1997

**Some biochemical aspects of nitric oxide in biological systems.**

Christopher Michael. Howard  
*University of Windsor*

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SOME BIOCHEMICAL ASPECTS OF NITRIC OXIDE

IN BIOLOGICAL SYSTEMS

by

Christopher Michael Howard

A Thesis
Submitted to the Faculty of Graduate Studies and Research
through the Department of Chemistry and Biochemistry
in Partial Fulfillment of the Requirements for the
Degree of Master of Science at the
University of Windsor

Windsor, Ontario, Canada

1997
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ABSTRACT

SOME BIOCHEMICAL ASPECTS OF NITRIC OXIDE
IN BIOLOGICAL SYSTEMS

by

Christopher Michael Howard

The role of nitric oxide (NO) in biological systems is becoming increasingly important. NO has been linked in smooth muscle relaxation, inhibition of platelet aggregation, alterations in neurotransmission and cell-mediated cytotoxicity. Initially, the effect of NO on the transport of L-arginine into human platelets was studied. NO is known to have an effect on this system, and platelets have been shown to possess an isoform of the enzyme nitric oxide synthase. Two NO donors (S-nitrosoglutathione and S-nitroso-N-acetyl penicillamine), a source of NO$^-$ and a solution of authentic NO were used to measure the effect of NO on the L-arginine transport system. The results of these experiments implicate a role of GSNO and GSSG in affecting the transport of L-arginine into the platelet. The mechanism that we are proposing supports our findings showing that GSNO or GSSG can increase the efficiency (Vmax/Km) of L-arginine transport, but the maximal increase is observed under conditions where GSNO and GSSG can act on this system.

The toxicity of nitric oxide can be due to its direct effects, or more likely through higher oxidation products such as peroxynitrite (OONO$^-\$). There is evidence for peroxynitrite production in vivo, and this has been linked to an assortment of pathological conditions. An indirect method of peroxynitrite detection is the formation nitrotyrosine.
We have found evidence for nitrotyrosine formation in platelet proteins from patients suffering from prolonged hyperglycemia. Calmodulin is one of the proteins implicated in this peroxynitrite modification. Alterations in calmodulin structure and function could provide some explanation in the hyperaggregation of platelets in diabetic individuals.

The effect of peroxynitrite on the enzyme glutathione S-transferase was also studied. Results show that incubation with a 100 molar excess of peroxynitrite inhibits the activity of the enzyme to approximately 55% of the native enzyme. Pre-incubation of either of the substrates with the enzyme offers some protection to this peroxynitrite modification, indicating that the some of the residues being modified by peroxynitrite are located within the catalytic site of the enzyme.
DEDICATION

"Do not believe in anything simply because you have heard it. Do not believe in anything simply because it is spoken and rumored by many. Do not believe in anything simply because it is found written in your religious books. Do not believe in anything merely on the authority of your teachers and elders. Do not believe in traditions because they have been handed down for many generations. But after observation and analysis, when you find that anything agrees with reason and is conducive to the good and benefit of one and all, then accept it and live up to it."

Buddha

"Twenty years from now you will be more disappointed by the things that you didn't do than by the ones you did do. So throw off the bowlines. Sail away from the safe harbor. Catch the trade winds in your sails. Explore. Dream. Discover."

Mark Twain
ACKNOWLEDGEMENTS

First and foremost, I am indebted to my research supervisor, Dr. Bulent Mutus, not only for his ideas and insight, but for the support throughout my graduate career at the University of Windsor. I would also like to thank the members of my supervisory committee, Dr. James Green and Dr. Alden Warner for their ideas and constructive criticisms.

During the course of this work, the staff at Hotel-Dieu/Grace hospital was extremely helpful in always providing blood samples whenever I requested. A special thanks must go to Mrs. Sally Lewis for her assistance in setting up blood collections from diabetic patients, and her care for me as a patient in her clinic.

My time here would not have been nearly as interesting without the people that I encountered along the way. I would like to thank my labmates Marie Tannous and Peter Pathrose for making my time in the lab a memorable one. I would also like to thank all of the other students who I met in the department, especially Chris Deslippe, Domenic Perri, Dave Michels, and all the members of our softball dynasty. Victory is sweet.

Finally I would like to thank my family, both my “nuclear” family and my “adopted” family here in Windsor. I could never have made it this far without your support Mom, Dad and Jen, and I appreciate all that you have done for me. I never would have made it through my time in Windsor without the kind support and hospitality extended to me by the Khayat family; Michel, Nadia, Roula, Said and Reama. And of course, to Zayna, who kept me going when I could go no more. A greater person I have never met.
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<tr>
<td>ALS</td>
<td>amyotrophic lateral sclerosis</td>
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<tr>
<td>BCIP</td>
<td>5-bromo-4-chloro-3-indoyl phosphate</td>
</tr>
<tr>
<td>CaM</td>
<td>calmodulin</td>
</tr>
<tr>
<td>CDNB</td>
<td>1-chloro-2,4-dinitrobenzene</td>
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<tr>
<td>GSH</td>
<td>glutathione (reduced)</td>
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<td>GSNO</td>
<td>S-nitrosoglutathione</td>
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<tr>
<td>GSSG</td>
<td>glutathione (oxidized)</td>
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<td>GST</td>
<td>glutathione S-transferase</td>
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<td>HEPES</td>
<td>N-(2-hydroxyethyl)piperazine-N’-(2-ethanesulfonic acid)</td>
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<td>IDDM</td>
<td>insulin-dependent diabetes mellitus</td>
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<tr>
<td>NBT</td>
<td>nitroblue tetrazolium</td>
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<td>NOBF₄</td>
<td>nitrosonium tetrafluoroborate</td>
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<td>NOS</td>
<td>nitric oxide synthase</td>
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<td>polyacrylamide gel electrophoresis</td>
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<td>PBS</td>
<td>phosphate buffered saline</td>
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<td>SDS</td>
<td>sodium dodecyl sulfate</td>
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<td>SNAP</td>
<td>S-nitroso-N-acetyl penicillamine</td>
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<td>SOD</td>
<td>superoxide dismutase</td>
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Chapter 1

INTRODUCTION

1.1. Nitric Oxide

For many years, free radicals were thought to be too reactive to participate in normal biochemical processes. However, the discovery of the enzyme superoxide dismutase (SOD) (McCord & Fridovich, 1969) led to a new and vast area of research. SOD catalyses the reaction forming hydrogen peroxide from superoxide:

\[ O_2 + e^- \xrightarrow{SOD} O_2^{\cdot \cdot} \xrightarrow{SOD} 1/2 O_2 + 1/2 H_2O_2 \]

With this revelation, it naturally follows that other free radicals would also be found that participate in a variety of biochemical reactions. One such free radical is the inorganic gas nitric oxide ($\cdot\cdot$NO).

Nitric oxide is a colourless gas and contains fifteen electrons, seven from nitrogen and eight from oxygen. This leaves one unpaired electron, making NO a paramagnetic molecule. The solubility of NO in water at 25°C and 1 atm is $1.8 \times 10^{-3}$ M, which remains unchanged over pH 2-13 (Butler and Williams, 1993). NO can be considered to have three
redox states: the radical NO₂⁺, removal of one electron resulting in NO⁻ (nitrosonium) and the addition of one electron yielding NO⁻ (nitroxide). The instability of free radicals makes NO extremely reactive, and under physiological conditions NO can convert between its three different redox states. Due to this reactivity, NO can participate in many physiological processes.

The biological half-life of nitric oxide was initially assumed to be low, due to its radical nature. It is now known that the half-life of NO depends on its initial concentration (Stamler et al, 1992b). In PBS the half-life of NO is approximately 130 seconds, when [NO] is 10-20 μM and [O₂] is in 5-10 fold excess (Ford et al, 1993). In the gas phase the reaction of NO with O₂ is second order with respect to NO, r = k[NO]²[O₂] (Stamler et al, 1992b). The rate law remains identical in solution and k = 5 x 10⁶ dm⁶ mol⁻² s⁻¹. Therefore, NO at a concentration of 10⁻⁷M in aqueous solution has a half-life of nearly 3 hours (Butler and Williams, 1993). NO reacts with O₂ as follows:

\[
\begin{align*}
\text{NO} + \frac{1}{2} \text{O}_2 & \rightarrow \text{NO}_2 \\
\text{NO}_2 + \text{NO} & \rightarrow \text{N}_2\text{O}_3 \\
\text{N}_2\text{O}_3 + \text{H}_2\text{O} & \rightarrow 2 \text{HNO}_2 \rightarrow 2 \text{H}^+ + 2 \text{NO}_2^- \end{align*}
\]

NO acts as a widespread second messenger that exerts its effects through the stimulation of the enzyme guanylate cyclase. NO binds to the heme moiety of this enzyme. The stimulation of this enzyme leads to an increase in cGMP concentration which in turn lowers intracellular calcium levels. This causes smooth muscle relaxation, inhibition of
platelet aggregation, and alterations in neurotransmission (Moncada et al., 1991). NO is a diffusible molecule, therefore it can be produced in one cell and exert its actions in another (Fig. 1). The ability of NO to readily diffuse also means that no specific transporter is required for its entry into a cell.

![Diagram of L-arginine to NO pathway]

**Fig. 1** - The L-arginine/nitric oxide pathway. Abbreviations: NOS-nitric oxide synthase; Heme GC-heme moiety of guanylate cyclase; GTP-guanosine triphosphate; cGMP-cyclic guanosine monophosphate (MacAllister and Vallance, 1996).

