UNIVERSITY OF CALGARY

The Importance of Nitric Oxide Adducts in Biological Fluids

by

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ABSTRACT

This research aimed at identifying how inhaled nitric oxide (NO) was being delivered to the periphery, and assessing the mechanisms by which inhaled NO impacted on pathology during ischemia-reperfusion. We have developed sensitive and reproducible methodologies for the detection of plasma S-nitrosoglutathione, S-nitroso-albumin, and nitrite / nitrate. We have demonstrated that S-nitroso-albumin was preferentially formed during NO inhalation compared to S-nitrosoglutathione. When NO inhalation was terminated, S-nitroso-albumin dropped and plasma nitrite / nitrate were maintained. Nitrite production was detected in plasma proteins and not red blood cells under aerobic conditions *in vitro*, suggesting that the dominant source of NO was plasma. Moreover, supplementation of cysteine enhanced the decomposition of S-nitroso-albumin; thus, modified NO production *in vitro*. Finally, we observed that inhaled NO improved intestinal blood flow during ischemia-reperfusion, coincident with the presence of arterial-venous gradients in S-nitroso-albumin. We conclude that S-nitroso-albumin was potentially responsible for intravascular NO delivery during pathology.

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DEDICATION

I would like to dedicate this work to the memory of my father, Hing Chiu Ng, who passed away during my graduate studies. I wish he could see me graduate. I thank him for teaching me not to give up. I hope my completion would make him proud.

This thesis is also dedicated to my mother, Janet, and my sisters, Lisa and Ada. I thank Lisa for giving me unconditional support and guidance over the past years. She has also encouraged me to be strong and taught me how to handle many unpleasant situations.

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LIST OF ABBREVIATIONS

ACh	acetylcholine
BH_4	tetrahydrobiopterin
BSA	bovine serum albumin
Ca^{2+}	calcium
Cu^{+}	copper (I)
Cu^{2+}	copper (II)
CysNO	S-nitrosocysteine
DAN	2,3-diaminonaphthalene
DTPA	diethylenetriaminepentaacetic acid
EDRF	endothelium-derived relaxing factor
EDTA	ethylenediaminetetraacetic acid
eNOS	endothelial nitric oxide synthase
FAD	flavin adenine dinucleotide
FMN	flavin mononucleotide
GSH	reduced glutathione
GSNO	S-nitrosoglutathione
GSSG	glutathione disulfide
Hb	hemoglobin
HbNO	nitrosylhemoglobin
HbO_2	oxyhemoglobin
HBSS	Hank's balanced salt solution
$HgCl_2$	mercuric chloride

HPLC	High Performance Liquid Chromatography
iNOS	inducible nitric oxide synthase
K_2HPO_4	anhydrous potassium phosphate dibasic
L-NAME	N^G-nitro-L-arginine-methyl ester
L-NMMA	N^G-monomethyl-L-arginine
MetHb	methemoglobin
MP	mobile phase
N_2	nitrogen
N_2O_3	dinitrogen trioxide
NADPH	reduced nicotinamide adenine dinucleotide phosphate
$NaNO_2$	sodium nitrite
$NaNO_3$	sodium nitrate
NaOH	sodium hydroxide
NEM	N-ethylmaleimide
nNOS	neuronal nitric oxide synthase
NO	nitric oxide
NO_2^-	nitrite ion
NO_2	nitrogen dioxide
NO_3^-	nitrate ion
NOS	nitric oxide synthase
NS	non-significant
NTA	1-[H]-naphthotriazole
O_2	oxygen

O_2^-	superoxide anion
$ONOO^-$	peroxynitrite
PBS	phosphate buffered saline
RBCs	red blood cells
RSNO	S-nitrosothiol
SEM	standard error of the mean
SMA	superior mesenteric artery
SMV	superior mesenteric vein
SNO-Alb	S-nitroso-albumin
SNO-Hb	S-nitrosohemoglobin
SP	stationary phase
SSA	5-sulfosalicylic acid

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 INTRODUCTION

Nitric oxide (NO) is a diatomic free radical with a single unpaired electron. It was previously considered as an atmospheric pollutant, which is produced during the combustion of fossil fuels (8;51). However, NO has now been implicated in many physiological processes, including blood pressure regulation, host defense and neurotransmission. In addition, NO has been postulated to be involved in pathophysiological conditions such as septic shock (93). Although NO has beneficial as well as harmful effects, NO delivered in a regulated manner has great potential to act as a therapeutic agent and may lead to new approaches to the prevention and treatment of diseases. Indeed, NO itself can be administered directly by inhalation, and has been used as a therapeutic option for the treatment of pulmonary hypertension in newborn infants (57;115) and acute respiratory distress syndrome in adults (57;93).

The effects of inhaled NO are purported to be limited to the pulmonary circulation since NO is inactivated by hemoglobin within the bloodstream that restricts its remote effects. However, recent studies have shown that NO interacts with peptides or proteins to form NO adducts called S-nitrosothiols (RSNOs), which may function as NO carrying systems, and thus transfer NO to the distal target sites (128;130). These RSNOs may serve as important intermediates that are responsible for the long-distance effects of NO. In fact, much work has been performed to demonstrate that inhaled NO can have an impact on the peripheral microvasculature (19;20;64), but there is insufficient data for identifying how inhaled NO is stabilized and delivered to the peripheral circulation.

This series of studies were designed to assess how inhaled NO is being carried within the circulation by RSNOs, and to examine the mechanisms by which inhaled NO

can impact on pathophysiological conditions. It is believed that an effective therapeutic delivery system for inhaled NO can be established as a potential means of treating disease in the periphery.

1.2 BACKGROUND: A HISTORICAL OVERVIEW OF NITRIC OXIDE

NO research has received tremendous amounts of attention over the last decades. The discovery of the roles of NO in biological systems dates back to 1980 when Furchgott and Zawadzki (22) demonstrated that acetylcholine (ACh) induced vascular relaxation in the presence of intact endothelium, and they provided evidence that this effect was mediated by a labile diffusible factor, which Furchgott later called endothelium-derived relaxing factor (EDRF). In 1987, different groups of researchers (21;103) suggested that perhaps EDRF is NO or may be some labile nitroso compounds that decompose with the liberation of NO (42). Indeed, several investigators postulated that nitrosylated compounds such as RSNOs are more likely to account for the EDRF activity (97;117). Since then, numerous studies have been directed toward understanding the roles of NO, with particular attention given to those RSNOs in biological systems.

1.3 SOURCES OF NITRIC OXIDE

In characterizing the fundamental roles of NO, the first question asked is the source of this species in a physiological setting. Indeed, NO is synthesized endogenously from the amino acid L-arginine by a family of enzymes called nitric oxide synthases (NOSs). NOSs exist as three different isoforms that are named on the basis of their locations. These enzymes include neuronal NOS (nNOS), inducible NOS (iNOS) and

endothelial NOS (eNOS) (90;98). nNOS is mainly found in neural cells of the central nervous system, where NO acts as a neurotransmitter. eNOS is present in endothelial cells of blood vessels, and is responsible for the vasodilator tone that is essential for the regulation of blood pressure. Both nNOS and eNOS are constitutively expressed, and their activities are regulated by Ca^{2+} concentrations and by subsequent binding of calmodulin. Under normal circumstances, nNOS and eNOS generate low levels of NO. In contrast, NO is produced in large quantities by iNOS, which is found in many cell types including macrophages, neutrophils and smooth muscle cells. iNOS is expressed after stimulation of inflammatory or immunological reactions, such as endotoxin, lipopolysaccharides (LPS) and cytokines, including interleukin-1, tumor necrosis factor (TNF) and γ-interferon (95). iNOS does not require Ca^{2+} for enzyme activation because it already has calmodulin tightly bound as a subunit. Its activity is controlled by regulation of mRNA transcription and translation, and several hours (4 – 6 hours) (58) are required for the production of NO by iNOS because it is a complex process that requires the synthesis of iNOS protein. Once iNOS is expressed, it produces copious quantities of NO for prolonged periods (58;70;81;149). Although these three enzymes are different by their locations, regulation of activity and functional roles, they all catalyze the same reaction by utilizing L-arginine as a substrate, and convert this amino acid to NO and a by-product, L-citrulline. This process involves oxygen (O_2) and reduced nicotinamide adenine dinucleotide phosphate (NADPH) as co-substrates. In addition, tetrahydrobiopterin (BH_4), flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN) are important co-factors for the activity of the enzymes during NO synthesis (68;93) (Figure 1.1). Nevertheless, the activity of nNOS, eNOS and iNOS

can be inhibited by derivatives of L-arginine, such as N^G-monomethyl-L-arginine (L-NMMA) or N^G-nitro-L-arginine-methyl ester (L-NAME). These NOS inhibitors are useful tools for studying the L-arginine-NO pathway *in vitro* and *in vivo* (93;144).

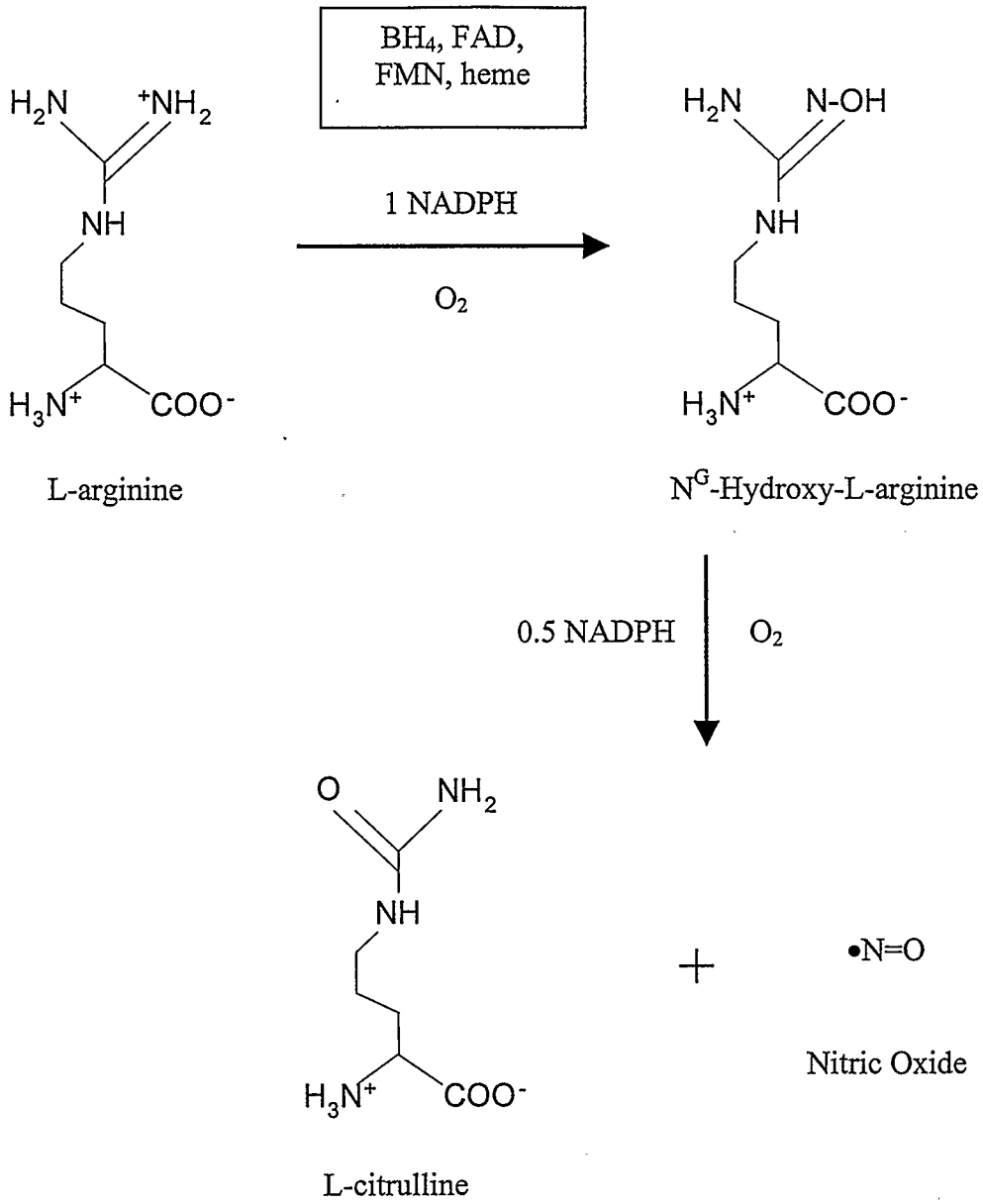


Figure 1.1. Conversion of L-arginine to L-citrulline and nitric oxide. The reaction is catalyzed by nitric oxide synthase (NOS), which utilizes NADPH and O_2 as co-substrates. Important co-factors include BH_4, FAD, FMN and heme. The 1st step involves hydroxylation of L-arginine that forms N^G-hydroxy-L-arginine as an intermediate. The 2nd step converts N^G-hydroxy-L-arginine to products NO and L-citrulline.

1.4 FUNCTIONS OF NITRIC OXIDE – FRIEND OR FOE?

NO plays essential roles in physiology and pathophysiology. At low concentrations, it is involved in physiological regulation and / or anti-inflammatory processes that are beneficial to biological systems; however, at high concentrations for long periods, NO may mediate deleterious effects (35). Therefore, it is important to understand the dual actions of NO because if the protective actions of NO can be maintained or enhanced, and the pathophysiological roles can be reduced or abolished, then NO may act as a potential therapeutic agent (94). The physiological and pathophysiological roles of NO, as well as its therapeutic applications are described below.

1.4.1 The Physiological Roles of Nitric Oxide

NO produced by eNOS is involved in homeostatic processes in biological systems. For example, the release of NO from endothelium has been shown to diffuse freely to vascular smooth muscle cells and promote vasodilation (41;103). On the other hand, research has demonstrated that using either a NOS synthase inhibitor (L-NAME or L-NMMA), or knocking out the genes encoding eNOS, an increase in mean arterial blood pressure and a reduction in blood flow occur. This suggests the importance of NO as being a vasodilator to maintain blood flow (93). Moreover, NO has significant effects on platelets and leukocytes. This radical not only reduces platelet and leukocyte adhesion to the endothelium, but also inhibits leukocyte activation and platelet aggregation (65;93). Thus, NO participates in anti-inflammatory functions, as well as optimizes blood flow regulation in the circulation. In the nervous system, NO is released by nNOS that also

contributes to the regulation of blood flow and pressure (93). In addition, NO acts as a neurotransmitter both in the peripheral and central nervous systems. In the central nervous system, NO is involved in several functions including learning and the formation of memory, whereas in the peripheral nervous system, it may contribute to sensory transmission and may act as a modulator in nonadrenergic and noncholinergic nerves (58;93).

1.4.2 The Pathophysiological Roles of Nitric Oxide

Although NO has important physiological functions, it may have detrimental effects in biological systems when it is over expressed by iNOS. Normally, the immunological induction of NO synthesis in macrophages appears to be an important part of the host defense mechanism because NO released in this situation may serve as an anti-oxidant and possess cytotoxic properties that are capable of killing bacteria, viruses and protozoa, as well as tumor cells (58). However, when NO is synthesized excessively by iNOS, and under conditions where both NO and superoxide (O_2^-) are generated, highly reactive and harmful species, such as peroxynitrite ($ONOO^-$) (111) and hydroxyl radicals (6;37) are produced. These species may mediate deleterious effects, such as inhibition of mitochondria respiration (125), lipid peroxidation (155) and DNA damage (100). In particular, the overproduction of NO and the formation of $ONOO^-$ are thought to contribute to tissue damage in pathology such as sepsis and septic shock, which are the most common causes of death in intensive care units (18;61;76). In fact, several mechanisms have been proposed for the high mortality of sepsis and septic shock. For example, prolonged vasodilation and low systemic vascular resistance caused by the

excessive production of NO may consequently lead to persistent systemic hypotension and perfusion abnormalities. These in turn result in multiple organ dysfunction and death (38;133). Moreover, the tissue injury in septic patients may also be attributed to the reactive nitrogen oxide species derived from the interaction between NO and O_2^- (61).

1.4.3 Therapeutic Implications – Nitric Oxide Inhalation Therapy

It is clear that NO has both protective and harmful effects. The discussion about these effects remains controversial (51). Nevertheless, the beneficial roles of NO in the cardiovascular, central and peripheral nervous systems are unequivocal. Studies have suggested that impaired generation of NO from the endothelium may lead to disease states, such as hypertension or endothelial dysfunction derived from ischemia-reperfusion (73;93). Therefore, the actions of NO may be useful in patients in certain cases, and may be therapeutic (94). In fact, NO itself can be administered directly by inhalation, and has been used in the treatment of infants with pulmonary hypertension (57;115) and in adults with respiratory distress syndrome (57;93). Therapeutically, inhaled NO acts as a local pulmonary vasodilator, which improves oxygenation and reduces pulmonary hypertension (48;148). Several investigators have shown that NO gas, when inhaled at 5 – 80 ppm, can completely reverse the pulmonary vasoconstriction (114;115). This demonstrates an important role for inhaled NO in the respiratory field. In general, the effects of NO inhalation are believed to be restricted to the lungs only because NO is short-lived and rapidly inactivated in blood by the reaction with oxyhemoglobin (HbO_2) (52). However, several lines of evidence suggest that the beneficial effects of NO not only last throughout the inhalation period, but in some cases persist after the termination

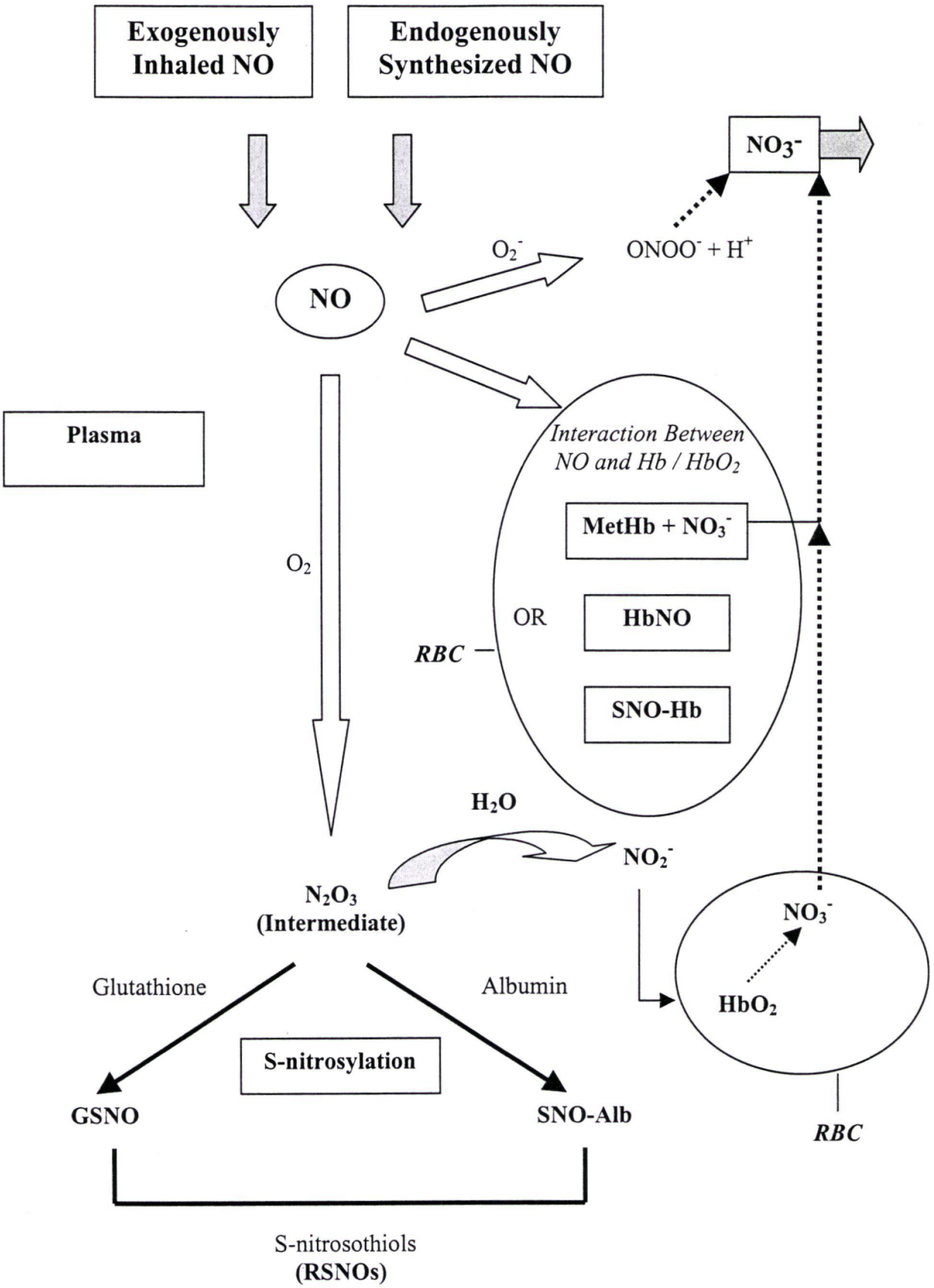
of treatment (19;20;28;64;94). Apparently, inhaled NO may extend its effects far beyond the pulmonary vasculature, and may act as a therapeutic delivery system to the systemic circulation. For example, Fox-Robichaud et al. (19) and Kubes et al. (64) demonstrated that inhaled NO could indeed reverse the vasoconstriction and leukocyte recruitment in the mesentery during NO synthesis inhibition. Moreover, they showed that the endothelial dysfunction, which is derived from a severe decrease in NO synthesis from the endothelium during ischemia-reperfusion, could be prevented by NO inhalation (19;64). These phenomena illustrate that inhaled NO exerts effects outside the lungs, and can actually reach the peripheral microvasculature to cause tremendous efficacy in reducing pathophysiological conditions (10;19;20;28;64). Accordingly, there must be a mechanism by which inhaled NO impacts upon the peripheral microvasculature. Perhaps some intermediate molecular species serve to stabilize NO and transfer NO to the target sites. To assess how inhaled NO is being carried within the circulation, it is important to understand the metabolic pathways of NO in biological systems.

1.5 METABOLISM OF NITRIC OXIDE IN ERYTHROCYTES AND PLASMA

As a free radical, NO is highly reactive and short-lived. For example, the half-life of NO in blood is only 0.05 – 1 s (52;55;128). In general, NO reacts rapidly with O_2, heme groups and various functional groups on biological molecules including thiols under physiological conditions (24). The metabolic pathways of NO in plasma differ somewhat from those in erythrocytes. In red blood cells (RBCs), most NO is oxidized by oxyhemoglobin (HbO_2) to form methemoglobin (MetHb) and nitrate (NO_3^-). This reaction has been considered as the major route for the destruction of NO *in vivo*

(5;55;152). Moreover, a small proportion of NO may react with hemoglobin (Hb) to form nitrosylhemoglobin (HbNO), or with the 93-cysteine residue of the β-subunit to form S-nitrosohemoglobin (SNO-Hb). The fraction of each is dependent on the ratio of oxygenated and deoxygenated hemoglobin within the erythrocytes (46;52;55). In the aqueous phase of plasma, NO may react with O_2 to form higher oxides of nitrogen (N_2O_3), which leads to the formation of nitrite (NO_2^-). NO_2^- remains stable for several hours in aqueous solutions (52;53), but in the presence of oxidizing species such as HbO_2, NO_2^- is converted to NO_3^- (43), with the latter being the major metabolite in urine (52;151). On the other hand, NO may interact with superoxide (O_2^-) to form $ONOO^-$, which decays rapidly once protonated and subsequently forms NO_3^- (6;52;55). Alternatively, the intermediate, N_2O_3, formed from the autoxidation of NO may undergo S-nitrosylation with molecules containing thiol groups in amino acids, peptides or proteins, such as cysteine, glutathione or albumin to form NO adducts called S-nitrosothiols (RSNOs) (56;130;131) (Figure 1.2). Examples of RSNOs include low molecular weight S-nitrosocysteine (CysNO), S-nitrosoglutathione (GSNO) and high molecular weight S-nitroso-albumin (SNO-Alb). Among these RSNOs, SNO-Alb tends to be more stable than low molecular weight molecules (9;127;128;130). In the present study, particular attention was given to plasma GSNO and SNO-Alb.

Figure 1.2. Simplified scheme of possible metabolic pathways of NO in erythrocytes and plasma. Endogenously synthesized NO and exogenously applied NO (inhaled NO) may reach the erythrocytes to react with either oxyhemoglobin (HbO_2) to form methemoglobin (MetHb) and nitrate (NO_3^-), or with hemoglobin (Hb) to form nitrosylhemoglobin (HbNO), or with the 93-cysteine residue of the β-subunit to form S-nitrosohemoglobin (SNO-Hb) depending on the ratio of oxygenated and deoxygenated Hb in RBCs. In plasma, NO may react with oxygen (O_2) and consequently hydrolyze to form nitrite (NO_2^-). NO_2^- may convert to NO_3^- in the presence of HbO_2. NO may also react with superoxide (O_2^-) to form peroxynitrite ($ONOO^-$). The subsequent decomposition of $ONOO^-$ may also lead to the formation of NO_3^-, which is then eliminated from the circulation. Alternatively, NO may undergo autoxidation to form an intermediate, dinitrogen trioxide (N_2O_3), which may then react with thiols (glutathione or albumin) to form S-nitrosothiols (GSNO or SNO-Alb).


Exogenously Inhaled NO
Endogenously Synthesized NO
NO3-
O2-
ONOO- + H+
NO
Plasma
Interaction Between NO and Hb / HbO2
MetHb + NO3-
O2
OR
HbNO
RBC
SNO-Hb
H2O
NO2-
N2O3 (Intermediate)
NO3-
HbO2
Glutathione
Albumin
S-nitrosylation
GSNO
SNO-Alb
RBC
S-nitrosothiols (RSNOs)

1.6 IMPORTANCE OF S-NITROSOTHIOLS AS NITRIC OXIDE CARRIERS

Despite the well-characterized interactions between the biological thiols and the derivative oxides of nitrogen (N_2O_3), the exact mechanisms by which RSNOs are formed *in vivo* remain uncertain due to the presence of other competitive reactions in biological systems (56;84). Nevertheless, RSNOs are found in the circulation. For example, GSNO has been identified in human physiological fluids (11;25). In addition, Stamler et al. (130) have detected that NO circulates in plasma as RSNO species, and SNO-Alb is principally formed among those molecules (128). More importantly, RSNOs have been shown to possess EDRF-like vasorelaxant effects (97;117), and anti-platelet properties (50;112;128;130), suggesting a mechanism by which the biological activity of NO is preserved in the circulation, perhaps through the formation of these intermediate RSNOs.

RSNOs are relatively more stable than NO (130). They act as NO storage molecules, which prolong the physiological half-life of NO and preserve the bioactivity of NO. Therefore, RSNOs are of great significance in NO metabolism in biological systems. They may play an important role in NO transport, and may have therapeutic potential as NO donors because they serve as important intermediates for NO in plasma from which NO is stored, transported and then released to the target sites (7;126). Essentially, RSNOs function as long-distance NO vehicles, which liberate NO in the presence of contaminant transition metals (in particular copper) and reducing agents (101;112;123;154). Moreover, RSNOs may also participate in transnitrosation reactions in which the NO group is transferred to another thiol that leads to the release of NO if the resultant compound is more susceptible to decomposition than the parent compound

(122). Both of these mechanisms are thought to attribute to the NO delivery in biological systems, but remain to be investigated.

The contention that inhaled NO can only affect the pulmonary vasculature has to be reassessed if plasma RSNOs are responsible for the stabilization, transport and delivery of NO in biological systems. Presumably, the delivery of NO to blood in the form of inhaled NO may produce RSNOs, and as a result, these intermediates may deliver small amounts of NO to the periphery. The present studies were undertaken to determine whether inhaled NO is being carried within the circulation by RSNOs in plasma, and to assess whether these species can deliver NO to impact the peripheral tissues. These work require the availability of analytical techniques that are reliable and capable of detecting the concentrations of RSNOs, including GSNO and SNO-Alb, as well as the NO metabolites, NO_2^- and NO_3^-, which serve as indicators of NO release from RSNOs in biological fluids.

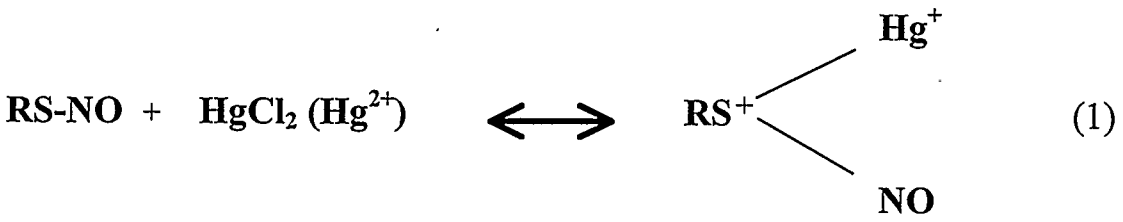
1.7 TECHNIQUES USED TO MEASURE S-NITROSOTHIOLS AND NITRIC OXIDE METABOLITES

1.7.1 Determination of SNO-Alb and GSNO in Biological Fluids

Plasma RSNOs have been detected *in vivo* with varying range of concentrations reported (30;81;85;91;120;128;130). For example, Stamler and colleagues (128) initially reported that the endogenous normal plasma levels of RSNOs (particularly SNO-Alb) were 7 μM. In addition, a recent study by Tyurin et al. (143) showed that the RSNO levels were also in micromolar range in human plasma. However, these findings have been questioned by researchers (138) because many other groups, such as Goldman et al.

(30), Jourd'heuil et al. (47), Marley et al. (83) and Rossi et al. (116) have found that the levels are several-fold lower (28 – 250 nM) than Stamler's originally reported values. The variations in concentration may reflect the differences in species, as well as the analytical techniques used. In the past, RSNOs in biological samples have been measured by a wide spectrum of different techniques. For instance, the Saville assay (119), which utilizes mercuric chloride ($HgCl_2$) to disrupt the S-NO bond of RSNOs, and then followed by quantitative detection of nitrous acid in sulfanilamide and naphthylethylenediamine dihydrochloride (Griess reagent), was initially employed. However, there are a few drawbacks regarding the use of this technique. First, this method is not sufficiently sensitive to detect RSNOs in biological fluids at nanomolar concentrations. Secondly, the procedures may be hindered by free thiols and the background concentration of NO_2^- present in biological samples, which may lead to artifactual formation of RSNOs (83). In addition, the NO_2^- contamination in all reagents and water may also interfere with the detection of RSNOs. Therefore, it is important to eliminate these concerns prior to the quantification of RSNOs in order to obtain reliable information. In the current study, we have developed techniques based on those described by Park et al. (104) and Marley et al. (83) for the analysis of SNO-Alb. We have employed a fluorescence assay that can enhance sensitivity and reproducibility for the measurement of SNO-Alb. This procedure is a variation of the Saville method in combination with fluorescence technique, which is based on the detection of a fluorescent compound, 1-[H]-naphthotriazole (NTA), formed between the acidified 2,3-diaminonaphthalene (DAN) and nitrous acid (protonated NO_2^- ion) released from SNO-Alb by the treatment with $HgCl_2$ (104;147) (Figure 1.3). Consequently, the intensity of

the fluorescent signal produced by NTA is measured. Moreover, we have eliminated the interference caused by free thiols and NO_2^- in biological samples by the addition of N-ethylmaleimide (NEM) and sulfanilamide respectively. Particularly, NEM can alkylate free thiols, which may stimulate the degradation of SNO-Alb. Thus, the use of NEM can increase the stability of SNO-Alb. Furthermore, the presence of NEM prior to the addition of sulfanilamide prevents the artifactual SNO-Alb generation from NO_2^-. Under the treatment with these reagents, results obtained should be trustworthy and reproducible.



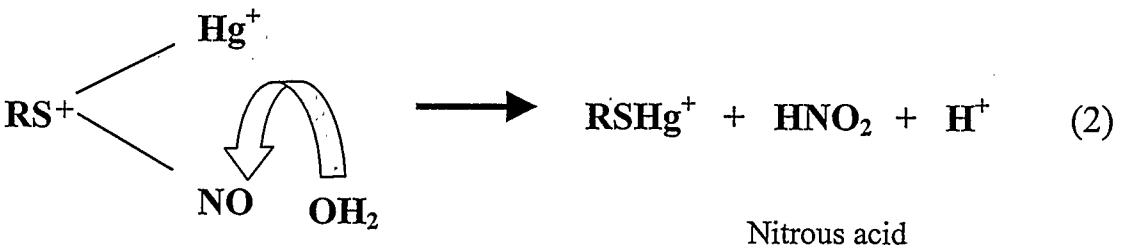


Figure 1.3. A simplified scheme for the detection of RSNO (SNO-Alb) using DAN-fluorescence assay. Mercury (II) ion (Hg^{2+}) catalyzes the decomposition of RSNO by breaking the S-NO bond, which results in the release of nitrous acid (HNO_2) (Equations 1 & 2). The protonated NO_2^- ion (nitrous acid) is then added to 2,3-diaminonaphthalene (DAN) to form a fluorescent compound 1-[H]-naphthotriazole (NTA) for fluorescence detection (Equation 3).

