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The cellular membrane : properties, influence and proximal events relevant to vascular physiology

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The Cellular Membrane: Properties, Influence and Proximal Events Relevant to Vascular Physiology

by

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ABSTRACT

The discovery of nitric oxide as a ubiquitous biochemical messenger makes understanding of its signaling properties imperative. The finding that nitric oxide is enzymatically liberated from physiological S-nitrosothiols by protein disulfide isomerase (PDI) proximal to cell membranes suggests 1) that •NO release is influenced by enzyme redox status and 2) that •NO traverses biological membranes to elicit cellular signals.

This study was undertaken to determine 1) how vascular redox conditions influence membrane-associated PDI activities and 2) whether membrane cholesterol influences •NO-dependent signals. This study further benefited from application of a therapeutic agent to mitigate redox changes in platelet functions mediated by PDI.

Objective 1 was achieved using cells derived from an animal model of the pre-diabetic state. Upon induction of a pre-diabetic state, cellular ROS generation was enhanced and redox-dependent changes in platelet functions mediated by PDI were detected. Importantly, these changes 1) could be mimicked in platelets by treatment with exogenous ROS and 2) could be remediated by administration of a hypocholesterolemic agent.

Similarly, platelets derived human diabetic subjects exhibited altered metabolism. The effects of varying redox conditions on psPDI-redox status, -activity and aggregation were characterized. Our studies demonstrate the redox susceptibility of psPDI resulting in inhibition of denitrosation and disulfide reductase activities. Redox-dependent increases in rates of aggregation, however, could not be attributed to psPDI active site status based on the redox-independence of aggregation rates upon removal and controlled replacement of psPDI.

Lastly, diffusion and reactivity of •NO generated or released proximal to the membrane determines subsequent •NO-dependent signals. The role of hydrophobic domains in accelerated •NO autooxidation is recognized; however the role of cellular lipid composition in fine-tuning •NO signals requires further attention. Model systems and cells bearing a genetic defect that manifests as membrane sterol accumulation were used to investigate the role of cholesterol in modulating •NO-based signals. Studies show that 1) cholesterol alters the diffusion properties of •NO in model systems, 2) cellular sterol accumulation diminished the response of guanylate cyclase to exogenous •NO and could be mimicked by acute cholesterol loading and that 3) cellular metabolism of exogenous •NO, measured by DAF nitrosation is also altered in a cholesterol-dependent manner. Collectively, this study suggests that cholesterol influences the transmission of •NO-dependent signals.

Dedicated to my mother for wanting me to have something better

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ACD	– acid citrate dextrose
CaM	– calmodulin
DEA/NO	– Sodium (Z)-1-(N,N-Diethylamino)diazen-1-ium-1,2-diolate
DMPC	– dimyristoylphosphatidyl choline
DPI	– diphenyleneiodonium
DPPC	– dipalmitoylphosphatidyl choline
DTNB	– 5,5'-dithiobis(2-nitrobenzoic acid)
DTT	– dithiothreitol
eNOS	– endothelial nitric oxide synthase
EPR	– electron paramagnetic resonance spectroscopy
ER	– endoplasmic reticulum
Fb	– fibrinogen
FAD	– flavin adenine dinucleotide
FMN	– flavin mononucleotide
GSH	– reduced glutathione
GSNO	– S-nitrosoglutathione
GSSG	– oxidized glutathione
HDL	– high-density lipoprotein
HMG-CoA reductase	– 3-hydroxy-3-methylglutaryl Co-A reductase
iNOS	– inducible nitric oxide synthase
IR	– insulin receptor
IRS	– insulin receptor substrate
l_d	– liquid-disordered
l_o	– liquid ordered
LDL	– low-density lipoprotein
LDLR	– low-density lipoprotein receptor
L-NMMA	– N^G-monomethyl-L-arginine
msPDI	– microparticle surface protein disulfide isomerase
NAD(P)H	– nicotinamide adenine dinucleotide phosphate
nNOS	– neuronal nitric oxide synthase
•NO	– nitric oxide (nitrogen monoxide)
NOS	– nitric oxide synthase
NPC1	– Niemann-Pick Type C1 protein
PDI	– protein disulfide isomerase
PDZ	– post-synaptic density protein-95, discs-large, ZO-1
PIP3	– phosphatidyl inositol 3,4,5 phosphate
PM	– plasma membrane
Proli/NO	– Disodium 1-[(2-Carboxylato)pyrrolidin-1-yl]diazen-1-ium-1,2-diolate
PRP	– platelet-rich plasma
psPDI	– platelet surface protein disulfide isomerase
RBC	– red blood cell
ROS	– reactive oxygen species
SCAP	– SREBP cleavage-activating protein

SCP2	– sterol-carrier protein 2
sGC	– soluble guanylate cyclase
SNAP	– S-nitrosoacetylpenicillamine
SOD	– superoxide dismutase
StAR	– steroidogenic acute regulatory protein
T2D	– Type II diabetes

PART I

CHAPTER 1

GENERAL INTRODUCTION

1.1 Nitric Oxide

1.1.1 Introduction

In the 1980's and early 1990's, education of students on the properties and chemistry of nitric oxide focused on its existence as a deleterious component of automobile exhaust and air pollution. The evolution of a brown haze of nitrogen dioxide as air was introduced into a syringe of pure nitric oxide was a memorable pedagogical tool. However, it failed to convey the discovery of the seemingly ubiquitous nature and broad bioactivity of this remarkable free radical.

Since the identification of •NO as the endothelium-derived relaxation factor (ERDF) by Moncada [Plamer *et al.,* 1987] and Ignarro [Ignarro, *et al.,* 1987], there has been a virtual explosion of interest in the properties and physiological interactions of nitric oxide. In retrospect we can now appreciate the fact that nitric oxide plays a role in nearly every major aspect of human physiology including immune responses [Marletta *et al.*, 1988], neurotransmission [Garthwaite *et al.,* 1988], erectile dysfunction [Toda *et al.,* 2005], platelet aggregation [Bassenge *et al.,* 1991], myocardial function [Champion *et al.,* 2003], respiration [Mason *et al.,* 2006], reproduction [Lapointe *et al.,* 2006], cyto-protection [Wang *et al.,* 2002a] and –toxicity [Pacher *et al.,* 2007] , angiogenesis [Roberts *et al.,* 2007] and cellular proliferation [Contestabile *et al.,* 2004]. Thus, this section will discuss various facets of •NO chemistry, beginning with its biosynthesis, followed by the physical and chemical properties which govern its ability to transmit biological signals, insights from cellular reactivity experiments and close with a brief overview of the paradigm of •NO signaling.

1.1.2 Biosynthesis

Elucidation of the biosynthetic path of nitric oxide formation is reported to have resulted from the convergence of three separate lines of investigation [Ignarro *et al.,* 2000]. Clues to the agents responsible for formation of •NO came from 1) studies on the mechanism of agonist-induced relaxation of isolated blood vessels [Furchgott *et al.,* 1980, Griffith *et al.,* 1984, Furchgott *et al.,* 1984], 2) studies of nervous transmission in the central nervous system [Garthwaite *et al.,* 1988, Garthwaite *et al.,* 1987] and 3) the disposition of nitrate from humans and animal models of infection and macrophage cytotoxicity [Marletta *et al.,* 1988, Iyengar *et al.,* 1987, Hibbs *et al.,*1987]. In concert, these studies confirmed that •NO was endogenously produced in multiple tissues and cells in response to physiological agents, synthesized enzymatically from L-arginine and excreted in oxidized form as either nitrite (NO_2^-) or nitrate (NO_3^-). Several groups simultaneously noted the NADPH-dependence of production of nitrogen oxides from L-arginine [Marletta *et al.,* 1988, Knowles *et al.,* 1989], thus paving the way for affinity isolation of the neuronal nitric oxide synthase (nNOS) from rat cerebellum and demonstration of its Ca^{2+} / calmodulin dependence [Bredt *et al.,* 1990]. Shown in Fig. 1.1.1 is the overall reaction in which L-arginine undergoes a five-electron oxidation to L-citrulline with consumption of two moles of oxygen and 1.5 moles of NAD(P)H and generating one mole of •NO. Mechanistic studies have shown that 1 mole of NADPH is consumed in the conversion of L-arginine to N^{ω}-hydroxy-L-arginine and 0.5 moles are consumed in the conversion of N^{ω}-hydroxy-L-arginine to L-citrulline [Stuehr *et al.*, 1991b].

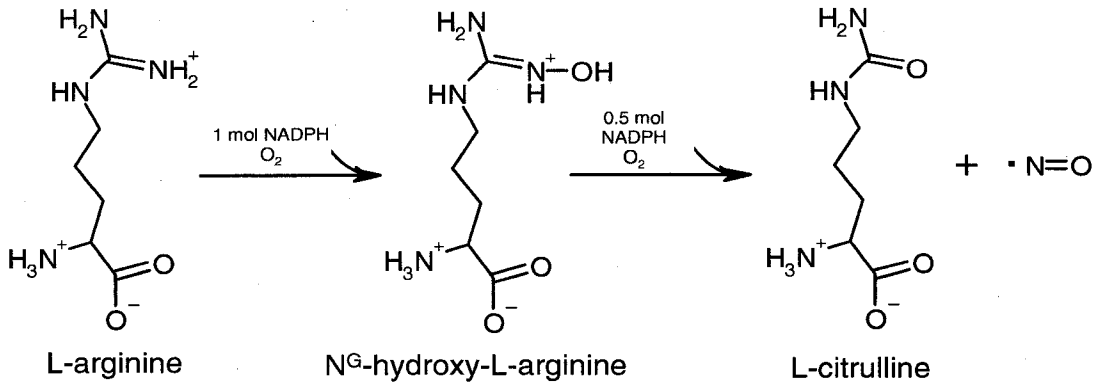


Figure 1.1.1 Generation of •NO from L-arginine

It has since been shown that nitric oxide synthase exists as neuronal (nNOS / NOS1), endothelial (eNOS / NOS3) and inducible (iNOS / NOS2) isoforms related by various prosthetic requirements (tetrahydrobiopterin, flavin mononucleotide, haem, flavin adenine dinucleotide, Zn) and mechanism but distinguished by structure [Li *et al.*, 2005], localization [Dudzinski *et al.*, 2007] and the factors which govern their activity [Govers *et al.*, 2001, Papapetropoulos *et al.*, 1999, Hattori *et al.*, 1994]. Each of the three NOS isoforms bear binding sites for NADPH, FAD, FMN and calmodulin (CaM) and require homodimerization for activity [Kone, *et al.*, 2000]. However, only nNOS and eNOS are allosterically controlled by the Ca^{2+}-dependent association of CaM, and as shown in Figure 1.1.2 below, bear unique consensus sequences in the N-terminal domain (PDZ for nNOS [Kone, *et al.*, 2000] and acylation for eNOS [Kone, *et al.*, 2000]) which are thought to facilitate subcellular localization [Sessa *et al.*, 1993, Garcia-Cardena *et al.*, 1996b, Brenman *et al.*, 1997].

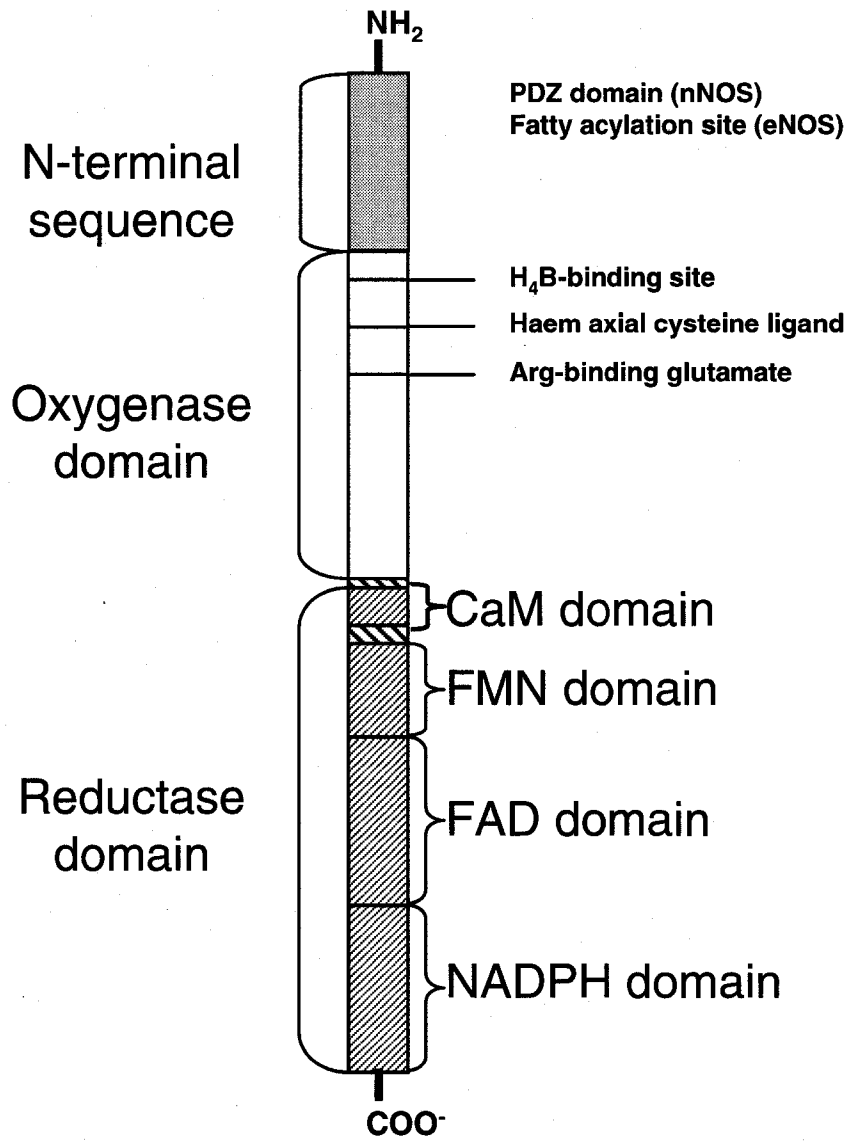


Figure 1.1.2 – Generalized NOS domain structure

Adapted from [Hemmens *et al.,* 1999]

Both nNOS and eNOS are constitutively expressed [Moncada *et al.,* 2006, Bredt *et al.,* 1999], whereas iNOS is inducibly expressed in response to various cytokines produced by lymphocytes upon detection of a pathogen [Stuehr *et al.,* 1991a, Lowenstein *et al.,* 1992].

eNOS and nNOS can be stimulated by increases in intracellular calcium [Fleming *et al.,* 1997] to produce submicromolar quantities of •NO and at this level are thought to interact primarily with metal centres and other radicals evoking normal regulatory mechanisms under physiological conditions. However, the widespread prevalence of *in vivo* protein S-nitrosothiols under normal conditions [Bryan *et al.,* 2004] suggests mechanisms by which either low levels of •NO produced are activated or thiyl radicals are formed to facilitate protein S-nitrosation/nitrosylation. This particular topic will be discussed further at a later point in the thesis.

Alternately, iNOS isoforms, induced by an array of inflammatory cytokines (such as IL-1, IL-6, TNF-α) are capable of generating localized μM levels of •NO and are considered to contribute largely to nitrosative and oxidative stress in pathophysiological scenarios [Bredt *et al.,* 1999].

Notably, studies also indicate that •NO can be formed from both nitrite (NO_2^-) [Lundberg *et al.,* 2005, Huang *et al.,* 2005, Grubina *et al.,* 2007] and nitrate (NO_3^-) [Lundberg *et al.,* 2004] and thus require consideration in any comprehensive understanding of the dynamics of nitrosation / nitrosylation.

1.1.3 Reactivity, regulation and physiological targets

The study of nitric oxide as a signaling molecule presents many challenges to an investigator due to the unique factors that regulate its bioactivity. As a diffusible, paramagnetic free radical with a half-life on the order of seconds [Viinikka *et al.,* 1996], features such as its spatial generation, diffusion characteristics, reactivity with other species and stability of resultant •NO adducts play a critical role in its physiological and pathophysiological activity. In a cellular milieu, the biological activity of •NO is highly dependent upon variations in both the chemical and physical environments encountered during its lifetime. Thus, a thorough consideration of both the chemical reactions in which it participates and its physical properties under an array of potential cellular conditions are key to understanding how this short-lived free radical can convey information *in vivo.*

Herein are described the major aspects of nitric oxide chemistry in a biological milieu with particular emphasis on its regulation, role and physiological targets in the vasculature.

1.1.4 Spatial and temporal regulation

As a short-lived free radical, both the cellular locale of nitric oxide production and the temporal nature of its generation bear significant influence on the range of its biological function and those species with which it is subsequently capable of interacting, thus representing an additional level of regulation of •NO signaling.

Recent reports have indicated that the subcellular localization of various NOS isoforms in cardiac tissue dictate the species with which •NO will interact and hence its

overall effect on cardiac structure and function [Barouch *et al.,* 2002]. Isoform-specific NOS knockouts are reported to exhibit distinct phenotypes affecting Ca^{++} flux at either the sarcoplasmic reticulum (nNOS) or the plasma membrane (eNOS), thus supporting the notion that regulation of •NO-dependent signals is affected by the compartmentalization of •NO-generating systems. Importantly, this study shows that the locale of •NO synthesis contributes to fine tuning of its signals within distinct microdomains of the cell acting as an autocrine rather than paracrine effector. This, of course is ostensibly in conflict with the notion that •NO at physiological levels diffuses widely (ie tens to hundreds of μm) and that diffusion rates are sufficiently fast that, on average, •NO will diffuse out of the cell faster than reacting within [Lancaster *et al.*, 1996]. However, rapid reactions that occur due to colocalization (or even coupling) of •NO generating systems with that of species exhibiting high reactivity toward NO, may restrict its action to subcellular compartments.

In extension, eNOS has been shown to interact with caveolin potently inhibiting the production of nitric oxide [Feron *et al.,* 1996] by binding to the same region as CaM. Upon stimulation with either acetylcholine (Ach) or platelet activating factor (PAF), eNOS was further shown to dissociate from caveolae to different cellular locales, thus determining its distinct activities as either a vasodilatory- or permeability-enhancing moiety [Sanchez *et al.,* 2006].

In a recent publication, the Loscalzo group employed a modified version of the biotin switch assay [Jaffrey *et al.,* 2001] to visualize intracellular protein S-nitrosation [Yang *et al.,* 2005]. Their results indicate that protein S-nitrosation in endothelial cells 1) occurs under the conditions of endogenous •NO synthesis (stimulated by the addition

of calcium ionophore and L-arginine), 2) appears to be localized to mitochondria and peri-mitochondrial space and 3) is diminished by the use of inhibitors of the electron transport chain, superoxide scavengers (Mn(III)TMPyp), N_2O_3 scavengers (azide) and superoxide/peroxynitrite scavengers (FeTmPyp). This study thus implicates both nitrosation and oxidative nitrosylation events in protein S-nitrosation [Yang *et al.,* 2005].

Although these studies collectively support the idea that the cellular locale of •NO generation profoundly influences the subsequent signals it mediates, few studies have addressed the role of lipid composition and hydrophobic compartments in modulating *cellular* •NO signals. Alternately, proteomics studies aimed at identifying motifs which facilitate protein S-nitrosation support the notion that hydrophobic domains may contribute to post-translational modification of protein thiols with •NO [Greco *et al.,* 2006].

1.1.5 Physical properties

As a dynamic, highly mobile entity, much effort has been applied to creating a conceptual framework that describes the spatial movement of •NO [Lancaster *et al.,* 1994, Lancaster *et al.,* 1997]. This framework requires that we consider both the solubility and diffusional movement of •NO in different biological environments to understand the factors influencing its subsequent reactions. In order to inform our understanding of the motion and reactivity of nitric oxide, we will also consider the properties of oxygen given both its importance in auto-oxidation of nitric oxide and similarity in physical properties (molecular weight, paramagnetism, solubility etc.).

1.1.5.1 Solubility

Nitric oxide is known to have a solubility of $1.94 \pm 0.03 \times 10^{-6}$ mol mL^{-1} atm^{-1} at 25°C [Zacharia *et al.*, 2005] in pure water which decreases to $1.75 \pm 0.02 \times 10^{-6}$ mol mL^{-1} atm^{-1} in PBS at the same temperature; however, as a hydrophobic radical, •NO is reported to be 6-9 times more soluble in organic rather than aqueous solvents [Malinski, T., *et al.* 1993, Shaw, A. *et al.* 1997]. Alternately, partition coefficients of 4.4 [Möller *et al.*, 2005] and 200 [Rafikova *et al.*, 2004] have been reported for •NO in lipoproteins and perfluorocarbons, respectively. One notes that the lipophilicity of •NO bears important implications for its ultimate reactivity and signaling properties.

Subczynski has measured the partition coefficient (K_p) of oxygen in dimyristoylphosphatidylcholine (DMPC) vesicles by monitoring the resolution of the super-hyperfine coupling of a spin-labeled probe [Subczynski *et al.*, 1983]. It was found that the lipid environment greatly influences the partition coefficient of oxygen, reducing it from a value of ~3 above the transition temperature of the phospholipid to less than 0.2 below the pre-transition temperature (in the crystalline state), indicating exclusion of oxygen. Importantly, these results corroborate the notion that the physical state of membrane can substantially affect the physical processes of small gaseous radicals.

The partitioning of •NO and O_2 into lipoprotein and phospholipid vesicles was investigated by Möller *et al.* employing an equilibrium shift assay to determine coefficients of K_p = 3.8±0.8 and 4.8±0.5 for •NO in LDL and egg yolk phosphatidylcholine (EYPC) vesicles, respectively [Möller *et al.*, 2005]. Partition coefficients for O_2 were determined similarly and found to be 3.2±0.4 and 4.2±0.4 in LDL and egg yolk phosphatidylcholine (EYPC) vesicles, respectively.

That both nitric oxide and dioxygen partition into hydrophobic environements is of particular importance when one considers the prevalence of lipoproteins and biomembranes in the vasculature. Seminal studies conducted within the Lancaster lab noted the accelerated autooxidation of •NO in hydrophobic environments and the dependence of this 'lens effect' on the aggregate structure of lipid environments [Liu *et al.,* 1998a].

The partitioning of both •NO and O_2 were represented in combined form in order to model and quantitatively describe the acceleration of autooxidation as follows:

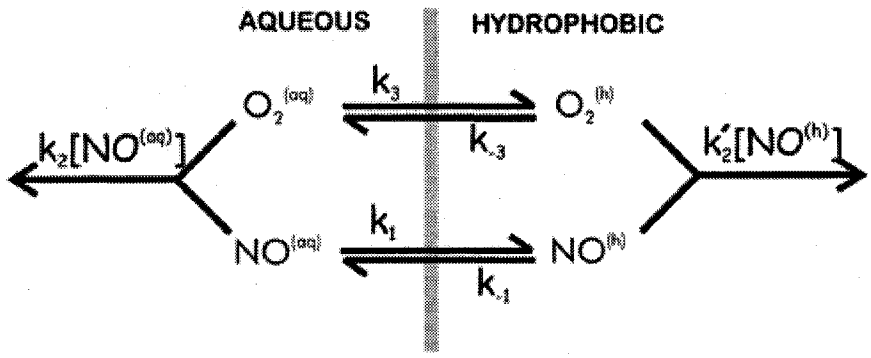


Figure 1.1.3 Two-Compartment model of NO auto-oxidation

An acceleration factor (λ) was thus calculated as rate of reaction between •NO and O_2 in hydrophobic environments using the expression $\lambda = (k_3/k_{-3})(k_1/k_{-1})^2(k_2'/k_2)$ [Liu *et al.,* 1998]. Notably, the authors concluded that ~90% of the autooxidation of •NO would take place in the membrane as opposed to the cytosol. However, despite a 10% decrease in the acceleration factor (taking into consideration hydrophobic volumes ($v_h\lambda_h$)) from synthetic liposomes (322±2) to hepatocyte membranes (291±3), that 'presence of proteins and other components in biological membranes does not appreciably alter the

rate of disappearance away from that predicted only on the basis of the hydrophobic partitioning'. In light of the fact that subtle changes in •NO signaling can substantially alter secondary signals (such as the generation of cGMP), it is precisely this assumption at which we hoped to look more closely.

1.1.5.2 Diffusion

•NO diffusion coefficients have been measured in various lipid environments at 37^0C including synthetic liposomes (1.7 X 10^{-5} cm^2 s^{-1}) [Denicola *et al.,* 1996b], RBC plasma membrane-derived liposomes (0.5 X 10^{-5} cm^2 s^{-1}) [Denicola *et al.,* 1996b], lipoproteins (3.9 x 10^{-6} cm^2 s^{-1}) [Möller *et al.,*2005], (2 x 10^{-5} cm^2 s^{-1}) [Denicola *et al.,* 2002] and in situ in the aorta wall (3.3 X 10^{-5} cm^2 s^{-1}) [Malinski *et al.,* 1993].

Similarly, diffusion coefficients were measured for oxygen and reported as 4.1 X 10^{-5} cm^2 s^{-1} in dipalmitoyl lecithin vesicles, 6.7 X 10^{-5} cm^2 s^{-1} dimyristoyl lecithin vesicles, 4.3 X 10^{-5} cm^2 s^{-1} in 3:1 dimyristoyl lecithin cholesterol vesicles and 3.2 X 10^{-5} cm^2 s^{-1} in erythrocyte plasma membranes [Fischkoff et al., 1975].

Using measured diffusion coefficients, it is thus possible to calculate the transit time for a diffusible species through a defined distance according to the Einstein-Smoluchowski equation [Einstein *et al.*, 1905, Lancaster *et al.,*2000].

(1) $$\langle x^2 \rangle = 2Dt$$

Once generated from a source, its ability to convey messages is dependent upon its movement from that source and its interaction with a target. The movement of a free radical can be expressed as a diffusion constant (D) using Stokes law in which,

(2) $D = kT/4\pi\eta r$ [Lancaster *et al.*, 2000]

where k is the Boltzmann constant, T the absolute temperature, η the viscosity of the medium and r the radius of the diffusing species. Alternately, it has been shown that the inverse relationship between viscosity and the diffusion constant holds only for solute molecules which are large in comparison to solvent [Evans *et al.*, 1981] and thus can be ignored in the case of ·NO (and of O_2).

Fick's first law of diffusion states that the rate of diffusion (or flux) is proportional to the concentration gradient of a diffusing species such that:

(3) $J = -D\,(dC/dx)$ [Porterfield *et al.*, 2001]

where J is the rate of diffusion per unit area of diffusion, C is the concentration, x is the position from a point of generation and D, the diffusion coefficient. The negative sign is needed if we assume the concentration decreases as x increases. A consequence of this is that as •NO is being consumed intracellularly that the flux would increase as a result of an increase in the magnitude of the gradients.

Alternately, as a comparative model authors have invoked steady-state conditions

(in which •NO is stable) to model the spherical diffusion of •NO and estimate the radius it would diffuse by the expression:

(4) $C(r) = C_oR_o/r$ [Porterfield *et al.,* 2001]

where C(r) is the concentration at a radius r, C_o the initial concentration of diffusing species and R_o is a finite radius value. As we see, as r increases, the concentration of the diffusing species goes to zero and $C_r = C_o$ when $R_o = r$.

Although useful for comparison, •NO does not diffuse spherically (ie unidirectionally in all directions) but moves in all directions equally and randomly subject to local thermal fluctuations [Einstein *et al.,* 1905, Lancaster, *et al.,* 1997]. As we know •NO is reactive and disappears in a manner that has been *estimated* by first order decay such that:

(5) $C_t/C_o = \exp^{(-kt)}$ [Porterfield *et al.,* 2001]

where C_t is the concentration of •NO at time t and C_o is the concentration at time zero. Combining this with Einstein's equation in (1) and the equation modeling spherical diffusion of •NO in (4), the authors estimated the flux of •NO at various distances for comparison to measured fluxes of •NO. Authors concluded that •NO flux was reduced by ~50% at ~20 μm from a source of •NO. These results are in the range of those suggested by Lancaster (150 -300 μm) [Lancaster *et al.,* 1994, Lancaster *et al.,* 1997] and suggest that not only will •NO act primarily as a paracrine effector but that it will traverse a number of cellular membranes before reacting.

Studies on the reaction of nitric oxide with erythrocyte-housed haemoglobin have spurred a great deal of debate in an attempt to explain the large disparity in the rates of reaction between •NO and free and RBC-encapsulated haemoglobin [Han *et al.,* 2002]. Several theories have been advanced to explain this including 1) that the erythrocyte membrane poses an intrinsic barrier to •NO diffusion and permeability [Huang *et al.,* 2001, El-Farra *et al.,* 2003, Vaughn *et al.,* 2001, Vaughn *et al.,* 2000], 2) that the unstirred layer surrounding the RBC membrane poses a barrier to diffusion [Liu *et al.,* 1998, Liu *et al.*, 2002b, Azarov *et al.*, 2005], 3) that underlying submembranous cytoskeleton mediates •NO bioavailability [Han *et al.,* 2005, Huang *et al.,* 2001, El-Farra *et al.,* 2003, Vaughn *et al.,* 2000, Huang *et al.,* 2001] or that an oxygen-dependent intrinsic property of the membrane contributes to the barrier effect [Azarov *et al.,* 2005, Huang *et al.,* 2007]. The ongoing debate serves to underscore the complexity of the problem at hand.

1.1.5.3 Permeability

Permeability of a species is determined as the product of the partition coefficient and the diffusion coefficient divided by thickness of the membrane (l) such that $P = (K_pD)/l$ [Dzikovskiet al., 2003, Malinski *et al.,* 1993b]. This permeability term is proportional to spin-relaxation enhancements of a paramagnetic species and thus has been exploited by Subczynski *et al.* to measure •NO permeability through model lipid bilayers by electron paramagnetic resonance (EPR) spectroscopy in one of the only known papers to address this feature of •NO. In this paper, it was concluded that the presence of 30% cholesterol in fluid-phase bilayers comprised of egg yolk phosphatidyl

choline did not pose a permeability barrier to the transmembrane diffusion of NO [Subczynski *et al.,* 1996].

In contrast, earlier studies conducted by Subczynski found that the oxygen permeability coefficients in the presence and absence of 50 mol % cholesterol are 22.7 and 125.2 cm/s, respectively, for dimyristoylphosphatidyl choline ([Myr2]PtdCho) membranes, and 54.7 and 114.2 cm/s, respectively, for dioleoylphosphatidyl choline ([Ole2]PtdCho) membranes. It was thus concluded that in the absence of cholesterol, bilayer membranes did not pose a barrier to transport. However, in the presence of cholesterol that oxygen permeability decreases by a factor of 5 and 2.5 for [Myr2]PtdCho and [Ole2]PtdCho membranes respectively [Subczynski *et al.,* 1989, Subczynski *et al.,* 1991].

By extension, cellular studies have correlated the magnitude of cellular oxygen gradients (difference between extra- and intracellular oxygen concentration) with the levels of PM cholesterol [Khan *et al.*, 2003]. These studies measured intracellular oxygen levels in cells bearing defects in cholesterol metabolism by electron paramagnetic resonance (EPR)-based oximetry and found oxygen gradients could be enhanced by the addition of PM cholesterol or nearly abolished by its removal. It is unknown how this would affect the reaction of nano- to micromolar fluxes of •NO and the resultant •NO based signaling. However, recent reports investigating the mechanism by which cells liberate nitric oxide equivalents from S-nitrosothiols and take up resultant •NO provides evidence which supports the formation of N_2O_3 in the membrane [Ramachandran *et al.,* 2001].

1.1.6 Reaction with radicals

An interesting aspect of nitric oxide chemistry is the relative unreactive nature of •NO to most biological molecules, excluding other free radicals and metal-centres. It has been demonstrated that •NO will not nitrosate low molecular weight thiols such as cysteine and glutathione in the absence of oxygen and must be `activated` in order to participate in *S*-nitrosation reactions; thus, a primary route of •NO `activation` is via reaction with molecular oxygen. The reaction of •NO with oxygen in an aqueous environment is as follows:

(6) $4\ \bullet NO + O_2 + 2H_2O \rightarrow 4NO_2^- + 4H^+$ ($k = 2X\ 10^6\ M^{-2}s^{-1}$ at 25°C)

[Ford *et al.*, 1993]

and the rate of disappearance of •NO has been determined to be second order with respect to [•NO] and third order overall.

This assertion bears several implications; firstly, that this reaction will predominate at higher fluxes of •NO; secondly, that at low oxygen concentrations, oxygen will act to limit the reaction; and thirdly that •NO and O_2, both being hydrophobic gases, will concentrate within the hydrophobic interior of the biological compartments, thus facilitating reaction. Indeed, it has been suggested by Liu *et al.*, that the auto-oxidation of nitric oxide is confined (> 90%) to hydrophobic compartments [Liu *et al.*, 1998a]. This overall reaction is comprised of several intermediate reactions, represented as follows:

(7) $2 \bullet NO + O_2 \rightarrow 2 \bullet NO_2$ [Stamler *et al.*, 1992]

$\bullet NO_2$ is then capable of reacting with another molecule of nitric oxide to form nitrous anhydride or with another molecule of itself to form dinitrogen tetroxide:

(8) $NO + \bullet NO_2 \rightarrow N_2O_3$ ($k = 1.1 \times 10^9 M^{-1}s^{-1}$) [Fukuto, *et al.*, 2000]

(9) $\bullet NO_2 + \bullet NO_2 \rightarrow N_2O_4$ ($k = 4.5 \times 10^8 M^{-1}s^{-1}$)[Fukuto, *et al.*, 2000]

Although it is generally held that m N_2O_4 is not a physiologically relevant species, in light of the low levels of $\bullet NO_2$ at any given time, both N_2O_3 and N_2O_4 are potent nitrosating agents capable of transferring an $\bullet NO$ moiety to nucleophilic species including thiols and amines given by the rate equation:

(10) $rate = k[Nuc][N_2O_3]$

(11) $rate = k[Nuc][N_2O_4]$

However, in any aqueous reaction, both species are subject to hydrolysis (shown in the following reactions) and depending on rates, may compete with nitrosation.

(12) $N_2O_3 + H_2O \rightarrow 2NO_2^- + 2H^+$ ($k = 1 \times 10^3 s^{-1}$)

[Gobert *et al.*, 1999]

(13) $N_2O_4 + H_2O \rightarrow NO_2^- + NO_3^- + 2H^+$ ($k = 1 \times 10^3 s^{-1}$)

[Fukuto, *et al.*, 2000]

N_2O_3 is also formed by the reaction of protonated nitrite in acidic microenvironments (such as peroxisomes and lysosomes). The reaction is essentially a dehydration reaction in which two molecules of nitrous acid lose water and generate the nitrosating species:

(14) $H^+ + NO_2^- \rightarrow HNO_2$ [Fukuto, *et al.*, 2000]

(15) $2HNO_2 \rightarrow N_2O_3 + H_2O$ [Fukuto, *et al.*, 2000]

As indicated, •NO auto-oxidation proceeds via a reaction having second order dependence on nitric oxide. Thus, this reaction would proceed primarily under circumstances of increased localized concentration of nitric oxide. Given the stoichiometric limitations of this reaction, other reaction pathways for the consumption / activation of nitric oxide may play a significant role in its chemical transformation. Importantly, •NO reacts with superoxide at diffusion-limited rates forming peroxynitrite ($OONO^-$):

(16) $\bullet NO + O_2^{\bullet -} \rightarrow OONO^-$ ($k = 6.7 \times 10^9 M^{-1}sec^{-1}$) [Huie, *et al.*, 1999]

The physiological relevance of this reaction is supported by evidence that nitric oxide synthases can, under conditions of arginine scarcity, produce superoxide as well as nitric oxide [Vasquez-Vivar *et al.*, 1998]. Once protonated, peroxynitrite forms peroxynitrous acid which can undergo homolytic decomposition forming hydroxyl radical and nitrogen

dioxide [Merényi *et al.,* 1998], however the likelihood of this reaction has been questioned [Koppenol *et al.,* 1998]. Alternately, protonated peroxynitrite can then decompose via a rearrangement forming nitrate [Chen *et al.,* 1998] but can also decompose to form nitrite with concomitant release of oxygen [Pfeiffer *et al.,* 1997].