1.2. *EDRF and Nitric Oxide*

An endothelium-derived substance was found to cause relaxation of isolated blood vessels (Furchgott & Zawadzki, 1980). Previously, the vascular endothelium had been thought of as an inert barrier which separated the blood from the vascular smooth muscle. Relaxation was observed when the endothelium was intact, and did not occur if the endothelium was removed. The relaxant effect was restored if an intact endothelium was juxtaposed with the blood vessel. This suggested that there was some "factor" that was released from the endothelium and diffused to the blood vessel, and this was termed the
"endothelium-derived relaxing factor" (EDRF). These findings helped explain the results of previous in vitro studies, which failed to consistently observe vasodilation, and which could now be explained by accidental removal of the endothelium. The identity of EDRF remained elusive for a number of years, but a breakthrough came in 1987. Moncada's group demonstrated that cultured endothelial cells synthesize nitric oxide (Palmer et al., 1987). It was also determined that EDRF and NO are pharmacologically identical and that NO accounts for the activity of EDRF. It was later shown that NO is formed following the stereo-specific oxidation of one of the terminal guanidino nitrogens of L-arginine (Moncada, 1991), and that the reaction is catalyzed by the enzyme nitric oxide synthase (NOS) (E.C. 1.14.13.39) (Fig. 2).

1.3. Nitric Oxide Synthase (NOS)

With the findings implicating nitric oxide in the relaxation of vascular smooth muscle, the role(s) of L-arginine and nitric oxide synthase take on a greater importance. It had previously been shown that L-arginine was essential for full macrophage cytotoxicity and to stimulate the activity of neuronal guanylate cyclase (Moncada, 1991). Both of these functions can be attributed to the production of NO from L-arginine, and it is now known that a variety of cells synthesize NO via this pathway:

\[
\text{L-arginine} + 1.5 \text{ NADPH} \rightarrow \text{L-citrulline} + \text{NO} + 1.5 \text{ NADP}^+ 
\]

NOS requires the presence of the four following prosthetic groups for full activity; FAD, FMN, tetrahydrobiopterin and a heme group. The required 1.5 equivalents of NADPH transfers three electrons, two at a time. The two flavin groups can store up to four
Fig. 2- Reaction mechanism of nitric oxide synthase. PPIX refers to protoporphyrin IX which, when conjugated to iron forms the catalytically important heme group present in NOS. The symbol ^ is used to represent a free radical. (Sexton, 1994)
electrons, so it is thought that the fourth electron is stored here until the next catalytic cycle. The role of tetrahydrobiopterin is unclear at the present time, but it appears to be important structurally for electron transfer (Marletta, 1993).

There are three isoforms of the enzyme nitric oxide synthase (reviewed in Knowles & Moncada, 1994):

1. nNOS, first identified in neuronal cells of the central and peripheral nervous systems;
2. eNOS, first identified in vascular endothelial cells; and
3. iNOS, or inducible nitric oxide synthase.

Both of the isoforms nNOS and eNOS are constitutively expressed (and are collectively referred to as "constitutive NOS"), whereas iNOS can be induced in a wide variety of cells. The activity of iNOS is calcium independent, unlike the other two isoforms. The induction of iNOS has been observed in response to a wide variety of bacterial products and cytokines and thus, the major role of iNOS appears to be in the development of non-specific immunity. The nNOS isoform appears to act mainly as a neurotransmitter/neuromodulator, whereas the eNOS isoform is responsible for the effects seen in the cardiovascular system (smooth muscle relaxation, inhibition of platelet aggregation). A platelet NOS has been reported (Malinski et al, 1993; Radomski et al, 1990) and was confirmed in our laboratory by the purification of the platelet constitutive isoform to homogeneity (Muruganandam and Mutus, 1994). Quite obviously, the regulation of NO production by this enzyme must be tightly controlled. Overproduction of NO is observed in a variety of pathological conditions and treatment by targeting NOS is of current therapeutic interest (Moncada, 1995).
1.4. *L-Arginine Transport*

As a precursor to nitric oxide, L-arginine is of critical importance in signaling processes. Other fates of L-arginine include protein synthesis and involvement in the urea cycle. The transport of L-arginine to areas where NO is known to have an effect is obviously of importance to the regulation of NO production by NOS. L-Arginine transport across the plasma membrane has been observed and characterized in many mammalian cells (reviewed in White, 1985) and has been classified as system $y^\ast$. The substrates for this transporter are the cationic amino acids L-arginine, L-lysine and L-ornithine. These substrates are all transported with an approximate equal affinity. System $y^\ast$ has several characteristics which can be used to identify its presence in the cell membrane. It is Na$^-\$independent, saturatable by its substrates, inhibited by substrate analogs and other cationic amino acids, sensitive to membrane polarization, pH insensitive and susceptible to trans-stimulation (Sexton, 1994). The steady state kinetics of L-arginine transport by system $y^\ast$ has been described by an iso uni uni mechanism, which involves only one substrate at either side of the plasma membrane and an isomerization step that moves the protein binding site from one face to the other (White and Christensen, 1982). Cationic amino acids have been shown to accumulate in fibroblasts at intracellular:extracellular distribution ratios between 10 to 20 (White *et al*, 1982). To maintain this asymmetric distribution the influx of the amino acid must be greater than the efflux. The determination of the kinetic parameters of this system, in fibroblasts and hepatoma cells, showed that the efficiency of influx ($V_{max}/K_m$) was approximately 10 times greater than the efficiency of the efflux (White and Christensen, 1982). It has been proposed that the energy required to
maintain the distribution may be due to the membrane potential, but the importance of the potential has been refuted based on thermodynamic calculations (White, 1985).

The elucidation of system $y^-$ was done prior to the discovery of the role of L-arginine in NO biosynthesis. There are many cells known to produce NO, and now shown to display system $y^-$ activity include human endothelial cells, human polymorphonuclear leukocytes, human T lymphocytes, rat aorta vascular smooth muscle cells, porcine pulmonary artery endothelial cells, murine macrophages, and murine neurons (Bussolati et al, 1993; Low et al, 1993; Greene et al, 1993; Sato et al, 1991; Stoll et al, 1993; Boyd, 1992). Many of these cells show an increase in system $y^-$ activity by mechanisms that involve increased protein synthesis, NO-dependent stimulation or both.

Studies on the structure of the $y^-$ system are few. It has been found that the cDNA corresponding to the system $y^-$ found in mice is composed of 622 amino acids, 14 potential membrane spanning domains and a combined molecular weight of 67 kDa (Albritton et al, 1989). In cells isolated from the rat kidney cortex, radiation inactivation analysis has shown that the system $y^-$ activity corresponds to a 90 kDa protein (Beliveau et al, 1990). The reported differences in molecular weight of system $y^-$ suggest some heterogeneity between species.

The importance of the L-arginine/nitric oxide pathway as a mediator in the regulatory system controlling platelet adhesion and aggregation is expanding as it has been shown that platelets are exposed to the action of NO and can produce NO themselves. It has been shown that aggregation induced by collagen, ADP, arachidonic acid and thrombin is reduced in the presence of L-arginine (Radomski et al, 1990a; Radomski et al, 1990b), which is known to be the substrate for NO production. Also, the synthesis of NO
has been shown to increase in the presence of collagen and increased further by the addition of L-arginine (Malinski et al, 1993). In vivo experiments have also shown that the inhibition of L-arginine transport and NO synthase activity induces platelet activation (Bodzenta-Lukaszyk et al, 1994). Further evidence of endogenous NO production in platelets is given by the observation that L-arginine inhibits the 12-lipoxygenase pathway of arachidonic acid metabolism (Nakatsuka and Osawa, 1994). These observations are consistent with the activation of the L-arginine/NO pathway in platelets during aggregation, and indicate a critical role for L-arginine availability to support NO production by platelets (Vasta et al, 1995). The mechanism of L-arginine uptake may therefore represent a potential regulatory point in the L-arginine/NO pathway.

1.5. Peroxynitrite

Nitric oxide has been shown to be cytotoxic at high levels (Sexton et al, 1994). The pathological conditions brought about by the overproduction of NO can arise from two different pathways. The first and most obvious route is a direct effect such as the competitive inhibition of mitochondrial respiration at cytochrome c oxidase. However, many of the effects of NO are thought to be due to its oxidation products, and not due to NO itself. Peroxynitrite (\( ^{-}\text{OONO} \)) is one such molecule.

The formation of peroxynitrite was first proposed in 1990 (Beckman et al, 1990). Peroxynitrite is formed from the reaction of superoxide with NO:

\[
^\cdot\text{NO} + ^\cdot\text{O}_2 \rightarrow ^{-}\text{OONO}
\]
Knowing that some cells (such as activated macrophages) produce both of these radicals (Ischiropoulos et al, 1992a), it seemed likely that they could react to form peroxynitrite in vivo. The rate of formation of peroxynitrite has been reported as $6.7 \times 10^9 \text{ M}^{-1}\text{s}^{-1}$ (Huie and Padmaja, 1993). For this reaction to occur to a significant level, the rate of formation of peroxynitrite must exceed the rate of reaction of superoxide with SOD and the rate of reaction of NO with heme compounds (McCord and Fridovich, 1988; Traylor and Sharma, 1992). The rate of peroxynitrite formation is approximately 30 times faster than the reaction of NO with the heme moiety of oxyhemoglobin, and 3 times faster than the reaction between superoxide and SOD (Ischiropoulos, 1995). In fact, the formation of peroxynitrite is the only reaction known to outcompete SOD for superoxide. Peroxynitrite is stable at alkaline pH; however protonation of the peroxynitrite anion leads to the formation of the conjugate acid, peroxynitrous acid (HOONO). This acid is relatively unstable, and the decomposition of peroxynitrous acid yields a number of products, including the hydroxyl radical ("OH) and the nitronium ion (NO$_2^-$).

