaccharide (LPS) and cytokines produced large amounts of nitrogen oxides ($NO_x$), which were involved in the destruction of microbes and tumor cells (Stuehr and Marletta, 1985; Nathan and Hibbs, Jr., 1991). One token of the biological significance of this novel signal molecule is its selection by the journal Science as 'The Molecule of the Year' in 1992 (Koshland, Jr., 1992). In 1998, Furchgott, Ignarro and Murad were awarded the Nobel Prize 'for their discoveries concerning nitric oxide as a signalling molecule in the cardiovascular system'.

The NO synthase (NOS) family of enzymes generate NO from L-arginine, which acts as an effector molecule in various physiological and pathophysiological processes. The known biological functions of NO include vasodilatation, inhibition of platelet aggregation, neurotransmission and regulation of immune response (Moncada and Higgs, 1995; Moilanen et al., 1999). Three NOS isoforms have evolved to function in animals. The constitutively expressed enzymes are neuronal NOS (nNOS) and endothelial NOS (eNOS) (Knowles and Moncada, 1994). An inducible NOS (iNOS) is synthesized in response to inflammatory or proinflammatory mediators. Expression of iNOS may be benefical in host defence or in modulating the immune response, but its

expression is also linked to a number of inflammatory diseases such as asthma and rheumatoid arthritis. Selective pharmacological control of the NOSs should help to clarify their significance in human health and disease (Stuehr, 1999).

NO reacts rapidly with superoxide anion ($O_2^{\cdot -}$) to form peroxynitrite. This is not a free radical but a short-lived and far more reactive molecule than its precursors (Beckman et al., 1990). It reacts with and damages many important biological molecules including thiols, lipids, proteins and nucleic acids by a number of mechanisms (Pryor and Squadrito, 1995; Murphy et al., 1998).

Even after more than a century of therapeutic use the exact mode of action of organic nitrates remained a mystery. It is now clear that most of their biological activity is related to the formation of NO. NO-donors are being applied as pharmacological tools in studying biological effects mediated by NO (Feelisch, 1998), and NO-donors are believed to have therapeutic potential in a range of cardiovascular and other diseases (Moncada and Higgs, 1995). In the present study we characterised NO- and peroxynitrite–releasing properties of 3-aryl substituted oxatriazole-5-imine derivatives, a novel group of NO-donors.

Expression of iNOS is very differently regulated in human inflammatory cells than in murine cells. NO is a well-documented effector molecule in rodent phagocytes, whereas its synthesis in human neutrophils and other inflammatory cells has been controversial (Moilanen et al., 1999). However, NO overproduction seems to be important in the pathogenesis of various inflammatory diseases, including arthritis. In the present study we investigated NO production in activated human neutrophils and in inflamed joints.

## 2. NITRIC OXIDE (NO)

The enzymes responsible for the synthesis of NO from L-arginine in mammalian tissues are known as NO synthases. NO synthesis from L-arginine involves two separate steps. At the first, one molecule of L-arginine is oxidized to produce $N^G$-hydroxy-L-arginine (OH-L-Arg) as an intermediate product. This reaction requires NADPH and $O_2$, which are converted to $NADP^+$ and $H_2O$. At the second step, OH-L-Arg is further oxidized to yield one molecule of NO and L-citrulline each. Two molecules of $O_2$ and one molecule of NADPH are converted to two molecules of $H_2O$ and one molecule of $NADP^+$ (Fig. 1). The detailed chemical mechanisms involved in these reactions are as yet unknown (Stuehr et al., 1991; Knowles, 1997; Stuehr, 1999).

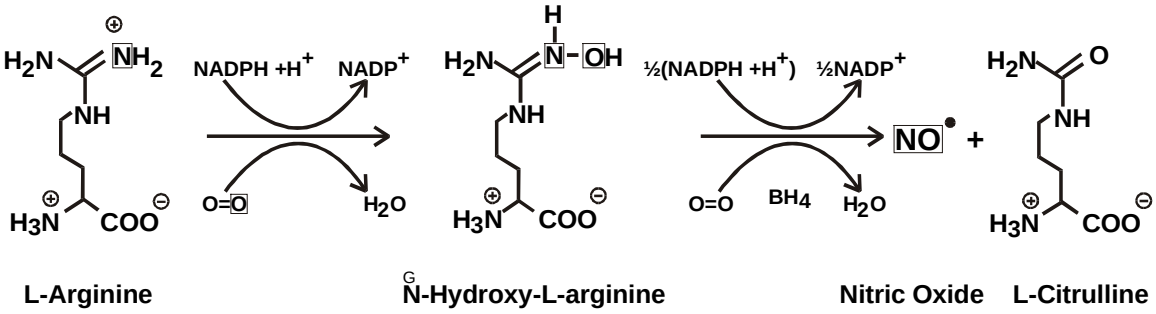


Figure 1. Biosynthetic pathway for production of NO from L-arginine in mammals.

### *2.1. Isoenzymes of nitric oxide synthase (NOS)*

The mammalian systems in which NO synthesis was first demonstrated were the vascular endothelium (Ignarro et al., 1987; Palmer et al., 1987), the brain (Garthwaite et al., 1988) and activated macrophages (Marletta et al., 1988). NO synthases evince homology with the cytochrome $P_{450}$ –type enzymic group. Three different NO synthase isoenzymes have been characterised. For historical reasons, these enzymes are named constitutively expressed nNOS, originally identified in rat neurons (also known as type I NOS); constitutively expressed eNOS, originally identified in bovine endothelial cells

(also known as type III NOS); and iNOS, which was originally identified as being inducible by cytokines in rodent macrophages and hepatocytes (also known as type II NOS) (Knowles, 1997). The first isoenzyme to be purified and cloned was the rat nNOS (Bredt et al., 1991). This enzyme is $Ca^{2+}$- and calmodulin-dependent and is constitutively expressed in the brain. The cDNA encoding the human nNOS has been cloned, and its mRNA shown to be expressed at high levels in the human brain and also in skeletal muscle (Nakane et al., 1993) as well as in the airway epithelium (Asano et al., 1994). The constitutively expressed NO synthase in vascular endothelial cells (eNOS) appears to be functionally close to nNOS and distinct from iNOS. In addition, eNOS is $Ca^{2+}$- and calmodulin-dependent. The molecular mass of purified eNOS (135 kDa) is lower than that of nNOS (155 kDa) (Bredt and Snyder, 1990; Pollock et al., 1991). Despite the very similar function, only 57 % of the amino acid sequence is identical between the two human constitutive NOSs. Rodent macrophages have been the source of the most extensively studied inducible $Ca^{2+}$ -independent NO synthase (iNOS). Murine macrophage iNOS has a molecular weight of 130 kDa and shares only 51 and 54 % sequence identity with eNOS and nNOS, respectively (Yui et al., 1991; Stuehr et al., 1991a). NOS proteins have a bidomain structure: they have oxygenase activity in an amino-terminal half and reductase activity in a carboxyl-terminal half (Stuehr, 1999).

Cells containing constitutive nNOS or eNOS produce physiological amounts of NO in response to agonists such as acetylcoline in the vascular endothelium, glutamate in the brain, or collagen in platelets. These receptor-mediated responses result in an increase in the intracellular concentrations of calcium, which is critical for activation of constitutive NOS. NO produced by constitutively expressed NOSs regulates physiological functions such as vascular tone, platelet aggregation, immune response and neurotransmission (Moncada and Higgs, 1995; Moilanen et al., 1999). INOS is expressed after exposure to stimuli such as inflammatory cytokines e.g.interleukin–1 (IL-1) tumor necrosis factor-$\alpha$ (TNF-$\alpha$) interferon-$\gamma$ (IFN-$\gamma$) and bacterial endotoxins. Once stimulated, iNOS, which

can be activated in many different cell types, may generate up to 1000 times more NO than constitutively expressed eNOS and nNOS (MacMicking et al., 1997; Clancy et al., 1998; Amin et al., 1999). NO synthesised in high amounts by activated magrophages possesses cytotoxic properties implicated in the ability of these cells to kill bacteria, viruses, protozoa and tumor cells. Although this mechanism is important in host defence, it is also proinflammatory and destructive, and is involved in the pathogenesis of various inflammatory and other pathological conditions (Grisham et al., 1999; Moilanen et al., 1999).

The chromosomal location of the nNOS, eNOS and iNOS genes has been determined. The nNOS gene has been localized to human chromosome 12 (Kishimoto et al., 1992; Xu et al., 1993). The eNOS gene maps to chromosome 7 and iNOS gene to chromosome 17 (Xu et al., 1994). The NO synthase genes therefore represent a dispersed gene family on three different chromosomes. NOS synthase has a sequence close to that of cytochrome $P_{450}$ reductase, and both enzymes have a heme centre with the same kind of spectral properties (Bredt et al., 1991; Klatt et al., 1992; White and Marletta, 1992; Stuehr and Ikeda-Saito, 1992b).

### *2.2. Cofactors of NOS*

NO synthesis requires as cofactors heme, tetrahydrobiopterin ($BH_4$), flavin mononucleotide (FMN), flavin adenine dinucleotide (FAD) and the presence of calmodulin (CaM) (MacMicking et al., 1997). Mammalian NOSs are functionally active only in the dimeric form. $BH_4$ is a critical cofactor, since it is needed for the dimerization of iNOS (Stuehr, 1999). Mouse iNOS residues Gly450 and Ala453 are critical for the binding of $BH_4$, since mutation of these residues has been shown to prevent the dimerization (Cho et al., 1995). In $BH_4$-deficient cells, iNOS protein is expressed in an inactive monomeric form, which is converted to the active, dimeric form in the presence of $BH_4$ (Tzeng et al., 1995).

All three isoforms of NOS require CaM as a cofactor. CaM, which binds at the amino-terminal side of the reductase, is essential for the electron flow from the reductase to the oxygenase domain. An important difference between the isoenzymes is that, while in eNOS and nNOS CaM binding and activation occur in response to physiological changes in $Ca^{2+}$, iNOS contains tightly bound CaM and is fully active at the low $Ca^{2+}$ concentrations encountered *in vivo* (Cho et al., 1992; Salerno et al., 1997). Once expressed, therefore, iNOS could theoretically continue to synthesize NO indefinitely until substrate and other cofactors become limiting (MacMicking et al., 1997).

*2.3. Inhibitors of NOS*

A range of structurally related arginine analogues have been found to inhibit the NO synthases. Interest in NOS inhibitors predated the discovery of NOS itself. In 1987 Hibbs and co-workers identified that N-monomethyl-L-arginine (L-NMMA) is an inhibitor of the arginine-dependent cytotoxic response of murine macrophages before it was determined that the cytotoxic factor was NO and that the arginine-dependent enzyme was iNOS (McCall et al., 1991a). L-NMMA, the prototypic NOS inhibitor, evinces effective activity against all NOS isoforms. L-NMMA and other arginine analogues are competitive inhibitors of NOS. L-NMMA and NN-dimethyl-L-arginine (L-ADMA) are both naturally occurring compounds although their normal plasma concentrations are low. Although L-NMMA appears to be a non-selective inhibitor of NOS, some other inhibitors exhibit isoenzyme selectivity. $N^G$-nitro-L-arginine (L-NAME) shows considerable potency and selectivity toward the constitutive isoforms of NOS (Dwyer et al. 1991). L-Canavine, N-amino-L-arginine and N-iminoethyl-l-ornithine (L-NIO) inhibit the macrophage iNOS more potently than the brain nNOS and eNOS (McCall et al., 1991a; Knowles and Moncada, 1994). Also more selective inhibitors of iNOS have been described, for example L-N-iminoethyl lysine (L-NIL) (Moore et al., 1994). A recent report published by a research group from Glaxo Wellcome indicated that 1400W is the most selective iNOS inhibitor presently available (Garvey et al., 1997).

Some cofactor antagonists also bring about inhibition of NO synthesis. Aromatic iodonium compounds such as diphenyleneiodonium (DPI), which inhibits nucleotide-requiring flavoproteins, irreversibly inhibit macrophage iNOS as well as eNOS activity (Stuehr et al., 1991b). DPI competes with both NADPH and FAD for specific binding sites on a protein target and it also inhibits some other NADPH-enzymes like $O_2^{\cdot-}$-generating NADPH oxidase and mitochondrial NADPH oxidase (Knowles and Moncada, 1994). Another way to inhibit NOS is to limit the supply of one of its substrates or cofactors. Inhibitors of $BH_4$ synthesis such as 2,4, -diamino-6-hydroxypyrimide (DAPH) have been used (Werner-Felmayer et al., 1990). CaM antagonists such as trifluoperazine and W7 have been shown to inhibit NOSs (Förstermann et al., 1991), including the constitutive $Ca^{2+}$ -dependent nNOS and eNOS, and iNOS in the rat liver (Evans et al., 1992).

Cytokine–stimulated iNOS gene expression is dependent on nuclear factor kappa B (NFκB) activation. Thus, modulation of NFκB activity affects the induction of iNOS. The iNOS promoter possesses a NFκB binding site at position –76 to 85 (Xie et al., 1994). Anti-inflammatory steroids block iNOS expression in many cell types (Palmer et al., 1992) (Di Rosa et al., 1990; Radomski et al., 1990; O'Connor and Moncada, 1991; De Vera et al., 1997) possibly through the inhibition of NFκB activation resulting in decreased iNOS transcription (Kleinert et al., 1996; De Vera et al., 1997). Glucocorticoids also inhibit NO production by inhibiting $BH_4$ synthesis and L-arginine transport (Gross and Levi, 1992; Simmons et al., 1996; Saura et al., 1996). Cyclosporin A has been reported to inhibit induction of iNOS rather than its catalytic activity (Muhl et al., 1993; Hattori and Nakanishi, 1995).