In light of the high levels and omnipresence of carbon dioxide (CO_2), the reaction between $OONO^-$ and CO_2 forming nitrosoperoxocarbonate ($OONOCO_2^-$) is thought to be of critical importance:

(17) $OONO^- + CO_2 \rightarrow OONOCO_2^-$ ($k = 5.8 \times 10^4\ M^{-1}\ s^{-1}$)

[Chen *et al.*, 1973]

Nitrosoperoxocarbonate possess a half-life of ~ 1 μs [Merenyi *et al.*, 1997] and decomposes according to two pathways:

(18) $OONOCO_2^- \rightarrow {}^{\bullet}NO_2 + CO_3^{\bullet-}$ [Lymar *et al.*, 1996]

(19) $OONOCO_2^- \rightarrow NO_2^- + CO_3^-$ [Lymar *et al.*, 1996]

Species formed in (18) are both potent, one-electron oxidants ($E^{o}_{\bullet NO2} = 1.04V$) [Stanbury *et al.*, 1989] and $E^{o}_{CO3\bullet-} = 1.78$ V [Bonini *et al.*, 2001]) and are thought to contribute significantly to nitration events.

Nitric oxide is capable of reaction with lipid (L•), lipid-oxo (LO•) and lipid-peroxyl (LOO•) radicals at diffusion-limited rates [O'Donnell *et al.,* 2001]. It is this ability to terminate the propagation of lipid radical reactions that is attributed responsibility for the anti-oxidant potential of nitric oxide [Hayashi *et al.,* 1995, Yates *et al.,* 1992]. Conversely, nitric oxide auto-oxidation products such as nitrogen dioxide and

peroxynitrite are potent oxidizing agents capable of initiating lipid oxidation [Radi *et al.,* 1994].

It is becoming apparent that there are a wide array of nitrated lipid products formed in reactions mentioned above [O'Donnell *et al.,* 1999], some of which have been isolated from *in vivo* sources [Baker *et al.,* 2005]. That these lipids are capable of inducing signaling event similar to that of nitric oxide [Cui *et al.,* 2006] underscores the importance of nitric oxide reactions in lipid environments.

1.1.7 Reaction with thiols

S-nitrosothiols represent a pool of nitric oxide donors that effectively stabilize the relatively short-lived free radical, extending the lifetime of the active •NO species [Tsikas *et al.,* 1999]. Any small molecule or protein that bears a free thiol moiety can, in principle, be S-nitrosated; however, as has been demonstrated, endogenous nitrosation of protein thiols appears more often selective [Greco *et al.,* 2006, Hess *et al.,* 2005]. Formation and degradation of *S*-nitrosothiols is a dynamic process that is influenced largely by the prevailing redox environment, oxygen and metal ion availability and thiol reactivity [Miersch *et al.,* 2005].

Considering the limited direct reaction between •NO and thiols, nitrosothiol formation is generally preceded by reaction with other species and thus an understanding of protein *S*-nitrosation is predicated upon knowledge of the chemistry of nitric oxide and its redox congeners with oxygen, metal ions and reactive oxygen species.

As stated above the reaction of •NO with oxygen generates the potent nitrosating species N_2O_3. The rate constants for the nitrosation of low molecular weight thiols by

N_2O_3 [Keshive *et al.,* 1996] have been determined for reactions generating low molecular *S*-nitrosothiols via the general reaction scheme:

(17) $N_2O_3 + RSH \rightarrow RSNO + NO_2^- + H^+$ [Keshive *et al.,* 1996]

A rate constant of $k = 6.6 \times 10^7 M^{-1}s^{-1}$ was provided for glutathione, however it is expected that protein microenvironments, whether hydrophobic/hydrophilic, acidic/basic would greatly influence the rate of formation of nitrosated protein thiols. Dinitrogen tetroxide is similarly capable of nitrosating thiols (or other nucleophilic species) according to the reaction:

(18) $N_2O_4 + RSH \rightarrow RSNO + NO_3^- + H^+$ [Fukuto, *et al.,* 2000]

In light of the relatively high concentrations of •NO required for auto-oxidation reactions, Gow *et al* have provided evidence suggesting an alternate mechanism that would operate at physiological •NO concentrations. This mechanism, shown below, proceeds through sequential reactions in NO reacts directly with a reduced thiol forming a radical intermediate which donates an electron to an electron acceptor, thus producing nitrosothiols:

(19) $RSH + \bullet NO \rightarrow RSN\bullet\text{-}OH$ [Gow *et al.,* 1997]

(20) $RSN\bullet\text{-}OH + NAD^+ \rightarrow RSN{=}O + NADH$ [Gow *et al.,* 1997]

One implication of this mechanism is that reaction will proceed under anaerobic conditions, however only in the presence of an electron acceptor such as NAD^+. Studies have shown that •NO is capable of interacting directly with thiols under anaerobic conditions in which •NO oxidizes thiols to the corresponding disulfide with concomitant generation of N_2O via a RS-N-OH intermediate as seen in the following reaction:

(21) $2\,RSH + 2\,{\bullet}NO \rightarrow RSSR + N_2O + H_2O$ [Pryor *et al.,* 1982]

The nitric oxide moiety of S-nitrosothiols exchanges readily with other thiols, preferentially over nitrogen or carbon, via nucleophilic attack of the S–NO nitrogen by thiolate anion:

(22) $R_1SH + R_2SNO \rightarrow R_1SNO + R_2SH$ [Barnett *et al.,* 1994]

Thus, reactivity of thiols correlates well with increased acidity of the thiol group [Wang *et al.,* 2001] and would thus have significant effect on the transnitrosation of protein thiols from small molecular weight nitrosothiols, given the influence of protein microenvironment. Spectral studies conducted to investigate the kinetics of transnitrosation between low molecular weight thiols, such as glutathione and cysteine, and SNO donors such as S-nitrosoacetylpenicillamine (SNAP), revealed that rates of transnitrosation were faster than decomposition yielding SNO at physiological pH [Arnelle *et al.,* 1995]. The ease with which transfer of the NO-moiety occurs increases

the likelihood that thiol-based storage pools of nitric oxide are involved in regulation of protein function by S-nitrosation reactions.

Peroxynitrite ($OONO^-$) is also capable of nitrosating thiols by a mechanism that is believed to occur via direct thiol nitrosation coupled to the elimination of HOO^- [van der Vliet *et al.,* 1998] seen below and corroborated by the experimental observation of concomitant peroxide generation:

(23) $GS^- + ONOOH \rightarrow GSNO + HOO^-$

($k_{Cys} = 5.9 \text{ X}10^3 \text{ M}^{-1} \text{ s}^{-1}$, $k_{GSH} = 1.5 \text{ X}10^3 \text{ M}^{-1} \text{ s}^{-1}$ [van der Vliet *et al.*, 1998]

$OONO^-$ also reacts with carbon dioxide [Denicola *et al.*, 1996a] and protein-bound metal centres [Thomson *et al.* 1995, Floris *et al.* 1993] at reaction rates 1-3 orders of magnitude higher than with thiols and thus likely competitors for reaction.

Research efforts have now linked nitrosation of specific proteins to cellular regions in which one would expect to see concomitant generation of superoxide and nitric oxide and thus $OONO^-$. They have shown that treatment with superoxide dismutase mimetics alter the extent of nitrosation [Yang *et al.,* 2005] thus underscoring the role of $OONO^-$ in protein S-nitrosation. Iron-based metal–nitrosyl complexes can nitrosate thiols through a redox reaction in which a ferric–nitrosyl is reduced to its ferrous state with concomitant transfer of nitrosonium to a free thiol:

(24) $Fe_3^+(haem) + \bullet NO \rightarrow Fe_2^+ - (NO^+)(haem) + RSH$

$\rightarrow RSNO + Fe_2^+(haem) + H^+$ [Wade *et al.,* 1990]

S-nitrosothiols are generally considered to be stable compounds when protected from light and in the presence of metal-ion chelators [Singh *et al.*, 1996]. However, photolytic decomposition of the SNO bond is known to occur upon exposure to either 340 or 540 nm wavelength light effecting bond homolysis, thus yielding both thiyl and nitric oxide radicals [Sexton *et al.*, 1994]. S-nitrosothiols are also susceptible to metal-mediated decomposition by mercury [Goldman *et al.*, 1998], iron [Vanin *et al.*, 1997, Vanin *et al.*, 2004] or copper [Singh *et al.*, 1996]. Perhaps the best studied mode of decomposition is the copper-mediated mechanism:

(25) $RSNO + Cu^{+} + H^{+} \rightarrow RSH + \bullet NO \quad (k = 9.4 \pm 2.0 \times 10^{7}\ M^{-1}s^{-1})$ [Burg *et al.*, 2000]

(26) $2RSH + Cu^{2+} \rightarrow RSSR + Cu^{+}$ [Burg *et al.*, 2000]

In the case of copper, both Cu^{2+} and Cu^{+} are both effective in decomposing S-nitrosothiols; however the monovalent cation has been shown to be far more efficient that the bivalent cation providing the basis for a redox-cycling mechanism in which Cu^{2+} can be reduced by physiological reducing agents including GSH and ascorbate, thus accelerating decomposition of RSNOs [Singh *et al.*, 1996]. Computational studies investigating interactions of Cu^{+} to the S and N centres of the SNO bond and found that binding to the S-centre both lengthened (and weakened) the S-N bond and strengthened the NO bond consistent with its role in catalyzing decomposition [Baciu *et al.*, 2005].

1.1.8 Reaction with transition metals

The coordination of •NO by metals forming metal–nitrosyl complexes has been well studied and haemoproteins (guanylate cyclase, haemoglobin, cytochrome c oxidase

and cytochrome P450) constitute some of the best characterized systems. As a metal ligand, •NO is capable of assuming either electrophilic (by donating an electron to the metal) or nucleophilic (by accepting an electron from the metal) character [Collman *et al.*, 1987]. The geometry of the metal-nitrosyl complex can form either bent or linear modes. In general, donation of an electron from •NO to an electropositive centre thus forming NO^+ is associated with a linear bond geometry. In contrast, donation of an electron from the metal centre to nitric oxide forming NO^- is associated with a bent geometry. This geometry / electronic status of metal-bound •NO may be able to predict its subsequent chemistry with either nucleophilic or electrophilic species.

•NO is known to react with oxyhaemoglobin yielding nitrate and methaemoglobin with a second order rate constant of 89 +/- 3 x 10^6 M^{-1} s^{-1} for oxyhaemoglobin according to the reaction:

(25) Hb-Fe(II)-O_2 + •NO → Hb-Fe(III) + NO_3^- [Doyle *et al.*, 1981]

Additionally, •NO is known to react with both met- and deoxy-forms of haemoglobin, as shown:

(26) Hb-Fe(III) + •NO → Hb-Fe(III)-OH_2 + NO_2^- [Eich *et al.*, 1996]

(27) Hb-Fe(II) + •NO → Hb-Fe(II)-NO [Eich *et al.*, 1996]

As indicated, iron-nitrosyl compounds thus formed are thought to be capable of transferring nitrosonium equivalents to available thiols as shown in reaction (24) and,

although contentious, is considered by some to be and important reaction in auto-catalytic S-nitrosation of Cysβ93 in human haemoglobin [Jia *et al.,* 1996, Stamler *et al.*, 1997].

Of equal importance in vascular cells is the metal-mediated reaction of nitric oxide with guanylate cyclase generating the key second messenger – cGMP [Arnold *et al.,* 1977]. Interaction of •NO with guanylate cyclase increases the affinity of the enzyme for its substrate - guanosine triphosphate (GTP) ~3-fold and increases the V_{max} ~ 100 to 200-fold [Stone *et al.,* 1996]. It does so by formation of a metal-nitrosyl complex with the haem-centre, inducing a conformational change in the metalloporphyrin moiety which exposes the catalytic site [Zhao *et al.,* 1998]. Transient increases in cGMP activate cGMP-dependent ion channels, kinases and phosphodiesterases which mediate physiological effects of the •NO signal such as platelet inhibition [Moncada *et al.,* 2006], smooth muscle relaxation [Moncada *et al.,* 2006] and regulation of neurotransmission [Krumenacker *et al.,* 2004] and ion flux [Lee *et al.,* 2001].

Ceruloplasmin, a multi-copper containing oxidase protein found abundantly in plasma, has been shown to efficiently catalyze the S-nitrosation of low molecular weight thiol compounds such as glutathione and N-acetyl cysteine and may thus be important in establishing stabilized nitric oxide pools. Experiments suggest that S-nitrosothiol adducts are formed through reaction between a free thiol and nitrosonium ion generated upon the one electron oxidation of nitric oxide by protein-bound copper [Inoue *et al.,* 1999].

•NO can also react with free transition metal ions of which copper and iron are perhaps the best studied. Experiments have shown rapid reaction of •NO with protein thiols in both bovine serum albumin and human hemoglobin upon oxidation by Cu^{2+} [Stubauer *et al.,* 1999], forming near stoichiometric amounts of protein S–NO in either

oxygenated or deoxygenated solution. This reaction is thought to proceed via a metal-nitrosyl intermediate, rather than via nitrosonium directly in that addition of Cu^{2+} was not found to cause to rapid loss of •NO.

(28) $Cu^{2+} + \bullet NO \rightarrow Cu^{1+}\text{-}NO^{+} + RSH \rightarrow RSNO$ [Stubauer *et al.*, 1999]

1.1.9 Nitric oxide donors

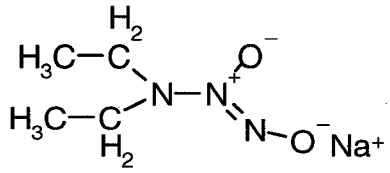
The reactivity of nitric oxide in a biological milieu suggests that there are mechanisms by which it is stabilized *in vivo* to preserve its biological functions. Indeed there exist a number of physiologically stable •NO adducts that can donate authentic •NO or its redox congeners. Practical application of •NO donors as therapeutics in the mitigation of disease and as diagnostics and research tools have spurred the development of a number of synthetic donors including organic nitrites and nitrates [Wang *et al.*, 2002b], sodium nitroprusside [Thatcher *et al.*, 2005], diazeniumdiolates (NONOates) [Megson *et al.*, 2002], S-nitrosothiols [Al-Sa'doni *et al.*, 2000], novel NO-hybrid drugs [Miller *et al.*, 2007] and NO-binding zeolites [Miller *et al.*, 2007] which vary in their physical properties, rates of decomposition and products generated. Although a comprehensive discussion of available •NO donors is beyond the scope of this thesis, a brief discussion of the properties of donors used in subsequent investigations is provided.

1.1.9.1 *S*-nitrosothiols

S-nitrosated thiols functionally extend the lifetime and bioactivity of •NO from time scales of seconds to minutes, hours and perhaps longer depending upon its environment [Hogg *et al.*, 2000]. Physiological *S*-nitrosothiol-based •NO donors are derived from low molecular weight thiols such as glutathione. This S-nitrosated species bears a characteristic SNO absorption band between 330 – 370 nm with ε = 980 L $mol^{-1}cm^{-1}$ [Xian *et al.*, 2000]. •NO (or a redox congener) is subsequently released from these species via the action of thiols [Hu *et al.*, 2006], metals [Hogg *et al.*, 2000] or enzymes capable of cleaving the SNO bond [Sliskovic *et al.*, 2005], evidenced by the loss of absorbance at the absorption maxima. Other nitrosothiols are also easily prepared and useful for investigation including S-nitrosocysteine, S-nitrosohomocysteine and *S*-nitrosopenicillamine, each of which possesses unique decomposition profiles [Grossi *et al.*, 2002] and susceptibility to metal-mediated decomposition [Noble *et al.*, 2000].

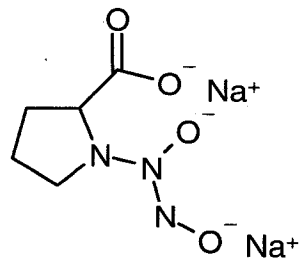
1.1.9.2 Diazeniumdiolates

Another useful class of NO-donors are the diazeniumdiolates (commonly known as NONOates) that bear the general structure of X-[N(O)NO](¯) . Two commonly used examples (Sodium (Z)-1-(N,N-Diethylamino)diazen-1-ium-1,2-diolate (DEA/NO) [Davies *et al.*, 2001] and Disodium 1-[(2-Carboxylato)pyrrolidin-1-yl]diazen-1-ium-1,2-diolate (Proli-NO) [Waterhouse *et al.*, 2006]) are shown below:



DEA/NO

$t_{1/2}$ = 2 minutes



Proli/NO

$t_{1/2}$ = 2 seconds

Figure 1.1.4 Structures of two commonly used diazeniumdiolates

This class of compounds decomposes spontaneously (ie without metabolism or redox activation) by hydrolysis generating •NO, nitrite ion as well as original and N-nitrosated base [Keefer *et al.,* 2005]. These species decompose according to first-order kinetics with half-lives in the range of minutes to hours, dependent upon their structure, thus making very useful tools for the controlled delivery of •NO in the study of nitric oxide signaling.

1.1.10 Use of diaminofluoresceins in the detection of redox activated nitric oxide

The diaminofluoresceins are a class of fluorophores that have been used to detect the products of nitric oxide oxidation products, participating in reactions that result in the formation of the highly fluorescent triazolofluoresceins [Nakatsubo *et al.*, 1998, Kojima *et al.*, 1998]. The triazolofluoresceins can be formed by nitrosative reactions in which a species capable of transferring a nitrosonium moiety (such as N_2O_3) nitrosates an aromatic nitrogen as shown below:

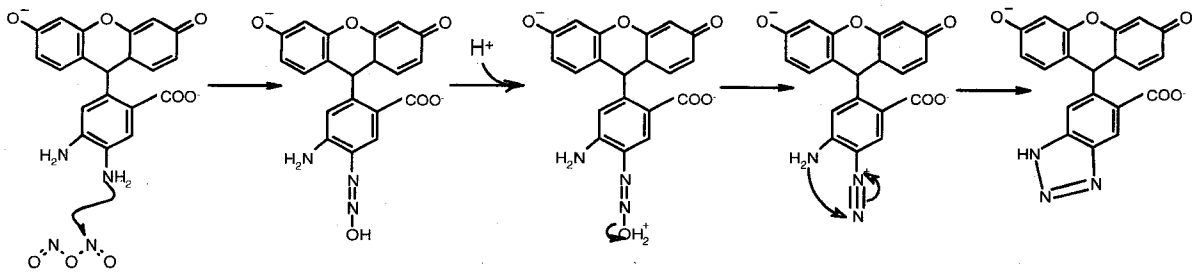


Fig 1.1.5A Nitrosation of diaminofluorescein by N_2O_3

Alternately, triazolofluorescein can be formed by oxidative nitrosylation in which one of the aromatic nitrogens undergoes one-electron oxidation (by species such as $\bullet NO_2$ or $CO_3^{\bullet-}$) followed by reaction with radical nitric oxide as shown below.

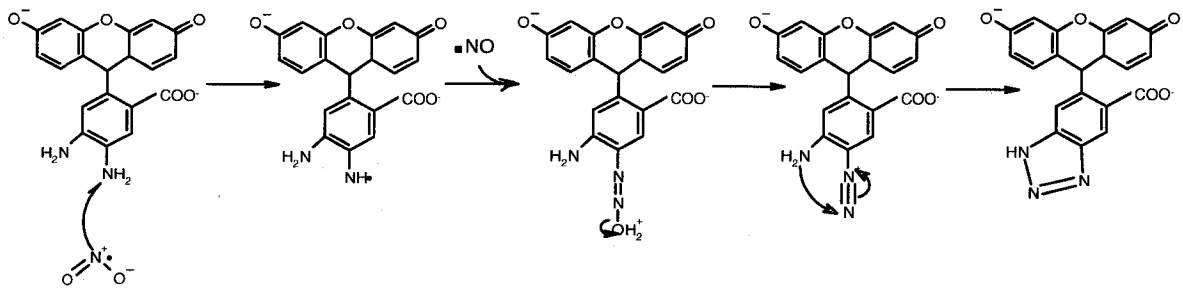


Fig 1.1.5B Oxidative nitrosylation of diaminofluorescein by $\bullet NO_2$

1.1.11 Nitric Oxide Signaling

The paradigm of cellular signaling pathways mediated by •NO and its redox congeners (NO_x) generally envisions transduction of signals through reaction with protein-bound metals, protein thiols and more recently via lipid species [O'donnell *et al.,* 1999]. Through these interactions, reports indicate that NO_x are capable of influencing diverse cellular events that regulate transcription [Ckless *et al.,* 2007], protein-folding [Uehara *et al.,* 2006], contractility [Cartwright *et al.,* 2007], cell death [Mitchell *et al.,* 2005], proliferation [Moreno-López *et al.,* 2004] and many more.

Although the cellular signals transmitted by metal-nitrosyl complexes have been well studied, signals based upon nitrosation have lagged due to methodological hurdles. Recent technological advances have enabled the study of thiol nitrosation and revealed this as another post-translational means of chemically modifying and functionally altering proteins. Changes in protein function, which persist on a physiologically relevant time scale, effectively transmit biological signals and thus provide a framework for elucidating signaling networks.

In order to characterize the relative contribution of nitrosation (addition of NO^+ to nucleophilic species) versus nitrosylation (radical-radical reaction with or coordination of •NO) reactions on a global scale, Feelisch measured S-nitrosation, N-nitrosation and metal–nitrosyl formation in rat plasma, red blood cells, heart, lung, aorta, liver, kidney and brain tissues [Bryan *et al.,* 2004]. Their study demonstrated that nitrosation products were found at levels comparable to nitrosylation products and that the majority of nitrosation products were associated with the protein fraction. The results of their study reveal that protein S-nitrosation is a ubiquitous occurrence that rivals the competing process of metal nitrosylation, despite appearances of being kinetically disfavored.

Although the authors concluded that the prevalence of comparable levels of nitrate and nitrite argues in favour of an oxidative nitrosylation mechanism (by peroxynitrite) rather than nitrosation (by nitrous anhydride), the observation that nitric oxide can be formed by nitrite [Lundberg *et al.,* 2005] complicates interpretation of data.

An alternate explanation for this observation may lie in the observations that hydrophobic environments (cellular membranes, lipoproteins and protein microdomains) promote the 1) auto-oxidation of nitric oxide [Liu *et al.,* 1998a] by sequestering lipophilic

oxygen and nitrogen and 2) nitrosation of protein residues [Nedospasov *et al.,* 2000, Rafikova *et al.,* 2004]. Although our understanding of •NO reactivity in hydrophobic environments has benefited greatly from these insights, the complex behaviour of lipids and their ability to influence cellular physiology warrants closer consideration of how •NO signaling is potentially altered by membrane lipid composition.

1.2 Cholesterol

1.2.1 Introduction

Biochemical processes that contribute to the metabolism of cholesterol in health and disease have occupied the scientific imagination for the last century. From the distant determination of its molecular structure [Crowfoot *et al.,* 1943] to the elucidation of regulatory mechanisms governing cholesterol synthesis and trafficking [Brown *et al.,* 1997], to its more recent role in membrane organization and dynamics [Brown *et al.,* 1998, Simons *et al.,* 2000], modern life sciences continue to probe various aspects of this vitally important sterol and its biosynthetic intermediates. Cholesterol contributes to both human health and the pathogenesis of disease through its role as a structural lipid with substantial influence over the properties of biological membranes and as a precursor molecule for bile acid and steroid hormone synthesis. Intermediates in the biosynthetic pathway of cholesterol also play a pivotal role in human health and disease independent of their role in cholesterol production through their ability to exert effects on a variety of cell-signaling pathways.

This section will provide an overview of various features of cholesterol biosynthesis, cellular homeostasis and transport, as well as its influence on membrane properties and the pathological consequences of its accumulation with emphasis on aspects relevant to the studies within this thesis.

1.2.2 Biosynthesis

Early studies into its biosynthesis revealed that cholesterol is assembled from 2 carbon acetate precursors earning Konrad Bloch the 1964 Nobel Prize in Chemistry. In

the years to follow, it was discovered that the assembly of cholesterol is orchestrated by a complex array of soluble and membrane-bound enzymes and that this process is tightly regulated by transcriptional [Moore *et al.,* 1991, Reynolds *et al.,* 1984] and translational mechanisms [Peffley *et al.,* 1985], feedback inhibition and hormonal regulation [Ness *et al.,* 2000], post-translational modification [Hinson *et al.,* 1997] and regulated degradation of synthetic enzymes [Shimomura *et al.,* 1997].

Under normal circumstances most extra-hepatic tissues derive required cholesterol by *de novo* synthesis from acetyl coenzyme A and acetoacetyl coenzyme A in a series of steps involving various enzymes and intermediates as depicted in Figure 1.2.1. adapted from [Liscum *et al.,* 2001].

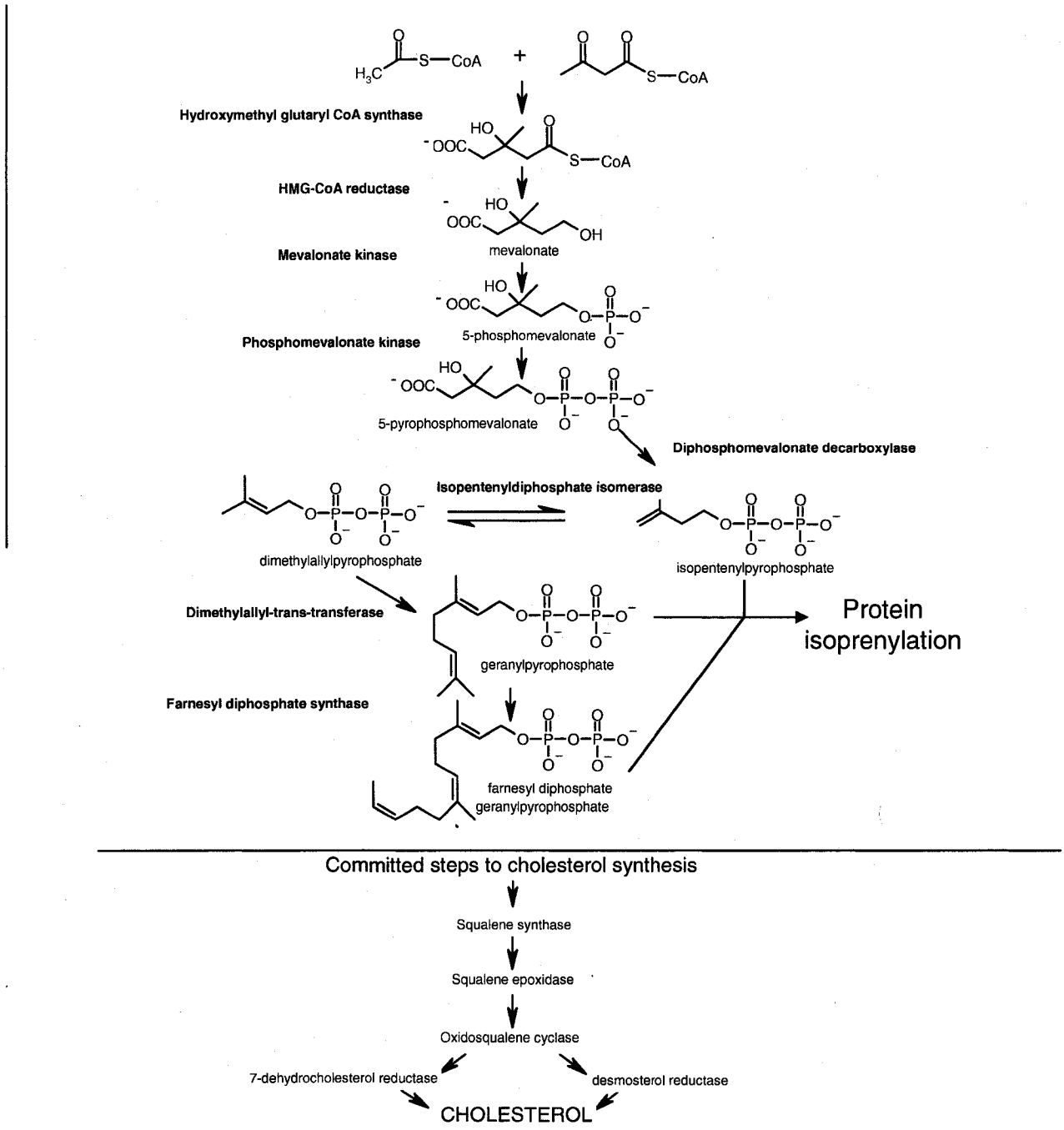


Figure 1.2.1 Biosynthesis of cholesterol

A key step of the pathway is catalyzed by the enzyme 3-hydroxy-3-methylglutaryl Co-A reductase (HMG-CoA reductase) and as the rate limiting step, has been the focus of intense attention from scientists, clinicians and pharmaceutical companies alike. This enzyme is localized as a tetramer to the ER membrane, its C-terminal catalytic domain situated on the cytosolic rather than luminal face of the ER [Liscum *et al., 2001*]. Here, HMG-CoA reductase converts 3-hydroxymethylglutaryl-CoA to mevalonate. In addition to the catalytic domain, HMG-CoA reductase possesses a sterol-sensing domain comprised of membrane-spanning helices 2 to 5, which confer cholesterol-sensitivity (changes in enzyme function sensitive to [cholesterol]) upon the enzyme thus limiting biosynthesis during times of cholesterol availability. It is of interest to note that this domain is similarly possessed by several other key enzymes of the cholesterol biosynthetic and trafficking pathways (including NPC1 [Carstea *et al.,* 1997], sterol responsive element binding protein (SREBP) cleavage activating protein (SCAP) [Hua *et al.,* 1996] and 7-dehydrocholesterol Δ7-reductase [Bae *et al.,* 1999]), suggesting that it has been evolutionarily conserved for multiple cholesterol-dependent functions.

The product of this reaction – mevalonate is then converted via decarboxylation, dehydration and isomerization to isopententenyl-diphosphate, geranyl-diphosphate and ultimately to farnesyl-diphosphate in a series of reactions. These species, in addition to serving as substrates for the subsequent and final committed steps of cholesterol biosynthesis, have also been shown to act as important bioactive intermediates which fulfill critical cellular roles independent of cholesterol synthesis. To illustrate, investigators have shown that complete inhibition of cholesterol synthesis at the rate-limiting step induced cell death [Mosley *et al.,* 1983]. This could not be mitigated by the

addition of exogenous cholesterol alone but required mevalonate as well, thus emphasizing the biological necessity of the subsequently produced intermediates.

It has since been demonstrated that non-sterol isoprenoids can post-translationally modify proteins through isoprenylation (the addition of farnesyl, geranyl or geranylgeranyl moieties). These modifications can facilitate the targeting of modified proteins to biomembranes and other proteins [Resh *et al.,* 2006, Gelb *et al.,* 2006] and are critical components of functional enzymes [Magee *et al.,* 1999]. Notably, isoprenoids represent an important link between the cholesterol biosynthetic machinery and generation of deleterious reactive oxygen species (ROS) by NAD(P)H oxidase in a pathological setting [Bedard *et al.,* 2007]. These areas are functionally related through the isoprenylation of Rac1 (a G-protein involved in the activation of NAD(P)H oxidase), and have thus become key areas in the study of both the molecular basis of signaling and disease, as well as an attractive target for therapeutic intervention. As such, this topic will be discussed in greater detail under the aegis of cholesterol-lowering therapies.

In summary, the biosynthetic pathway of cholesterol not only gives rise to this vitally important sterol but also generates a variety of crucial, non-sterol intermediates bearing important biological roles in human health and disease. Understanding how to modulate this pathway and the consequences of modulation on cellular function is imperative in the development of pharmacological agents that mitigate the effects of cholesterol dysregulation and associated pathologies.

1.2.3 Cholesterol Homeostasis

Cellular cholesterol levels are maintained by a complex system of mechanisms involving synthesis, uptake, storage and efflux [Maxfield *et al.,* 2005]. As indicated above, cholesterol biosynthesis occurs primarily at the ER and within peroxisomes and is exquisitely regulated at a number of points which sense and respond to prevailing cholesterol levels. Despite this fact, ER-cholesterol content is relatively low, estimated to possess only 0.5-1.0% of total cellular cholesterol [Lange *et al.,* 1999] and endogenously synthesized cholesterol must leave the ER to exert many of its biological effects.

Following synthesis, cholesterol rapidly distributes throughout the cell in a non-homogenous manner. Transport occurs against an increasing cholesterol gradient as traverses from the ER to the highly enriched plasma membrane gradually increasing in prevalence from the ER to the *cis* golgi apparatus [Blanchette-Mackie *et al.,* 2000], from the *cis* to the *trans*-golgi compartments and from the *trans* golgi to the plasma membrane. The majority of newly synthesized cholesterol is thought be transported from the ER to the PM by an energy-dependent mechanism [Liscum *et al.,* 1999, Miriam R. Kaplan, Simoni *et al.,* 1985] involving either sterol-carrier protein-2 (SCP-2) [Puglielli *et al.,* 1996] or a caveolin complex which bypasses the golgi apparatus [Uittenbogaard *et al.,* 2000, Smart *et al.,* 1996] with a $t_{1/2}$ of arrival at the plasma membrane of about 10 minutes [DeGrella *et al.,* 1982]. Alternately, a lesser fraction is thought to follow the secretory pathway via the trans-golgi network [Maxfield *et al.,* 2002].

It is estimated that 50-90% of total cellular cholesterol resides in the plasma membrane [Liscum *et al.,* 1999], and that 30-40% of plasma membrane lipids are cholesterol [Maxfield *et al.,* 2002]. The influence of cholesterol on the properties of

cellular membranes is well appreciated in the literature [Pencer *et al.,* 2005, London *et al.,* 2002, Niu *et al.,* 2002, Huster *et al.,* 1998, Subczynski *et al.,* 1989] and will be discussed below.

In addition to *de novo* synthesis, cellular cholesterol is derived from the uptake plasma lipoproteins predominately in the form of low-density lipoprotein (LDL). Lipoproteins are soluble aggregates of protein (apo-lipoprotein) and lipids packaged to facilitate their systemic transport to and from peripheral tissues. They are *de novo* synthesized in the liver and intestines but can also be formed in peripheral tissues by the modification of other lipoproteins or from the lipidation of free apo-lipoproteins. Once formed, lipoproteins are cleared primarily by the liver through receptor-mediated uptake, but can also be catabolized by peripheral tissues.

Lipoproteins contain various lipid classes including glycerolipids, fatty acids, phospholipids, lysophospholipids, sphingomyelin, free cholesterol and cholesterol esters. Lipoproteins are defined on the basis of both their characteristic lipid (triglyceride, cholesterol and phospolipid) content and hence density, as well as by their association with a variety of apo-lipoproteins.

Cells derive exogenous cholesterol primarily from LDL-receptor (LDLR)-mediated internalization of LDL and respond to its uptake by down-regulating the expression of the receptor (LDLR). Conversely, cells deprived of exogenous cholesterol have been reported to increase LDLR expression up to 20-fold [Brown *et al.,* 1981]. LDL taken up by endocytosis is dismantled within the endosomal and lysosomal compartments and resultant free cholesterol subsequently directed towards either the plasma membrane in a protein-dependent mechanism in involving the cholesterol-binding

protein - NPC1 [Wojtanik *et al.,* 2003] or acylated and directed back to the ER [Slotte *et al.,* 1989].

Conversely, excess cellular cholesterol is effluxed from the cell via the process of reverse cholesterol transport, in which HDL binds to the cell surface scavenger receptor-B1 (SRB1) and can act as a cholesterol acceptor, thus facilitating the efflux of cellular cholesterol [Fielding *et al.,* 1995, Ji *et al.,* 1997] and its return to the liver for its excretion as oxysterols. The process of efflux, although known to occur by spontaneous desorption [Rothblat *et al.,* 1992], is also mediated by the membrane-resident - ATP binding cassette 1 (ABC1) transporter protein [Wengen *et al.,* 2000], thus delivering cholesterol to physiological acceptors such as high-density lipoprotein (HDL) or human serum albumin [Ha *et al.,* 2003].

The field of intracellular cholesterol trafficking is a complex, multi-faceted area, the progress of which has lagged behind the understanding of cholesterol metabolism. Here, I have provided only a cursory overview of the various mechanisms responsible for cholesterol transport within the cell. In the next section I will focus exclusively on the area of aberrant intracellular cholesterol trafficking mediated by NPC1 and its cellular consequences.