The peroxynitrite anion has two geometric isomers, cis- and trans-. Calculations have shown that the cis- conformation is more stable than the trans- conformation (Tsai et al, 1994), due to the partial delocalization of the negative charge in the cis- conformation over the entire peroxynitrite molecule. The apparent pK$_a$ of cis-peroxynitrite is 6.8 (Beckman, 1996). Therefore, only approximately 20% of peroxynitrite will be protonated to peroxynitrous acid at physiological pH. After protonation, peroxynitrite can undergo isomerization to form the trans-conformation (Fig. 3). The trans-isomer can then form an
"excited state" form, which can rationalize the apparent hydroxyl-like radical formation of peroxynitrite.

\[
\begin{align*}
O_2^- \cdot OH & \leftrightarrow O_2^- \cdot N-OH & \leftrightarrow O_2^- \cdot N-O^- \\
\text{cis} & \quad \text{trans} & \quad \text{H}_2O
\end{align*}
\]

Fig. 3-Peroxynitrite isomerization. Peroxynitrite is most stable when folded into a cis-conformation. Protonation of the anion will allow it to isomerize to trans-peroxynitrous acid, which can form an excited state which can react like the hydroxyl radical and nitrogen dioxide (Beckman, 1996).

There is evidence for the production of peroxynitrite in vivo. It has been observed that superoxide can reduce the effects of NO, by forming peroxynitrite (Gryglewski et al., 1986; Beckman and Crow, 1993b). Scavengers of superoxide, such as SOD, can therefore enhance the biological activity of NO by preventing its reaction with superoxide (Assreuy et al., 1994). Also, looking at this from the other side of the equation, NO can reduce the effects of superoxide (Wink et al., 1993).

1.6. Nitration of Tyrosine Residues

An indirect method for the detection of peroxynitrite in vivo is the formation of nitrotyrosine residues. As previously mentioned, the decomposition of peroxynitrous acid by a heterolytic bond cleavage can yield the nitronium ion (NO₂⁺) which is a potent nitrating agent of phenolic groups. This reaction is catalyzed by transition metals,
including the metal centers of SOD (Ischiropoulos et al, 1992b). Tyrosine is an amino acid with the phenolic group as its side-chain, so the formation of nitrotyrosine residues in proteins would indicate the presence of in vivo peroxynitrite formation.

![Nitration of a tyrosine residue](image)

**Fig. 4-** Nitration of a tyrosine residue. (Beckman, 1994a)

Nitrotyrosine residues have been detected in a variety of pathological conditions, including human atherosclerotic coronary arteries (Beckman et al, 1994b), placental tissues from pregnant women with preeclampsia (Myatt et al, 1996), human lung biopsy (Haddad et al, 1994) and autopsy samples from patients who had suffered from sepsis, pneumonia or adult respiratory distress syndrome (Kooy et al, 1995). Since tyrosine cannot be nitrated by nitric oxide itself (Van Der Vliet et al, 1994) nitrotyrosine formation provides convincing evidence of peroxynitrite formation.

Besides the pathological consequences to nitration of tyrosine residues, the possible role(s) in cellular function are also being investigated (Gow et al, 1996). The phosphorylation of tyrosine residues is an important regulator of signal transduction in cells. The formation of nitrotyrosine prevents the formation of phosphotyrosine by tyrosine kinases (Martin et al, 1990) and therefore interferes with the normal signal
transduction pathways. This mechanism has been proposed to account for the pathological action of SOD mutations in ALS (Beckman et al., 1993a). The formation of nitrotyrosine residues has also been used to study the role of tyrosine in the function of proteins. Nitration has been shown to inactivate nearly 140 mammalian proteins whose activity is dependent on tyrosine residues (Nielsen, 1995).

1.7. *S*-Nitrosothiols

Because of the reactivity of nitric oxide, many experiments utilize NO generating systems, including S-nitrosothiols to produce NO. Some of these compounds have been hypothesized to be involved in the storage and/or transport of NO *in vivo*. Many *S*-nitrosothiols have been shown to possess the biological properties of NO itself, such as smooth muscle relaxation (Ignarro et al., 1981; Jia and Furchgott, 1993; Feelisch et al., 1994; Mathews and Kerr, 1993), inhibition of platelet aggregation (Radomski et al., 1992a; Simon et al., 1993), immunosupression (Merryman et al., 1993) and neurotransmission (Talman et al., 1993).

A variety of NO donors are utilized to slowly release nitric oxide in the desired fashion. One such class of these compounds are the *S*-nitrosothiols (RSNO). *S*-Nitrosothiols are formed by the electrophilic nitrosation of thiols:

\[ \text{RSH} + \text{XNO} \leftrightarrow \text{RSNO} + \text{H}^+ + \text{X}^- \]

Until relatively recently few examples of *S*-nitrosothiols were known, mainly because of their instability. With the discovery of the biology of nitric oxide, many more stable
examples have been prepared and characterized. S-Nitrosothiols have been found *in vivo*, including S-nitrosoalbumin in human plasma (Stamler *et al.*, 1992), hemoglobin (Gruetter *et al.*, 1980) and S-nitrosoglutathione (GSNO) in bronchial lavage fluid (Gaston *et al.*, 1993). The formation of S-nitrosothiols may be important in such processes as signal transduction, DNA repair, and blood-pressure regulation (Gow *et al.*, 1997). There is a considerable therapeutic potential for exogenous RSNO in areas such as the regulation of blood pressure, prevention of blood clotting, bronchodilation and smooth muscle relaxation for surgical procedures (Meyer *et al.*, 1994). Previous studies in our laboratory have shown that the selective release of NO from RSNO could be utilized as an effective photochemotherapeutic agent (Sexton *et al.*, 1994). Two of the more frequently used examples are S-nitrosoglutathione (GSNO) and S-nitroso-N-acetyl penicillamine (SNAP) (Fig. 5).

\[\begin{align*}
\text{SNAP} & \quad \text{GSNO}
\end{align*}\]

*Fig. 5*- Structures of some common S-nitrosothiols.

Tertiary RSNOs (such as SNAP) are usually green compounds and primary structures (such as GSNO) are reddish. These compounds have visible absorption spectra
corresponding to the $n_N \rightarrow \pi^*$ transition between 540-590 nm with a low extinction coefficient (10-20 L mol$^{-1}$ cm$^{-1}$) and a UV absorption corresponding to the $n_O \rightarrow \pi^*$ transition around 340 nm with an extinction coefficient of approximately 1000 L mol$^{-1}$ cm$^{-1}$ (Williams, 1996). Nitrosothiols are known to break down thermally and photochemically to release nitric oxide:

$$2 \text{RSNO} \rightarrow \text{RSSR} + 2 \text{NO}^*$$

It is thought that the mechanism of action of RSNO is similar to that of NO itself since the activation of guanylate cyclase and resultant rise in cGMP is common to both compounds (Moncada et al., 1991; Radomski et al., 1992). The thermal decomposition of RSNO has recently been shown to be dependent on the presence of a copper catalyst (Williams, 1996):

$$[\text{RSNO} + 2 \text{OH}^- \leftrightarrow \text{RS}^- + \text{NO}_2^- + \text{H}_2\text{O}]$$

$$\text{Cu}^{2+} + \text{RS}^- \rightarrow \text{Cu}^+ + \text{RS}^*$$

$$\text{Cu}^+ + \text{RSNO} \rightarrow \text{Cu}^{2+} + \text{RS}^- + \text{NO}$$

$$2 \text{RS}^* \rightarrow \text{RSSR}$$

RSNO is thought to undergo very rapid (Williams, 1988) or instantaneous (Park, 1988) SNO-SH exchange reactions with other thiols. With some RSNO, the presence of another
thiol is required for the RSNO to be biologically active (Pietraforte et al., 1995). The two thiols undergo a transnitrosation reaction:

$$\text{R'}\text{S-NO} + \text{RSH} \leftrightarrow \text{R'SH} + \text{RS-NO}$$

This reaction makes the analysis of the effects of a specific RSNO on a physiological system very difficult to define (Henry et al., 1989). In the extracellular environment where the thiol concentration is low, the SNO-SH exchange would be relatively unimportant and the S-nitroso compound should be stable. If, however, the compound has to enter a cell, conversion to GSNO is likely and any effects seen would likely be due to GSNO (Meyer et al., 1994).

1.8. *Glutathione*

As previously mentioned, the formation of S-nitrosothiols *in vivo* has been hypothesized to be involved in the storage and transport of NO. Glutathione (L-γ-glutamyl-L-cysteinylglycine) (GSH) is the most abundant intracellular non-protein thiol, so it would follow that under the proper conditions GSNO could be formed. The reaction of GSH with an electrophile (NO\textsuperscript{−}) to form GSNO greatly prolongs the biological life of NO. GSH is utilized in cells to perform a variety of functions, including the protection against oxidative damage, the detoxification of foreign compounds, the involvement in protein disulfide formation and the maintenance of an intracellular reducing environment for redox sensitive enzymes (reviewed in Meister, 1992; Deneke, 1989). There are a variety of enzymes that work in conjunction with GSH, including glutathione reductase, glutathione
peroxidase and glutathione S-transferase. An extensive study previously conducted in our laboratory (Francescutti et al., 1996) has shown the effects of peroxynitrite in part of this system. The catalytic properties of the enzyme glutathione reductase were altered following exposure to peroxynitrite. These alterations are thought to result from the formation of a nitrotyrosine residue, which was detected by immunological methods, at or near the glutathione disulphide binding site.