A few approaches have been described for the determination of GSNO, including the decomposition of S-NO bond to NO and its subsequent detection by chemiluminescence (128) or electrochemical detection with an NO electrode (108) or gas chromatography-mass spectrometry (141). Nevertheless, GSNO can be assessed with the aid of reversed-phase high performance liquid chromatography (HPLC) using UV-Visible detection, which we have employed in the present study. In fact, HPLC offers several advantages. It allows distinctive separation of GSNO from a complex mixture, plasma, and provides the capability of detecting the compound of interest directly. Moreover, this method requires only minimal sample preparation with only a single ultrafiltration step for GSNO. In general, the two major components on HPLC, namely the mobile phase (MP) and the stationary phase (SP), facilitate the separation. The extent or degree of separation is mostly determined by the choice of MP and SP that are dependent on the chemical and physical properties of the compounds. When a mixture is dissolved in the MP, the solution passes through the SP (chromatographic column), where separation occurs. Separation process is governed by the distribution of substances between two phases that is based on their different migration rates through the column. Compounds that are weakly held by the SP travel faster through the column. As a result, the compound that emerges from the column is detected by UV-Visible light absorbance detector (62). This technique allows the detection of GSNO from hundreds of nanomolar to thousands of nanomolar concentration range.

1.7.2 Determination of Nitrite and Nitrate in Biological Fluids

The oxidation products of NO, NO_2^- and NO_3^-, have been used as markers of NO production in biological systems (9;52;93;99). Changes in the concentration of these ions in biological fluids may be associated with the NO release by RSNOs within the circulation. The Griess assay by spectrophotometry is most frequently used for the determination of NO_2^- (17). This technique is simple, which requires only the addition of a reagent mixture to the sample. However, the Griess method is not sensitive enough to detect nanomolar concentrations of NO_2^-, which may be produced through the release of NO by RSNOs (also in nanomolar concentration) *in vivo* and *in vitro*. Moreover, this method does not pick up NO_3^-, unless samples are subjected to either chemical or enzymatic reduction (121). On the other hand, NO_2^- and NO_3^- are present at different concentrations in biological fluids. Therefore, methods aimed at quantifying NO_2^- and NO_3^- have to cover the low nanomolar concentration range for NO_2^- and high micromolar range for NO_3^-. In this case, employing two distinctive techniques may be helpful for the determination of these ions. In particular, the chromatographic technique described in this research may offer the advantage of measuring NO_3^- because this method does not require a reduction step. In addition, sample manipulation is small because it requires only a deproteinization step prior to chromatography. For the detection of NO_2^-, the DAN fluorescence detection can be applied. This method, as described by Misko et al. (92) is more sensitive than the well-known Griess assay because fluorescence detection allows for exquisite sensitivity for NO_2^- in nanomolar concentration. As NO_2^- formation was measured in this study as an index of NO production, and the concentration of this

ion was expected to be in nanomolar range; therefore, a highly sensitive DAN fluorescence technique was chosen for the detection of NO_2^- in biological fluids.

1.8 STATEMENT OF HYPOTHESIS AND OBJECTIVES

The first objective of this study was to develop analytical assays for the measurement of NO_2^-, NO_3^-, GSNO and SNO-Alb in plasma.

The main focus of this study was to determine how inhaled NO was stabilized and delivered to the peripheral circulation, and to investigate whether inhaled NO could impact on pathology. Additionally, we sought to identify whether plasma RSNOs were the dominant source of NO compared to RBCs, and to examine whether supplementation of an NO carrier could enhance the NO release from RSNOs. To properly address these issues, we quantitatively measured the concentrations of NO_2^-, NO_3^-, GSNO and SNO-Alb in plasma.

Hypothesis 1: S-nitroso-albumin is the dominant S-nitrosothiol over S-nitrosoglutathione in plasma during NO inhalation.

Objectives:

1) To determine whether the levels of NO_2^- and NO_3^- in cat plasma change during NO inhalation and after NO inhalation is terminated.
2) To measure the levels of GSNO and SNO-Alb during inhaled NO and after inhaled NO is discontinued, and to examine which RSNO (GSNO or SNO-Alb) is primarily formed in cat plasma during NO inhalation.

Hypothesis 2: NO is mostly released from plasma proteins compared to red blood cells in oxygenated milieu *in vitro*.

Objectives:

1) To determine whether SNO-Alb is the source of NO release by measuring the *in vitro* plasma NO_2^- levels over 60 min incubation in the presence of oxygen.

2) To determine if RBCs are sources of NO by measuring the *in vitro* levels of NO_2^- and NO_3^- in buffer incubated in RBCs from NO breathing animals.

3) To examine whether the release of NO occurs in whole blood from NO breathing animals by measuring the *in vitro* levels of NO_2^- and NO_3^- in plasma.

Hypothesis 3: Supplementation of an NO carrier, cysteine, increases NO release from S-nitroso-albumin *in vitro*.

Objectives:

1) To determine whether supplementation of cysteine affects the plasma concentration of SNO-Alb *in vitro*.

2) To determine if levels of NO_2^- change in plasma with the treatment of cysteine over 60 min incubation *in vitro*.

Hypothesis 4: Inhaled NO can impact on pathology, such as ischemia-reperfusion by extracting NO from SNO-Alb across the post-ischemic vasculature.

Objectives:

1) To determine the effect of inhaled NO on intestinal blood flow before and after ischemia-reperfusion.

2) To measure the arterial-venous differences for SNO-Alb in order to determine whether the post-ischemic tissue removes NO from this NO carrier.

3) To investigate whether NO is released from SNO-Alb across the post-ischemic vasculature by measuring the arterial-venous differences for plasma NO_2^- and NO_3^-.

CHAPTER 2

METHODS AND MATERIALS

2.1 EXPERIMENTAL MODELS

2.1.1 Protocols for Cat Surgery

Cats of approximately 2.5-3 kg in size (not significantly different in size than premature human neonates) were selected as animal models in this research because NO inhalation could be easily regulated in these animals, and blood sampling was not limited by volume. Moreover, we were able to cannulate relevant blood vessels in these animals.

The surgery procedures used in this research have been described previously in details (19;63;64). Animal protocols were approved by the University of Calgary Animal Care Committee and met the Canadian Guidelines for Animal Research. In brief, cats (2.5-3 kg) were fasted for 24 h and initially anesthetized with ketamine hydrochloride (75 mg intramuscularly). The jugular vein was cannulated, and anesthesia was maintained by the administration of sodium pentobarbital. Systemic arterial pressure was monitored using a pressure transducer (Statham P23A, Gould, Oxnard, CA) connected to a catheter in the left carotid artery. A tracheotomy was performed to support breathing by artificial ventilation. NO at 80 ppm was delivered from a certified grade NO / balance N_2 gas cylinder to the inhalation line of a Harvard ventilator via a high-accuracy Matheson flowmeter (Matheson Gas Products Canada, Edmonton, Alberta, Canada), and NO and NO_2 were measured with a Pulmonox II NO and NO_2 electrochemical analyzer (Pulmonox Research and Development, Tofield, Alberta, Canada). Throughout the experiments, exhaled NO_2 was read online to ensure that levels were < 5 ppm. This NO delivery setup was similar to the one used to deliver NO to newborn infants with respiratory distress in the neonatal intensive care unit of the Foothills Medical Centre

(University of Calgary), except in our system the cats were not provided with supplemental oxygen but rather ventilated on room air.

A midline abdominal incision was made, and a segment of small intestine was isolated from the ligament of Treitz to the ileocecal valve. The remainder of the small and large intestine was extirpated. Body temperature was maintained at 37°C using an infrared heat lamp. All exposed tissues were moistened with saline-soaked gauze to prevent evaporation. Heparin sodium (10, 000 U, Leo Pharma Inc. Ajax, Ontario, Canada) was administered, then an arterial circuit was established between the superior mesenteric artery (SMA) and left femoral artery. The SMA blood flow was continuously monitored using an electromagnetic flowmeter (Carolina Medical Electronics, King, NC), and blood pressures were recorded with a physiological recorder (Grass Instruments, Quincy, MA) (64).

2.2 EXPERIMENTAL PROTOCOLS

2.2.1 Major S-nitrosothiols in Plasma During NO Inhalation

In the first series of experiments, baseline blood samples were collected from the SMA of cats, and the plasma portions were analyzed. To investigate whether the formation of RSNOs occurred during NO inhalation, and whether these molecules released NO after inhaled NO was terminated, 80 ppm inhaled NO was given to cats and continued for 60 min before blood samples were taken. Inhaled NO was then discontinued for 60 min, and blood samples were again drawn. Concentrations of GSNO, SNO-Alb, NO_2^- and NO_3^- were measured in plasma before, during and after NO inhalation. Figure 2.1 shows the flow chart of the experimental design.

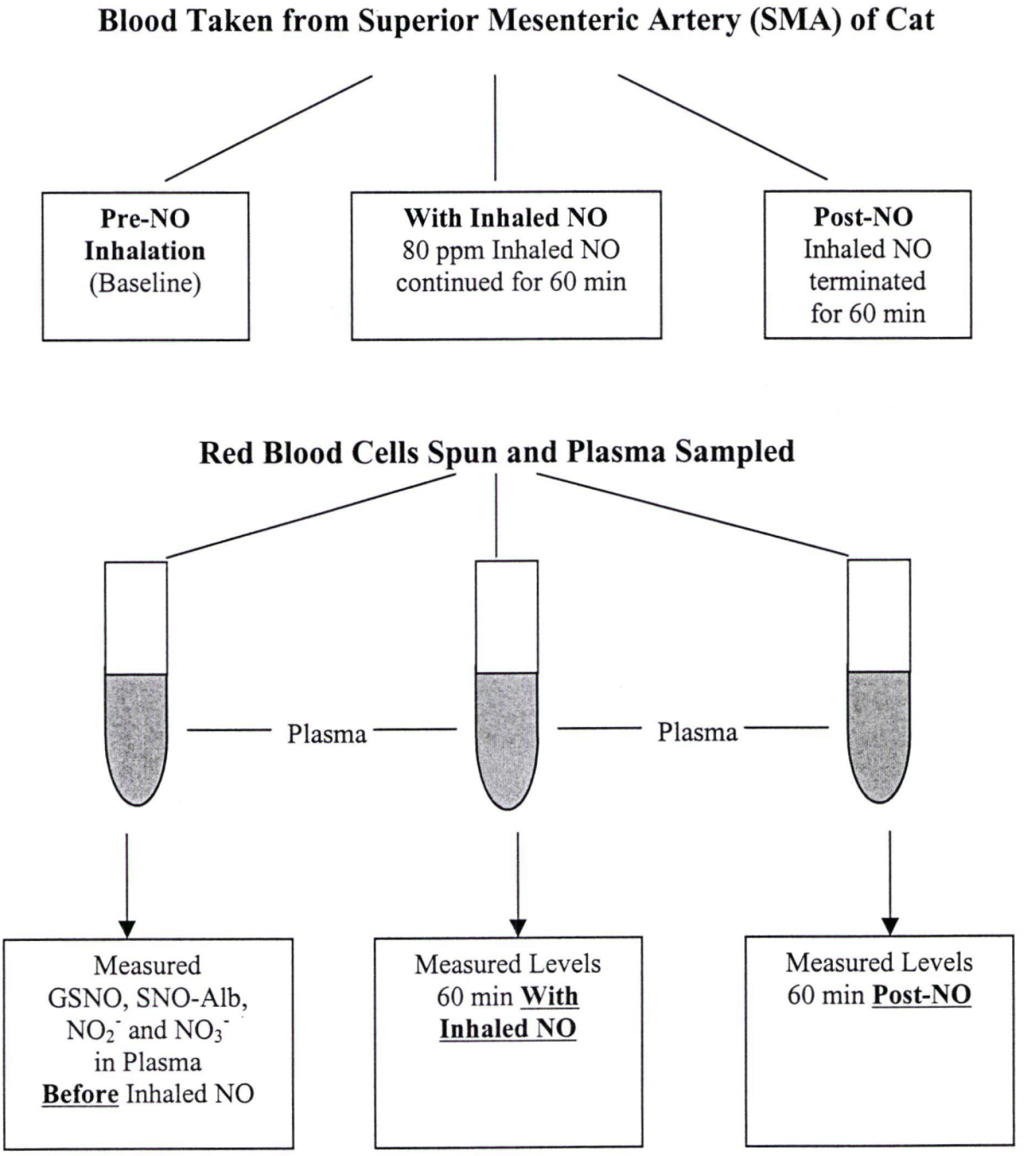


Figure 2.1 Experimental design for the measurement of GSNO, SNO-Alb, NO_2^- and NO_3^- in cat plasma before, during and after NO inhalation.

2.2.2 Liberation of NO from Molecules in Different Compartments of Blood *in vitro*

To identify whether molecules in RBCs or RSNOs in plasma that had been exposed to inhaled NO over an hour would release NO *in vitro*, the compartments of whole blood were separated into two fractions, namely RBCs and plasma. Blood samples being exposed to inhaled NO for 60 min were collected as described in Section 2.2.1. They were then centrifuged to plasma. In the first part of the experiments, plasma from cats on inhaled NO was transferred to another tube (Figure 2.2) and incubated in the dark for an hour under aerobic conditions (with ambient oxygen). Plasma was analyzed at the start (0 min) and end of the 60 min incubation to examine the levels of SNO-Alb, NO_2^- (primary oxidation product of NO *in vitro*) and NO_3^- ions. An increase in NO_2^- and NO_3^- concentrations might represent the generation of NO from RSNOs in plasma. Figure 2.2 illustrates the experimental protocols for part 1 of the experiments.

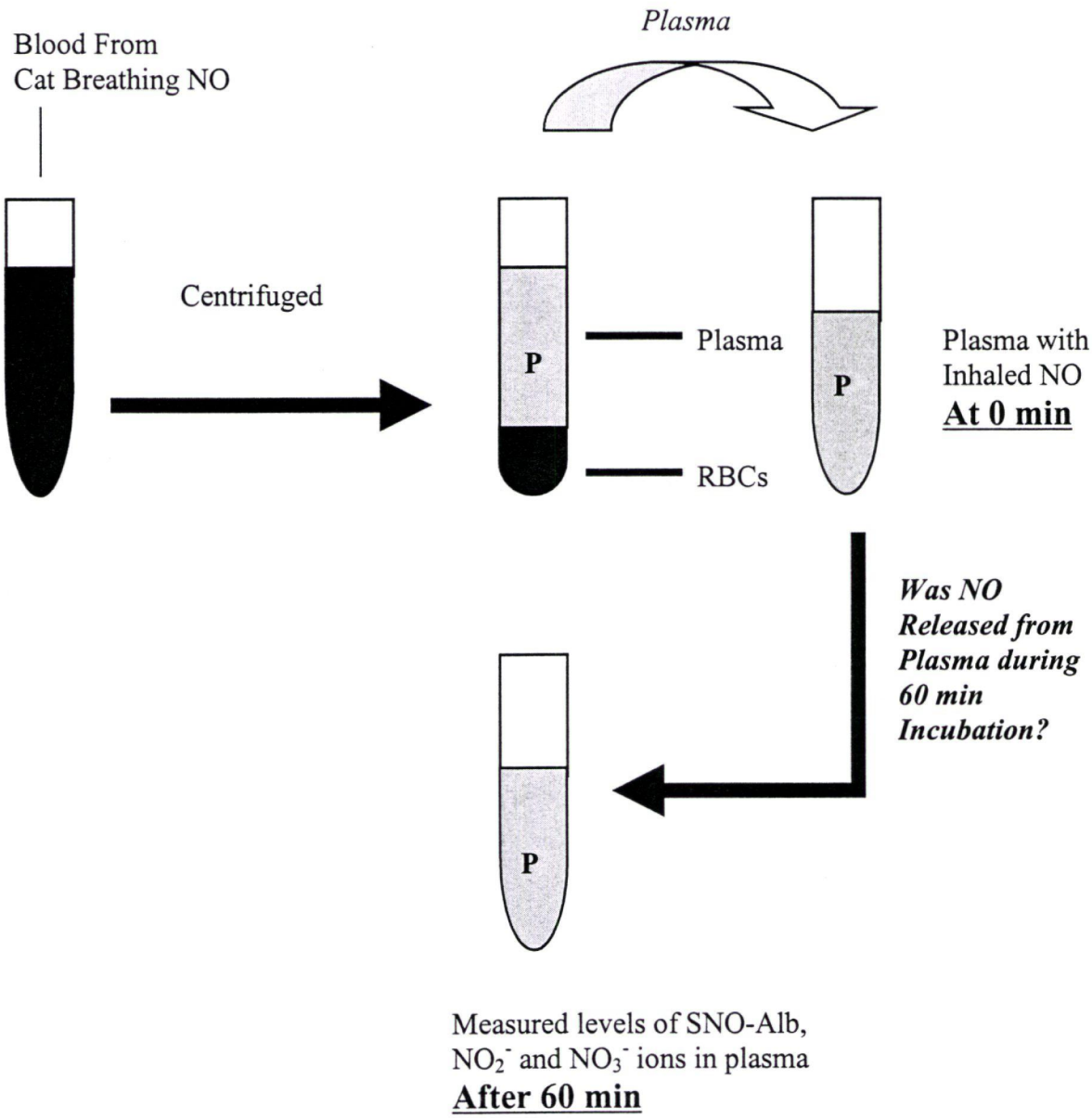


Figure 2.2 Part 1: Experimental design for the evaluation of NO release from S-nitrosothiols in plasma proteins *in vitro*. SNO-Alb, NO_2^- and NO_3^- concentrations in plasma were measured. Note: P = plasma

To evaluate whether RBCs released NO *in vitro*, RBCs from cats breathing NO were reconstituted in Hank's balanced salt solution (HBSS) (Life Technologies, NY, US) and incubated for an hour. Concentrations of NO_2^- and NO_3^- in the buffer solution were measured before and after the incubation (Figure 2.3, Part 2). Both ions were analyzed because there was a possibility that NO released from RBCs might convert to NO_2^- and NO_3^-, and that NO_2^- might be further oxidized to NO_3^- by HbO_2 within the RBCs (53;55).

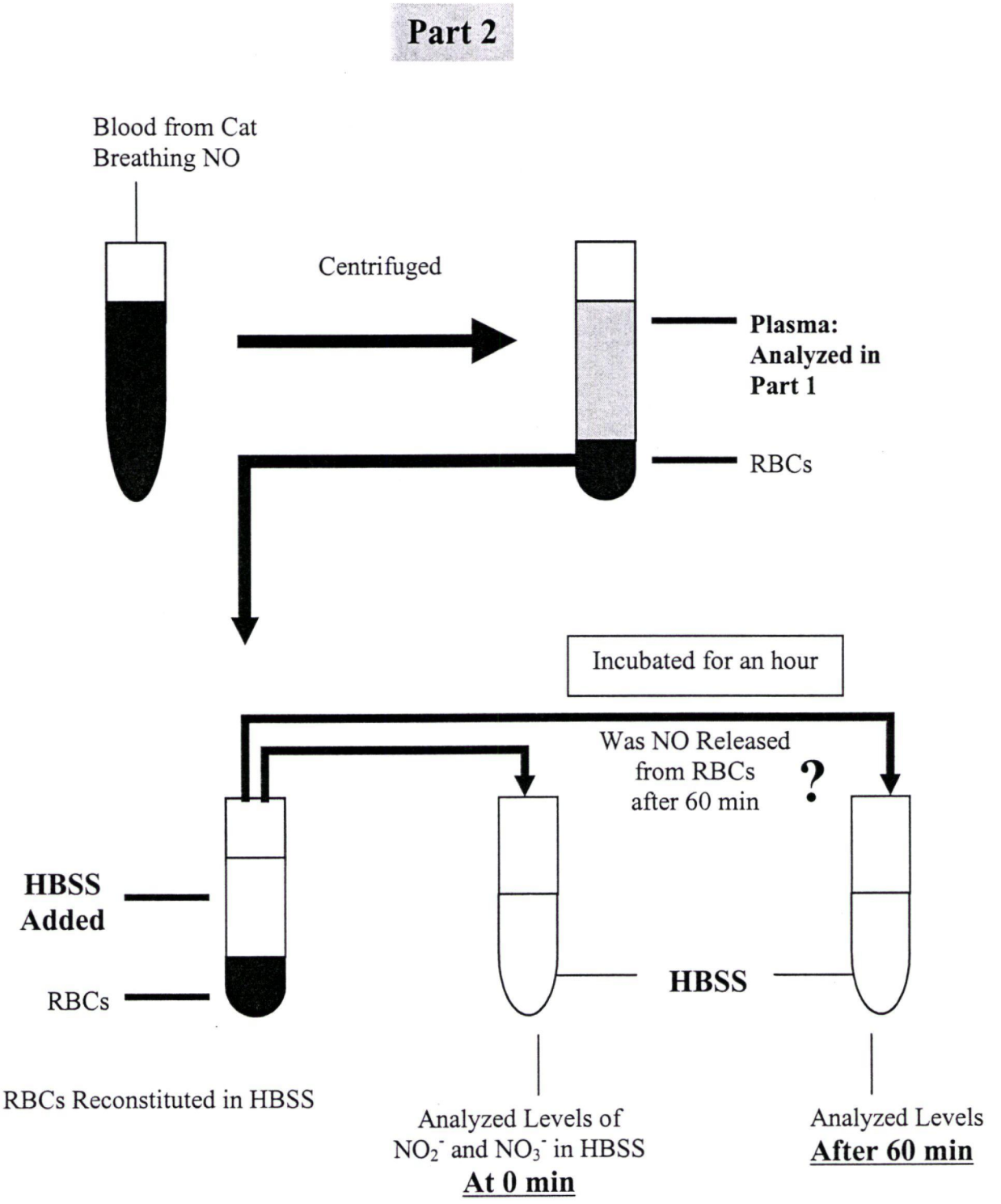


Figure 2.3 Part 2: Experimental Protocols to investigate the NO release from RBCs before and after incubation in HBSS *in vitro*. NO_2^- and NO_3^- concentrations in HBSS were measured.

In a separate set of experiments, we examined whether thiol groups in plasma could enhance the NO release from RBCs *in vitro*. Experiments similar to Part 2 were performed; however, instead of adding HBSS to RBCs, fresh naïve plasma from a cat not breathing NO was added to RBCs from a cat breathing NO. The mixture was then incubated in the dark for an hour in the presence of ambient oxygen. Levels of NO_2^-, NO_3^- and SNO-Alb in plasma were analyzed before and after the incubation.

In the last series of experiments, we investigated whether NO was released from whole blood *in vitro*. Whole blood from cats breathing NO was incubated in the dark over an hour in the presence of ambient oxygen without any manipulation. Then, whole blood was spun to plasma after the incubation, and the plasma levels of NO_2^-, NO_3^- and SNO-Alb were measured.

2.2.3 Supplementation of an NO Carrier to S-nitroso-albumin *in vitro*

Blood with inhaled NO was spun to plasma, which was then transferred to two separate tubes (Figure 2.4). In one tube of the plasma, levels of NO_2^-, NO_3^- and SNO-Alb were examined over 0, 5, 30, 60 min time intervals, and treated as a control. In another tube, 100 μM cysteine (Sigma Chemical Company, Ontario, Canada) was added and analyzed over the same time intervals. Both treated and untreated groups were incubated at 37°C in the dark. Plasma levels of NO_2^- and NO_3^- were measured in order to investigate whether the addition of cysteine could increase the NO production from SNO-Alb *in vitro*. Figure 2.4 shows the experimental design for this part of the experiments.

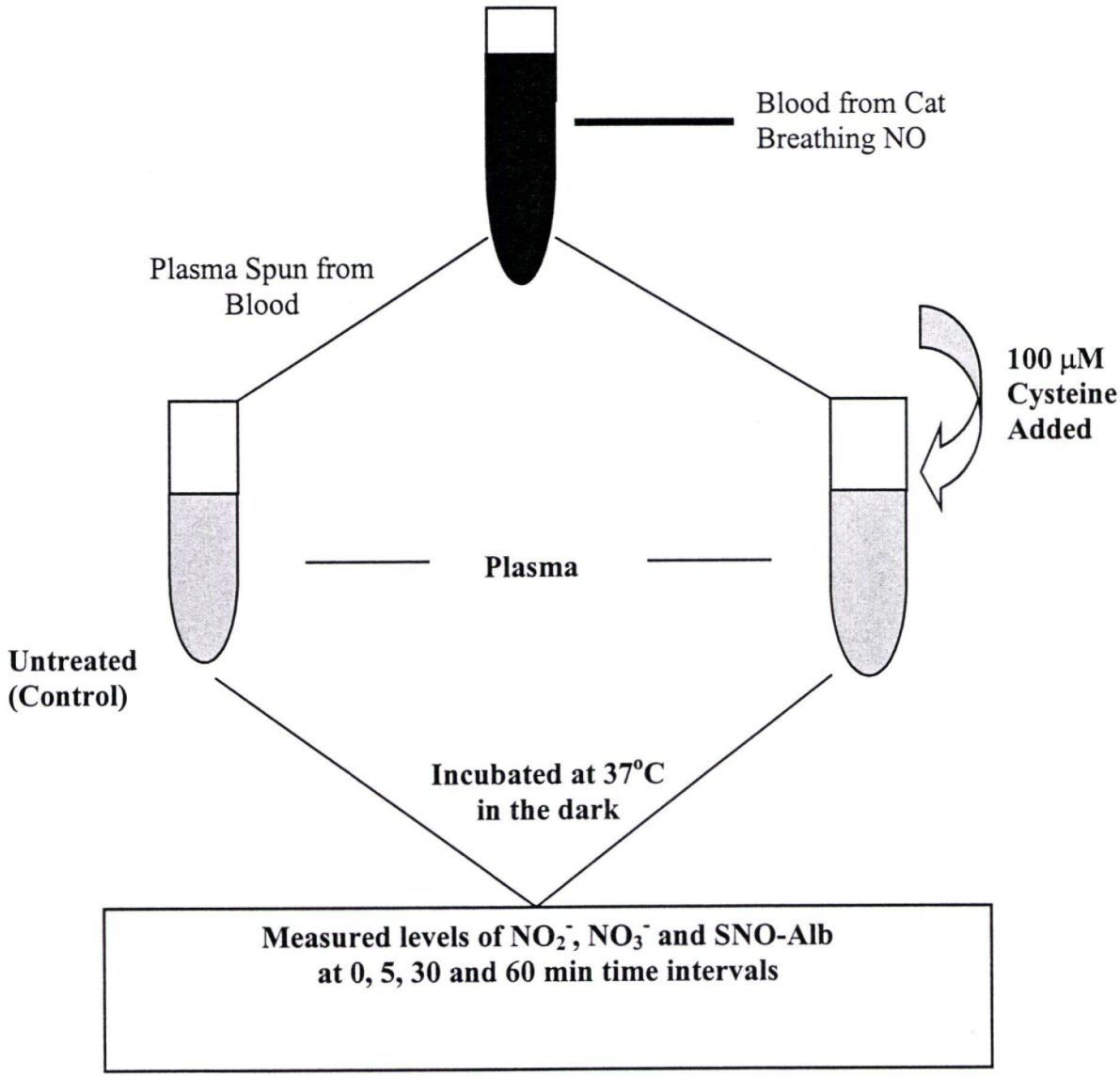


Figure 2.4 Experimental design to evaluate whether supplementation of cysteine to SNO-Alb could increase NO release *in vitro*.

2.2.4 Inhaled NO-Induced NO Delivery During Ischemia-Reperfusion

In the first set of experiments, we determined whether the NO releasing capacity occurred in healthy animals during NO inhalation by analyzing the arterial and venous differences for SNO-Alb, NO_2^- and NO_3^- across the intestinal microvasculature. Arterial blood samples were drawn in the same way as described in Section 2.1.1, whereas venous blood samples were taken through a catheter placed in a small branch off the superior mesenteric vein (SMV) before and after animals were made to breathe 80 ppm NO for 60 min. Concentrations of SNO-Alb, NO_2^- and NO_3^- in plasma obtained from arterial and venous blood samples were measured before and after NO inhalation. To examine the effect of inhaled NO on intestinal blood flow in healthy animals, SMA blood flow was recorded before and during 60 min of NO inhalation.

In a second set of experiments, we evaluated whether NO was delivered by SNO-Alb across the intestinal microvasculature during a pathological condition, i.e., ischemia-reperfusion, in cats inhaling 80 ppm NO. Baseline measurements of NO_2^-, NO_3^- and SNO-Alb in arterial and venous plasma obtained from blood samples were first examined. Experiments were carried out in which ischemia was induced by mechanically occluding the SMA blood flow with a Gaskell clamp (C-clamp) (64). Blood flow was reduced to 20% of the pre-ischemic value (control) for an hour, and then the clamp was removed to allow reperfusion. 80 ppm NO was given to cats right at the onset of reperfusion, and blood samples were collected at 30 and 60 min after reperfusion with inhaled NO. Measurements of blood pressure and SMA blood flow were also obtained at the aforementioned times at either 0 (room air ventilation) or 80 ppm NO during ischemia-reperfusion.

2.2.5 Sample Treatments

All blood samples collected from cats were immediately added to 10 mL culture tubes containing ethylenediaminetetraacetic acid (EDTA) (final concentration 2 mM) obtained from Sigma. They were centrifuged at 3000 rpm for 10 min at 4°C. Plasma samples used for measurement of RSNOs were transferred to tubes that contained diethylenetriaminepentaacetic acid (DTPA) and N-ethylmaleinimide (NEM) (final concentration 5 mM) in order to chelate transition metals and to alkylate excess thiol groups present in plasma respectively (83).

2.2.6 Nitrite, Nitrate, S-nitrosoglutathione and S-nitroso-albumin Assays

The measurement of NO_2^-, NO_3^-, GSNO and SNO-Alb in plasma will be discussed in details in the next chapter.

2.3 STATISTICAL ANALYSIS

Standard curves and regression analysis for method development were performed using Microsoft® Excel 2000 software. The coefficient of variation (% CV) was calculated as standard deviation divided by the mean multiplied by 100.

All experimental data are expressed as mean ± standard error of the mean. One-sided P values were calculated by Mann-Whitney test using standard statistical program. Probability values of $p < 0.05$ were considered statistically significant.

CHAPTER 3

METHOD DEVELOPMENT FOR THE DETERMINATION OF NITRITE, NITRATE, S-NITROSOGLUTATHIONE AND S-NITROSO-ALBUMIN IN PLASMA

Objectives:

To develop analytical assays for the measurement of nitrite, nitrate, S-nitrosoglutathione and S-nitroso-albumin in plasma.

3.1 INTRODUCTION

The present study describes quantitative procedures for the detection of NO_2^-, NO_3^-, GSNO and SNO-Alb in biological fluids. The methodologies developed here provide a rapid, sensitive and direct determination of these ions and compounds in a complex matrix, plasma. Validation of the assays is included in each section shown below.

3.2 ANALYSIS OF S-NITROSOGLUTATHIONE BY HPLC

3.2.1 Materials

Reduced glutathione (GSH), glutathione disulfide (GSSG), sodium nitrite ($NaNO_2$), benzoic acid, N-ethylmaleinimide (NEM), diethylenetriaminepentaacetic acid (DTPA) and anhydrous sodium phosphate monobasic were obtained from Sigma Chemical Company (Ontario, Canada). Hydrochloric acid was a product of BDH Inc. (Ontario, Canada). 30K Nanosep® centrifugal devices were purchased from Pall Gelman (Ontario, Canada). Millipore Milli-Q ultra-pure deionized water was used throughout the research.

3.2.2 Synthesis of S-nitrosoglutathione

Stock solution of GSNO was freshly prepared by reacting equimolar $NaNO_2$ with GSH in 1 M hydrochloric acid, and incubated at room temperature for 15 min in the dark (45). DTPA was added as a chelating agent to prevent degradation, since GSNO decomposes in the presence of metal ions in solutions or in biological samples (123). Stock solution of GSNO was diluted using phosphate buffered saline (PBS) containing 50 μM DTPA (pH 7.4), and the standard curves for GSNO were prepared by adding known amounts of GSNO to either PBS or plasma (see Section 3.2.5).

3.2.3 Extraction Procedures for S-nitrosoglutathione

Benzoic acid (~ 0.082 mM) was spiked into 200 μL of plasma as an internal standard. 200 μL of 5 mM NEM was added to alkylate any unreacted thiols in plasma at room temperature. Alkylation of thiol groups by NEM could stabilize RSNOs in plasma by preventing transnitrosation reactions (83). Moreover, the treatment with NEM could minimize the artifactual formation of RSNOs through the interaction between NO_2^- and thiols in plasma (83). Plasma samples were ultrafiltrated using Pall Gelman Nanosep, 30,000 MW cut-off filters to remove high molecular weight RSNOs. The filtrate was collected and injected onto the HPLC.

3.2.4 Reversed-Phase High Performance Liquid Chromatography

Concentrations of GSNO in plasma were measured using HPLC 1100 Series (Agilent Technologies, US) equipped with a binary pump. The column employed was Hypersil BDS C_{18} column, 125 × 4 mm, 5μm (Agilent Technologies, US) connected with

a guard holder that contained an octadecyl cartridge, 4 × 3.0 mm (Phenomenex, U.S.). The purpose of using the guard cartridge was to prevent the impurities of samples from entering the analytical column. In this case, the contaminants that were irreversibly adsorbed by the stationary phase of the column would remain in the guard cartridge. The mobile phase was 99% 50 mM phosphate buffer (pH 5.5) and 1% acetonitrile. The gradient run was carried out at a flow rate of 0.55 mL/min. Detection was by absorbance at 336 and 214 nm using a UV-Visible (UV-Vis) multiple wavelength detector. Figures 3.1 and 3.2 illustrate the HPLC instrumentation (2) and the parameters for the analysis of GSNO respectively.