1.2.4 The Role of NPC1 in cellular cholesterol trafficking and the etiology of disease

In light of the non-homogenous distribution of cholesterol within the cell and the critical role that it plays in membrane properties it comes as no surprise that the cell has evolved a number of mechanisms for sterol trafficking. Although there still exists significant ambiguity in the means by which cholesterol is transported throughout the

cell, studies have provided evidence that this process is mediated by a number of proteins including caveolin, sterol-carrier protein 2 (SCP2), Niemann-Pick Type C1 protein (NPC1) and steroidogenic acute regulatory protein (StAR) [Schroeder *et al.,* 2001].

In particular, the role of NPC1 in cholesterol trafficking has been substantially aided by the study of the genetic disorder that gives rise to molecular defects in the protein. This disorder known as Niemann-Pick disease Type C1 is an autosomal recessive, neurodegenerative condition that results in intracellular lipid accumulation. Discovery of the npc1 gene led to the observation that, like both HMG-CoA reductase and SCAP, NPC1 possessed a sterol-sensing region [Carstea *et al.,* 1997]. Shortly thereafter reports asserted a putative role for NPC1 in cholesterol transport, confirmed the role of the sterol-sensing domain in cholesterol binding [Ohgami *et al.,* 2004] and localized NPC1 to the membrane of late endosomes and lysosomes [Ko *et al.,* 2001].

It was later noted that mutations in the npc1 gene were responsible for the dysfunctional transport of cholesterol and resultant pathology of Niemann-Pick disease which manifests clinically as neonatal jaundice, hepatosplenomegaly, vertical gaze palsy, ataxia, dystonia, and progressive neurodegeneration [Garver *et al.,* 2007ab, Raymond *et al.,* 2004]. As history shows (and the case of Niemann-Pick disease is no exception), the study of a disease whose molecular origins are known provides not only valuable insights into the pathogenesis of disease but also provides clues regarding its function under normal cicrumstances.

NPC1 is expressed in virtually all tissues of the body, however is most highly expressed in the liver. On a molecular level, homozygous npc1 deficient (-/-) mice, exhibit a reported 4-fold accumulation of cholesterol, thought to arise from the

dysfunctional transport of cholesterol from late endosomes/lysosomes to other cellular compartments [Garver *et al.,* 2002]. NPC1, normally localized to endosomal bodies is hypothesized to act in LDL-derived cholesterol trafficking, transporting it from late endosomes to the plasma membrane. Thus NPC1-deficient cells instead accumulate LDL-derived, unesterified cholesterol in the late endosomes and lysosomes [Reid *et al.,* 2004, Liscum *et al.,* 1989] and are thought to exhibit delayed arrival of LDL-derived cholesterol at the plasma in comparison to normal cells [Wojtanik *et al.,* 2003].

Several papers have, despite evidence of diminished cholesterol levels in calveolar structures, reported increased cholesterol / phospholipid ratios and overall cholesterol levels [Vainio *et al.,* 2005, Koike *et al.,* 1998]. This may be due to increased non-protein-mediated sterol transfer or spontaneous desorption from cholesterol-enriched intracellular organelles in close proximity to the plasma membrane [Putney *et al.,* 1999].

Although by no means the only protein which regulates intracellular trafficking of cholesterol and the content of the cellular plasma membrane, NPC1 apparently fulfills a critical function in transport, arrival and maintenance of membrane cholesterol levels and thus membrane properties. As NPC1-deficient cells were used as a model to investigate the role of lipid accumulation in •NO metabolism and signaling, discussion will be limited to only this particular trafficking protein.

1.2.5 Role of cholesterol in membrane properties

In recent years, the early concept of the membrane as a homogenous mixture of lipids and associated proteins, both capable of exhibiting unfettered motion [Singer and Nicholson] has been challenged by various observations [Simons *et al.,* 1997, Zegers *et*

al., 1998]. The model currently under exploration envisions the membrane as an inhomogeneous mixture in which distinct liquid-ordered (l_o) microdomains co-exist in equilibrium with liquid-disordered (l_d) regions. These 'rafts' are hypothesized to act as triage centres facilitating the interaction of signaling proteins which similarly localize to these domains for transduction of cellular signals.

Cholesterol, in particular, has been shown to play a major role in membrane architecture, capable of inducing the formation of l_o domains in model membranes composed of 1:1 or 2:1 ratios of porcine brain phosphatidylcholine to sphingomyelin [Crane *et al.,* 2004]. It is the most abundant sterol in eukaryotic plasma membranes, reported to span 17 Å into one leaf of a 60 Å thick bilayer [Vanderkooi *et al.,* 1994] or to approximately the C9 position along an acyl chain. Studies on the influence of cholesterol on the physical structure of lipid bilayers have revealed interesting observations. Notably, both computational and experimental data suggest that cholesterol induces an increase in the overall thickness of a bilayer [Mason *et al.,* 2003, Pencer *et al.,* 2005] expanding it vertically from an average thickness of 52.5 Å to 58 Å [Tulenko *et al.,* 1998] and concomitantly inducing a decrease in the acyl chain molecular volume [Greenwood *et al.,* 2006, Cournia *et al.,* 2007].

Cholesterol has also been shown to exert substantial influence over the domain architecture of cellular membranes inducing the formation of phase-separated domains [London *et al.,* 2002, Niu *et al.,* 2002, Huster *et al.,* 1998] which exhibit altered physical properties [Pandit *et al.,* 2007, Henriksen *et al.,* 2006] including changes in fluidity [Kusumi *et al.,* 1983] and mobility [Pucadyil *et al.,* 2006], hydrophobicity [Subczynski, *et al.,* 1994], decreased oxygen permeability [Subczynski *et al.,* 1989, Buchwald *et al.,*

2000a, Buchwald *et al.,* 2000b], and further, to alter the activity / localization of integral membrane proteins such as the insulin-receptor [Vanio *et al.,* 2005], Na^+-K^+ ATPase, adenylate cyclase, alkaline phosphatase, rhodopsin and transporters for glucose, organic anions and thymidine [Tabas, *et al.,* 2002]. Importantly, it has been demonstrated that cholesterol-feeding can induce physical changes in the membranes of arterial smooth muscle cells derived from rabbit models of atherosclerosis [Tulenko *et al,* 1998, Chen *et al.,* 1995a], (consistent with observations from model systems) thus representing an extension of insights derived from model systems to *in vivo* systems.

1.2.6 Type II Diabetes, insulin resistance and hypercholesterolemia

The prevalence of diabetes is reaching epidemic proportions in North America rising in prevalence an average of 6.2% per year between the years of 1995 and 2005 in Canada alone [Lipscombe *et al.,* 2007]. Ninety percent of diabetes cases are of the Type 2, characterized by decreasing sensitivity of peripheral tissues to the insulin signal that normally induces the cellular uptake of glucose thus resulting in elevated blood glucose levels. In addition to hyperglycemia, insulin resistance is associated with a host of metabolic disturbances that manifest as hyperinsulinemia [Olefsky *et al.,* 1981], lipid abnormalities [Frohlich *et al.,* 2000, Krentz *et al.,* 2003] (including hypertriglyceridemia decreased HDL levels and increased prevalence of smaller LDL particles), oxidative stress [Penckofer *et al.,* 2002], inflammation [Dandona *et al.,* 2002] and a hypercoaguable or pro-thrombostic state [Frohlich *et al.,* 2000, Krentz *et al.,* 2003, Grant *et al.,* 2007]. Long term complications of poorly controlled diabetes include cardiovascular disorders including atherosclerosis, hypertension and cardiomyopathies,

often resulting in clinical events such as myocardial infarction, congestive heart failure or thromboembolic stroke [Carr *et al.,* 2001].

As a key feature of T2D, the causes and consequences of insulin resistance have been widely investigated. Insulin is a 5700 Da molecular weight peptide hormone synthesized in the β-islet cells of the pancreas as an inactive, single-chain, pre-protein with an N-terminal signal sequence which directs it towards secretory granules. Following removal of the signal sequence and introduction of three disulfide bonds, proinsulin remains stored in granules until blood glucose rises. Spikes in blood glucose then trigger the proteolytic excision of a peptide producing C-peptide and mature insulin for export from β-pancreatic cells into the blood stream where it is estimated to reach high picomolar to low nanomolar levels [Kiyokawa *et al.,* 1989].

Insulin receptor (IR) is an $\alpha_2\beta_2$ heterotetramer expressed in a variety of tissues including liver [Sell *et al.,* 1994], skeletal muscle [Mosthaf *et al.,* 1993], adipocytes [Marshall *et al.,* 1980] and vascular cells [Rakugi *et al.,* 2002] and upon arrival of the insulin signal at target tissue, receptor ligation triggers autophosphorylation of cytofacial Tyr residues in the β-subunit. This is the first step in a phosphorylation cascade propagated from the insulin receptor to various isoforms of insulin receptor substrate (IRS1/2/3/4) [Sykiotis *et al.,* 2001]. The kinase activity of IRS activates phosphatidyl inositol-3-kinase (PI3K) [Asano *et al.,* 2005, Duan *et al.,* 2004] producing (PIP3) and providing the substrate used by PDK-1/2 to phosphorylate Akt/PKB [Persad *et al.,* 2001]. From this point, Akt/PKB acts to modulate cell-specific signaling cascades that govern diverse processes including NO production [Zeng *et al.,* 1996], protein synthesis, glucose

uptake, cholesterol and fatty acid biosynthesis [Porstmann *et al.*, 2005] and cell survival and apoptosis [Persad *et al.*, 2001] as illustrated in Figure 1.2.2 below:

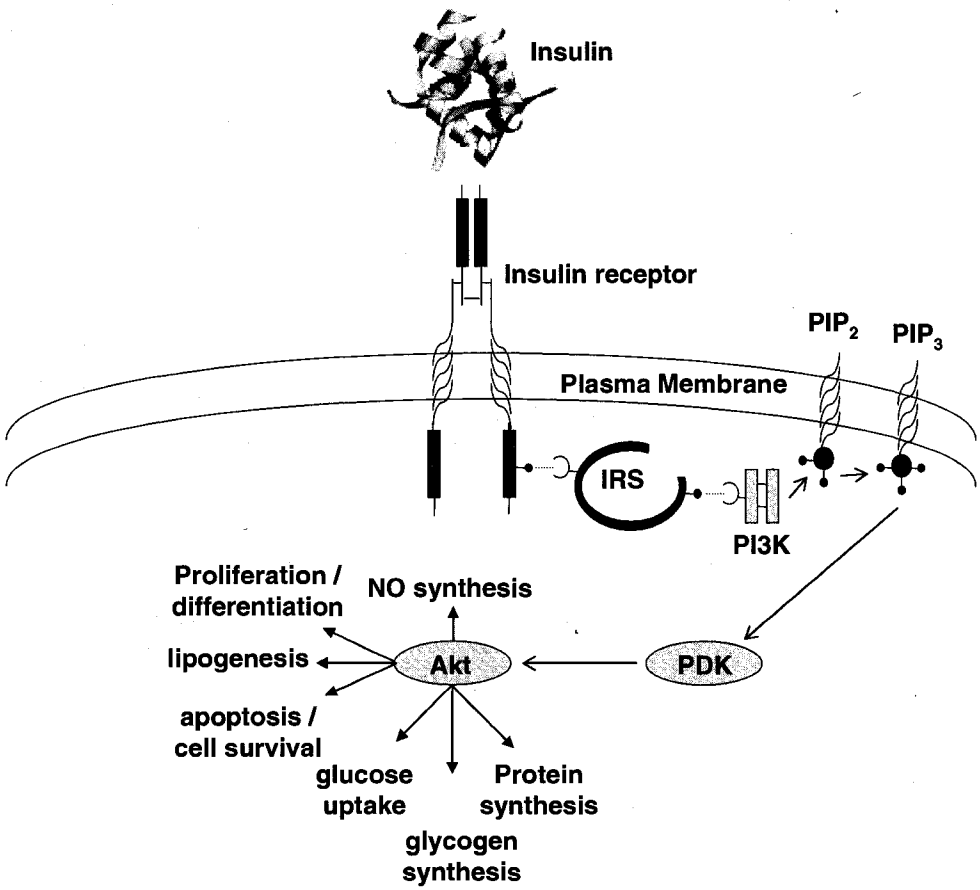


Figure 1.2.2 Role of Akt in insulin signaling events
Image adapted from [Whiteman *et al.*, 2002]

Mechanisms of insulin resistance are thus thought to arise from alterations in receptor expression [Ondinone *et al.*, 1997], phosphorylation state [Draznin *et al.*, 2006] and/or kinase activity [Yasukawa *et al.*, 2005] and can account for many insulin-resistance phenotypes [Pessin *et al.*, 2000]. Notably there are a number of studies which suggest that conditions associated with the diabetic milieu, such as hyperglycemia [Oku,

et al., 2001] and oxidative [Houstis *et al.,* 2006] and nitrosative [Kaneki *et al.,* 2007] stress can contribute to insulin resistance through the aforementioned mechanisms.

Another key feature of T2D is the dysregulation of cholesterol homeostasis which results in altered lipoprotein levels and properties. Normal total cholesterol levels in the vasculature are in the range of ~200 mg/dl (5mM) and it is estimated that 60-70% of total cholesterol is derived from apo-B containing LDL and 20-30% is derived from apo-A containing HDL. Optimum levels are considered to be <100 mg/dL and >50mg/dL for LDL- and for HDL-derived cholesterol respectively [NIH Publication No. 02-5215, 2002].

Hypercholesterolemia is a known contributor to endothelial and platelet dysfunction which is characterized by decreased bioavailability of •NO [Warnholtz *et al.,* 2004], altered expression of cellular adhesion molecules [Sampietro *et al.,* 1997, Utsumi *et al.,* 2007], generation of reactive oxygen [Stokes *et al.,* 2007 J, Stokes *et al.,* 2001, Kitayama *et al.,* 2007] and nitrogen species [Förstermann *et al.,* 2006, Pacher *ert al.,* 2005], and increased secretion of matrix proteins [Fan *et al.,* 2003]. Collectively these changes influence platelet- and leukocyte-endothelial interactions promoting adhesion [Stokes *et al.,* 2001] and thrombogenicity [Lacoste *et al.,* 1995, Stokes *et al.,* 2007, Stokes *et al.,* 2001], tip vasomotor tone toward constriction [Praticò *et al.,* 2005] and spur the development of atherosclerosis [Collin *et al.,* 2007].

Studies have suggested a number of mechanisms by which hypercholesterolemia limits the bioavailability of •NO including alterations in expression of the •NO target - guanylate cyclase [Melichar *et al.,* 2004], cholesterol-induced increases in generation of reactive oxygen species resulting in •NO consumption [Stokes *et al.,* 2002] and resultant

protein-thiol oxidation [Miersch *et al.,* 2007], diminished availability of the eNOS cofactor – tetrahydrobiopterin, [Kawashima *et al.,* 2004] and inhibition of endothelial nitric oxide synthase (eNOS) due to enhanced inhibitory interaction with caveolin [Feron, *et al.,* 1999].

However, in light of the ability of increased cholesterol levels to alter the physical properties of cellular membranes and the generation of reactive oxygen species, primary questions we asked were 1) whether physical changes in the membrane could contribute to diminished bioavailability of •NO and 2) whether redox-altered, cell-mediated liberation of •NO from physiological •NO pools could be restored using cholesterol-lowering drugs by pleiotropic mechanisms.

1.2.7 Therapeutic Options for Treating Hypercholesterolemia

Recent years have seen the development of a number of approaches to lowering cholesterol levels. Of course, restricting dietary intake is an obvious means, however pharmacologic agents provide the clinician with options when altering an individuals diet proves to be ineffective or infeasible. Each of the available agents marketed to lower cholesterol does so by interrupting key points of either sterol uptake or biosynthesis.

For instance, a relatively novel drug known as ezetimibe, is capable of lowering circulating cholesterol levels by 15-20% [Bays *et al.,* 2001, Dujovne *et al.,* 2002] and does so by interacting with Niemann-Pick C1-like 1 protein, believed to mediate cholesterol absorption from brush border intestinal epithelial cells [Altmann *et al.,* 2004, Garcia-Calvo *et al.,* 2005].

Alternately, bile-acid binding resins (such as cholestyramine) are polymeric resins which act to sequester bile acids in the gastrointestinal tract thus preventing reintroduction into enterohepatic circulation [Insull *et al.,* 2006]. They do so by exchanging anions for negatively charged bile acids forming an insoluble complex that is subsequently excreted. Given that bile acids are synthesized from a cholesterol precursor, these agents show efficacy in lowering circulating cholesterol levels with mean reductions in LDL up 20% [Aldridge *et al.,* 2001].

Another class of drugs known as statins (HMG-CoA reductase inhibitors), are perhaps the most widely used hypolipidemic agents on the market today, likely due to both efficacy in lowering plasma cholesterol levels as well as additional beneficial effects not fully explained by their lipid lowering capacity. It is precisely these additional benefits or 'pleiotropic effects' of statins that were the focus of our investigation.

There are a number of statins currently on the market (including lovastatin, pravastatin, rosuvastatin, simvastatin, atorvastatin and fluvastatin). These drugs share the common feature of a HMG-like moiety and a bulky, hydrophobic group (illustrated in Figure 1.2.3) which bind to the HMG-CoA binding site and competitively inhibit enzymatic activity [Istvan *et al.,* 2002] with inhibition constants in the range of 0.1 – 250 nM [Holdgate *et al.,* 2003, Carbonell *et al.,* 2005, Istvan *et al.,* 2002].

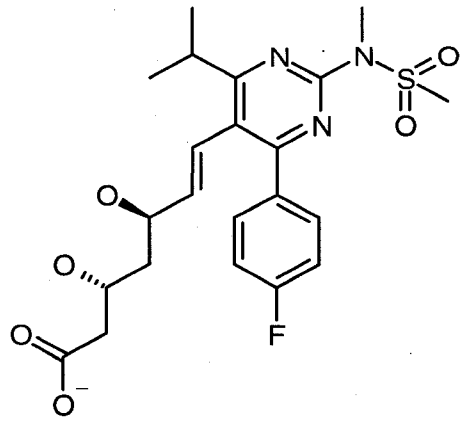


Figure 1.2.3 Structure of rosuvastatin

Although, statins are well-documented to effectively lower plasma [LDL] [Istvan *et al.,* 2002], investigators have noted that the beneficial effects of statins are often noted prior to the observation of the lipid-lowering effects [Tsunekawa *et al.,* 2001, John *et al.,* 2005] and have led to studies on the pleiotropic effects of statins and their ability to mitigate vascular dysfunction.

Studies have shown that lipid-lowering therapies are capable of lowering erythrocyte and platelet membrane cholesterol [Lijnen *et al.,* 1996], improving endothelial dysfunction [Davignon *et al.,* 2004], increasing nitric oxide bioavailability [Feron *et al.,* 2001], exerting antioxidant properties [Aikawa *et al.,* 2002, Thallinger *et al.,* 2005], inhibiting inflammatory responses [Davignon *et al.,* 2004, Ridker *et al.,* 2005], stabilizing atherosclerotic plaques [Fukumoto *et al.,* 2001, Llorente-Cortés *et al.,* 1998] and more recently restoring platelet function [Miersch *et al.,* 2007, Haramaki *et al.,* 2007] Although mechanisms for the variety of observed effects are not completely elucidated, investigations in to the molecular basis for at least one aspect of the pleiotropic effects of statins reveal that isoprenoid intermediates produced subsequent to

the reduction of HMG-CoA are critical to the function of a number of Ras family, G-proteins most of which are isoprenylated, including the small GTPase, subunit of NAD(P)H oxidase – Rac1 [Di-Poï *et al.,* 2001]. Studies have shown that Rac1 isoprenylation is required for its interaction with Rho-guanosine dissociation inhibitor (Rho-GDI) [Grizot *et al.,* 2001] and consequently the ability of the Rac-RhoGDI complex to associate with p67 and activate NAD(P)H oxidase.

NAD(P)H oxidase (NOX) is a membrane-associated, multi-subunit enzyme complex that catalyzes the one-electron reduction of molecular oxygen forming the more reactive oxygen species – superoxide ($O_2^{\bullet -}$). In phagocytes, catalysis is accomplished by a membrane-resident heterodimer comprised of p22phox and gp91phox which together form the α- and β- subunits of the catalytic cytochrome b_{558}. The catalytic activity is then regulated by a host of cytosolic proteins including p47 (organizer protein), p67 (activator protein) and small GTPases - rap1 [Quinn *et al.,* 1989] and rac1 [Miyano *et al.,* 2007] which are thought to be required for membrane localization and formation of a functional NAD(P)H complex [Miyano *et al.,* 2007] as shown in Figure 1.2.4.

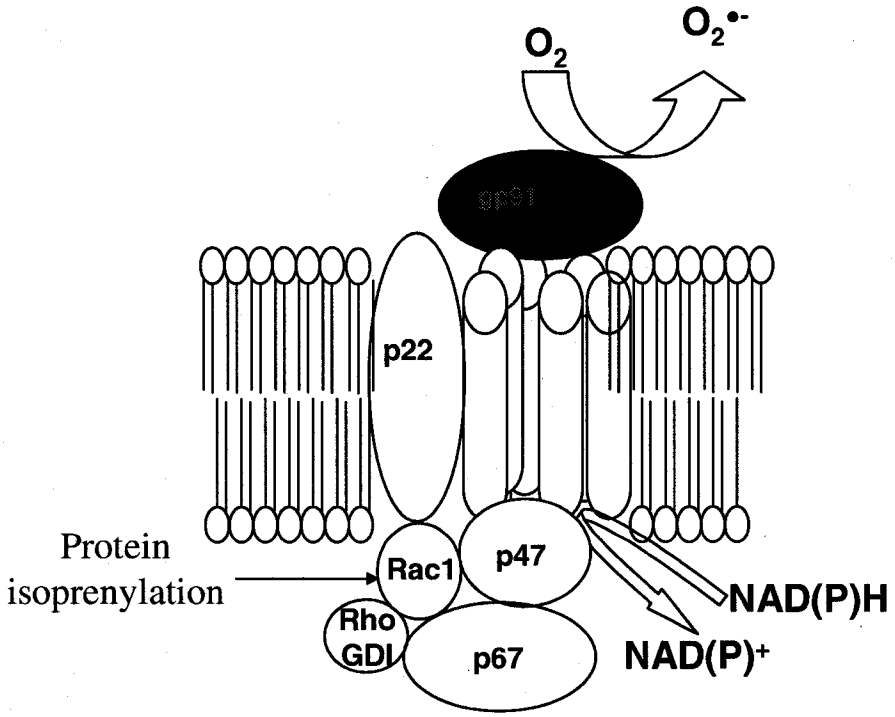


Figure 1.2.4 Quaternary structure of a functional NAD(P)H oxidase complex

Importantly, NAD(P)H oxidase is present in non-phagocytic cells including both endothelial cells [Li *et al.,* 2004] and platelets [Plumb *et al.,* 2005] in which it is reported to be induced by a hypercholesterolemic milieu [Ohara *et al.,* 1993, Stokes *et al.,* 2007, Sanguigni *et al.,* 2002].

In light of these findings and that HMG-CoA reductase inhibitors would likely limit the supply of isoprenoids critical for the formation of a functional oxidase complex, we hypothesized that the increase in reactive species produced by NAD(P)H oxidase in platelets and endothelial cells would alter various aspects of platelet function toward a pro-thrombogenic state and could be remediated by statin treatment.

1.3 Protein Disulfide Isomerase

1.3.1 Overview

Protein disulfide isomerase is a ubiquitous, 58kDa soluble protein, localized to the lumen of the endoplasmic reticulum [Luz *et al.,* 1996] where it assists in the oxidative folding of nascent proteins and the attainment of native disulfide bonding patterns [Gruber *et al.,* 2006] and stress responses [Uehara *et al.,* 2006, Azfer *et al.,* 2006]. In addition to its role in protein folding, it is also a subunit of enzyme complexes including prolyl 4-hydroxylase [Koivunen *et al.,* 2005] and the microsomal triglyceride transfer protein [Levy *et al,* 2002].

PDI has been the focus of intense study in light of both its abundance (~0.4% of total cellular protein [Noiva *et al.,* 1992]) and prevalence in nearly every tissue. Although it was long considered a strictly ER-resident protein localized by way of its C-terminal KDEL retention signal [Cabrera *et al.,* 2003, Luz J *et al.,* 1996], it has since come to light that PDI can also be found in several non-ER locations [Essex *et al.,* 1995, Goplen *et al.,* 2006, Akagi *et al.,* 1988] and in particular on the exofacial surface of a wide variety of cells [Turano *et al.,* 2002]. This observation suggests that PDI may possess different physiological functions in the milieu of the cell surface and was thus both the impetus and a primary focus of the experiments within this thesis.

1.3.2 Domain structure and catalytic activity

PDI possesses four thioredoxin (Tr_x) folds with an overall domain structure of **a-b-b'-a'-c.** For the last ten years, solution NMR structures have been available for isolated a and b domains of recombinant human PDI [Kemmink *et al.,* 1996, Kemmink

et al., 1997], a complete crystal structure of the yeast homologue of PDI has now been determined [Tian *et al.,* 2006] and has shed additional light on the structure-function relationship of this enzyme. X-ray structures reveal that, as predicted, each of the a and a' domains bear the structure of the canonical thioredoxin fold with a repeating β-α-β motif of the sequence- βαβαβαββα. Active site thiols are found toward the N-terminus of the second alpha helix in the a and a' domains arranged in a thioredoxin consensus sequence - CXXC, capable of catalyzing thiol oxidation, reduction and isomerization reactions by way of the redox active cysteines. In contrast, the thioredoxin modules of the b and b' domains possess atypical structural features [Tian *et al.,* 2006] and are catalytically inactive [Kemmink *et al.,* 1997].

Acquisition of the crystal structure also demonstrates that PDI has an overall 'U-shaped' structure in which a fold occurs between the b and b' domains such that the a and a' domains and the enzyme active sites oppose one another separated by a cleft [Fig. 1.3.1] and thus lending credence to evidence of co-operative activity between the two active sites [Sliskovic *et al.,* 2005, Hawkins *et al.,* 1991].

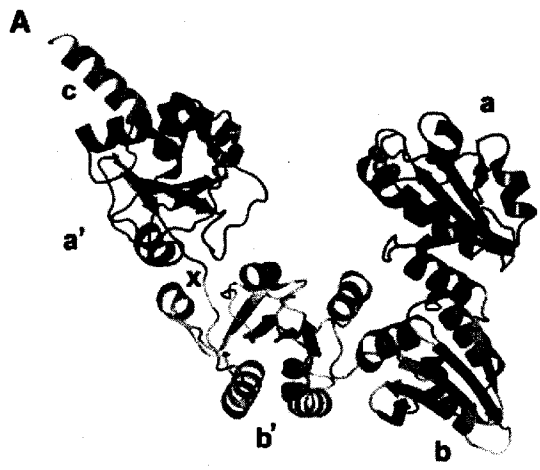


Figure 1.3.1 Crystallographic structure of yeast PDI
Permission to use image acquired from corresponding author [Tian *et al.,* 2006].

As stated, the a and a' domains both possess catalytically active vicinal thiols within the general Cys-XX-Cys motif. In eukaryotic sources the intervening residues are glycine and histidine, the latter of which is reported to stabilize the thiolate anion of the adjacent cysteine residue. It is thus attributed responsibility for the anomalously low Cys pKa of 4.6 [Lambert *et al.,* 1983] which is thought to confer redox activity to the active site.

Studies on the role of the b and b' domains in protein function support a role in substrate binding for which the b' domain is considered to be the primary binding site [Klappa *et al.,* 1998, Pirneskoski *et al.,* 2004, Tian *et al.,* 2006]. Structural studies draw attention to the significant hydrophobic character along the interior of the U-shaped fold within the b and b' domains. These patches appear to be contiguous with similar patches in the a and a' domains are thought to provide an optimal surface to accommodate and bind substrates [Tian *et al.,* 2006]. Experimental studies further suggest that multiple

domains (a, b' and a') contribute to its association with other proteins as a subunit of a functional enzyme [Koivunen *et al.,* 2005].

The c domain possesses both the KDEL-ER retention sequence and a low-affinity, high-capacity Ca^{2+}-binding site which is reported to affect neither its chaperone nor its isomerase activity [Koivunen *et al.,* 1999] and whose role in enzyme function is unknown [Macer *et al.,* 1988], but may be involved in interactions with other proteins or peptides [Noiva *et al.,* 1993, Klappa *et al.*, 1998].

1.3.3 Redox dependence of PDI catalytic activity

As stated, PDI bears two active sites, one within each of the a and a' domains. Studies on PDI redox properties show that the reduction potential of PDI active sites (-175 mV) [Lundström *et al.,* 1993] are more oxidizing than those of human thioredoxin (-270 mV) [Aslund *et al.,* 1997], but less oxidizing than those of the prokaryotic homologue of PDI – Dsb (-89 mV) [Wunderlich *et al.,* 1993] and that the redox status of the active site is maintained by a redox buffer such as glutathione and its disulfide [Walker *et al.,* 1996]. This is an important property of PDI to be considered in assessing its function at the surface of vascular cells.

PDI is capable of catalyzing reduction, oxidation and isomerization as shown in Fig. 1.3.2 depending upon the oxidation state of its active site thiols [Wilkinson *et al.,* 2004].

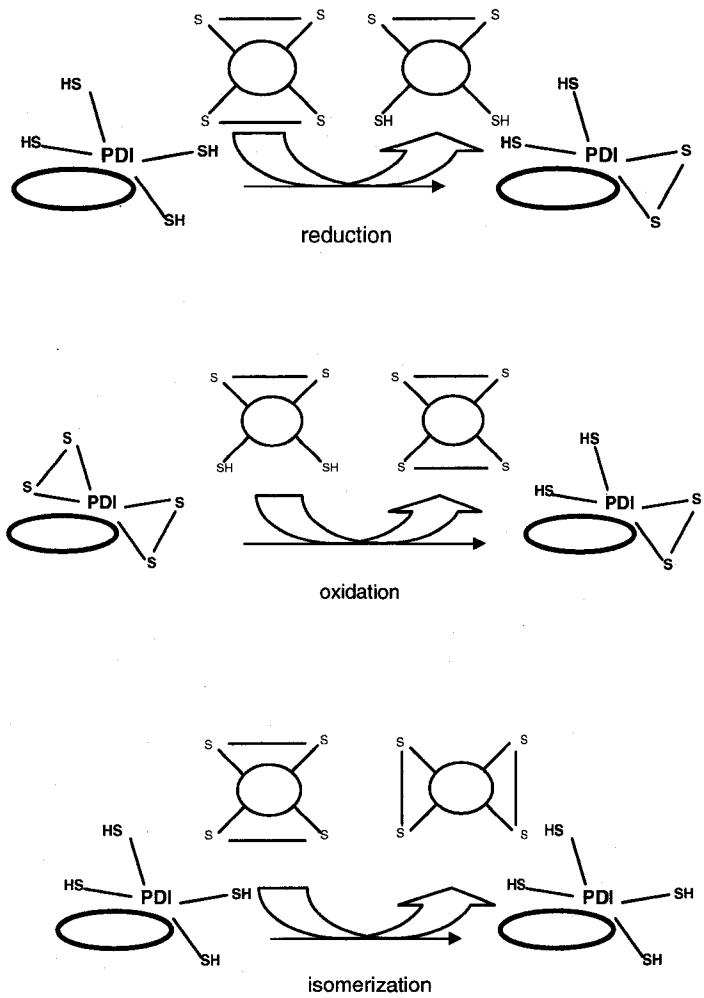


Figure 1.3.2 Redox-dependent activities of protein disulfide isomerase

Mechanistically, PDI is thought to initiate nucleophilic attack of a substrate via its thiolate anion, forming a transient mixed disulfide. Reduced substrate is then released by formation of the disulfide with the adjacent active-site thiol.

The redox state of PDI is generally maintained by the prevailing [GSH]/[GSSG] of the compartment in which it is found [Raturi *et al.,* 2007, Hawkins *et al.,* 1991], but can also be reduced by thioredoxin reductase [Lundström *et al.,* 1990] and perhaps by

other enzymatic systems. In the ER the [GSH]/[GSSG] is thought to be ~3:1 thus maintaining PDI a partially oxidized state and is further thought to be oxidized to it disulfide form by its natural oxidizing agent - Ero1p; a flavoenzyme Ero1p that produces disulfide bonds for oxidative protein folding in the endoplasmic reticulum [Kulp *et al.*, 2006, Gross *et al.*, 2004]. In contrast, cell surface PDI in the vasculature may be maintained by both the cysteine and glutathione couples [Go *et al.*, 2005] and also possibly by a membrane-bound oxidoreductase system that reduces the active site thiols via the transfer of intracellular reducing equivalents [Berridge *et al.*, 2000, Essex *et al.*, 2004].

PDI active site thiols have also been shown to be oxidized by auto-oxidation [Kozarova *et al.*, 2007], reactive oxygen species such as H_2O_2 [Kim *et al.*, 2000, Miersch *et al.*, 2007], lipid peroxidation products [Carbone *et al.*, 2005] and of course, by oxidized glutathione [Raturi *et al.*, 2007].

Studies on the isomerization activity of PDI show that substitution of the N-terminal cysteines almost completely abrogates enzyme activity [Walker *et al.*, 1996], suggesting that the initial step in catalysis is blocked. Alternately, enzyme possessing serine substituted for the C-terminal cysteine may maintain up to 50% of its isomerase activity but show less than 5% of its oxidase activity [Walker *et al.*, 1996]. This suggests 1) the C-terminal cysteine provides an escape mechanism that is not absolutely essential and 2) both cysteines are required for oxidation likely by directly oxidizing substrate.

1.3.4 PDI Denitrosation Activity

An interesting aspect of PDI function is its ability, like that of thioredoxin [Stoyanovsky *et al.,* 2005, Sengupta *et al.,* 2007], to catalyze the reduction of nitrosated thiols [Sliskovic *et al.,* 2005].

Although both PDI and thioredoxin require a reduced active site to elicit denitrosation, the NO_x species released as the product of reaction differ between the two enzymes. Recent characterization of the denitrosation activity of the individual enzymes have shown that thioredoxin appears to release nitroxyl (NO^-) as the product of its reaction [Stoyanovsky *et al.,* 2005], whereas PDI was shown to release authentic •NO [Sliskovic *et al.,* 2005].

Mechanistic studies on the catalysis of denitrosation by PDI further suggest that catalytic denitrosation reactions are initiated by nucleophilic attack of the free thiolate ion on the •NO moiety of the nitrosothiol resulting in transnitrosation of enzyme thiols, formation of an enzyme nitroxyl disulfide intermediate and expulsion of nitric oxide which leaves the active site in an oxidized state [Sliskovic *et al.,* 2005] as shown in Fig 1.3.3. In an alternate possibility the radical disulfide formed in the a' domain could either donate or accept an electron from •NO yielding either 1) nitroxyl (NO^-) whose formation is highly unfavourable [Bartberger *et al.* 2002] or 2) nitrosonium (NO^+) which could presumably renitrosate formed thiols. Neverltheless, the demonstation that PDI denitrosation activity yields radical •NO is an important consideration when evaluating the role of cell surface PDI and the downstream targets of liberated nitric oxide redox congeners.

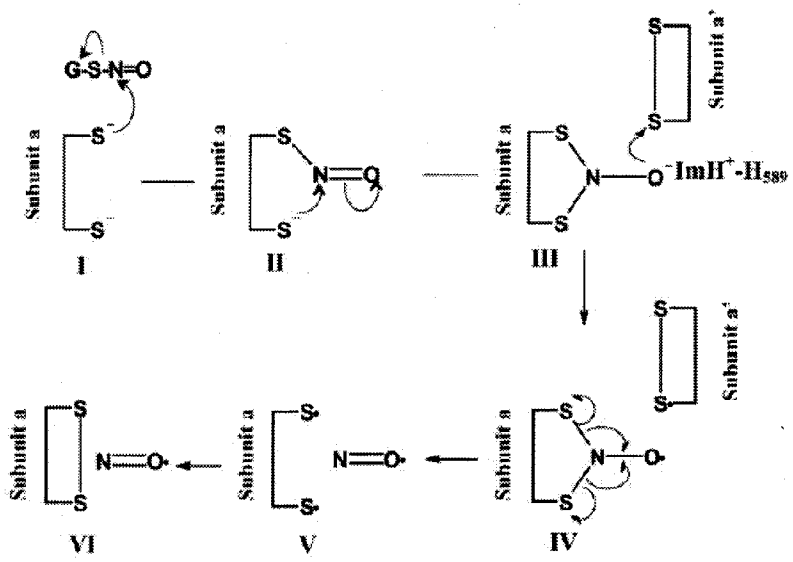


Figure 1.3.3 Proposed catalytic mechanism for PDI-mediated denitrosation activity
Permission to use image acquired from corresponding author [Sliskovic *et al.,* 2005].