The enzyme glutathione-S-transferase (GST) is a group of ubiquitous enzymes believed to serve essential functions in the biotransformation of xenobiotics (Jakoby and Habig, 1980), acting to detoxify the cell. It catalyzes the conjugation of various electrophiles with GSH. The major part of the enzyme activity is located in the cytosol of all tissues investigated, and the concentration of the enzymes amounts to several percent of the cytosolic proteins in many tissues. There are a number of isoforms of this enzyme which are classified according to their isoelectric point and substrate affinity. Glutathione transferases with basic isoelectric points have been purified from human liver (Kamisaka et al., 1975) (isoforms α–ε and μ), and an acidic form from human erythrocytes (Marcus et al., 1978). An acidic form has also been found in high concentrations in human placenta (Guthenberg et al., 1979; Polidoro et al., 1980) and has been labeled as the π isoform. This isoform is composed of two subunits and has a molecular weight of 47 kDa. The isoelectric point of the π isoform is 4.8 (Mannervik and Guthenberg, 1981).

Most cells contain GSH at a concentration between 0.1-10 mM (Till et al., 1988). It would be expected that a change in the intracellular concentration of GSH would affect the thiol/disulfide ratio of proteins that could lead to an alteration in protein function (Gilbert, 1990; Ziegler, 1985). Therefore, maintenance of intracellular GSH levels is
extremely important in the viability of the cell. The intracellular GSH level has been implicated in the regulation of platelet function by influencing platelet aggregation (Bosia et al, 1985; Hofmann et al, 1980; Matsuda et al, 1979). Previous studies in our laboratory (Sexton and Mutus, 1995) have characterized the uptake of glutathione by human platelets. The mechanism of this uptake was determined to be Na⁺-independent and saturable. GSH analogs inhibited the uptake of GSH while membrane depolarization elevated the rate of uptake of GSH. It was also found that depletion of intracellular GSH lead to a near 40% increase in GSH uptake.

1.9. Nitric Oxide and Diabetes

As has been previously discussed, nitric oxide (and its oxidation products) have been implicated in many pathological conditions. The wide assortment of conditions implicating NO is increasing and spans many different areas of medicine. One such condition known to have NO as a contributing factor to development is insulin-dependent diabetes mellitus (IDDM) (Burkart et al, 1994). Animal models for many NO-linked diseases show that high local levels of NO can lead to tissue destruction during the developmental stages of these conditions. There are two possible pathways to this destruction, either by direct induction of cell damage by NO (Kroncke et al, 1995) or by an altered regulation in the immune response (Wei et al, 1995). Autoimmune diabetes is brought about by selective destruction of the insulin-secreting β cells, leading to hyperglycemia. Several animal models have shown that activated macrophages are the first immunocytes to infiltrate the pancreatic cells during the development of diabetes, and they
have been shown to participate in the destruction of the β cells (Kolb-Bachofen et al., 1988; Lee et al., 1988; Hanenberg et al., 1989).

Prolonged hyperglycemia has many chronic and well known effects. During diabetes, persistent hyperglycemia causes increased production of free radicals via autooxidation of glucose (Ceriello et al., 1996) and non-enzymatic protein glycation. This would lead to a possible localized production of peroxynitrite in the vascular system, putting blood cells at risk of peroxynitrite modification. Platelets are blood cells that serve an important physiological role. Perhaps the most important function of platelets is adhesion and aggregation, forming a plug to prevent loss of blood through a vascular break. There is a great deal of information on the molecular mechanism of platelet activation, but by no means has the entire process been elucidated (Siess, 1989). Patients suffering from diabetes mellitus are known to be highly susceptible to accelerated vascular diseases. Much of this has previously been attributed to the hemostatic imbalance (Meade et al., 1985; Roeson et al., 1993). This imbalance has been shown to perpetuate vascular disease by a number of factors, including platelet aggregation, platelet dysfunction and increased blood coagulability (Kwaan et al., 1992). A variety of platelet components show abnormal function in diabetic patients, including membranes, granules, intermediary metabolism and platelet coagulation factors (Winocour, 1992). Of great pathological importance are the findings that platelets from diabetic patients are hypersensitive to platelet agonists, including ADP, collagen, arachidonic acid and thrombin in vitro (Colwell et al., 1981). This hypersensitivity could lead to unwanted thrombus formation, capable of producing stroke and ultimately death.
1.10. *Aim of Study*

The goal of the experiments described herein is to further the understanding of nitric oxide, especially in its relation to biological systems. To achieve this goal, three areas were studied with relation to nitric oxide biochemistry. The first part of this study involves the effect of NO on the transport of L-arginine into human platelets. Since L-arginine has clearly been shown to be a required substrate for NO production (section 1.3.), its transport into the cell is of great regulatory importance. Several different sources of NO are utilized to study its effect on this system, and include two NO donor compounds (GSNO and SNAP), a solution of authentic NO and a source of NO$^-$ (NOBF$_4$). The second part of this study involves the detection of nitrotyrosine residues in platelet proteins from diabetic patients. The impairment of platelet function, coupled with the fact that local production of peroxynitrite is possible leads to the possibility that NO, through peroxynitrite, is modifying the platelet proteins and forming nitrotyrosine residues, as seen in other pathological conditions associated with an overproduction of NO (section 1.6.). The third part of this study looks at the effects of peroxynitrite on the activity of the enzyme glutathione-S-transferase. Modification of this protein by peroxynitrite, producing nitrotyrosine residues, could alter the activity due to changes in protein shape and/or perturbation of the binding site.
Chapter 2

MATERIALS AND EQUIPMENT

2.1. Materials

Citrated whole blood was provided by both the hematology and chemistry departments at the Hotel-Dieu/Grace hospital in Windsor, Ontario.

Distilled deionized water was prepared using a Barnstead Fi-Stream distiller from Sybron Corporation.

The anti-nitrotyrosine antibody was purchased from Upstate Biotechnology Incorporated and the anti-calmodulin antibody was purchased from ICN Biomedicals, Inc. [2,3-\textsuperscript{3}H]-L-arginine was obtained from New England Nuclear. Diisononyl phthalate and dibutyl phthalate were purchased from Aldrich.

The following compounds were obtained from Sigma Chemical Co.: IgG conjugated to alkaline phosphatase, β-mercaptoethanol, 1-chloro-2,4-dinitrobenzene, 5-bromo-4-chloro-3-indoyl phosphate, Coomassie brilliant blue R-250, glutathione, L-arginine, N-(2-hydroxyethyl)piperazine-N’-(2-ethanesulfonic acid) (HEPES), nitroblue tetrazolium, placenta GST, polyvinylpyrrolidine, and Sigma-Fluor (scintillation fluid).

Purchased from Bio-Rad Laboratories were: ammonium persulfate, Ponceau S, SDS-PAGE molecular weight standards, Tween-20, 30% acrylamide/bis solution (29:1).

All other reagents were of reagent grade or better.
2.2. Equipment

The following is a list of equipment used in the performance of the described experiments:

300 W quartz halogen lamp from an overhead projector;

Beckman J-6B centrifuge;

Beckman LS 6500 multi-purpose scintillation counter;

Bio-Rad Mini Trans-Blot® electrophoretic transfer cell apparatus;

Bio-Rad model GS-670 imaging densitometer;

Bio-Rad power supply model Power Pac 300;

Fisher Scientific micro-centrifuge model 59A;

Kontes hand held homogenizer;

Mettler AJ100 balance;

Orion 420A pH meter

Shimadzu UV-160 visible recording spectrophotometer equipped with a thermostated cell holder.
Chapter 3

METHODS

3.1. *Protein Estimation* (Bradford, 1976)

The Bio-Rad protein estimation kit was used in determining the amount of protein. Standard curves were constructed using (1 µg - 10 µg) bovine serum albumin as the standard. The samples were incubated for 20 minutes at room temperature. The absorbance was then measured at 595 nm.

3.2. *Preparation of Authentic NO Solutions*

Phosphate buffered saline (137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄ and 1.8 mM KH₂PO₄) (PBS) was first deoxygenated by sonication under vacuum for 1 hour followed by purging with helium for 2 hours. NO gas was passed through saturated KOH before being introduced into the deoxygenated PBS.

3.3. *Platelet Isolation*

Human blood was obtained in citrated vacucontainers from a local hospital. Platelets were isolated by centrifugation similarly to that described in Muruganandam *et al* (1993). Whole blood was first spun at 130 x g for 20 minutes. The platelet rich plasma was then centrifuged at 3300 x g to pellet the platelets. This was followed by 1 wash with wash buffer (10 mM HEPES, 137 mM NaCl, 3.7 mM KCl, 5.5 mM glucose at pH 6.8) at 1800 x g. The platelet pellet was then resuspended in incubation buffer (10 mM HEPES, 137 mM NaCl, 3.7 mM KCl, 5.5 mM glucose at pH 7.4).
3.4. *Measurement of L-Arginine Uptake*

The uptake of $[^{3}H]$-L-arginine by intact platelets ($3 \times 10^{11}$ platelets/250 μL) was performed in incubation buffer (10 mM HEPES, 137 mM NaCl, 3.7 mM KCl, 5.5 mM glucose at pH 7.4) at 37°C in a final volume of 250 μL. At the specified time the platelet suspension was layered over a mixture of dibutyl phthalate/diisononyl phthalate (1.3 :1) and centrifuged for 30 seconds at 9500 x g in a swing-out rotor micro-centrifuge. The supernatant and most of the oil layer was aspirated away and the remaining platelet pellet was dissolved in 250 μL of 2 % SDS before transferring to scintillation fluid for radioactive counting. Exposure to NO donors (100 μM final concentration) was done for five minutes, in the presence or absence of light. Platelet suspensions used to measure the effects of copper were incubated with CuCl$_2$ at a final concentration of 20 μM.