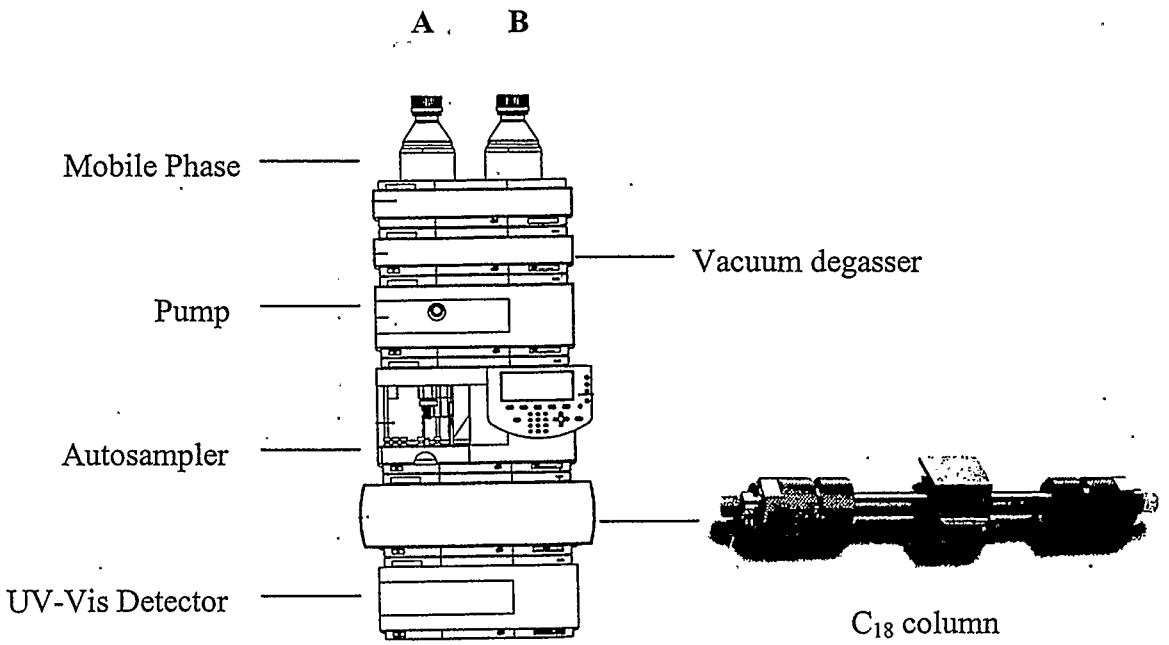


Figure 3.1. HPLC instrumentation for the analysis of GSNO (Taken from the reference manual of Agilent Technologies, 1999) (2).

Figure 3.2. HPLC conditions and parameters for the analysis of GSNO.

Column: Hypersil BDS C_{18} column, 125 × 4 mm, 5μm

Guard Holder: SecurityGuard™ cartridge (ODS, 4 × 3.0 mm)

Solvents: A: 99% 50 mM phosphate buffer (pH 5.5),

B: 1% Acetonitrile

Flow rate: 0.55 mL/min

Gradient:

Time (min)	**Solvent B (%)**	**Flow Rate (mL/min)**
0.00	1.0	0.55
8.00	1.0	0.55
8.01	10.0	1.00
18.00	10.0	1.00
18.01	1.0	0.55
26.00	1.0	0.55

Injection volume: 100 μL

Run time: 26 minutes

UV-Vis detection: λ = 336, 214 nm

The retention times of GSNO and the internal standard, benzoic acid, are listed in Table 3.1. GSNO and benzoic acid are detected at 336 nm and 214 nm respectively. Chromatograms of GSNO and benzoic acid in PBS and plasma are shown in Figure 3.3.

Compound	**Wavelength (nm)**	**Retention Time (min)**	**Relative Retention Time**
GSNO	336	3.594	0.40
Benzoic acid	214	9.029	1.00

Table 3.1. Retention times of GSNO and benzoic acid in plasma on HPLC. Relative retention time of GSNO is calculated with respect to benzoic acid, i.e., the retention time of the target compound divided by that of the internal standard.

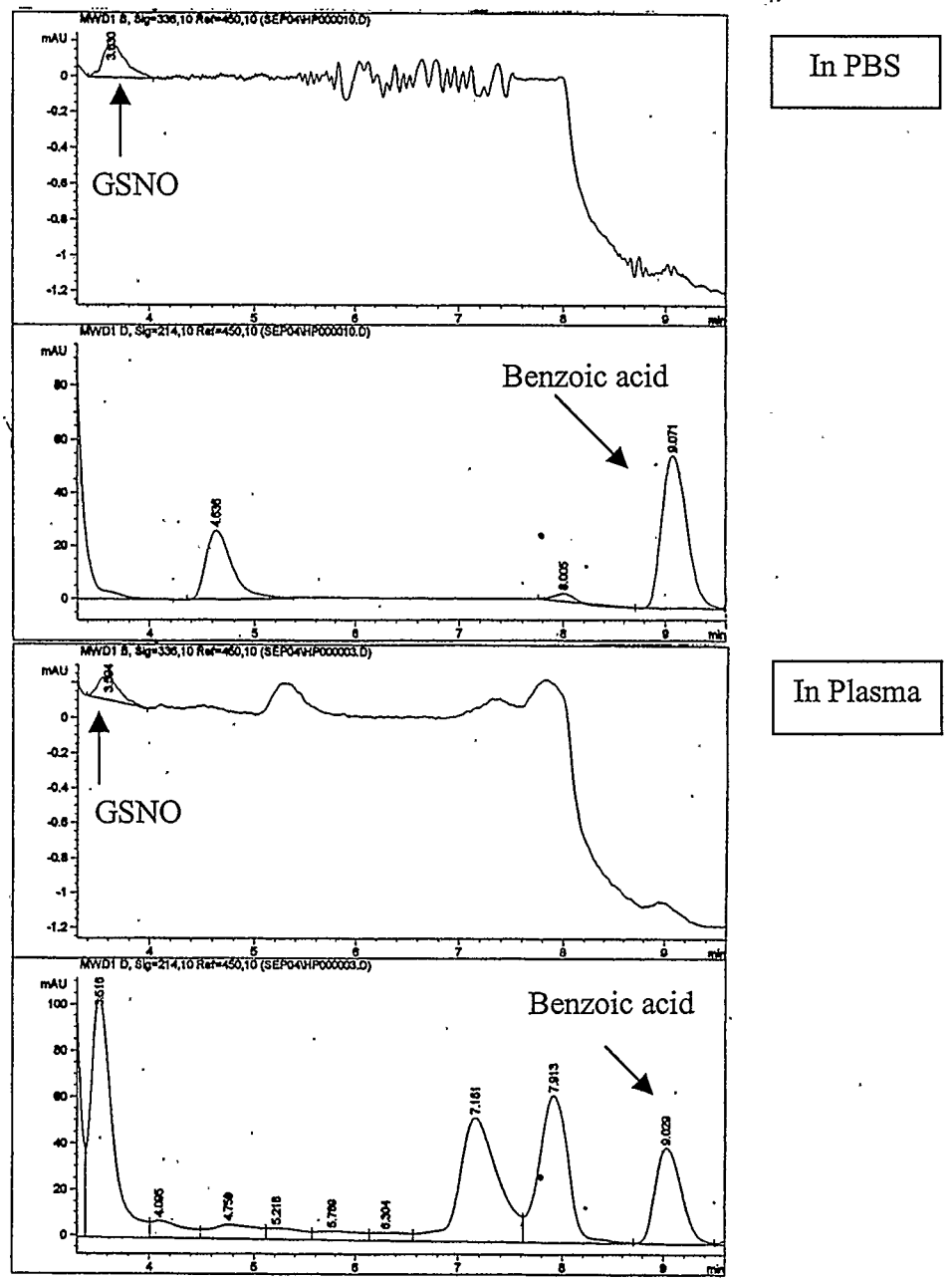
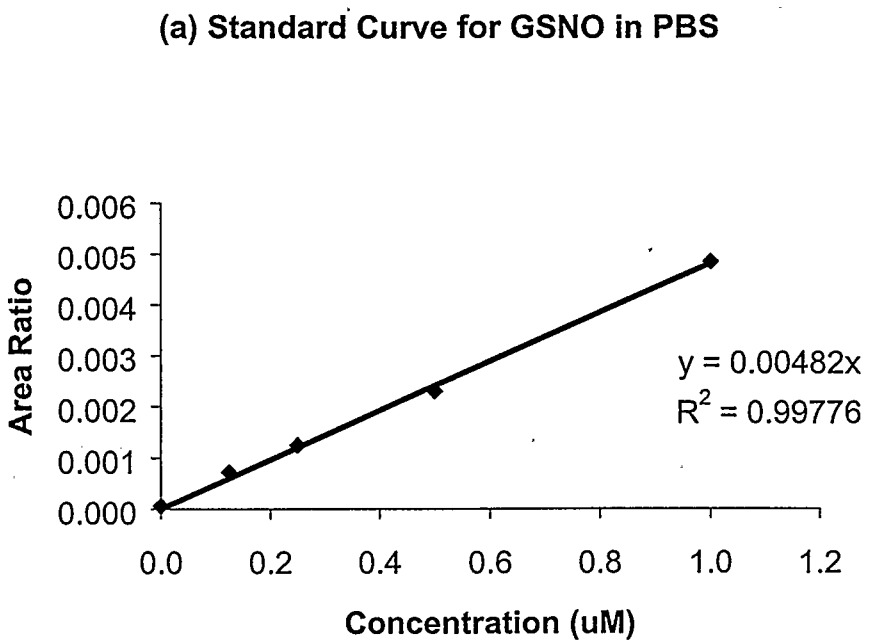


Figure 3.3. Chromatograms of 0.5 μM GSNO and benzoic acid in PBS and plasma. Top two panels: GSNO at 3.630 min (λ = 336 nm) and benzoic acid at 9.071 min (λ = 214 nm) in PBS. Bottom two panels: GSNO at 3.594 min and benzoic acid at 9.029 min in plasma. Both spiked with 0.5 μM GSNO and benzoic acid (~ 0.082 mM) and then extracted as described in Section 3.2.3.

3.2.5 Assay Validation: Standard Curves for GSNO in PBS and Plasma

In order to demonstrate that plasma matrix did not cause interference with the detection of GSNO, and the measurement of this compound was not different from that in an aqueous solution, standard curves for GSNO were prepared in both PBS and plasma for comparison. The curves were obtained at concentrations 0, 0.125, 0.25, 0.5 and 1.0 μM by adding appropriate amounts of GSNO to either PBS (pH = 7.4) or plasma followed by the extraction procedures as described in Section 3.2.3. The slope of GSNO in plasma was compared to that in PBS. As demonstrated in Figure 3.4 (a & b), linear curves in each matrix were obtained, and the detection limit for GSNO in plasma was 0.125 μM. The y-intercept of the standard curve in PBS is zero because this buffer contained no GSNO at 0 μM, whereas that in plasma is shifted upward probably due to the background concentrations of GSNO in this matrix. Moreover, the slope of GSNO in PBS resembles that in plasma ($y = 0.00482 \cdot x$ versus $y = 0.00461 \cdot x$). The similarity of the slopes suggests that the detection of GSNO in plasma was not different from that in PBS. One explanation is that the addition of NEM (included in the extraction procedures) was able to increase the stability of GSNO that would otherwise decompose when combined with other thiol groups in plasma (83).



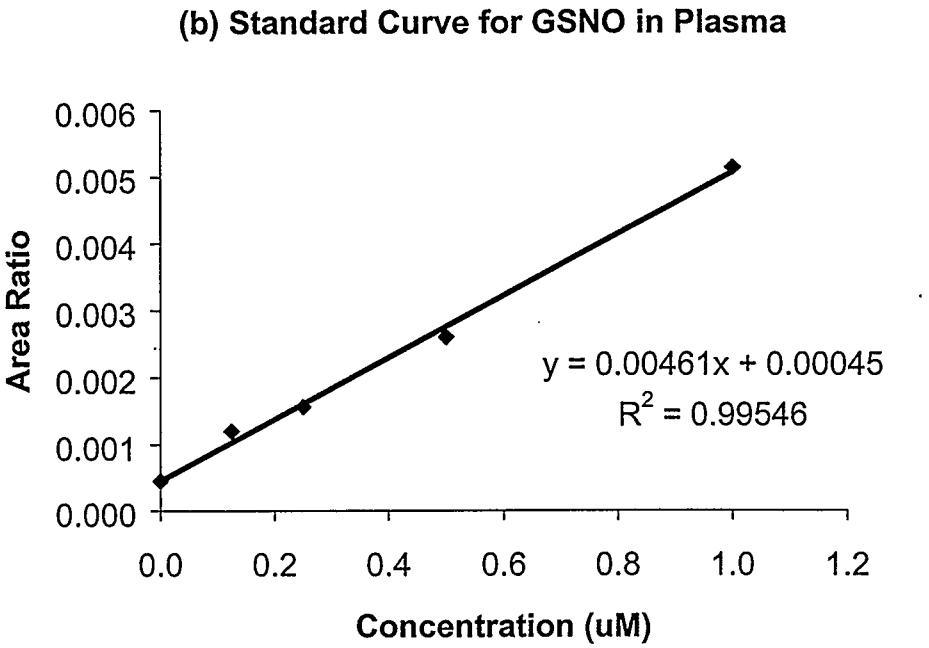


Figure 3.4. (a) Standard curves for GSNO in PBS and (b) in plasma at concentrations 0, 0.125, 0.25, 0.5 and 1.0 μM.

3.2.6 Confirmation of GSNO Peak

Plasma is a complex solution. Many other species that are present in plasma may interfere with the actual identification of the components of interest. Therefore, it is necessary to confirm whether the peak of GSNO detected on the HPLC was authentic, and not from other forms of glutathione such as glutathione disulfide and the reduced form of glutathione (GSSG and GSH respectively). Pure standards of GSSG and GSH were first prepared in PBS, and then separated on the HPLC system in order to determine their retention times (Figure 3.5). In one set of tubes, freshly synthesized GSNO was spiked in PBS or plasma and analyzed on the HPLC as a control (Figure 3.6). In another set of tubes, 2 mM $HgCl_2$ was added to plasma that contained 500 μM GSNO, and then incubated at room temperature in the dark for 10 min. After the incubation, the mixture was eluted through the HPLC column (Figure 3.7). It has been shown that the addition of $HgCl_2$ disrupts the S-NO bond of GSNO (104). The decomposition of this compound produces glutathione disulfide, GSSG (123;147). As a result, the chromatogram for the mixture of $HgCl_2$ and GSNO in plasma should contain only the peak of GSSG on the HPLC (Figure 3.7). In other words, GSNO should disappear after the treatment with $HgCl_2$. This should confirm the peak of GSNO, and show the validity of this HPLC assay. Table 3.2 summarizes the retention times of GSSG, GSH and GSNO. The absorbance of GSSG and GSH were measured at both 214 and 220 nm, whereas that of GSNO was monitored at 336 nm. The retention times of each compound demonstrate that there is no overlap between the peaks of GSNO and GSSG or GSH. Chromatograms of 500 μM GSSG, GSH and GSNO are shown in Figures 3.5 and 3.6 respectively, illustrating that the peak of GSNO is distinguishable from other peaks. Figure 3.7 shows

that GSNO disappeared after the treatment with $HgCl_2$, and GSSG was left behind after the reaction.

Table 3.2. Retention times of 500 μM GSSG, GSH and GSNO in PBS or plasma. The absorbance of GSSG and GSH were measured at both 214 and 220 nm; GSNO was monitored at 336 nm. It should be noted that GSSG and GSH do not co-elute with GSNO.

Compounds	**Wavelength (nm)**	**Retention Time (min)**
500 μM GSSG	214	2.484
	220	2.484
500 μM GSH	214	2.609
	220	2.608
500 μM GSNO in PBS	336	3.878
500 μM GSNO in Plasma	336	3.739

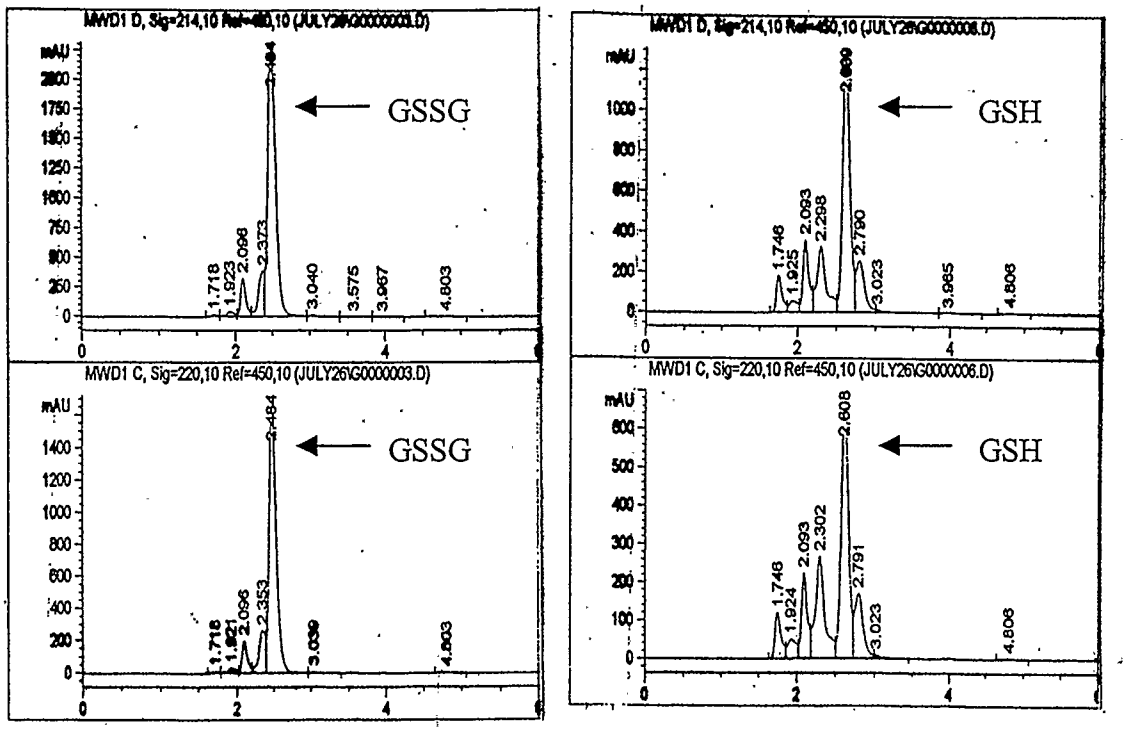


Figure 3.5. Chromatograms for GSSG and GSH in PBS.

Left panel: 500 μM GSSG prepared in PBS. This compound elutes at 2.484 min at λ = 214 and 220 nm respectively. Right panel: 500 μM GSH prepared in PBS. This compound elutes at 2.609 and 2.608 min at λ = 214 and 220 nm respectively.

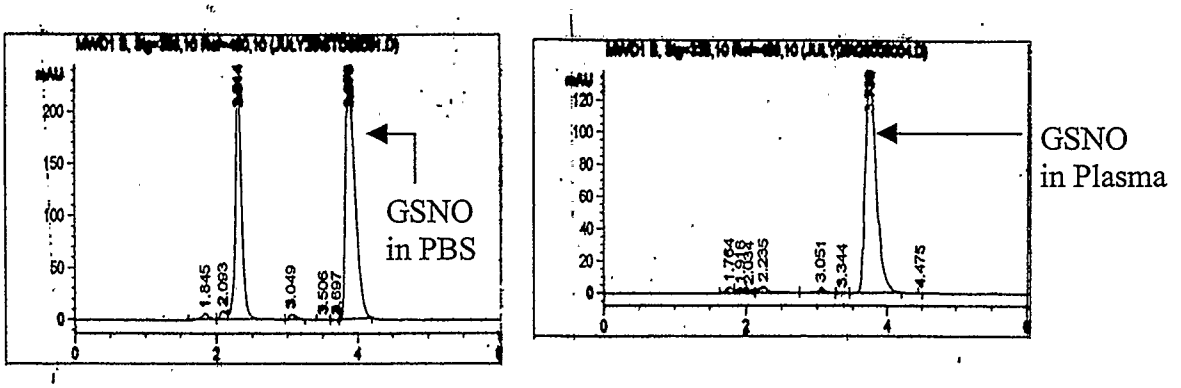
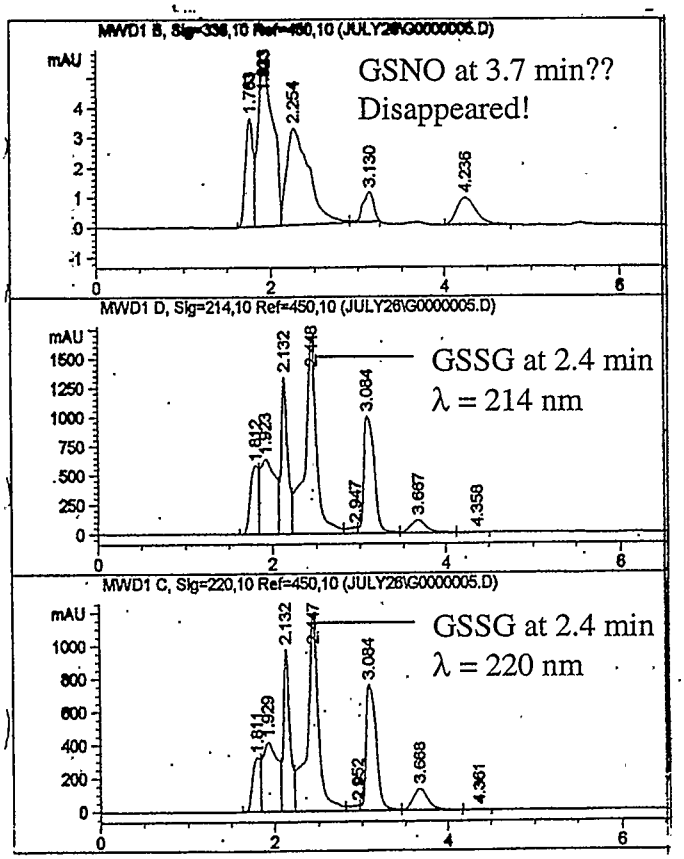


Figure 3.6. Chromatograms for GSNO in PBS and plasma.

Left panel: 500 μM GSNO spiked in PBS at 3.878 min (λ = 336 nm).

Right panel: 500 μM GSNO spiked in plasma at 3.739 min (λ = 336 nm).



GSNO should be seen at ~ 3.7 min at 336 nm, but disappeared after the addition of $HgCl_2$.

After the reaction with 2 mM $HgCl_2$, GSSG was formed, which showed up at ~ 2.4 min at both 214 and 220 nm.

Figure 3.7. GSNO (500 μM) disappeared in plasma (λ = 336 nm) after the addition of 2 mM $HgCl_2$. The appearance of GSSG is clearly shown at wavelength 214 and 220 nm after the reaction with $HgCl_2$.

3.2.7 Recovery of GSNO in PBS and Plasma

Some of the GSNO could be retained on the filters during ultrafiltration in the extraction procedures. It is important to determine how much GSNO in plasma was recovered using the centrifugal devices. The percent recovery was examined by spiking 0.5 μM freshly synthesized GSNO in plasma followed by the extraction procedures, and compared to the same amount of pure standard of GSNO without performing extraction (i.e., without using the filters). The same process was done in PBS as a comparison. Table 3.3 shows the results of recovery for GSNO in PBS and plasma. Results demonstrate that ~ 85% of GSNO was recovered in plasma.

Sample	**Recovery (%)** **Mean ± SEM**
GSNO in PBS	80.14 ± 0.35
GSNO in Plasma	84.88 ± 0.90

Table 3.3. Percent recovery for GSNO in PBS and plasma. Results obtained by dividing the area ratio of 0.5 μM GSNO spiked in either PBS or plasma after extraction against the internal standard, over the area ratio of pure standard of 0.5 μM GSNO without extraction and then multiplied by 100. Results are presented as mean ± SEM (n = 3).

3.3 ANALYSIS OF NITRATE BY HPLC

3.3.1 Materials

Sodium nitrate ($NaNO_3$), sodium chloride (NaCl) and salicylic acid were obtained from Sigma Chemical Company (Ontario, Canada). Acetonitrile was purchased from VWR Canlab (Ontario, Canada).

3.3.2 Preparation of Pure Standards of Nitrate and Salicylic Acid

13.71 mg of $NaNO_3$ was weighed into a 10 mL volumetric flask and dissolved thoroughly in ultra-pure deionized water. The internal standard, salicylic acid, was prepared by adding 11.68 mg into the volumetric flask containing the same type of water.

3.3.3 Extraction Procedures for Nitrate in Plasma

500 μL of plasma sample was spiked with the internal standard, salicylic acid. 1 mL of acetonitrile was added for protein precipitation, and the mixture was centrifuged at 3000 rpm for 5 min. 200 μL of the supernatant was then transferred to another culture tube, and dried until it was evaporated. Finally, it was reconstituted into 200 μL of 50:50 (Solvent A:B) mobile phase (see below), and injected onto the HPLC.

3.3.4 Anion-Exchange High Performance Liquid Chromatography

The anion-exchange chromatographic system was similar to that described above for GSNO, except the column used was an anion-exchange column, IonPac AS4A-SC (4 × 250 mm) coupled with a guard column IonPac AG4A-SC (4 × 50 mm), were both obtained from Dionex Corporation (CA, U.S). The mobile phase consisted of water/20%

acetonitrile and 0.1% NaCl/20% acetonitrile with gradient elution (see HPLC conditions below) at a flow rate of 1.5 mL/min. The effluent was monitored at 214 nm using a UV detector. The HPLC parameters are presented in Figure 3.8.

Figure 3.8. HPLC parameters for the analysis of NO_3^- in plasma.

Column: Dionex IonPac AS4A-SC (4 × 250 mm)

Guard column: Dionex IonPac AG4A-SC (4 × 50 mm)

Solvents: A: water/20% acetonitrile

B: 0.1 % sodium chloride/20% acetonitrile

Flow Rate: 1.5 mL/min

Gradient:

Time (min)	**Solvent B (%)**	**Flow Rate (mL/min)**
0.00	0.0	1.5
5.00	0.0	1.5
5.01	20.0	1.5
25.00	100.0	1.5
25.01	100.0	2.0
35.00	100.0	2.0
35.01	0.0	1.5
45.00	0.0	1.5

Injection volume: 50 μL

Run time: 45 minutes

UV detection: $\lambda = 214$ nm

Table 3.4 shows the retention times of NO_3^- and the internal standard, salicylic acid. Both are detected by absorbance at 214 nm. Chromatograms of NO_3^- and salicylic acid in water and plasma are shown in Figure 3.9.

Compound	Wavelength (nm)	Retention Time (min)	Relative Retention Time
NO_3^-	214	14.22	0.70
Salicylic acid	214	20.34	1.00

Table 3.4. Retention times of NO_3^- and salicylic acid in plasma on HPLC. Relative retention time of NO_3^- is obtained from the ratio of the retention time of NO_3^- to that of salicylic acid.

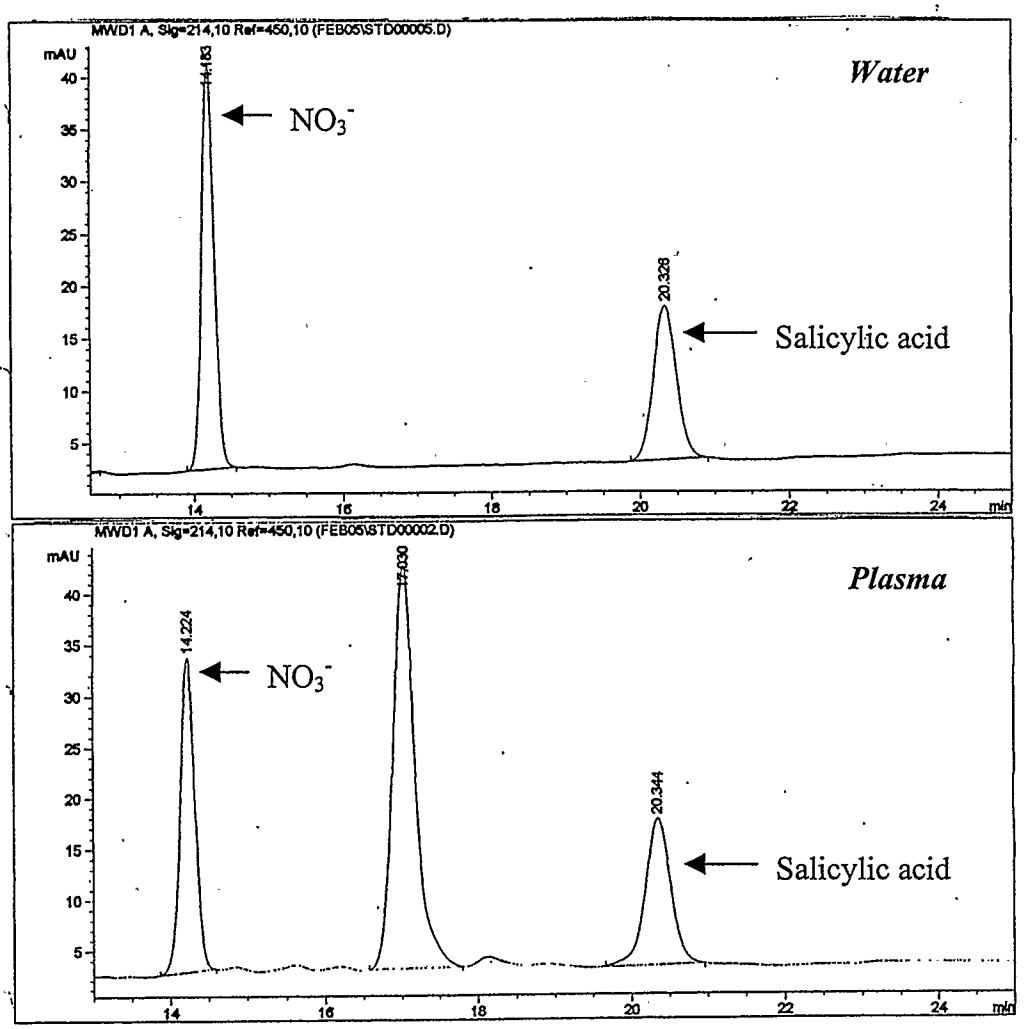


Figure 3.9. Chromatograms of NO_3^- and salicylic acid in water and plasma. Top panel: 16 μM (1 μg/mL) of NO_3^- was spiked in water. Bottom panel: 16 μM (1 μg/mL) of NO_3^- was spiked in plasma. Both spiked with salicylic acid as an internal standard and extracted as described in Section 3.3.3.

3.3.5 Assay Validation: Standard Curve

A standard curve for NO_3^- was prepared over the range of 4 μM to 161 μM (0.25 μg/mL - 10 μg/mL) by adding known amounts of aqueous standards of $NaNO_3$ to plasma. Linear regression was obtained with the equation: $y = 0.6302 \cdot x + 0.704$ ($r^2 = 0.9964$) calculated against the internal standard, salicylic acid. The detection limit for NO_3^- in plasma was 4 μM. Reproducibility of the standard curve was observed by generating the same range of calibration curves three times.

3.3.6 Precision

Precision was carried out in order to determine the random error of the assay (i.e., whether the data obtained were reproducible) by spiking known amounts of NO_3^- (40 μM, the theoretical concentration in Table 3.5) in either water or plasma. Three aliquots of each solution were prepared and then extracted based on the assay described previously (Section 3.3.3). The concentrations of NO_3^- in each solution (measured concentration in Table 3.5) were calculated using the standard curve obtained in Section 3.3.5. The standard deviation and the coefficient of variation (%CV) were examined. The precision results are shown in Table 3.5.

Table 3.5. Precision for NO_3^- in water and plasma based on the HPLC procedures.

Theoretical Concentration of NO_3^- (μM)	**Measured Concentration of NO_3^- Mean Value (μM)**	**Standard Deviation (SD)**	**Standard Error of the Mean (SEM)**	**% CV**
40 μM NO_3^- in water	41.42	1.84	1.06	4.44
40 μM NO_3^- in plasma	45.73	2.72	1.57	5.94

3.3.7 Recovery for Nitrate in Plasma

The recovery for NO_3^- was investigated by spiking 16 μM (1 μg/mL) pure standard of NO_3^- in plasma followed by the extraction procedures and injected onto the HPLC. Same amount of pure standard of NO_3^- was also run on the HPLC without performing extractions. The percent recovery was calculated by dividing the concentration recovered after extractions by the concentration of pure standard without extractions.

$$\% \text{ Recovery} = \frac{\text{Concentration of } NO_3^- \text{ with extraction}}{\text{Concentration of } NO_3^- \text{ without extraction}} \times 100$$

It was found that 90.38 ± 2.41% of NO_3^- had been recovered in plasma.