*2.4. Biochemistry of NO*

Reactions of NO in biological systems depend on timing, location and the rate of its production. NO contains an unpaired electron which allows rapid reactions with other free radicals, and can form strong ligands to transition metals. NO reacts in biological systems with $O_2$, $O_2^{\cdot -}$ and transition metals. The products of these reactions e.g. peroxynitrite and metal-nitrosyl adducts have various toxic and biological effects (Wink and Mitchell, 1998). Such reactive nitrogen oxide species are produced when fluxes of NO are enhanced. These types of reactions may predominate during active inflammation. *In vivo*, the formation of these reaction products of NO is kept low by the rapid removal of NO through reactions with oxyHb in red blood cells and by scavenging of $O_2^{\cdot -}$ by superoxide dismutase (SOD) (Gryglewski et al., 1986; Beckman and Koppenol, 1996). NO interacts directly with physiological targets and this is thought to occur under conditions where the rates of NO production are low. These types of reactions may serve regulatory and / or anti-inflammatory functions (Grisham et al., 1999).

The gas-phase auto-oxidation of NO has long been of interest by reason of the importance of NO as a component in air pollution. The reaction product is nitrogen dioxide ($NO_2$), one substance associated with the brown haze in smog (Schwartz and White, 1983).

$$2\ NO + O_2 \rightarrow 2\ NO_2$$
$$NO_2 + NO \rightarrow N_2O_3$$

Free $NO_2$ formed in the gas phase reaction is not formed in aqueous media. $N_2O_3$ formed undergoes hydrolysis in an aqueous solution to form nitrite, as shown below.

$$N_2O_3 + H_2O \rightarrow 2\ NO_2^-$$

### *2.4.1.Reactions with metal-containing proteins*

The reaction of NO with metals to form nitrosyl complexes occurs *in vivo* with iron-containing proteins. The most facile reaction of NO to form stable nitrosyl adducts is with proteins which contain a heme moiety. Most notable here is the reaction of NO with guanylate cyclase, which stimulates the formation of cyclic guanosine 3´:5´ - monophosphate (cGMP) from guanosine 5´ triphosphate (GTP). Synthesis of cGMP has a number of effects, among them regulation of vascular tone and inhibition of platelet aggregation (Ignarro, 1990; Moncada et al., 1991; Grisham et al., 1999). NO can also inhibit other metalloproteins such as NOS, cytochrome oxidase, catalase (Wink and Mitchell, 1998) and modulate cyclooxygenase (COX) activity (Salvemini et al., 1993; Kosonen et al., 1998; Grisham et al., 1999).

### *2.4.2. Reactions with thiols*

S-nitrosothiol adducts are formed during the autooxidation of NO via interaction between $N_2O_3$ and certain thiols. Nitrosation of thiols in proteins may have a variety of effects. Nitrosothiols have been suggested to act as intermediates in the action of various nitrovasodilating compounds such as sodium nitroprusside and nitroglycerin (Ignarro, 1990). Also, it is thought that EDRF may be a nitrosothiol adduct (Stamler et al., 1992a). S-nitroso-albumin is the most abundant nitrosothiol in human plasma and it may function as a “strorage-form” of NO (Stamler, 1994).

### *2.4.3. Metabolism of NO in human blood*

*In vivo*, at low concentrations of NO, the diffusion of NO into blood vessels is rapid enough to limit its opportunity to react with $O_2$ to form reactive species. NO released from endothelial cells into the vessel lumen is rapidly oxidized to nitrite in the plasma at the immediate site of its release. Nitrite is converted to nitrate via an autocatalytic reaction with oxyhemoglobin (oxyHb) within the erythrocyte. Alternatively, a small

proportion of NO may reach the erythrocytes directly, where it forms either nitrosylhemoglobin or oxidizes to nitrate and methemoglobin (metHb), the fraction of each depending on the ratio of oxygenated and non-oxygenated hemoglobin within the erythrocyte. Thus nitrate represents the final, but rather unspecific, end product, whereas nitrite is more specific but rather unstable, intermediate of NO metabolism in human blood (Doyle and Hoekstra, 1981; Feelisch, 1991; Kelm and Yoshida, 1996). Due to the high concentration of oxyHb and its rapid reaction with NO, this is one of the primary metabolic fates, as well as primary detoxification mechanism for NO (Grisham et al., 1999).

*2.4.4.Reactions with lipids*

In addition to metal nitrosyl formation, NO may react directly with free radicals. It will rapidly react with alkoxyl (RO·) and alkyl hydroperoxyl (ROO·) radicals. These types of reactions may be critical in modulating metal or enzyme-catalyzed lipid peroxidation (Rubbo et al., 1994). NO seems to inhibit lipid peroxidation and thus may be important in modulating the inflammatory response by inhibiting the formation of proinflammatory lipids (Rubbo et al., 1994).

*2.4.5. Reaction with superoxide anion ($O_2^{\cdot -}$)*

The reaction between $O_2^{\cdot -}$ and NO has been shown to occur with a rate constant of $7 * 10^9$ $M^{-1}s^{-1}$ (Huie and Padmaja, 1993). The product is a powerful oxidant, peroxynitrite (Beckman et al., 1993; Pryor and Squadrito, 1995).

$NO + O_2^{\cdot -} \rightarrow ONOO^-$

The biological properties of peroxynitrite are discussed in greater detail in chapter 3.

*2.5. Nonenzymatic NO production*

Nonenzymatic NO production has been demonstrated, e.g. in the stomach (Lundberg et al., 1994), on the surface of the skin (Weller et al., 1996), in the ischemic heart (Zweier et al., 1995), and in urine (Weitzberg and Lundberg, 1998). This nonenzymatic NO production results from chemical reduction of nitrite in an acidic environment. At a low pH, the nitrite ion ($NO_2^-$) will be converted to nitrous acid and then the nitrogen oxides including NO:

$$NO_2^- + H^+ \rightleftharpoons HNO_2$$
$$2\ HNO_2 \rightleftharpoons N_2O_3 + H_2O$$
$$N_2O_3 \rightleftharpoons NO + NO_2$$

Dietary nitrate is absorbed in the gastrointestinal tract and up to 25 % of circulating nitrate is actively taken up by the salivary glands and secreted in saliva (Spiegelhalder et al., 1976). Bacteria in the oral cavity reduce parts of the salivary nitrate to nitrite, which is further reduced to NO in the acidic environment in the stomach (Lundberg et al., 1994). Interestingly, it may be noted that NO formed nonenzymatically in the stomach may in part be derived from NO produced earlier by NOS activity elsewhere in the body. NO synthesized, e.g. in the endothelium is oxidized in the blood to nitrate, which may be taken up by the salivary glands and then turn up again as NO in the stomach. Weitzberg and Lundberg suggested that intragastric NO may be involved in host defence against swallowed pathogens as well as in the regulation of superficial mucosal blood flow and mucus secretion (Weitzberg and Lundberg, 1998). A very similar production route to that in the stomach has been described in the skin. Bacteria in the skin convert nitrate, (present in sweat) to nitrite, which is further reduced to NO on the slightly acidic surface of the skin (Weller et al., 1996). Also, bacteria present in infected urine will reduce nitrate to nitrite by enzymatic reduction by nitrate reductase. Under reducing conditions urinary nitrite will be further converted to NO and other reactive

nitrogen intermediates, which may exert antibacterial effects. Urinary NO production may be autoregulated *in vivo*, since NO is produced only when bacteria are present (Lundberg et al., 1997). Increased NOS-independent NO production may also occur during ischemia-reperfusion injury. Ischemia or hypoxia leads to acidic and highly reducing conditions, which may lead to nonenzymatic production of NO (Zweier et al., 1995).

### *2.6. NO synthesis from $N^G$-hydroxy-L-arginine (OH-L-Arg)*

OH-L-Arg, produced through the N-hydroxylation of one of the nitrogens of L-arginine is the only amino-acid intermediate which has been identified in the reaction catalysed by NOS. NADPH and $O_2$ are required in both oxidation reactions catalysed by NOS, that is from L-arginine to OH-L-Arg and then from OH-L-Arg to NO and citrulline (Stuehr et al., 1991; Pufahl et al., 1992). Hydrogen peroxide ($H_2O_2$) has been reported to support citrulline and nitrite/nitrate formation, in place of $O_2$ and NAPDH, by murine macrophage NOS. Chemiluminescence experiments have failed to detect NO as a product of this reaction. However, spectral experiments with OH-L-Arg and $H_2O_2$ under anaerobic conditions have demonstrated the appearance of a ferrous heme-NO complex, which suggests that in these conditions NO is formed (Pufahl et al., 1995). Also, nNOS catalyses the oxidation of OH-L-Arg by $H_2O_2$. The amino acid products were identified as citrulline and cyanoornithine, whereas it is not clear whether NO was formed (Clague et al., 1997). Modolell and co-workers have reported that phagocytic cells produce NO through a respiratory burst if OH-L-Arg is present, without expression of NOS (Modolell et al., 1997).

Cytochrome $P_{450}$3A has been shown to catalyse oxidation of OH-L-Arg to nitrogen oxides and citrulline in the rat liver (Renaud et al., 1993). It seems that although the oxidation of L-arginine to OH-L-Arg is exclusively effected by NOS, the second oxidative step, the conversion of OH-L-Arg into citrulline and nitrogen oxides may also be accomplished by other oxidases/oxidative products (Renaud et al., 1993; Pufahl et al., 1995; Modolell et al., 1997).

*2.7. Methods for measuring NO*

The method most commonly used for the determination of nitrite is a spectrophotometric assay based on the Griess reaction This procedure is based on the two-step diazotization reaction in which acidified nitrite produces a nitrosating agent which reacts with sulfanilic acid to produce the diazonium ion. This ion is then coupled to N-(1-naphthyl)ethylenediamine to form a chromophoric azo derivative (Miles et al., 1996). Its advantage is its simplicity; it usually requires only the addition of a reagent mix to the sample. The Griess method can also be used for measuring nitrite and nitrate ($NO_x$) in biological samples. The procedure comprises two parts, the enzymatic or chemical reduction of nitrate to nitrite followed by spectrophotometric detection of nitrite. In biological fluids $NO_x$ may originate from various sources in addition to NO. It may be ingested with food or inhaled as NO / $NO_2$ or be produced by gut or airway bacteria. The pollution caused by heavy traffic as well as smoking will ensure that people inhale large amounts of $NO_x$ (Green et al., 1982; Schmidt et al., 1992).

Determination of NO by the chemiluminescence reaction with ozone ($O_3$) is the basis of one of the most accurate NO assays available. In the early 1960s it was described that light emission accompanies the reaction of $O_3$ or singlet oxygen with gases such as NO, $NO_2$, CO and $SO_2$. It was found that $O_3$ reacts most readily with NO. This is the basis of the selectivity of $O_3$ chemiluminescence assay for NO (Clyne et al., 1964; Hampl et al., 1996). The reaction of NO with $O_3$ yields nitrogen dioxide, some of which is in the excited state ($NO_2^*$)

$$NO + O_3 \rightarrow NO_2^* + O_2$$

In the excited state the electrons are unstable and dissipate energy as they regain their original stable ground state. The excess energy of $NO_2^*$ is released as a photon (*hv*) (Clyne et al., 1964).

$NO_2^* \rightarrow NO_2 + h\nu$

The light is emitted in the red and infrared region of the spectrum (~640-3000 nm) and photomultipliers are used to detect it (Hampl et al., 1996).

The NO + $O_3$ reaction takes place in the gas phase. This makes chemiluminescence convenient for NO measurements in gaseous samples such as exhaled air of patients with asthma (Alving et al., 1993; Lehtimäki et al., 2000). In addition to measuring NO itself, the NO chemiluminescence assay can be used to measure the intermediates and end products of NO oxidation (s-nitrosothiols, nitrite and nitrate). This is useful, as the method can be applied to measure nitrite and nitrate in biological samples, e.g. cell culture medium, serum and synovial fluid (Hampl et al., 1996).

Electron spin resonance (ESR) spectroscopy provides a specific probe of molecules with unpaired electrons whose energy levels are altered in the presence of a magnetic field. Spin-traps are good tools for altering short-lived free radicals to less reactive form. For instance the heme group (in heme proteins) can be used as a spin trap for NO. The heme –iron –nitrosyl complex formed can be detected by ESR (Singel and Lancaster, 1996).

The oxyHb assay is based on the reaction of oxyHb with NO to form metHb and nitrate. The same reaction also largely accounts for the inhibitory effect of hemoglobin on the biological effects of endogenously formed or exogenously applied NO.

oxyHb + NO $\rightarrow$ metHb + $NO_3^-$

The formation of metHb can be detected by the spectrophotometer from changes in the spectrum of an oxyHb-containing solution upon NO-mediated conversion to metHb (Feelisch and Noack, 1987). The rate of reaction between oxyHb and NO is 26 times

faster than the auto-oxidation of NO in aqueous solution. The trapping of NO by oxyHb is almost stoichiometric under most experimental conditions (Feelisch et al., 1996). The only exception to this is the presence of high concomitant formation of $O_2^{\cdot -}$. Thus under such conditions the addition of SOD is recommended (Sutton et al., 1976; Feelisch et al., 1996).