3.5. *Synthesis of Peroxynitrite*

Peroxynitrite was synthesized in a quenched-flow reactor. Solutions of (i) 0.6 M NaNO$_2$ and (ii) 0.6 M HCl/0.7 M H$_2$O$_2$ were pumped into a tee-junction and mixed in a 3-mm diameter by 2.5 cm glass tube. The acid-catalyzed reaction of nitrous acid with H$_2$O$_2$ to form peroxynitrous acid was quenched by pumping 1.5 M NaOH at the same rate into a second tee-junction at the end of the glass tubing. The solution was then frozen at -20°C.
3.6. *Synthesis of S-Nitrosoglutathione*

To an ice-cold GSH solution (1.5 g) in 8 mL of H₂O and 2.5 mL 2 M HCl, 0.345 g of sodium nitrite was added. The mixture was stirred at 4°C for 40 minutes, treated with 10 mL of acetone and stirred for an additional 10 minutes. The pink precipitated product was obtained by vacuum filtration and washed successively with ice-cold H₂O (5 x 1 mL), acetone (3 x 10 mL) and diethyl ether (3 x 10 mL). The product was dried by placement in an evacuated dessicator. The reaction was performed in the dark. GSNO was quantified according to its absorbance at 545 nm (ε=15.9 M⁻¹ cm⁻¹).

3.7. *Western Immunoblotting of Human Platelet Proteins*

The platelet samples were run on a 10% w/v SDS-polyacrylamide gel, according to the method of Laemmli (Laemmli, 1970), along with a set of molecular weight markers (broad range) and BSA (0.20 mg/mL), to act as a negative control. The proteins were then transferred electrophoretically to nitrocellulose membranes (S&S BA-S nitrocellulose). Transfer was checked by staining the transferred gel with Coomassie Blue staining solution. The nitrocellulose membranes were then immersed in blocking solution (50 mM Tris, pH 7.4, containing 0.5 M NaCl and 2% polyvinylpyrrolidone) and left overnight with gentle agitation. Following this incubation, the membranes were rinsed extensively with washing solution (TBS containing 0.05% Tween-20, pH 7.5). The membranes were then probed with either the anti-nitrotyrosine antibody (1:400 dilution in TBS containing 0.05% Tween-20) or the anti-calmodulin antibody (1:200 dilution) for 1 hour, again with gentle agitation. After this incubation the membranes were washed twice, for 10 minutes
each time, with washing solution (TBS containing 0.05% Tween-20, pH 7.5). The membranes were then probed with an anti-rabbit IgG antibody conjugated to alkaline phosphatase (1:3000 dilution), with gentle agitation, for 1 hour. The membranes were rinsed again with washing solution for 2 washes of 10 minutes each. The washing solution was then decanted off and the membranes were then washed in TBS for a further 10 minutes. The bromochloroindolyl phosphate/nitroblue tetrazolium substrate was then added and the blot was allowed to develop until bands became visible. Colour development was stopped using 20 mM EDTA. The blots were then analyzed using a Bio-Rad model GS-670 imaging densitometer.

3.8. *Glutathione S-Transferase Kinetic Assay*

Enzyme activity was determined spectrophotometrically at 340 nm by measuring the formation of the conjugate of glutathione (GSH) and 1-chloro-2,4-dinitrobenzene (CDNB). To a 1-mL cuvette was added 500 µL of buffer (0.2 M sodium phosphate, pH 7.0), 50 µL of GSH (20 mM), and a suitable amount of enzyme and deionized water to a final volume of 1 mL. The reaction, carried out at 30°C, was started by addition of 50 µL of CDNB (20 mM in 95% ethanol). The reaction was monitored spectrophotometrically by the increase in absorbance at 340 nm ($e_{340}=10$ mM$^{-1}$cm$^{-1}$). To determine the effects of peroxynitrite on the enzyme, a 100 times molar excess was added to the enzyme and the assay repeated as above. In substrate protection studies, the enzyme was pre-incubated with 100 times molar excess of the appropriate substrate, and the assay repeated as above.
3.9. *Nitric Oxide Determination*

The amount of nitric oxide was determined via a colorometric assay (Boehringer Mannheim) quantifying the amount of nitrate present in solution. NO donors (SNAP 14.1 mM, GSNO 5.87 mM) were exposed to either visible light or CuCl₂ for five minutes; platelet suspensions were studied in the presence/absence of visible light. All experiments were performed in triplicate.

3.10. *Statistical Analyses*

The analyses of membrane transport data was performed by fitting the data to a rectangular hyperbola function of the equation:

\[ v = \frac{V_{\text{max}} \cdot [S]}{K_m + [S]} \]

The Michaelis-Menten enzyme kinetic parameters, \( K_m \) and \( V_{\text{max}} \), were calculated by a non-linear regression analysis using the computer program SigmaPlot. To determine statistical significance, an unpaired t-test was employed (SigmaPlot), with \( P < 0.05 \) being taken as statistically significant. In analyzing the GST kinetic data, the enzyme kinetic parameters were calculated from the linear regression data and extending the best fit line to the axis of a double-reciprocal plot.
Chapter 4

RESULTS

4.1. *GSNO and L-Arginine Transport.*

Initially the effect of nitric oxide on the transport of L-arginine into platelets was studied. Previous studies in our laboratory have alluded to a role of NO in this process. Therefore GSNO was used as a NO donor to observe any effects on the transport of L-arginine into the platelet. GSH was used as a blank in these experiments, so that any effects due to the glutathione carrier of GSNO would be eliminated. NO was liberated from the donor (GSNO) by photolysis of the S-NO bond, which has been well studied in our laboratory (Sexton *et al.*, 1994). The experiment was done both in the dark (Fig. 6) and with a five minute exposure to visible light (Fig. 7) to release the NO. The Michaelis-Menten kinetic parameters for each trial were calculated, and as a tool for studying the effect of NO the efficiency of this system was calculated (Vmax/Km) (summarized in Table 1). The results of these two experiments show that in the dark there was no significant difference in L-arginine transport in the presence of GSNO, but after a five minute exposure to visible light the efficiency of this transport increased from 2.936 +/- 0.558 pmol/min/μmol (dark) to 10.89 +/- 1.331 pmol/min/μmol (light). During the time these experiments were being performed an article was published linking the thermal decomposition of S-nitrosothiols to the presence of copper (Williams, 1996). Therefore the same experiments were repeated, however the platelet samples were pre-incubated with 20 μM CuCl₂. In this case we see an increase in the efficiency in the samples with GSH, both in the dark (Fig. 8) and in the light (Fig. 9), though there is no significant
Table 1-Kinetic parameters of platelet L-arginine transport. Results are expressed as the mean +/- standard error.

<table>
<thead>
<tr>
<th></th>
<th>Vmax (pmol/min)</th>
<th>Km (μM)</th>
<th>Efficiency (Vmax/Km)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank, Dark (+ Cu)</td>
<td>492.7 +/- 144.9</td>
<td>320.2 +/- 137.1</td>
<td>1.539 +/- 0.659</td>
</tr>
<tr>
<td>Blank, Light (+ Cu)</td>
<td>237.4 +/- 15.8</td>
<td>74.87 +/- 11.6</td>
<td>3.171 +/- 0.491</td>
</tr>
<tr>
<td>SNAP, Dark (+ Cu)</td>
<td>1197 +/- 278.6</td>
<td>652.9 +/- 188.9</td>
<td>1.833 +/- 0.530</td>
</tr>
<tr>
<td>SNAP, Light (+ Cu)</td>
<td>623.5 +/- 81.4</td>
<td>143.1 +/- 34.7</td>
<td>4.357 +/- 1.057</td>
</tr>
<tr>
<td>GSH, Dark (+ Cu)</td>
<td>467.9 +/- 32.4</td>
<td>128.0 +/- 17.1</td>
<td>3.655 +/- 0.488</td>
</tr>
<tr>
<td>GSH, Light (+ Cu)</td>
<td>469.9 +/- 132</td>
<td>101.5 +/- 59.4</td>
<td>4.630 +/- 1.310</td>
</tr>
<tr>
<td>GSNO, Dark (+ Cu)</td>
<td>321.0 +/- 46.2</td>
<td>177.7 +/- 44.3</td>
<td>1.806 +/- 0.450</td>
</tr>
<tr>
<td>GSNO, Light (+ Cu)</td>
<td>602.8 +/- 119.5</td>
<td>119.5 +/- 81.2</td>
<td>5.044 +/- 1.427</td>
</tr>
<tr>
<td>NOBF3 (blank)</td>
<td>292.2 +/- 22.3</td>
<td>165.9 +/- 22.4</td>
<td>1.761 +/- 0.238</td>
</tr>
<tr>
<td>NO (aq) (blank)</td>
<td>653.6 +/- 87.5</td>
<td>126.3 +/- 32.8</td>
<td>5.175 +/- 1.344</td>
</tr>
<tr>
<td>NOBF3 (blank)</td>
<td>728.2 +/- 88.5</td>
<td>248.0 +/- 47.1</td>
<td>2.936 +/- 0.558</td>
</tr>
<tr>
<td>GSSG (blank)</td>
<td>891.7 +/- 129.9</td>
<td>106.2 +/- 31.8</td>
<td>8.396 +/- 1.514</td>
</tr>
<tr>
<td>GSNO, Light (+ Cu)</td>
<td>579.4 +/- 27.2</td>
<td>53.2 +/- 6.5</td>
<td>10.89 +/- 1.331</td>
</tr>
<tr>
<td>GSSG (blank)</td>
<td>887.6 +/- 96.0</td>
<td>100.9 +/- 22.8</td>
<td>8.797 +/- 1.588</td>
</tr>
<tr>
<td>NOBF3 (blank)</td>
<td>822.2 +/- 79.0</td>
<td>204.0 +/- 32.5</td>
<td>4.030 +/- 0.642</td>
</tr>
<tr>
<td>GSSG (blank)</td>
<td>829.1 +/- 122.2</td>
<td>199.7 +/- 49.2</td>
<td>4.152 +/- 0.823</td>
</tr>
<tr>
<td>NO (aq) (blank)</td>
<td>346.6 +/- 26.8</td>
<td>177.5 +/- 23.7</td>
<td>1.953 +/- 0.261</td>
</tr>
<tr>
<td>NOBF3 (blank)</td>
<td>413.6 +/- 102.3</td>
<td>213.2 +/- 86.3</td>
<td>1.940 +/- 0.785</td>
</tr>
<tr>
<td>GSSG (blank)</td>
<td>609.1 +/- 92.5</td>
<td>141.9 +/- 40.2</td>
<td>4.292 +/- 0.652</td>
</tr>
<tr>
<td>NOBF3 (blank)</td>
<td>528.3 +/- 51.7</td>
<td>210.4 +/- 33.8</td>
<td>2.011 +/- 0.197</td>
</tr>
</tbody>
</table>
Fig. 6-Effect of GSNO on platelet L-arginine transport. The experiment was performed as outlined in section 3.4. Platelet samples were incubated with either 100 μM GSH or 100 μM GSNO for 5 minutes in the absence of light. Results are expressed as the mean ± standard error.
Fig. 7- Effect of GSNO and light on platelet L-arginine transport. The experiment was performed as outlined in section 3.4. Platelet samples were incubated with either 100 μM GSH or 100 μM GSNO for 5 minutes in the presence of visible light. Results are expressed as the mean ± standard error.
Fig. 8- Effect of GSNO and Cu$^{2+}$ on platelet L-arginine transport. The experiment was performed as outlined in section 3.4. Platelet samples (pre-incubated with 20 μM CuCl$_2$) were incubated with either 100 μM GSH or 100 μM GSNO for 5 minutes in the absence of light. Results are expressed as the mean ± standard error.
Fig. 9 - Effect of GSNO, light and Cu²⁺ on platelet L-arginine transport. The experiment was performed as outlined in section 3.4. Platelet samples (pre-incubated with 20 μM CuCl₂) were incubated with either 100 μM GSH or 100 μM GSNO for 5 minutes in the presence of visible light. Results are expressed as the mean ± standard error.
difference in the two (5.044 +/- 1.427 pmol/min/μmol and 5.175 +/- 1.344 pmol/min/μmol respectively). Samples exposed to GSNO (pre-incubated with 20 μM CuCl₂) showed a dramatic increase both in the dark (8.396 +/- 1.514 pmol/min/μmol) and in the light (8.797 +/- 1.588 pmol/min/μmol), though again there is no significant difference between the two.