3.4 FLUOROMETRIC ANALYSIS OF NITRITE

3.4.1 Materials

Sodium nitrite ($NaNO_2$), 2,3-diaminonaphthalene (DAN), anhydrous potassium phosphate dibasic (K_2HPO_4), N,N-dimethyl-formamide (DMF) and 5-sulfosalicylic acid (SSA) were obtained from Sigma Chemical Company (Ontario, Canada). Sodium hydroxide (NaOH) was purchased from BDH Inc. (Ontario, Canada).

3.4.2 Instrumentation

An Aminco SPF-500 Spectrofluorometer (SLM Instruments Inc., U.S) was used for NO_2^- detection. Fluorometric readings were taken at excitation and emission wavelengths of 380 and 450 nm, respectively.

3.4.3 Preparation of Stock Sodium Nitrite

Stock solution of NO_2^- was prepared by dissolving $NaNO_2$ in ultra-pure deionized water. Standards were made by diluting the stock solution, and were used in setting up the standard curves as described below.

3.4.4 Nitrite Determination in Plasma: Standard Curve

DAN fluorescence detection was employed for the measurement of NO_2^- because this technique allows for exquisite sensitivity for NO_2^- in nanomolar concentration (92). The fluorometric assay for NO_2^- was a modification of the procedures described by Misko et al. (92). The method was based on the reaction of NO_2^- with DAN under acidic conditions to yield a highly fluorescent compound 2,3-naphthotriazoleiazole (NTA) (92) (Figure 3.10). The intensity of the fluorescent signal produced by NTA was determined.

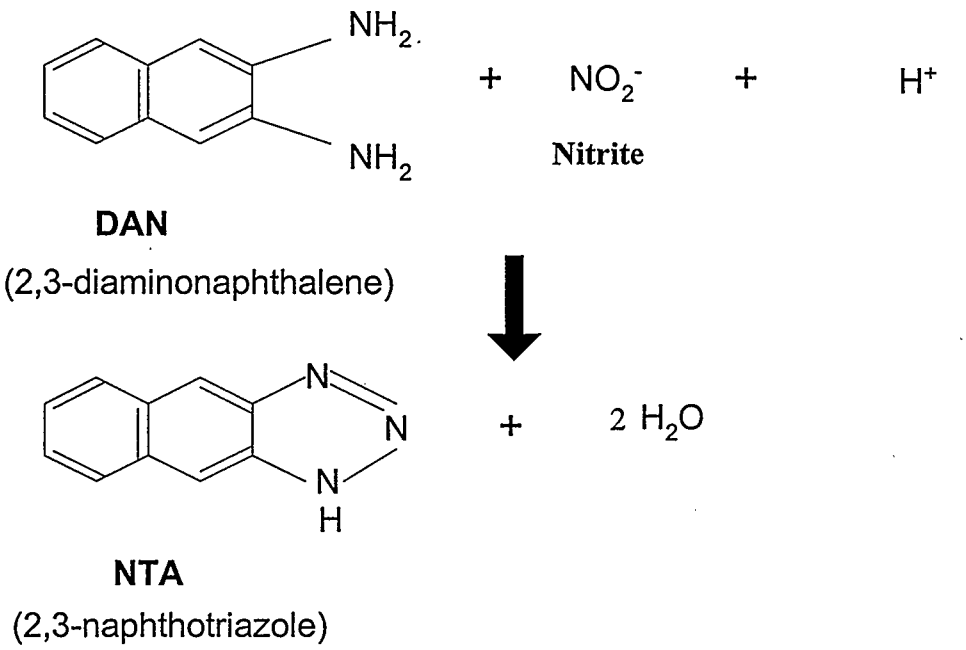


Figure 3.10. Reaction of NO_2^- with 2,3-diaminonaphthalene (DAN) to form 2,3-naphthotriazole (NTA) under acidic conditions.

Standard curves for NO_2^- were prepared by adding pure standards of $NaNO_2$ over the range of 100 – 1000 nM to 500 μL of plasma. 500 μL of ultra-pure deionized water was then added to dilute the plasma samples followed by the addition of 50 μL of 5 mM NEM. The reason why NEM had to be added was to remove any thiol groups in plasma that could react with NO_2^- to form artifactual RSNOs (83). After the treatment with NEM, 50 μL of 100 μg/mL freshly prepared DAN that dissolved in 0.62 M hydrochloric acid was added to the plasma. The mixture was protected from light and incubated at 37°C for 15 min. To precipitate proteins in plasma, 50 μL of 1 M K_2HPO_4 was first added to neutralize the acidic condition after the incubation with acidified DAN, followed by the addition of 150 μL of 0.5 M SSA (SSA is an inefficient protein-precipitating agent when added to samples at acidic pH) (104). Samples were kept on ice and waited for 10 min to allow sufficient time for precipitation of protein by SSA. The precipitate was removed by centrifugation at 3000 rpm for 10 min. Supernatant was transferred to another tube and 50 μL of 2.8 M NaOH was added in order to maximize the fluorescent signal by the alkaline solution (92). Fluorometric readings using excitation at 380 nm and emission wavelength at 450 nm were taken 10 min after stabilization with the base. The detection limit for NO_2^- in plasma was 100 nM.

3.4.5 Validation of Assay

The presence of free low molecular weight thiols in plasma might cause interference with the determination of NO_2^- in the fluorometric assay. With the addition of NEM, the low molecular weight thiols should be eliminated. To confirm whether NEM was capable of removing thiol groups in plasma, a standard curve was established

by spiking a very high concentration of thiol, GSH (100 μM), in plasma followed by the procedures described in Section 3.4.4 with the treatment of NEM (Figure 3.11). A similar curve was also prepared except the addition of GSH was omitted for result comparison (Figure 3.12). The slope in Figure 3.11 (spiked with GSH) is similar to that in Figure 3.12 (without addition of GSH), indicating that the low molecular weight thiol (100 μM GSH) was successfully eliminated by the treatment of 5 mM NEM. It should be noted that the y-intercepts in both standard curves are most likely due to the background concentrations of NO_2^- in plasma.

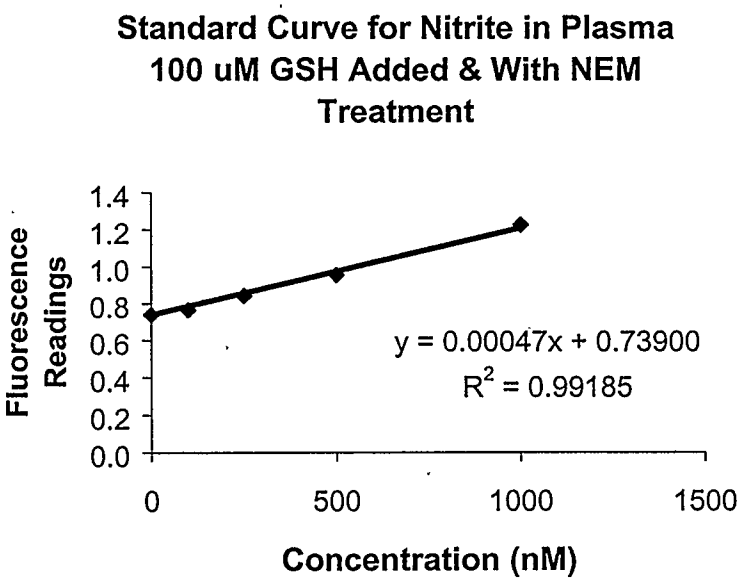


Figure 3.11. Standard curve for NO_2^- in plasma spiked with 100 μM GSH and treated with 5 mM NEM.

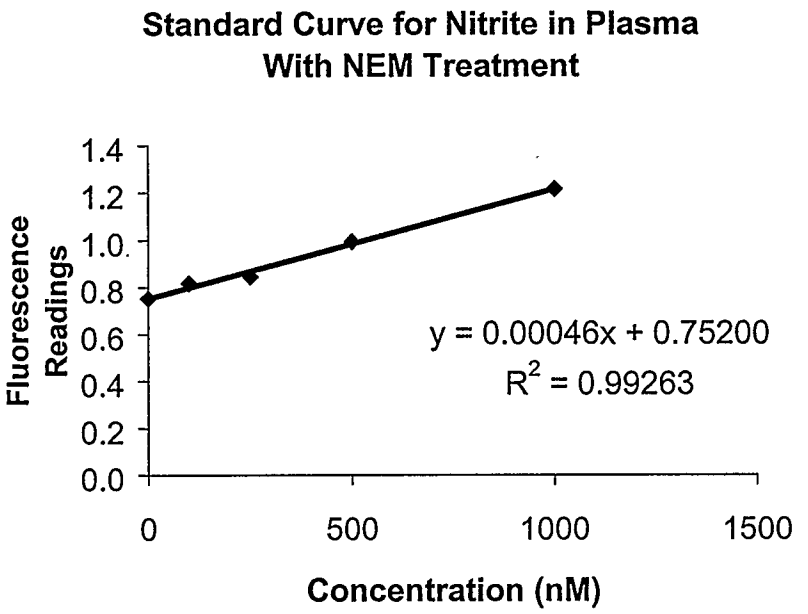


Figure 3.12. Standard curve for NO_2^- in plasma with treatment of NEM.

To further demonstrate the validity of this assay, 1 μM freshly synthesized GSNO was added to plasma spiked with 1 μM $NaNO_2$, followed by the procedures described in Section 3.4.4 in order to test whether GSNO (i.e., S-nitrosothiols) interfered with the determination of NO_2^-. The fluorometric readings (triplicates were performed) were found to be similar to the sample without addition of GSNO. Results confirmed that GSNO did not cause any interference with the detection of NO_2^- in the assay.

3.5 FLUOROMETRIC ANALYSIS OF S-NITROSO-ALBUMIN

3.5.1 Materials

Bovine serum albumin (fatty acid free), ammonium sulfamate, sulfanilamide, mercuric chloride ($HgCl_2$) and N-(1-naphthyl)-ethyl-enediamine dihydrochloride (NEDD) were purchased from Sigma Chemical Company (Ontario, Canada). Econo-Pac® 10 DG disposable chromatography columns were obtained from Bio-Rad Laboratories (CA, U.S.).

3.5.2 Instrumentation

Determination of the stock concentration of SNO-Alb was carried out on a Hitachi U-2000 Spectrophotometer. Fluorometric detection was conducted using Spectrofluorometer (SLM Instruments Inc., U.S.) as described in the analysis for NO_2^-.

3.5.3 Synthesis of Stock Solution of S-nitroso-albumin

Bovine serum albumin (BSA, 47.4 mg/mL) was first dissolved in PBS together with 25 μM DTPA, and SNO-Alb was then synthesized by reacting BSA with sevenfold-

molar excess of acidified $NaNO_2$ (104). The mixture was incubated for two hours at room temperature in the dark. After the incubation, the stock solution of SNO-Alb was treated with 25 μM DTPA and 1% ammonium sulfamate for 10 min at room temperature in order to reduce the rate of degradation of SNO-Alb and to remove unreacted nitrous acid respectively. The stock concentration of SNO-Alb was determined by spectrophotometer at 540 nm as described below.

3.5.4 Determination of Stock SNO-Alb by Spectrophotometer

400 μL of stock SNO-Alb was added to a culture tube followed by the addition of 40 μL of 50 mM $HgCl_2$. The mixture was incubated at 37°C for 15 min. After the incubation, it was diluted five times with PBS. In 400 μL of the diluted solution, equal volume of 1% sulfanilamide and NEDD were added in the same tube followed by the incubation at room temperature for 10 min. The absorbance of the mixture was taken at 540 nm using spectrophotometer. Quantification of stock SNO-Alb was calculated based on a standard curve of NO_2^- over the range of 1 μM to 100 μM using the same method described in this section, except the addition of $HgCl_2$ was omitted, since there were no RSNOs present in the aqueous standards of NO_2^- ($HgCl_2$ is used to break the S-NO bond of RSNOs). It was found that the stock solution contained approximately 75 μM SNO-Alb. The stock solution was stored at –20°C until it was used for preparation of standard curves (see below).

3.5.5 Determination of SNO-Alb in Plasma Samples

Concentrations of SNO-Alb in plasma samples were determined using similar procedures as described by Park et al. (104), except a few pre-treatment steps were performed prior to the addition of DAN. First, 500 μL of plasma samples was diluted with equal volume of water. 50 μL of 5 mM NEM was immediately added to plasma in order to alkylate free thiol groups that might cause transnitrosation with SNO-Alb. Transnitrosation involves the transfer of NO group from RSNOs present in plasma to the surrounding thiol groups (142). This reaction may induce the rate of degradation of SNO-Alb. In the presence of NEM, SNO-Alb is stabilized, since the occurrence of transnitrosation is minimized. After the addition of NEM, 50 μL of 0.5% sulfanilamide in 0.1 M hydrochloric acid was added to remove any NO_2^- that was originally present in plasma to prevent artifactual RSNO generation from NO_2^-. Next, plasma samples were desalted using Bio-Rad Econo-Pac® 10 DG columns. The purposes of using these chromatography columns were to eliminate low molecular weight compounds, and to ensure that the only thing that came out of the columns was the high molecular weight fraction from the plasma samples. After the high molecular weight plasma fraction was collected, 10 μL of 20 mM $HgCl_2$ was added. The use of $HgCl_2$ was to mediate the breakdown of S-NO bond from SNO-Alb so that the releasable nitrous acid produced was able to react with DAN (104). After the addition of $HgCl_2$, the mixture was incubated at room temperature for 10 min in the dark followed by the addition of K_2HPO_4, SSA and NaOH as mentioned in the NO_2^- procedures (92;104). Fluorometric readings were again taken using excitation at 380 nm and emission wavelength at 450 nm.

3.5.6 Standard Curves for SNO-Alb in PBS and Plasma

Stock solution of SNO-Alb (75 μM) was diluted using PBS containing 25 μM DTPA. Standard curves were obtained over the range of 50 nM – 1000 nM by the addition of known amounts of SNO-Alb to either PBS or plasma, followed by the procedures described in Section 3.5.5, except the additions of NEM and sulfanilamide were omitted initially. The effects of these reagents will be compared in the next section (validation of assay). In setting up the standard curve for SNO-Alb in PBS, the desalting process, as well as the addition of K_2HPO_4 and SSA were eliminated because only a small amount of protein had to be dealt with when spiking SNO-Alb in PBS. It was, therefore, not necessary to precipitate the protein. On the other hand, these steps were important for the removal of protein or protein-containing particles in plasma samples (104). The standard curves for SNO-Alb in PBS and plasma were compared in order to show that the additional steps used in plasma did not affect the SNO-Alb assay. Figure 3.13 shows the standard curves for SNO-Alb in PBS (top panel) and plasma (bottom panel) without treatment of NEM and sulfanilamide. Linear curves were obtained in both matrices over the concentration range of 50 nM to 1000 nM, and the detection limit for SNO-Alb in plasma was 50 nM (Figure 3.13, bottom panel). The y-intercepts of the standard curves in PBS and plasma illustrate the background fluorescence signals. In fact, the y-intercept for plasma is slightly higher than that for PBS. It is most likely caused by the endogenously present SNO-Alb in plasma that results in an additional increase in the background signal. It should be noted that the y-intercept has to be subtracted when calculating the concentrations of SNO-Alb in plasma samples in order to eliminate the background interference.

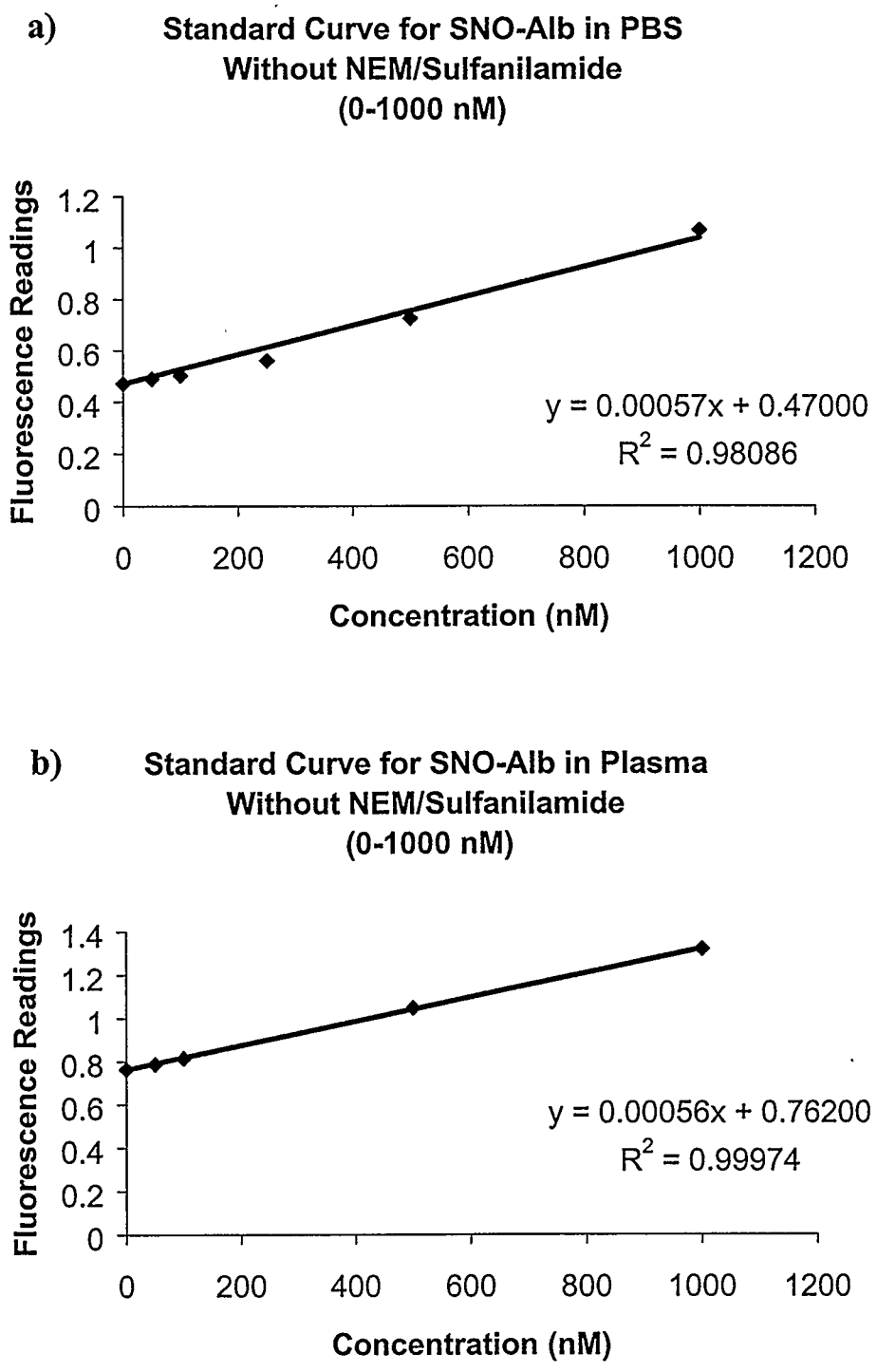


Figure 3.13. Standard curves for SNO-Alb in a) PBS (top panel) and b) plasma (bottom panel) without addition of NEM and sulfanilamide. In PBS, the desalting process and the addition of K_2HPO_4 and SSA were omitted.

3.5.7 Validation of Assay: Treatment with NEM and Sulfanilamide

In our preliminary tests, we observed that the addition of GSH and $NaNO_2$ interfered with the analysis of SNO-Alb in plasma without the treatment with NEM and sulfanilamide. Therefore, we tested whether free thiols could be alkylated by NEM and free NO_2^- could be eliminated by sulfanilamide by adding 100 μM GSH and 10 μM $NaNO_2$ to plasma that contained spiked SNO-Alb prior to the treatment with NEM and sulfanilamide. The mixture was then desalted to recover the high molecular weight plasma fraction, followed by the procedures described in Section 3.5.5. The standard curve in Figure 3.14 is compared to the one shown in Figure 3.13 (bottom panel) in the absence of NEM and sulfanilamide. Figure 3.14 demonstrates two important observations. First, the addition of NEM and sulfanilamide did not interfere with the SNO-Alb assay, as the treatment with these reagents did not modify the results. Secondly, NEM and sulfanilamide were able to remove the high concentrations of GSH and NO_2^- in plasma since the slope of the standard curve in Figure 3.14 is similar to that in Figure 3.13 (bottom panel). Conceivably, the addition of NEM and sulfanilamide to plasma samples is essential to prevent free thiols and NO_2^- from interfering with the assay.

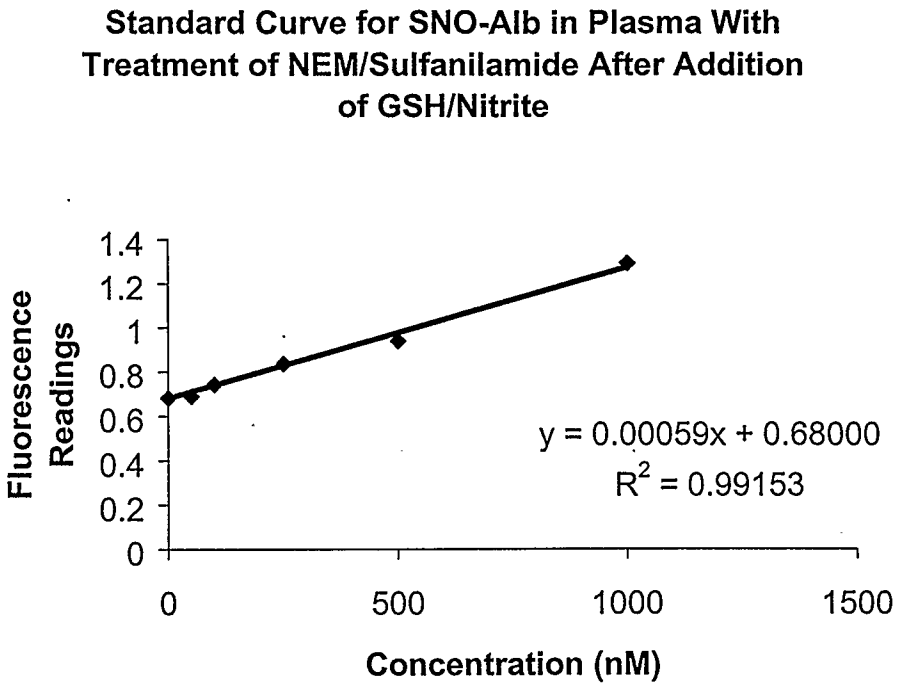


Figure 3.14. Standard curve for SNO-Alb in plasma treated with NEM/sulfanilamide after addition of 100 μM GSH and 10 μM $NaNO_2$. Slope of this curve is similar to the one in Figure 3.13 (bottom panel) without treatment of NEM/sulfanilamide.

3.5.8 Recovery

The desalting process was necessary in order to eliminate any low molecular weight fraction in plasma that could interfere with the determination of SNO-Alb. However, this process might affect the recovery of SNO-Alb. To determine whether the known amounts of SNO-Alb added to plasma could be recovered after the desalting step, the assay was done in the following ways:

1. 500 nM SNO-Alb was spiked in plasma without any NEM and sulfanilamide treatment and without desalting the samples, followed by the addition of $HgCl_2$, DAN and SSA as described in Section 3.5.5.
2. 100 μM GSH and 10 μM $NaNO_2$ were added to plasma. 500 nM SNO-Alb was spiked in plasma followed by the addition of 5 mM NEM and 0.5% sulfanilamide. Desalting process was then carried out.

Table 3.6 shows the average recovery results. It was found that 87 ± 0.7% of SNO-Alb could be recovered in plasma after performing the desalting process.

Concentration of SNO-Alb (nM)	Fluorescence Readings Without Desalting	Fluorescence Readings With Desalting	Recovery (%)
500 nM	1.00 ± 0.19	0.87 ± 0.16	86.90 ± 0.74

Table 3.6. Average recovery results for SNO-Alb in plasma (n = 3). Results are presented as mean ± SEM.

3.6 SUMMARY

Accurate and reproducible analytical assays have been established in this study for the detection of NO_2^-, NO_3^-, GSNO and SNO-Alb in plasma. These techniques should be useful for the quantification of NO metabolites, and investigations on the formation and distribution of RSNOs in biological fluids.

CHAPTER 4

IDENTIFICATION OF DOMINANT S-NITROSOTHIOLS IN PLASMA DURING NO INHALATION

Hypothesis: S-nitroso-albumin is the dominant S-nitrosothiol over S-nitrosoglutathione in plasma during NO inhalation.

Objectives:

1) To determine whether the levels of NO_2^- and NO_3^- in cat plasma change during NO inhalation and after NO inhalation is terminated.
2) To measure the levels of GSNO and SNO-Alb during inhaled NO and after inhaled NO is discontinued, and to examine which RSNO (GSNO or SNO-Alb) is primarily formed in cat plasma during NO inhalation.

Having established the analytical techniques for the measurement of NO_2^-, NO_3^-, GSNO and SNO-Alb in plasma, our next objective was to determine the levels of these ions and molecules before, during and after cats breathing NO. Additionally, whether GSNO or SNO-Alb was a more abundant RSNO formed in the circulation was identified.

4.1 RESULTS

Levels of Nitrite and Nitrate Increase During NO Inhalation But Do Not Drop After Termination of Inhaled NO.

It has been previously shown that the primary decomposition product of NO in oxygenated fluid is NO_2^-, which is stable for up to several hours in aqueous solution and in plasma. However, NO_2^- is further oxidized to NO_3^- in the presence of oxidizing species such as HbO_2 (43;52;53;151). Therefore, any delay in blood sample processing may lead to the transformation of NO_2^- to NO_3^-. For this reason, the total concentrations of NO_2^- and NO_3^- in plasma are reported for proper characterization of NO metabolism in cat blood.

In our preliminary work, we measured the baseline levels of NO_2^- and NO_3^- in cat plasma for 2 hours prior to NO inhalation. We observed that the concentrations of these ions did not change until animals were allowed to breathe 80 ppm NO. Moreover, levels of NO_2^- and NO_3^- reached very high levels within 1 hour of NO inhalation, and then remained at the same level for at least another 60 min after the termination of inhaled NO. When these levels were examined for an additional 2 hours (3 hours in total) after inhaled NO was discontinued, we noticed that the concentrations of both ions gradually reduced. Based on these preliminary data, we have chosen to study the levels of NO_2^- and NO_3^- when they were at steady state, that is, at 60 min of inhaled NO and 60 min after NO inhalation was terminated.

Figure 4.1 demonstrates the sum of NO_2^- and NO_3^- in cat plasma under control, and during 60 min of NO inhalation, and then for 60 min without inhaled NO. The total concentrations of these ions were on average 15 ± 2 μM under normal conditions (before

NO inhalation). When cats were given 80 ppm NO to breathe for 60 min, the sum of NO_2^- and NO_3^- significantly elevated (n = 9, *P < 0.0001); their levels were twice as much as the baseline values (the pre-NO values in Figure 4.1). Inhaled NO was then discontinued for an hour. Interestingly, concentrations of both ions remained almost the same after NO inhalation had been terminated for an hour.

Previous work in our laboratory had suggested that cats were capable of clearing 90% of NO_2^- and NO_3^- very efficiently from blood 60 min following injection of a high concentration (400 μM) of $NaNO_2$ solution (64). Accordingly, NO_2^- and NO_3^- should be constantly removed from the circulation and consequently excreted as NO_3^-, which is the major metabolite in urine (52;159). In this regard, we have examined the urinary levels of NO_3^- in cats in our pilot experiments. Although quantitative results for NO_3^- in urine are not included, as the analytical assay for urine measurement has not been validated, we observed that the concentrations of NO_3^- in urine progressively increased during NO inhalation and the levels continued to increase over 3 hours after inhaled NO was discontinued, suggesting an ongoing clearance of NO_2^- and NO_3^-. However, our data in the present study reveal that there was new synthesis of NO_2^- and NO_3^- in vasculature or perhaps an ongoing NO production from molecules in plasma (e.g., RSNOs) during the time period when NO inhalation was terminated.

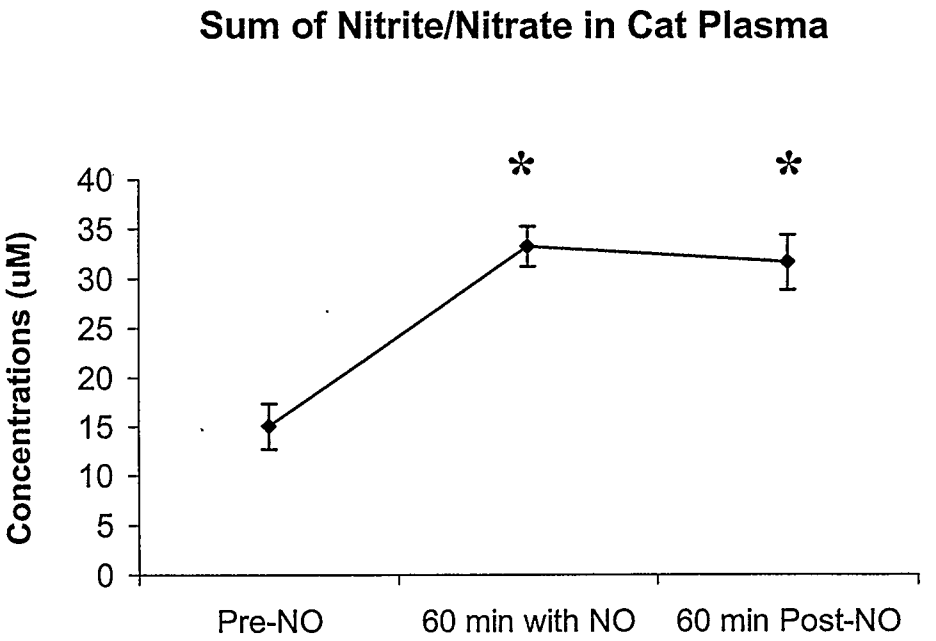


Figure 4.1. Sum of NO_2^- and NO_3^- in cat plasma before (pre-NO), during (60 min with NO) and after (60 min post-NO) NO inhalation (n = 9). *P < 0.0001 relative to pre-NO. Values are mean ± SEM of nine observations.

Concentrations of S-nitroso-albumin Elevate During NO Inhalation But Decrease After Termination of Inhaled NO.

Results from the previous section suggest that NO must be continuously produced in the circulation over 1 hour after the termination of inhaled NO. Our next question was to ask which molecules were releasing NO during the time period examined. Previous reports have shown that NO reacts with protein thiols in plasma to form RSNOs that conserve and stabilize NO bioactivity (128;130). For this reason, we measured the concentrations of GSNO and SNO-Alb in plasma before, during and after NO inhalation. Our data demonstrate that the baseline levels of GSNO were undetectable. During 60 min NO inhalation and at 60 min after post inhaled NO, only trace amounts of GSNO were observed. As depicted in Figure 4.2, the amount of GSNO in plasma during NO breathing (bottom panel in Figure 4.2) was lower than that with spiked GSNO (125 nM) (top panel in Figure 4.2). Our results suggest that during NO inhalation, the concentrations of GSNO were below the limit of detection of the analytical assay (i.e. < 125 nM). On the other hand, we measured the concentrations of SNO-Alb during each event. Although this compound was undetectable under control conditions, it significantly increased in concentrations (376 ± 58 nM on average) during NO inhalation (Figure 4.3, $n = 9$, $^{*}P < 0.0001$). Moreover, the levels of this species progressively decreased when inhaled NO was discontinued for an hour (Figure 4.3, $n = 9$, $^{\dagger}P < 0.0001$ relative to 60 min with NO). Our preliminary data show that the levels of SNO-Alb, indeed, reduced to the baseline values 3 hours after inhaled NO was terminated.

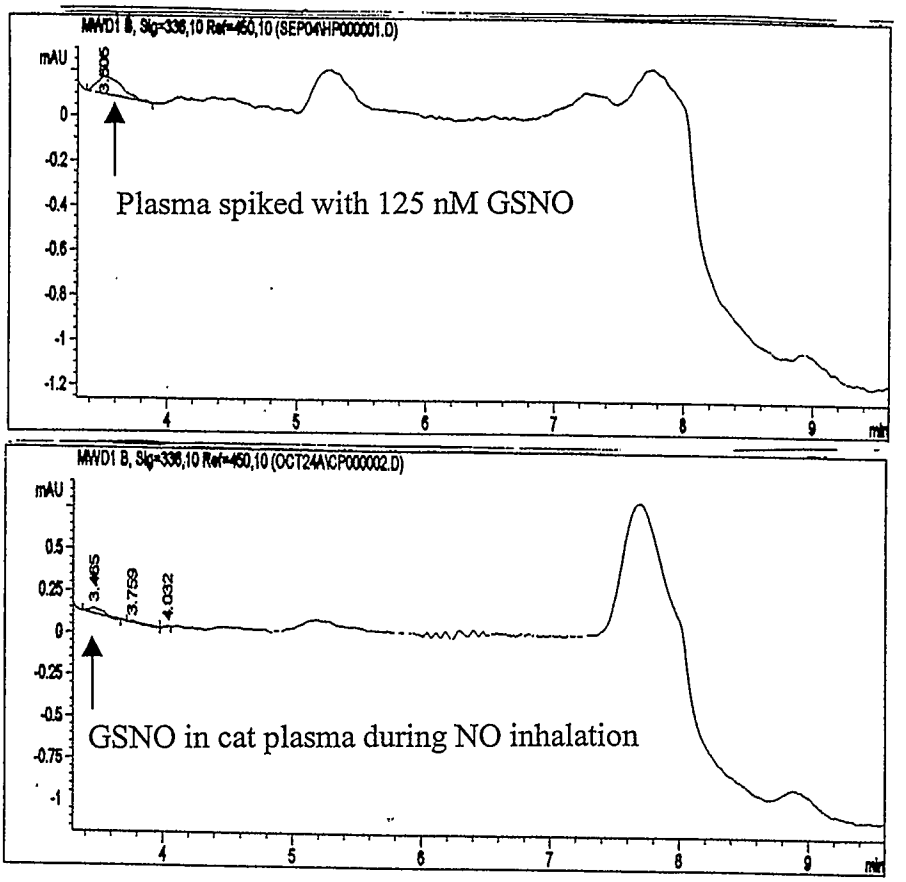


Figure 4.2. The chromatogram of GSNO in cat plasma during 60 min NO inhalation (bottom panel). Top panel: Plasma spiked with 125 nM GSNO. Bottom panel: GSNO in cat plasma during NO inhalation. Only trace amounts of GSNO were observed during NO inhalation in all experiments (n = 9). Comparing to the spiked GSNO, concentrations of this compound in animals breathing 80 ppm NO were < 125 nM.