1.3.5 PDI Chaperone Activity

In addition to its ability to assist in forming the native disulfide bonding patterns in nascent proteins and in liberating •NO from nitrosothiols, PDI also possesses chaperone activity which is independent of it catalytic activities. Notably, PDI mutants with no isomerase activity retain full chaperone activity, which is thought to be maintained by the peptide binding site of PDI [Wang *et al.,* 1998]. Thus, PDI interacts with incompletely- or unfolded proteins which expose unstable hydrophobic stretches to the aqueous environment, which would otherwise aggregate with similarly mis-folded proteins, thus stabilizing them until refolding takes place. This activity appears to be important in cell surface-localized PDI as well [Versteeg *et al.,* 2007].

1.3.6 Identified roles of cell surface PDI

PDI has been found on the exofacial surface of a variety of cells including NG108-15cells [Xiao *et al.,* 1999], pancreatic exocrine cells [Yoshimori *et al.*, 1990], lymphoid cells [Tager *et al.,* 1997, Park I et al., 2006, Barbouche et al., 2005], endothelial cells [Hotchkiss *et al.*, 1998], hepatocytes [Terada K *ert al.,* 1995] and blood platelets [Essex *et al.,* 1995].

A number of roles for cell-surface PDI have been identified including invasiveness of gliomas [Goplen *et al.,* 2006], transnitrosation of metallothionein in endothelial cells [Zhang *et al.,* 2006], promotion of HIV entry into lymphocytes [Fenouillet *et al.,* 2001], regulation of leukocyte adhesion [Bennett *et al.,* 2000] and in redox switching of tissue factor [Ahamed *et al.,* 2006].

It has further been demonstrated that cell surface PDI also plays a role in catalyzing the transfer of NO from exogenous S-nitrosothiols to the intracellular space [Zai *et al.,* 1999]. These results were subsequently confirmed and extended by showing that the uptake of NO_x from extracellular NO nitrosothiols correlated to the levels of PDI in HT1080 fibrosarcoma cells in which PDI had been over- and under-expressed. Importantly, pre-treatment of the cells with lipophilic antioxidant - tocopherol inhibited the uptake of NO_x suggesting the accumulation of N_2O_3 in the membrane [Ramachandran *et al.,* 2001, Zai *et al.,* 1999]. Studies have shown that incubation of cells with mM concentrations of the lipophilic antioxidant - α-tocopherol, results in the accumulation of mM concentrations within cellular membrane compartment [Jiang *et al.*, 2000]. At this concentration, tocopherol would likely act to quench potent oxidants such as N_2O_3 in the membrane space.

A similar role for cell surface PDI has now been demonstrated in platelets in which it catalyzes not only the known pro-aggregatory, -secretory and -adhesive reactions in platelets but also an anti-platelet denitrosation activity [Root *et al.,* 2004, Bell *et al.,* 2007, Shah *et al.,* 2007] and will be discussed further in the next section.

1.4 Platelets

1.4.1 Introduction

Although the role of platelets in the arrest of bleeding was first noted in the late 1800's [Bizzozero *et al.,* 1882], interest in their haemostatic function proliferated only in the mid-1950s. The study of platelets has since become a multi-pronged investigation revealing the complex signaling pathways which govern the activation, adhesion, secretion and morphological changes of platelets in both normal vascular response to injury and in the etiology of thrombotic disorders.

Platelets are small, anucleate, cellular structures less than 1 μm in diameter, derived from progenitor megakaryocytes via the process of thrombopoiesis. These cells circulate in the vasculature as discoid cells, numbering ~ 300, 000 / μL for 8 – 10 days before being transported to the spleen where they are dismantled via resident phagocytes.

The primary role of the platelet is in haemostasis, where the activation of platelets by humoral effectors or exposure to subendothelial proteins results in the execution of an elaborate array of signals culminating in morphological changes that promote adhesion to sites of vascular injury. Tethered platelets secrete vasoconstrictors and protein clotting factors to stem localized blood flow and provide materials that act as the scaffolding for construction of the platelet plug. Alternately, platelet dysfunction has been found to contribute to various pathological conditions which manifest as aberrant adhesion of platelets to the vessel wall, release of pro-inflammatory substances and thrombotic disorders resulting in death. Expectedly, contributing factors to mechanisms of platelet dysfunction have been the subject of intense research. This section will introduce the basics of platelet aggregation focusing on the link between thrombin-induced stimulation

of platelets and the role of thiol isomerases in aggregation. It will further discuss the pathological consequences of hypercholesterolemia and oxidative stress in platelet physiology.

1.4.2 Platelet structure and function

Although platelets were at one time considered to be mere artifacts formed during collection, and although lacking certain aspects of their progenitor cell, they have since been shown to possess specific internal architecture, an active metabolism and various specialized functions.

Platelets have a unique structure defined as an open-canalicular system [Escolar *et al.,* 1991], accessible to the surrounding aqueous environment which serves to increase their overall surface area facilitating exchange of materials and discharge of internal vesicles. Platelets contain a variety of intracellular, membrane-bound vesicles including α granules and dense granules whose contents are released during platelet activation at sites of vessel wall injury and thus play an important role in haemostasis and wound repair, as well an inflammation and thrombogenicity [Harrison *et al.,* 1993].

Upon receipt of the appropriate signal a series of signaling events trigger and elicit drastic morphological changes, mediated by platelet contractile proteins including actin [Fox *et al.,* 2001] and thrombasthenin which convert the platelet from a discoid- to a burr-like shape in which numerous extended pseudopods are apparent [Mattson *et al.,* 1985]. Morphological changes are accompanied by vesicular degranulation [Polasek *et al.,* 2004], display and activation of membrane receptors [Jurk *et al.,* 2005], shedding of platelet microparticles [Heijnen *et al.,* 1999, *et al.,* 2007] and metabolic changes [Wee

et al., 2006] which mediate vascular adhesion and platelet aggregation. Although these functions are critical to normal haemostasis, pathological changes in the vasculature are known to contribute to increased *in vivo* activation of platelets and shedding of pro-thrombotic particles [Martínez *et al.,* 2005]. The net result is a tipping of the balance between readiness and aggregation toward a pro-thrombogenic stance [Bigalke *et al.,* 2007, Cabeza *et al.,* 2004] which favours adhesion to and infiltration of the vessel wall and enhances interaction with other platelets.

1.4.3 Platelet agonists

Platelets are capable of responding to a variety of physiological agonists including thrombin (and other proteases), collagen, adenosine diphosphate, thromboxane A_2, arachidonic acid, cathepsin G [Selak *et al.,* 1988], antibody-antigen complexes, Ca^{2+}, as well as non-physiological agonists including the calcium ionophore (A23187) [Geroge *et al.,* 1985], convulxin [Jandrot-Perruset *et al.,* 1998] and latex particles [Miyamoto *et al.,* 1989]. In light of the fact that thrombin was throughout our studies, discussion of agonist-induced activation of platelets will be restricted this protease.

1.4.4 Thrombin-induced signaling and aggregation

Thrombin is a 36 kDa serine protease formed from a 72 kDa pro-thrombin zymogen following several reactions which occur upon interaction of membrane-bound tissue factor with circulating Factor Xa and its cofactor Va [Pattersonet *et al.,* 2001]. Proteolytically active thrombin subsequently acts to cleave between Arg 41 and Ser 42 of protease-activated receptor 1 (PAR1) or PAR4 in humans releasing a 6 aa peptide

(SFLLRN) and exposing a new N-terminus [Coughlin *et al.*, 2000]. This newly exposed N-terminus exhibits intramolecular affinity for the exofacial body of the receptor and upon binding triggering conformational changes that promote coupling with and activation of $G_{12/13}$, G_q, or G_i G-protein-coupled receptors (GPCR). GPCRs consequently route a multitude of cellular signals that elicit shape change through phospholipase C-mediated increases in calcium flux [Hubbard *et al.*, 2006] including integrin activation, metabolic responses, and secretions [Coughline *et al.*, 2000, Shankar *et al.*, 2006] as summarized in Figure 1.4.1.

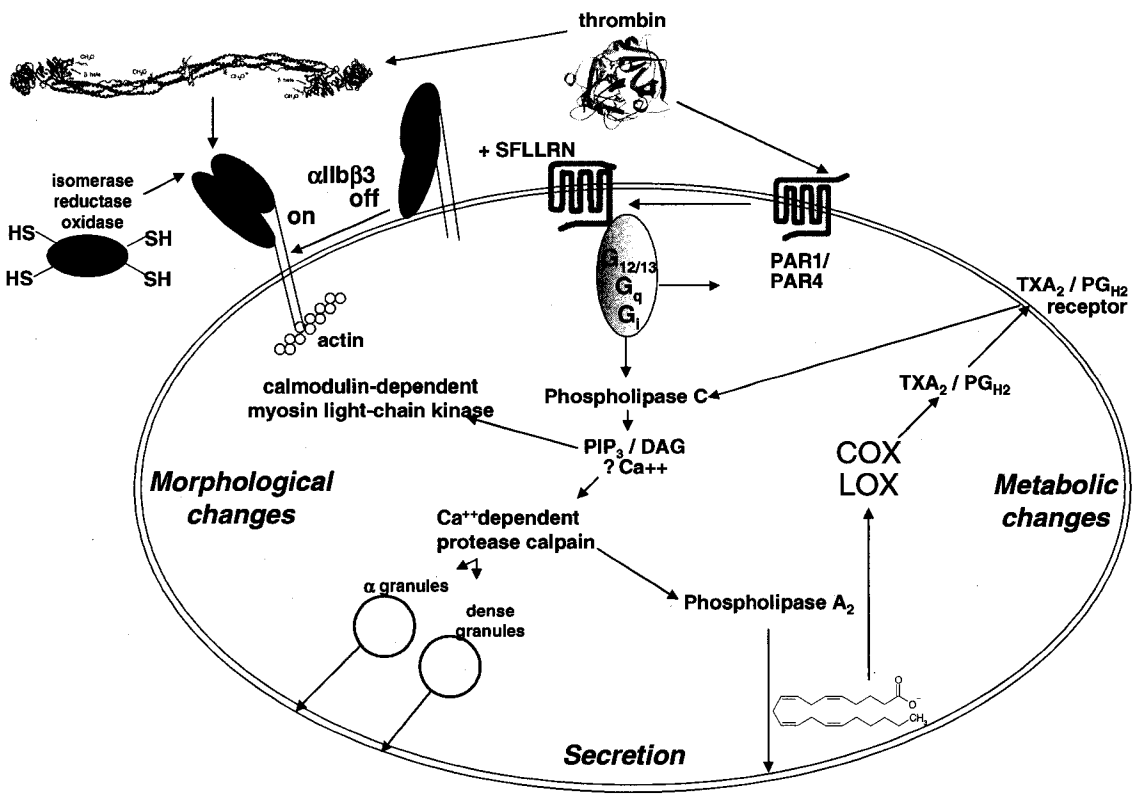


Figure 1.4.1 Signaling of thrombin-induced platelet aggregation - An Overview

1.4.5 Reactive oxygen species in the vasculature

Vascular cells including leukocytes, endothelial cells and platelets produce a variety of reactive oxygen species including superoxide ($O_2^{\bullet-}$) [Cerwinka *et al.,* 2003], hydrogen peroxide (H_2O_2) [Krotz *et al.,* 2004], hydroxyl radical (•OH) [Caccese *et al.,* 2000] and hypochlorous acid (HOCl) [Stocker *et al.,* 2004]. Although these species bear a role in normal cell signaling and physiology, they can also cause oxidative damage to proteins, lipids and nucleic acids when produced in deleterious quantities [Hawkins *et al.,* 2003] and thus contribute to the pathology of a variety of disease states including diabetes [Flora *et al.,* 2007, Ferroni *et al.,* 2004].

Superoxide is formed by the one-electron reduction of molecular oxygen by a variety of NAD(P)H oxidase isoforms [Orient *et al.,* 2007], xanthine oxidase [Vorbach, *et al.,* 2003], Complexes I, II and III of the mitochondrial electron transport chain [Adam-Vizi *et al.,* 2005], during the uncoupling of NOS [Förstermann *et al.,* 2006] and by cytochrome P450 enzymes [Coon *et al.,* 1992]. Although superoxide is not itself a strong oxidant (E^o=-0.160 V) [Wood *et al.,* 1987]), it can contribute to the generation of more potent oxidants such as hydrogen peroxide (via dismutation) [Cai *et al.,* 2000] and peroxynitrite (via radical-radical reaction with nitric oxide) [Huie *et al.,* 1993].

In addition to its formation by the dismutation of superoxide, hydrogen peroxide is also generated by a variety of peroxisomal oxidative enzymes during catalysis [Kunduzova *et al.,* 2002, Schrader *et al.,* 2006]. In platelets especially, cyclooxygenases and lipoxygenases are also considered be key sources ROS including hydrogen peroxide [Miller *et al.,* 2006].

1.4.6 Role of superoxide and peroxide in platelets

Studies have demonstrated the production of superoxide by platelets and progenitor megakaryocytes, corroborated by the presence of components of a functional NAD(P)H oxidase [Seno, *et al.,* 2001].

Subsequent efforts have further shown that NAD(P)H oxidase-derived superoxide is generated in response to both thrombin and collagen agonists and plays a pivotal role in platelet physiology regulating adhesion [Begonja *et al.,* 2006] (via the αIIbβ3 integrin) [Begonja *et al.,* 200, Gregg *et al.,* 2004], aggregation [Chlopicki *et al.,* 2004] and recruitment [Krötz *et al.,* 2002].

Suggestively, platelets derived from T2D patients are reported not only to produce increased quantities of H_2O_2 [Leoncini *et al.,* 1997] and superoxide [Dixon *et al.,* 2005], but also exhibit to hyperaggregability [Mandal *et al.,* 1993]. This can be in part, explained by observations that platelets treated with superoxide or peroxide exhibit increased intracellular calcium flux [Schaeffer *et al.,* 1999, Redondo *et al.,* 2005]. Undoubtedly, there are additional redox-dependent mechanisms that contribute to hyperaggregability observed under the conditions of diabetes and associated risk factors for cardiovascular disease.

1.4.7 Integrin activation and aggregation

Integrin activation is believed to be a key event in the adhesion of platelets to vascular and extra-vascular proteins such as fibrinogen, von Willebrand factor and collagen thus contributing to aggregation. Integrin αIIbβ3 (the fibrinogen receptor) is perhaps the best-studied integrin to date and is often referred to as the 'canonical'

integrin. Although the αIIbβ3 integrin was initially thought to be entirely in the oxidized state [Calvete *et al.,* 1991], recent studies have shown that it bears not only free thiols [Yan *et al.,* 2000], but that disruption of disulfide bonds may be involved in conversion to a high affinity state [Sun *et al.,* 2002, Ruiz *et al.,* 2001].

Activation of αIIbβ3 is known to occur upon agonist-induced stimulation and involves a conformational change from a low to high affinity state [Shimaoka *et al.,* 2002] via an inside-out signaling mechanism [Payrastre *et al.,* 2000] initiated via G-protein coupled receptors [Coughlin *et al.,* 2000, Shankar *et al.,* 2006]. The αIIbβ3 integrin cytoplasmic domain is associated with a number of cytoskeletal proteins, adaptor and signaling proteins, kinases and phosphatases [Ma *et al.,* 2007]. These species undoubtedly contribute to induction of structural changes in the extracellular region of the heterodimer that convert it from a compact globular to an extended, ligand-binding conformation (in what has been referred to as the 'switchblade' hypothesis) [Ma *et al.,* 2007]. Numerous studies have also suggested that platelet exofacial free thiols [Essex *et al.,* 2004b] and disulfide bond rearrangements [Yan *et al.,* 2000] are intimately involved in this process and contribute to sustained integrin ligation of both collagen [Essex *et al.,* 2004b] and fibrinogen [Lahav *et al.,* 2002], 'irreversible' platelet aggregation, outside-in signaling events [Payrastre *et al.,* 2000] and perhaps to activation of the integrin itself [Chen *et al.,* 1992b]. It is here that PDI is thought to play a primary role in thrombin-induced platelet aggregation.

1.4.8 PDI-mediated aspects of platelet physiology and redox control

Early platelet investigations demonstrated that a variety of both membrane penetrating and cell-impermeant thiol-modifying agents were capable of potently inhibiting adhesion [Jocelyn *et al.,* 1968] and aggregation [Aledort *et al.,* 1968, McIntyre, *et al.,* 1977] of platelets. This work laid the ground for later studies that demonstrated the presence of protein disulfide isomerase on the surface of platelets [Essex *et al.,* 1995] and spurred the investigation of exofacial thiol/disulfide-dependent processes in platelet physiology. In 1992 it was reported that a PDI activity capable of renaturing scrambled RNase was observed in supernatants of activated platelets [Chen *et al.,* 1992b] and later that enzymatically active PDI could be found on the surface of platelets [Essex, *et al.,* 1995]. This observation undoubtedly led to evidence of a role for PDI in platelet aggregation and secretion [Essex *et al.,* 1999].

Although it is known that there are at least 17 thiol isomerases in the ER [Maattanen *et al.,* 2006], evidence for roles in platelet function have only been provided for PDI [Root *et al.,* 2004], Erp5 [Jordan *et al.,* 2005] and the αIIbβ3 integrin receptor [O'Neill *et al.,* 2000]

As stated prior, PDI is a redox-sensitive, vicinal thiol-dependent enzyme bearing reductase, oxidase and isomerase (as well as chaperone and denitrosation) activities depending upon the prevailing redox environment. Redox balance in the vasculature is maintained primarily by several thiol-based redox couples including the GSH/GSSG, Cys/Cys_2 and couples that are generally believed to exist at low μM levels and vary from ratios of ~13:1 under normal conditions to ~1:1 under conditions of oxidative stress [Go *et al.,* 2005]. Oxidative stress also manifests as an imbalance between the production of

ROS, RNS and their neutralization by antioxidants and antioxidant enzymes. The redox environment established by the interplay between these various species exerts sway over vascular physiology by altering the functional activity of redox sensitive proteins. Thus, the central role of platelet surface thiols in aggregation and secretion events have made readily apparent the redox dependence of platelet physiology, noted by some investigators [Essex *et al.,* 2003, Raturi *et al.,* 2007]

Notably it has been shown that platelet aggregation is potentiated by the addition of low (μM) quantities of GSH which appears to introduce free thiols in to the β_3 subunit of the integrin $\alpha IIb\beta_3$ subunit [Essex *et al.*,2003], previously thought to possess no free thiols. Although this study confirms a role for PDI by showing inhibition by PDI antagonists, it however does not directly assess the redox status of PDI or its potential involvement in directly catalyzing integrin reduction. This study further investigated the functional role and possible interactions of PDI and the fibrinogen receptor - αIIbβ3 integrin [Essex *et al.,* 2005] using a peptide reported to directly activate the integrin by inducing a conformational change [Derrick *et al.,* 1997] ostensibly in the absence of any redox change. Interestingly, aggregation stimulated in this manner could be inhibited by thiol-blocking agents, but not by inhibitors of PDI, leading authors to conclude that PDI may be acting upstream of the fibrinogen receptor and supports earlier evidence which showed that inhibition of PDI was coincident with diminished 'activated' αIIbβ3 as measured PAC antibody binding (which recognizes only the activated integrin) [Chen *et al.,* 1992b].

1.4.9 Platelet-surface, PDI-mediated denitrosation activity

It is generally recognized that PDI (and possible other thiol-isomerases [Jordan *et al.,* 2005, Robinson *et al.,* 2006]) mediate integrin-mediated adhesion events in platelet adhesion [Lahav *et al.,* 2003.], secretion [Essex *et al.,* 1999] and aggregation [Lahav *et al.,* 2000, Lahav *et al.,* 2002, Lahav *et al.,* 2003]

Another equally important aspect of platelet physiology mediated by PDI is brought to light by a recent *in vitro* study on the denitrosation activity of PDI [Sliskovic, *et al.,* 2005]. It was found that isolated PDI was capable of liberating authentic nitric oxide from S-nitrosoglutathione in a mechanism which invoked a nitroxyl-disulfide intermediate and co-operative enzyme activity, but required a reduced active site in order to effectively catalyze the reaction. The ability of •NO to interact with platelet guanylate cyclase has been recognized for some time [Radomski *et al.,* 1987] thus resulting in the generation of cGMP which is known to regulate platelet activation [Doni *et al.,* 1991, Mellion *et al.,* 1983] and adhesion [Radomski *et al.,* 1987]. It was recognized early that S-nitrosothiols were also capable of activating guanylate cyclase in platelets however the solution stability of various nitrosothiols did not correlate with their anti-platelet activity, thus leading authors to believe that platelets must possess some means of active metabolism [Mathews *et al.,* 1993]. Subsequent studies demonstrated the ability of platelet-surface PDI to metabolize low-molecular weight S-nitrosothiols and inhibit platelet aggregation in a guanylate cyclase-dependent fashion [Root *et al.,* 2005, Bell *et al.,* 2007].

It is apparent that cell surface PDI plays a critical role in both platelet aggregation events and anti-platelet denitrosation activity however, the influence of PDI redox status

on these events remains largely uncharacterized. Given the coincidence of lipid disorders with oxidative stress [Stokes *et al.,* 2007, Sanguigni *et al.,* 2002] and platelet hyperaggregability and in various pathological milieu [Watanabe *et al.,* 1998, Korporaal *et al.,* 2006] including T2D [Manda *et al.,* 1993, Ferroni *et al.,* 2004] this is presumably a necessary task.

Work investigating the role of redox environment in pro-atherogenic events and monocytes adhesion, measured the major thiol-disulfide redox couples in human plasma and calculated ranges of redox potential (E_h) of -120 to -20 mV for the Cys/Cys_2 couple and -150 to -50 mV for the GSH/GSSG couple [Go *et al.,* 2005]. Suggestively, increasing oxidizing environment was associated with increasing adhesion of monocytes to endothelial cells. In light of these studies, the obvious question is how pathological changes in redox environment contribute to platelet aggregation and inhibition events mediated by PDI.

PART II

CHAPTER 2[1]

ANTI-OXIDANT AND ANTI-PLATELET EFFECTS OF ROSUVASTATIN IN A HAMSTER MODEL OF PRE-DIABETES

[1]Published as original research as Miersch *et al.* 2007.

2.1 Introduction

Elevated reactive oxygen species (ROS) production in diabetes-related cardiovascular disease has been recognized in both diabetic humans [Spitale *et al.,* 2002] and animal models of diabetes [Sonta *et al.,* 2004]. ROS have been shown to alter platelet physiology in a variety of ways [Gregg *et al.,* 2004, Redondo *et al.,* 2005, Walsh *et al.,* 2004, Chlopicki *et al.,* 2004], thus promoting hyperaggregability. Thrombotic complications in diabetes remain a leading cause of mortality [Beckman *et al.,* 2002], thus warranting the investigation and adoption of therapeutic strategies that mitigate this enhanced potential for thrombogenesis observed in Type II diabetes (T2DM) [Ferroni *et al.,* 2004].

We have recently made the novel and interesting observation that platelets derived from human Type II diabetics display both elevated NADPH oxidase (NOX)–dependent ROS production and a concomitant decrease in platelet-mediated liberation of nitric oxide from low molecular weight *S*-nitrosothiols (termed denitrosation) [Miersch, *et al.,* manuscript in preparation]. Indeed, we have shown that healthy human platelets treated with exogenous ROS exhibit a similar loss in ability to metabolize *S*-nitrosothiols, as well as an enhanced potential for aggregation. Central to both of these events is the enzyme protein disulfide isomerase. This enzyme is known to localize to the platelet surface (psPDI) where it is catalytically active and responsible for both proaggregatory disulfide exchange reactions [Lahav *et al.,* 2000, Lahav *et al.,* 2002, Lahav *et al.,* 2003] and antiaggregatory liberation of nitric oxide from low molecular weight *S*-nitrosothiols [Root *et al.,* 2004].

We have thus attributed the observed loss in platelet metabolism of *S*-nitrosothiols to the oxidation of psPDI active-site thiols by increased oxidant load and have sought

within this paper to investigate therapeutic avenues for mitigating platelet hyperaggregability. Inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA reductase) have shown efficacy in lowering circulating lipid levels and in reducing cardiovascular mortality [Maron *et al.,* 2000]. However, it has recently come to light that this class of drugs, referred to as statins, may have additional vasculoprotective benefits that are not derived directly from cholesterol lowering. This assertion stems from the observations that lower serum cholesterol levels positively correlate with improved risk of coronary vascular events, but not with risk of stroke [Collins *et al.,* 2004]. Alternatively, statin treatment has been shown to reduce not only coronary events but also ischemic stroke [Amarenco *et al.,* 2004].

Investigation of the "pleiotropic effects" of statins is based, in part, on the hypothesis that they bear antioxidant properties and are capable of inhibiting the production of reactive oxygen species from enzyme complexes such as NAD(P)H oxidase.

Other "vascular NOX isozymes" found in endothelial and smooth muscle cells have been implicated in the promotion of atherogenesis [Laufs *et al.,* 2005] and in animal models of diabetes [Sonta *et al.,* 2004]. In light of the potential role of NOX in diabetes and cardiovascular disease, we hypothesized that rosuvastatin would attenuate NOX activity and perhaps normalize platelet activity by protecting psPDI from excess ROS, thus providing the impetus for our study of the potential antithrombotic and antiatherogenic benefits in the vasculature.

We employed the Syrian Golden hamster, which becomes metabolically disturbed upon fructose feeding for 7 days such that it develops hyperinsulinemia and

hyperlipidemia in the absence of overt hyperglycemia, thus representing a viable model of the prediabetic, insulin-resistant state [Taghibiglou *et al.,* 2002]. We observed that platelets and aortic endothelial cells (AEC) from fructose-fed (FF) animals exhibit enhanced production of NOX-dependent ROS. Further, platelets showed diminished psPDI-denitrosation activity and hyperaggregability in comparison to non-fructose-fed (NFF) control animals. These observations could be mimicked by treatment of platelets from NFF animals with exogenous ROS.

As expected, rosuvastatin exerts antioxidant properties in both endothelial cells and platelets derived from fructose-fed animals. Most importantly, we conclude that rosuvastatin shows efficacy in reversing ROS-mediated inhibition of platelet denitrosation activity and hyperaggregability.

2.2 Materials and equipment

2.2.1 Materials

Ellman's reagent (DTNB), glutathione (GSH), sodium nitrite, trisodium citrate dihydrate, citric acid monohydrate, dextrose, apyrase, sodium chloride (NaCl), dibasic sodium phosphate (Na_2HPO_4), dibasic potassium phosphate (KH_2PO_4), potassium chloride (KCl), sodium bicarbonate ($NaHCO_3$), magnesium chloride hexahydrate ($MgCl_2 \cdot 6H_20$), calcium chloride hexahydrate ($CaCl_2 \cdot 6H_20$), fructose, BSA, F12K media, endothelial cell growth supplement (ECGS), porcine heparin, xanthine, xanthine oxidase, thrombin, diphenyleneiodonium chloride (DPI), and mevalonate were purchased from Sigma-Aldrich Canada (Oakville, ON). Isofluorane was purchased from the Leamington Animal Clinic (Leamington, ON). Medical grade oxygen was purchased from BOC Gases (Windsor, ON). 10-Acetyl-3,7-dihydroxyphenoxazine (Amplex red) and

horseradish peroxidase (HRP) were obtained from Molecular Probes (Eugene, OR). Mn(III)tetrakis(1-methyl-4-pyridyl)porphyrin pentachloride (Mn(III)TMPyP) was obtained from Cedarlane Labs (Hornby, Ontario). Matrigel was purchased from BD Biosciences (Mississauga, ON). Rosuvastatin was provided by AstraZeneca (UK).

2.2.2 Equipment

Tech3 Isoflurane Key-Fill Vaporizer, Harvard Appartus, Holliston, MA

EBA 12 Desktop Centrifuge, Fisher Sceintific, Mississauga, ON

Popper 3" Curved Feeding Needles, Fisher Scientific, Mississauga, ON

Agilent 8453 UV-VIS Spectrophotometer; Agilent Technologies Canada Inc., Mississauga, ON

Apollo 4000 Free Radical Analyzer; World Precision Instruments USA, Sarasota, FL

Biotek E_x808 UV/Vis Plate Reader, Biotek Instruments, Inc., Winooski, VT

Haemocytometer; Reichert Co, Buffalo, NY

ISO-NOPF200 200 μM Flex NO Sensor; World Precision Instruments USA, Sarasota, FL

Jouan CR3i Centrifuge, Jouan Inc., Winchester, Virgnia

Mettler AJ1000 Balance; Mettler, Toledo Canada, Mississauga, ON

Microtitre 96-well Solid Plate, Thermo Electron Corp., Canada, Burlington, ON

NUAIRE Biological Safety Cabinet Class II Type A/B3; Thermo Electron Corp. Canada, Burlington, ON

Orion Model 420A pH Meter, Thermo Electron Corp. Canada, Burlington, ON

Tissue Culture Flask, T-75, Sardstedt Inc., Montreal, PQ

Wallac 1420 Victor^3 Fluorescent Plate Reader, Perkin Elmer, Woodbridge, ON

2.3 Methods

2.3.1 Synthesis of GSNO

S-nitrosoglutathione was prepared as previously described [Root *et al.*, 2004]. Briefly, GSH [free thiol] content was determined with DTNB [Riddles *et al.*, 1983]. Acidified $NaNO_2$ was added to GSH solution in a stoichiometric amount and reacted for 30 min at 4°C. The final pH of the solution was adjusted to 7.4. GSNO was further recrystallized by the slow addition of ice-cold acetone and resuspended in the appropriate buffer.

2.3.2 Animal care

All experimental procedures performed on the Syrian hamsters were approved by the University of Windsor Research Ethics and Animal Care Committees and in accordance with the CCAC *Guide to the Care and Use of Experimental Animals.* Syrian Golden hamsters were obtained from Charles River Canada (Montreal, PQ). Animals were housed one per cage and given free access to food and water. Animals were divided into two groups: water feeding (control) and 10% fructose feeding. After 2 weeks of fructose feeding, animals were subdivided into four groups as follows: control with no rosuvastatin, control with 10 mg/kg rosuvastatin, fructose-fed with no rosuvastatin, and fructose-fed with 10 mg/kg rosuvastatin. Rosuvastatin was prepared in water and administered daily to animals by oral gavage for 7 days.

2.3.3 Determination of rosuvastatin pharmacokinetics

Plasma concentration (C_p) of rosuvastatin was determined by collecting 200-μL samples of blood from non-fructose-fed animals only at 1-h intervals post-oral gavage for 24 h. These samples were analyzed for their rosuvastatin content by reverse-phase HPLC with electrospray ionization tandem mass spectrometric detection at AstraZeneca (Wilmington, DE). Primary cultures of endothelial cell explants were cultivated in the presence of a range of rosuvastatin concentrations comparable to the $C_{p\ max}$ values in order to simulate *in vivo* exposure to the drug.

2.3.4 Isolation of whole blood and tissues from hamsters

Hamsters were anesthetized using isoflurane delivered with 1% oxygen through an isoflurane vaporizer in an induction chamber and then transferred to a heating pad in the supine position and fitted with a rodent circuit. The thorax and abdomen were then rinsed with 70% isopropanol and hamsters were euthanized by exsanguination. Blood was removed from the inferior vena cava using a 25 3/8 gauge syringe needle and mixed with ACD in a 9:1 ratio. The heart was then removed by severing the aorta just below the iliac bifurcation. It was flushed with PBS and an approximately 3-mm section of aorta excised from just above the left ventricle. This was placed in F12K media warmed to 37°C.

2.3.5 Isolation and primary culture of aortic endothelial cells

Aortic segments were cleaned of remaining adventitial tissue, cut into 2 × 2-mm squares, and placed endothelium down on Matrigel-coated petri dishes in a similar

manner to previously described methods [Suh *et al.*, 1999]. Cultivation in F12K media at 37°C in 5% carbon dioxide, with or without rosuvastatin, resulted in growth of endothelial cells outward from the aortic square which continued to proliferate after removal of the fragment. Cells were grown to 85% confluence and used within three passages. Adherent endothelial cells were washed twice with ice-cold PBS, pH 7.4, then gently scraped off using a rubber cell scraper and collected for experimentation by centrifugation at 100 *g*.

2.3.6 Isolation and preparation of platelets from whole blood

Washed Syrian hamster platelets were obtained by centrifuging whole blood for 15 min at 190 *g* at 37°C, to yield platelet-rich plasma (PRP). Platelets were obtained from PRP and washed three times in Tyrodes-albumin solution by successive rounds of centrifugation for 15 min at 900 *g* and 37°C. The first wash was supplemented with 2 U/mL heparin and 1 U/mL apyrase, the second with 1 U/mL apyrase and the third wash contained Tyrodes-albumin solution only without heparin or apyrase. Platelet count was performed using a hemocytometer. Suspensions of washed Syrian hamster platelets were kept in the incubator at 37°C and used within 4 h of isolation.

2.3.7 Analysis of platelet aggregation

Aggregations were performed in triplicate and monitored spectrophotometrically as the rate of turbidity clearance at 630 nm following initiation of aggregation by the addition of 0.5 U thrombin. This was the amount of thrombin determined by titration to yield maximal rates of initial aggregation. Reactions were followed for 15 min at 37°C and initial rates of aggregation compared to a blank prepared without thrombin.

2.3.8 Analysis of platelet denitrosation

An aliquot of platelet suspension was added to a stirred cuvette containing 100 mM phosphate buffer, pH 7.2, with 1 mM EDTA and 100 μM GSNO in the presence or absence of 50 μM of the cell-permeant superoxide dismutase mimetic, Mn(III)TMPyP. Prior studies have shown that platelet-mediated consumption of GSNO can be blocked by anti-PDI antibodies [Root *et al.*, 2005] and although the [GSNO] used is 1-2 orders of magnitude larger than found physiologically, this is ~2 times the K_m determined for PDI in *in vitro* studies [Sliskovic *et al.*, 2006]. Evolution of nitric oxide was monitored amperometrically using a Clark-type electrode (ISO-NOP, World Precision Instruments, Sarasota, FL) interfaced with the Apollo 400 free radical analyzer (WPI, Sarasota, FL). Signal was measured following addition of platelets as the change in current and [NO] released interpolated from a standard curve. Platelet denitrosation activity was assessed by monitoring the platelet-mediated release of •NO from a solution of 100 μM GSNO.

2.3.9 Determination of cellular NAD(P)H oxidase-derived ROS

Cells were split into equal portions and ROS measured in the presence and absence of 40 μM of the flavoprotein inhibitor, diphenyleneiodonium [O'Donnell *et al.*, 1993] using 15 μM 10-acetyl-3,7-dihydroxyphenoxazine and 1 U/mL horseradish peroxidase as a reporter system for hydrogen peroxide (H_2O_2) and ROS which ultimately form peroxide [Zhou *et al.*, 1997]. NOX generates superoxide, which is rapidly dismuted to hydrogen peroxide [Cai *et al.*, 2005]. Fluorescence in the assay is generated through oxidation of the substrate to the highly fluorescent resorufin by H_2O_2 in a horseradish-peroxidase (HRP) -coupled reaction. Signal was obtained on a Spectramax Gemini XS fluorescent microplate reader (Molecular Devices, Sunnyvale, CA) using

excitation/emission of 544/590 nm over 60 min at 37°C. DPI-inhibitable ROS-dependent signal was calculated by the subtraction of total ROS minus ROS generated in the presence of DPI, thus yielding the contribution of ROS derived from NOX.

2.3.10 Superoxide treatment of *ex vivo* platelets

Platelets were isolated as indicated and treated with a range of xanthine concentrations and 1.5 mU/mL xanthine oxidase or xanthine alone for 30 min. The reaction proceeded until completion (~30 min as confirmed by Amplex assay) and 10^8 platelets were suspended for use in aggregation and denitrosation experiments

2.3.11 Statistical analysis

Error bars represent standard deviation of the mean. Statistical analysis was performed using ANOVA with readings deemed to be significant at the 95% confidence interval and determined by pairwise comparison using Tukey's post hoc test.