4.2. SNAP and L-Arginine Transport

To further study the effects of NO on the transport of L-arginine into platelets, another NO donor (SNAP) was used. This compound was chosen due to its availability and its lack of function within the platelet (as opposed to GSH). Again, the transport was measured in the dark (Fig. 10) or after exposure to visible light for 5 minutes (Fig. 11). As with the studies utilizing GSNO no significant change in the efficiency of L-arginine transport was observed in the dark. However, when visible light was used to photolyse the S-NO bond in SNAP the efficiency increased from 1.532 +/- 0.544 pmol/min/μmol (dark) to 4.357 +/- 1.057 pmol/min/μmol (light). These experiments were repeated as with GSNO, in the presence of 20 μM CuCl₂. In these experiments, the efficiency was increased to 3.655 +/- 0.488 pmol/min/μmol (dark) (Fig. 12) and 4.630 +/- 1. pmol/min/μmol (light) (Fig. 13).

In an attempt to quantify the amount of NO given off by each of these S-nitrosothiols, a nitrate assay was performed. The results are given in Table 2.
Fig. 10- Effect of SNAP on platelet L-arginine transport. The experiment was performed as outlined in section 3.4. Platelet samples were incubated with either buffer or 100 µM SNAP for 5 minutes in the absence of light. Results are expressed as the mean ± standard error.
Fig. 11- Effect of SNAP and light on platelet L-arginine transport. The experiment was performed as outlined in section 3.4. Platelet samples were incubated with either buffer or 100 μM SNAP for 5 minutes in the presence of visible light. Results are expressed as the mean ± standard error.
Fig. 12- Effect of SNAP and Cu$^{2+}$ on platelet L-arginine transport. The experiment was performed as outlined in section 3.4. Platelet samples (pre-incubated with 20 μM CuCl$_2$) were incubated with either buffer or 100 μM SNAP for 5 minutes in the absence of light. Results are expressed as the mean ± standard error.
Fig. 13- Effect of SNAP, light and Cu$^{2+}$ on platelet L-arginine transport. The experiment was performed as outlined in section 3.4. Platelet samples (pre-incubated with 20 μM CuCl$_2$) were incubated with either buffer or 100 μM SNAP for 5 minutes in the presence of visible light. Results are expressed as the mean ± standard error.
Table 2: Amount of NO released from S-nitrosothiols. Results are expressed as the mean ± standard error.

<table>
<thead>
<tr>
<th>Sample</th>
<th>[Nitrate] (µM)</th>
<th>% Released</th>
</tr>
</thead>
<tbody>
<tr>
<td>SNAP, dark</td>
<td>11.50 ± 1.33</td>
<td>not detected</td>
</tr>
<tr>
<td>SNAP, dark, Cu</td>
<td>162.5 ± 6.47</td>
<td>1.18 ± 0.05</td>
</tr>
<tr>
<td>SNAP, light</td>
<td>172.6 ± 7.63</td>
<td>1.25 ± 0.06</td>
</tr>
<tr>
<td>GSNO, dark</td>
<td>21.56 ± 2.15</td>
<td>0.30 ± 0.03</td>
</tr>
<tr>
<td>GSNO, dark, Cu</td>
<td>130.5 ± 8.00</td>
<td>2.20 ± 0.14</td>
</tr>
<tr>
<td>GSNO, light</td>
<td>167.3 ± 5.60</td>
<td>2.80 ± 0.09</td>
</tr>
</tbody>
</table>
4.3. Other Sources of NO and L-Arginine Transport

To examine the effects of a different redox state of NO, another source of NO was utilized. NO$^-$(from NOBF$_4$) was used in this instance. A 100 μM solution of this compound (Fig. 14) showed no significant change in the efficiency (4.030 +/- 0.642 pmol/min/μmol) of L-arginine transport compared to that of a control (4.152 +/- 0.823 pmol/min/μmol). An aqueous solution of NO$_{aq}$ was prepared and its effect on L-arginine transport was studied (Fig. 15). Again, a 1 μM solution showed no significant difference in the efficiency (1.953 +/- 0.261 pmol/min/μmol) of L-arginine transport in comparison to a control (1.940 +/- 0.785 pmol/min/μmol).

4.4. Glutathione and L-Arginine Transport

The effect of GSNO on the efficiency of L-arginine transport (section 4.1.) possibly implicates GSSG in this process. GSSG would be formed in the decomposition of GSNO in the presence of Cu$^{2+}$ (Williams, 1996). Therefore, the effect of GSSG on the transport of L-arginine into platelets was studied (Fig. 16). It was found that GSSG significantly increased the efficiency of L-arginine transport (4.292 +/- 0.652 pmol/min/μmol) as compared to the control (2.011 +/- 0.197 pmol/min/μmol).

The efficiency results of all the trials studying the transport of L-arginine are graphically summarized in Fig. 17.
Fig. 14- Effect of NOBF₄ on platelet L-arginine transport. The experiment was performed as outlined in section 3.4. Platelet samples were incubated with either buffer or 100 μM NOBF₄ for 5 minutes. Results are expressed as the mean ± standard error.
Fig. 15-Effect of authentic NO on platelet L-arginine transport. The experiment was performed as outlined in section 3.4. Platelet samples were incubated with either buffer or 1 μM NOₐq for 5 minutes. Results are expressed as the mean ± standard error.
Fig. 16- Effect GSSG on platelet L-arginine transport. The experiment was performed as outlined in section 3.4. Platelet samples were incubated with either buffer or 100 μM GSSG for 5 minutes. Results are expressed as the mean ± standard error.
Fig. 17-Catalytic efficiency of L-arginine transport. Results are expressed as the mean ± percent error. * indicates a statistically significant difference between conditions (p< 0.05). Filled bars indicate samples pre-incubated with 20 μM CuCl₂.
4.5. **Immunological Analysis of Platelet Proteins**

The platelets of patients from a local hospital were subjected to an immunological analysis to determine the presence of nitrotyrosine residues. To see if patients suffering from diabetes have an increased incidence of nitrotyrosine formation, the blood samples were pooled according to the reported glycated hemoglobin value, as an index of glycemic control. The samples were first probed with an anti-nitrotyrosine antibody (Fig. 18). Two bands were evident on the resulting Western immunoblot, both in the lane corresponding to the blood samples from patients having a glycated hemoglobin value over 12% (poor glycemic control). No bands were observed in any of the other lanes. With the previous interest in the peptide calmodulin in our laboratory, it was noted that the molecular weight of one of these bands roughly corresponded to that of calmodulin. Therefore the platelet samples were again subjected to a Western blot, but this time using an anti-calmodulin antibody. This time only one band was evident, with a molecular weight corresponding to one of the bands in the anti-nitrotyrosine blot (approximately 17 kDa), which also correlates to the known molecular weight of calmodulin (16.7 kDa).
Fig. 18- Western immunoblot of platelet proteins. The platelet proteins were probed with either an anti-nitrotyrosine (1:400 dilution) or an anti-calmodulin (1:200 dilution) antibody. An anti-IgG conjugated to alkaline phosphatase (1:3000 dilution) was used to detect these antibodies, and NBT/BCIP was used to visualize. Blood samples were pooled according to their reported glycated hemoglobin values (<8% to >12%). Size of pre-stained MW standards (from bottom): 7.5 kDa, 19.2 kDa, 29 kDa, 34.1 kDa, and 51.6 kDa.
4.6 Peroxynitrite and GST Kinetics