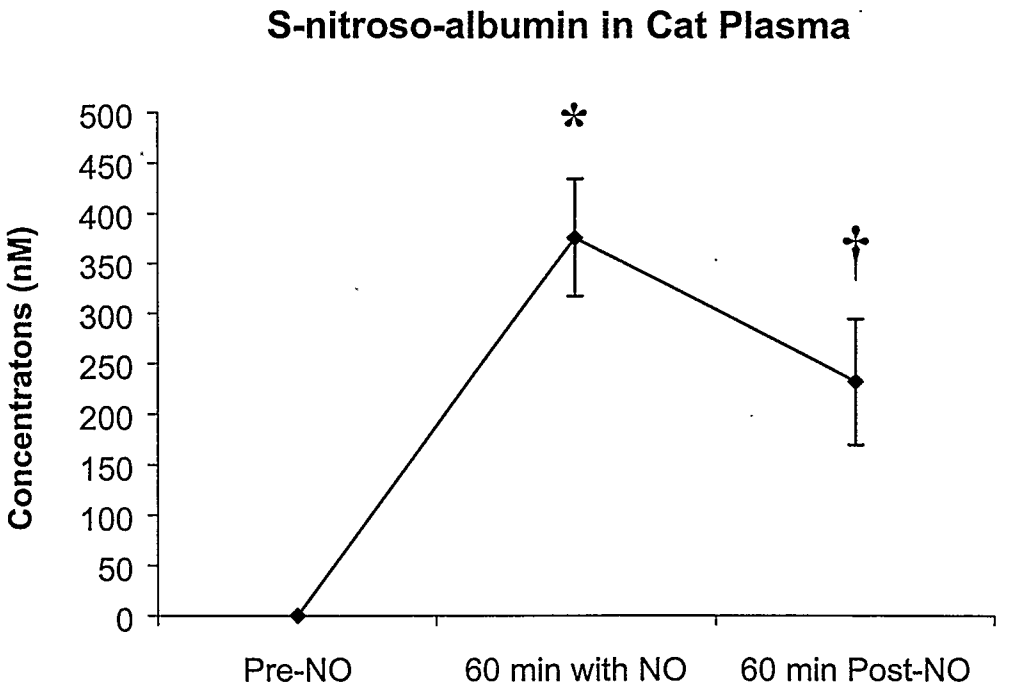


Figure 4.3. SNO-Alb in cat plasma before (pre-NO), during and after NO inhalation for 60 min (n = 9). *P < 0.0001 relative to pre-NO; †P < 0.0001 relative to 60 min with NO. Values are mean ± SEM of nine observations.

S-nitroso-albumin is Predominant Over S-nitrosoglutathione During NO Inhalation.

As demonstrated in Figure 4.4, high nanomolar concentrations of SNO-Alb were detected in plasma during 60 min of inhaled NO. In comparison with the trace amounts of GSNO, it is clear that during NO inhalation SNO-Alb was primarily formed within the circulation. Nevertheless, there are other RSNOs that exist in biological systems.

It should be mentioned that only the levels of SNO-Alb were measured in all subsequent studies as representative of RSNOs. GSNO was analyzed in all succeeding experiments, but its levels never rose above the level of detection. Therefore, we have chosen not to report these data.

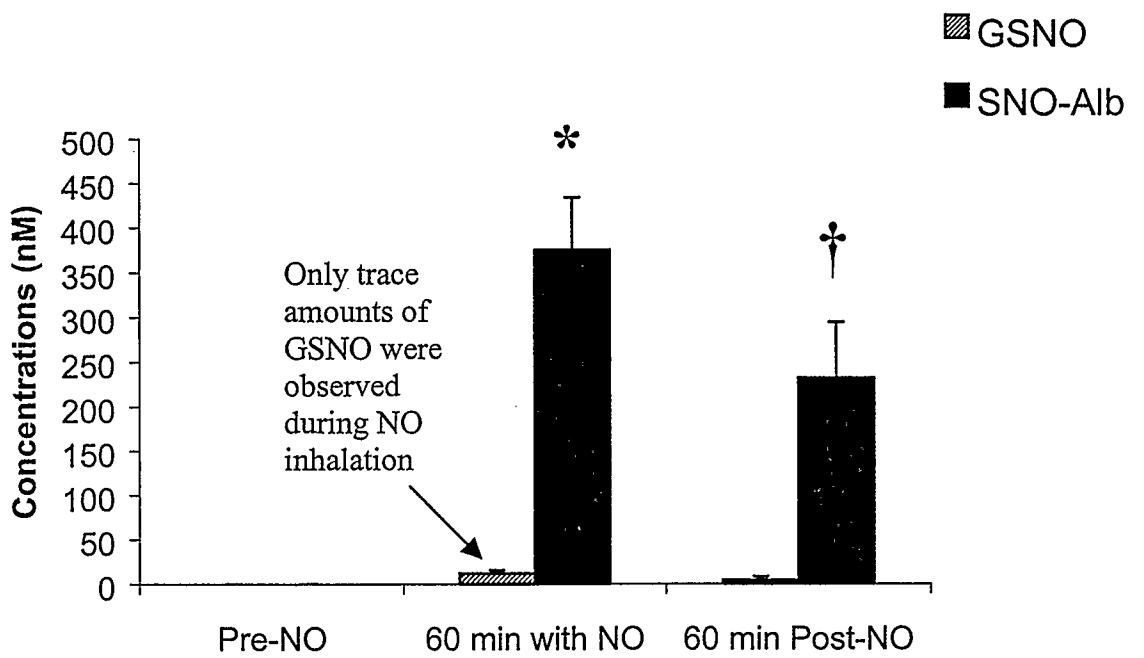


Figure 4.4. A comparison between the concentrations of GSNO and SNO-Alb in cat plasma during 60 min NO inhalation. For SNO-Alb, *P < 0.0001 relative to pre-NO; †P < 0.0001 relative to 60 min with NO. Values for SNO-Alb are mean ± SEM of nine observations. Trace amounts of GSNO are shown in the figure to illustrate that SNO-Alb was preferentially formed during NO inhalation compared to GSNO.

4.2 DISCUSSION

Inhaled NO has been used therapeutically as a local vasodilator in the pulmonary vasculature for treatment of pulmonary hypertension or other respiratory problems (106). Its effects are thought to be limited to the lungs due to the short half-life of this radical in biological systems (64). However, Stamler and colleagues (130) have reported that RSNOs are formed readily under physiologic conditions to stabilize NO, and possess vasodilation and platelet inhibition effects. These molecules may serve as intermediates that play important roles in transport, storage and metabolism of NO (47;131). Based on this contention, we hypothesize that RSNOs, such as GSNO and SNO-Alb may act as reservoirs for inhaled NO in plasma because they have NO-carrying capacity. Therefore, continuous delivery of NO to blood in the form of inhaled NO may produce RSNOs or perhaps other NO-adducts, and thereby deliver small amounts of NO to peripheral tissues. As a result, inhaled NO may have an impact on the periphery.

In the present study, we have demonstrated that the major decomposition product of NO *in vivo*, namely NO_2^- and NO_3^-, increased during NO inhalation, but remained unchanged for 60 min after NO inhalation was terminated, indicating that NO had been released by molecules in plasma such that the levels of these ions were maintained. Under normal circumstances, NO_2^- and NO_3^- are rapidly cleared from blood. This contention has been supported by several investigators, who found that both inhaled NO and injected NO_2^- are quickly oxidized by RBCs to produce NO_3^- as a final by-product, and then subsequently excreted in urine of mammalian organisms (52;124;151;158;159). Indeed, a cursory examination in our pilot study show that when urine samples were taken from cats, clearance of NO_3^- in urine successively increased over time, whereas the

production of NO_2^- and NO_3^- in plasma continued for an hour after the termination of inhaled NO. This strongly supports the view that NO_2^- and NO_3^- were continuously delivered to urine, but these ions in plasma were continuously formed for an hour after inhaled NO was discontinued.

We hypothesize that the elevated levels of NO_2^- and NO_3^- in plasma were due to NO inhalation leading to RSNO formation and then release of NO from RSNOs that eventually oxidized to NO_2^- and NO_3^- within the circulation. However, one could argue that the elevated levels of these ions were perhaps due to other factors, such as the anesthesia given to the cats during the prolonged experimental protocol. Nevertheless, the baseline levels of NO_2^- and NO_3^- observed in our preliminary data demonstrate that cats that had anesthesia maintained for the same 2 hour duration did not result in an increase in NO_2^- and NO_3^- concentrations. Furthermore, once NO inhalation was terminated, NO_2^- and NO_3^- remained elevated, perhaps as a result of the NO liberation directly or indirectly from SNO-Alb. This compound decreased in concentrations after inhaled NO was discontinued, suggesting that it was broken down and generated NO in the circulation over the time period examined. Moreover, the release of NO from SNO-Alb persisted for, at least, an hour after inhaled NO was removed, but this molecule completely vanished after 3 hours. These data correlate well with the levels of NO_2^- and NO_3^- in plasma, which maintained for approximately an hour, and then began to decline once SNO-Alb was used up.

Our results reveal that SNO-Alb was formed within the circulation during NO inhalation because when animals were made to breathe 80 ppm NO, a high nanomolar range for SNO-Alb (150 - 770 nM) was found in plasma. However, the way in which

RSNOs are formed in the circulation has been highly controversial. One of the mechanisms proposed by investigators for the formation of RSNOs is the reaction of thiols with nitrogen oxide species formed from the autoxidation of NO (32;84;131). This process involves the presence of oxygen. Nevertheless, many researchers consider this reaction as too slow, and is limited by competing reactions between NO and HbO_2 (23;56;84). Our data agree inasmuch as SNO-Alb was not detectable under control conditions. Nonetheless, this species was measurable during inhaled NO. These results are consistent with the view that RSNOs (e.g., SNO-Alb) can be detected *in vivo* in spite of the low rate reaction of oxygen with NO and the presence of erythrocytes (30;84;85;91;120;128;130). Indeed, Marley et al. (84) have found that even in the presence of RBCs, significant amounts of plasma SNO-Alb are formed following treatment with an NO donor under aerobic conditions.

Our data demonstrate that the levels of GSNO are barely detectable. Therefore, we can conclude that the concentrations of GSNO in plasma during NO inhalation are below the detection limit of the analytical assay for this compound (< 125 nM). In comparison with SNO-Alb, our results strongly support the contention that during NO inhalation SNO-Alb was primarily formed in plasma. In other words, SNO-Alb was a more significant RSNO in plasma. A comparison was only made between two RSNOs, firstly because they are the most common compounds within plasma (9;128). Secondly, only a few RSNOs have been characterized; some of them are shown to be highly unstable (9;126), such as S-nitrosocysteine, which make the detection of these molecules difficult if not impossible. Despite the presence of other RSNOs in biological systems, the most prominent thiol present in plasma is albumin (~ 500 μM) (84), and this high

molecular weight compound constitutes more than 95% of total plasma thiols. It is, therefore, presumed that the absolute concentration of high molecular weight RSNOs generated is higher than low molecular weight RSNOs. In fact, the concentration of SNO-Alb has been consistently shown to exceed that of the low molecular weight RSNOs (30;84;91;128;130). For example, Welch et al. (150) have reported that SNO-Alb accounts for greater than 85% of the plasma RSNO pool under normal conditions. Data in our current study extend this view to very significant levels of SNO-Alb during NO inhalation.

RSNOs have been found to possess EDRF like-properties including vasodilation and inhibition of platelet aggregation (50;130). Therefore, once RSNOs are formed from inhaled NO, they may induce beneficial effects to the peripheral organs through the release of NO within the circulation. In fact, RSNOs are believed to decompose to liberate NO under appropriate conditions (9;44;123;126;154). The breakdown of RSNOs is likely to occur by a metal ion-catalyzed process *in vivo*. For example, Cu^{2+} acts as a catalyst to generate Cu^{+}, which promotes the decomposition of RSNOs for the release of NO (9;101;123;135). In addition, the degradation of RSNOs can be stimulated in the presence of reducing agents such as ascorbate, and thiols in plasma. These reducing agents facilitate the reduction of metal ions and then accelerate the metal ion-dependent decomposition of RSNOs (123). Moreover, thiols in plasma can also undergo a reaction called transnitrosation with either GSNO or SNO-Alb in giving off NO (101;122;123). Nevertheless, the mechanism(s) of NO release from RSNOs is still poorly understood and remains to be investigated.

It should be noted that the sum of NO_2^- and NO_3^- has been simultaneously measured in biological samples as an indicator of NO production (1;12;85;88;89;109;136;139). In general, NO is converted to NO_2^- and NO_3^- when inhaled or added to blood, and that NO_2^- is further oxidized to NO_3^- by the hemoglobin contained in RBCs (52;53). As a result, the determination of NO_2^- and NO_3^- *in vivo* may serve as a useful tool in studies on the production of NO (53;134). However, several investigators illustrate that NO_3^- could be an unreliable indicator of NO production, as this parameter is influenced by a variety of factors, such as dietary nitrate intake, saliva formation, bacterial nitrate synthesis within the bowels, denitrifying liver enzymes and inhalation of atmospheric gaseous nitrogen oxides (52;110). Moreover, the natural abundance of NO_3^- with a rather high background concentration in blood may hamper the exact evaluation of the amount of NO formed in the circulation (52;71). On the other hand, studies by different groups of researchers suggest that perhaps only plasma NO_2^- can accurately reflect the endogenous NO formation (54;71;110). Indeed, Lauer et al. (71) reported that only plasma NO_2^- increased during eNOS stimulation, but not NO_3^-, and they detected that very little amount of NO_2^- was converted to NO_3^- in the vasculature. These data suggest that NO_2^- is perhaps a more relevant parameter in the assessment of NO synthesis in the systemic circulation. More importantly, it has to be emphasized that for the *in vitro* setting, an increase in NO_2^- concentration is definitely an essential sign of NO production because the major product of NO in oxygenated fluids *in vitro* (i.e., in the absence of HbO_2) is NO_2^-.

Another important point that needs to be mentioned is that 80 ppm NO was chosen to administer to cats in this study rather than a lower concentration, such as 40

ppm or less that are mostly employed in many clinical studies. It is because lower concentration, such as 20 ppm was found insufficient to impact on the distal microvasculature (19). It was shown by Fox-Robichaud et al. (19) that 80 ppm would need to be used to cause an effect on the peripheral vasculature. 80 ppm inhaled NO was not necessary in previous studies, since they focused on the effects of inhaled NO at the level of the lungs. Although 80 ppm inhaled NO was employed throughout the current study, we did not find that 80 ppm NO had reached levels that are toxic, as NO_2 levels were continuously read on-line and levels never exceeded the permissible limit of 5 ppm. More importantly, 80 ppm has been used in human neonates studies for up to 10 hours without any detrimental effects (153).

In this chapter, we have demonstrated that SNO-Alb circulates in plasma during NO inhalation. SNO-Alb is a more significant species, as its abundance is higher than GSNO in plasma. SNO-Alb may release NO in the circulation such that the levels of NO metabolites, NO_2^- and NO_3^-, are maintained after the termination of NO inhalation.

CHAPTER 5

PLASMA PROTEINS RELEASE NO IN THE PRESENCE OF OXYGEN *IN VITRO*

Hypothesis: NO is mostly released from plasma proteins compared to red blood cells in oxygenated milieu *in vitro*.

Objectives:

1) To determine whether SNO-Alb is the source of NO release by measuring the *in vitro* plasma NO_2^- levels over 60 min incubation in the presence of oxygen.
2) To determine if RBCs are sources of NO by measuring the *in vitro* levels of NO_2^- and NO_3^- in buffer incubated in RBCs from NO breathing animals.
3) To examine whether the release of NO occurs in whole blood from NO breathing animals by measuring the *in vitro* levels of NO_2^- and NO_3^- in plasma.

Although SNO-Alb was shown to be the primary RSNOs in plasma compared to other molecules (50), such as GSNO, hemoglobin has also been proposed to play an important role in NO metabolism via the formation of S-nitrosohemoglobin (SNO-Hb) to preserve NO bioactivity (33;34;46;87;129). However, which compartment of blood (plasma or RBCs) is contributing mostly to the NO production in the circulation has never been addressed. In this study, simple separation techniques were established in order to investigate whether proteins in plasma or molecules in RBCs were responsible for the NO release *in vitro*. Levels of SNO-Alb and the oxidation product of NO (mainly NO_2^- in the absence of HbO_2) in plasma were examined by incubating samples over an

hour in the presence of oxygen in order to observe the changes in their concentrations. In addition, HBSS and intact plasma incubated in RBCs from NO breathing cats were measured for NO_2^- and NO_3^- in order to determine whether molecules in RBCs released NO during the time of incubation. Moreover, the effect of oxygen on the production of NO_2^- (indicator of NO release) from plasma proteins was also investigated.

5.1 RESULTS

Nitrite in Plasma of Inhaled NO Increases During Incubation.

As discussed in the previous chapter, NO_2^- is a more reliable parameter in assessing the production of NO, especially for the *in vitro* setting, since NO is primarily oxidized to NO_2^- in aerobic aqueous solution (in the absence of HbO_2) (14;43;52;53;71). Therefore, only plasma NO_2^- levels are reported in this section because a rise in levels of this ion most closely represents the NO release from plasma proteins *in vitro*.

In order to determine whether SNO-Alb in plasma that had been exposed to inhaled NO would contribute to the NO release *in vitro*, plasma was isolated from RBCs and incubated for 60 min in the dark in the presence of ambient oxygen. Levels of NO_2^- were examined before and after the incubation (at 0 and 60 min). Figure 5.1 shows the concentrations of NO_2^- in plasma from NO breathing animals during 60 min incubation *in vitro* in each individual experiment (dotted lines). The solid line represents the average results (Figure 5.1). Our data demonstrate that there was an increase in levels of NO_2^- in each single experiment, and the rise in concentrations of this ion was significant (Figure 5.1, $n = 9$, $^*P < 0.0001$), indicating that NO had been released from plasma, and consequently led to an augmentation in NO_2^- concentrations probably from the oxidation

process of NO over the time period examined. It should be mentioned that, although the levels of NO_3^- in plasma are not reported in this section, its levels significantly increased after 60 min incubation.

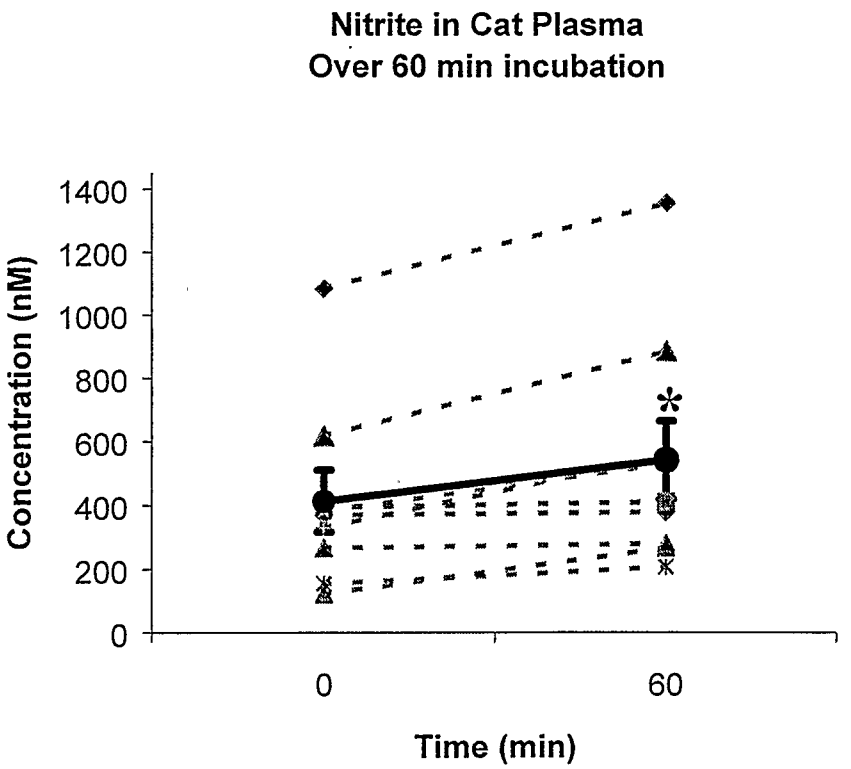


Figure 5.1. NO_2^- in cat plasma of inhaled NO before (0 min) and after (60 min) incubation. Plasma samples were incubated in the dark in the presence of ambient oxygen. Dotted lines are results from each individual experiment. Solid line represents the average results. Data are presented as mean ± SEM of nine observations. *P < 0.0001 relative to 0 min.

Plasma SNO-Alb Decreases During Incubation.

As shown in Figure 5.2, concentrations of SNO-Alb slowly decreased from 376 ± 58 nM to 190 ± 42 nM (n = 9, *P < 0.0001) over the 60 min incubation, suggesting a spontaneous release of NO from this compound. These data demonstrate that plasma SNO-Alb decomposed and liberated NO *in vitro*, and then NO in plasma was converted to NO_2^-. Results presented here support the rise in levels of NO_2^- shown in the previous section. It is conceivable that plasma SNO-Alb was contributing to the NO release *in vitro*.

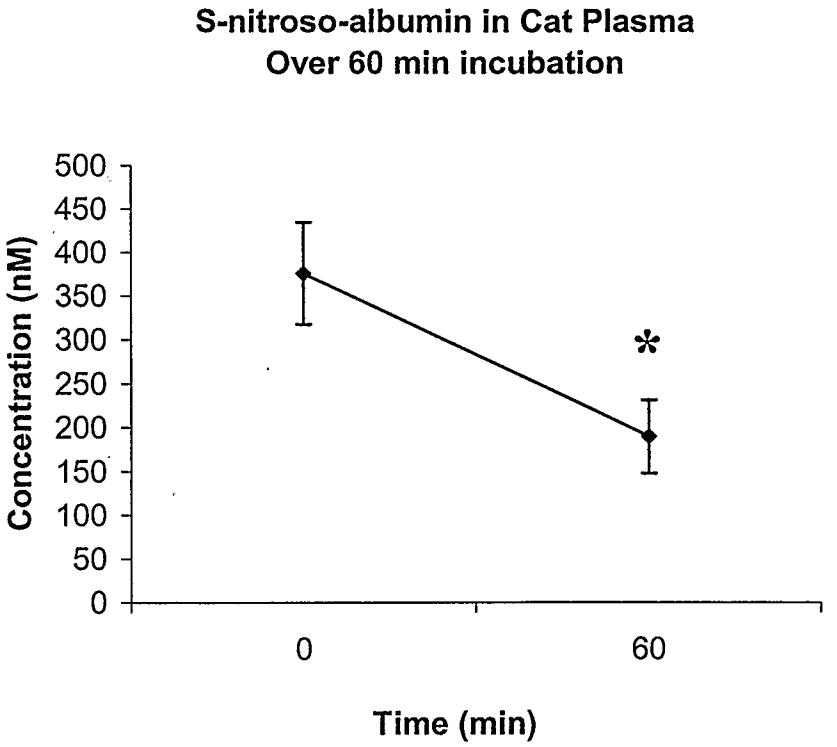


Figure 5.2. SNO-Alb in cat plasma of inhaled NO incubated in the presence of ambient oxygen in the dark for 60 min (n = 9). Data are presented as mean ± SEM. *P < 0.0001 relative to 0 min.

Levels of Nitrite and Nitrate in HBSS Reconstituted from RBCs Do Not Increase During Incubation.

In the following sections, the levels of NO_2^- and NO_3^- are reported because the incubation was performed together with RBCs. It is important to measure NO_2^- as well as NO_3^- because HbO_2 in RBCs may catalyze the oxidation of NO_2^- to NO_3^- (43;55). However, Lauer et al (71) suggested that the conversion of NO_2^- to NO_3^- could be a slow process. Therefore, the change in concentration of NO_2^- remains to be an important indicator of NO production.

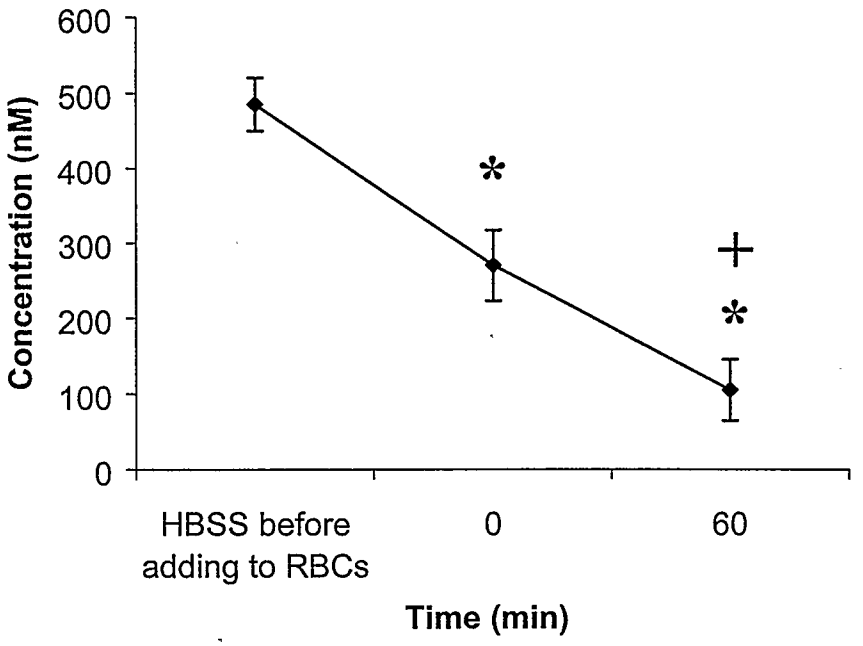
In this study, HBSS was prepared fresh prior to the addition to RBCs; however, 485 ± 35 nM NO_2^- and 6.13 ± 0.40 μM NO_3^- were detected in this solution. As depicted in Figure 5.3 (top panel), a drop in NO_2^- concentrations was observed once HBSS was added to RBCs from NO breathing animals at 0 min ($n = 9$, $^*P < 0.0001$), probably because NO_2^-, that was originally present in HBSS, reacted with HbO_2 in RBCs, and then converted itself to NO_3^-. Further decrease in levels of NO_2^- was seen during the 60 min incubation, perhaps due to the continuous conversion of NO_2^- to NO_3^- in the presence of RBCs. During the time of incubation, the levels of NO_2^- did not increase at all, and in fact, a significant drop ($n = 9$, $^+P < 0.0001$ relative to 0 min incubation) was obtained over time. Presumably, if NO was released from RBCs over 60 min, the concentrations of NO_2^- would be expected to be maintained or an increase in levels of NO_3^- should be anticipated during the time of incubation.

As demonstrated in Figure 5.3 (bottom panel), fresh HBSS originally contained ~ 6 μM NO_3^-. Our preliminary work shows that when this buffer was added to RBCs (without being exposed to inhaled NO), its levels were 17 ± 4 μM (not shown in the

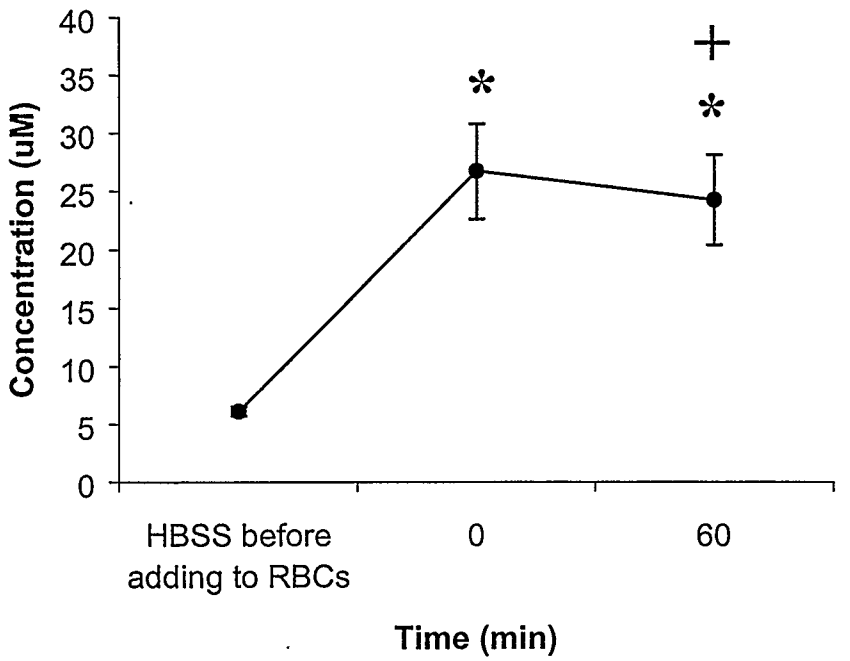
figure), indicating that intact RBCs contained a high background of NO_3^-. Upon addition of HBSS to RBCs that had been exposed to 80 ppm inhaled NO, the concentrations of NO_3^- increased to 27 ± 4 μM (at 0 min in Figure 5.3, bottom panel). One possible reason is that inhaled NO was rapidly taken up by RBCs, and was then converted to NO_3^-. This ion that was newly formed within the erythrocyte probably diffused to the surroundings (113). Although the concentrations of NO_3^- in HBSS increased initially at 0 min, during the 60 min incubation period, there was no augmentation in levels of this ion in HBSS at all. Indeed, the levels of NO_3^- significantly dropped (Figure 5.3, bottom panel, n = 9, $^+P < 0.0001$ relative to 0 min incubation), suggesting that molecules in RBCs were not producing NO; otherwise, NO would have changed to NO_3^- directly or indirectly from NO_2^- in the presence of RBCs, and resulted in a supplementary increase in levels of NO_3^- in HBSS. Data from this part of the experiments illustrate that inhaled NO had been taken up by RBCs or it might be stored in RBCs, but was not released to the surroundings in the presence of oxygen over the 60 min time course.

Figure 5.3. Levels of NO_2^- (top panel) and NO_3^- (bottom panel) in HBSS reconstituted from RBCs incubated in the dark for 60 min (n = 9). Data are presented as mean ± SEM. *P < 0.0001 relative to HBSS before adding to RBCs; $^+$P < 0.0001 relative to 0 min. Levels of NO_2^- and NO_3^- did not increase during 60 min incubation.

Nitrite in HBSS Reconstituted from RBCs



Nitrate in HBSS Reconstituted from RBCs



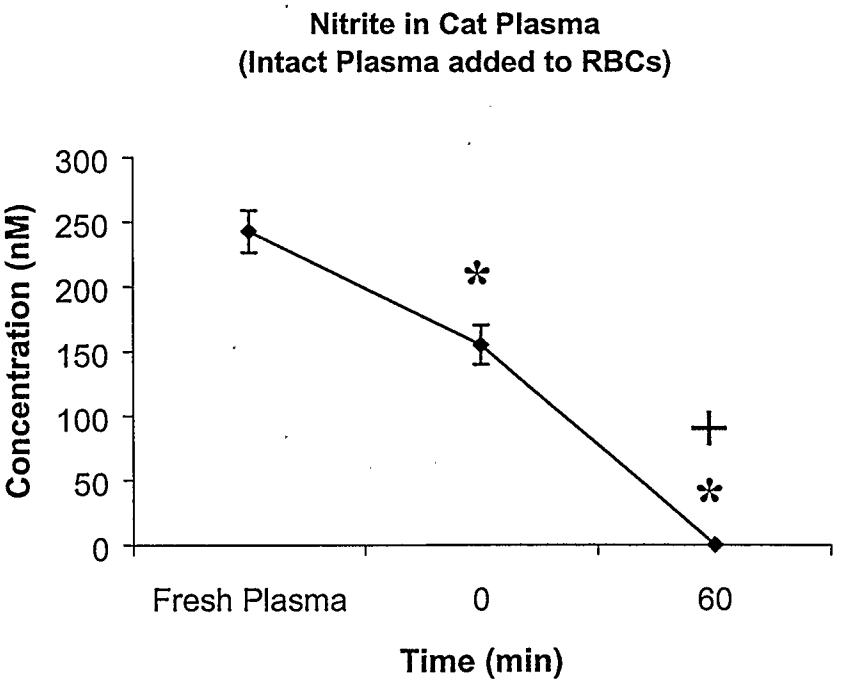
Nitrite, Nitrate and SNO-Alb in Fresh Plasma Added to RBCs from NO Breathing Animals.