2.4 Results

2.4.1 Pharmacokinetics

A time-course of C_p for rosuvastatin revealed that a maximum of about 20 nM was reached at 4–6 h following oral gavage and increased to ~30 nM at 24-h, potentially due to re-ingestion of drug by coprophagia (Fig 2.1).

2.4.2 Platelet generation of reactive oxygen species

Upon fructose feeding, platelet ROS generation increased by ~ 1.5-fold (Fig. 2.2) in comparison to controls. Daily administration of 10 mg/kg of rosuvastatin to FF hamsters for one week normalized production of ROS to levels similar to that of NFF control animal levels. Rosuvastatin treatment did not affect the ROS production of platelets derived from NFF animals in a statistically significant manner.

2.4.3 Platelet surface PDI denitrosation activity

psPDI •NO-releasing activity was ~ 45 % lower in platelets from FF hamsters when compared to those from NFF hamsters (Fig. 2.3). psPDI NO-releasing activity of FF animals was restored almost to control levels by rosuvastatin. Surprisingly, rosuvastatin administration to NFF hamsters decreased platelet-mediated denitrosation by ~15% and may reflect the role of hydrophobic interactions in PDI-platelet surface binding. Importantly, platelet denitrosation was measured in the presence of the superoxide dismutase mimetic, Mn(III)TMPyP. Comparison of the results ± superoxide dismutase (SOD) mimetic show little difference in denitrosation activity, suggesting that the

observed loss in denitrosation activity is not simply due to scavenging of liberated •NO by elevated levels of superoxide, but to a diminished capacity to metabolize nitrosothiol.

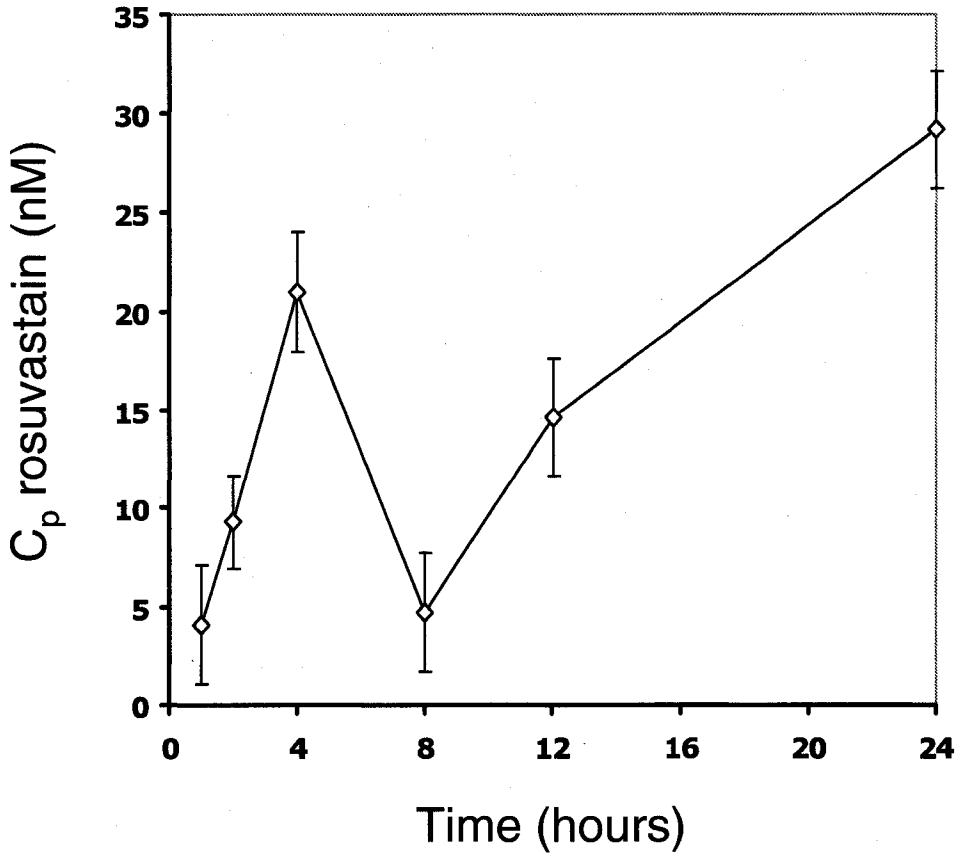


Figure 2.1 Monitoring pharmacokinetics of rosuvastatin in Syrian hamsters

Rosuvastatin concentrations in plasma were determined by taking 200 μL of blood at time intervals over 24 hours from the initial drug administration. Plasma concentrations of rosuvastatin were analyzed by reverse-phase HPLC with electrospray ionization tandem mass spectrometric detection.

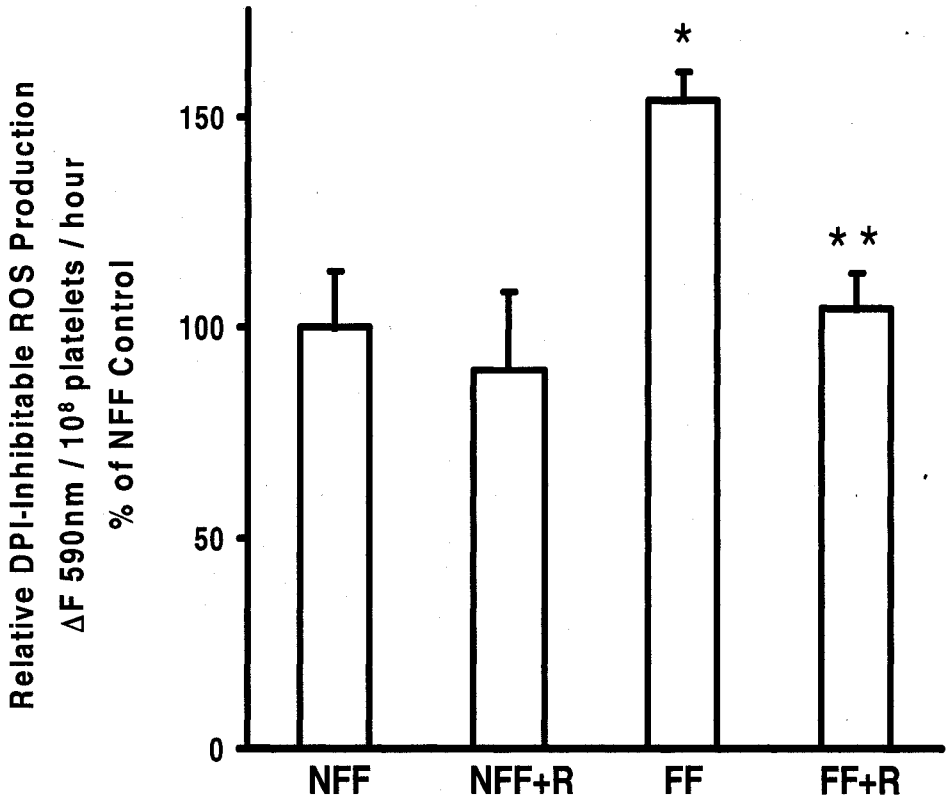


Figure 2.2 Monitoring platelet ROS-generating activity in fructose and rosuvastatin fed Syrian hamsters

Platelets from both control and fructose fed hamsters, with and without 10 mg/kg of rosuvastatin, were collected and prepared as described in Materials and Methods. DPI-inhibitable ROS production was monitored in the presence of 15 μM 10-acetyl-3,7-dihydroxyphenoxazine (Amplex Red) and 1 U/mL horseradish peroxidase. The formation of resorufin, indicative of reactive oxygen species production, was monitored as the increase in fluorescence at 590 nm Data shown represent percent difference in DPI-inhibitable ROS with respect to control, with n=13 study samples taken from 13 animals for each group of NFF and FF, with and without drug, run in triplicate. $P<0.05$, * with respect to NFF control, ** with respect to FF untreated with drug.

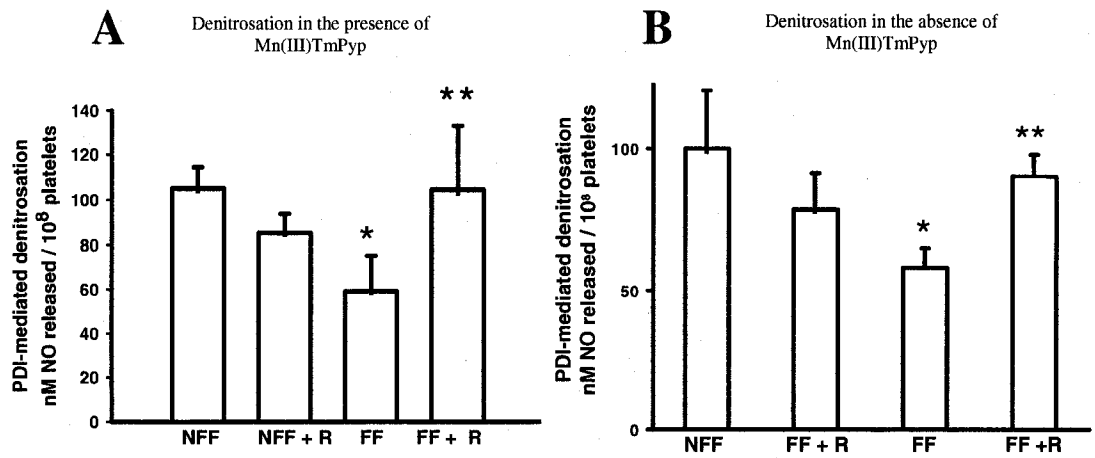


Figure 2.3 PDI denitrosation activity of platelets from fructose fed and non-fructose fed Syrian hamsters

Platelets from both control and fructose fed hamsters, with and without 10mg/kg of rosuvastatin, were collected and prepared as described in Materials and Methods. PDI denitrosation activity was monitored amperometrically immediately following injection of the platelet suspension in to a solution of 100 μM GSNO in the absence (B) or presence of 50 μM Mn(III)TMPyP (A). Data shown represent the percent difference in absolute amounts of •NO released per 10^8 platelets, for n=6 study samples taken from 6 animals for each group of NFF and FF, with and without drug, run in triplicate. $P<0.05$, * with respect to control, ** with respect to FF untreated with drug. Platelets injected as blank into solution in the absence of GSNO and buffer injected into GSNO exhibited no measurable evolution of •NO.

2.4.4 Platelet aggregation

Representative examples of the aggregation profiles are shown in Fig. 2.4 and compiled aggregation data is presented in the inset (n=13, per group). As expected, the initial rates of thrombin-induced aggregation in FF animals were ~ 1.5-fold larger than controls (inset). Rosuvastatin decreased the initial rates of aggregation in NFF platelets by ~20%. In the case of FF animals receiving rosuvastatin, the initial rates of aggregation normalized returning to near those observed for NFF hamsters receiving rosuvastatin.

2.4.5 Effect of fructose feeding on GSNO inhibition of platelet aggregation

To assess the implications of lost denitrosation activity, we further monitored the ability of GSNO to inhibit platelet aggregation at time points prior to and following commencement of fructose feeding. Results indicate a progressively diminished ability of GSNO to inhibit thrombin-induced aggregation of washed platelets with the duration of fructose feeding. Figs. 2.5A and B show representative aggregation plots in the absence (○) or presence (●) of 12.5 μM GSNO compared to unstimulated platelets (▼) at 0 and 2 weeks of fructose feeding. Observations are summarized as percent inhibition of initial rates of with GNSO in Fig. 2.5C and reveal a ~15% loss in inhibition at the second week. Treatment of platelets from NFF animals with increasing amounts of X/XO show a similar decline, up to ~20% inhibition of platelet aggregation induced by GSNO (Fig. 2.5D) at 10 μM xanthine, suggesting that the loss of GSNO anti-platelet effects with fructose feeding may be due to ROS-induced changes in PDI-mediated liberation of •NO.

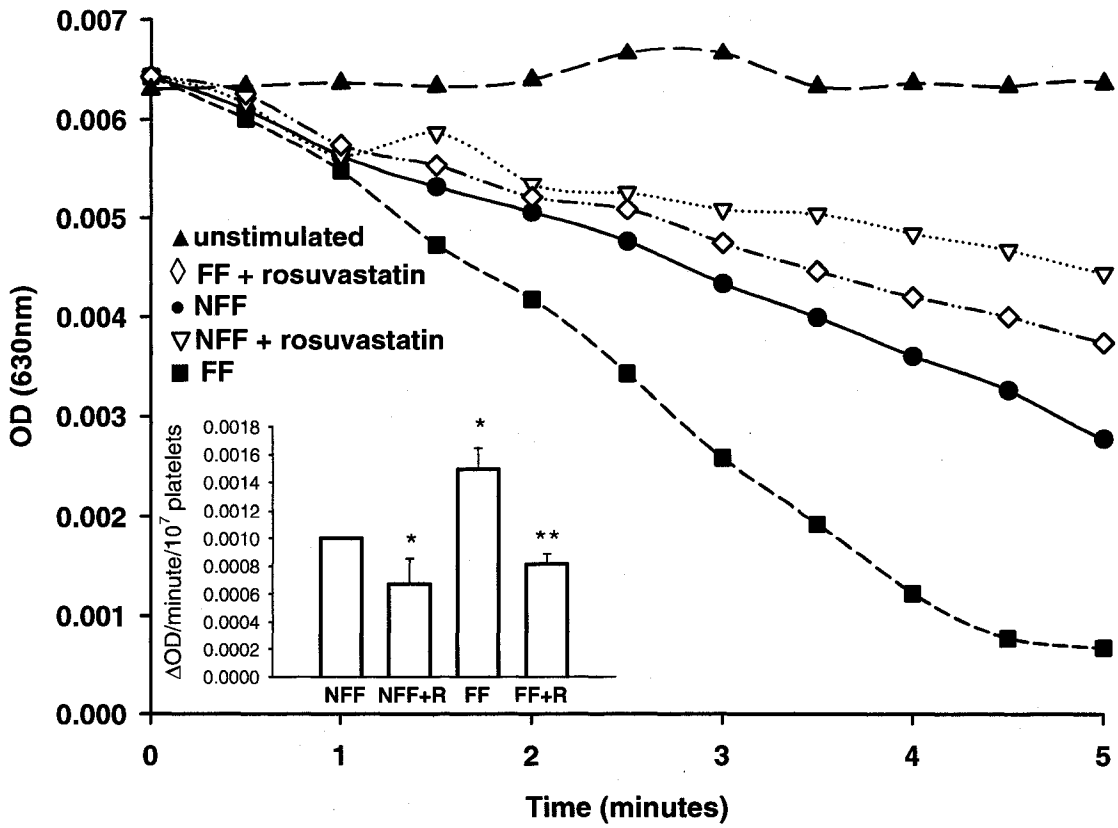


Figure 2.4 Effects of fructose feeding and rosuvastatin administration on aggregation rates of Syrian hamster platelets

Platelets from four study groups of hamsters were collected and prepared as outlined in Materials and Methods. Aggregation of platelets in Tyrodes-albumin solution (10^8) was monitored at 630 nm after stimulation with 0.5 U/mL thrombin over a 10-minute time period. Representative traces are shown for platelet aggregation profiles from NFF animals (●), NFF animals treated with 10mg/kg rosuvastatin (▽), FF animals (■) and FF animals treated with 10mg/kg rosuvastatin (◊). Unstimulated platelets (▲) are shown as blank. Inset data shown represent percent difference in aggregation rates with respect to control. n=13 study samples taken from 13 animals for each group of NFF and FF, with and without drug, run in triplicate, $P<0.05$, * with respect to thrombin-stimulated NFF control, and ** with respect to thrombin-stimulated FF hamster platelets.

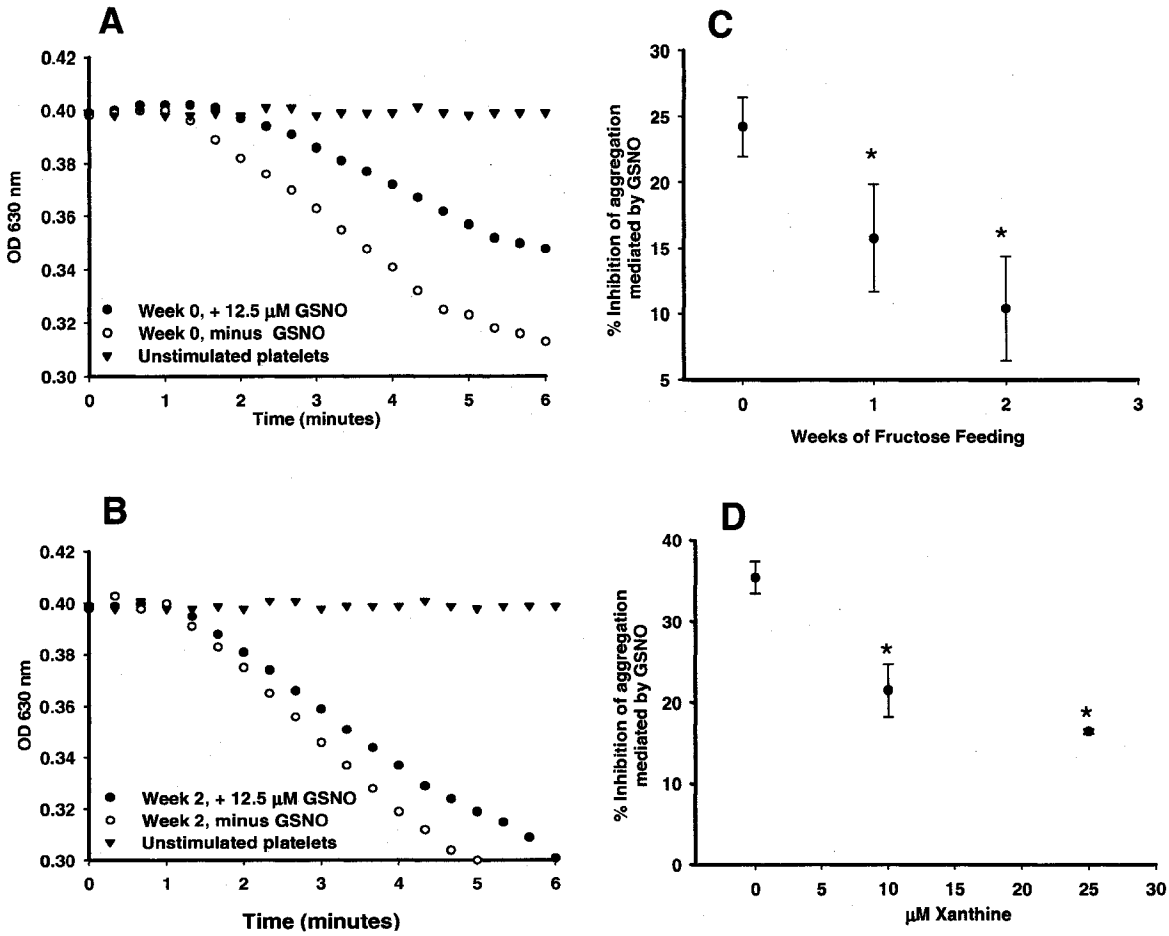


Figure 2.5 Effect of fructose feeding on GSNO inhibition of platelet aggregation

Platelets from hamsters were collected and prepared as outlined under Materials and Methods at 0, 1, and 2 weeks of fructose feeding. Aggregation of platelets in Tyrodes-albumin solution was monitored at 630 nm after stimulation with 0.5 U/mL thrombin in the presence or absence of 12.5 µM GSNO over a 10-min time period. Representative traces are shown for platelet aggregation profiles from NFF animals in the presence (●) and absence (○) of the indicated [GSNO]. Unstimulated platelets (▼) are shown as blank. Percentage inhibition of aggregation was calculated as the ratio of the GSNO-inhibited to uninhibited initial rate of aggregation and summarized in panel C at Weeks 0, 1, and 2. n = 6 study samples taken at each time point and run in triplicate, $p < 0.05$, * with respect to thrombin-stimulated NFF control. Similarly, platelets isolated from NFF-fed animals were suspended in Tyrodes-albumin solution and exposed to a transient (30 min) flux of

superoxide generated from 0 to 10 μM xanthine and 1.5 mU/mL xanthine oxidase, verified to be complete within the allotted time by the Amplex assay. Aggregation of platelets was then monitored at 630 nm after stimulation with 0.5 U/mL thrombin in the presence or absence of 12.5 μM GSNO over a 10-min time period. Percent inhibition of aggregation was calculated the same as above.

2.4.6 The effects of X/XO-generated superoxide on NFF platelet function

The question arises as to whether the observed attenuation of the •NO-releasing activity of psPDI and potentiation of the initial rates of aggregation could be the result of increased ROS production observed in FF hamsters. In order to test this hypothesis, platelets isolated from control (NFF) hamsters were exposed to a combination of X and XO. Xanthine oxidase is known to generate $O_2^{\bullet-}$ and H_2O_2 through the enzymatic transfer of electrons from xanthine to molecular oxygen [Vorbach *et al.*, 2003] and subsequent spontaneous dismutation of $O_2^{\bullet-}$ to H_2O_2. In these experiments, generation of ROS was controlled to ensure completion of the reaction within 30 min as assessed by the Amplex assay. The platelets were then probed for psPDI denitrosation activity and thrombin-induced aggregation *in vitro*.

2.4.7 Effect of ROS on psPDI denitrosation activity from NFF animals

Platelets from NFF animals were treated with X and XO to mimic the effects of excess vascular ROS derived from superoxide and then assessed for psPDI denitrosation activity. The initial rate of psPDI-catalyzed GSNO denitrosation was measured as the current evolved from a •NO-specific electrode (Fig 2.6) in a solution of buffered GSNO into which a suspension of ROS-treated platelets from NFF animals was injected. Results revealed dose-dependent inhibition following treatment with increasing concentrations of X in the presence of XO versus both controls with X (in the absence of XO) and in the absence of X. It was observed that pre-treatment of platelet from NFF animals with ROS generated from 25 μM X and 1.5 mU/mL XO resulted in 60 ± 11.2% inhibition of denitrosation activity.

Notably, ROS generation from the X/XO system was verified to have ceased prior to assessment of denitrosation. Catalytically-formed superoxide can react with liberated •NO during denitrosation experiments, thus appearing to inhibit denitrosation. Thus, this was a necessary control to show that diminished denitrosation activity was not due merely to consumption of released •NO by reaction with platelet superoxide, but instead due to a loss in the enzymatic liberation of •NO from S-nitrosoglutathione. Given that platelets from NFF animals produce normal levels of ROS, but displayed inhibition of denitrosation activity upon treatment with exogenous ROS, this confirms the loss of psPDI-mediated activity.

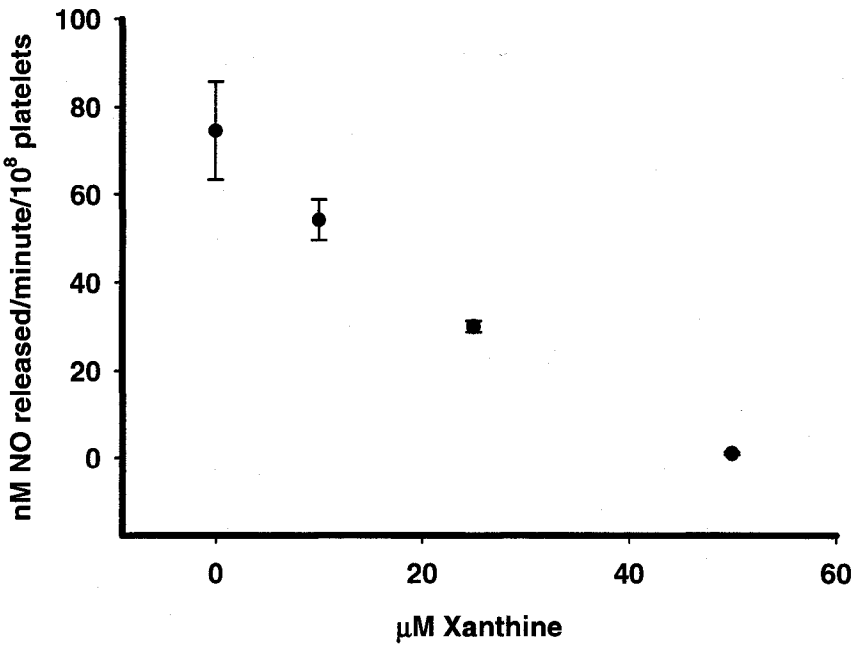


Figure 2.6 Effect of transient platelet exposure to superoxide on psPDI denitrosation activity

Washed platelets were obtained from NFF hamsters by centrifugation and then exposed to a transient (30 min) flux of superoxide generated from 25 μM xanthine and 1.5 mU/mL xanthine oxidase, verified to be complete by Amplex assay before assessment of the denitrosation activity. Release of •NO was measured amperometrically following injection of treated platelets into a stirred cuvette containing 1 mM EDTA and 100 μM GSNO. Data shown represent the absolute amount of •NO released per minute for each of n = 4 study samples with standard deviation and indicate an IC_{50} of ~25 μM xanthine.

2.4.8 The effects of ROS on NFF platelet aggregation

The initial rates of thrombin-induced aggregation of NFF animal platelets with (●,) or without (○,) 25 μM xanthine exposure (30 min) were nearly identical (-0.022 ± –0.002 /min) as shown in Fig 2.7. Upon exposure to 25 μM X plus 1.5 mU/mL XO (30 min) the initial rates increased by ~1.4-fold (-0.032 ± –0.003 /min) (▲, Fig. 5B) versus those treated with X alone (●). Unstimulated platelets are shown as control (∇).

2.4.9 NOX activity in 1° culture of hamster AEC

Recent evidence points also to the constitutive expression of NOX in endothelial cells resulting in the generation of low levels of superoxide [Li *et al.*, 2004]. Elevations in NOX activity have been observed in the pathophysiology of vascular diseases including diabetes [Beckman *et al.*, 2002], hypertension [Paravicini *et al.*, 2004] and atherosclerosis [Rueckschloss *et al.*, 2003, Laufs *et al.*, 2005]. In these series of experiments, aortic endothelial cell explants taken from NFF and FF animals were exposed to 0 to 100 nM concentrations of rosuvastatin in culture (Fig 2.8). The FF group had ~1.6 fold larger NOX activity than NFF. The exposure of the NFF group to increasing concentrations of rosuvastatin had no statistically significant effect on the basal NOX activity. However, elevated NOX activity of endothelial cells derived from FF animals decreased in a rosuvastatin-dose dependent manner and was statistically identical to the basal NFF-NOX activity in the presence of 50 nM rosuvastatin. This is very significant as 50 nM is in range of the *in vivo* C_p (~30 nM) of the drug determined in this study. Further, in the presence of 200 μM mevalonate, the anti-oxidant effects of

100 nM rosuvastatin were partially lost suggesting that restoration of isoprenoid biosynthesis was associated with restoration of NOX function.

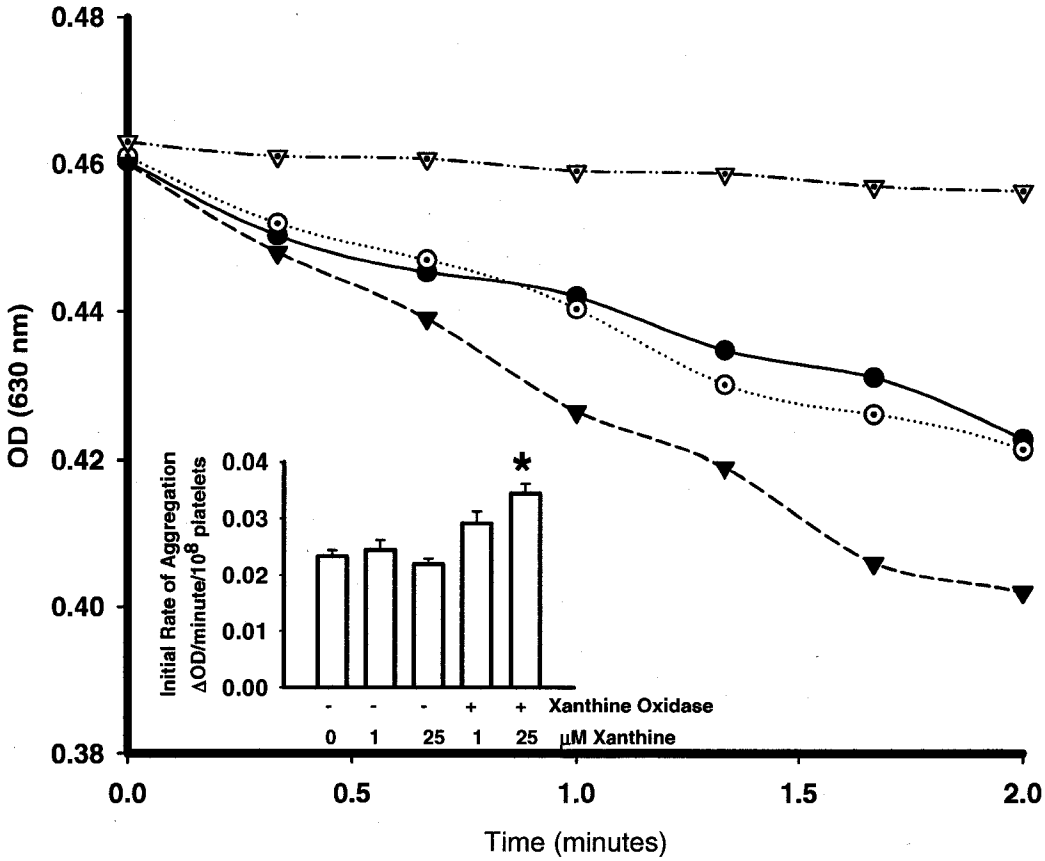


Figure 2.7 Effect of transient platelet exposure to superoxide on platelet aggregation

Washed platelets were obtained from NFF hamsters by centrifugation and then exposed to a transient (30 minute) flux of superoxide generated from 25 μM xanthine and 1.5 mU/mL xanthine oxidase. Platelets (10^8) were suspended in Tyrodes-albumin buffer in triplicate and aggregation initiated by the addition of 0.5 U/mL thrombin. Traces are representative of the initial aggregation in triplicate run in triplicate from n = 4 experiments and show that platelets treated with X/XO (▼) exhibit an approximately 1.4-fold increase in the initial rates of aggregation versus either untreated platelets (○) or platelets treated with xanthine alone (●) all compared to unstimulated platelets (▽). $p < 0.05$, * with respect to thrombin-stimulated control treated with 25 μM xanthine alone.

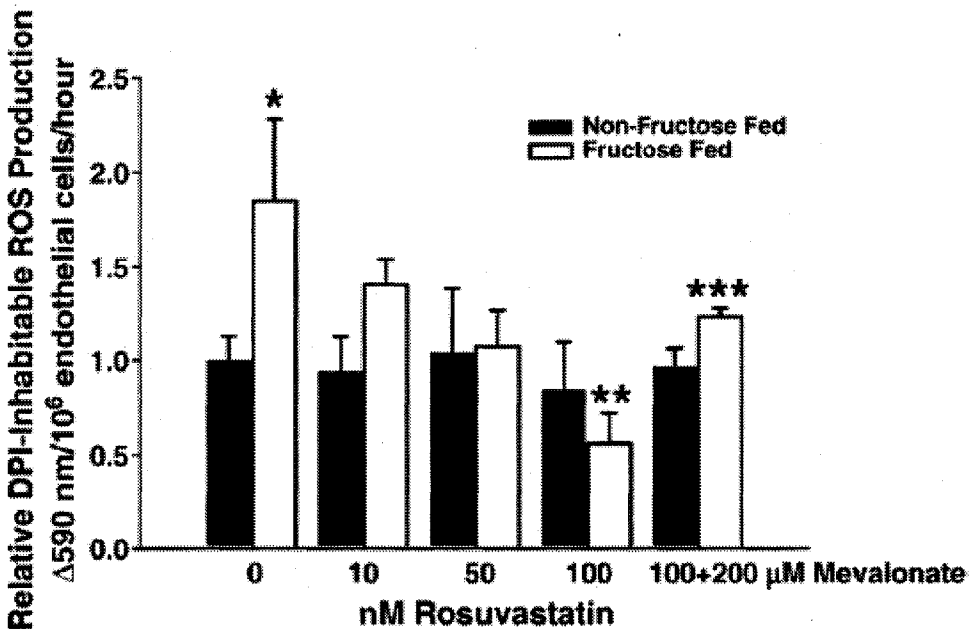


Figure 2.8 Effect of varying rosuvastatin on primary hamster AEC

Hamster aortas were collected as indicated from FF and NFF hamsters. Primary AEC were cultivated until 85% confluent in the presence of varying levels of drug for one week at which time they were harvested for measurement of DPI-inhibitable reactive oxygen species. Data shown represent the % difference in DPI-inhibitable ROS produced in one hour as measured by Amplex fluorescence emission at 590 nm. n = 6 study samples derived from 6 animals for each group of NFF and FF, with and without drug and run in triplicate. $P < 0.05$ * with respect to NFF controls without drug and ** with respect to FF without drug and *** with respect to FF treated with 100 nM rosuvastatin.

2.5 Discussion

The present study was initiated by our observations that both in humans with T2DM and in an animal model of pre-diabetic insulin resistance, elevated platelet production of ROS is observed concomitantly with a decrease in psPDI denitrosation activity and enhanced aggregatory potential. Studies within our lab suggest that elevated vascular ROS oxidize active-site thiols of psPDI, thus contributing to both inhibition of the anti-aggregatory nitric oxide liberating activity and an increased potential for aggregation. The hypothetical mechanism of ROS-mediated inhibition of psPDI RSNO-denitrosation activity is thought to be as follows: ROS is produced by enzymes such as NOX or other ROS-producing enzymes and is rapidly converted to H_2O_2 by extracellular superoxide dismutase (SOD) or by spontaneous dismutation. H_2O_2 readily oxidizes vicinal dithiols [Conway *et al.,* 2002, Kim *et al.,* 2000], such as those found at the active site of PDI, thus inactivating the RSNO-denitrosation activity of PDI and contributing to enhanced potential for aggregation through provision of oxidative equivalents. Bolstering this hypothesis is the recent report indicating that psPDI and NOX are in close spatial contact in the platelet PM [Janiszewski *et al.,* 2004].

Another crucial piece of evidence supporting our hypothesis is our recent demonstration [Root *et al.,* 2004] that the RSNO-denitrosation activity of psPDI inhibits platelet aggregation by two routes: first, PDI denitrosates RSNOs, releasing •NO that attenuates platelet activation via the guanylate cyclase/G-kinase route; secondly, RSNOs are denitrosated at the same PDI-active site that catalyses the disulphide bond formation between integrins and their ligands [Lahav *et al.,* 2000, Lahav *et al.,* 2002, Lahav *et al.,* 2003], thereby attenuating irreversible aggregation. In addition to the demonstration that

inhibitors of platelet PDI inhibit the consumption of S-nitrosoglutathione [Root *et al.*, 2004], others have shown that inhibition of platelet PDI also impedes the accumulation of cGMP and intracellular reaction of delivered nitric oxide equivalents with DAF [Bell *et al.*, 2007]. In contrast, inhibitors of other putative nitrosothiol-degrading enzymes (glutathione peroxidase) or nitrosothiol transporter (L-amino acid transporter) were found to exert only a minimal effect on •NO-signaling [Bell *et al.*, 2007], suggesting that psPDI may be the most physiologically relevant system for delivering •NO-equivalents into platelets.

In the past five years several isozymes of NOX have been identified in non-phagocytic cells like platelets [Krotz *et al.*, 2002], smooth muscle cells [Hilenski *et al.*, 2004), neurons [Hilburger *et al.*, 2005], endothelial cells, [Griendling *et al.*, 2000] and fibroblasts [Griendling *et al.*, 2000]. In platelets, NOX is believed to be plasma-membrane associated [Krotz *et al.*, 2004], however, in endothelial cells the NOX subunits are largely assembled and found associated with the intracellular cytoskeleton and distributed perinuclearly [Li *et al.*, 2002]. Vascular NOX isoforms display low (nM) constitutive $O_2^{\bullet-}$ generation and this basal activity is reported to be potentiated by a variety of stimuli common to type II diabetes pathology, including increased insulin [Kashiwagi *et al.*, 1999], glucose [Inoguchi *et al.*, 2000], oxidized LDL [Rueckschloss *et al.*, 2001] and hyperlipidemia [Li *et al.*, 2003].