The NO-induced increase in intracellular cGMP has become a standard assay for the biochemical activity of NO. The bioassay has the drawback that it is indirect and relies on the presence of activity of soluble guanylyl cyclase. cGMP levels can be assayed by radioimmunoassay (RIA) (Steiner et al., 1972; Moilanen et al., 1993) or enzyme-linked immunosorbant assay (ELISA) (Föstermann and Ishii, 1996).

Purification of native NOS proteins, as well as cDNA isolation and expression, has allowed the generation of antibodies to whole proteins or peptide sequences of NOS, and DNA probes for NOS gene sequences. Using these antibodies, the presence of the NOS isoforms has been detected by immunohistochemistry in cells and tissues throughout the organism (Förstermann and Dun, 1996; Moilanen et al., 1997) or Western blotting (Lähde et al., 2000). Reverse transcriptase polymerase chain reaction (RT-PCR) and other molecular biological methods are used for mRNA measurements of NOS (Lähde et al., 2000).

## 3. PEROXYNITRITE

Peroxynitrite is a short-lived and damaging oxidant rapidly formed in the reaction between NO and $O_2^{\cdot -}$ (Murphy et al., 1998). $O_2^{\cdot -}$ is produced continuously in various biological reactions, e.g in mitochondrial respiration and especially in inflammation by NADPH oxidase in activated macrophages and neutrophils (Baggiolini and Wymann, 1990; Shigenaga et al., 1994).

*3.1. Chemistry*

Because both NO And $O_2^{\cdot -}$ are free radicals, they react together extremely rapidly at close to the diffusion limit to form peroxynitrite (Huie and Padmaja, 1993)

$$O_2^{\cdot -} + NO \rightarrow ONOO^-$$

Peroxynitrite is not a free radical but a short–lived and highly reactive molecule (Beckman et al., 1990). It is relatively stable in alkaline solutions, slowly decomposing to nitrite and oxygen (Goldstein et al., 1996; Pfeiffer et al., 1997).

$$2\ ONOO^- \rightarrow 2\ NO_2^- + O_2$$

At around neutral pH peroxynitrite rapidly decomposes ($t_{½}$<1sec) to peroxynitrous acid (ONOOH), which has pKa of 6.75 at 37 °C. It is unstable and rapidly isomerizes to nitrate (Koppenol et al., 1992).

$$ONOO^- \rightleftharpoons ONOOH \rightarrow H^+ + NO_3^-$$

Peroxynitrite and ONOOH are strong oxidants in reactions of both one and two electron mechanisms.

$$ONOO^- + 2H^+ + e^- \rightarrow NO_2^{\cdot}(aq) + H_2O$$
$$ONOO^- + 2H^+ + 2e^- \rightarrow NO_2^- + H_2O$$

In the presence of biological targets these reactions lead to a range of oxidized products. Peroxynitrite also reacts rapidly with $CO_2$ to form the nitrosoperoxycarbonate anion (Denicola et al., 1996). The high reactivity of peroxynitrite toward aqueous $CO_2$ together with the high biological concentration of $CO_2$ (about 1 mM $CO_2$) makes the

reaction with $CO_2$ one of its prevalent reactions. The speed of the reaction of peroxynitrite with $CO_2$ in biological systems is faster than uncatalyzed decomposition of peroxynitrite. Thus, many of the reactions of peroxynitrite *in vivo* are more likely to be mediated by reactive intermediates derived from its reaction with $CO_2$ than from peroxynitrite itself (Squadrito and Pryor, 1998).

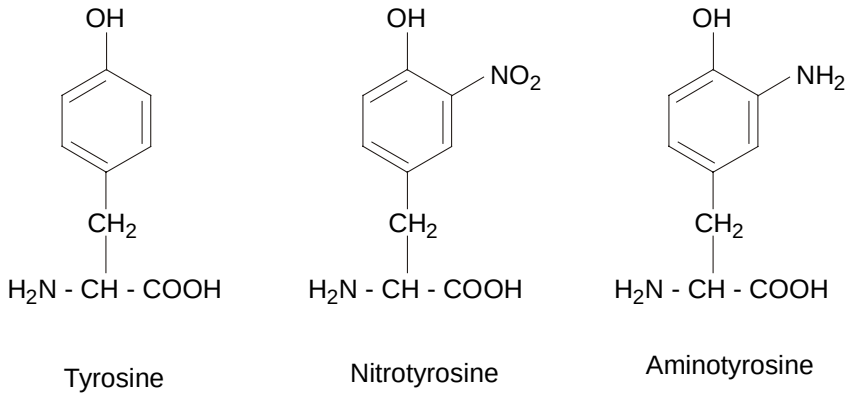


Figure 2.

Tyrosine residues are nitrated by peroxynitrite to form 3-nitrotyrosine (Ischiropoulos et al., 1992) and nitrotyrosine or its derivative aminotyrosine is used as a marker of peroxynitrite production in biological samples (Fig. 2).

The reaction of peroxynitrite with thiols forms two main products: in alkaline pH the main reaction is oxidation to form an unstable derivative which reacts further with other thiols to form a disulfide. At acidic pH the predominant reaction is oxidation by ONOOH or ONOOH* to form the thiyl radical (Quijano et al., 1997). At physiological pH values, oxidation leading to disulphide formation may dominate, but also thiyl radical formation is possible (Quijano et al., 1997; Murphy et al., 1998).

By reason of their very rapid reaction, whenever NO and $O_2^{\cdot -}$ are present simultaneously *in vivo* the peroxynitrite may form. As a consequence of normal aerobic respiration, mitochondria consume $O_2$, reducing it by sequential steps to produce $H_2O$. As a by-product of this process $O_2^{\cdot -}$ is produced (Ames et al., 1993). Also, NADPH oxidases produce $O_2^{\cdot -}$ (Baggiolini and Wymann, 1990). Under normal conditions the amount of SOD is sufficient to channel all $O_2^{\cdot -}$ towards the dismutation products $O_2$ and $H_2O_2$. The steady state concentration of $O_2^{\cdot -}$ is normally kept low, in the range of 10-100 pM (Gardner and Fridovich, 1991). The rate of the reaction between NO and $O_2^{\cdot -}$ is two to three times faster than that of $O_2^{\cdot -}$ with SOD. However, *in vivo* SOD concentrations are far higher (5-10 μM) than physiological levels of NO (10-100 nM) and it therefore limits peroxynitrite formation (Beckman, 1994; Murphy et al., 1998). Whenever the NO concentration increases, it will compete with SOD for $O_2^{\cdot -}$ and form peroxynitrite. Because NO is hydrophobic and diffuses easily through membranes, it can diffuse several cell diameters from its site of synthesis. In contrast, $O_2^{\cdot -}$ can only diffuse a short distance within a cell before being degraded by SOD, and it will not cross lipid bilayers rapidly. Thus, intracellular peroxynitrite formation will arise from elevated production of NO and its subsequent diffusion to sites of $O_2^{\cdot -}$ formation. In the extracellular environment $O_2^{\cdot -}$ will live longer (Halliwell and Gutteridge, 1989), whereas NO will be degraded more rapidly (Murphy et al., 1998). The first indication that peroxynitrite formation was likely *in vivo* was that SOD increased the lifetime of NO in biological systems (Gryglewski et al., 1986). In cell culture, peroxynitrite formation has been demonstrated e.g. in activated macrophages (Ischiropoulos et al., 1992) and in endothelial cells (Kooy et al., 1994).

### *3.2. Biological effects of peroxynitrite formation*

Human tissues are continually exposed to a number of damaging reactive oxygen species. As a strong oxidant and nitrating agent peroxynitrite is more reactive and harmful than its precursors and is therefore more toxic to cells than $O_2^{\cdot -}$, $H_2O_2$, or NO.

### *3.2.1. Low density lipoprotein (LDL) oxidation*

The first reports demonstrating an oxidative reaction of peroxynitrite towards LDL used the sydnonimine SIN-1 (Darley-Usmar et al., 1992; Jessup et al., 1992). This compound simultaneously releases NO and $O_2^{\cdot -}$, which rapidly react to form peroxynitrite (Feelisch et al., 1989). Incubation of LDL with SIN-1 resulted in the depletion of LDL antioxidants and the formation of both lipid hydroperoxides and thiobarbituric acid reactive substances (TBARS), indicating that lipid peroxidation was occurring (Darley-Usmar et al., 1992; Malo-Ranta et al., 1994; Hogg and Kalyanaraman, 1998). In the absence of $O_2^{\cdot -}$, NO will inhibit lipid oxidation; in the presence of $O_2^{\cdot -}$, NO will be scavenged to form peroxynitrite, which will initiate lipid peroxidation. The balance between the levels of NO and $O_2^{\cdot -}$ becomes a critical determinant of whether NO exhibits pro-oxidant or anti-oxidant behaviour (Hogg and Kalyanaraman, 1998).

### *3.2.2. Tyrosine nitration*

The primary effect of peroxynitrite on proteins is nitration of tyrosine residues (Beckman et al., 1992). Nitrated tyrosine has been found in endothelium, foamy macrophages and inflammatory cells in the atherosclerotic human artery, indicating that peroxynitrite is produced *in vivo* (Beckmann et al., 1994). Peroxynitrite has also been shown to induce tyrosine nitration in human platelets (Mondoro et al., 1997) and endothelial cells (Gow et al., 1996).

Tyrosine phosphorylation of enzymes is a common mechanism mediating cellular signaling. Tyrosine nitration inhibits tyrosine phosphorylation in endothelial cells and modulates tyrosine phosphorylation in human platelets (Mondoro et al., 1997) and human neuroblastoma cells (Li et al., 1998). Moreover, nitrated tyrosine residues in peptides cannot be phosphorylated by tyrosine kinases *in vitro*. These data indicate that nitration of proteins can prevent or modulate cellular signal transduction, which requires tyrosine phosphorylation (Kong et al., 1996; Mondoro et al., 1997).

### *3.2.3. DNA damage*

Peroxynitrite initiates a number of DNA modifications. The reaction of nucleobases with peroxynitrite forms several new compounds, indicating that peroxynitrite induces base modifications, e.g. formation of 8-nitroguanine from guaninine (Szabo and Ohshima, 1997). SIN-1 that simultaneously generates NO and $O_2^{\cdot -}$ has been used to study peroxynitrite-induced DNA damage. SIN-1 was shown to increase the production of 8-oxoguanine in DNA treated with SIN-1 (Inoue and Kawanishi, 1995).

Peroxynitrite also induces single strand breaks in DNA in various systems. For instance, peroxynitrite can induce DNA strand breaks *in vitro* in plasmid DNA, converting the supercoiled form to relaxed or linear forms (Szabo and Ohshima, 1997). DNA cleavage caused by peroxynitrite or SIN-1 was observed at almost every nucleotide with a small dominance of guanine residues (Inoue and Kawanishi, 1995).

### *3.2.4. Reactions with thiols*

Inside the cell, peroxynitrite will preferentially react with free thiols because of the fast rate of this reaction and the high intracellular thiol concentration. Glutathione is the predominant intracellular thiol, and it has been assumed that the glutathione system may protect cells against peroxynitrite–induced cytotoxicity (Radi et al., 1991; Murphy et al., 1998). Since the plasma thiol concentration is one tenth lower than inside cells (Ellman and Lysko, 1979), detoxification of peroxynitrite by thiols will be less effective (Murphy et al., 1998).

### *3.2.5. Apoptosis and necrosis*

Exposure to large amounts of peroxynitrite leads to necrotic cell death, due to disruption of cellular metabolism and membrane integrity (Bonfoco et al., 1995). Peroxynitrite is also a potent inducer of apoptotic (programmed) cell death and causes apoptosis in many cell types (Murphy et al., 1998). Addition of smaller amounts of peroxynitrite, too small to cause necrosis, may still induce an apoptotic program. This mode of cell death is relevant to the damage caused by peroxynitrite *in vivo*, since chronic production of small amounts of peroxynitrite may arise in chronic inflammation (Slater et al., 1995; Murphy et al., 1998).

## *3.3. Methods to measure peroxynitrite*

### *3.3.1. Methods to measure nitrotyrosine*

Peroxynitrite reacts with free or protein-pound tyrosine residues, producing a stable product, 3-nitrotyrosine. This reaction requires the addition of nitronium ($NO_2^+$) to the tyrosine residues, a reaction which cannot be produced by NO itself (van der Vliet et al., 1994). Because of its stability, nitrotyrosine has been held to be a good marker for peroxynitrite production and NO-mediated tissue damage (Ischiropoulos et al., 1992).

Kaur and Halliwell developed an HPLC method with UV detection for the analysis of nitrotyrosine at 274 nm in serum and synovial fluid from patients with rheumatoid arthritis. The limit of the detection was 0.2 µM (Kaur and Halliwell, 1994).

Using commercially available polyclonal and monoclonal antibodies against nitrotyrosine, Western blotting and immunohistochemistry has been carried out in several studies. These antibodies against nitrotyrosine have usually been raised by immunization of rabbits using peroxynitrite-treated bovine serum albumin (Herce-Pagliai et al., 1998).

Khan and associates have developed a competitive ELISA method to assay nitrated proteins in biological fluids and homogenates. This approach yields semiquantitative results (Khan et al., 1998).

Nitrotyrosine is considered to be a specific fingerprint marker of peroxynitrite. In certain conditions also nitrite can nitrate tyrosine in a peroxynitrite-independent manner. In that pathway nitrite reacts first with hypochlorous acid (HOCl) to form nitryl chlorine as an intermediate which then modifies tyrosine (Kettle et al., 1997). Also, in the presence of $H_2O_2$ and nitrite, heme peroxidases can form nitrating species, which nitrate tyrosine (van der Vliet et al., 1997).