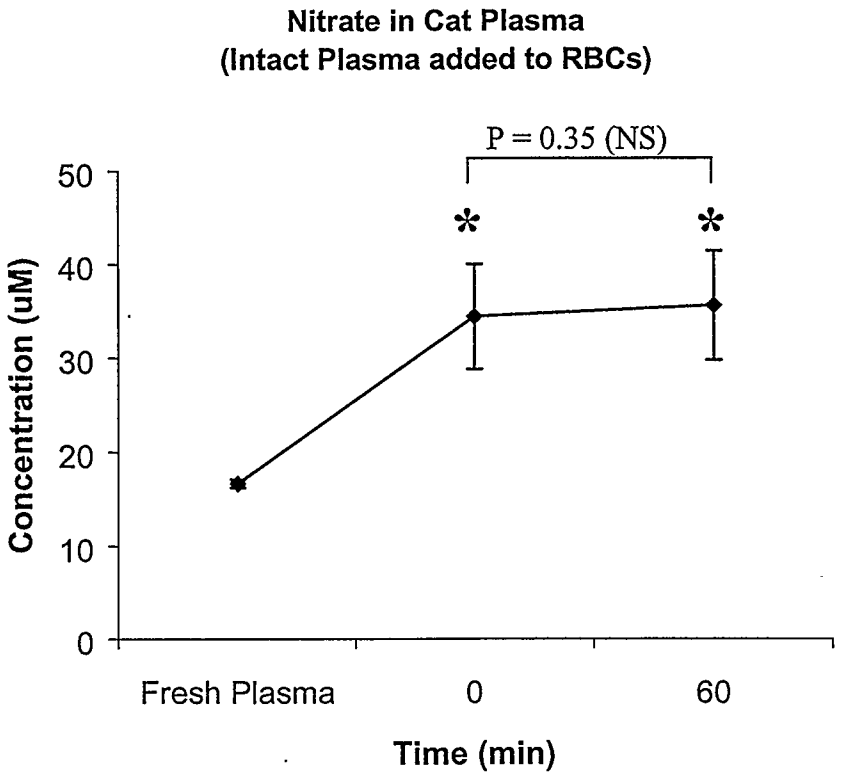
In the previous section, we have demonstrated that RBCs do not release NO under normal circumstances in the presence of oxygen *in vitro*. However, it has been suggested by several investigators that molecule(s) in RBCs (most likely SNO-Hb) may transfer its NO group to GSH or other thiols to form RSNOs, which may then promote the NO release and elicit vessel relaxation *in vivo* (46;129). For this reason, we investigated whether thiols in plasma could enhance the NO production from molecule(s) in RBCs. Instead of using HBSS, fresh intact plasma was added and incubated in RBCs from NO breathing animals for 60 min. As depicted in Figure 5.4 (top panel), fresh plasma originally contained 243 ± 16 nM NO_2^-. After adding plasma to RBCs that had been exposed to inhaled NO, NO_2^- levels dropped to 155 ± 15 nM ($n = 3$, $^*P < 0.05$), and then continued to decrease during the 60 min incubation ($n = 3$, $^+P < 0.05$ relative to 0 min incubation). In the case of NO_3^- (Figure 5.4, bottom panel), 16.7 ± 9.0 μM NO_3^- was detected in intact plasma. A rise in concentrations of this ion was observed at 0 min ($n = 3$, $^*P < 0.05$ relative to fresh plasma), probably due to the formation of NO_3^- from the interaction between inhaled NO and HbO_2 within RBCs, and thus diffused to plasma. However, no additional increase was seen over the 60 min incubation ($n = 3$, $P = 0.35$ relative to 0 min incubation). Results obtained from NO_2^- and NO_3^- suggest that molecule(s) in RBCs did not liberate NO in the presence of thiols in plasma. In other words, thiols did not promote the NO release from RBCs *in vitro*.

Moreover, we measured the concentrations of SNO-Alb in intact plasma that had been incubated in RBCs with inhaled NO for 60 min. The underlying reason for this was

to determine whether plasma proteins could extract NO from molecule(s) (e.g., SNO-Hb) in RBCs. As demonstrated in Figure 5.5, SNO-Alb was not detected in intact plasma. The addition of intact plasma to RBCs that had been exposed to 80 ppm inhaled NO did not cause an increase in levels of SNO-Alb at either 0 or 60 min during the incubation. Indeed, SNO-Alb was not detectable at any time point. Data suggest that NO was not withdrawn from molecules in RBCs in the presence of plasma.

Figure 5.4. Levels of NO_2^- (top panel) and NO_3^- (bottom panel) in intact plasma incubated in RBCs with inhaled NO in the dark for 60 min (n = 3). Data are presented as mean ± SEM. *P < 0.05 relative to fresh plasma before adding to RBCs; $^+$P < 0.05 relative to 0 min. Levels of NO_2^- and NO_3^- did not increase during 60 min incubation. NS = non-significant


Nitrite in Cat Plasma
(Intact Plasma added to RBCs)
Concentration (nM)
300
250
200
150
100
50
0
*
+
*
Fresh Plasma
0
60
Time (min)


Nitrate in Cat Plasma
(Intact Plasma added to RBCs)
P = 0.35 (NS)
Concentration (uM)
50
40
30
20
10
0
*
*
Fresh Plasma
0
60
Time (min)

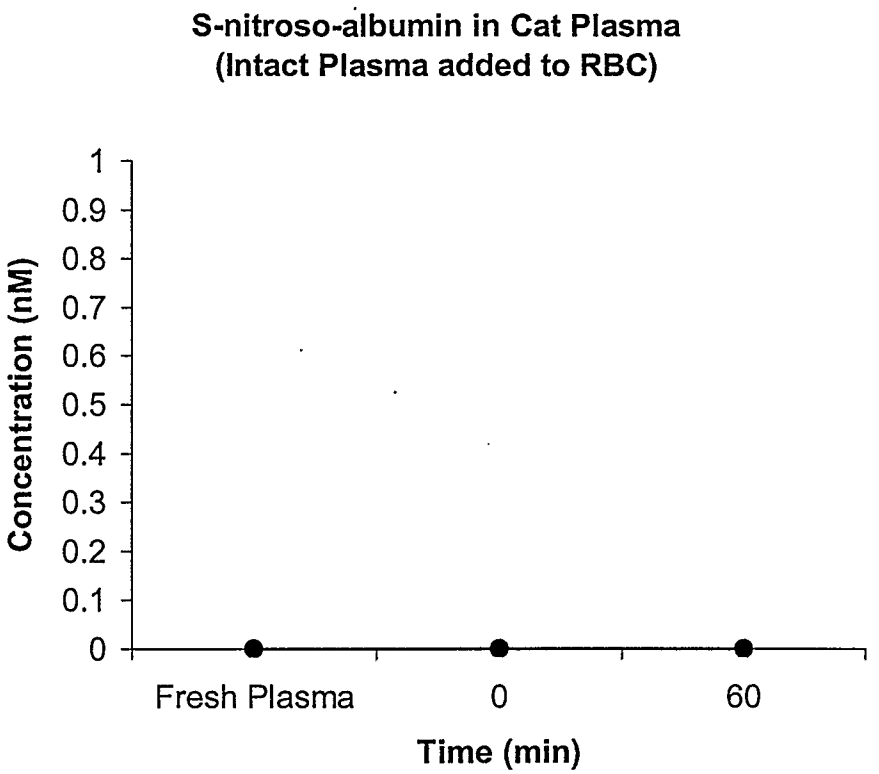


Figure 5.5. Levels of SNO-Alb in intact plasma incubated in RBCs with inhaled NO in the dark for 60 min (n = 3). Data are presented as mean ± SEM.

Nitrite, Nitrate and SNO-Alb in Whole Blood.

For comparison of results, we have examined whether the production of NO from plasma or RBCs occurs in a similar fashion in whole blood. Blood that had been exposed to 80 ppm inhaled NO was incubated in the dark in the presence of ambient oxygen for 60 min, after which the plasma was analyzed for NO_2^-, NO_3^- and SNO-Alb content. As depicted in Figure 5.6, NO_2^- concentrations decreased (top panel, $n = 8$, $^*P < 0.05$), whereas NO_3^- levels did not change significantly over the 60 min incubation (Figure 5.6, bottom panel). Levels of SNO-Alb decreased during the same time period from 366 ± 65 nM to 196 ± 70 nM (Figure 5.7). The change in SNO-Alb was non-significant ($n = 8$, $P = 0.065$), since in one out of the eight measurements investigated, there was an increase in SNO-Alb concentrations. The reason for this discrepancy was unclear. It should be noted that the drop of NO_2^- in plasma suggests a sequestration of NO by RBCs.

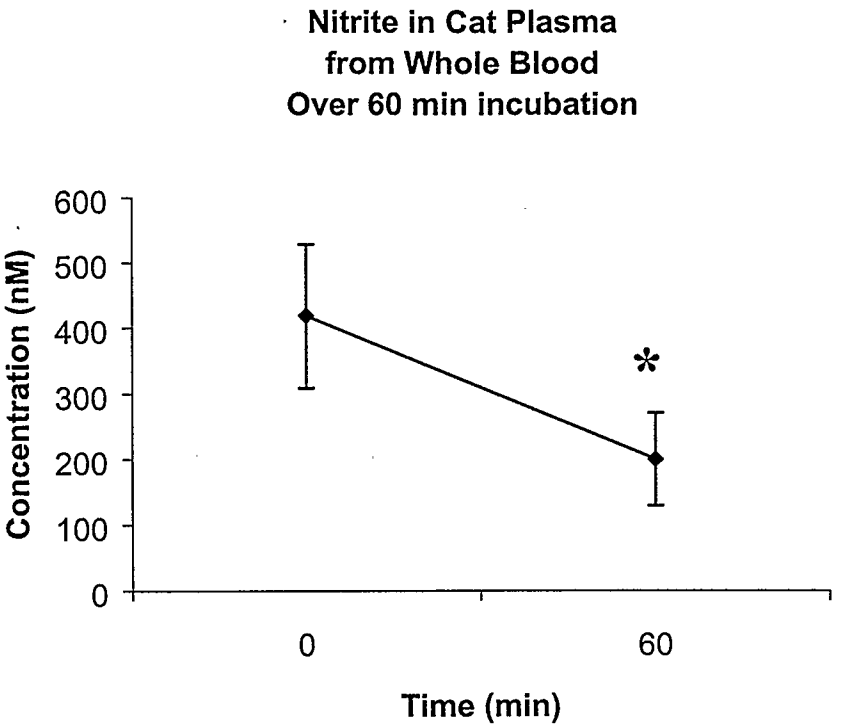
Figure 5.6. NO_2^- and NO_3^- in cat plasma from whole blood incubation.

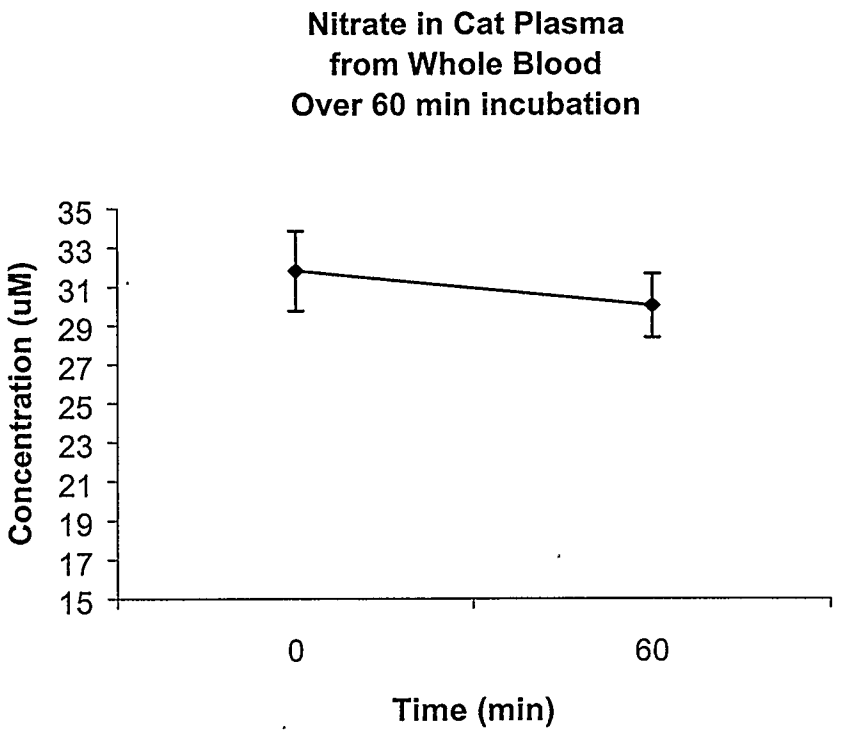
Top panel:

Whole blood was incubated in the presence of oxygen in the dark for 60 min, after which NO_2^- in plasma was analyzed (n = 8). Data are presented as mean ± SEM. *P < 0.05 relative to 0 min.

Bottom panel:

Whole blood was incubated in the presence of oxygen in the dark for 60 min, after which NO_3^- in plasma was analyzed (n = 8, P = NS). Data are presented as mean ± SEM.


Nitrite in Cat Plasma
from Whole Blood
Over 60 min incubation
Concentration (nM)
600
500
400
300
200
100
0
*
0
60
Time (min)


Nitrate in Cat Plasma
from Whole Blood
Over 60 min incubation
Concentration (uM)
35
33
31
29
27
25
23
21
19
17
15
0
60
Time (min)

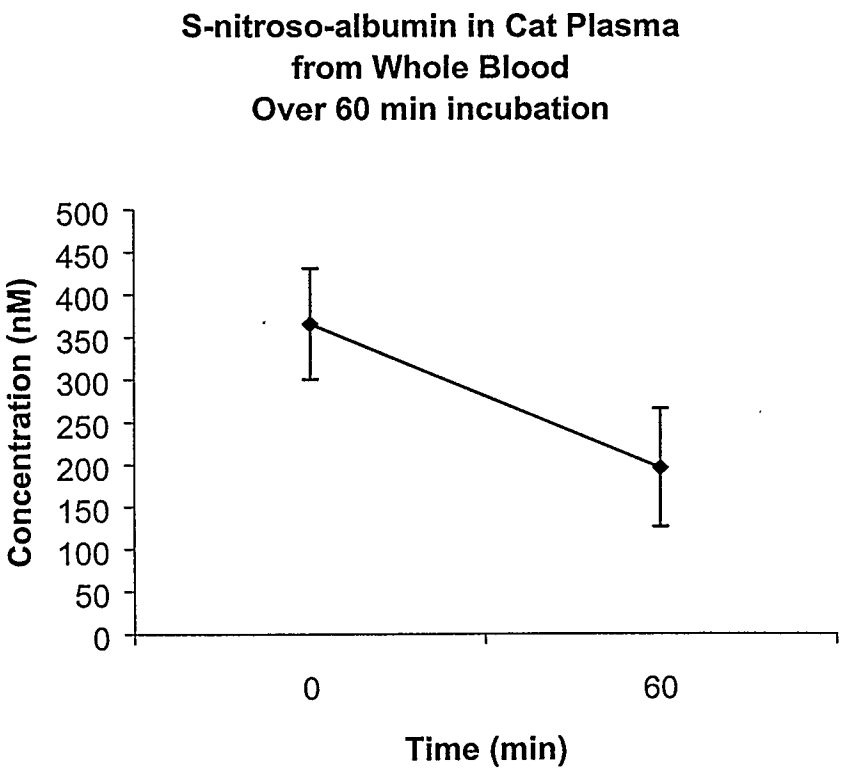


Figure 5.7. SNO-Alb in cat plasma from whole blood incubation.

Whole blood was incubated in the presence of oxygen in the dark for 60 min, after which SNO-Alb in plasma was analyzed (n = 8, P = 0.065, NS). Data are presented as mean ± SEM.

Formation of Nitrite in Plasma is Oxygen-Dependent.

We have demonstrated that the levels of NO_2^- increased only in plasma during 60 min incubation. Therefore, we hypothesize that NO is mostly released from plasma proteins in the presence of ambient oxygen *in vitro*. To determine the effect of oxygen on the formation of NO_2^-, plasma from NO breathing animals was deoxygenated very gently under a stream of nitrogen in a sealed culture tube before incubating in the dark for 60 min, and then plasma NO_2^- and SNO-Alb were measured thereafter. Baseline levels of SNO-Alb were not detectable (not shown in the figure), but with NO breathing high nanomolar concentrations of this compound in plasma were observed (Figure 5.9, levels at 0 min). Incubation of the deoxygenated plasma for 60 min resulted in a significant drop in SNO-Alb over time (Figure 5.9, $n = 3$, $^*P < 0.05$). Therefore, a rise in levels of NO_2^- should be anticipated. However, there was no augmentation in levels of NO_2^- after the incubation (Figure 5.8). One possible explanation may be that NO released from SNO-Alb in plasma could not convert to NO_2^- in the absence of oxygen. According to Feelisch (14), NO can quickly oxidize to NO_2^- in an aqueous medium *in vitro*, but the conversion requires oxygen. In this case, oxygen had been removed from plasma, and therefore, the levels of NO_2^- did not increase. These data further support the view that the formation of NO_2^- comes from the NO liberation in plasma, and that the process is oxygen-dependent.

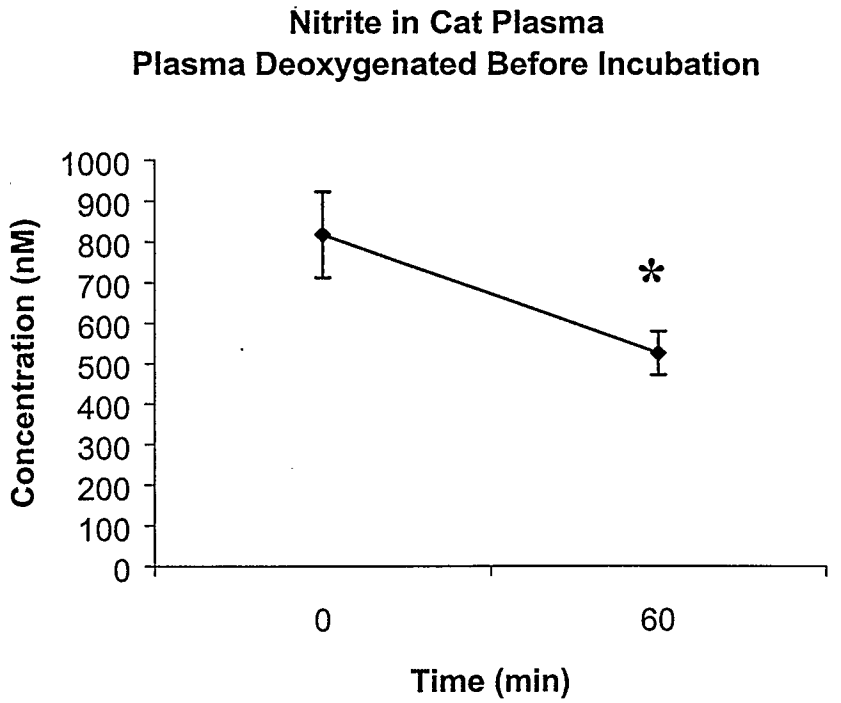


Figure 5.8. Levels of NO_2^- in plasma from NO breathing animals before and after deoxygenation under a stream of nitrogen. After plasma was deoxygenated, it was incubated in the dark for 60 min, and then analyzed for NO_2^- content (n = 3). *P < 0.05 relative to 0 min. Data are presented as mean ± SEM.

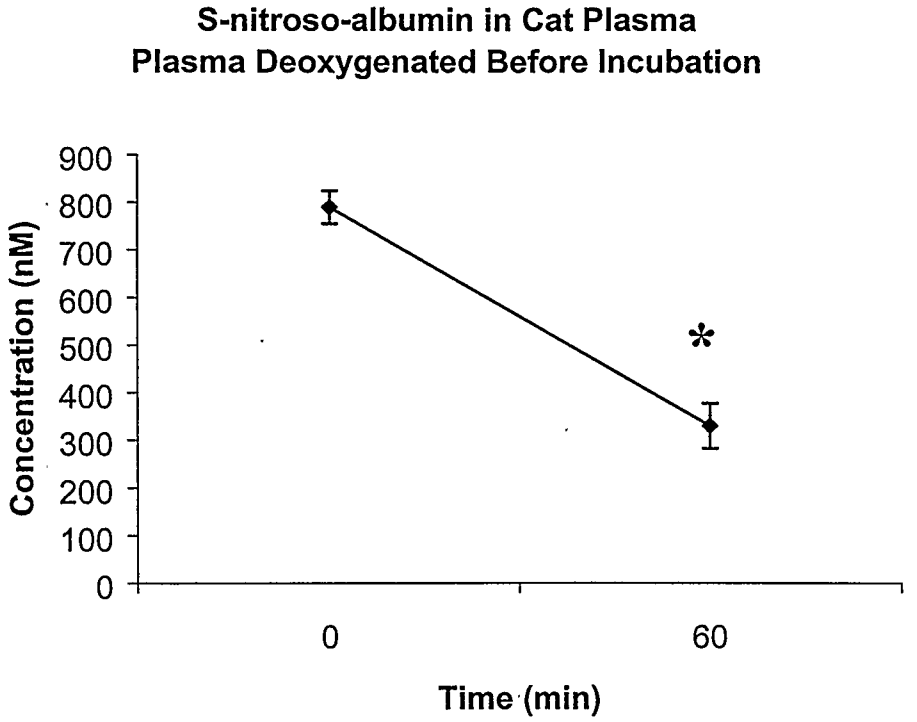


Figure 5.9. Levels of SNO-Alb in plasma from NO breathing animals before and after deoxygenation under a stream of nitrogen. After plasma was deoxygenated, it was incubated in the dark for 60 min, and then analyzed for SNO-Alb content (n = 3). *P < 0.05 relative to 0 min. Data are presented as mean ± SEM.

5.2 DISCUSSION

We have shown in the previous chapter that SNO-Alb was primarily formed in the circulation compared to GSNO during NO breathing in cats. SNO-Alb appears to be responsible for the NO release, as observed in the unaltered levels of NO_2^- and NO_3^- in plasma during 1 h post inhaled NO. However, recent work by Stamler and colleagues (33) suggests that besides plasma SNO-Alb and other RSNOs, NO may bind to the heme and cysteine residue (at position 93 on the β-globin chain) of Hb forming NO-heme adducts, including iron nitrosylhemoglobin (HbNO) and S-nitrosohemoglobin (SNO-Hb) respectively (26). Specifically, SNO-Hb has been proposed to play a part in transporting NO within the circulation, and preferentially liberating NO under certain conditions, for example, in the presence of low molecular weight thiols (129;156), and at sites of very low oxygen tension, where Hb exists mainly in the deoxygenated state (46;107;129). Nevertheless, the theory regarding SNO-Hb as an NO carrier has been controversial. Several investigators have raised doubts about whether SNO-Hb is produced and whether NO is generated from this molecule (69;105;145). Indeed, Hb has been considered as an NO scavenger due to its iron-containing heme group that destroys NO (82). In particular, NO interacts rapidly with HbO_2 to produce NO_3^- and methemoglobin (MetHb). This reaction has been treated as the major pathway for NO elimination from the body (55). Therefore, if NO is scavenged instantaneously by Hb that would limit the formation of SNO-Hb, the question arises as to whether RBCs are sources of NO in the circulation. Our data suggest they are not and that plasma proteins are the dominant source of NO.

In support of our contention, we identified whether inhaled NO was carried by plasma proteins or molecules in RBCs, and hence liberated NO over the time of

incubation in an oxygenated environment *in vitro*. Using simple separation techniques, we divided the compartments of blood, namely the RBCs and plasma, and investigated the release of NO in each isolated compartment by measuring the NO_2^- levels in plasma, and the NO_2^- and NO_3^- production in RBCs. Indeed, we have demonstrated that the levels of NO_2^-, the major NO metabolite *in vitro*, increased in plasma during incubation only when RBCs were removed. Moreover, once plasma proteins were removed (i.e. when RBCs remained), the production of NO_2^- and NO_3^- was eliminated during the time of incubation. Furthermore, our results reveal that SNO-Alb was most likely contributing to the NO release in plasma in the presence of oxygen because a drop in levels of this compound was observed over 60 min incubation *in vitro* (Figure 5.2). In fact, our data are supported by Hogg (36), who suggested that the low levels of SNO-Alb are possibly responsible for maintaining the bioactive state of NO that may contribute to the control of vascular tone *in vivo* (36).

In addition, our data demonstrate that NO was not released from RBCs because there was no increase in the levels of NO_2^- in HBSS over the time of incubation. One possible explanation for this phenomenon is that most of the inhaled NO has been taken up by HbO_2 in RBCs, which is then converted to NO_3^-, but not NO_2^-. On the other hand, it may be possible that an appreciable amount of NO has transferred to the cysteine residue of Hb, which results in the formation of SNO-Hb, as suggested by Vaughn and co-workers (145). Assuming that SNO-Hb is formed and releases NO, the levels of NO_2^- should, at least, be maintained or an increase in NO_3^- should be detected in HBSS in the presence of RBCs during the incubation; however, this was not observed in our study. Therefore, we can infer that RBCs do not liberate NO under our experimental conditions.

Indeed, Gladwin et al. (27) reported that only minimal amount of SNO-Hb was formed during NO breathing in healthy individuals. They suggest that the transport of NO using the SNO-Hb pathway possibly exists, but it may not be the dominant process. In addition, a recent study by Huang and colleagues (39) actually provide strong evidence that the primary reaction product resulting from the introduction of NO in oxygenated blood is MetHb, but not the NO-heme adducts. These results strongly support our observations that the dominant source of NO may not be the RBCs.

It should be noted that the levels of NO_2^- decreased, whereas that of NO_3^- increased initially upon the addition of HBSS to RBCs from NO breathing animals (Figures 5.3). Although it is tempting to suggest that the sudden rise in concentrations of NO_3^- might represent an instantaneous release of NO from RBCs, this would also require an increase in NO_2^-. It is our theory that the high NO_3^- content detected instantly upon the addition of buffer to RBCs was mainly due to the release of stored NO_3^-, which accumulated during NO inhalation. Indeed, there is evidence to support our contention. First, Gladwin et al. (26) reported that the principal products (i.e., NO_3^-) in volunteers inhaling 80 ppm NO gas were those from the interaction between NO and HbO_2 in blood. Secondly, Recchia and colleagues (113) have demonstrated that this interaction occurs inside the RBCs, and the NO_3^- that is formed from the oxidation with hemoglobin can rapidly leave the erythrocyte and then export through the erythrocyte membrane to plasma in less than 10 seconds at 0°C (113). It is presumed in our experiments that NO_3^- formed from the interaction between inhaled NO and HbO_2 diffused from RBCs to HBSS, and therefore increased the amounts of NO_3^- in buffer. Equation 1 illustrates the interaction between NO and HbO_2 (43;55):

$NO + HbO_2 \rightarrow NO_3^- + MetHb$ (Equation 1)

Released out of RBCs

Moreover, the increase in NO_3^- concentrations accompanied with a drop in levels of NO_2^- initially before incubation started. This can be explained by the interaction between NO_2^- and HbO_2 in RBCs. Although the amounts of NO_2^-, that was originally present in fresh HBSS (~ 485 nM), were small, they might have converted to NO_3^- in the presence of RBCs due to the oxidation reaction (Equation 2) (55):

$4\,NO_2^- + 4\,HbO_2 + 4\,H^+ \leftrightarrow 4\,NO_3^- + 4\,Hb^+ + O_2 + 2\,H_2O$ (Equation 2)

Formation of NO_3^- from the interaction between NO_2^- and HbO_2

It is very likely that the aforementioned reactions (Equations 1 and 2) collectively lead to the instantaneous increase in NO_3^- concentrations in the beginning. As a result, NO_3^- in HBSS rose initially prior to incubation. Nevertheless, the levels of NO_2^- and NO_3^- did not increase during the 60 min incubation, suggesting that NO may not be released from molecules in RBCs.

Despite the lack of increase of NO_2^- and NO_3^- in HBSS over the incubation period, we asked whether the addition of intact plasma to RBCs would encourage the NO liberation from RBCs. It has been suggested by several investigators that SNO-Hb can

react with thiols in plasma that may mediate the release of NO (46;129;156). Intact plasma was therefore added because thiols in plasma might undergo NO exchange reactions with molecules in RBCs that could promote the NO generation, and thus an increase in either plasma NO_2^- or SNO-Alb (if NO exchange reaction occurred with albumin in plasma) should be anticipated. However, the data obtained from intact plasma that had been incubated in RBCs from NO breathing animals, once again, did not show an augmentation in NO_2^- production, implying that NO was not released from molecules in RBCs. Apparently, thiols in plasma are incompetent to assist the process of NO liberation from RBCs under our *in vitro* conditions.

Our whole blood experiments demonstrate the importance of separating the compartments of blood in order to identify which component is mostly responsible for the NO release *in vitro*. NO inhalation did not induce an increase in plasma NO_2^- that was incubated in whole blood. These results were different from those obtained in the isolated plasma, where an increase in NO_2^- was observed. These data show that we were unable to distinguish whether NO was generated from plasma proteins or RBCs unless the blood compartments were separated, and this could only be accomplished by performing *in vitro* tests. It is our contention that the NO generated by SNO-Alb is quickly sequestered by RBCs. *In vivo*, a competition between RBCs and other cells (endothelial cells and smooth muscle cells) would permit biological responses to SNO-Alb. In our test tube system, only RBCs were available for NO uptake. Nevertheless, the interaction of inhaled NO with the components of blood is clearly complex *in vivo* and needs further investigation (36).

Up to now, we have postulated that NO is only released from plasma, but not from molecules in RBCs. In explaining why there was an increase in NO_2^- production in isolated plasma that had been exposed to 80 ppm NO after 60 min incubation, we examined the effect of oxygen on the production of this ion. When oxygen was removed from isolated plasma by deoxygenation, opposite trends were observed (i.e., no NO_2^- production), indicating that oxygen was an important factor to cause the augmentation in levels of NO_2^-. In other words, the formation of NO_2^- from NO is oxygen-dependent. Indeed, several investigators have stated that NO reacts with oxygen (O_2) to form NO_2, which combines with another NO to yield N_2O_3. This species then hydrolyzes spontaneously in aqueous solutions to form NO_2^- anion. Therefore, the production of NO_2^- in isolated plasma over the time of incubation can be elucidated by the following sequences of reactions (14;43):

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

$$NO_2 + NO \leftrightarrow N_2O_3$$

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

Accordingly, NO_2^- is produced from NO in the presence of oxygen and water in plasma.

It may be difficult to reconcile that while NO is scavenged by Hb, considerable amount of SNO-Alb can be formed in the circulation. Although this contradiction is still incompletely understood (36;105), recent studies have attempted to clarify the paradox. Several researchers have found that intravascular flow generates a red-cell-free zone near

the vessel wall, and it is likely that this area compensates to some extent for scavenging of NO by Hb in RBCs. It is possible that while the majority of NO is oxidized by HbO_2, the amount remaining, although small, may still be sufficient to nitrosate with thiols in plasma, and therefore, allow the formation of SNO-Alb through the reaction of NO with oxygen at sites where erythrocytes and Hb are excluded (77;105). Once SNO-Alb in plasma is formed, it acts as an NO carrier and releases NO via several mechanisms, including transnitrosation and reductive reactions within the circulation (84;123). This plasma layer is located at the endothelial interface perhaps allowing for some delivery of NO to the vessel wall.

In summary, our results have demonstrated that NO_2^- production only occurs in plasma. Once plasma proteins are removed, this production is eliminated. These data imply that plasma is probably a chief source of NO, which is then converted to NO_2^-, at least, in the presence of oxygen. Although several mechanisms could be involved in the NO release from different compartments of blood, SNO-Alb represents an important intermediate in the storage, transport and release of NO within the circulation.

CHAPTER 6

SUPPLEMENTATION OF CYSTEINE MODIFIES NO PRODUCTION FROM S-NITROSO-ALBUMIN *IN VITRO*

Hypothesis: Supplementation of an NO carrier, cysteine, increases NO release from S-nitroso-albumin *in vitro*.

Objectives:

1) To determine whether supplementation of cysteine affects the plasma concentration of SNO-Alb *in vitro*.
2) To determine if levels of NO_2^- change in plasma with the treatment of cysteine over 60 min incubation *in vitro*.

In chapter four, we have demonstrated that SNO-Alb was the dominant RSNO over GSNO in plasma during NO inhalation. Our next target was to investigate whether the addition of a low molecular weight thiol, cysteine, could enhance the decomposition of SNO-Alb, which in turn increased the NO production from this compound *in vitro*. The change in NO_2^- concentrations in plasma was used as an indicator to identify if supplementation of cysteine could modify the NO release from SNO-Alb. Observations from this study could provide us useful information on whether low molecular weight thiols were capable of promoting the NO delivery to the periphery to affect the vasculature.

6.1 RESULTS

Supplementation of Cysteine Enhances the Decomposition of S-nitroso-albumin *in vitro*.