NADPH oxidases (NOX) catalyze the one electron reduction of molecular oxygen to yield superoxide anion ($O_2^{\bullet-}$). NOX, initially identified in neutrophils, is a multimeric protein composed of plasma membrane (PM) catalytic subunits $p22^{phox}$ and $p91^{phox}$ containing cytochrome b_{558}, cytosolic regulatory subunits $p47^{phox}$, $p40^{phox}$, $p67^{phox}$, and the

small G-protein Rac1 or Rac2, which translocate to the PM to activate the catalytic subunits . Assembly of a functional enzyme complex requires association of Rac GTPase and this association appears to be regulated by its carboxyterminal geranylgeranylation (isoprenylation) [Ando *et al.,* 1992]. Biosynthesis of isoprenoids, as well as cholesterol requires mevalonate, thus NOX activity can be attenuated with HMG-CoA inhibitors [Bandoh *et al.,* 2003, Takayama *et al.,* 2004] like rosuvastatin which inhibit the rate-limiting conversion of hydroxymethylglutaryl CoA to mevalonate.

Therefore, the first aim of this study was to determine the effects of rosuvastatin on platelet PDI and NOX activities and on *in vitro* platelet aggregation in the animal model of pre-diabetes – the Syrian Golden hamster. Previous studies have demonstrated that fructose feeding of hamsters for 2-3 weeks induces insulin resistance, as confirmed by *in vivo* euglycemic hyperinsulinemic clamp studies [Taghibiglou *et al.,* 2002]. Fructose feeding is associated with hyperinsulinemia, hyperlipidemia but a normoglycemic state, suggesting the development of a pre-diabetic, insulin resistant state. Although the mechanism by which hamsters develop insulin resistance and resultant dyslipidemia is not known, several groups have noted this phenomenon and use this model to investigate carbohydrate-induced chages in lipid metabolism [Rémillard *et al.*, 2001, Kasim-Karakas *et al.*, 1996]

Fructose feeding of hamsters resulted in a ~60% increase in platelet ROS production in comparison to NFF controls (Fig. 2.2) a concomitant ~42 % reduction in psPDI RSNO-denitrosation activity (Fig. 2.3) and thrombin-induced aggregation rates (Fig. 4) that were on average ~1.5-fold larger than platelets from NFF hamsters. Fructose feeding of hamsters was also found to result in a progressive loss in inhibition of platelet

aggregation mediated by GSNO that varied with the duration of feeding (Fig. 2.5C) to a maximum of <15% loss at the second week. Similar results were obtained in platelets from NFF animals treated with X/XO revealing a <20% loss in GSNO-mediated platelet inhibition at 10 μM xanthine (Fig. 2.5D), further supporting our hypothesis that ROS readily oxidizes the active-site vicinal dithiols of PDI, blocking denitrosation activity and thus limiting the anti-platelet effects of GSNO.

The second aim of the study was to determine whether the observed attenuation of the •NO-releasing activity of psPDI and potentiation of the initial rates of aggregation could be the result of increased NOX activity, i.e. superoxide overproduction, observed in platelets of FF hamsters. To test this, we exposed platelets from NFF animals to the xanthine / xanthine oxidase superoxide-generating system and in support of our hypothesis, platelets exposed to a range of xanthine concentrations and 1.5 mU/mL xanthine oxidase over 30 min 1) inhibited the psPDI-dependent RSNO denitrosation activity by ~50% at 25 μM xanthine (Fig. 2.6) and 2) potentiated the initial rates of thrombin-induced aggregation by ~1.4-fold (Fig. 2.7). These effects are very similar to the FF platelet results lending credence to our hypothesis.

The third aim was to determine the effects of short-term rosuvastatin treatment on the platelet-parameters tested here. Rosuvastatin had no statistically significant effect on the platelet ROS production by NFF hamsters. However, drug administered to FF animals attenuated the generation of ROS to control levels (Fig.2.2). In the case of psPDI, rosuvastatin decreased the NFF-psPDI denitrosation activity by ~20% (Fig 2.3). Although PDI is generally considered to bind to the plasma membrane through electrostatic interactions, we have recently investigated and provided evidence that

interaction with the platelet plasma membrane is mediated, in part, by hydrophobic interactions (Raturi, manuscript submitted). It is then possible that cholesterol lowering induced by rosuvastatin in NFF animals alters the hydrophobic character of the platelet plasma membrane potentially diminishing the bound enzyme and accounting for this ~20% loss in denitrosation activity.

Alternately, exposure of FF animals to rosuvastatin resulted in the restoration of the psPDI activity to near control levels. These results suggest that rosuvastatin at nM C_p is effective in blunting the production of ROS by platelets, and thus permitting psPDI-catalyzed release of sufficient •NO from RSNOs to inhibit platelets. This was borne out in the aggregation studies in which rosuvastatin treatment slightly decreased (~20%) the initial rates of aggregation in NFF animals and significantly decreased (~50%) the initial rates in FF animals (Fig. 2.4). Results obtained from the aggregation study suggest that rosuvastatin treatment of FF animals, not only contributes to the normalization of platelet ROS production and psPDI activities, but also restores normal platelet aggregability.

Numerous studies have established links between an elevated pathologic endothelial NOX activity and atherogenesis [Cai *et al.,* 2005, Li *et al.,* 2004, Redondo *et al.,* 2005, Spitaler *et al.,* 2002], thus the fourth aim of this study was to examine the effect of rosuvastatin on NFF and FF hamster aortic endothelial cell NOX activity (Fig. 2.8). To this end, aortic explants from NFF and FF animals were grown in primary culture and exposed to varying nM concentrations of rosuvastatin. NFF NOX-activity was insensitive to *in vitro* rosuvastatin exposure. In contrast, FF animals displayed aortic endothelial NOX activity that was on average ~1.8-fold larger than controls and could be attenuated to near control levels by 50 nM drug. At 100 nM rosuvastatin, FF-NOX-

dependent ROS production was ~40% less than NFF controls, an effect which could be partially reversed by the addition of 200 μM mevalonate. That ROS production was not fully reversed by mevalonate, suggests that other sources may also contribute to DPI-inhibitable ROS. Diphenyleneiodonium is a flavoprotein inhibitor and will inhibit other enzymes such as nitric oxide synthase which has been shown to produce superoxide in platelets of patients with coexisting hypertension and diabetes [Dixon *et al.,* 2005]. However, that mevalonate could in part reverse the anti-oxidant effects of rosuvastatin in AEC, corroborates the assertion that drug effects are possibly elicited by the inhibition of isoprenoid formation.

The relative insensitivity of AECs derived from NFF animals compared to the mitigation of ROS production in those derived from FF animals at the same level of drug suggests that perhaps a different NOX isozyme is being induced in the animal model of pre-diabetes. It can be postulated that the rosuvastatin insensitive NFF-isoform of NOX possesses a higher affinity for geranylgeranylate or the geranylgeranyl transferase in comparison to the FF-isoform of NOX. The net result would be that NFF-NOX would remain active even if rosuvastatin attenuated geranylgeranylation, whereas the activity of the FF-NOX would diminish with decreasing levels of geranylgeranylate.

In summary, this study elaborates on the novel finding that increased ROS production in animal models of diabetes is accompanied by an attenuation of psPDI denitrosation activity that likely contributes to platelet hyperaggregability and thrombogenesis. Treatment of hamsters with 10 mg / kg rosuvastatin shows efficacy in normalizing both of these activities in this animal model of pre-diabetes. Further investigation of ROS generated from primary endothelial cells similarly shows a

significant increase in fructose-fed versus non fructose-fed hamsters that would perturb normal intracellular signaling and contribute to endothelial dysfunction. Treatment of primary endothelial cells with levels of rosuvastatin in the range of those found *in vivo* results in a restoration of ROS production to control levels.

Based on the demonstrated efficacy of rosuvastatin to restore platelet and endothelial ROS levels, as well as normalizing platelet aggregation and PDI-denitrosation activity in models of pre-diabetes, broadening of the pathological states to which this drug is utilized may be beneficial in preventing secondary atherothrombotic complications associated with non-insulin dependent diabetes mellitus.

PART II

CHAPTER 3[2]

NITRIC OXIDE SIGNALING IS AFFECTED BY VARIATIONS IN THE CHOLESTEROL CONTENT OF THE PLASMA MEMBRANE

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3.1 Introduction

Nitric oxide signaling is a complex process governed by numerous factors including its partitioning into hydrophobic cellular environments [Liu *et al.,* 1998a], its diffusional path from a point of generation [Lancaster *et al.,* 2000, Wood *et al.,* 1994, Philippides *et al.,* 2004] and reaction with oxygen [Caccia *et al.,* 1999, Lewis *et al.,* 1994], other radicals [Kissner *et al.,* 1998, Lima *et al.,* 2003] and metal centres [Chiang *et al.,* 2006, Mason *et al.,* 2006], all of which will contribute to its spatial heterogeneity of biological activity. Although there are a number of explanations for the general decline in •NO bioavailability under conditions of hypercholesterolemia [Stokes *et al.,* 2002, Munzel *et al.,* 2005], there have been relatively few papers that have sought to question the role of cellular cholesterol in altering the diffusional properties of •NO [Subczynski, *et al.,* 1998], despite 1) prompts to do so from researchers in the literature [Denicola *et al.*, 1996b], 2) evidence of the critical role of lipids in determining the lifetime and reactivity of •NO [Liu *et al.,* 1998a] and 3) reports noting the role of cellular lipids in defining the diffusional field of •NO [Porterfield *et al.,* 2001].

Nevertheless, there exists a growing appreciation of cholesterol (Chol) as a major determinant of membrane properties and architecture (Subczynski *et al.,* 1996, Nedeianu *et al.,* 2004, Pucadyil *et al.,* 2006] and as a contributing factor to pathophysiological changes [Hao *et al.,* 2001, Rukmini *et al.,* 2001, Preston *et al.,* 2003, Chen *et al.,* 1995a, Qin *et al.,* 2006].

Experiments conducted in liposomal and bilayer lipid membrane model systems to evaluate the contribution of changes in the diffusional properties of •NO demonstrate the existence of cholesterol-dependent changes in membrane transit times and suggest

significance to overall cellular metabolism. This may be of particular importance in •NO -responsive cells exhibiting lipid disorders such as Niemann-Pick disease and cells such as RBCs and platelets in which membrane cholesterol is believed to vary passively [Cazzola *et al.,* 2004].

Niemann-Pick type C1 (NPC1) disease is a fatal neural disorder whose molecular origins are believed to result from mutations in an intracellular sterol transporter (NPC1 and 2) that manifest as the accumulation of unesterified Chol, glycosphingolipids, sphingomyelin in late endosomal and lysosomal compartments and progresses towards neurodegeneration and loss of neural cells [Patterson *et al.,* 1991]. Investigations of the cellular consequences of aberrant lipid trafficking have found disturbances in both intracellular and plasma membrane Chol distribution that affect cellular function and contribute to the pathogenesis of disease. Several studies have shown that despite bearing diminished Chol within caveolar structures [Garver *et al.,* 2002], NPC1-deficient cells exhibit a marked increase in the plasma membrane Chol content that alters membrane bilayer properties and fluidity [Vainio *et al.,* 2005, Koike *et a.,* 1998] as well as functional signaling [Vainio *et al.,* 2005].

In this study, we sought to evaluate the influence of cholesterol on the cellular metabolism of •NO in NPC1-deficient fibroblasts in which the levels and distribution of cholesterol are known to be altered in comparison to normal (NPC+/+) human fibroblasts. Our study demonstrates a clear and significant role for cellular cholesterol in altering both guanylate cyclase-mediated •NO -dependent generation of cGMP and the modulation of reactions capable of nitrosating nucleophilic species such as amines, thiols and hydroxyl residues [Espey *et al.,* 2001]. To assess the generality of our observations,

•NO metabolism was similarly monitored and confirmed to show cholesterol-dependence in platelets, RBCs and MCF-7 cells.

In concert, these observations raise the possibility that pathological alterations in levels and distribution of cellular cholesterol constitute a common, but as yet under-investigated mechanism of errant •NO-signaling.

3.2 Materials and equipment

3.2.1 Materials

Tris-Base, EDTA, PMSF, N^G-monomethyl-L-arginine (L-NMMA), diaminofluorescein diacetate, dimyristoylphosphatidyl choline, dipalmitoylphosphatidyl choline, Chol, Dulbecco's modified eagle's media (DMEM), diethylaminetriamine pentaacetic acid (DTPA), hemoglobin, sodium dithionite, malachite green, ammonium molybdate and performic acid was obtained from Sigma, Mississauga, ON. Alexa 532 succinimidyl ester was obtained from Invitrogen, Burlington, ON. 1-hexadecanoyl-2-(1-pyrenedecanoyl)- *sn*-glycero-3-phosphocholine (β-py-C_{10}-HPC) from Cayman Chemicals, Ann Arbor, MI. Mn(III)tetrakis(1-methyl-4-pyridyl) porphyrin pentachloride (Mn(III)TMPyP) from Cedarlane Labs, Hornby, ON. cGMP enzyme immunoassay kit was from Amersham Biosciences, Baie d'Urfé, Québec. •NO(g), 10% with argon balance (BOC gases, Mississauga Ontario), was purified by bubbling though 1M KOH solution and used directly for experiments or dissolved in deoxygenated PBS with 500 μM DTPA and quantified spectrophotometrically by hemoglobin assay as previously described (Sliskovic, I., Raturi, A. & Mutus, B. (2005) J Biol Chem 280, 8733-41.). Normal human fibroblasts (HFF-1) were obtained from ATCC and NPC1 fibroblasts (GM17923) from the Coriell Cell Repository and maintained in Dulbecco's minimal essential medium (DMEM) supplemented with 10% fetal calf serum and cultivated at 37°C under an atmosphere of 5% CO_2.

3.2.2 Equipment

Agilent 8453 UV-VIS Spectrophotometer; Agilent Technologies Canada Inc., Mississauga, ON

Apollo 4000 Free Radical Analyzer; World Precision Instruments USA, Sarasota, FL

BBA-01A BLM amplifier; Eastern Scientific LLC, Rockville MD, USA.

BBS-100 Basic BLM setup; Eastern Scientific LLC, Rockville MD, USA. (See Figure 3.1

Haemocytometer; Reichert Co, Buffalo, NY

ISO-NOPF200 200 μM Flex NO Sensor; World Precision Instruments USA, Sarasota, FL

Mettler AJ1000 Balance; Mettler, Toledo Canada, Mississauga, ON

MB-1 BLM testing circuit; Eastern Scientific LLC, Rockville MD, USA.

Northern Eclipse 6.0 Imaging Software; Empix Imaging Inc., Mississauga, ON

NUAIRE Biological Safety Cabinet Class II Type A/B3; Thermo Electron Corp. Canada, Burlington, ON

Orion Model 420A pH Meter, Thermo Electron Corp. Canada, Burlington, ON

Varian Eclipse Fluorescence Spectrophotometer, Varian Canada, Mississauga, ON

Wallac 1420 Victor^3 Fluorescent Plate Reader, Perkin Elmer, Woodbridge, ON

Zeiss Axiovert 200 Inverted Fluorescence Microscope, Empix Imaging Inc., Mississauga, ON

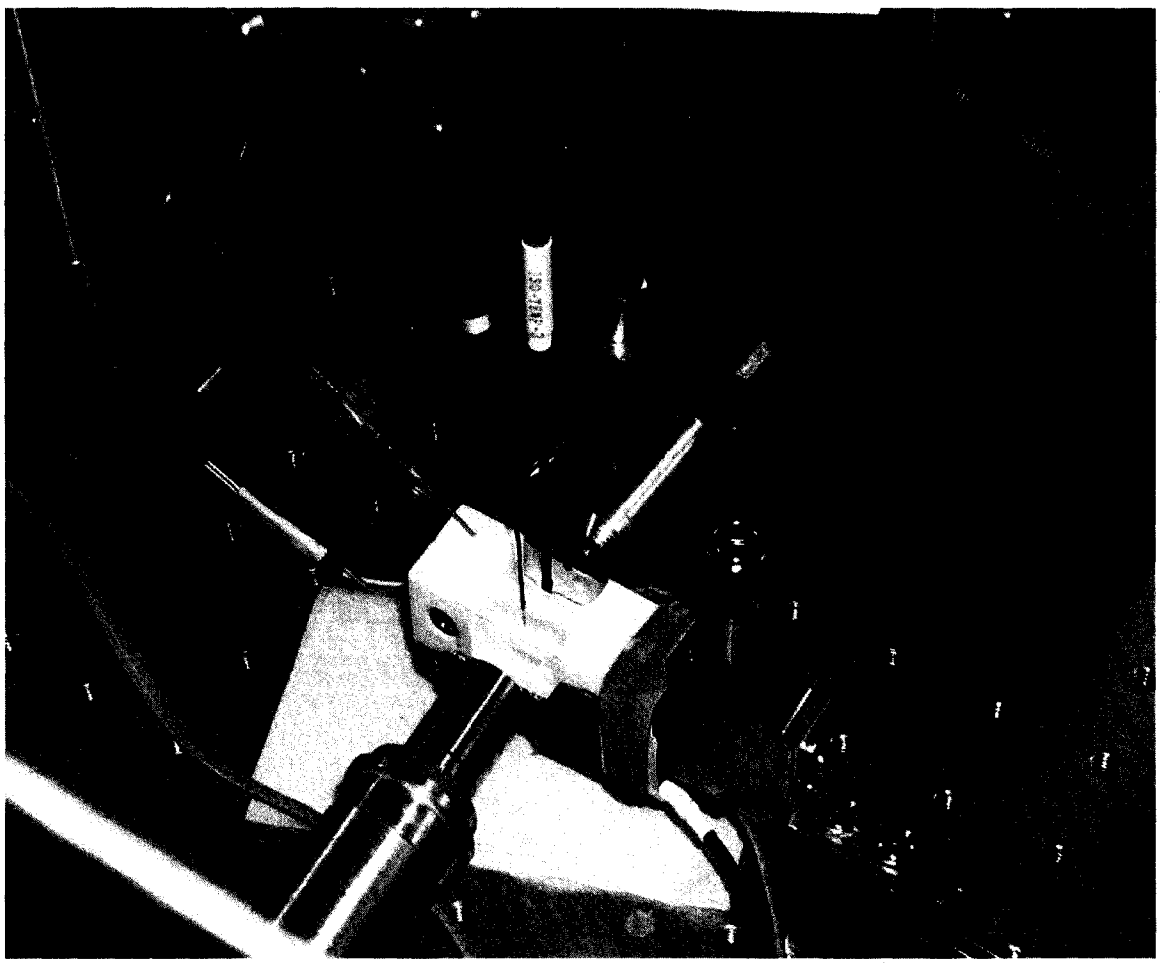


Figure 3.1 Experimental setup of the bilayer lipid membrane chamber

A teflon divider separates the right and left sides of the chamber. Clear plastic windows allow real-time visualization and monitoring of bilayer integrity. On the right side of the chamber, a temperature probe monitors temperature at 37°C. On the left side, a 0.7 mm nitric oxide probe monitors nitric oxide which traverses the bilayer, which is delivered from a tank of 1% nitric oxide in argon by a high-accuracy, low flow gas regulator.

3.3 METHODS

3.3.1 Quantification of NO diffusion by pyrene quenching

The interaction of excited-state pyrene moieties with paramagnetic species such as NO and O_2 are diffusion controlled processes [Birks *et al.*,1970] and have previously been used to monitor the diffusion of nitric oxide in hydrophobic environments allowing determination of apparent diffusion coefficients [Denicola *et al.*, 1996b, Denicola *et al.*, 2002, Möller *et al.*, 2005]. We thus prepared liposomes of either dimyristoyl- or dipalmitoyl-phosphatidylcholine in the presence or absence of 33 mol % cholesterol doped with 1-hexadecanoyl-2-(1-pyrenedecanoyl)-*sn*-glycero-3-phosphocholine (β-py-C_{10}-HPC) as previously described. Briefly, KOH-scrubbed nitric oxide from a 10% gas mixture (balance argon) was bubbled into oxygen-free liposome samples in sealed cuvettes equilibrated to 37°C while monitoring fluorescence quenching (λ_{ex} 337 nm, λ_{em} = 376 nm) in a Cary Eclipse fluorescence spectrometer (Varian, Cary, Mississauga). 10 μL aliquots were removed with a gastight syringe and •NO determined by a •NO-specific electrode and comparison to a standard curve. Stern-Volmer plots were constructed by plotting the fractional quenching observed against the measured [•NO] for not less than 5 replicate runs each with a coefficient of linearity >0.95. Quenching constants (k_q) were then determined from the slope using previously published fluorescence lifetimes (τ_o) of 178 and 194 ns for DMPC and DMPC:Chol and 163 ns in DPPC and 178 ns in DPPC:Chol [Fischkoff *et al.*, 1975]. Diffusion coefficients were calculated from the Einstein-Smoluchowski equation ($D=\mu*kT/e$) [Denicola *et al.*, 1996b] and subsequently used to calculate membrane transit times using the equation $\langle x \rangle^2 = 2Dt$ [Einstein *et al.* 1905].

3.3.2 Measurement of NO diffusion across synthetic BLMs

The BLM chamber was purchased from Eastern Scientific, Rockville MD. The desired ratios of chol:phospholipids were prepared in decane in a vial sealed with a septum and purged with $N_2(g)$. A 5 μL aliquot of this solution was withdrawn and placed on the 1 mm hole in the Teflon divider in the BLM chamber. The both sides of the chamber were then filled with PBS at a rate of 500 μL/ min. The entire BLM chamber was maintained at 37°C and both sides of the chamber were stirred with the aid of micro stir-bars. The formation of the lipid bilayer was monitored by the saturation of the capacitance and by visual inspection. A constant amount of $\bullet NO_{(g)}$ (ultrapure 1%, 99% argon) 200 μL/ min was metered out with a Matheson gas regulator (FM-1050) and introduced to one side of the chamber. The amount of $\bullet NO_{(aq)}$ diffusing through the BLM was measured with Clark-type NO electrode (WPI, Inc) as a function of time and recorded digitally via Duo-18 software (WPI, Inc).

3.3.3 Synthesis, purification and Alexa 532-labeling of PFO-D4

Plasmid pRT10 containing the perfringolysin O gene (pfoA) was used to construct the pfoA derivative D4 encoding the C-terminal region of the toxin. The DNA fragment containing the D4 (K391-N500) - encoding region was PCR amplified using pRT10 plasmid as the PCR template and ligated into the pTrcHisB expression vector inserting it between the BamHI and KpNI restriction sites on the 3' end of the sequence encoding the His-tag site. The polymerase chain reaction primers used were:

forward primer 5'- CCCGGATCCGTCTACAGAGTATTCTAAGG -3' and

reverse primer 5'- CCCGGTACCGGATTGTAAGTAATACTAGATCC-3'.

His-tagged PFO-D4 was isolated on a Ni^{2+} affinity column after successive washes totaling 8 column volumes with 30 mM imidazole in 100 mM Tris buffer pH 8.0. PFO-D4 was eluted with 100 mM imidazole in pH 8.0 100 mM Tris buffer, concentrated and purity demonstrated to be >95% pure by image analysis on a 15% SDS-PAGE gel. Isolated PFO-D4 extensively dialyzed and labeled over night with a five fold molar excess of Alexa 532 succinimidyl ester (epsilon = 81000 L $mol^{-1}cm^{-1}$) at 4°C, separated from unreacted dye on a Sepahdex-G25 column and extent of labeling calculated as the molar ratio of dye to protein.

3.3.4 Preparation of Chol-loaded cyclodextrins

Cyclodextrin was charged with cholesterol as per previously described methods [Christian *et al.*, 1997].

3.3.5 Cellular membrane cholesterol labeling by Alexa532-PFO-D4

Cells were fixed for 15 minutes at room temperature with 4% paraformaldehyde in HEPES buffer, washed 3 times and stained with 400 nM Alexa 532-labeled D4 for 30 minutes with gentle shaking. Cells were washed again 3 times and imaged on an Axiovert epifluorescence microscope with 535 nm/550 nm excitation/emission under identical exposure conditions. Images were pseudo-coloured red using Northern Eclipse 6.0 imaging software and the average intensity per unit cell area measured for 50–80 cells from a number of separate images.

3.3.6 Isolation of fibroblast plasma membranes

The cells of 3 T75 flasks of either normal or NPC-1 deficient fibroblasts at ~85% confluency were trypsinized, washed once with 100 mM 1X Tris buffer pH 7.4 and then once with 10 mM hypotonic Tris buffer pH 7.4 containing 2mM EDTA and 1 mg PMSF. A 1 mL suspension was then homogenized by 50 strokes in a Dounce. Mechanical disruption was verified by microscopic inspection and the homogenate was spun at 190g for ten minutes. Subsequent isolation of plasma membrane lipids was performed as previously described [Vainio *et al.*, 2005]. Lipid suspension isolated in buffer was extracted 2X with 200 µL 1:1 MeOH/$CHCl_3$, back-extracted once with 1M NaCl and the solvent evaporated in a glass tube under a gentle stream of nitrogen.

3.3.7 Determination of plasma membrane cholesterol and phospholipid content

3.3.7.1 Phospholipid Analysis

Lipid film was dissolved in 200 µL $HClO_3$, and digested for 60 minutes at 180°C. Aliquots of the acidified digest mixed with Malachite Green and ammonium molybdate and the absorbance measured at 630 nm as per previously published methods [Zhou *et al.*, 1992]. Phosphorous concentration was interpolated from a standard curve. Phospholipid content was estimated by multiplying by 25 the moles of phosphorous and expressed as µmol PL / 10^6 cells.

3.3.7.2 Cholesterol Analysis

Lipid film was sonicated in 200 µL CHAPS:Triton:NaCholate buffer and Chol quantified using a Chol oxidase enzymatic assay (Invitrogen) and interpolation from a standard curve and expressed as µmol PL / 10^6 cells.

3.3.8 Cellular NO responsiveness by cGMP determination

Cells were cultivated to approximately 85% confluency, collected by trypsinization and washed once with PBS containing 500 µM DTPA. Cells counts were adjusted, treated with bolus authentic nitric oxide from a gastight syringe in the presence of 50 µM Mn(III)TMPyP and incubated for 2 minutes at 37°C. Cells were immediately spun down and lysed with lysis buffer (containing phosphodiesterase inhibitors) and lysates prepared as per cGMP EIA kit (Amersham Biosciences) instructions.

3.3.9 Measurement of NO_x-mediated extracellular nitrosation by cell impermeant DAF

•NO uptake and metabolism to species capable of nitrosating DAF was also monitored by the reaction of extracellular DAF-2 with •NO supplied by exogenous •NO - donor in the presence of intact cells. Cells are capable of acting as 'NO sinks' and will take up a portion of •NO from an extracellular donor, altering subsequent reactions with a NO_x sensitive fluorophore, such as DAF-2. This approach has the additional benefit of circumventing potential intracellular interferences which can contribute to artifactual DAF-2T signals [Zhang *et al.*, 2002, Espey *et al.*, 2002 *et al.*, Jourd'heuil D *et al.*, 2002]. Adherent NHF and NPC1-deficient fibroblasts and MCF-7 cells were trypsinized, collected, washed once with and suspended in PBS containing 500 µM DTPA (used

throughout DAF experiments). Human RBCs and platelets were isolated from whole blood by differential centrifugation, washed with 200 volumes of buffer and resuspended. Membrane cholesterol was then augmented or depleted using increasing concentrations of the cholesterol-loaded adduct of MβCD (Chol: MβCD) or MβCD (or cholesterol oxidase) alone with gentle nutation for one hour. Cells were removed from excess cholesterol adduct, cyclodextrin or enzyme by centrifugation, washed once and suspended in a 10 μM DAF solution in PBS containing 0.5 mM DTPA. Cells counts were optimized to consume approximately 50% of delivered •NO.and then treated with the NO donor (either DEA/NO or Proli/NO) for monitoring of evolved fluorescence over time. Cell viability was measured by Trypan blue exclusion to be greater than 90% in all cases following treatment.

3.4 RESULTS

3.4.1 Chol attenuates NO-induced pyrene quenching in liposomes

Incorporation of cholesterol into DPPC and DMPC liposomes induced a ~14% and ~30% decrease respectively in the slopes of the Stern-Volmer plot indicating a decrease in the frequency of quenching interactions between excited-state pyrene and •NO (Fig, 3.2A, 3.2B, repectively). The bimolecular quenching constant (k_q) for each liposome preparation was then determined ($k_q=K_{sv}/\tau_o$) from the slope (K_{sv}) of the Stern-Volmer plot for each experiment using previously published fluorescence lifetimes for each in the various lipid mixtures (see Materials and Methods). Apparent diffusion coefficients were then estimated from the Einstein-Smoluchowski equation (k_q = $4\pi RDN$) [Fischkoff *et al.,* 1975]. These calculations indicate that the presence of Chol in DPPC and DMPC liposomes decreases the diffusion coefficients measured at approximately the C9 position of acyl chains in the membrane by ~27% and ~29% respectively, suggesting a substantial influence of cholesterol over the diffusion of nitric oxide in a lipid environment. Assuming a membrane thickness of 10 nm, transit times were calculated using the measured diffusion coefficients using the equation $\langle x \rangle^2 = 2Dt$ [Einstein *et al.,* 1905] and are shown in Table 1.

3.4.2 The diffusion of NO across bilayer lipid membranes decreases as a function of increasing Chol

In these studies, the 1mm diameter hole of the BLM chamber (Fig. 3.1) was "painted" with either dipalmitoylphosphatidylcholine (DPPC) or to varying Chol to DDPC mixtures. Authentic $\bullet NO_{(g)}$ was bubbled at a rate of 200 μL of 1% •NO in argon/min on one side of the chamber with stirring and the arrival of •NO that had crossed the

membrane bilayer measured with a •NO-electrode was placed in the *trans* chamber. Rates were calculated as the initial rate of increase in [•NO] measured by the electrode during the approach to steady state. The average diffusion rate of •NO across the DPPC bilayers decreased by ~48 % upon introduction of 17.3 mol% Chol and 67 % to 75 % when cholesterol levels were increased to ~30 mol% and ~37 mol%, respectively (Fig. 3.3). These results illustrate the dramatic manner in which Chol modulates •NO - diffusion in the model bilayer employed here. Although the changes in transit time calculated in the pyrene quenching experiments are substantial, in order to account for experimental observations, the increase in membrane residence time would have to alter the net diffusion time required to reach the electrode. Alternately, if the changes in membrane properties and resultant changes in •NO diffusion altered the potential for reaction within the membrane, this may account for observed differences in rates of arrival at the electrode.

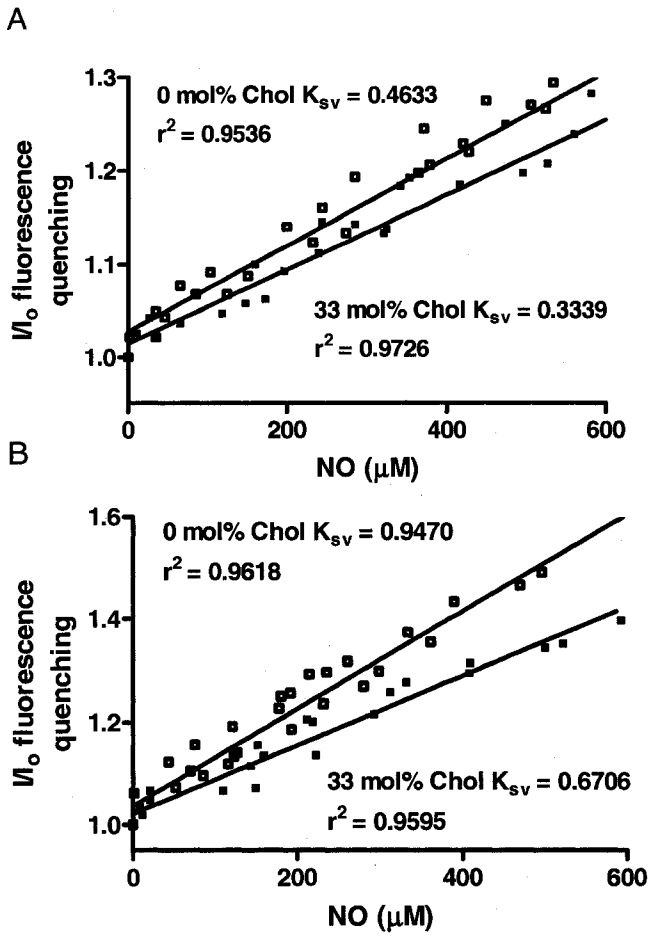


Figure 3.2 Quenching of pyrene phospholipids in liposomes

Stern-Volmer plots were obtained for deoxygenated suspensions of both DPPC (**A**) and DMPC (**B**) liposomes doped with 0.02 mol% β-py-C_{10}-HPC in the presence (filled squares) and absence (hollow squares) of 33 mol% cholesterol containing 1.35 μmol total lipid. Experiments were conducted at 37°C in PBS with 500 μM DTPA and illustrate the fractional quenching of the excited state pyrene moiety at various [•NO] in the presence and absence of cholesterol.

Table 1

Measured diffusion coefficients and membrane transit times for liposomes of determined cholesterol:phospholipid composition by pyrene quenching

	kQ ($M^{-1}s^{-1}$)	D(quencher) $cm^2\ s^{-1}$	transit time (μseconds)
DPPC	3.07E+09	6.76E-06	1.48
DPPC + 30% Chol	2.25E+09	4.95E-06	2.02
DMPC	5.06E+09	1.11E-05	0.89
DMPC + 30% Chol	3.61E+09	7.95E-06	1.25

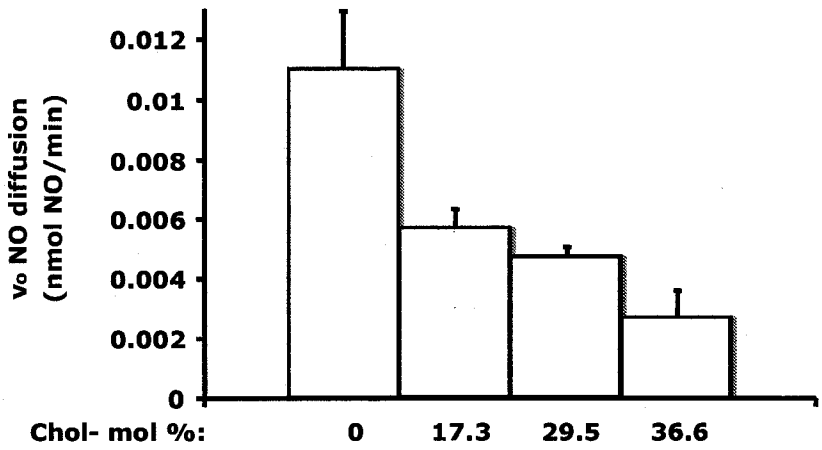


Figure 3.3 Rates of •NO diffusion across a synthetic lipid bilayer

Rates of •NO diffusion across the BLM as a function of BLM-Chol composition (mol%) (n=12, $p<0.05$ Tukey's post hoc test).

3.4.3 Quantitation of NPC1 and normal human fibroblast plasma membrane (PM) cholesterol content

Studies have shown that despite bearing diminished Chol within caveolar structures [Garver *et al.,* 2002], NPC1-deficient fibroblasts exhibit a marked increase in plasma membrane (PM) Chol content [Vainio *et al.,* 2005] and exhibit a concomitant decrease in membrane fluidity [Vainio *et al.,* 2005, Koike *et al.,* 1998]. To verify previous assertions, the PM-Chol content of both cell lines was determined and compared by two independent methods: i) direct analyses of phospholipid and Chol content of isolated plasma membranes and ii) by probing intact cells with a fluorescent-labeled (Alexa 532) perfringolysin domain 4 (PFO-D4), a PM-directed, cholesterol-rich domain-specific probe [Shimada *et al.,* 2002, Waheed *et al.,* 2001].