### *3.3.2. Dihydrorhodamine oxidation to rhodamine*

The oxidation of nonfluorescent dihydrorhodamine to its fluorescent product rhodamine 123 is relatively selective for peroxynitrite over $O_2^{\cdot -}$, $H_2O_2$, or NO and it has been used in isolated organelles, cells, and intact animals to measure peroxynitrite production (Kooy et al., 1994; Packer and Murphy, 1995; Zingarelli et al., 1996). However, like nitrotyrosine, it suffers from the lack of specificity. Many other biologically significant oxidants, for example hypochlorous acid and $OH^{\cdot}$, are also able to oxidize dihydrorhodamine (Kettle et al., 1997).

### *3.3.3. Luminol enhanced chemiluminescence*

Another technique to measure the continuous formation of peroxynitrite is luminol-enhanced chemiluminescence, which shows some selectivity for peroxynitrite over other oxidants. Peroxynitrite reacts with luminol to yield chemiluminescence, which is greatly enhanced by bicarbonate present in the reaction mixture (Radi et al., 1993). However, this method is not very specific for peroxynitrite, because many other biological oxidants and peroxidases may cause increased chemiluminescence (Murphy et al., 1998).

## 4. NO-DONORS

NO-donors are compounds, which produce NO when applied to biological systems and are thus able either to mimic an endogenous NO-related response or substitute for an endogenous NO deficiency (Fig. 3). The pathways leading to the formation of NO differ greatly among the various groups of compounds, some of which require enzymatic catalysis while others produce NO non-enzymatically. NO-donors are believed to have therapeutic potential in a range of cardiovascular, gastrointestinal and other diseases (Moncada and Higgs, 1995; Feelisch, 1998; Muscara and Wallace, 1999).

### *4.1. Organic nitrates*

Organic nitrates are nitric acid esters of mono- and polyhydric alcohols and most of these compounds are only sparingly soluble in water. Organic nitrates are susceptible to alkaline hydrolysis, but are generally stable in neutral or weakly acidic solutions. Until the late seventies, after more than a century of therapeutic use, the exact mode of action of organic nitrites remained a mystery. In 1977 Ferid Murad analysed the mechanism of action of glyceryl trinitrate and other related vasodilatators. He suggested that these compounds release NO, which enhances cGMP production and relaxes smooth muscle (Arnold et al., 1977; Katsuki et al., 1977a; Katsuki et al., 1977b). Nonenzymatic formation of NO from nitrate esters requires interaction with sulfhydryl (SH) groups. In the course of this reaction, thiols (RSHs) are oxidized to their respective disulfides (RSSRs) and nitrate is released as the major nitrogenous metabolite. Classical organic nitrovasodilators such as glyceryl nitrate and isosorbide mono- or dinitrates are established as reliable drugs for the treatment and prevention of anginal attacks and cardiac ishemia. Their chronic administration may lead to development of tolerance because of the exhaustion of cysteine, which is required for metabolic reduction of nitro groups to NO (Axelsson and Karlsson, 1984; Feelisch, 1993). The site of enzymatic biotransformation of organic nitrates has not yet been identified (Feelisch, 1998).

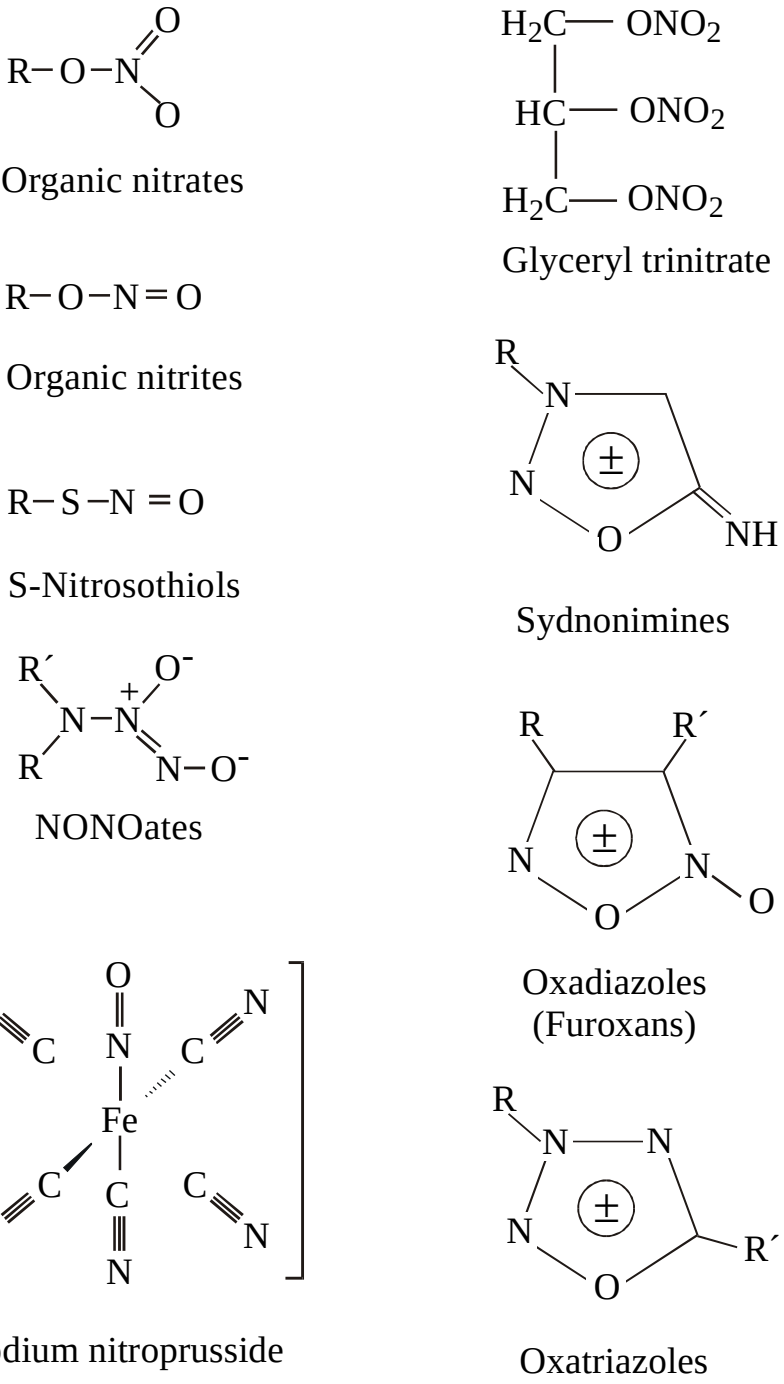


Figure 3. The chemical structure of NO releasing compounds.

### *4.2. S-Nitrosothiols*

S-Nitrosothiols are colored solids or liquids, which are obtained by S-nitrosation of thiols. The cysteine residue of a number of proteins can also be nitrosated (Stamler et al., 1992b). Both S-nitrosylated low-molecular weight thiols (such as S-nitrosoglutathione) and protein thiols (in particular S-nitrosoalbumin) have been detected in biological fluids as a consequence of endogenous NO metabolism or exogenous administration of NO, e.g. therapy with inhaled NO gas (Gaston et al., 1993). Only a few nitrosothiols have been isolated in solid form, these including S-nitroso-*N*-acetylpenicillamine (SNAP) (Feelisch, 1993), which is widely used as an experimental tool, and S-nitrosoglutathione (GSNO) (Feelisch, 1998). *In vitro*, S-nitrosothiols decompose to yield the corresponding disulfides (RSSR) and NO by cleavage of the S-N bond. Another important process to consider in the context of S-nitrosothiols is the transamination reaction, i.e. the transfer of bound NO from one thiol group to another. This feature distinguishes S-nitrosothiols from other NO-donor classes.

### *4.3. NONOates*

NONOates are adducts of NO with nucleophils such as an amine and sulfite, in which a NO dimer is bound to the nucleophilic residue via a nitrogen atom, thus forming a functional group of the structure X-[N(O)NO]$^-$. It is thought that NONOates generates NO by acid-catalysed dissociation with regeneration of the free nucleophile and NO, although enzymatic metabolism *in vivo* cannot be excluded. Almost all NONOates are highly soluble in water. The decomposition of NONOates is pH-dependent. The reaction follows first-order kinetics, yielding up to 2 moles of NO per mole of NONOate. At higher concentrations of NONOates and at physiological pH, the yield is usually lower (Feelisch, 1998).

*4.4. Sodium nitroprusside*

The most widely studied of the iron nitrosyls is sodium nitroprusside (SNP; sodium pentacyanonitrosyl ferrate (II)). The compound is used clinically to reduce blood pressure, e.g. in hypertensive emergencies. The mechanism of NO release from SNP is not completely understood. In biological systems both non-enzymatic and enzymatic NO release from SNP may occur. Thiolate anions may attact SNP, leading to decomposition of the complex with formation of NO, disulfide and cyanide ($CN^-$). A membrane-bound enzyme may be involved in the generation of NO from SNP in biological tissues, and either NADH or NADPH appears to be required as a cofactor (Bates et al., 1991; Feelisch, 1998).

*4.5. Sydnonimines*

The most widely studied compound of sydnonimines is molsidomine (N-ethoxycarbonyl-3-morpholino-sydnonimine). Molsidomine is a prodrug, which is converted by liver esterases to the active metabolite 3-morpholine-sydnonimine (SIN-1). SIN-1 is a potent vasorelaxant and anti-aggregatory agent. These activities are thought to be mediated largely by the release of NO, which activates soluble guanylyl cyclase (Feelisch et al., 1989; Noack and Feelisch, 1989; Feelisch, 1998). However, not all of the biological effects of SIN-1 can be attributed to NO. For instance, SIN-1-induced suppression of neutrophil degranulation was been shown not to be mediated by cGMP, NO or peroxynitrite, but by the NO-lacking metabolite SIN-1C (Kankaanranta et al., 1997). SIN-1 decomposes to produce NO in an oxygen-dependent process. SIN-1 undergoes rapid nonenzymatic hydrolysis to the ring-open form SIN-1A. Oxygen promotes oxidative conversion to a cation radical intermediate from which NO is released and more stable SIN-1C is formed. In the course of this reaction stoichiometric amounts of $O_2^{\cdot -}$ are formed as a result of oxygen reduction (Feelisch et al., 1989). The release of $O_2^{\cdot -}$ together with NO may lead to the formation of peroxynitrite, for which reason SIN-1 is often regarded as a peroxynitrite-donor rather than a NO-donor. In

addition, in anaerobic condition, oxidation of SIN-1 is supported by cytochrome c to release NO but peroxynitrite is not formed (Feelisch, 1998).

*4.6. Oxadiazoles (Furoxans)*

In synthetic organic chemistry, furoxans (1,2,5-oxadiazole-2-oxides) are used in the preparation of various heterocyclic compounds. Some representatives of this class have been shown to exert a variety of NO-related bioactivites, including bacteriostasis, cytotoxicity, mutagenity, immunosuppression and direct vasodilatator and blood pressure-lowering activities (Feelisch et al., 1992; Ferioli et al., 1995; Feelisch and Stamler, 1996). Furoxans liberate NO after reacting with sulphydryl groups of low molelular weight thiols and proteins. Some furoxan derivatives have been shown to release NO spontaneously (Hecker et al., 1995). Furoxans have attracted commercial interest as potential antiplatelet and antianginal agents (Civelli et al., 1994; Bohn et al., 1995). However, their mutagenity may be a significant drawback in drug development (Feelisch and Stamler, 1996).

*4.7. 1,2,3,4 –oxatriazole-5-imines and their derivatives*

At the beginning of 1990s the GEA Laboratories synthesized a new group of NO-donors, 3-aryl- 1,2,3,4-oxatriazole-5-imines, structurally related to sydnonimines. Four different types of 3-aryl- 1,2,3,4-oxatriazole-5-imines were synthesized, i.e. unsubstituted imines (e.g. GEA 3162), amides (e.g.GEA 3233), sulfonamides (e.g. GEA 3175) and ureas (e.g. GEA 5339). For further studies only those GEA compounds were selected which showed low or no mutagenic properties in the Ames test.