Plasma of cats that had been exposed to 80 ppm inhaled NO was analyzed for levels of SNO-Alb before and after addition of cysteine over 60 min time intervals. Results were compared with each other. A previous study reported optimal effects with 100 μM cysteine to induce the degradation of SNO-Alb (31;84). We, therefore, have chosen to add 100 and 1000 μM cysteine to SNO-Alb. Similar results were obtained from both concentrations of cysteine. Hence, 100 μM cysteine was employed in all subsequent experiments. The upper curve (hollow circle) in Figure 6.1 illustrates the concentrations of SNO-Alb at 0, 5, 30 and 60 min after incubating plasma samples at 37°C in the dark. Results demonstrate that plasma SNO-Alb steadily decomposed *in vitro* over an hour. The drop in concentrations was significant at 30 and 60 min compared to that at 0 min (Figure 6.1, $n = 4$, $^{*}P < 0.05$). By contrast, the lower curve (solid circle) in the same figure shows the concentrations of SNO-Alb after supplementation of 100 μM cysteine. Levels of SNO-Alb significantly decreased ($n = 4$, $^{*}P < 0.05$) over the 60 min time interval in the presence of cysteine, and the drop was larger at all time points. At 60 min, the decrease was significant in the presence of cysteine compared to that without cysteine ($n = 4$, $^{+}P < 0.05$). In fact, the release of NO from SNO-Alb was faster with the treatment of cysteine; without cysteine a 25 min delay was noted. Although the change in levels of SNO-Alb was not more than 20% at any time, the net amount of NO release (next section) was quite significant.

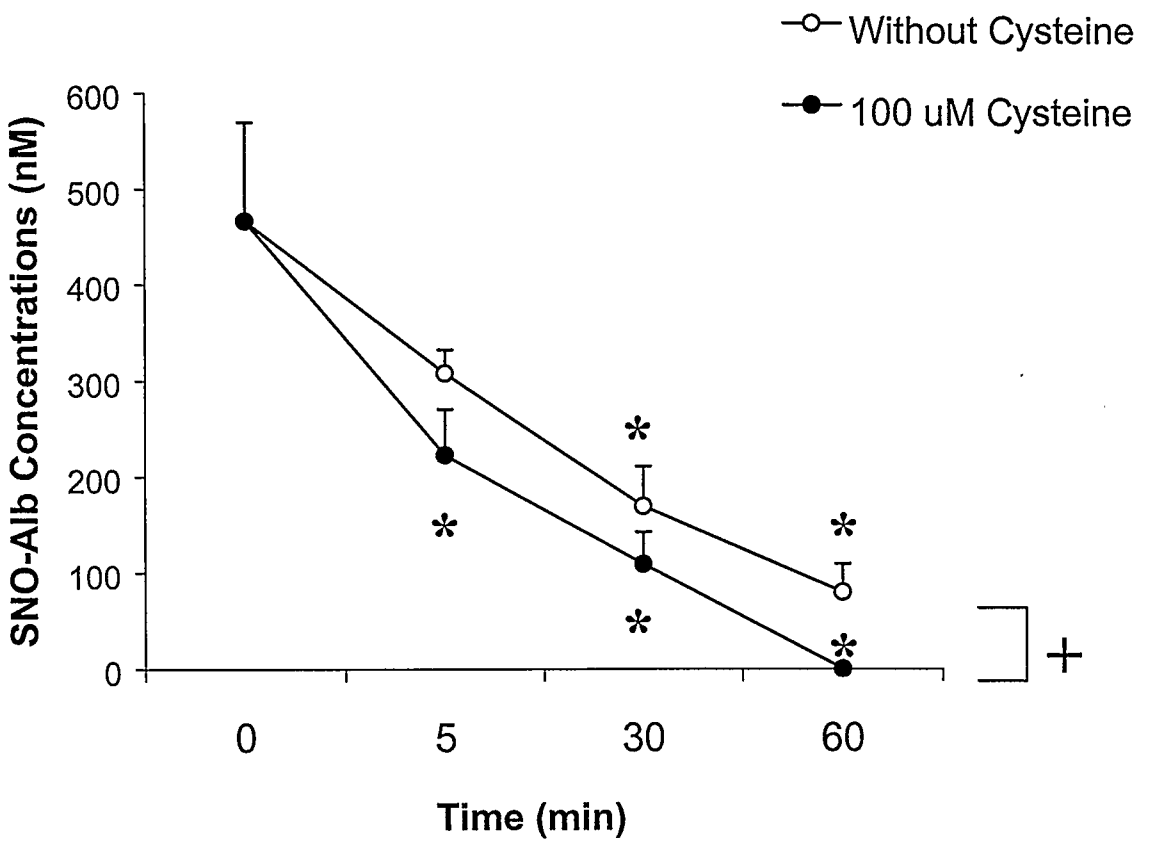


Figure 6.1. Levels of SNO-Alb in plasma from NO breathing animals with and without addition of 100 μM cysteine (n = 4). *P < 0.05 relative to respective control at 0 min; $^{+}$P < 0.05 relative to untreated group (without cysteine). Data are presented as mean ± SEM. Both were measured at 0, 5, 30 and 60 min.

Plasma Nitrite Levels Increase with Treatment of Cysteine *in vitro*.

Results in the previous chapter illustrate that the production of NO from plasma SNO-Alb leads to an increase in levels of NO metabolite, that is, NO_2^- *in vitro*. Moreover, data presented in the previous section show that SNO-Alb decomposed in an hour, and the degradation of this compound occurred to a greater extent with the treatment of cysteine. Therefore, if additional amounts of NO had been released, the levels of NO_2^- detected in plasma with addition of cysteine should be higher than those without cysteine. Figure 6.2 clearly shows the concentrations of NO_2^- in plasma in the presence and absence of 100 μM cysteine. The bottom curve in Figure 6.2 represents the levels of NO_2^- in plasma incubated at 37°C in the dark over 60 min. Under normal circumstances, NO_2^- levels increased with time, indicating that NO was slowly produced from various plasma, constituents including SNO-Alb. Although the increase was significant at 30 and 60 min after the incubation, a more prominent increase in levels of NO_2^- were observed for samples with supplementation of 100 μM cysteine (upper curve in Figure 6.2). The augmentation was significantly larger at 30 and 60 min in the presence of cysteine ($n = 4$, $^+P < 0.05$) than that in the absence of cysteine, suggesting that there was probably more NO release from plasma proteins (i.e., SNO-Alb) during the observation period. Again, the release of NO from SNO-Alb appeared to be expedited by about 30 min. It should be mentioned that the levels of NO_3^- were also measured in our experiments. Our results show that the overall change in levels of NO_3^- after 60 min incubation was higher in the presence of cysteine than that without cysteine.

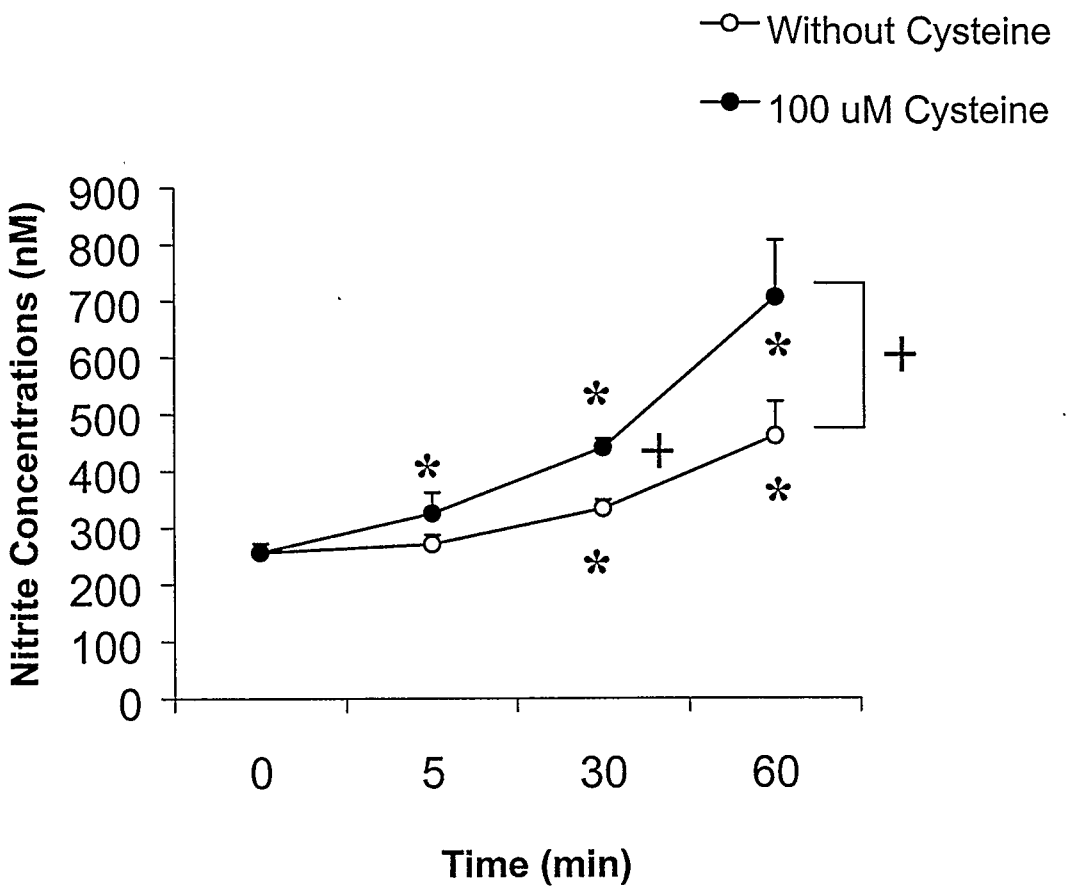


Figure 6.2. Levels of NO_2^- in plasma from NO breathing animals with and without addition of 100 μM cysteine (n = 4). *P < 0.05 relative to respective control at 0 min; $^+$P < 0.05 relative to untreated group (without cysteine). Data are presented as mean ± SEM. Both were measured at 0, 5, 30 and 60 min.

6.2 DISCUSSION

The data presented earlier in our study demonstrate that SNO-Alb releases NO *in vitro*, and *in vivo*. However, little is known about the exact role of SNO-Alb in the metabolism of NO, and it has been imprecisely defined how SNO-Alb releases NO in vivo (120). One of the concerns is whether SNO-Alb can deliver NO out of the vasculature due to its enormous size. As reported by Keaney et al. (50), large plasma proteins do not readily cross the endothelial cell membranes. Therefore, SNO-Alb is unlikely to deliver NO directly to the extravascular milieu to affect the peripheral tissues because SNO-Alb is generally restricted to pass through the endothelial barrier (64;120).

Several investigators believe that SNO-Alb may act as a reservoir for NO in plasma from which NO can be transferred to smaller molecular weight compounds that are capable of traversing the endothelial cell membranes. This NO exchange reaction has been referred to in the literature as transnitrosation (3;84;122;146). Transnitrosation is defined as the transfer of the nitroso functional group from a RSNO to a thiol (47). In principle, the S-nitrosothiol-thiol exchange reaction can be written as follows (142):

RSNO	+	R'SH	↔	RSH	+	R'SNO
S-nitrosothiol		Thiol				

Essentially, NO is transferred from a high molecular weight RSNO to a thiol group, R'SH, to form the thiol RSH and the corresponding low molecular weight R'SNO.

In the current study, we hypothesize that in the presence of low molecular weight thiols, such as cysteine, NO production from SNO-Alb can be enhanced. This contention

is based on the work by Scharfstein et al. (120), who reported that after infusion of SNO-Alb in rabbits, blood pressure decreased due to a transfer of NO from this species to a low molecular weight thiol (mainly L-cysteine in their system), which strongly enhanced vascular sensitivity. Their study suggests that L-cysteine may control the metabolism of SNO-Alb, possibly through transnitrosation reactions to accelerate the NO release from SNO-Alb. Although the exact mechanism of transnitrosation reaction is still incompletely understood (140;142), it appears that the transfer of NO from SNO-Alb to smaller molecular weight thiols can improve the biological effectiveness of SNO-Alb.

In light of our data, which focus on the effects of inhaled NO, we have observed that SNO-Alb decomposed slowly, but continuously in plasma under normal circumstances *in vitro*. Addition of a low molecular weight thiol, 100 μM cysteine, promoted the loss of SNO-Alb, and probably enhanced the NO release. This is evidenced by the increase in levels of NO_2^- in plasma within 60 min after supplementation of cysteine. Apparently, the NO-transfer from SNO-Alb to cysteine that eventually caused the production of NO might be more rapid than the decomposition of SNO-Alb by itself. One possible explanation is that cysteine extracts NO from SNO-Alb through transnitrosation. Once transnitrosation has occurred, the corresponding RSNO, in this case S-nitrosocysteine (CysNO), is formed. The newly produced low molecular weight RSNO is rather unstable, which may then undergo degradation in the presence of transition metal ions in plasma to release NO. Consequently, the concentrations of NO metabolite, that is, NO_2^- increase. Transnitrosation is a very rapid process, as suggested by Tsikas et al. (142); therefore, the increase in NO_2^- in samples treated with cysteine is higher than the untreated samples. The proposed concepts are illustrated in Figure 6.3.

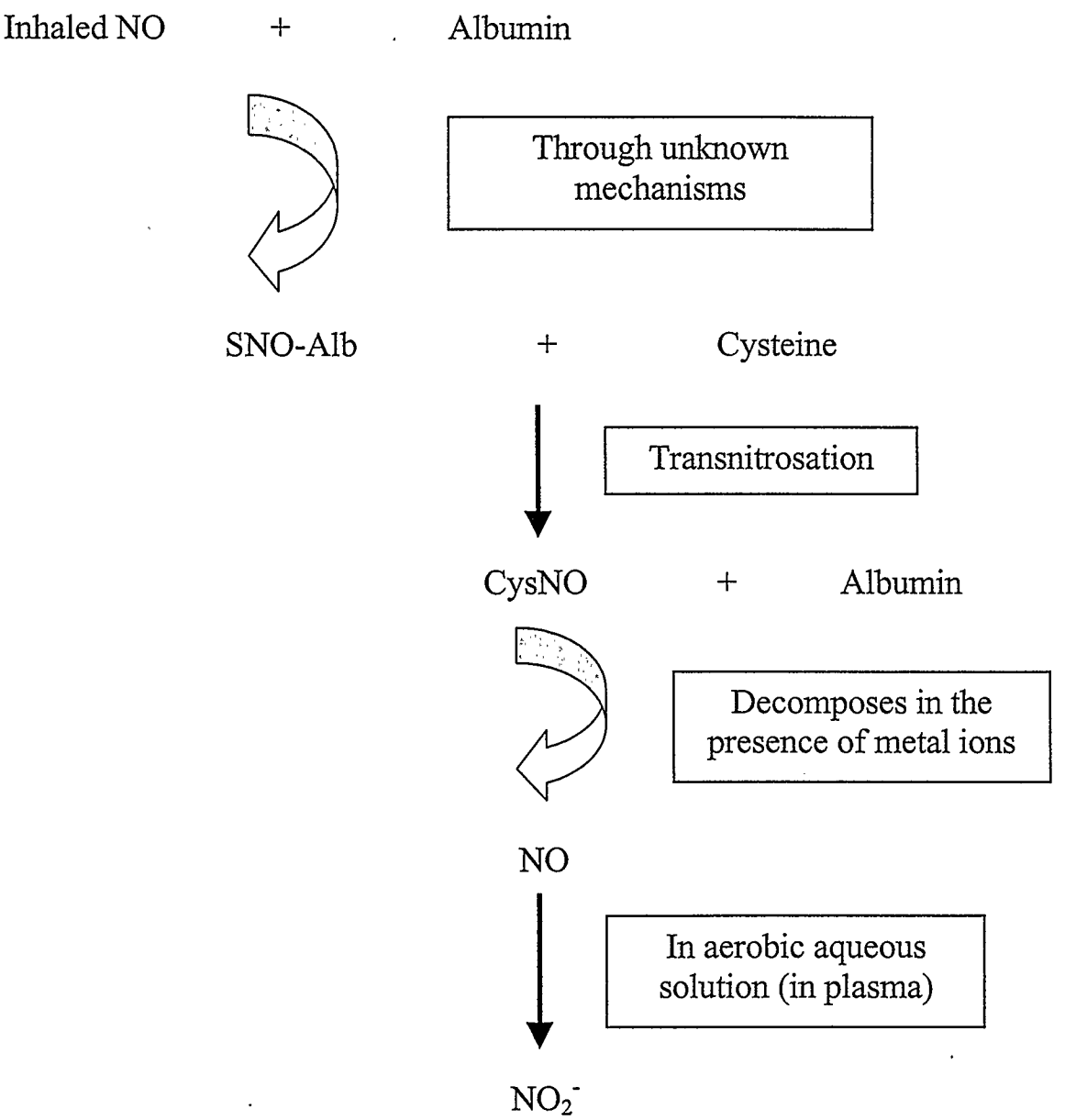


Figure 6.3. Schematic representation of interactions between inhaled NO and albumin. Formation of SNO-Alb probably occurs through unknown mechanisms. SNO-Alb may undergo transnitrosation reaction with cysteine to produce CysNO. This compound then decomposes in the presence of metal ions to release NO. NO is converted to NO_2^- in oxygenated fluids (in plasma) *in vitro*.

Moreover, our data demonstrate that SNO-Alb completely disappeared *in vitro* with the treatment of cysteine after 60 min. Under normal circumstances, the half-life of SNO-Alb is approximately 40 min in plasma, as reported by Stamler et al. (130). In addition, Gluckman and colleagues (29) found that SNO-Alb steadily released NO over a 6 h period when they studied the kinetics of NO release from SNO-Alb *in vitro*. Apparently, the rate of degradation of this species seems to be dependent on assay conditions (16). In our experiments, we demonstrated that the half-life of SNO-Alb in plasma was at least 60 min, and the decomposition of this species was greatly enhanced by cysteine. Results reflect that cysteine shortens the half-life of SNO-Alb because this low molecular weight thiol catalyzes the slower decomposition of SNO-Alb. Clearly, supplementation of cysteine modifies the NO production from SNO-Alb; it also represents a more efficient and rapid release of NO *in vitro*.

It should be noted that researchers have found that the induction of NO release from SNO-Alb by the endogenous cysteine is insufficient to cause major decomposition from SNO-Alb (122). Supplementation of high thiol concentrations is perhaps necessary to promote the loss of NO from SNO-Alb. The need for cysteine supplementation may be due to transnitrosation reactions being too slow at low thiol concentrations (101). Therefore, adding a concentration that is higher than the endogenous levels would be more appropriate in facilitating the NO production from SNO-Alb. The endogenous biologic thiols present in mammalian plasma are in low micromolar range. It has been reported that approximately 20 μM glutathione and 10 – 30 μM cysteine exist in human plasma (9;52;132). In our experiments, 100 μM cysteine was selected because this amount exceeds the theoretical endogenous value, which should enable us to observe the

effect of NO transfer. Nevertheless, we have attempted to use 1000 μM cysteine in our pilot study, but similar results were obtained as with 100 μM cysteine. It is, therefore, concluded that 100 μM was adequate to enhance the NO production from SNO-Alb. In other words, 100 μM cysteine was probably within the optimal concentration range for this investigation.

Furthermore, cysteine was chosen in this study, firstly, because it is a small molecule (smaller than glutathione with respect to the molecular weight) (52); its NO derivative, CysNO, has been proposed to be capable of crossing the endothelial cell membranes (120). Secondly, according to Kelm et al. (52), CysNO is very unstable. It has a shorter half-life than other RSNOs including GSNO and SNO-Alb. CysNO decomposes quickly once it is produced through transnitrosation reaction. More importantly, it is well known that CysNO is more reactive towards NO loss than GSNO so that this could make for an ideal delivery system for NO (101).

In summary, our findings demonstrate that the addition of a low molecular weight thiol, cysteine, enhances the decomposition of SNO-Alb. The concentration of 100 μM cysteine is able to accelerate the decay of SNO-Alb, promote the liberation of NO from this molecule, and consequently convert NO to NO_2^- *in vitro*. Definitely, supplementation of an NO carrier, in the case of cysteine, can modify the NO production from SNO-Alb in plasma *in vitro*. Although the exact mechanisms are unclear, both transnitrosation and reductive reactions by metal ions have been reported as the most probable pathways for the NO release (47;84). The observations obtained in the current study provide potentially important therapeutic implications for NO delivery to the periphery.

CHAPTER 7

INHALED NO INDUCES S-NITROSO-ALBUMIN TO DELIVER NITRIC OXIDE DURING ISCHEMIA-REPERFUSION

Hypothesis: Inhaled NO can impact on pathology, such as ischemia-reperfusion by extracting NO from SNO-Alb across the post-ischemic vasculature.

Objectives:

1) To determine the effect of inhaled NO on intestinal blood flow before and after ischemia-reperfusion.
2) To measure the arterial-venous differences for SNO-Alb in order to determine whether the post-ischemic tissue removes NO from this NO carrier.
3) To investigate whether NO is released from SNO-Alb across the post-ischemic vasculature by measuring the arterial-venous differences for plasma NO_2^- and NO_3^-.

In this study, we investigated whether inhaled NO could impact on pathology in situations like ischemia-reperfusion by monitoring the intestinal blood flow in animals before and after ischemia-reperfusion in the presence and absence of inhaled NO. We demonstrated that NO inhalation did not affect intestinal blood flow under normal conditions. However, during ischemia-reperfusion, blood flow was maintained at the pre-ischemic values only when NO inhalation was administered. In addition, we measured the arterial and venous levels of SNO-Alb to determine whether this NO carrier was responsible for the NO release in the circulation following ischemia-reperfusion. Moreover, we examined the venous-arterial differences for NO_2^- and NO_3^- in plasma to

support our contention that NO was being released from SNO-Alb in the periphery during ischemia-reperfusion in the presence of NO inhalation.

7.1 RESULTS

Inhaled NO Improves Intestinal Blood Flow During Ischemia-Reperfusion.

We tested the effect of inhaled NO on intestinal blood flow in normal unperturbed microvasculature. Figure 7.1 demonstrates that in animals that were made to breathe 80 ppm NO, intestinal blood flow was the same as in animals not breathing NO, suggesting that inhaled NO does not affect intestinal blood flow under normal circumstances.

To determine the role of inhaled NO in disease conditions such as ischemia-reperfusion, we examined the intestinal blood flow before and after ischemia-reperfusion in the presence and absence of NO inhalation. In the first set of experiments, we observed that intestinal blood flow under normal conditions was ~ 80 mL min^{-1} 100 g^{-1} in control animals breathing room air (Figure 7.2). Ischemia was induced by occluding the SMA blood flow to 20% of the control values for an hour using a Gaskell clamp (19). After removal of the clamp to allow reperfusion, blood flow increased, but it was still below the pre-ischemic values at 30 and 60 min after reperfusion in the untreated group (without NO inhalation) (Open bars in Figure 7.2).

In a second set of experiments, when 80 ppm inhaled NO was given to the animals right at the onset of reperfusion, the intestinal blood flow progressively rose. Within 60 min, the blood flow was recovered to 73 mL min^{-1} 100 g^{-1} (Figure 7.2, striped bar). The increase in blood flow at 60 min after ischemia-reperfusion in the presence of

inhaled NO was significantly higher (Figure 7.2, n = 3, $^{+}P < 0.05$) compared to the untreated group in which NO inhalation was not administered.

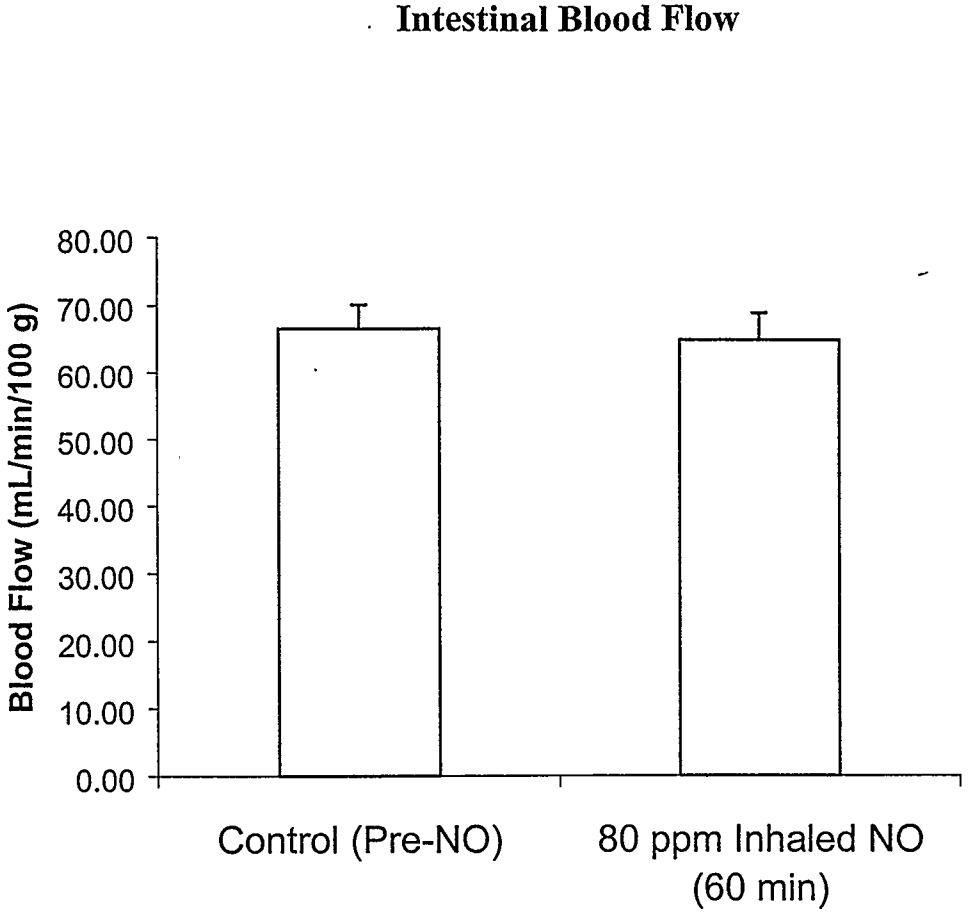


Figure 7.1. Intestinal blood flow under control (pre-NO) and during 60 min in animals inhaling 80 ppm NO (n = 3). Data are presented as mean ± SEM. Blood flow was not different between untreated animals and those with NO inhalation.

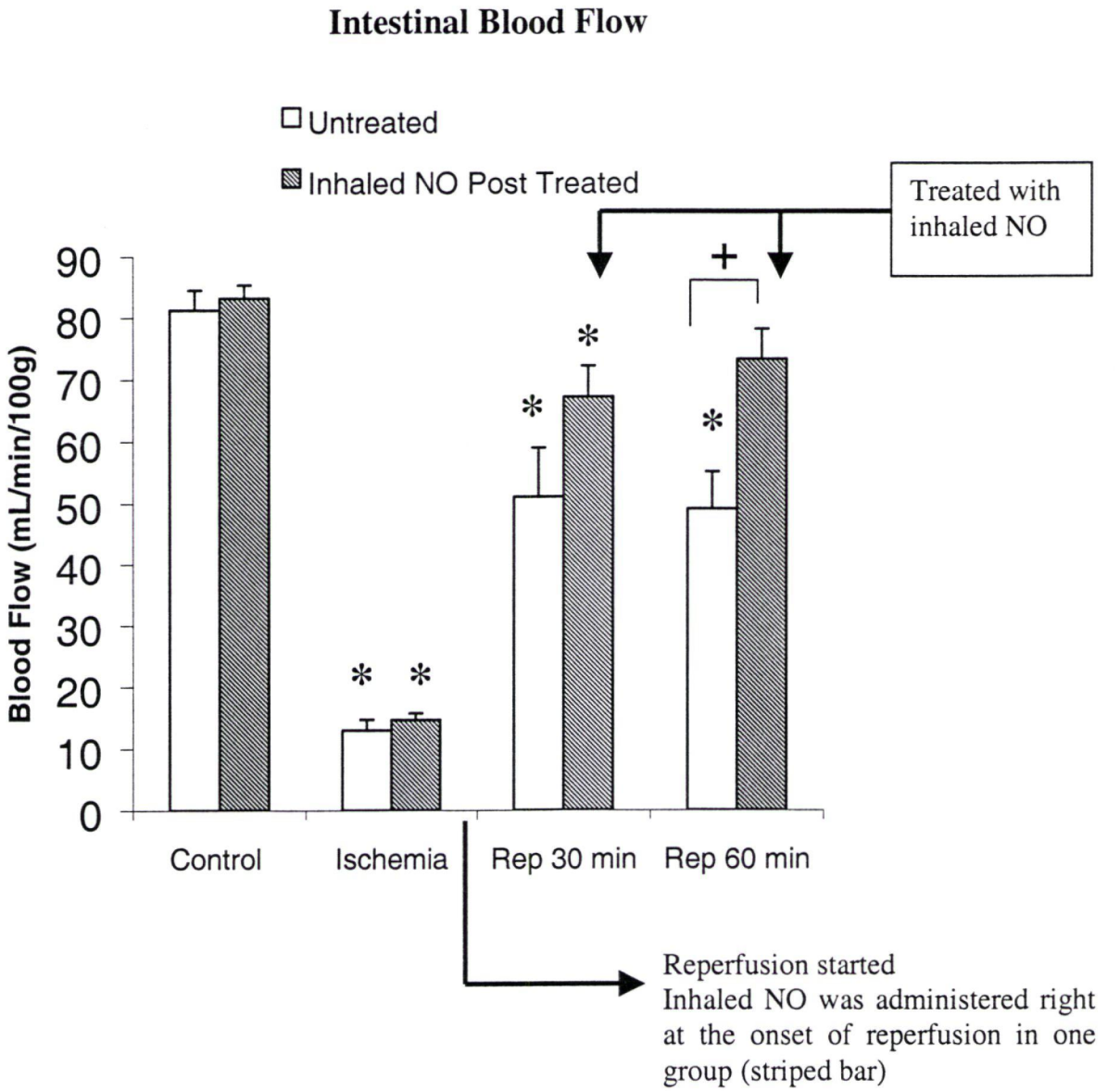


Figure 7.2. Intestinal blood flow under control, during 60 min of ischemia and at 30 and 60 min after reperfusion (Rep) in untreated cats (n = 3), and cats inhaling 80 ppm NO (n = 3). 80 ppm inhaled NO was given to animals right at the onset of reperfusion. *P < 0.05 relative to respective control. $^{+}$P < 0.05 relative to untreated group (without inhaled NO). At 60 min after reperfusion, intestinal blood flow was maintained at pre-ischemic values in animals breathing NO.

Plasma SNO-Alb Decreases Across The Post-ischemic Vasculature.

It was demonstrated in the previous section that intestinal blood flow could be restored in the presence of 80 ppm inhaled NO after ischemia-reperfusion. Our next aim was to determine whether SNO-Alb was being extracted across the post-ischemic vasculature by measuring the arterial and venous differences for this compound. As depicted in Figure 7.3, baseline levels of SNO-Alb were undetectable in arterial and venous plasma in animals ventilated on room air. In the first series of experiments, when animals were made to breathe 80 ppm NO for 60 min before ischemia-reperfusion, significant amounts of arterial and venous SNO-Alb were found (Figure 7.3, $n = 3$, $^{*}P < 0.05$). The arterial levels of SNO-Alb (382 ± 66 nM) were higher than the venous levels (207 ± 35 nM) (Figure 7.3, $n = 3$, $^{+}P < 0.05$) during 60 min NO inhalation, suggesting delivery or metabolism of NO in the peripheral circulation. In a second set of experiments, ischemia was induced for 60 min. No plasma samples were collected during ischemia. Then, samples were analyzed at 30 and 60 min following ischemia-reperfusion in cats breathing NO. We observed that the arterial concentrations of SNO-Alb continued to increase until 60 min after ischemia-reperfusion with inhaled NO. In fact, the amounts of arterial SNO-Alb at 60 min were significantly higher than those at 30 min (Figure 7.3, $n = 3$, $^{\phi}P < 0.05$), and were even higher than those with NO breathing alone ($n = 3$, $^{\#}P < 0.05$). Moreover, the arterial-venous gradients in SNO-Alb were observed at each time point after ischemia-reperfusion when NO inhalation was administered ($n = 3$, $^{+}P < 0.05$).

Table 8.1 summarizes the arterial-venous differences for SNO-Alb during NO inhalation alone, and at 30 and 60 min after ischemia-reperfusion in cats breathing NO.

As demonstrated in this table, the arterial-venous gradients in SNO-Alb at 60 min (724 ± 102 nM) were significantly higher than those at 30 min (Table 7.1, n = 3, $^{\#}P < 0.05$) after ischemia-reperfusion when inhaled NO was administered. The differences at 60 min were even higher than those in animals with NO inhalation alone (Table 7.1, n = 3, $^{+}P < 0.05$), suggesting a bigger NO extraction from SNO-Alb during ischemia-reperfusion in the presence of inhaled NO.