To further study the effects of peroxynitrite in a biological system, a study of the enzyme glutathione S-transferase (π isoform) was undertaken. The reaction between GSH and CDNB (catalyzed by GST) was monitored using a spectrophotometer. The steady state kinetic parameters were determined by the linear regression analysis of a double-reciprocal plot (Fig. 19), and the results are summarized in Table 2. The first step in this experiment was determining the Michaelis-Menten kinetic parameters of the unmodified enzyme. The efficiency (Vmax/Km) of this enzyme was determined to be $9.70 \times 10^{-3} \pm 8.3 \times 10^{-4}$. To determine the effect(s) of peroxynitrite, the enzyme was incubated for 5 minutes in a 100 molar excess of peroxynitrite. This incubation lead to a drastic decrease in the efficiency of the enzyme ($5.33 \times 10^{-3} \pm 4.2 \times 10^{-4}$). To determine if the substrates (GSH and CDNB) offered any protection to this peroxynitrite modification, the enzyme was pre-incubated with a 100 molar excess of the appropriate substrate before exposure to peroxynitrite. A decrease in efficiency was still observed, both with pre-incubation with GSH ($8.51 \times 10^{-3} \pm 3.9 \times 10^{-4}$) or CDNB ($7.96 \times 10^{-3} \pm 4.8 \times 10^{-4}$), though the decrease was not as drastic as that observed with just peroxynitrite.
Fig. 19 - Glutathione S-transferase kinetic assay. The experiment was performed as outlined in section 3.8. To determine the effects of OONO the enzyme was incubated with a 100 molar excess. In protection studies, the enzyme was pre-incubated with a 100 molar excess of the appropriate substrate (CDNB or GSH). Results are expressed as the mean ± standard error.
Table 3- Kinetic parameters of glutathione S-transferase activity. Results are expressed as the mean ± standard error.

<table>
<thead>
<tr>
<th></th>
<th>Vmax (dA/s)</th>
<th>Km (mM)</th>
<th>Efficiency (Vmax/Km)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GST</td>
<td>1.30x10^{-2} ± 1.5x10^{-3}</td>
<td>1.34 ± 0.06</td>
<td>9.70x10^{-3} ± 8.3x10^{-4}</td>
</tr>
<tr>
<td>GST + OONO</td>
<td>8.31x10^{-3} ± 1.0x10^{-3}</td>
<td>1.56 ± 0.11</td>
<td>5.33x10^{-3} ± 4.2x10^{-4}</td>
</tr>
<tr>
<td>CDNB protection</td>
<td>7.08x10^{-3} ± 9.1x10^{-4}</td>
<td>0.89 ± 0.05</td>
<td>7.96x10^{-3} ± 3.9x10^{-4}</td>
</tr>
<tr>
<td>GSH protection</td>
<td>8.17x10^{-3} ± 9.8x10^{-4}</td>
<td>0.96 ± 0.04</td>
<td>8.51x10^{-3} ± 4.8x10^{-4}</td>
</tr>
</tbody>
</table>
5.1. *L*-Arginine Transport

In this study, the steady-state kinetic parameters (Vmax/Km) for L-arginine transport were determined under a variety of conditions (summarized in Fig. 17). Since Cu²⁺ or light was used to release NO from S-nitrosothiols, initially L-arginine kinetics were performed in the presence and absence of Cu²⁺ or visible light. The Vmax/Km for L-arginine transport obtained ± light were not statistically different (approximately 1.7 pmol/min/μmol) (Fig. 17). When the kinetics were performed in the presence of Cu²⁺ (20 μM) a statistically significant increase in Vmax/Km to approximately 3.0 pmol/min/μmol was observed. L-Arginine transport was not affected by a 5 minute pre-incubation of the platelets ± GSH (100 μM) and ± light (Fig. 6 and Fig. 7). However when the GSH experiments were repeated in the presence of Cu²⁺ the Vmax/Km values increased to approximately 4 pmol/min/μmol (Fig. 8 and Fig. 9). Pre-incubation with GSNO (100 μM) in the dark elevated the Vmax/Km values to approximately 3 pmol/min/μmol (Fig. 6), which is very close to that obtained with Cu²⁺ incubation. However the largest enhancements in the Vmax/Km values were obtained when GSNO was incubated with Cu²⁺ (Fig. 8) or when GSNO was incubated with the platelets in the presence of light (Fig. 7 and Fig. 9). Incubation of platelets with authentic NO (Fig. 15) or NOBF₄ (Fig. 14), a source of NO⁻, had no discernible effect on the transport parameters. S-nitroso-N-acetyl penicillamine (SNAP) also had no effect on the transport parameters (Fig. 10). Upon
inclusion of Cu$^{2+}$ in the SNAP solutions (± light) the Vmax/Km were elevated to values observed with GSNO or with Cu$^{2+}$ alone, approximately 3 to 4 pmol/min/μmol.

The amount of NO released by either Cu$^{2+}$ or visible light from the NO-donors was determined as that of the nitrate (NO$_2^-$) ion (Table 2). This was done since $^\ast$NO released from S-nitrosothiols in aerobic solutions ends up as NO$_2^-$ (Pietraforte et al, 1995). These experiments indicate that approximately 1 to 3 % (which corresponds to approximately 1 to 3 μM) of the S-NO is lost as NO during the 5 min incubation in the presence of light or Cu$^{2+}$.

To rationalize the fact that authentic NO failed to affect a response we postulate that the observed alterations in L-arginine transport efficiency are elicited by a combination of GSNO and GSSG. This hypothesis is summarized in Fig. 20. In this hypothesis, the platelet L-arginine transporter has a GSNO specific site and a GSSG specific site. Binding of GSNO to its specific site transnitrosylates the SH groups, which rapidly lose 2 NO and forms RSSR. This is the mechanism previously proposed for NO loss from stable S-nitrosothiols such as GSNO (Pietraforte et al, 1995). The RSSR formation at the transnitrosylation site partially simulates transport efficiency to values obtained with GSNO alone (approximately 3.0 pmol/min/μmol). RSSR formation at this site can also be induced by incubation of platelets by Cu$^{2+}$ (Vmax/Km approximately 3.0 pmol/min/μmol). The addition of Cu$^{2+}$ allows GSH to be rapidly oxidized to GSSG. Therefore, GSSG combining at the GSSG site induces a conformation that gives rise to Vmax/Km values of approximately 4 to 5 pmol/min/μmol. GSSG can also be produced from GSNO in the presence of Cu$^{2+}$ by an alternate mechanism (Williams, 1996):
Fig. 20- Proposed scheme of GSNO/GSSG effect on L-arginine transport.
\[
[\text{RSNO} + 2 \text{OH}^- \leftrightarrow \text{RS}^- + \text{NO}_2^- + \text{H}_2\text{O}]
\]

\[
\text{Cu}^{2+} + \text{RS}^- \rightarrow \text{Cu}^+ + \text{RS}^*
\]

\[
\text{Cu}^+ + \text{RSNO} \rightarrow \text{Cu}^{2+} + \text{RS}^- + \text{NO}
\]

\[
2 \text{RS}^* \rightarrow \text{RSSR}
\]

In order to test our hypothesis concerning GSSG the L-arginine transport kinetics were determined in the presence of 140 μM GSSG (Fig. 16). As predicted the \(V_{max}/K_M\) values were increased to approximately 4.3 pmol/min/μmol. We have also shown that GSSG is one of the major products of GSNO photolysis of GSNO (Wood et al., 1996).

According to our hypothesis the maximum efficiency of transport is obtained when the transnitrosylation site thiols are oxidized and GSSG is bound to the GSSG site. These conditions can be satisfied with GSNO plus Cu\(^{2+}\) or GSNO plus light. Under both conditions there is sufficient GSNO left to transnitrosylate the GSNO site thiols and enough GSSG formed to fill the GSSG site.

Data from recent studies independent from our laboratory corroborates different aspects of the scheme that we propose. It has been shown that incubation of GSNO with serum albumin leads to the formation of S-nitroso-albumin, which implicates the transnitrosylation reaction which we have proposed (Freedman et al., 1995). Also, the release of NO from GSNO has been described as either an NO transfer to a free thiol, or by an enzymatic cleavage (Askew et al., 1995). The authors conclude that transnitrosation or enzymatic cleavage are obligatory steps in the mechanism of NO release from GSNO, whereas SNAP does not follow this process. The S-nitrosation of cellular thiols has been shown to increase levels of cGMP in endothelial cells (Mayer et al., 1995). It has also been
proposed that the potency and platelet-selectivity of GSNO may result from targeted NO release at the platelet surface (Gordge et al, 1996). GSNO is currently being considered for clinical use for its anti-thrombic property. The use of NO donors in this area has been limited because with the inhibition of platelet activation comes the resulting hypotensive effect due to NO. However, it has been found that GSNO has a significant anti-aggregatory effect at doses that cause only a small decrease in blood pressure in rats (Ramsay et al, 1995).

5.2. Immunological Analysis of Platelet Proteins

Analysis of the Western immunoblot results using the anti-nitrotyrosine antibody yields interesting results. Platelets isolated from patients with normal (<8% glycated hemoglobin) or moderately controlled hyperglycemia do not show any evidence of nitrotyrosylated proteins. However, platelets isolated from patients with serious hyperglycemia (>12% glycated hemoglobin) show evidence of two proteins with significant nitrotyrosine formation. Comparing the migration of these proteins to standard proteins of known molecular weights indicates that these two proteins have approximate molecular weights of 10 kDa and 17 kDa respectively. The formation of these nitrotyrosine residues implicates the in vivo production of peroxynitrite, as evidenced in other pathological states, due to a simultaneous overproduction of "NO and O_2". The nitration of tyrosine residues is a convenient marker of reactive nitrogen-centered oxidants being produced. It is not necessarily due to the formation of peroxynitrite, but peroxynitrite is the most likely source in vivo (Beckman et al, 1994b). Other reactive species, such as nitrogen dioxide, can also form nitrotyrosine in simple solutions. Acidified
nitrite can also produce nitrotyrosine if left for several days in contact with a protein (Beckman and Koppenol, 1996). However, the amounts of nitrogen dioxide or nitrite that are present in vivo are far lower than that necessary to cause significant nitration in vitro. In a complex medium, such as that found in a cell, the reaction of superoxide dismutase with peroxynitrite becomes important. This superoxide dismutase-peroxynitrite pathway directs a more selective nitration of tyrosines on certain proteins (Beckman and Koppenol, 1996). Considering the vast number of alternative targets in biological systems, this superoxide dismutase-peroxynitrite direction of nitration becomes even more selective.