*In vitro* the rate of spontaneous release of NO from GEA compounds decreases in the following order: imines > amides ~sulfonamides > ureas. The fast type includes mainly the unsubstituted 5-imine derivatives, which at physiological pH are rapidly hydrolysed with a subsequent release of NO. The slow type of NO releasers comprises GEA

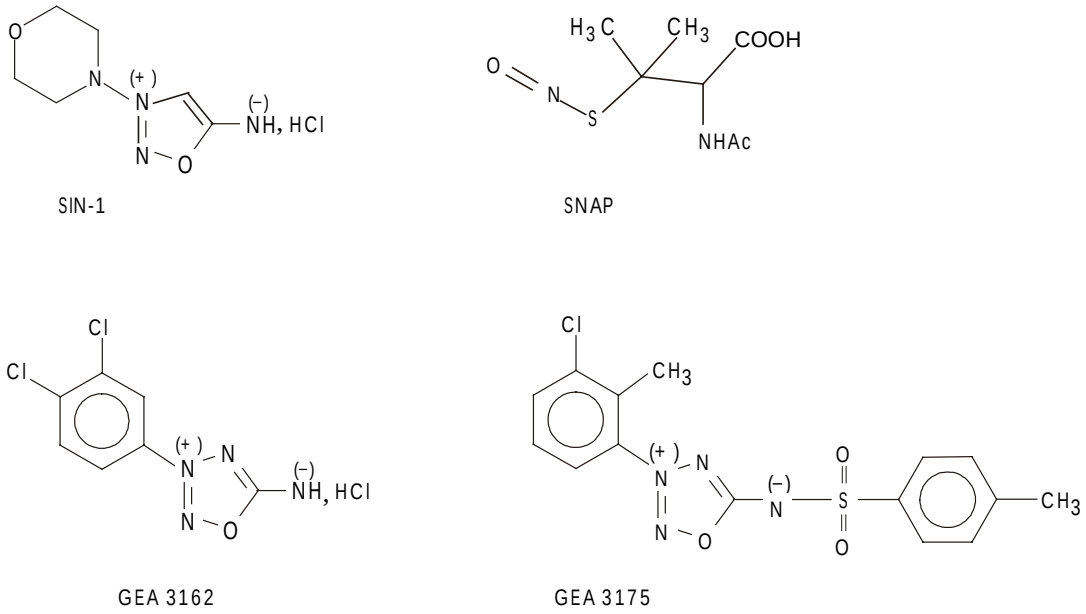


Figure 4. The chemical structures of GEA 3162, GEA 3175, SIN-1 and SNAP.

compounds substituted at the 5-imino position of the amide, sulfonamide and urea types. The mechanism of NO release from these compounds remains speculative and is not understood in detail. GEA compounds consume oxygen only when used at high concentrations, and thereby an oxygen-dependent mechanism of NO-release, similar to that found for sydnonimine, SIN-1 (Feelisch et al., 1989), is unlikely to be involved in the decomposition process of GEA compounds (Karup et al., 1994). It is suggested that GEA compounds react with glutathione and other thiols in blood and tissues and thus form nitrosothiols which release NO more slowly than the GEA compounds itself (Karup et al., 1994; Cohen et al., 1995). Such a mechanism has also been proposed for the furoxane NO-donors (Feelisch et al., 1992).

Pharmacological effects of GEA compounds are associated with a rise in cGMP. GEA compounds suppress platelet aggregation in platelet-rich plasma, and evince vasodilator, fibrinolytic and thrombolytic activities (Corell et al., 1994) and induce relaxation of bronchioles (Corell et al., 1994; Vaali et al., 1996; Hernandez et al., 1998) and trachea

(Vaali et al., 1998). GEA compounds are antibacterial (Virta et al., 1994), suppress tumor cell growth (Vilpo et al., 1997) and regulate glycosaminoglygan synthesis in articular cartilage (Järvinen et al., 1995). GEA 3162 and GEA 5024 increase cGMP production in human polymorphonuclear leukocytes (PMNs) and concomitantly inhibit PMN functions, i.e. leukotriene $B_4$ synthesis, degranulation, chemotaxis and $O_2^{\cdot -}$ release (Moilanen et al., 1993; Wanikiat et al., 1997). GEA 3162 inhibits endothelial cell-mediated oxidation of LDL (Malo-Ranta et al., 1994). GEA 3162 and GEA 3175 suppress human peripheral blood mononuclear cell proliferation by a cGMP-independent manner (Kosonen et al., 1997; Kosonen et al., 1998). GEA 3175 inhibits LPS-induced production of prostacyclin in human endothelial cells (Kosonen et al., 1998), and also, GEA 3175 inhibits leukocyte adhesion to human endothelial cells (Kosonen et al., 1999; Kosonen et al., 2000). Low doses of intragastrically administred GEA 3162 inhibit gastric ulceration induced by ethanol in anesthetized rats. In constrast, higher doses of GEA 3162 administered i.g. exacerbate the damage (Asmawi et al., 1999).

In general, NO-donors are applied as pharmacological tools in studying biological effects mediated by NO. However, the metabolites or decomposition products of the respective compounds may have biological effects of their own. It is advisable always to include proper controls to confirm that the effects found have indeed been elicited by NO.

## 5. NO AND PEROXYNITRITE PRODUCTION BY INFLAMMATORY CELLS

### *5.1. NO in inflammation*

NO plays a vital role in host defence and immunity, including the modulation of inflammatory responses. Upregulation of the iNOS generates excessive NO production during a variety of inflammatory diseases, including sepsis, asthma, ulcerative colitis, psoriasis, giant cell arteritis, Sjögren's syndrome, rheumatoid arthritis and osteoarthritis (Amin et al., 1999; Moilanen et al., 1999). Although the inhibition of NO synthesis improves the outcome in experimentally induced inflammation, the role of NO in the pathogenesis of the respective human diseases is unclear (Grisham et al., 1999; Amin et al., 1999). Induction of iNOS and NO production are differently regulated in human than in rodent cells, which have been the most extensively studied so far. Unlike murine iNOS, human iNOS in most cells is not induced in response to LPS or a single cytokine; a combination of cytokines or more complex treatments are needed, and the regulation of human iNOS expression in inflammatory cells remains for a most part obscure (MacMicking et al., 1997; Moilanen et al., 1999). There are, however, fragmentary data suggesting that NO production takes place in the sites of inflammation, e.g. in inflamed joints in patients with rheumatoid arthritis (Garthwaite et al., 1988; Yui et al., 1991; Charles et al., 1993; Sakurai et al., 1995; Moilanen et al., 1997).

In contrast, several *in vitro* and *in vivo* studies have suggesed that NO may also function as an anti-inflammatory mediator. Depending on the type and phase of the inflammatory reaction and the individual vascular or cellular responses studied NO can exert both proinflammatory and anti-inflammatory properties (Moilanen et al., 1999). NO has direct effects on the synthesis of some inflammatory mediators, e.g. it inhibits the activity of 5-lipoxygenase (Kanner et al., 1992) an enzyme which produces leukotrienes, and also inhibits NADPH oxidase (Clancy et al., 1992), which forms $O_2^{\cdot -}$ . NO released by NO-donors has been shown to reduce cytokine-induced leukocyte adhesion to endothelial cells (Kosonen et al., 1999) and to inhibit leukocyte functions

(Moilanen et al., 1993; Kosonen et al., 1997; Kosonen et al., 1998). In some instances peroxynitrite formation can be regarded as an mechanism of inactivation of NO and $O_2^{\cdot -}$ (Gryglewski et al., 1986). However, because peroxynitrite is a strong oxidant and nitrating agent, there is increasing evidence to support its role as a derivative of NO which mediates tissue damage in inflammation (Beckmann et al., 1994; Salvemini et al., 1998). Peroxynitrite formation has been detected at sites of inflammation in experimental animals and in humans (Kaur and Halliwell, 1994; Miller et al., 1995; Kooy et al., 1995; Szabo et al., 1995a; Szabo et al., 1995b).

*5.2. NO and macrophages*

NO is an important effector molecule in murine macrophage-mediated host defence (MacMicking et al., 1997). Expression of iNOS requires exposure of cells to immune or inflammatory stimuli. This phenomenon was first demonstrated with mouse peritoneal macrophages treated with bacterial LPS, in which NO production was enhanced synergistically by the addition of IFN-γ (Stuehr and Marletta, 1987; Nathan and Hibbs, Jr., 1991). In response to LPS and IFN-γ murine macrophages produce high amounts of NO which contribute to their cytotoxic action against tumor cells, bacteria, helminth, fungal or protozoan infections and replication of viruses (MacMicking et al., 1997; Moilanen et al., 1999). The significance of NO synthesis in rodent macrophages is well established, although it is not certain whether this can be extended to human monocytes/macrophages. Human monocyte magrophages do not express iNOS in response to cytokines or cytokine combinations known to induce NO synthesis in mouse macrophages (Schneemann et al., 1993; Bermudez, 1993). Thus far, there have been some reports on human monocytes/macrophages synthesizing NO. However, the expression and regulation of iNOS in human monocytes/macrophages still remains controversial. Cells from patients with diseases such as malaria (Anstey et al., 1996), rheumatoid arthritis (St Clair et al., 1996) and tuberculosis (Nicholson et al., 1996) contain mononuclear phagocytes which express iNOS and produce NO. Treatment of

human monocytes or mononuclear cells with IFN-α *in vitro* has been reported to cause an increase in monocyte iNOS activity and NO production (Sharara et al., 1997). Also, induction of human monocytes has been detected after infection with *Mucobacterium avium* (Denis, 1991) and after activation through surface receptor CD69 (De Maria et al., 1994).

### *5.3. NO and lymphocytes*

NO production in murine lymphocytes has been demonstrated and seems to be subtype-specific. Data from the murine cell modell show that T helper type 1 (Th1) cells but not T helper type 2 (Th2) cells can be induced to produce NO, which serves as a regulatory mechanism leading to suppressed function of Th1 lymphocytes (Taylor-Robinson et al., 1994). Similar results have not been obtained in human lymphocytes. There are, however, data supporting the regulatory role of NO produced by activated macrophages or NO-donors, GEA 3162 and GEA 3175 and SNAP, on lymphocyte proliferation (Albina and Henry, Jr., 1991; Kosonen et al., 1997; Kosonen et al., 1998).

### *5.4. NO and neutrophils*

Rat neutrophils express an inducible calcium-independent form of NOS in response to LPS and cytokines IFN-γ, and TNF-α. Anti-inflammatory steroids prevent the expression of iNOS in these cells (McCall et al., 1991b; Kolls et al., 1994; Moilanen et al., 1999). The production of NO in human neutrophils is a subject of controversy and the iNOS gene in human neutrophils is under very different regulation than the rat gene. Inflammatory stimuli known to induce iNOS in rat neutrophils do not exert the same action on human neutrophils, not at least in *in vitro* conditions (Miles et al., 1996). There are, however, some reports on NO production also in human neutrophils. These were first reported to inhibit platelet aggregation by releasing a NO-like factor (Salvemini et al., 1989; Faint et al., 1991). NO synthesis in human neutrophils has been

reported to be triggered by n-formyl-L-methionyl-L-leucyl-L-phenylalanine (FMLP) or by direct activators of protein kinase C (Schmidt et al., 1989; Faint et al., 1991; Goode et al., 1994; Lärfars and Gyllenhammar, 1995). Also, iNOS activity has been demonstrated in human neutrophils isolated from the oral cavity (Sato et al., 1996) or from urine from patients with urinary track infections (Wheeler et al., 1997). However, there are several studies in which no NO synthesis in human neutrophils has been found (Yan et al., 1994; Padgett and Pruett, 1995; Miles et al., 1996; McBride and Brown, 1997).

Polymorphonuclear leukocytes are a particularly important cell type in acute inflammation. When human neutrophils are activated with an agonist like FMLP, platelet activating factor (PAF) or leukotriene $B_4$ ($LTB_4$), the $O_2^{\cdot -}$ production by plasma membrane–bound NADPH oxidase (respiratory burst) is also activated.

$$2\ O_2 + NADPH \rightarrow 2\ O_2^{\cdot -} + NADP^+ + H^+$$

The generation of $O_2^{\cdot -}$ by human neutrophils is thought to be an essential component of their microbicidal and cytotoxic action. However although $O_2^{\cdot -}$ can be directly toxic, some studies have shown a limited reactivity with biological molecules (Beckman et al., 1990), suggesting that production of more reactive secondary intermediates may be important. NO reacts rapidly with $O_2^{\cdot -}$ to form peroxynitrite, and generation of peroxynitrite during the respiratory burst of human neutrophils has been reported (Carreras et al., 1994; Evans et al., 1996).

*5.5. NO in rheumatoid arthritis*

Rheumatoid arthritis (RA) is a chronic inflammatory disease characterized by prolonged synovitis in multiple joints. Inflamed synovium is composed of a variety of cell types, including lymphocytes, macrophage- and fibroblast –type synovial cells and mast cells (Bromley et al., 1984; Firestein and Zvaifler, 1990; Remmers et al., 1991). In exacerbated phases, exudative synovial fluid contains high numbers of neutrophils and mononuclear cells (Kavanaugh and Lipsky, 1999). In addition to the inflammatory mediators produced by the rheumatoid synovium, polymorphonuclear cells accumulating in the joint release lysosomal enzymes and produce toxic oxygen radicals and lipid-class inflammatory mediators. These result in the cartilage and bone destruction in RA (Brown, 1988; Moilanen, 1994; Kankaanranta, 1995).

Farrell and associates reported increased nitrite concentrations in serum and synovial fluid from RA patients and suggested a possible role of NO in the inflammatory responses seen in arthritis (Farrell et al., 1992). The urinary excretion of nitrate, a stable metabolite of NO, is increased in patients with RA and its concentration is reduced towards normal levels after commencement of prednisolone treatment. Furthermore, enhanced NO production and iNOS expression has been reported in several animal models for arthritis (Stichtenoth et al., 1994; Stefanovic-Racic et al., 1994; Weinberg et al., 1994) and NOS inhibitors have been shown to prevent the clinical symptoms of the disease (Stefanovic-Racic et al., 1994).

iNOS expression is induced when human articular chondrocytes are stimulated with IL-1ß, TNF-α or endotoxin, and human iNOS was cloned from human chondrocytes stimulated with IL-1ß. Indeed, human chondrocytes were the second cell type in which human iNOS was cloned and characterized (Charles et al., 1993; Maier et al., 1994). Immunohistochemical studies and in situ hybridization analysis have revealed that both

iNOS protein and mRNA are expressed in synovial cells, in endothelial cells, in inflamed synovium and in chondrocytes (Sakurai et al., 1995).