When intact cells were fixed and exposed to Alexa532-PFO-D4, the labeling of the NHFs was not uniform but punctate in appearance (Fig. 3.4A). In contrast, the NPC1 fibroblasts were labeled more uniformly across the surface of the cellular membrane (Fig. 3.4C). In order to confirm the cholesterol-specific binding of the Alexa532-PFO-D4 probe, NHFs were incubated with cholesterol-loaded MβCD to augment PM-Chol [Christian *et al.,* 1997]. Upon Chol-loading, the Alexa532-PFO-D4 labeling increased in uniformity (Fig. 3.4B) resembling the staining pattern of NPC1 cells (Fig 3.4C). In addition, raft-associated Chol was depleted from the PM of NPC1 cells by exposure to MβCD [Christian *et al.,* 1997] (Fig. 3.4D), resulting in loss of the uniform labeling pattern and development of a staining pattern resembling that of Alexa532-PFO-D4-labelled NHFs (Fig. 3.4A). In the case of NPC1 fibroblasts, incubation of the cells with MβCD resulted in a ~2.7 fold decrease in staining intensity. In a further experiment to confirm the specificity of PFO-D4, the NPC1 cells were first incubated with unlabelled

PFO-D4, washed, fixed then exposed to Alexa532-PFO-D4. Under these conditions the amount of cell surface-bound label decreased to levels obtained with NPC1 cells treated with 2 mM MβCD (Fig. 3.4E, right most bar). Image density plots of the data (Fig. 3.4E; summary of n = 9 experiments) revealed that NPC1 fibroblasts stained ~3.7-fold more intensely upon staining with 400 nM Alexa532-PFO-D4 label than NHFs and that upon enhancement of the NHF-PM with Chol-loaded MβCD (100 μM), cholesterol-specific labeling increased by ~2.5 fold.

In agreement with previous reports, plasma membranes isolated from NHF and NPC1 fibroblasts were positive for PM-resident proteins (data not shown), had similar phospholipid content (Fig **3.5**) but exhibited ~2-fold higher total Chol content in the PM of NPC1-deficient versus NHFs (Fig. 3.**5**). The difference in relative cholesterol levels between methodologies (~2-fold by cholesterol isolation vs ~3.7 by D4-labeling of fixed cells) may reflect the previously reported ability of cell fixation to increase cholesterol accessibility [Lange *et al.,* 1983], however these experiments clearly illustrate that NPC1-deificient fibroblasts contain PM-Chol than their normal counterparts.

Noting the importance of hydrophobic environments in •NO signaling we questioned whether cholesterol, as a critically important and abundant component of the plasma membrane, was capable of influencing •NO diffusion and reactivity and resultant NO-signaling (i.e. cGMP production and nitrosation).

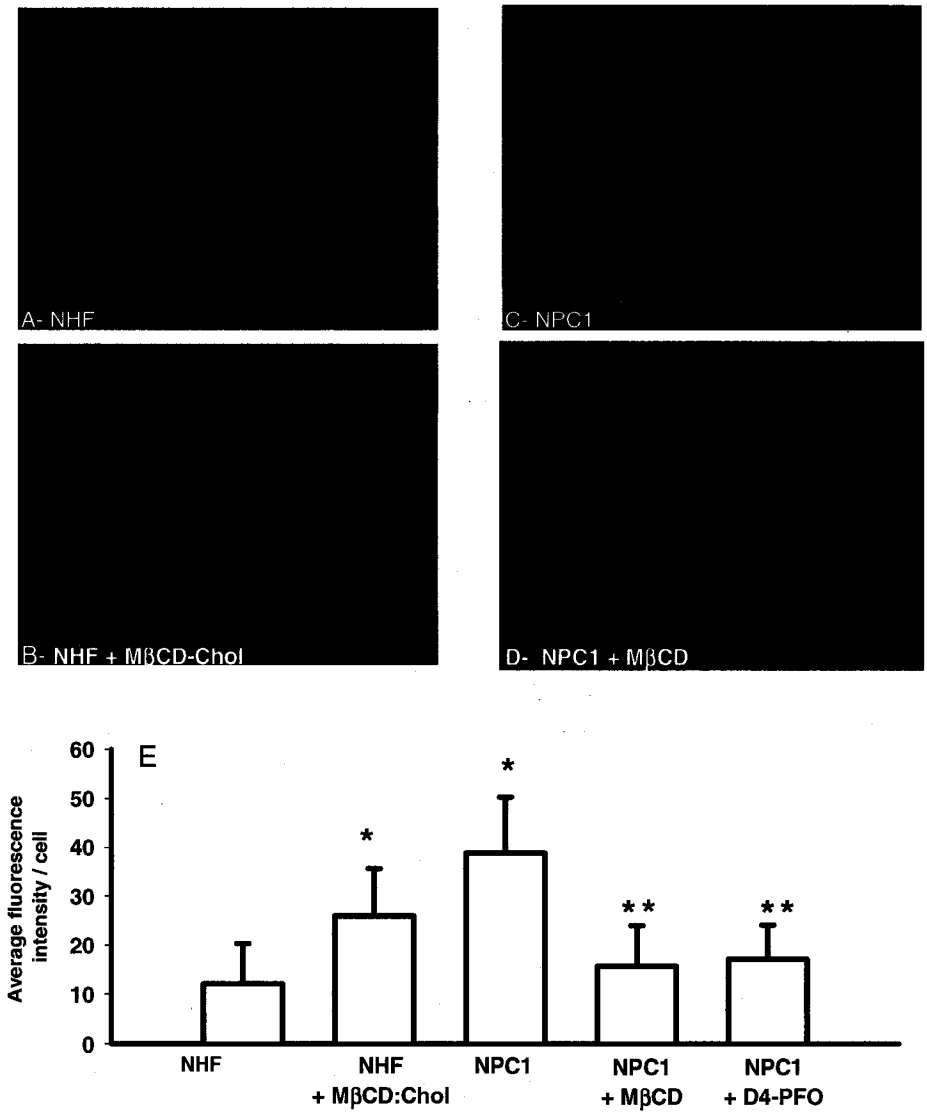


Figure 3.4 PM cholesterol of NHF vs NPC1 fibroblasts by fluorescent imaging of D4-labeling

Fixed normal (A) and NPC1-deficient fibroblasts (C) stained with 400 nM Alexa 532-labeled D4 and imaged under identical exposure conditions exhibited cholesterol-dependent fluorescence. Normal human fibroblasts repeatedly stained less intensely than their NPC1-deficient counterpart. Importantly, treatment of NHFs with 100 μM cholesterol-loaded MβCD was associated with an increase in cellular staining (B) and staining observed in NPC1-deficient cells could be largely abrogated by treatment with 10 mM MβCD prior to fixation (D). Data taken from multiple images in nine separate experiments are summarized numerically from average intensities per cell for not less than 50 cells (E). Errors represent standard deviation from the mean. *$P < 0.005$ compared to NHF. **$P < 0.001$ compared to NPC1.

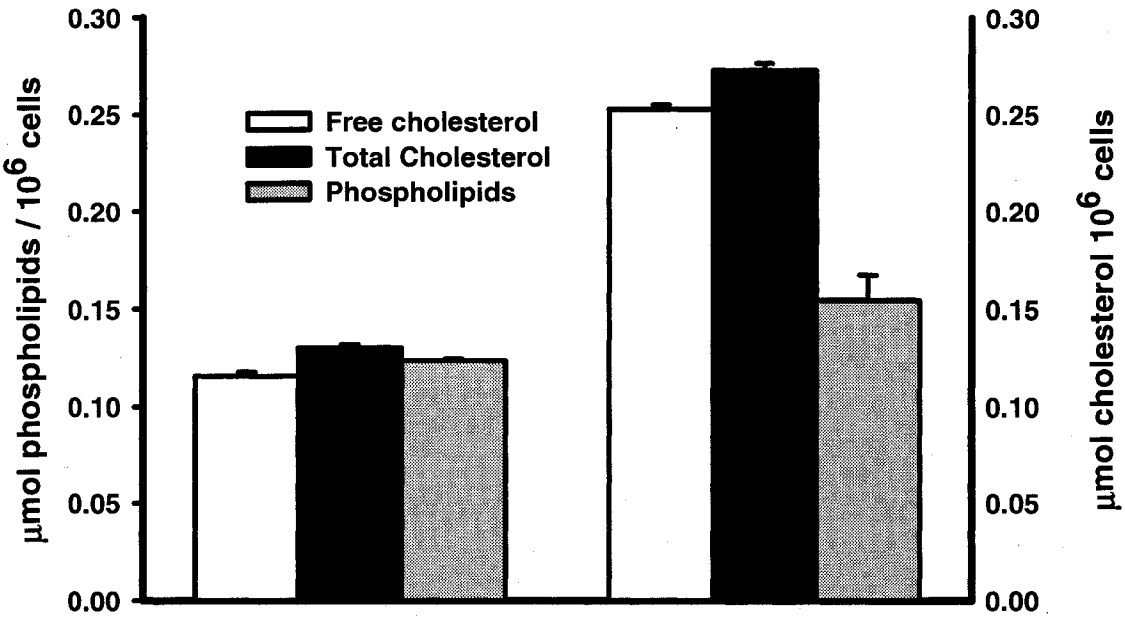


Figure 3.5 PM cholesterol:phospholipid of NHF vs NPC1 fibroblasts by analysis of lipid isolates

Cholesterol and phospholipid levels were determined from plasma membrane isolates obtained by sucrose gradient ultracentrifugation and are representative of two separate experiments with error shown as the standard deviation of the mean.

3.4.4 Responsiveness of NPC1-deficient and NHFs to bolus •NO by cGMP response

NPC1-deficient and normal human fibroblasts were used as a model to compare whether variations in the cellular cholesterol content and localization affected NO-dependent reactions in intact cells.

We first determined whether NO-signaling, as assessed by •NO-mediated cGMP production, was altered in NPC1-deficient cells versus NHFs. •NO-dependent cGMP generation in NPC1 fibroblasts was 44.6±5.3% (n=3 experiments, ***$p<0.005$) of that accumulated in NHFs in response to 3 μM of extracellular NO (Fig. 3.6A). NHF cGMP production could be brought to near NPC1-levels by exposing the cells to 100 μM Chol-loaded MβCD (Fig. 3.6B). The lower response in NPC1 fibroblasts was not due to lower guanylate cyclase activity as lysates derived from equivalent numbers of both NPC1 and NHF cells produced nearly identical amounts of cGMP when stimulated with bolus •NO following, rather than prior to cell lysis (Fig. 3.6C).

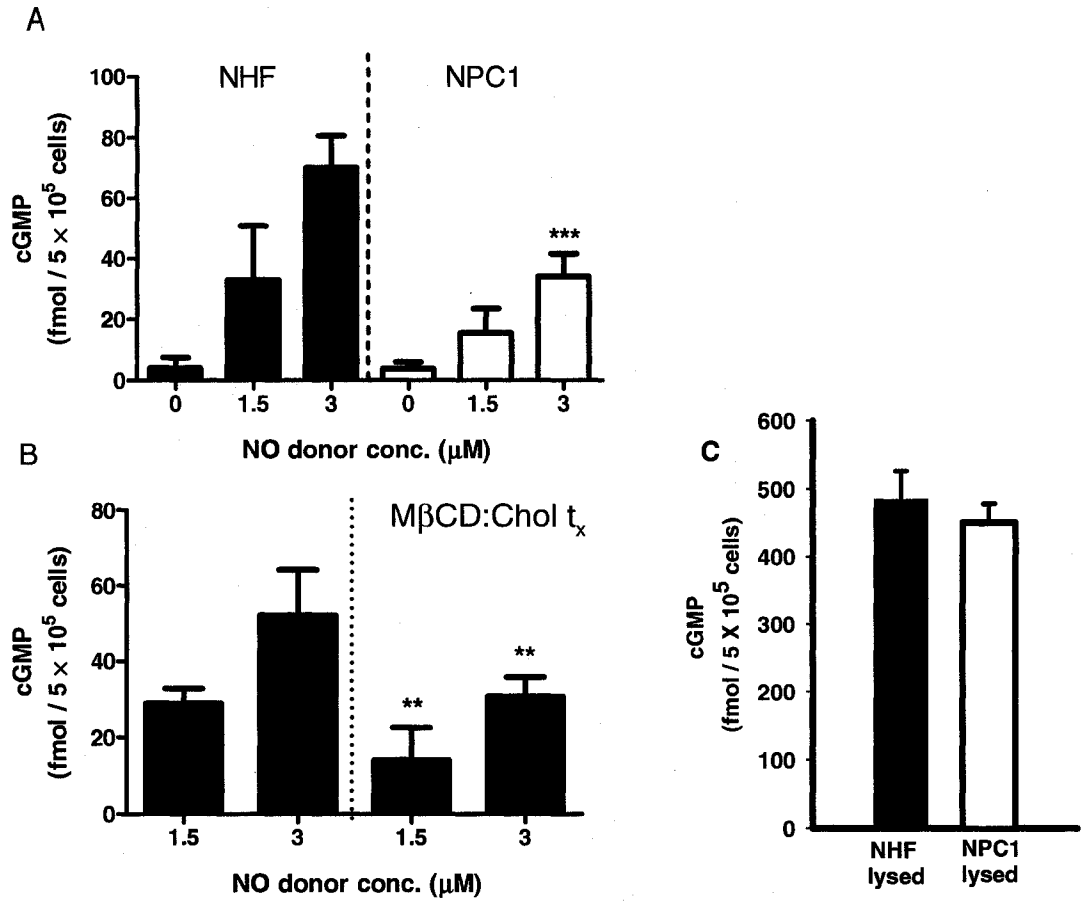


Figure 3.6 Comparison of the NHF vs NPC1 cGMP response mediated by bolus •NO

Cyclic guanosine monophosphate was measured by enzyme immunoassay following stimulation of normalized cell counts of both NHF and NPC1-deficient fibroblasts with identical concentrations of bolus authentic nitric oxide and compared in (A). ***P<0.005 versus NHFs at the same [•NO], n = 3 experiments. Normal human fibroblasts were then treated with 100 μM cholesterol-loaded MβCD to increase PM cholesterol and similarly stimulated with •NO prior to measurement of cGMP for comparison to NHFs bearing normal PM cholesterol (B) **P<0.05 versus untreated NHFs at the same [•NO], n = 3 experiments. As a control, cGMP was measured following •NO treatment of lysates of both normal human and NPC1-deficient fibroblasts and found not to be statistically different in n = 2 experiments (C).

3.4.5 Influence of cholesterol on the nitrosation of extracellular DAF in the presence of either NPC1-deficient or NHFs

Several papers have noted drawbacks in the imaging of intracellular •NO reactions in DAF-loaded cells [Zhang *et al.,* 2002, Espey *et al.,* 2002, Jourd'heuil D *et al.,* 2002]. Alternately, *extracellular* DAF (DAF-2) will compete for nitrosating equivalents with intact cells delivered from physiological fluxes of •NO generated upon decomposition of DEA/NO [Espey *et al.,* 2001]. With this experimental design, we were able to obtain insight into the factors influencing the cellular metabolism of •NO and resultant extracellular nitrosative reactions while circumventing noted interferences.

In extension to the observed cholesterol-dependence of •NO-induced cGMP accumulation, 5 X 10^5 NPC1-deficient fibroblasts/mL exhibited ~3-fold larger nitrosative response when treated with 250 nM DEA/NO in the presence of cell-impermeant 10 μM DAF-2 versus an identical suspension of NHFs (Fig. 3.7A) in n = 3 experiments. Error bars are expressed as the standard error of mean naphthatriazole accumulation for quadruplicate samples following completion of decomposition (n = 3 experiments, $p < .001$) in the presence of NHFs vs NPC1s in all three experiments. Upon inspection of the fluorescence traces of naphthatriazole (DAF-2T) accumulation in the presence and absence of cells, one notes that in the initial stages of NO flux, that the rate of DAF-2T formation is unity in the presence or absence of cells. Only after the initial flux of •NO has been delivered, are the divergent rates observable. Higher fluxes of NO achieved during early time points promotes nitrosative reactions and only after the slowing of NO delivery do differences in competing processes manifest. Results thus more likely reflect variation in the net cellular metabolism of •NO arising from differences in sterol levels or localization.

Further, it was observed that a one hour treatment with 0.2 units/mL cholesterol oxidase resulted in diminished DAF nitrosation in the presence of either NHF (86.5 ± 4.0%, n=3, $p<0.05$) or NPC1-deficient fibroblasts (74.9 ± 14.2%, $p<0.05$) versus buffer-treated controls (Fig. 3.7B). Measurement of membrane cholesterol by enzymatic assay confirmed a 47.6±7.3% and 33.2±1.4% decrease in membrane cholesterol NHF and NPC1 cells, respectively. Alternately, treatment of each cell type with 100 μM MβCD:cholesterol adduct for one hour enhanced membrane cholesterol 4.25±.25 fold in NHFs and 2.39±.19-fold in NPC1-deficient cells and resulted in modest increases in DAF nitrosation in the case of NHFs (12.9± 7.1%, n=3, $p<0.05$) that were, however, not statistically significant in the presence of NPC1-deficient cells (10.8±7.9%, n=3, $p>0.5$) versus buffer-treated controls (Fig. 3.7B). Taken together these observations are consistent with the hypothesis that membrane cholesterol is capable of modulating nitrosation reactions in the presence of nanomolar to micromolar fluxes of •NO.

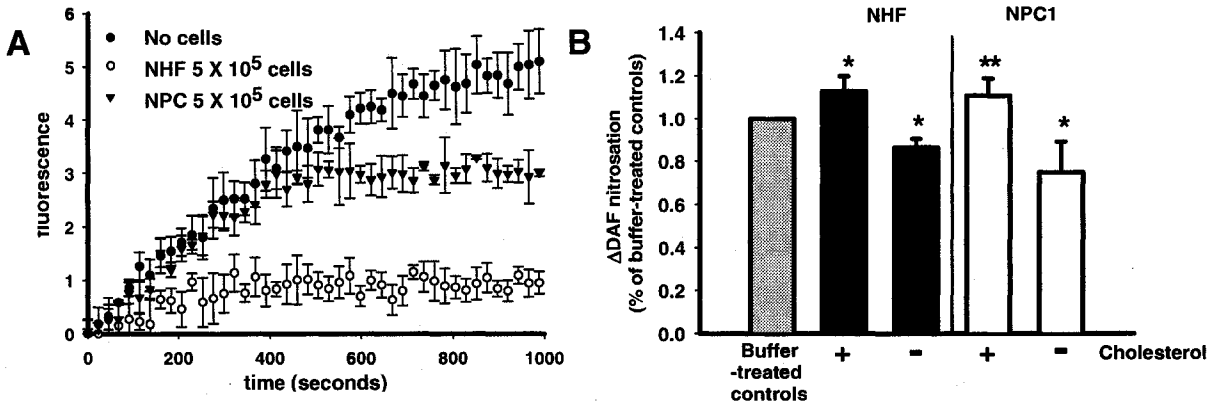


Figure 3.7 DAF nitrosation is altered in the presence of NHF vs NPC1 fibroblasts upon modulation of plasma membrane cholesterol

Cell suspensions containing 5 X 10^5 cells/mL with 10 μM DAF were treated with 250 nM DEA/NO in quadruplicate in a microwell plate and the evolved fluorescence intermittently monitored with shaking over the course of DEA/NO decomposition at 37^0C. Shown are representative traces from n = 3 experiments with errors bars calculated as the standard error of n = 4 samples and $p < 0.001$ comparing the extent of nitrosation in the presence of NPC1 versus normal human fibroblasts (A) following the completion of •NO delivery. Collected results from experiments in which the membrane cholesterol was either enhanced (with 100 μM MβCD:Chol) or depleted (with 0.2 U/mL cholesterol oxidase) in both NHF (black bars) and NPC1-deficient cells (white bars). Shown are collected results from n = 3 experiments with errors bars calculated as the standard error accumulated naphthatriazole of n = 12 samples and *$P < 0.05$ versus buffer-treated controls (grey bars). Note that **$P > 0.05$ in the case of cholesterol-enhanced NPC1 cells (B). Experiments performed in the presence of the superoxide dismutase mimetic Mn(III)TmPyp were nearly identical (data not shown).

3.4.6 Comparison of the superoxide-independent effects of cholesterol modulation on extracellular DAF nitrosation in platelets, erythrocytes and MCF-7 cells

In order to test the generality of our observation, several cell types were treated to deplete/enhance membrane cholesterol and assessed for their ability to modulate DAF-nitrosation. Experiments were performed in the presence of the SOD-mimetic Mn(III)TMPyP to exclude potential changes in superoxide production upon depletion or enhancement of cholesterol that could alter nitrosative potential. In all three cell types tested, modulation of plasma membrane cholesterol induced similar patterns of DAF-nitrosation upon treatment with •NO donor in the presence of 5 μM Mn(III)TMPyP. Depletion of membrane cholesterol in the various cells impeded DAF-nitrosation between 10 and 33% in comparison to cells in which membrane cholesterol was enriched (Table 2). Collectively, these data are in accord with the notion that increased that cholesterol is capable of shifting the chemical equilibrium of NO towards formation of nitrosating species such as N_2O_3.

Table 1.2
Comparison of the superoxide-independent effects of cholesterol on DAF-nitrosation in the presence in various cells

150×10^5 / mL erythrocyte		1×10^6 / mL MCF-7 adenocarcinoma		5×10^5 / mL platelets	
% nitrosation – cholesterol depleted vs cholesterol enhanced cells					
81.1%	±16.9%	90.0	±6.3%	67.6	±17.5%
n = 3	p = 0.052	n = 4	p = 0.0129	n = 3	p < 0.005

3.5 Discussion

Although recent claims may challenge this long-standing axiom of nitric oxide behaviour [Herrera *et al.*, 2006], •NO is generally held to traverse the membrane freely [Subczynski *et al.*, 1996]. Oddly, although •NO and O_2 are similarly paramagnetic, lipophilic and low in molecular weight, membrane cholesterol is reported to act as a barrier to oxygen in model systems [Subczynski *et al.*, 1989] and as a determinant in oxygen gradients and uptake in cellular systems [Khan *et al.*, 2003], but is reported to have no effect on •NO permeation [Subczynski *et al.*, 1996]. The basis for this difference is, at this point, unknown but may reflect inadvertent differences in model membrane structure that arise as a result the domain-forming, lateral associations of cholesterol [Veatch *et al.*, 2003].

We thus investigated the effects of membrane cholesterol on •NO diffusion in artificial liposomal membranes doped with β-py-C_{10}-HPC. Preparation of either DPPC or DMPC liposomes bearing 30% cholesterol decreased the rate of transbilayer diffusion ~30% in comparison to liposome comprised of phospholipid alone, mirrors previous results obtained using oxygen [Fischkoff *et al.*, 1975]. These studies revealed that O_2 diffusion rates, like the rates measured for nitric oxide, were inversely related to cholesterol content. Suggestively, in the study by Denicola, diffusion coefficients for •NO in liposomes derived from erythrocyte plasma membranes were 13 – 70% less than those determined in liposomes comprised of egg phosphatidyl choline alone [Denicola *et al.*, 1996b].

Cholesterol is known to induce the 'condensation' of phospholipids acyl chains [Radhakrishnan *et al.*, 2005] and thus our observations are thought to result from lower

diffusivity of •NO through the more tightly packed bilayer. Similar results were obtained using planar bilayers, in which as little as 17 mol% Chol inhibited the transbilayer diffusion rates of •NO as much as 50% and increasing the Chol fraction to 37mol% resulted in a 75% decrease in diffusion rates (Fig. 7). These data show that manipulation of cholesterol levels can have a significant impact on transbilayer diffusion of •NO, altering the membrane transit time and possible intra-membrane reactivity.

In an attempt to assess the role of membrane lipids in modulating •NO and NO_x signals, we used human fibroblasts which bear a molecular defect in the sterol trafficking protein - NPC1. These cells consequently accumulate cholesterol in comparison to normal fibroblasts and have been previously reported to contain increased levels of PM Chol. Consistent with previous reports it was determined that NPC1-deficient fibroblasts exhibited ~2-fold greater [Chol]/[phospholipid] in plasma membrane isolates [Vainio *et al.,* 2005], but further exhibited ~3-fold higher content of cholesterol-rich lipid raft in their PM than in intact NHFs (as determined by the specific labeling of the fluorophore-tagged cholesterol-binding domain of PFO) [Ohno-Iwashita *et al.,* 2004].

Our experiments show that plasma membrane cholesterol exerts influence over both the cellular generation of cGMP induced by bolus authentic •NO as well as the cellular metabolism of •NO supplied from synthetic •NO donors as measured by extracellular DAF nitrosation. Inclusion of a SOD-mimetic would presumably circumvent changes in nitrosylation and nitrosation patterns that arise from reaction of NO with $O_2^{\bullet-}$, thus suggesting that results obtained may reflect physical differences or the formation of reactive intermediates that depend upon cholesterol.

More specifically, NPC1-deficient cells bearing high levels of plasma membrane cholesterol exhibited diminished cGMP accumulation, but enhanced extracellular DAF nitrosation versus their normal counterparts. Both cGMP accumulation and DAF-nitrosation could be modulated by altering plasma membrane cholesterol levels. These results extended to other cell types including platelets, erythrocytes and MCF-7 cells which showed varying degrees of change in DAF nitrosation in response to alterations in membrane cholesterol, thus emphasizing the generality of our observations.

Since guanylate cyclase and DAF undergo reaction with distinct •NO species (•NO vs N_2O_3 / $•NO_2$ respectively), any explanation of the apparent relationship between plasma membrane cholesterol and •NO signaling requires consideration of the identity of the nitrosating / nitrosylating species. Attempts were made to distinguish between observed effects arising from nitrosation versus those arising from the reaction of NO with superoxide by inclusion of a SOD mimetic. Although this would presumably obviate $O_2^{•-}$-mediated changes in DAF nitrosation, it certainly does not exclude alterations in •NO metabolism that arise from other radicals that could facilitate nitrosation reactions and thus must be considered in any explanation of our observations.

DAF nitrosation is not directly mediated by authentic •NO, but by 'activated' redox congeners of •NO including N_2O_3 and $•NO_2$. These reactions occur by fundamentally different mechanisms (nitrosation versus oxidation followed by nitrosylation as shown in Figure 1.1.5A and 1.1.5B) and thus, alterations in DAF nitrosation induced by a cellular system must be preceded by reactions which form one of these nitrosating species. Importantly, these experiments were also conducted in the presence of a superoxide oxide dismutase mimetic – Mn(III)TmPyP to prevent the

diffusion-limited reaction of •NO with the radical anion – superoxide. Consumption of •NO by $O_2^{\bullet-}$ (when contemporaneously produced) yields $OONO^-$ which is capable of reacting with DAF by oxidative nitrosylation forming the fluorescent DAF-2T [Espey MG *et al.,* 2002]. In excluding reaction with superoxide, observed changes in DAF nitrosation must be attributed to other causes.

Indeed it has been shown that cholesterol reduces the permeability of oxygen to model bilayer membranes by a factor of 5 [Subczynski *et al.,* 1989], substantially affects oxygen uptake in RBC [Buchwald *et al.,* 2000a, Buchwald *et al.,* 2000b] and is an important factor in determining the magnitude of oxygen gradients observed across the cell membrane [Khan *et al.,* 2003]. In these studies, incorporation of cholesterol into the plasma membrane was shown to increase the magnitude of the oxygen gradient. Conversely, transmembrane gradients were diminished upon depletion of plasma membrane cholesterol by resultant increases in intracellular oxygen levels.

We thus propose, in accord with our observations, that spatial control exerted by membrane cholesterol over •NO shifts the balance of •NO reactivity from intracellular nitrosylation of haem targets such as guanylate cyclase to extracellular nitrosation of nucleophiles potentially via cholesterol-dependent changes in membrane structure / properties which affect the apparent reactivity of nitric oxide. This is consistent with our results which show that upon depletion of plasma membrane cholesterol in both NPC1-deficient and NHFs that nitrosation of extracellular DAF is diminished. In addition, loading of cellular plasma membranes was found to enhance nitrosation of extracellular DAF consistent with the notion that by increasing membrane transit times, membrane cholesterol appears to alter subsequent reactions of NO promote formation of nitrosating

and modification of extracellular targets perhaps through enhanced reactivity with oxygen. At this time however, there is no evidence to suggest that the changes in transit times are rate-limiting and thus other mechanisms cannot be excluded.

In terms of the physiological significance of these studies, alterations in •NO - mediated accumulation of cGMP observed between normal and diseased states in human [Aydin *et al.,* 2001, Lucotti *et al.,* 2006] are in the range of those observed within this study and are thus believed to be of physiological significance. Further, altered •NO-diffusion and signaling may contribute to the etiology of Niemann-Pick disease. Recent studies have provided evidence that NPC1 cells exhibit defective •NO-dependent deamination of glypican-1 heparin sulphate which may arise from restricted import/export of nitric oxide [Mani *et al.,* 2006].

The current dogma is that cellular PM-lipid composition is tightly controlled and need not be considered in altered nitric oxide signaling. However, both the existence of diseases of cholesterol metabolism and trafficking and experimental evidence that suggests that elevated serum cholesterol levels may alter the plasma membrane content of critical cells [Kim *et al.,* 1991] reconsideration of this assumption may be warranted. The studies herein suggest that PM cholesterol levels as well as lipid composition can affect the transbilayer diffusion rates of •NO and therefore •NO-mediated intracellular signaling. In light of reports of similar findings for cellular oxygen levels [Khan *et al.,* 2003] a re-examination of the relationship between vascular disease, cholesterol and fatty acid composition of plasma membranes and their effect on the bioavailability of not only •NO, but also O_2 needs to be addressed.

PART II

CHAPTER 4

PLATELET SURFACE PDI REDOX STATUS AND PLATELET EVENTS IN DIABETES

4.1 Introduction

A number of studies now published emphasize the importance of platelet surface thiol isomerases in redox-dependent platelet events [Lahav *et al.,* 2003, Jordan *et al.,* 2005, O'Neill *et al.,* 2000]. Thiol isomerases are structurally-related members of the PDI family of proteins which possess both redox-dependent activity (attributed to the common thioredoxin consensus sequence – CXXC), as well as chaperone activity [Versteeg *et al.,* 2007]. Vicinal thiols within the CXXC motif exist in equilibrium with the disulfide form and depending upon the status of the active site can catalyze reduction, oxidation or isomerization [Wilkinson *et al.,* 2004]. Although the necessity of a particular redox potential in platelet aggregation has been noted [Essex *et al.,* 2003], little has been done to directly explore specifically the role of thiol isomerase redox status and its influence on platelet physiology.

The best studied member of this family is the 56 kDa protein disulfide isomerase which has been localized to the surface of platelets where it is catalytically active and critically involved not only in aggregation, adhesion, and secretion, but also in inhibition of these events [Root *et al.,* 2005, Miersch *et al.,* 2007]. Notably, monoclonal antibodies to PDI have been shown to inhibit thrombin-stimulated activation of the αIIbβ3 integrin (measured by PAC-1 binding) [Essex *et al.,* 1999], adhesion to fibrinogen [Lahav *et al.,* 2000] and aggregation [Lahav *et al.,* 2002] in comparison to treatment with non-specific immunoglobulins.

Although there have been numerous studies into the redox-dependent behaviour of PDI, its influence in the context of platelet function requires characterization. Although it is generally held that free thiols are required for platelet aggregation [Lahav

et al., 2002], these assertions 1) are based upon experiments in which all free thiols were irreversibly blocked, 2) do not consider the possibility of reversible oxidation to the disulfide and 3) require more detailed interpretation in the context of the both redox dependence of aggregation and pathological changes in the vasculature.

PDI active site thiols are susceptible to oxidation by hydrogen peroxide [Kim *et al.,* 2000, Miersch *et al.,* 2007], oxidized glutathione [Raturi *et al.,* 2007] and S-nitrosothiols (with concomitant release of nitric oxide) [Root *et al.,* 2005, Raturi *et al.,* 2007]. We have recently shown that in animal models of pre-diabetes that a diet-induced increase in platelet and endothelial ROS production resulted in enhanced initial rates of aggregation and diminished platelet metabolism of S-nitrosoglutathione [Miersch *et al.,* 2007]. Importantly, these characteristics could be normalized by treatment with the HMG-CoA reductase inhibitor – rosuvastatin.

We now extend these studies comparing various characteristics of platelets from human normal and diabetic subjects. Further in vitro experiments were conducted to gain insight into the influence of human platelet-surface PDI (psPDI) redox status.

We have found, in agreement with others [Signorello, *et al.,* 2007] that platelets from diabetic subjects exhibited significantly elevated production of ROS compared to those from normal human subjects. Suggestively, they also exhibited diminished psPDI-mediated denitrosation and disulfide reductase activities but enhanced aggregation potential.

In vitro experimentation show that the oxidation of psPDI active site thiols using either H_2O_2 or decreasing ratios of GSH/GSSG results in 1) loss of platelet denitrosation activity 2) loss of disulfide reductase activity and 3) enhanced rates of initial aggregation.

Under these conditions we demonstrate the specific loss of psPDI thiols by maleimide labeling. However, our attempts to investigate specifically the role of psPDI-redox status on platelet aggregation, demonstrate that inhibition of aggregation associated with the loss of psPDI can be restored with exogenous enzyme, however, in a redox-insensitive manner. Consequently, increased rates of platelet aggregation observed under oxidizing conditions must be attributed to factors other than redox-dependent changes in psPDI.

4.2 Materials and equipment

4.2.1 Materials

Glutathione (GSH), oxidized glutathione (GSSG), dithiothreitol (DTT), EDTA, PMSF,trisodium citrate dihydrate, citric acid monohydrate, dextrose, NaCl, Na_2HPO_4, KH_2 PO_4, KCl, $NaHCO_3$, $MgCl_2 \cdot 6H20$, $CaCl_2 \cdot 6H_20$, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), Sephadex G-10 and G-100, apocynin, 4,5-diaminofluorescein (DAF-2), eosin isothiocyanate, bacitracin, thrombin and horseradish peroxidase (HRP) were purchased from Sigma-Aldrich Canada (Oakville, ON). Prostaglandin E_1 was obtained from Avanti Polar Lipids (Alabaster, AL). 10-acetyl-3,7-dihydroxyphenoxazine (Amplex Red) and Alexa 532 succinimidyl ester were obtained from Invitrogen (Burlington, ON). Mn(III)tetrakis(1-methyl-4-pyridyl) porphyrin pentachloride (Mn(III)TMPyP) from Cedarlane Labs (Hornby, ON). RL90 anti-PDI mouse monoclonal antibodies were obtained from Abcam (Cambridge, MA). Immobilized Protein G agarose beads were obtained from Pierce Biotechnology, (Rockford, IL).

4.2.2 Equipment

Agilent 8453 UV-VIS Spectrophotometer; Agilent Technologies Canada Inc., Mississauga, ON

Apollo 4000 Free Radical Analyzer; World Precision Instruments USA, Sarasota, FL

E_x808 Biotek Absorbance Microplate Reader, Biotek Instrument Inc., Winooski, VT

Haemocytometer; Reichert Co, Buffalo, NY

ISO-NOPF200 200 μM Flex NO Sensor; World Precision Instruments USA, Sarasota, FL

Mettler AJ1000 Balance; Mettler, Toledo Canada, Mississauga, ON

Orion Model 420A pH Meter, Thermo Electron Corp. Canada, Burlington, ON

Varian Eclipse Fluorescence Spectrophotometer, Varian Canada, Mississauga, ON

Wallac 1420 Victor^3 Fluorescent Plate Reader, Perkin Elmer, Woodbridge, ON

4.3 METHODS

4.3.1 Platelet isolation from whole blood

Whole blood was collected from the antecubital vein of healthy donors into ACD collection tubes. PRP was collected from whole blood after centrifugation at 900 g and treated with 1 μM prostaglandin E_1 to inhibit platelet activation.

4.3.2 Purification of recombinant human protein disulfide isomerase

PDI was expressed as the entire human sequence fused to an N-terminal His_6 tag in *Escherichia coli* strain BL21(DE3) using a pET-28a vector and isolated according to previously published methods [Sliskovic, *et al.,* 2005]. PDI was either fully oxidized or reduced by incubation with 20 mM GSSG or 20 mM DTT respectively for 2 hours at room temperature, purified over a G-25 column and stored at -80°C until use. Fully reduced enyme was further blocked by treatment with 20 mM iodoacetamide at pH 8.5 for 4 hrs at room temperature. Unreacted iodoacetamide was removed over a G-25 column and concentrated in a centrifugal concentrator. Enzyme redox status was verified by monitoring the disulfide reductase activity as indicated below following separation from reductant/oxidant/alkylating agent and immediately prior to use.