When the platelet samples are probed with an anti-calmodulin antibody, only one band is observed, with an approximate weight of 17 kDa. Calmodulin is a peptide with a molecular mass of 16.7 kDa, so this would seem to indicate that calmodulin, a ubiquitous eukaryotic calcium-binding protein, is one of the proteins being modified by peroxynitrite. Calcium is involved in the regulation of platelet function, as membrane associated calcium is involved in maintaining the integrity of the fibrinogen receptor and is thus indirectly involved in the aggregation of platelets (West, 1990). Free calcium in the cytoplasm is responsible for shape change, granular secretion, and is therefore also involved in platelet aggregation (Siess, 1989). Therefore, any change in the structure of calmodulin could possibly affect its binding to calcium, which would affect the regulation of platelet activity. Also, calmodulin is important in the regulation of the enzyme nitric oxide synthase, so again alterations in calmodulin structure could affect the regulation of this enzyme, and thus "NO production, which is known to be decreased in the diabetic state. The identity of the other protein remains unclear at this time.
5.3. Peroxynitrite and GST Kinetics

It is well established that peroxynitrite can react with several amino acid side chains in proteins. One of these side chains is the thiol group found in cysteine, producing disulfides and sulphenic acids (Radi et al, 1991). Peroxynitrite is also capable of oxidizing the indole side chain of tryptophan residues (Ischiropoulos and Al-Mehdi, 1995) and nitrating the aromatic side chains of phenylalanine and tyrosine (Beckman et al, 1992). The \( \pi \) isoform of glutathione S-transferase contains 56 tyrosine residues, 47 phenylalanine residues and 16 tryptophan residues, so there are many possible sites for peroxynitrite modification.

From the data it is apparent that peroxynitrite is reducing the efficiency of glutathione S-transferase (Fig. 19). The Michaelis-Menten kinetic parameters were calculated from a linear regression analysis of the double-reciprocal plot (Table 3). It is observed that after exposure to peroxynitrite, the efficiency of the enzyme is reduced to approximately 55% of that of the native enzyme. There are a number of possibilities as to how peroxynitrite is modifying the enzyme to alter its efficiency. Peroxynitrite can react with the thiol groups of cysteine, oxidize the indole group of tryptophan, and nitrate the aromatic groups of phenylalanine and tyrosine. Reaction with the thiol group can lead to inhibition of disulfide bond formation within the enzyme which could alter the catalytic properties of the enzyme. Nitration of the phenolic groups lowers the \( pK_a \) of the Tyr-OH from 10 to 7.2 (Creighton, 1993). This rather significant alteration in \( pK_a \) is proposed to lead to a loss in hydrogen bonding between Tyr-OH and potential hydrogen bond acceptors, which would destabilize the protein. It is also possible that the introduction of
the nitro group onto the tyrosine residue is hindered by steric constraints. The formation of nitrotyrosines could force an alteration in the structure of the protein due to the steric factors involved. Also, nitration of tyrosine residues changes a normally hydrophobic residue into a negatively charged hydrophilic residue, which would disrupt the assembly of the protein (Beckman and Koppenol, 1996).

When the enzyme was pre-incubated with a 100 molar excess of either substrate (CDNB or GSH) the effect of peroxynitrite was reduced, so that the catalytic efficiency was approximately 85% of that of the native enzyme. These numbers support the notion that the substrates are "protecting" the enzyme from the effects of peroxynitrite. This would lead to the hypothesis that at least part of the peroxynitrite modification is occurring in the substrate binding domains. Pre-incubation with the substrates would lead to binding in the catalytic sites, which would in principle block the access of peroxynitrite to any reactive centers in these areas. Further studies would be required to conclusively identify the sites of peroxynitrite modification. One such study would be studying the protein, and looking for signs of peroxynitrite modifications. Using fluorescence techniques, the oxidation of tryptophan residues and the nitration of tyrosine residues could be identified (as performed in Francescutti et al, 1996). Also, immunological methods could be used to determine the presence of nitrotyrosine residues, and any protection afforded by the substrates.
CONCLUSIONS

The investigation into the role of NO into the transport of L-arginine into human platelets suggests a combined role for GSNO and GSSG in the regulation of L-arginine uptake (diagrammed in Fig. 20). The rationale behind this is as follows. No significant change was observed in the kinetic parameters \( V_{\text{max}}/K_m \) of L-arginine transport after a five minute exposure to light (approximately 1.7 pmol/min/\( \mu \)mol). Incubation of the platelet samples with 20 \( \mu \)M \( \text{CuCl}_2 \) lead to an increase to approximately 3 pmol/min/\( \mu \)mol in the \( V_{\text{max}}/K_m \) value. When the platelet samples were incubated with 100 \( \mu \)M GSH (both in the presence and absence of visible light), no significant change in \( V_{\text{max}}/K_m \) was observed. However, when the samples were pre-incubated with \( \text{CuCl}_2 \), the \( V_{\text{max}}/K_m \) value rose to approximately 4 pmol/min/\( \mu \)mol. These results point to the involvement of GSSG, formed by the oxidation of GSH by \( \text{Cu}^{2+} \).

Incubation of the platelet samples with 100 \( \mu \)M GSNO raises the \( V_{\text{max}}/K_m \) to nearly 3 pmol/min/\( \mu \)mol. Pre-incubation with \( \text{CuCl}_2 \), or exposure to visible light, caused the \( V_{\text{max}}/K_m \) value to increase to between 8-10 pmol/min/\( \mu \)mol. As previously illustrated, GSNO in the presence of \( \text{Cu}^{2+} \) or visible light can lead to the production of GSSG. Measurement of the amount of NO released from GSNO (as nitrite) shows that only approximately 2-3 % of the NO is released (Table 2). The remaining GSNO is available for the transnitrosylation reaction outlined in our reaction scheme (Fig. 20). Therefore, the maximum effect is seen in the presence of both GSNO and GSSG.

To further study the effect on this system, another NO donor (SNAP) was used. SNAP itself had no significant effect on the \( V_{\text{max}}/K_m \), but pre-incubation of the platelets with \( \text{CuCl}_2 \) (± light) caused the \( V_{\text{max}}/K_m \) value to increase to between 3-4
pmol/min/μmol. This is the level attained with exposure to GSNO, or incubation with Cu²⁻ alone. Exposure of the platelets to authentic NO or NO⁻ did not significantly affect the kinetic parameters. To confirm the involvement of GSSG in this process, the effect of GSSG on the kinetic parameters was studied. It was found that the addition of GSSG to the platelets caused the Vmax/Km to increase to approximately 4 pmol/min/μmol.

The hypothesis that we have put forward can rationalize the results we have found. When GSNO or GSSG can bind to its specific site on the transporter, the Vmax/Km value increases to approximately 3-4 pmol/min/μmol. However, when both GSNO and GSSG can bind to the transporter, the Vmax/Km value is increased further, to approximately 8-10 pmol/min/μmol. The importance of the transnitrosylation reaction has been demonstrated in the reactions of GSNO (Freedman et al., 1995; Askew et al., 1995) but is not evident in the reactions of SNAP. It has also been proposed that the potency of GSNO on platelets may be due to its targeted release of NO at the platelet surface (Gordge et al., 1996) so binding of GSNO to the platelet surface would help to explain this hypothesis.

Human platelets do not show evidence for nitrotyrosine formation in vivo. However, in patients suffering from prolonged hyperglycemia (glycated hemoglobin > 12%) show two bands in a Western blot with the anti-nitrotyrosine antibody (Fig. 18). The formation of nitrotyrosine residues in proteins is a marker for the in vivo production of peroxynitrite. The formation of these residues is evidenced in other pathological states, due to the simultaneous overproduction of NO and O₂⁻. Comparing the migration of these proteins to those of known molecular weights indicates the these two proteins have approximate molecular weights of 10 and 17 kDa. Performing the Western immunoblot experiment again, but exposing the proteins to an anti-calmodulin antibody yields only one
band. Calmodulin has a molecular weight of 16.7 kDa, so it appears that calmodulin is one of the proteins being modified by peroxynitrite to form nitrotyrosine residues. Calmodulin is known to be involved in the regulation of platelet function, due to its interaction with calcium. Therefore, any change in the structure of calmodulin could alter its activity, and therefore alter the activity of platelets. This may help to explain the alteration in platelet function (hyperaggregation) in diabetic patients. The identity of the other protein is unclear at this time.

Peroxynitrite was observed to decrease the Vmax/Km of the reaction of glutathione S-transferase to approximately 55% of the native enzyme. It is now well established that peroxynitrite can modify proteins in several places, including the formation of nitrotyrosine residues or the oxidation of the indole side-chain found in tryptophan. Pre-incubation of the enzyme with either substrate afforded some protection from the effects of peroxynitrite (approximately 85% activity). This implies that peroxynitrite is modifying residues located within the catalytic site(s) of this enzyme, as binding of the substrate prevents the reaction of peroxynitrite with these residues.
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