NO is one of the constituents of the complex network of cytokines and other inflammatory mediators produced in inflamed joints (Moilanen et al., 1999). Cytokines exert a number of effector functions in normal homeostasis and in the development of immune and inflammatory responses. In autoimmune systemic disorders such as RA, there may be an imbalance in the relative quantities of specific cytokines synthesized at the sites of inflammation. Such cytokine dysregulation may be an important contributor to the pathophysiologic mechanism in RA (Lipsky et al., 1989). Whether excessive NO production in inflammatory arthritides is pro-inflammatory or anti-inflammatory is not clear. Pro-inflammatory effects of NO include the generation of destructive free radicals such as peroxynitrite and hydroxyl radical. NO induces the production of angiogenic cytokines and activates matrix metalloproteases (Miyasaka and Hirata, 1997; Moilanen et al., 1999). It degrades cartilage matrix by activating metalloproteases (Murrell et al., 1995), and by suppressing collagen and proteoglygan synthesis (Hauselmann et al., 1994; Taskiran et al., 1994; Murrell et al., 1995; Järvinen et al., 1995). In contrast, it inhibits lymphocyte proliferation and neutrophil function (Moilanen et al., 1993; Kosonen et al., 1997; Kosonen et al., 1998). NO also shows a cytoprotective effect through inactivation of oxygen radicals (Rubanyi et al., 1991). So far, there are only fragmentary data on the production and actions of NO and its derivative peroxynitrite in inflamed joints, suggesting that further studies are needed to understand its role in human arthritis.

## AIMS OF THE STUDY

The aim of the present study was to investigate NO and peroxynitrite production by NO-donors and inflammatory cells.

In detail the aims were:

1. To verify and characterize the NO-releasing properties of GEA 3162 and GEA 3175 (I, II).

2. To characterize the NO, $O_2^{\cdot -}$ and peroxynitrite -producing properties of GEA 3162 as compared to the earlier known NO-donors SIN-1 and SNAP (II).

3. To study NO synthesis from L-arginine and OH-L-Arg in human neutrophils following stimulation with the chemoattractant FMLP (III).

4. To investigate markers of NO and peroxynitrite production in inflamed joints in patients with arthritis, and to elucidate the cytokines and the inflammatory cells which may be involved in NO synthesis in inflamed joints (IV).

## MATERIALS AND METHODS

*1. Platelet aggregation (I)*

Platelet-rich plasma (PRP) was obtained by centrifugation (150 g, 20 min, 20$^{o}$C) from citrated venous blood drawn from healthy volunteers who abstained from all drugs for at least 10 days before sampling. GEA 3162, GEA 3175, SIN-1 or SNAP was added to the PRP (200-400 * $10^6$ platelets $ml^{-1}$) 5 min before platelet aggregation was induced by the addition of adenosine 5'-diphosphate (ADP) at concentrations of 2 – 8 µM (Kankaanranta et al., 1993; Corell et al., 1994).

*2. Neutrophil isolation and treatments (III)*

Neutrophils were isolated from blood samples obtained from healthy adult donors who had abstained from any drugs for at least 14 days before sampling. Buffy coat preparations were obtained from the Finnish Red Cross Blood Transfusion Service. Neutrophils were isolated as follows: a buffy-coat preparation of citrated blood was layered on Ficoll-Paque and centrifuged for 10 min at 3400 *g*, RT. Red blood cells were removed by dextran sedimentation followed by lysis of the remaining red blood cells with Tris-buffered 0.15 M $NH_4Cl$, pH 7.2. Neutrophils were washed twice with Dulbecco's phosphate-buffered saline (DPBS) (Moilanen et al., 1993).

The cell suspensions ($1*10^7 ml^{-1}$ of DPBS) were incubated for 30 min at 37°C with OH-L-Arg or L-arginine. For cGMP measurements, incubations were carried out in the absence or presence of a nonselective phosphodiesterase inhibitor IBMX (250 µM) and zaprinast (10 µM), an inhibitor of cGMP specific –type V phosphodiesterase.

*3. Cyclic GMP (I, III)*

Platelet and neutrophil incubations with NO-donors and/or NOS substrates (L-arginine and OH-L-Arg) were terminated by adding ice-cold trichloroacetic acid and samples were washed four times with water-saturated ethyl ether. They were then neutralized and dissolved with an equal volume of 100 mM sodium acetate buffer, pH 6.2. cGMP was measured by radioimmunoassay (RIA) as previously described (Axelsson et al., 1988; Moilanen et al., 1993).

*4. Conversion of oxyhemoglobin to methemoglobin (I)*

The increase in the concentration of metHb reflects the rate of NO formation. NO-donors were incubated with oxyHb in 50 mM Tris-HCl buffer (pH 7.7). Conversion of oxyHb to metHb was recorded with a double-wavelength spectrophotometer at 406 nm and 411.5 nm (Feelisch and Noack, 1987).

*5. Nitrite and nitrate*

5a) HPLC (I)

NO-donors were incubated in 10 mM phosphate buffer, pH 7.4, 37 $^{o}$C. The nitrite and nitrate formed were measured by HPLC with a UV-detector at 225 nm. We used an ODS 2 reverse phase column with 100 nM NaCl and 10 mM octylamine in $H_2O$, pH 6.2 as a mobile phase, flow rate 1 ml $min^{-1}$.

5b) NO, nitrite and nitrate by ozone-chemiluminescence analyser (I, II, IV)

The freshly dissolved NO-donors (work I and II) were incubated in 50mM phosphate buffer in a glass vial connected to the ozone-chemiluminescence analyser via a Tygontube.

In order to measure the nitrite + nitrate concentrations in serum and synovial fluid samples (study IV), vanadium (III) chloride in hydrochloric acid was used to convert nitrite and nitrate to NO, which was then quantitated by the ozone-chemiluminescence method (Braman and Hendrix, 1989). The samples were first treated with two volumes of ethanol at –20 °C for two hours to precipitate proteins. An aliquot of 20 µl was injected into a cylinder containing saturated solution of $VCl_3$ in 1 M HCl (0.8 g $VCl_3$ per 100 ml of 1 M HCl) at 95 °C and NO formed in these reducing conditions was measured by the NO analyser NOA 280 (Sievers Instruments Inc., Boulder, CO, USA) using sodium nitrate as a standard. When nitrite concentrations were measured, deproteinized samples were injected into a cylinder containing sodium iodide (1 % wt/vol) in acetic acid at room temperature to convert nitrite to NO, which was measured by NO analyser NOA 280 using sodium nitrite as a standard.

5c) Griess reaction (III)

In neutrophil incubations, nitrite was measured acording to Green and associates (Green et al., 1982). Griess reagent (100 µl) was added to an aliquot of sample medium (100 µl). The optical density at 540 nm was measured using a microplate reader. The total nitrite + nitrate concentration in the supernatant was determined by reducing nitrate by nitrate reductase and NADPH (Schmidt et al., 1992; Moshage et al., 1995).

*6. Detection of HbNO complex (I)*

NO-donors were incubated with venous blood on ice. Erythrocytes were transferred to electron paramagnetic resonance (EPR) tubes, and the EPR spectra recorded with an EPR spectrometer (Wennmalm et al., 1990). EPR analyses were made by Ann-Sofi Petersson in the University of Göteborg, Sweden.

*7. Detection of superoxide (II)*

NO-donors were incubated with nitro blue tetrazolium (NBT) in a spectrophotometer cuvette and the reduction of NBT was monitored at 560 nm at 37 $^{o}$C. SOD (100 µM) was added during the linear increase in absorbance (Auclair and Voisin, 1985).

*8. Nitrotyrosine (II,IV) and aminotyrosine (II) by HPLC*

NO-donors were incubated with 100 µM tyrosine in 10 mM phosphate buffer, pH 7.4, 37$^{o}$C for 30 min. The incubates were measured for nitrotyrosine concentration (Kaur and Halliwell, 1994) or reduced by sodium dithionite and acidified by HCl before aminotyrosine measurements. Aminotyrosine was analysed by HPLC with an EC detector. The eluent was 50 mM potassium phosphate buffer (pH 3.0) with 20 % (in study II) or 10 % (in study IV) MeOH (v:v) (Kaur and Halliwell, 1994), and 1.2 g/l heptanesulfonic acid sodium salt monohydrate for aminotyrosine measurements (II). Serum and synovial fluid samples (in study IV) were filtrated through Amicon Centrifree columns to remove proteins before HPLC separation.

*9. Oxygen consumption (II)*

NO-donors were incubated in 10 mM phosphate buffer at 37$^{o}$C and oxygen consumption was measured by a Clark electrode (Clark and Wolf, 1993). The drugs were first dissolved in DMSO and the DMSO-induced low oxygen consumption was subtracted from counted values.

*10. Nitrotyrosine Western blotting (III, IV)*

Neutrophils (III), serum and synovial fluid (IV) were incubated for 15 min in extraction buffer containing proteinase inhibitors at +4$^{o}$C. After centrifugation the supernatants were mixed in sample buffer. Proteins were separated by SDS-PAGE on 8 %

polyacrylamine gels and then transferred to a nitrocellulose filter. The nitrocellulose was blocked with non-fat dry milk. The primary antibody was against nitrotyrosine (rabbit) (1/1000), and the secondary antibody used was anti-rabbit IgG linked to horseradish peroxidase (HRP) (1/3000). The filter was illuminated with ECL chemiluminescence reagent and then exposed to film.

*11. iNOS Western blotting (IV)*

For iNOS Western blotting synovial cells were handled and the blotting done in the same way as for the samples for nitrotyrosine Western blotting (see above). The primary antibody used was against human iNOS (N20) (rabbit) (1/2000) and the secondary was the same anti-rabbit IgG –HPR as for nitrotyrosine (1/3000).

*12. Pre-albumin Western blotting (IV)*

The pre-albumin Western blotting was done from the same samples as nitrotyrosine Western blotting. The primary antibody was human pre-albumin antibody (rabbit) (1/1000), the secondary the same as for nitrotyrosine and iNOS Western blotting (see above) (1/3000).

*13. Amino acids (III, IV)*

Proteins were precipitated by adding 5 % sulfosalicylic acid to the samples (cell sample supernatant in study III and serum and synovial fluid in study IV), centrifuged, and mixed with 0.2M lithium citrate buffer, pH 2.2. Amino acids were separated by ion-exchange chromatography using an automated amino acid analyser. The assays were carried out by means of post column derivatization with o-phthaldialdehyde and subsequent fluorescence detection.

*14. Oxidation of dihydrorhodamine 123 to rhodamine 123 (III)*

Neutrophils were incubated with L-arginine or OH-L-Arg at 37 °C for 5 min and loaded with dihydrorhodamine 123. The cells were stimulated with FMLP for 10 min and the fluorescence measured with a spectrofluorometer at an excitation wavelength of 500nm and an emission wavelength of 536nm.

*15. RNA extraction and reverse transcriptase polymerace chain reaction (RT-PCR) (IV)*

Synovial fluid cell pellets and synovial membranes were purifield using a QIA shredder$^{TM}$, and RNA was extracted using the RNaesy kit. Synthesis of cDNA from extracted RNA and subsequent amplification of cDNA was carried out with the GeneAmp Thermostable r*Tth* Reverse Transcriptase RNA PCR Kit. Primers for amplification of ß-actin, which was used as reference, were 5'-TGACTGACTACCTCATGAAGATCCTCACCG-3'(sense) and 5'-CCACGTCACACTTCATGATGGAGTTG-3'(antisense) resulting in a 304 bp product. Primers for amplification of iNOS were 5'-ATGCCAGATGGCAGCATCAGA-3' (sense) and 5'-CACTTCCTCCAGGATGTTGTA-3'(antisense), resulting in a 372 bp product (Moilanen et al., 1997). Products were analysed on agarose gel containing ethidium bromide and visualized in UV light.

*16. Immunohistochemistry (IV)*

Immunolocalization of iNOS was carried out using cryostat sections of synovial membranes from 6 patients with rheumatoid arthritis. Cryostat sections (5-6 μm thick) were cut from 3-4 different blocks of tissue. The sections were mounted on "Polysine Slides" (BDH). After drying for 1 hour at room temperature, the sections were fixed briefly in acetone/methanol fixative (50/50) at -20°C and washed with 0.05 M Tris-HCl buffered saline pH 7.6, then immunostained by the biotin-streptavidin alkaline

phosphatase technique. The sections were incubated for 18 hours with low concentrations of primary antibodies that included the polyclonal sheep IgG antibody to human iNOS (1/500) (Moilanen et al., 1997), or normal sheep IgG (1/1000) as a negative control. This was followed by 1 hour incubation with biotinylated rabbit anti sheep IgG antibody**.**

*17. Measurement of CRP, IL-1ß, IL-6, TNF-α and IFN-γ(IV).*

Aliquots of serum and SF were frozen until assays. The concentrations of IL-1ß, IL-6, TNF-α and IFN-γ were determined by ELISA using reagents from Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, the Netherlands. Serum C reactive protein (CRP) was determined immunoturbidimetrically with Cobas Integra Analysator.

*18. Patients (IV)*

Serum and SF samples were collected at a single visit from 31 patients with active synovitis and 21 age- and sex-matched healthy controls. Twenty-one of these patients were diagnosed as having RA and as rheumatoid factor-positive, five had seronegativ arthritis, and five had spondylarthropathy. Macroscopically inflamed synovial membranes were obtained from six RA patients during arthroscopic synovectomy. For immunohistochemistry macroscopically inflamed synovia was collected from six RA patients undergoing knee replacement surgery.