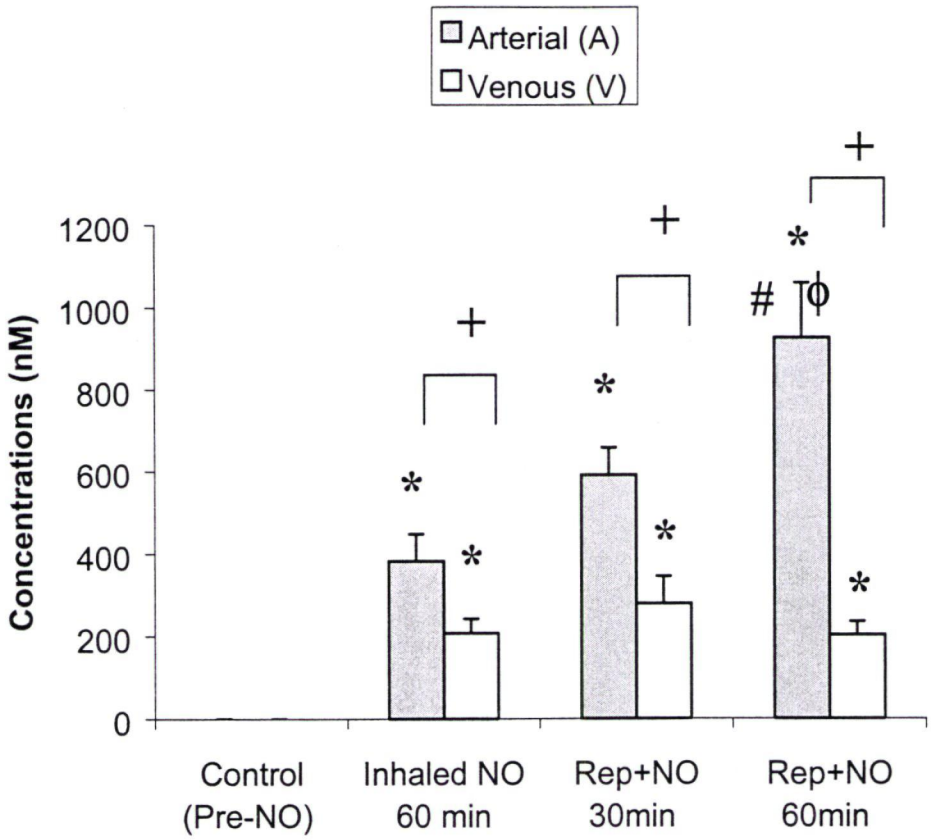


Figure 7.3. Arterial (A) and venous (V) levels of SNO-Alb under control, during 60 min inhaled NO, and at 30 and 60 min after reperfusion (Rep) in cats inhaling 80 ppm NO (n = 3). *P < 0.05 relative to control; $^{+}$P < 0.05 relative to respective arterial samples. $^{\#}$P < 0.05 relative to arterial samples of inhaled NO. $^{\phi}$P < 0.05 relative to arterial samples of rep + NO (30 min). Data are presented as mean ± SEM.

Samples	A - V for SNO-Alb Mean ± SEM (nM)
Control (Without NO inhalation)	0
Inhaled NO (60 min)	175 ± 31 (*)
Reperfusion + inhaled NO 30 min	312 ± 39 (*)
Reperfusion + inhaled NO 60 min	724 ± 102 (* $^{+\#}$)

Table 7.1. Differences in arterial (A) and venous (V) levels of SNO-Alb under control (without NO inhalation), during NO inhalation, and at 30 and 60 min after ischemia-reperfusion in animals inhaling 80 ppm NO (n = 3). *P < 0.05 relative to control; $^{+}$P < 0.05 relative to inhaled NO; $^{\#}$P < 0.05 relative to reperfusion + inhaled NO (30 min). Results are presented as mean ± SEM.

Consumption of Plasma SNO-Alb Increases During 60 min of Ischemia-Reperfusion in Cats Breathing NO.

To examine the total amounts of SNO-Alb being consumed by the post-ischemic vasculature after ischemia-reperfusion in the presence of NO inhalation, the arterial and venous SNO-Alb gradients (arterial SNO-Alb concentration minus venous SNO-Alb concentration) were multiplied by the blood flow measurements (blood flow per 100 g of tissue). All units are expressed as nanomole consumed per min per 100 g of tissue. As depicted in Figure 7.4, 12 ± 3 nanomole per min per 100 g of SNO-Alb was consumed during NO inhalation. The amounts of SNO-Alb used in the intestinal microcirculation significantly increased from 21 ± 4 to 54 ± 11 nanomole min^{-1} 100 g^{-1} during 30 and 60 min of ischemia-reperfusion in animals breathing NO (Figure 7.4, n = 3, *P < 0.05). Moreover, SNO-Alb consumption was significantly higher at 60 min than that at 30 min (n = 3, $^{\phi}P < 0.05$) when inhaled NO was administered during ischemia-reperfusion, and both values were much larger than that with NO inhalation alone (n = 3, $^{+}P < 0.05$), suggesting a demand for NO in the post-ischemic tissues.

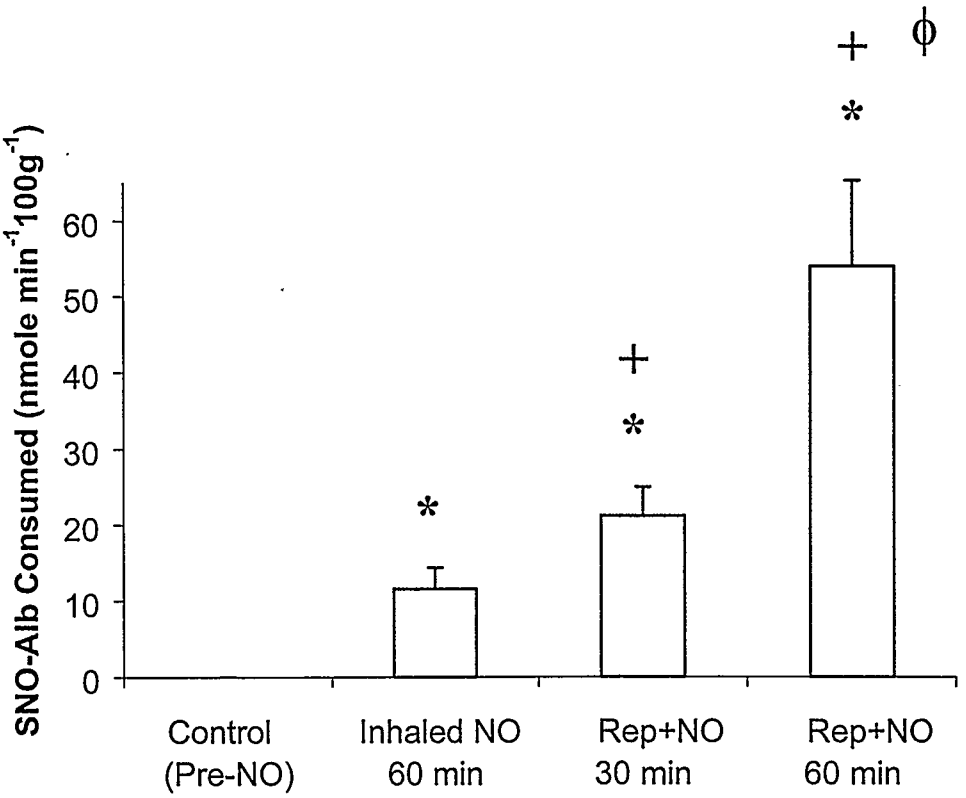


Figure 7.4. Amounts of SNO-Alb consumed under control, during 60 min inhaled NO, and at 30 and 60 min after reperfusion (Rep) in cats inhaling NO (n = 3). *P < 0.05 relative to control; $^{+}$P < 0.05 relative to inhaled NO; $^{\phi}$P < 0.05 relative to rep + NO (30 min). Data are presented as mean ± SEM.

Plasma Nitrite Increases Across The Post-ischemic Vasculature.

The levels of NO_2^- and NO_3^- in plasma are both reported in this section, as there is a possibility that NO_2^- may have converted to NO_3^- in blood. Nevertheless, researchers suggested that the conversion could be minimal on the passage through the circulation (71), and several investigators indicated that the arterial-venous differences for NO_3^- in plasma might not accurately reflect the production of NO across the systemic circulation. Therefore, NO_2^- remains the most reliable parameter in evaluating the NO release in the peripheral circulation (52;71;160).

As demonstrated in Figure 7.5, arterial and venous plasma NO_2^- did not change at baseline levels. When animals were made to breathe 80 ppm NO, arterial and venous NO_2^- levels significantly increased compared to the baseline values ($n = 3$, $^*P < 0.05$). Interestingly, the venous levels of NO_2^- (664 ± 53 nM) were higher than the arterial levels (489 ± 33 nM) during NO breathing ($n = 3$, $^+P < 0.05$). In addition, we determined the arterial and venous concentrations of NO_2^- at different time periods during ischemia-reperfusion in the presence of NO inhalation. We observed that the venous levels of NO_2^- were significantly higher at 60 min after ischemia-reperfusion in cats breathing NO compared to those at 30 min (Figure 7.5, $n = 3$, $^\theta P < 0.05$), as well as those with NO inhalation alone (Figure 7.5, $n = 3$, $^\phi P < 0.05$). These results correspond to the drop in venous SNO-Alb concentrations observed in the previous section, suggesting a release of NO from SNO-Alb and metabolism of NO in the peripheral circulation. Moreover, the venous levels of NO_2^- were significantly higher than the arterial levels at each time point of ischemia-reperfusion (Figure 7.5, $n = 3$, $^+P < 0.05$). Table 7.2 demonstrates the venous-arterial differences for plasma NO_2^- under control (without NO inhalation), during

60 min NO inhalation, and at 30 and 60 min after ischemia-reperfusion in cats inhaling NO. As shown in this table, the venous-arterial gradients in NO_2^- were larger at 30 and 60 min of ischemia-reperfusion with inhaled NO (339 ± 76 nM and 520 ± 67 nM respectively) than in animals with NO inhalation alone (176 ± 20 nM) (Table 7.2, n = 3, $^+P < 0.05$). Results suggest that the NO extraction is increased across the post-ischemic vasculature and leads to the formation of NO_2^-.

In the case of NO_3^-, an increase in the arterial and venous plasma NO_3^- was observed in each situation compared to the control (Figure 7.6). However, the arterial and venous NO_3^- levels were not significantly different under all experimental conditions, consistent with the inability of NO_3^- to reflect ongoing changes of NO release in the circulation (71).

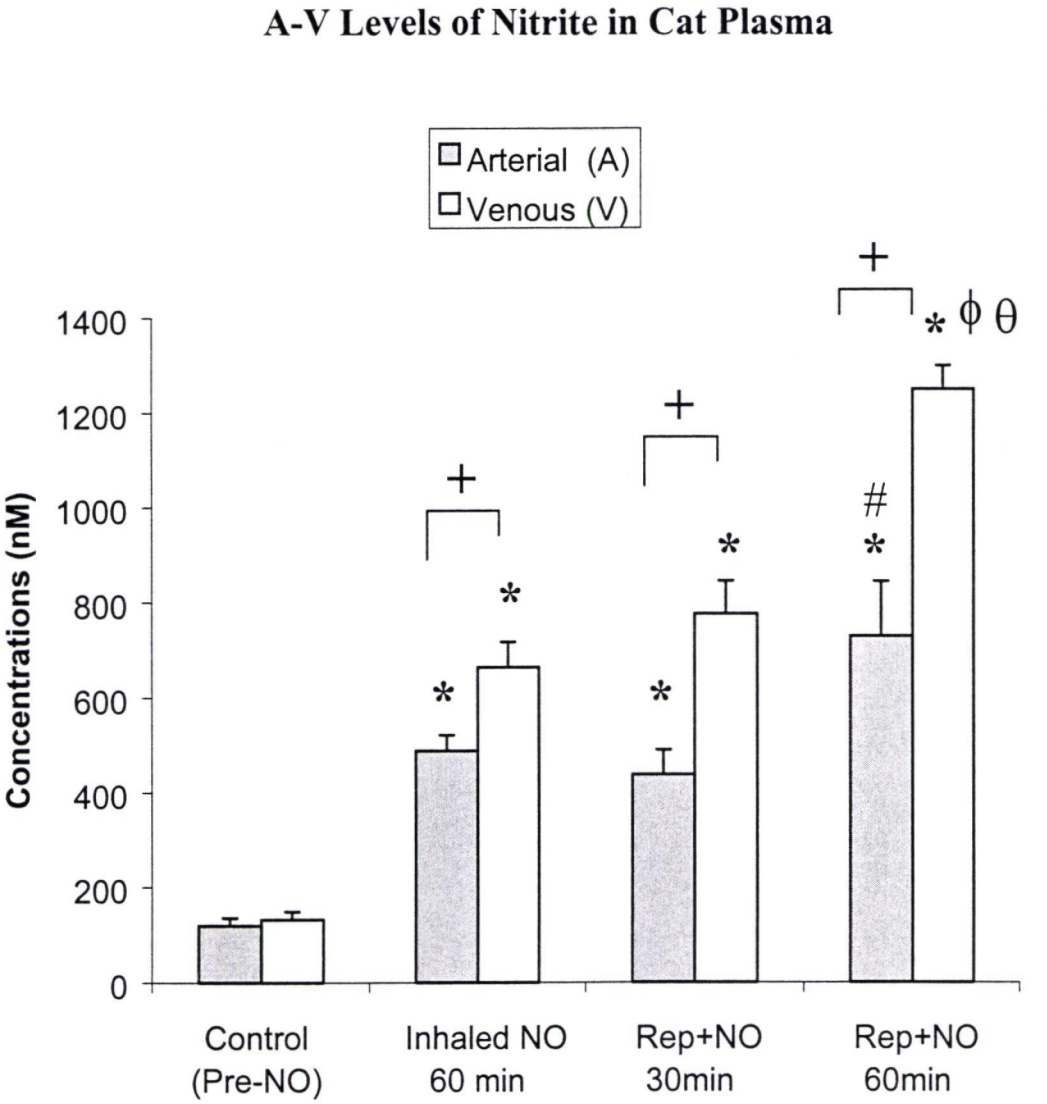


Figure 7.5. Arterial (A) and venous (V) levels of NO_2^- under control, during 60 min inhaled NO, and at 30 and 60 min after reperfusion (Rep) in cats inhaling 80 ppm NO (n = 3). *P < 0.05 relative to control; $^{+}$P < 0.05 relative to respective arterial samples. $^{\#}$P < 0.05 relative to arterial samples of inhaled NO. $^{\phi}$P < 0.05 relative to venous samples of inhaled NO. $^{\theta}$P < 0.05 relative to venous samples of rep + NO (30 min). Data are presented as mean ± SEM.

Samples	V – A for NO_2^- Mean ± SEM (nM)
Control (Without NO inhalation)	12.44 ± 8.58
Inhaled NO (60 min)	175.55 ± 20.08 (*)
Reperfusion + inhaled NO 30 min	338.78 ± 76.38 (* $^+$)
Reperfusion + inhaled NO 60 min	520.45 ± 66.94 (* $^+$)

Table 7.2. Venous (V) and arterial (A) differences for levels of NO_2^- under control (without NO inhalation), during NO inhalation, and at 30 and 60 min after ischemia-reperfusion in animals inhaling 80 ppm NO (n = 3). *P < 0.05 relative to control; $^+$P < 0.05 relative to inhaled NO. Results are presented as mean ± SEM.

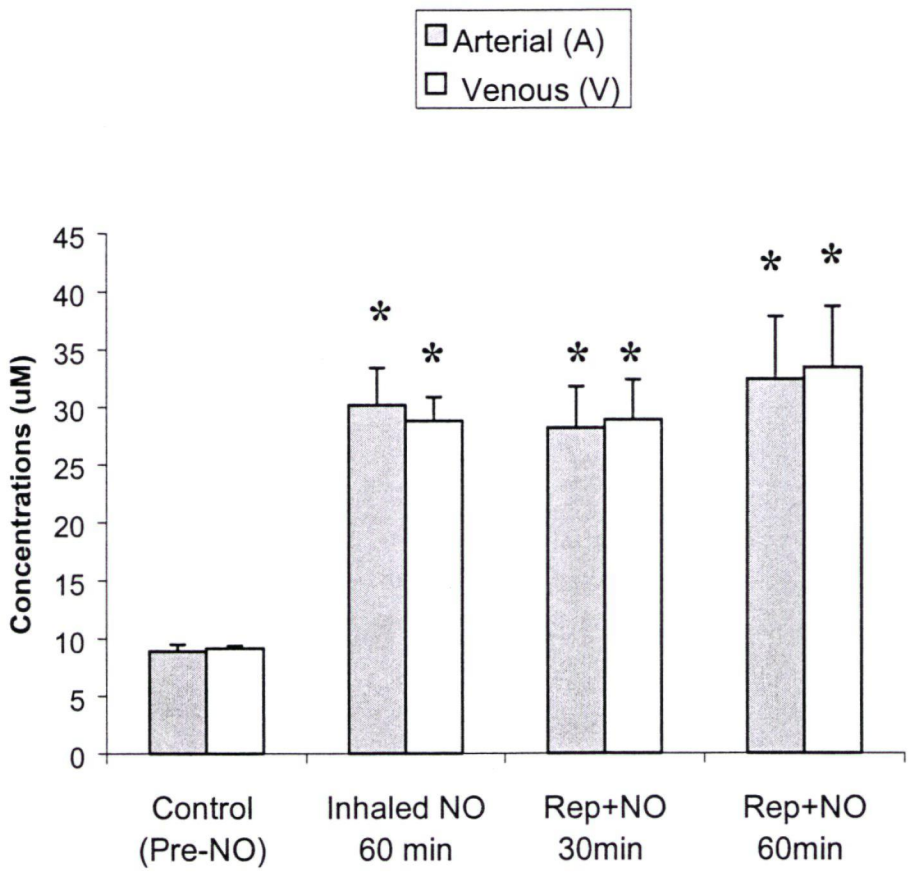


Figure 7.6. Arterial (A) and venous (V) levels of NO_3^- under control, during 60 min inhaled NO, and at 30 and 60 min after reperfusion (Rep) in cats inhaling 80 ppm NO (n = 3). *P < 0.05 relative to control. Data are presented as mean ± SEM.

7.2 DISCUSSION

Ischemia-reperfusion has been known to induce endothelial dysfunction characterized by a severe decrease in NO production by the endothelium (73). Many investigators have shown that the reduction of endothelial NO occurred as early as 2.5 min and was gradually worsened to more than 90% within 60 min after reperfusion (67;74;75;137). Moreover, the loss of NO during post-ischemic injury has been shown to be largely due to the generation of O_2^- radicals upon reintroduction of oxygen following ischemia (137) because NO is inactivated by O_2^- radicals. In addition, the deficit in NO release by the endothelium has been implicated to act as a trigger mechanism for the leukocyte-endothelial cell adhesion following SMA occlusion and reperfusion (4). This event may lead to migration of neutrophils across the endothelium and the release of their toxic mediators, and thus promote further endothelial dysfunction (4;73). Recently, inhaled NO has been demonstrated to be capable of reducing ischemia-reperfusion type injuries (19;64). For example, Fox-Robichaud et al. (19) and Kubes et al. (64) have reported that NO inhalation can interrupt and reverse the endothelial dysfunction and leukocyte recruitment in the mesentery induced by an ischemic episode. They proposed that the effects of inhaled NO on the post-ischemic microvasculature were perhaps due to the RSNOs that deliver NO to the periphery.

In this study, we further examined the potential role of RSNOs, particularly SNO-Alb, which might act as an NO carrier and participate in transporting NO to the peripheral circulation during mesenteric ischemia-reperfusion. We measured the arterial and venous levels of SNO-Alb, as well as the concentration differences for NO_2^- and NO_3^- across the systemic circulation during NO inhalation in healthy animals, and during

ischemia-reperfusion when inhaled NO was concurrently given to animals. We hypothesize, here, in the present study that the post-ischemic tissues would remove NO from SNO-Alb, and the NO extraction from SNO-Alb would increase during ischemia-reperfusion.

In the first part of our study, we demonstrated that intestinal blood flow did not change during NO inhalation, suggesting perhaps that plenty of NO was already present in normal intestine, such that inhaled NO had no effect on blood flow. However, within 60 min following ischemia-reperfusion, inhaled NO could entirely reverse the intestinal blood flow, whereas in the absence of inhaled NO, the blood flow was significantly below the pre-ischemic values. These data are consistent with the view that inhaled NO can reach distal vasculatures to effectively inhibit poor perfusion in disease conditions during ischemia-reperfusion. Moreover, our data support the idea that NO gas introduced in lungs is stabilized and transported to the periphery, perhaps by SNO-Alb, to modulate blood flow after ischemia-reperfusion, despite the rapid reaction with HbO_2 (15;55). This contention is verified by the arterial-venous gradients in SNO-Alb. Arterial levels of this compound were higher than the venous concentrations, suggesting a delivery source of NO across the peripheral circulation during ischemia-reperfusion. In contrast, the venous levels of NO_2^- were higher than the arterial levels. These data correlate well with the drop in concentrations of SNO-Alb, indicating that NO was extracted from this species and metabolized to NO_2^- in the peripheral circulation after ischemia-reperfusion. The release of NO from SNO-Alb perhaps restored intestinal blood flow back to the pre-ischemic values.

A very novel finding in this study was the augmented formation of arterial SNO-Alb in the circulation during ischemia-reperfusion when inhaled NO was simultaneously given to animals. The arterial levels were even higher than those with NO breathing alone. One possible route for the formation of SNO-Alb is via the reaction of thiols in albumin with nitrogen oxide species that are derived from the autoxidation of NO (explained earlier in this thesis) (32;126). Moreover, during ischemia-reperfusion, where O_2^- anions are produced (perhaps generated by activated neutrophils), these radicals may interact with NO to generate $ONOO^-$ (111), and the reaction between $ONOO^-$ and thiols in albumin may lead to the formation of SNO-Alb (60;155). Indeed, several investigators suggest that $ONOO^-$ can produce RSNOs (96;102;157). For example, Wu et al. (157) have demonstrated that $ONOO^-$ reacts with GSH to produce a nitrosothiol-like compound that relaxes pulmonary arteries *in vitro*. In addition, Moro et al. (96) reported that GSNO were formed in the reaction between $ONOO^-$ and GSH, and they suggested that $ONOO^-$ could convert to SNO-Alb through the interactions with albumin. Our data agree inasmuch as a rise in arterial SNO-Alb concentration was observed during ischemia-reperfusion with inhaled NO, perhaps due to the oxidation reaction between NO and oxygen, as well as the interaction between $ONOO^-$ and albumin in plasma.

It is interesting that far more SNO-Alb was consumed during ischemia-reperfusion and inhaled NO than during inhaled NO alone (Figure 7.4). In addition, the demand for NO from SNO-Alb in the post-ischemic vascular bed increased over time from 30 to 60 min following ischemia-reperfusion. The increased consumption of NO was associated with improved intestinal blood flow. Clearly, our results indicate that the post-ischemic tissues require a larger amount of NO being delivered by SNO-Alb to the

microvasculature than a normal unperturbed vascular bed. Moreover, our data would suggest a greater capacity for NO use, and perhaps even a greater need for NO. One possible explanation is that the large amounts of O_2^- radicals produced during ischemia-reperfusion may quickly quench the NO release from SNO-Alb, and thus results in higher consumption of this compound in the peripheral circulation. Indeed, the removal of O_2^- by reaction with NO may be considered beneficial (155) for the prevention of oxidative injury caused by the enhanced production of O_2^- during ischemia-reperfusion (80).

Our results suggest that SNO-Alb delivers significant amounts of NO to the periphery, and is then converted to NO_2^-, since the venous levels of plasma NO_2^- were higher than the arterial levels during ischemia-reperfusion when inhaled NO was administered. The venous-arterial differences observed in our study are consistent with those reported by Lauer and co-workers (71). This group reported that infusion of acetylcholine (ACh) stimulated the NO production from eNOS, which in turn increased the venous levels of plasma NO_2^-. Moreover, they found that the venous-arterial differences for NO_2^- increased at different dose levels of ACh, indicating that NO release in the vasculature was recovered on the venous side as NO_2^-. Our data agree inasmuch as negative arterial-venous gradients (i.e., V > A) exist for plasma NO_2^- across the systemic circulation.

In contrast to NO_2^-, venous-arterial gradients (V > A) for NO_3^- were not observed following ischemia-reperfusion when NO inhalation was administered. These findings do not come as a surprise because plasma NO_3^- levels are known to be influenced by a number of factors (52), as explained in Chapter 4. Moreover, the high background concentration of plasma NO_3^- (micromolar range) may underestimate the actual

production of NO_3^- or NO (may be in nanomolar range) across the systemic circulation (52;71;160). Although NO_2^- may convert to NO_3^- in the presence of HbO_2, the conversion may be sufficiently long that it is not detected in the venous blood immediately draining the organ. Therefore, the measurement of the arterial-venous NO_3^- difference may not reflect the production of NO across the circulation (52).

It should be noted that high concentrations of NO do not directly injure the intestinal vasculature to induce tissue dysfunction (49;66). Some investigators, however, have proposed that the reaction between NO and O_2^- at time of reperfusion may cause injury associated with intestinal ischemia-reperfusion through the formation of cytotoxic species, including $ONOO^-$ anion and hydroxyl radical (80;86). Nevertheless, Kanwar et al. (49) demonstrated that the addition of an NO donor at 1 hour of reperfusion did not cause any detrimental effect in the post-ischemic small intestine, and it did not induce further intestinal injury. Moreover, Kubes et al. (66) have reported that high levels of NO per se are not deleterious to tissue, and most importantly, they have shown that inhaled NO can impact very significantly on the post-ischemic microvasculature (64). Although $ONOO^-$ are purported to exert cytotoxic effects, its toxic actions are only remarkable at high doses (high micromolar to millimolar concentrations) (75). In addition, the effects of $ONOO^-$ can be influenced by the amount of NO available for reaction with O_2^-, as well as its interactions with other biological molecules such as thiols (80). Furthermore, $ONOO^-$ has been shown to mediate a number of physiological processes including vasodilation (78;157) and inhibition of leukocyte-endothelial cell interactions (72;102). Accordingly, low doses of $ONOO^-$ generated from the reaction between NO and O_2^- following ischemia-reperfusion may attenuate rather than enhance reperfusion injury.

Although we did not examine injury in our study, improved blood flow would certainly be viewed as beneficial in reperfusion through the use of inhaled NO.

This study also addresses a technical concern raised by others regarding the measurement of SNO-Alb. In a recent publication, Cannon III et al. (10) reported that the levels of SNO-Alb did not increase during NO breathing. They indicated that newer methodologies are required to preserve plasma SNO-Alb in order to prevent the rapid separation of albumin from low molecular weight thiol. In contrast, data presented in our current study (also in all experiments shown previously) demonstrate that SNO-Alb was being detected during NO inhalation. This is probably due to the additions of two important reagents at the start of our experiments, including DTPA and NEM to prevent the degradation and transnitrosation reaction of SNO-Alb. Clearly, the methodologies used in our study were compatible for the measurement of SNO-Alb.

In summary, in experimental conditions associated with mesenteric ischemia-reperfusion, inhaled NO may have an impact in the post-ischemic peripheral organs by transporting NO as RSNOs, such as SNO-Alb. Several investigators have found that the treatment with an NO donor, e.g., GSNO, can markedly attenuate ischemia-reperfusion injury (59;79). Our assertion that SNO-Alb formed from NO inhalation is responsible for the intravascular delivery of NO during ischemia-reperfusion is based on two lines of evidence. First, the variations in SNO-Alb levels correspond to changes in intestinal blood flow; and second, the venous-arterial gradients in NO_2^- represent an oxidation process of NO that indicates the release of NO from SNO-Alb within the circulation during ischemia-reperfusion. Our data strongly support the idea that inhaled NO may be an effective form of therapeutic interventions in post-ischemic injury in the periphery.

CHAPTER 8

SUMMARY AND CONCLUSIONS

Since the discovery of EDRF by Furchgott and Zawadzki in 1980 (22), and the subsequent demonstration that the factor is, indeed, NO (41;103), there has been a tremendous growth in research on NO and its properties over the last decade (40). Due to the instability and short half-life of NO, considerable recent interest has focused on the role of intravascular NO-derived molecules, including low and high molecular weight plasma RSNOs (128) that conserve and stabilize NO bioactivity (10;50;128), and may function as NO delivery systems and contribute to blood flow and oxygen delivery (10). The purpose of this research was to determine how inhaled NO was stabilized and delivered to the peripheral circulation by RSNOs. We sought to identify the major NO adduct that circulated in plasma during NO inhalation, and investigated whether NO was transported by this species to the periphery in pathological situations like ischemia-reperfusion. Moreover, using NO_2^- and / or NO_3^- as indicators of NO production, we examined the compartment of blood from which NO was released, and evaluated whether NO liberation could be modified by supplementation of a low molecular weight thiol.

In the first part of our study, we have described highly specific, sensitive, and artifact free analytical methods for the quantification of GSNO, SNO-Alb and the NO metabolites, plasma NO_2^- and NO_3^-. By employing the fluorometric techniques, we show that 80 ppm inhaled NO enabled significant amounts of SNO-Alb to be formed in the circulation. In addition, we demonstrate that this species was attenuated in concentration within 1 hour after the termination of NO inhalation, corresponding to the increase in plasma levels of NO_2^- and NO_3^-, which reflect the NO delivery by SNO-Alb in the circulation. These results collectively support the contention that NO is transported to the systemic circulation using inhaled NO via the formation of SNO-Alb or perhaps other

NO adducts, and thereby deliver small amounts of NO to sites distal from the lungs. Our data correlate well with the physiological findings reported by several investigators (19;20;64), who illustrated that inhaled NO could reach the periphery to prevent the vasoconstriction of arterioles and abolish leukocyte recruitment induced by local application of NO inhibitors in the mesenteric microvasculature in animals. Furthermore, in comparison with the low levels of GSNO, data in the present study demonstrate that the major circulating form of RSNO was present as SNO-Alb during NO inhalation. Our results are consistent with those reported by Stamler and colleagues (128), who found that the RSNO fraction was largely composed of the RSNO adduct of serum albumin.

Although SNO-Alb has been identified as a major reservoir of NO (30;91;128;143), and acts as a bioactive NO delivery agent to transport NO to a distal site (13;36;84), Hb has been proposed as a potential carrier of NO in the circulation (34;46;129). Nevertheless, a major concern is whether Hb can act as an NO transporter for the delivery of NO since Hb is an NO scavenger (82). Our *in vitro* experimental data suggest that NO was released from plasma proteins, but not from RBCs. Removal of the plasma compartment eliminated the production of NO_2^-, implying that RBCs were not sources of potential NO in the circulation. Additionally, an important finding in our study is that the release of NO and hence the formation of NO_2^- in plasma was an oxygen-dependent process. Although increases in NO_2^- were not seen in whole blood perhaps due to the rapid sequestration of NO by RBCs, *in vivo*, other cells (e.g., endothelial cells, smooth muscle cells) would also compete for the NO in the plasma rich space next to the vessel wall, and this would probably permit biological responses.

Indeed, our *in vivo* data suggest that inhaled NO did impact on intestinal blood flow, suggesting it was not entirely sequestered by RBCs.

SNO-Alb is an important NO donor that releases NO upon transnitrosation reactions with low molecular weight thiols (47;84). Our data demonstrate that the decomposition of SNO-Alb was enhanced by the addition of a high concentration (100 μM) of cysteine *in vitro*. Manipulating the concentration of cysteine catalyzed the NO release from SNO-Alb in concert with the increase in levels of NO_2^- in plasma. Our findings provide evidence that NO production from SNO-Alb can be modified and may be delivered in a more effective way by supplementation of a low molecular weight thiol.

The work presented in the last part of our study demonstrates that in normal unperturbed microvasculature, inhaled NO had no effect on intestinal blood flow, but it was critical for improvement of blood flow during ischemia-reperfusion when it has been documented that NO release is impaired. Clearly, the improved blood flow illustrates that the administration of NO inhalation may benefit reperfusion-type injuries of the cat mesenteric microvasculature. Moreover, we have shown that SNO-Alb was potentially responsible for the NO delivery across the systemic circulation following ischemia-reperfusion in NO breathing animals. To the best of our knowledge, this study is the first to show that arterial-venous gradients were present for SNO-Alb, indicating that this species released NO across the post-ischemic microvasculature to inhibit poor perfusion, and thus resulted in restoration of blood flow. This process was associated with a marked increase in NO_2^- production across the peripheral circulation, suggestive of an NO-mediated effect. Furthermore, SNO-Alb was used much more effectively during ischemia-reperfusion and inhaled NO than during NO inhalation alone, suggesting a

higher demand of NO extraction from SNO-Alb during pathological situations. The greater need for NO from SNO-Alb was perhaps due to the rapid interaction between NO and O_2^- produced during ischemia-reperfusion (80).

In conclusion, SNO-Alb is a potent vasodilator, whose action is related to its ability to release NO and assist in distribution of blood flow (118;143). The evaluation and the study of the concentration variation of this compound under pathophysiological conditions are of fundamental importance. The most important observation in this study is that inhaled NO can be stabilized and transported by SNO-Alb to the periphery to modulate blood flow and perhaps other events during ischemia-reperfusion. Additionally, our data reveal that the contribution of NO release from RBCs appears limited. It is conceivable that SNO-Alb formed from NO inhalation may act as a therapeutic delivery system for the treatment of post-ischemic injury in the periphery. However, the micro-environmental conditions *in vivo* may influence the stability and ability of SNO-Alb to act as a therapeutic agent. Based upon the data in our experiments, issues regarding the mechanisms of formation of SNO-Alb, as well as the mechanisms of NO release from this species *in vivo* are not fully understood. Additional research involving these questions should be conducted to achieve a complete understanding of the matter before NO inhalation can be used as a therapeutic approach for the treatment of diseases.

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