*19. Drugs and chemicals*

GEA 3162, GEA 3175, SIN-1 and SNAP were provided by GEA, Copenhagen, Denmark. They were always freshly prepared in DMSO immediately prior to the experiments. In experiments the final concentration of DMSO did not exceed 1 %. ADP, bovine hemoglobin, superoxide dismutase, tyrosine, 3-nitro-L-tyrosine, 3-amino-

L-tyrosine, nitrate reductase were ontained from Sigma, St. Louis, MO. $N^G$-hydroxy-L-arginine.monoacetate (Alexis, San Diego, CA) and heptanesulfonic acid sodium salt monohydrate (Fluka Chemie, Buchs, Switzerland) were obtained as indicated. Nitrotyrosine antibody was obtained from Upstate Biotechnology, Saranac Lake, NY. Pre-albumin was from Novocastra Laboratories Ltd, UK and iNOS antibody (rabbit polyclonal IgG, N20) and anti-rabbit IgG-HPR were obtained from Santa Cruz Biotechnology, Santa Cruz, CA. ECL chemiluminescence reagent was from Amersham International, Buckinghamshire, U.K.

*20. Statistics*

Results are expressed as mean ± SEM. Statistical significance was calculated by one-way analysis of variance supported by Bonferoni significance levels. Student's paired t-test and linear regression test were used in study IV. Differences were considered significant when $P < 0.05$.

## SUMMARY OF RESULTS

### 1. NO, $O_2^{\cdot -}$,and peroxynitrite releasing properties of GEA 3162 and GEA 3175 as compared to SIN-1 and SNAP (I,II)

Biological properties

GEA 3162, GEA 3175, SIN-1 and SNAP inhibited ADP-induced platelet aggregation and all four compounds enhanced cGMP production in platelets in a dose-dependent manner (Table 1.). The increase in cyclic GMP was potentiated about twofold by the phosphodiesterase inhibitor zaprinast and inhibited by the NO scavenger oxyHb.

Chemical properties

GEA 3162 and GEA 3175 as well as SIN-1 and SNAP induced conversion of oxyHb to metHb. Also, all compounds formed paramagnetic NO-hemoglobin complexes. All but GEA 3175 resulted in the formation of nitrite and nitrate in phosphate buffer. During 40 min incubation, GEA 3162, SIN-1 and SNAP (100µM) produced 50-70 µM nitrite and nitrate in phosphate buffer. GEA 3175 did not release detectable amounts of nitrite and nitrate in buffer solutions, but produced NO and nitrite when 1 % of plasma was added to the incubation buffer.

$O_2^{\cdot -}$ release from GEA 3162, SIN-1 and SNAP was measured by the reduction of nitro blue tetrazolium (Auclair and Voisin, 1985). SIN-1 caused a dose-dependent reduction of nitro blue tetrazolium which was inhibitable by addition of SOD. No reduction of nitro blue tetrazolium was detected in the solutions of GEA 3162 and SNAP. We measured nitrotyrosine and aminotyrosine as markers of peroxynitrite production. In the solutions of SIN-1 (100 µM, 500 µM), nitrotyrosine and aminotyrosine were clearly found. In the solutions of GEA 3162 up to 500 µM no nitrotyrosine was found and only trace amounts of aminotyrosine were detectable. Nitrotyrosine or aminotyrosine were not found in the solutions of SNAP.

| NO | GEA 3162 | GEA 3175 | SIN-1 | SNAP |
|---|---|---|---|---|
| cGMP in platelets | ↑↑↑↑ | ↑↑ | ↑ | ↑↑↑ |
| metHb formation | ↑↑↑↑ | ↑ | ↑↑ | ↑↑↑ |
| platelet aggregation | ↓↓↓↓ | ↓↓↓ | ↓ | ↓↓ |
| production of NO | ↑↑ | ↑↑↑* | ↑ | ↑↑↑↑ |
| **ONOO⁻** | | | | |
| $O_2^{\cdot-}$ | ↑ | NT | ↑↑↑↑ | ND |
| nitrotyrosine | ND | NT | ↑ | ND |
| aminotyrosine | ↑ | NT | ↑↑↑ | ND |
| oxygen consumption | ↑↑ | NT | ↑↑↑↑ | ↑ |

NT = not tested, ND = not detectable
* needs plasma for degradation

Table 1. The order of potency of NO-donors in biological and chemical measurements testing the NO-, $O_2^{\cdot-}$- and peroxynitrite-releasing properties of GEA 3162, GEA 3175, SIN-1 and SNAP.

**2. Lack of NO production by FMLP-activated neutrophils incubated with OH-L-Arg and L-arginine**

Incubation of FMLP-activated human peripheral blood neutrophils with OH-L-Arg (100-1000 µM) resulted in the production of nitrite, nitrate and citrulline in a dose-dependent manner. The production of these substances was not inhibitable by the NOS inhibitors L-NMMA (1mM) or L-NIO (1mM), nor by the cytochrome$P_{450}$ inhibitor troleandomycin (100 µM). There was no detectable nitrite, nitrate or citrulline production when OH-L-Arg was replaced with L-arginine (1000 µM), the common substrate for NOS. Incubation of FMLP-activated neutrophils with OH-L-Arg or L-arginine did not produce detectable amounts of cGMP even in the presence of

phosphodiesterase inhibitors or SOD. Moreover, no signs on peroxynitrite formation were found, as measured by tyrosine nitration and dihydrorhodamine 123 oxidation.

### 3. Markers of NO and peroxynitrite production in inflamed joints during exacerbation phases of arthritis

The concentration of nitrite was significantly higher in synovial fluid than in serum in patients with arthritis. There was a significant positive relationship between SF nitrite and IFN-γ concentrations ($r = 0.61$, $p < 0.001$). Synovial membrane samples from inflamed joints were positive for iNOS protein and mRNA. On the other hand, SF cells from arthritis patients were negative for iNOS mRNA and iNOS protein when studied by RT-PCR and Western blot, respectively. The serum concentrations of nitrite in patients with arthritis were significantly higher than those in healthy subjects. The citrulline concentrations in SF from patients with arthritis were significantly higher than those in serum (SF: 24.20 ± 1.79 μM; serum: 22.29 ± 1.35 μM; $p < 0.05$). The difference in the citrulline levels (1.91 ± 0.79 μM) was about the same as that in the $NO_x$ concentrations between SF and serum (1.40 ± 0.69 μM), suggesting that citrulline and nitrite (NO) are produced at equimolar concentrations in inflamed joints. Also, nitrated proteins were present in serum and SF samples from arthritis patients, and the amount of nitrated proteins in the serum was increased in the patients as compared to healthy controls.

## DISCUSSION

### 1. Methodology

In the present study several techniques were used to detect NO release from the novel mesoionic 3-aryl-substituted oxatriaxole-5-imine derivatives GEA 3162 and GEA 3175 as compared to SIN-1 and SNAP. To compare the biological activity of NO-donors and

to demonstrate the biological activity of NO the cGMP production in platelets (I) and neutrophils (III) was measured. Production of cGMP is a sensitive measure of NO synthesis even at the low physiological levels of NO production (Moncada et al., 1991). Our results show that NO-donors increased cGMP production in a dose-dependent manner in platelets. GEA 3162 and SNAP were more potent than equimolar concentrations of GEA 3175 and SIN-1 in increasing cyclic GMP concentrations. cGMP results suggested that NO was not produced from OH-L-Arg or L-arginine by FMLP -activated neutrophils. In these neutrophil experiments, NO-donors GEA 3162 and SNAP were used as the positive controls, which clearly enhanced cGMP production in neutrophils (Axelsson et al., 1988; Moilanen et al., 1993).

Determination of NO itself is difficult by reason of its radical nature and very short half-life (Knowles, 1997). Thus determination of the stable end products of NO, nitrite and nitrate, in plasma is most often used as a measure for the production of NO. The method most commonly used is based on the determination of nitrite by the Griess reaction (Moshage et al., 1995; Miles et al., 1996). In this simple assay a large number of samples can be processed in a short period of time. Nitrate can be measured as nitrite after enzymatic or chemical conversion. NO and nitrite in whole blood are rapidly oxidized to nitrate by hemoglobin in erythrocytes (Wennmalm et al., 1993), whereas in the absence of hemoglobin nitrite seems to be the principal metabolite in aqueous solutions (Ignarro et al., 1993). Thus the determination of nitrite + nitrate in plasma by the Griess assay may be used as an indicator for NO production (Moshage et al., 1995). The clinical value of nitrite+nitrate determinations in serum is limited because of variations in the dietary intake of nitrate. The nitrite levels in the body fluids, which do not contain hemoglobin, for example SF, may be a more reliable measure of local NO production. Low concentrations of nitrite in body fluids require a sensitive and spesific method like ozone chemiluminescence. Determination of NO by the ozone-chemiluminescence method is one of the most accurate NO assays available (Hampl et al., 1996). In the fourth study in our series we found elevated nitrite levels in SF

samples from patients with arthritis, which suggests local NO production in inflamed joints.

It has been proposed that the oxyHb method is not specific for NO since peroxynitirite has been found to indicate a similar spectral change in the assay (Schmidt et al., 1994). In our assay the peroxynitrite donor SIN-1 oxidized oxyHb in a similar manner as reported earlier (Feelisch and Noack, 1987). Thus oxyHb method does not rule out the possibility that the oxatriazole derivatives as well as SIN-1 release peroxynitrite.

One of the main purposes of this study was to establish whether GEA 3162 is a pure NO-donor or a SIN-1 -like peroxynitrite donor. We used methods for peroxynitrite measurement also in the third study to assess NO production by activated neutrophils: We found no increased cyclic GMP production in FMLP-stimulated neutrophils despite the production of nitrite, nitrate and citrulline. We thus sought to rule out the possibility that the NO formed was oxidized to peroxynitrite. To study the peroxynitrite formation by NO-donors and neutrophils and in patients with arthritis we needed to establish new methods in our laboratory. Peroxynitrite is a highly reactive molecule and there are no appropriate methods to measure it under physiological conditions. Peroxynitrite can be assayed indirectly by measuring nitrotyrosine (Kaur and Halliwell, 1994). Using the HPLC method with a UV detector we discerned some nitrotyrosine in the incubates of SIN-1 but not in those of GEA 3162 or SNAP. To increase sensitivity, we reduced nitrotyrosine to aminotyrosine, which could be quantitated by electrochemical detector. The sensitivity was enhanced by the factor of 60. By aminotyrosine method we were able to prove clearly that nitrotyrosine was formed in the incubations of SIN-1. In the incubation of GEA 3162 no nitrotyrosine and a barely detectable aminotyrosine peak was seen.

Oxidation of dihydrorhodamine 123 to rhodamine 123 has been successfully used as a marker of peroxynitrite production in various experiments (Kooy et al., 1994; Zingarelli et al., 1996). The technique is not, however, specific for peroxynitrite, since other

oxidating species such as hydrogen peroxide can result in the oxidation of dihydrorhodamine 123 to rhodamine 123. To overcome this problem, the measurements of NOS inhibitor –inhibitable oxidation of the probe and measurements of other markers of peroxynitrite, such as nitrotyrosine formation, have been recommended (Szabo, 1996). We developed a Western blotting method for measuring nitrotyrosine. Neither of these two methods demonstrated signs of peroxynitrite production in activated neutrophils incubated in the presence of OH-L-Arg or L-arginine under experimental conditions where nitrite, nitrate and citrulline production was evident. The Western blot of nitrotyrosine was successfully used in the fourth study. We were able to demonstrate that protein-bound nitrotyrosine was present in serum and SF from patients with arthritis, and that it is elevated in serum samples in arthritis patients as compared to healthy subjects.

**2. NO and peroxynitrite release from GEA 3162 and GEA 3175**

The first study was designed to verify and characterize the NO-releasing properties of the 3-aryl-substituted oxatriaxole-5-imine derivatives GEA 3162 and GEA 3175. For comparison we used two earlier known NO-releasing compounds, namely SIN-1 and SNAP. SIN-1 has been shown to release both NO and $O_2^{\cdot -}$, resulting in the formation of peroxynitrite, whereas nitrosothiol SNAP releases only NO (Feelisch, 1991; Hogg et al., 1992).

Platelet aggregation is known to be regulated by NO in a cGMP-dependent manner (Moncada et al., 1991; Wennmalm, 1994). GEA 3162, GEA 3175, SIN-1 and SNAP inhibited ADP-induced platelet aggregation, thus confirming their biological activity. Also, the ability of NO-donors to increase cGMP in platelets was studied. Both oxatriazole derivatives as well as SIN-1 and SNAP induced a marked increase in cGMP concentration in a dose-dependent manner. This increase was significantly potentiated by the specific type V phosphodiesterase inhibitor zaprinast, which prevents cGMP degradation by phosphodiesterases. Accordingly, the increase in cGMP concentration

was inhibited by oxyHb, which inhibits the action of NO by binding it to form metHb (Kelm and Yoshida, 1996). These are biochemical features of NO and suggest that the compounds release NO. Accordingly, GEA 3162, GEA 3175 as well as SIN-1 and SNAP induced the conversion of oxyHb to metHb and induced the formation of paramagnetic NO-hemoglobin complexes. These data strongly suggest that GEA 3162 and GEA 3175 release NO in functionally active form.

One of the main purposes of this study was to establish whether GEA 3162 is a pure NO-donor, not a SIN-1-like peroxynitrite donor. Reaction between NO and $O_2^{\cdot -}$ under physiological conditions leads to the formation of peroxynitrite. $O_2^{\cdot -}$ release from GEA 3162, SIN-1 and SNAP was measured by the reduction of nitro blue tetrazolium (Auclair and Voisin, 1985). SIN-1 was significantly different from GEA 3162 and SNAP in its ability to accomplish this reduction. The ability of SIN-1 to produce peroxynitrite was inhibited by SOD, suggesting that it was due to production of $O_2^{\cdot -}$. Our results confirm the earlier reported formation of $O_2^{\cdot -}$ from SIN-1 (Feelisch et al., 1989) and suggest that GEA 3162 and SNAP do not release $O_2^{\cdot -}$. Also, we measured nitrotyrosine and aminotyrosine as markers of peroxynitrite production. In the incubates of GEA 3162 no nitrotyrosine and barely detectable amounts of aminotyrosine were found and SNAP did not either trigger production of nitro- or aminotyrosine. SIN-1 produced both nitro- and aminotyrosine, which confirms the earlier documented formation of peroxynitrite in solutions of SIN-1 (Feelisch et al., 1989; Hogg et al., 1992).

GEA 3175 did not form measurable amounts of nitrite or nitrate in phosphate buffer, but was able to release NO and $NO_2$, even more than GEA 3162, when measured by ozone chemiluminescence in the presence of human plasma. The lack of formation of nitrite and nitrate in phosphate buffer, as well as the low activity in the oxyHb assay, may indicate a demand for enzymatic degradation of the sulphonamide moiety (Karup et al., 1994) or the presence of thiols (Cohen et al., 1995) before NO can be released. GEA

3162 differs from GEA 3175 in this respect, and our results suggest that GEA 3162 releases NO spontaneously in aqueous solutions.

SIN-1 decomposes to produce NO via an oxygen-dependent process. SIN-1 undergoes a rapid nonenzymatic hydrolysis to the ring-open form SIN-1A. Oxygen promotes oxidative conversion to a cation radical intermediate from which NO is released and more stable SIN-1C is formed. In the course of this reaction stoichiometric amounts of $O_2^{\cdot -}$ anions are formed as a result of oxygen reduction (Feelisch et al., 1989). We could also show that in a chemical process leading to NO release from SIN-1 oxygen is consumed from incubation solution. SIN-1 consumed 10 times more oxygen than GEA 3162 during the 20 min incubation, indicating that the mechanisms of NO release from these two compounds are different. The low oxygen consumption in solutions of GEA 3162 is in line with the low if any $O_2^{\cdot -}$ and peroxynitrite production during the NO-releasing process.

These finding suggest that GEA 3162 and GEA 3175 release NO by but probably by different mechanisms. GEA 3162 produces negligible amounts of $O_2^{\cdot -}$ and peroxynitrite along with NO release. This adds to the value of GEA 3162 as a useful tool in NO research.

### 3. OH-L-Arg metabolism in FMLP-stimulated human pheripheral blood neutrophils

The present data show that OH-L-Arg, an intermediate in the biosynthesis of NO, is metabolized to nitrite, nitrate and citrulline in the presence of activated human neutrophils. The same concentration of L-arginine, the substrate for NOS, did not result in significant formation of nitrite, nitrate of citrulline. NOS inhibitors L-NIO and L-NMMA did not inhibit the formation of these markers of NO synthesis from OH-L-Arg. Elevated concentrations of OH-L-Arg in serum have been reported in patients with RA (Wigand et al., 1997). Increased nitrite production was reported in the absence of NOS

activity in rat vascular smooth muscle cells supplemented with OH-L-Arg (Schott et al., 1994). Chytochrome $P_{450}$ has been shown to catalyse the oxidation of OH-L-Arg to NO and citrulline, suggesting another mechanism of NO formation from OH-L-Arg not involving NO synthase. According to these results we hypothesied that activated human neutrophils produce NO from exogenous OH-L-Arg by a mechanism not sensitive to NOS inhibitors. These data raise the possibility of the presence of a NO-producing mechanism different from the classical L-arginine/NO pathway in activated human neutrophils.

The critical step in testing this hypothesis was to confirm whether NO is produced under these incubation conditions. To demonstrate the biological activity of NO we measured cGMP production in neutrophils. No increase in cGMP production was found in activated neutrophils incubated in the presence of OH-L-Arg in conditions where nitrite, nitrate and citrulline production was evident, suggesting that NO was not produced. When human neutrophils are activated with FMLP, the $O_2^{\cdot -}$ generating system is activated (Schmidt et al., 1989; Goode et al., 1994). If neutrophils produce NO and $O_2^{\cdot -}$ simultaneously, the production of peroxynitrite is possible and it might explain the negative result of cGMP experiments. Therefore, formation of peroxynitrite was used as another measure of NO production in these experiments. However, we found no peroxynitrite production in activated human neutrophils.

NO production from OH-L-Arg in a NOS-independent manner has been reported. OH-L-Arg was transformed by $O_2^{\cdot -}$ to nitrite, nitrate and citrulline independently if $O_2^{\cdot -}$ was generated chemically or produced by activated murine macrophages. This mechanism is not possible in our study, because it has been reported (Vetrovsky et al., 1996) that NO is generated as an intermediate in the decomposition of OH-L-Arg by $O_2^{\cdot -}$. cGMP production and NO-hemoglobin complex formation evidenced this. Cytochrome $P_{450}$ has been shown to catalyse the formation of nitrite and citrulline from OH-L-Arg in rat liver (Boucher et al., 1992; Renaud et al., 1993). This mechanism is not a likely explanation for our findings, since troleandomycin, an inhibitor of $P_{450}$, was ineffective

in our experiments. This does not rule out a mechanism involving some other oxidative enzymes able to oxidise OH-L-Arg in FMLP-activated neutrophils. Another possible explanation arises from the findings of Clague and associates (Clague et al., 1997) and a group under Pufahl (Pufahl et al., 1995). They found that NOS catalyses the oxidation of OH-L-Arg but not L-arginine by hydrogen peroxide. When hydrogen peroxide acts as an oxidant, NOS produces cyano-ornithine, citrulline and $NO_x$ from OH-L-Arg. By contrast, when NADPH and $O_2$ are involved in the reaction, NOS catalyses the exclusive formation of citrulline and NO from both OH-L-Arg and L-arginine. The reaction with hydrogen peroxide might be involved in our experimental system, since hydrogen peroxide is produced by FMLP-activated neutrophils. Unfortunately, we were not able to measure cyano-ornithine; further experiments are thus needed to test whether this hypothesis might explain our observations of OH-L-Arg metabolism in activated human neutrophils.

We were unable in the present series to demonstrate NO production from FMLP-activated human neutrophils in the presence of OH-L-Arg or L-arginine. The lack of NO synthesis in human neutrophils confirms earlier findings elsewhere (Yan et al., 1994; Padgett and Pruett, 1995; Miles et al., 1995; McBride and Brown, 1997). Accordingly we were not able to detect iNOS mRNA or iNOS proteins in SF cells from patients with active synovitis. In contrast to our resuts, some authors have demonstrated markers of NO production in human neutrophils (Auclair and Voisin, 1985; Schmidt et al., 1989; Faint et al., 1991; Goode et al., 1994; Lärfars and Gyllenhammar, 1995; Nath and Powledge, 1997). It is not clear whether this is due to differences is experimental conditions or between blood donors. The presence of contaminating platelets could also explain positive NO production in neutrophil experiments. Our results clearly demonstrate the importance of the use of NOS inhibitors and different methods to confirm NO production.

## 4. NO production in arthritis

In the present study we sought an answer to the queston whether SF cells in inflamed joints produce NO via the iNOS pathway. Polymorphonuclear cells are associated with acute bacterial inflammation. Also, the exacerbation phases of arthritis are characterized by a large number of polymorphonuclear cells accumulating in the joint. As described above, the presence of iNOS and the role of NO in the function of human phagocytes has been difficult to demonstrate (Moilanen et al., 1997) even though it is well known that NO produced by the iNOS pathway in activated phagocytes is an important effector molecule in the rodent immune response (McCall et al., 1989; Kolls et al., 1994; MacMicking et al., 1997). iNOS activity has also been reported in human neutrophils isolated from the oral cavity (Sato et al., 1996) and in urine from patients with urinary track infections (Wheeler et al., 1997). The present results showed clearly that SF leukocytes were negative for iNOS protein and mRNA.

We also found that iNOS mRNA and protein were expressed in inflamed synovial membrane from patients with active rheumatoid synovitis. iNOS expression was clear in the pannus cells. This is in line with earlier findings indicating that in *ex vivo* organ cultures, both inflammatory synovium and cartilage explants from patients with RA, produce NO (Sakurai et al., 1995). In addition, bacterial products and inflammatory cytokines such as LPS, IL-1, TNF-α and IFN-γ have been shown to stimulate NO production in condrocytes, indicating that in addition to inflamed synovium also cartilage may be a significant source of NO in inflamed joints (Stadler et al., 1991; Charles et al., 1993; Palmer et al., 1993; Rediske J.J. et al., 1994; Järvinen et al., 1995).

Our results demonstrated a significant positive correlation between SF nitrite and IFN-γ concentrations. The correlation between NO production and IFN-γ levels is consistent with *in vitro* data showing that iNOS expression triggered by inflammatory stimuli is enhanced by IFN-γ (Xie et al., 1993; Kamijo et al., 1994; Spitsin et al., 1996). Both

transcriptional and post-transcriptional mechanisms for the enhancing effect of IFN-γ on iNOS expression have been described. The promoter region of the iNOS gene contains a binding site for interferon regulatory factor-1 (IFR-1), which is not necessary but rather an enhancing factor for iNOS transcription and responsible for the IFN-γ –dependent transcriptional regulation of iNOS (Martin et al., 1994). In addition, IFN-γ has been shown to prolong the half-life of iNOS mRNA possibly by preventing the synthesis of factors critical for the iNOS mRNA destabilization process (Spitsin et al., 1996). Therefore the correlation between NO production and IFN-γ concentrations in inflamed joints may be functionally significant.

The presence of nitrotyrosine, as a marker of peroxynitrite production, was studied in serum and SF proteins. There is an increasing amount of evidence to support the role for peroxynitrite as a mediator of tissue damage in inflammation (Beckmann et al., 1994; Kaur and Halliwell, 1994; Salvemini et al., 1998). A significant amount of nitrotyrosine was detected in a 55 kD protein, which according to its molecular weight might be pre-albumin. The amout of protein-bound nitrotyrosine was higher in serum samples from patients with arthritis than in those from healthy controls. SF also contained some free nitrotyrosine but the leves were not significantly different from those found in serum samples. These data show that protein-bound and free nitrotyrosine as a marker of peroxynitrite formation is present in serum and SF from arthritis patients.

## SUMMARY AND CONCLUSIONS

The purpose of the first part of the study was to verify and characterize the NO-, $O_2^{\cdot -}$- and peroxynitrite-releasing properties of the novel mesoionic 3-aryl-substituted oxatriazole-5-imine derivatives. In the further studies we measured L-arginine and OH-L-Arg metabolism in activated human neutrophils and evaluated the markers of NO and peroxynitrite production in inflamed joints.

The major findings and conclusions are as follows:

1. GEA 3162 GEA 3175, SIN-1 and SNAP inhibited platelet aggregation and induced a dose-dependent increase in cGMP in platelets. The increase in cGMP concentration was potentiated by phosphodiesterase inhibitors and inhibited by oxyhemoglobin. Platelet aggregation is known to be regulated by NO in a cGMP-dependent manner. Our results suggest that GEA 3162 and GEA 3175 as well as SNAP and SIN-1 release NO.

2. GEA 3162 releases NO spontaneously in aqueous solutions, whereas it produces negligible amounts of $O_2^{\cdot -}$, and peroxynitrite as compared to SIN-1 indicating that it resembles more the “clean” NO-donor SNAP. This adds to the value of GEA 3162 as a tool in NO research.

3. FMLP-stimulated human peripheral blood neutrophils did not produce detectable amounts of NO measured as cGMP production or as peroxynitrite formation in the presence of the NOS substrate L-arginine or its intermediate OH-L-Arg.

4. In the presence of OH-L-Arg but not in the presence of L-arginine FMLP-stimulated human peripheral blood neutrophils produced nitrite, nitrate and citrulline without signs of simultaneous NO synthesis. This metabolism of OH-L-Arg was not inhibitable by NOS or $P_{450}$ inhibitors, indicating that NOS or $P_{450}$ activity was not

involved in nitrite, nitrate or citrulline production from OH-L-Arg in human neutrophils.

5. In patients with arthritis, nitrite, $NO_x$ and citrulline concentrations were significantly higher in SF than in serum, indicating local NO production in inflamed joints. iNOS was expressed in synovial membrane, especially in the pannus, but not in SF cells. Compared to healthy subjects, serum concentrations of nitrite were significantly higher in samples from arthritis patients. In addition, protein bound nitrotyrosine, a marker of peroxynitrite production, was detected in serum and SF from patients with arthritis and its amount was elevated in serum samples from arthritis patients as compared to healthy controls. These data suggest that NO and peroxynitrite are formed in inflamed joints.

6. Synovial fluid nitrite concentrations were directly correlated with the IFN-γ levels, which is interesting because IFN-γ is involved in the upregulation of iNOS expression.

7. iNOS expression in human cells has been a matter of controversy. We were able to demonstrate that iNOS is involved in active synovitis, whereas neutrophils activated by FMLP did not produce NO despite the formation of nitrite, nitrate and citrulline, from OH-L-Arg.

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