4.3.3 Preparation of S-nitrosoglutathione

S-nitrosoglutathione was synthesized according to previously published methods [Root *et al.*, 2005].

4.3.4 Preparation of di-eosin GSSG

Di-eosin GSSG was synthesized according to previously published methods [Raturi *et al.*, 2007].

4.3.5 Diabetic Inclusion Criteria

Healthy human subjects, ages 35-70 years were chosen to participate in the study only if they showed no overt symptoms of disease and were taking no medication. Diabetic human subjects, ages 35-70 were chosen to fulfill the criteria of the Expert Committee on the Diagnosis and Classification of Diabetes Mellitus [Meltzer *et al.*, 1998]. Patients on diet treatment alone or diet treatment in combination with oral hypoglycemic agents were included in the study, however, subjects taking investigational agents, insulin or that were pregnant were excluded from the study. The experimental protocol was approved by the University of Windsor Research Ethics Board.

4.3.6 Platelet aggregation

Platelets from PRP centrifuged at 1500 g and washed 2X in HEPES:ACD pH 6 and exposed to either a range of [peroxide] or a decreasing ratios of GSH/GSSG for 30 minutes. Aggregation was stimulated by treatment with 0.5 U/mL thrombin under the indicated redox conditions and aggregation monitored as the decrease in OD at 630 nm.

4.3.7 Measurement of platelet NAD(P)H-derived ROS

Platelets were washed and suspended in 100 mM phosphate buffer pH 7.2 containing 2 mM EDTA and split into equal portions. ROS was then measured in the presence and absence of 100 μM of the NAD(P)H oxidase inhibitor, apocynin [Manning *et al.,* 2003] using 15 μM Amplex Red and 1 U/mL HRP as a reporter system [Zhou *et al.,* 1997] on a Spectramax Gemini XS Fluorescent Microplate Reader (Molecular Devices, Sunnyvale, CA) using excitation / emission of 544 / 590 nm over 60 minutes at 37°C. Thus, apocynin-inhibitable ROS was calculated by subtraction of total ROS minus ROS generated in the presence of apocynin, yielding the contribution of ROS derived from NAD(P)H oxidase.

4.3.8 Analysis of platelet denitrosation by •NO -electrode

An aliquot of platelet suspension was added to a stirred cuvette containing 100 mM phosphate buffer, pH 7.2, with 1 mM EDTA, 100 μM GSNO and 50 μM Mn(III)TmPyP. Evolution of nitric oxide was monitored amperometrically using a Clark-type electrode (ISO-NOP, World Precision Instruments, Sarasota, FL) interfaced with the Apollo 400 free radical analyzer (WPI, Sarasota, FL). Signal was measured following addition of platelets as the change in current and [•NO] released interpolated from a standard curve.

4.3.9 Platelet disulfide reductase activity

Platelets were isolated from PRP and treated exactly as above. Disulfide reductase activity was monitored using a novel probe (N, N',-dieosin-GSSG) designed

within our lab and synthesized according to previously published methods [Raturi *et al.*, 2007] and capable of detecting platelet disulfide reductase activity in the absence of exogenous reducing agent. Platelets were suspended under the indicated redox conditions in the presence of 150 nM N, N',-dieosin-GSSG with the appropriate blanks and the increase in fluorescence indicative of disulfide bond reduction was monitored using a 525 / 545 excitation / emission couple.

4.3.10 Redox modulation of platelets

Several papers have noted the redox dependency of events during platelet aggregation [Essex *et al.*, 2003, Gregg *et al.*, 2004]. To better understand specifically the role of PDI redox status in platelet physiology we assessed several platelet PDI-dependent activities including 1) aggregation by turbidimetry, 2) disulfide reductase activity using a novel fluorescent probe and 3) denitrosation activity each under varying redox conditions by the methods indicated above. To corroborate observations, the redox status of platelet PDI was further investigated by 1) labeling of active site thiols with a thiol-directed biotinylating agent, 2) assessing the effect isopropanol-assisted removal of psPDI on aggregation and 3) replacing liberated psPDI with exogenous PDI of known redox status and evaluating its influence on aggregation.

4.3.11 Measurement of platelet-mediated GSNO consumption with DAF

Isolated platelets were incubated with varying [H_2O_2] ± bacitracin for one hour, washed and suspended in 10 μM GSNO for 15 minutes along with a GSNO control. Platelets were then removed by centrifugation at 900 g for 5 minutes and remaining

supernatant GSNO solution added to wells of a microplate containing 10 μM DAF and 25 μM $CuCl_2$, resulting in Cu^{2+}-mediated decomposition of GSNO and DAF-nitrosation [Cook *et al.*, 1996].

4.3.12 Labeling of platelet surface PDI with biotin-PEO_4 maleimide

Unwashed platelet pellet was suspended in HEPES:ACD buffer pH 6.5 containing 3 mM biotin-PEO_4 maleimide and gently nutated for 90 minutes. Platelet-surface PDI was then liberated from the cell surface by suspending in HEPES:ACD containing 5% isopropanol and gentle vortexing for one minute. Labeled, liberated PDI was then immunoprecipitated using Protein G beads covalently coupled to RL90 anti-PDI antibodies for 24 hours at 4°C. Beads were washed 2X with TBS and protein eluted by boiling in 1X loading dye containing 20 mM DTT for separation by SDS-PAGE. Separated proteins were transferred to a PVDF membrane and blotted with both RL90 anti-PDI antibodies and streptavidin-HRP. Thiol-specificity of platelet labeling was demonstrated by pre-incubating platelets with 1 mM DTNB for 10 minutes. This treatment abolished subsequent labeling according to the above procedure (data not shown). Further, analysis of platelet washes in the absence of labeling revealed endogenous biotinylation of several proteins at >150 kDa, however no interferences in the size range of PDI (data not shown).

4.3.13 Removal and replacement of platelet surface PDI

Platelet surface PDI was removed from 10^8 platelets by treatment with HEPES:ACD containing 5% isopropanol (IP) for one minute with gentle vortexing.

Platelets were then centrifuged for 5 minutes at 500 g and the supernatant removed after which platelets were washed once and resuspended in HEPES:ACD alone for aggregation studies. PDI was replaced by the addition of either 1 μM oxidized or reduced PDI or an equimolar mixture of the two to the reaction mixture prior to addition of thrombin and monitoring of OD.

4.4 RESULTS

4.4.1 Effects of RL90 anti-PDI antibodies versus non-specific antibodies on platelet disulfide reductase activity aggregation

Washed platelets were incubated with 5 μg/mL of RL90 anti-PDI antibodies (in ascites fluid) or an equivalent amount of non-specific, reagent grade IgG at 37^0C for 10 minutes. In assessing the enzymatic source of platelet surface disulfide reductase activity, 10^7 platelets per mL were suspended in HEPES buffer in individual wells of a black fluorescent microplate. A final concentration of 150 nM dieosin-GSSG was added to the wells with platelets in triplicate (in the absence of any exogenous reducing agent), and the evolution of fluorescence monitored with intermittent shaking at 37^0C using an excitation / emission couple of 525 / 545 nm. Results representative of n = 3 experiments show that probe reduction is substantially inhibited upon antibody-mediated blockade of platelet surface PDI in comparison to treatment with non-specific antibodies. For aggregation studies 10^8 platelets/mL in individual wells of a 96-well microplate were stimulated with 0.5U thrombin in the presence of immunoglobulin and the optical density read in a UV/VIS microplate reader at 630 nm at 37^0C with shaking. Readings were performed in triplicate with results shown representative of n = 3 experiments and show that antibody-mediated blockade of PDI results in marked inhibition of both the initial rates and extent of aggregation.

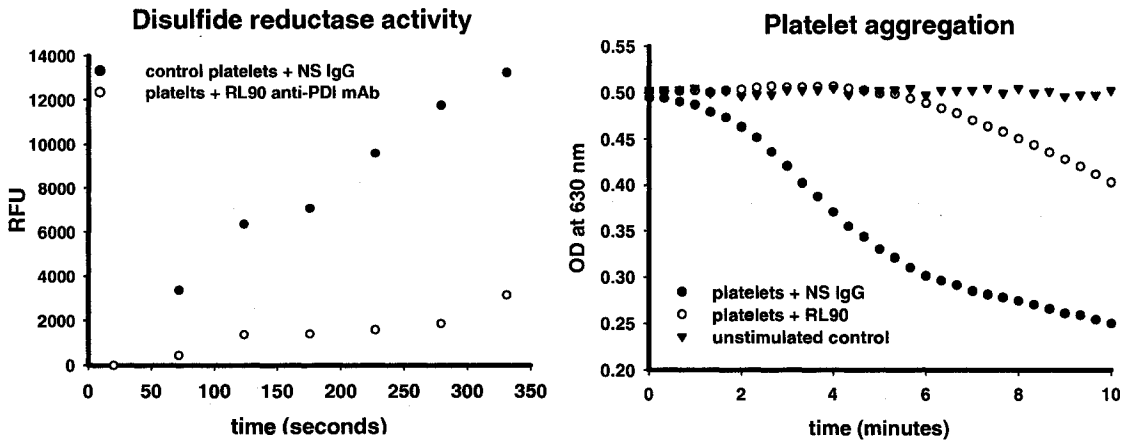


Figure 4.1 Effects of RL90 anti-PDI antibodies versus non-specific antibodies on platelet disulfide reductase activity and thrombin-stimulated aggregation

Fluorescence signal generated by platelet-mediated reduction of dieosin-GSSG was monitored in triplicate wells of a microplate following a brief incubation of platelets with anti-PDI antibodies (•) or an equivalent concentration of non-specific IgG (○)(left). The marked inhibition of disulfide reductase activity by anti-PDI mediated blockade suggests a central role for PDI in reducing disulfides at the platelets cell surface. As expected, anti-PDI antibodies also caused a significant decline in both the initial rates and overall extent of thrombin-stimulated aggregation (◊) in comparison to non-specific antibodies (♦). Controls run without thrombin stimulation (▼) confirm that the loss in OD is due to agonist-induced aggregation of platelet suspensions (right).

4.4.2 Platelets from diabetic subjects exhibit enhanced ROS production and aggregation but diminished disulfide reductase and denitrosation activity

Platelet NAD(P)H oxidase-derived ROS were measured by fluorometric assay. Platelet ROS generation was inhibited by apocynin [Manning RD Jr *et al.*, 2003] and the apocynin-inhibitable signal was subtracted from the total ROS signal, to obtain NAD(P)H oxidase-derived ROS. Results shown on left side of Fig. 4.1 were collected from n = 10 samples for both normal and T2D samples and indicate that platelets from T2D patients exhibited a 7-fold increase in ROS generation versus normal platelets ($P<0.005$). In contrast, results on the right show that psPDI on platelets derived from n=10 T2D patients released on average ~30% of the •NO release catalyzed by platelets derived from n=10 normal patients ($P<0.002$). As necessary controls, platelets injected into buffer alone or injection of buffer into a solution of 100 μM GSNO resulted in no significant generation of signal, confirming that in all cases we were observing platelet-mediated liberation of •NO. Previously, GSNO denitrosation by platelets was shown to be inhibited to >90% by anti-PDI antibodies and by the vicinal thiol blocker phenylarsine oxide (PAO) [Root *et al.*, 2005]

Aggregation profiles were also compared for n = 12 samples of normalized counts of platelets from both normal and diabetic subjects in Fig. 4.2 (left) and show a 35±8.2% ($P<0.05$) increase in initial rates of thrombin-induced aggregation of diabetic platelets confirming expectations. In contrast, comparison of the disulfide reductase activity for the same samples reveals a 42±9.3% decrease in psPDI-mediated activity in platelets derived from diabetic samples ($P<0.01$) as shown in Fig. 4.2 (right). In all cases error bars were calculated as the standard error of the mean and statistical analysis was performed using a paired *t*-test.

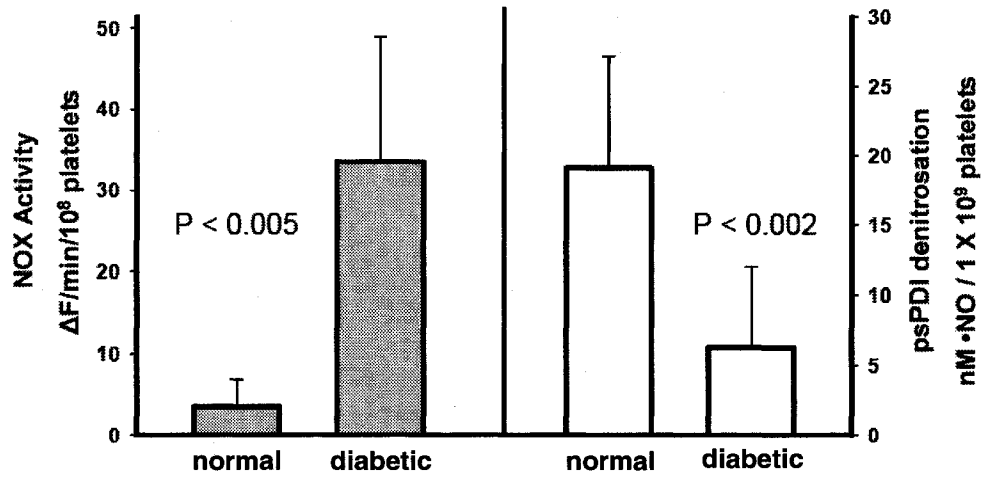


Figure 4.2 Comparison of platelet ROS and psPDI-mediated denitrosation activity of human normal versus diabetic platelets

NAD(P)H oxidase activity was measured using oxidation of the fluorophore, 10-acetyl-3,7-dihydroxyphenoxazine-HRP system as a reporter of ROS in the presence and absence of apocynin. NAD(P)H oxidase activity is shown on the left as the difference in fluorescence generated in the presence and absence of apocynin for n = 10 normal and n = 10 T2D platelet samples (P< 0.005 versus normal). In contrast, platelet PDI-mediated denitrosation activity in the presence of 50 μM Mn(III)TmPyP was measured amperometrically as the amount of •NO evolved per 10^9 platelets for both n = 10 normal and n = 10 T2D samples (P< 0.002 versus normal).

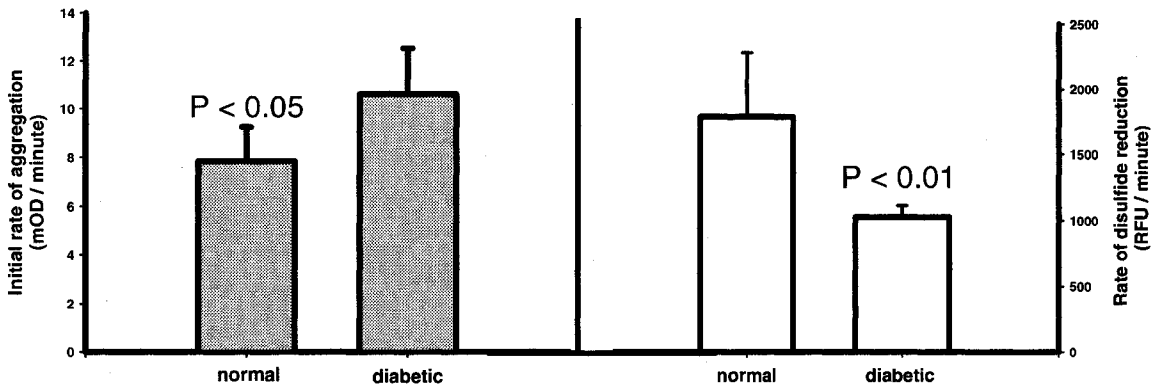


Figure 4.3 Comparison of the initial rates of aggregation and psPDI-mediated disulfide reductase activity in human normal vs diabetic platelets

Platelets were isolated from whole blood of diabetic and non-diabetic patients as indicated then suspended in HEPES buffer (pH 7.2). Triplicate platelet samples in microplate wells at a final concentration of 10^8/mL were stimulated with 0.5 thrombin/mL and aggregation monitored as the loss in OD at 630nm. Plots shown are average initial rates of aggregation measured for n = 12 diabetic and non-diabetic platelets with error bars representing the standard deviation of the mean ($P<0.05$) (left). Alternately, triplicate samples were suspended in HEPES buffer (pH 7.2) containing 150 nM dieosin GSSG in fluorescence microplate wells at a final concentration of 10^7 platelets/mL. The increase in fluorescence, indicative of intrinsic platelet disulfide reductase activity, was monitored using an excitation / emission couple of 525 / 545 nm in a fluorescence microplate reader. Plots shown are average initial rates of disulfide reduction measured for n = 12 diabetic and non-diabetic platelets with error bars representing the standard deviation of the mean ($P<0.01$) (right).

4.4.3 Platelet psPDI-mediated disulfide reductase activity is inhibited by decreasing GSH/GSSG and by increasing H_2O_2

Platelet disulfide reductase can be inhibited by the addition of anti-PDI antibodies (Figure 4.1). Although PDI is known to be susceptible to alterations in redox environment we sought to confirm this on the platelet cell surface. Treatment of platelets with either decreasing GSH/GSSG or with increasing H_2O_2 resulted in a dose-dependent inhibition of disulfide reductase activity measured by the reduction of dieosin-GSSG probe, likely due to oxidation of psPDI active-site thiols. In our studies, it is assumed that in light of the relatively low extracellular concentrations of GSH and GSSG (<200 μM total) and the brief incubation period (<5 min) that its effect on the intracellular GSH/GSSG ratio would be minimal. Importantly, treatment of 10^7 platelets / mL with equimolar GSH and GSSG (100 μM each) resulted in ~80% inhibition of platelet disulfide reductase activity (Fig 4.4A). This result is of physiological significance given that the thiol:disulfide ratio of low molecular weight plasma thiols is thought to vary between ~13:1 and ~1:1 depending on prevailing conditions [Go *et al.*, 2005].

Further, platelet disulfide reductase activity could be inhibited by increasing [H_2O_2] to a maximum of ~75% at 100 μM H_2O_2 (Fig 4.4B) suggesting that inhibition occurs through oxidation of the active site thiols of psPDI.

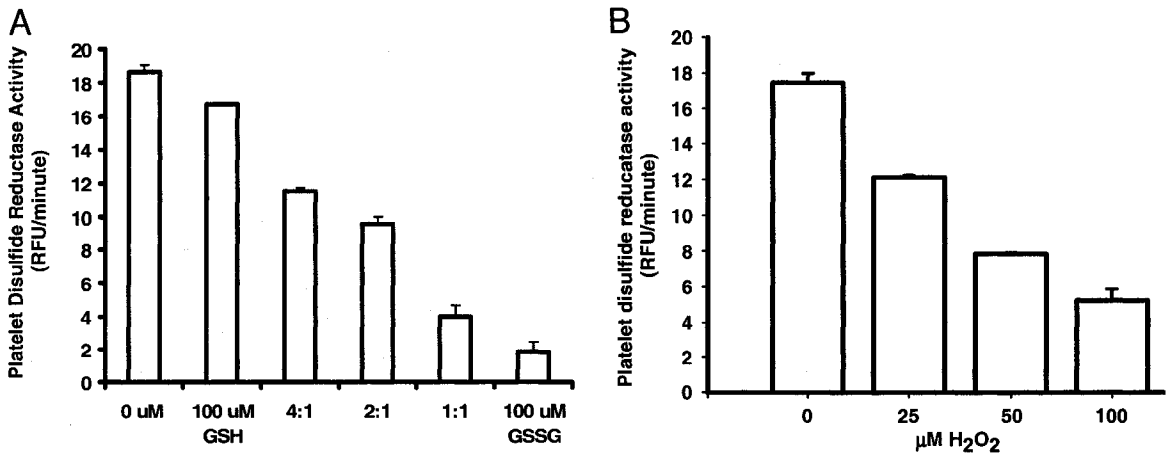


Figure 4.4 ***In Vitro*** **effects of varying redox conditions on psPDI-mediated disulfide reductase activity**

Washed platelets were suspended at a density of 10^7/mL in HEPES buffer (pH 7.2) in the presence of 150 nM dieosin-GSSG and the indicated concentration (or ratio) of reduced and oxidized glutathione (A) or hydrogen peroxide (B). Intrinsic platelet reductase activity was measured as the increase in fluorescence that occurs upon reduction of the probe in the presence of oxidant. Shown in both plots are values representing the initial rates of fluorescence increase for triplicate samples representing the results of n = 3 experiments. Error bars were calculated as the standard deviation of the mean. As a control, the fluorescence of reduced probe was monitored in the presence of the highest [oxidant] used and showed no evidence of quenching that would indicate oxidation of the probe back to the disulfide form.

4.4.4 Biphasic response of thrombin-stimulated platelet aggregation under conditions of decreasing GSH/GSSG correlates to oxidation of psPDI

In order to determine how the observed inhibition of platelet disulfide reductase activity under conditions of decreasing GSH/GSSG correlates with its influence on rates of platelet aggregation, washed platelet were suspended in identical ratios of glutathione and its oxidized disulfide for measurement of thrombin-induced aggregation. Interestingly, results shown in Fig. 4.4A demonstrate that initial rates of aggregation are maximal at both the highest and lowest ratios of GSH/GSSG. These results are in accord with reported observations in which aggregation could be potentiated by both the addition of low concentrations of either GSH or GSSG [Essex *et al.,* 2003]. However, this provides an alternate explanation for observations focused instead on the redox status of psPDI. Labeling of PDI thiols on the surface of intact platelets using a biotinylated-maleimide reagent (shown in Fig. 4.4B) confirms the oxidation of PDI on the platelet surface as suggested by the inhibition of disulfide reductase activity. This further suggests that the loss of psPDI-mediated disulfide reductase and denitrosation activity exhibited by platelets obtained from diabetic patients versus their normal counterparts could also be caused by ROS-dependent changes in the plasma thiol-disulfide balance.

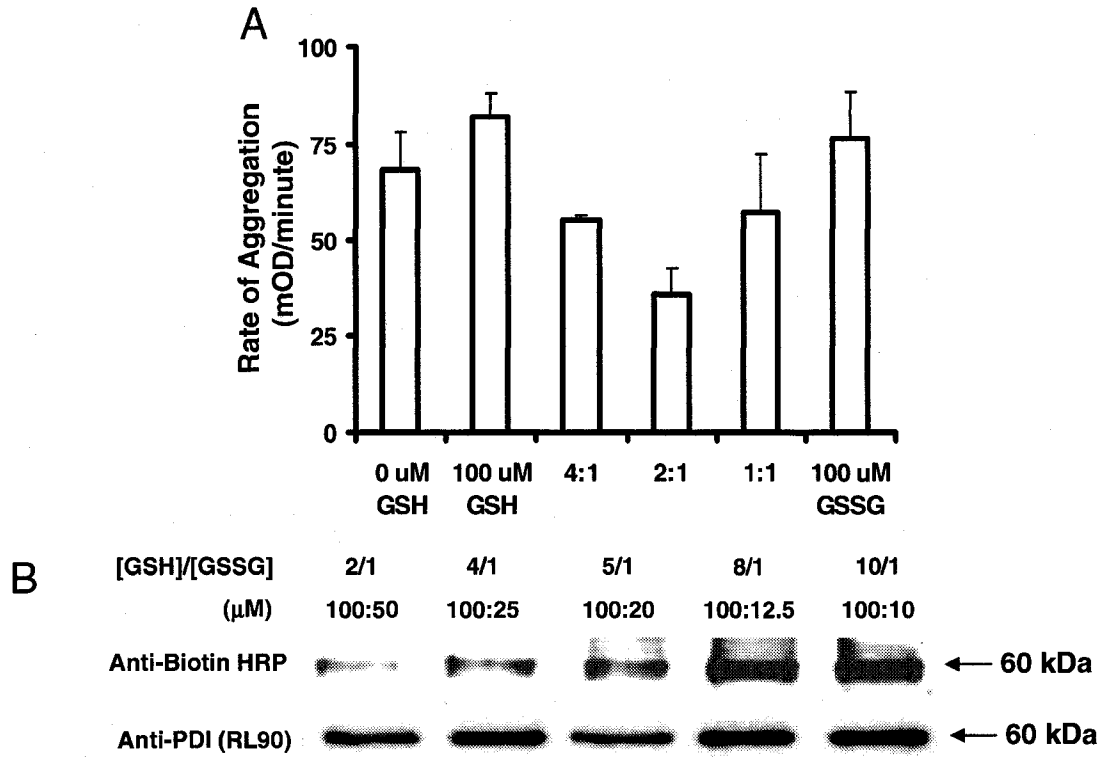


Figure 4.5 *In vitro* effects of varying redox conditions on psPDI-mediated aggregation events and redox status of psPDI

(A) Washed platelets were suspended in the wells of a microplate containing the indicated GSH/GSSG at a constant [GSH] of 100 μM in triplicate and stimulated with 0.5 U/mL thrombin. Plots shown are representative of n=5 experiments, calculated as the average initial rates of aggregation with error bars calculated as the standard error of the mean of three replicates. (B) Platelets were labeled with PEO_4-biotin maleimide at pH 6.5 for one hour at 37°C under the indicated GSH/GSSG. Platelets were then washed once with 100 μL of 5% IP as indicated and PDI immunoprecipitated from the supernatant using RL90 anti-PDI antibodies covalently bound to Protein G-Sepharose. Liberated PDI was run on an SDS-PAGE and transferred to a solid support for probing with both strepavadin-HRP and anti-PDI antibodies.

4.4.5 Bacitracin protects platelets from the ability of H_2O_2-treatment to potentiate thrombin-induced aggregation

Washed platelets exposed to increasing concentrations of H_2O_2 exhibit a progressive increase in the initial rates of thrombin-induced aggregation to ~2-fold increase at 25 μM H_2O_2 as observed in representative aggregation profiles shown in Fig. 4.5A. In order to implicate PDI in events contributing to the potentiated rates of aggregation, platelets were subsequently treated with 25 μM H_2O_2 in the presence or absence of the thiol-isomerase-specific inhibitor – bacitracin at a concentration known to inhibit platelet aggregation [Lahav *et al.*, 2002]. Although the mechanism by which bacitracin is capable of inhibiting thiol-isomerases is unknown, it is thought to inhibit multiple isomerases on the platelet cell surface [Robinson *et al.*, 2006]. Nevertheless, the rationale for this experiment predicted that if bacitracin is capable of inhibiting the thiol-isomerase activity of psPDI, that it could also protect active site thiols from oxidation by blocking access to the active site. By washing and diluting platelets following oxidant treatment in the presence or absence of bacitracin, excess bacitracin could be diluted to a level at which it would no longer inhibit platelet activity. This proved to be the case in that platelets treated with peroxide exhibited ~2-fold increase in initial rates of aggregation versus untreated controls. In the presence of bacitracin and 25 μM H_2O_2, rates of aggregation were restored to rates similar to untreated controls as shown in Fig. 4.5B. Importantly, this was not simply due to inhibition of potentiated rates given that platelets treated with bacitracin alone showed no evidence of inhibition compared to untreated controls, suggesting that the platelet wash step and dilution was effective in removing residual inhibitor.

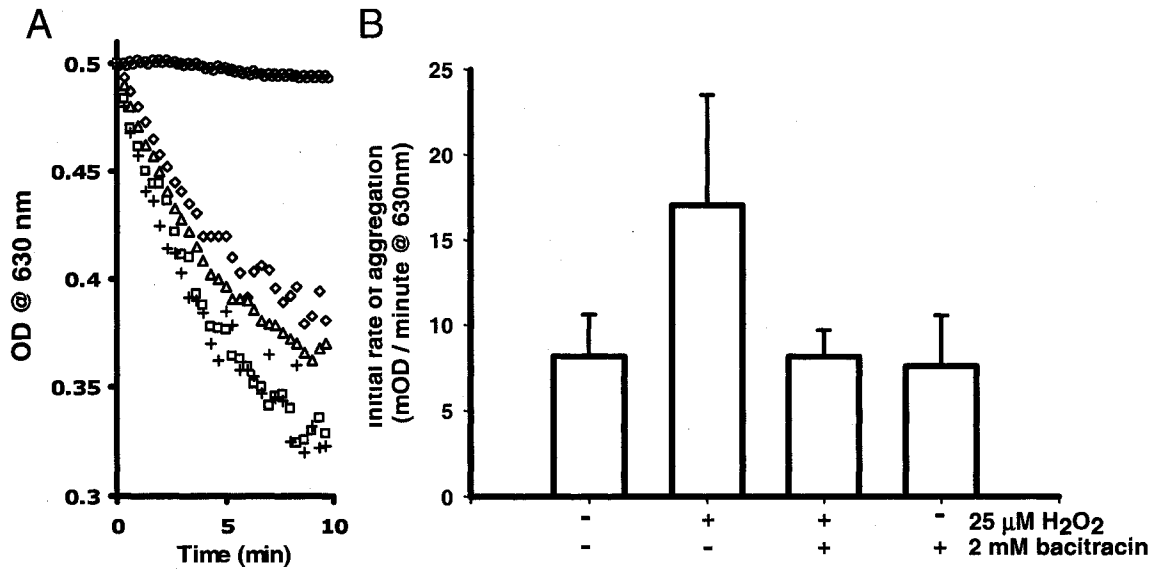


Figure 4.6 Bacitracin protects platelets from the ability of H_2O_2 treatment to potentiate thrombin-induced aggregation

Thrombin-induced platelet aggregation can be potentiated by treatment with H_2O_2. Representative aggregation traces are for experiments run in triplicate are shown in (A) for platelets treated with 0 (◊), 1 (▲), 10 (+) and 25 μM (□) H_2O_2 and stimulated by 0.5 U/mL thrombin versus unstimulated control platelets (○). Alternately, platelets suspended in HEPES:ACD buffer were incubated with the 25 μM H_2O_2 for 60 minutes in the presence or absence of 2 mM purified bacitracin. The platelet suspension was spun at 500 g for 5 minutes, the supernatant removed and platelets washed 1X with 20 volumes of HEPES:ACD buffer. Resuspended platelets were diluted 10X in HEPES buffer (pH 7.2) to a final concentration of 10^8/mL and stimulated with 0.5 U thrombin/mL. Change in OD was monitored at 630 nm for platelet samples run in triplicate for n = 3 experiments and shown as a representative plot of average initial rates of aggregation in (B). Error bars represent standard deviation of the mean for initial rates.

4.4.6 Bacitracin protects platelets from H_2O_2-mediated inhibition of psPDI-mediated denitrosation of S-nitrosoglutathione

We have shown previously that treatment of platelets with superoxide delivered from xanthine/xanthine oxidase effectively inhibited the ability of psPDI to liberate •NO from S-nitrosoglutathione [Miersch *et al.*, 2007]. Using human platelets we have similarly investigated the ability of H_2O_2 to inhibit metabolism of S-nitrosoglutathione. Incubation of platelets with increasing [H_2O_2] resulted in the progressive loss of GSNO consumption measured by the decomposition of residual GSNO by $CuCl_2$ in the presence of the NO_x-sensitive fluorophore – 4,5-diaminonaphthalene (DAF-2). Following treatment of platelets with the indicated [H_2O_2], platelets were washed once and resuspended in 10 μM GSNO for 15 minutes. Platelets were then removed by centrifugation and aliquots of supernatant containing GSNO reacted in the presence 25 μM Cu^{2+} and 10 μM DAF and residual GSNO and the evolved fluorescence compared to aliquots of an identical [GNSO] in the absence of platelets [Cook *et al.*, 1996]. Results shown in Fig. 4.6 demonstrate that as [H_2O_2] increases, platelet consumption of GSNO declines to near control levels at 100 μM H_2O_2. In order to further implicate psPDI in this activity, platelets were again treated with oxidant in the presence of 2 mM bacitracin. In similar fashion to results discussed in section 4.4.9, bacitracin protected denitrosation activity from the inhibitory effects of H_2O_2 bolstering evidence implicating psPDI as primarily responsible for platelet-mediated metabolism of GSNO. Importantly, bacitracin treatment in the absence of H_2O_2 did not affect platelet denitrosation activity confirming the effective removal of the inhibitor.

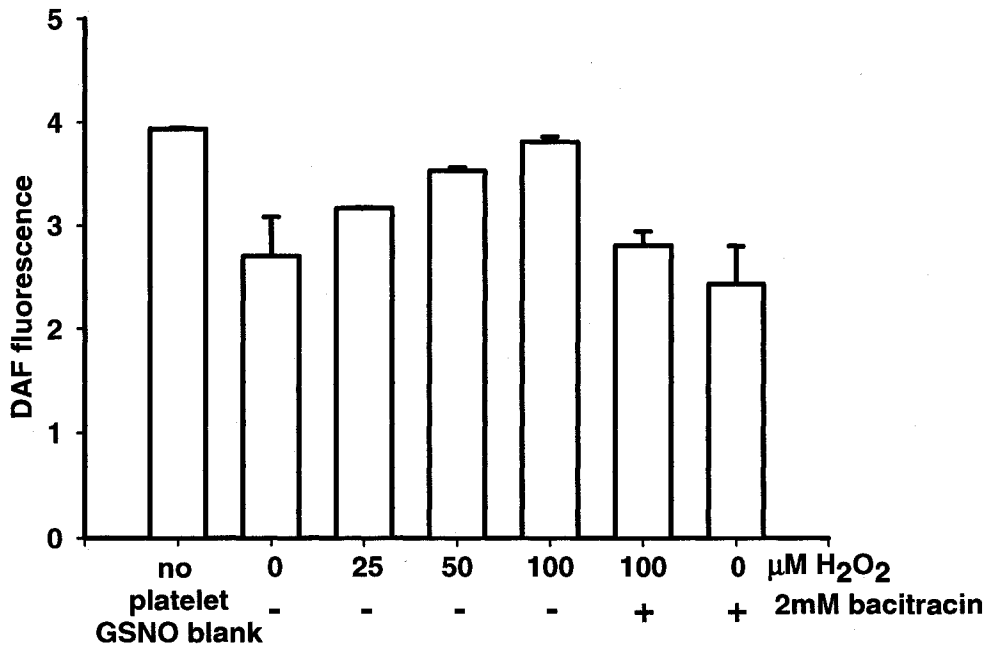


Figure 4.7 Bacitracin protects platelets from the ability of H_2O_2 treatment to inhibit psPDI-mediated denitrosation of S-nitrosoglutathione

Platelets suspended in HEPES:ACD buffer were incubated with the indicated [H2O2] for 60 minutes in the presence or absence of 2 mM purified bacitracin. The platelet suspension was spun at 500 g for 5 minutes, the supernatant removed and platelets washed 1X with 20 volumes of HEPES:ACD buffer. Platelets treated with peroxide and/or bacitracin as above, were resuspended in 10 μM GSNO and incubated for 15 minutes for comparison to GSNO alone (blank without platelets). To measure consumption of GSNO, platelets were removed by centrifugation at 500 g for 5 minutes. 20 μL aliquots of supernatant GSNO were decomposed by the addition of 25 mM Cu^{2+} in the presence of 10 μM DAF and evolved fluorescence measured in a microplate using 525 / 545 excitation / emission. Samples were run in triplicate and results shown are representative of three separate experiments.

4.4.7 Modulation of psPDI redox status by removal and replacement of cell surface enzyme – effects on aggregation

Studies have suggested that surface-bound PDI may interact with the cell exterior through electrostatic interactions [Terada *et al.,* 1995]. Alternately, it has been shown within our lab that brief, gentle washing of platelets with concentrations of isopropanol between 1 and 5% in HEPES:ACD result in an efficient, dose-dependent liberation of exofacial-bound platelet PDI by western blot (data not shown). We have exploited this observation to investigate specifically the role of PDI redox status in platelet aggregation.

Platelets were washed as indicated to effect removal of psPDI. Following isopropanol treatment platelets were separated by centrifugation, washed once with 20 volumes of HEPES:ACD and resuspended in wells of a microplate in quadruplicate. PDI (with the indicated redox status) was added to a total final concentration of 1 μM enzyme and platelets stimulated with 0.5 U/mL thrombin. Aggregation profiles representative of those obtained from 3 separate experiments are shown in Fig. 4.7A. Collected results with in Fig. 4.7B illustrate ~60% inhibition of aggregation which results with the loss of psPDI. It further illustrates the restoration of the initial rates of aggregation by replacement of liberated PDI with exogenous PDI, irregardless of the redox status of the enzyme, or even whether free thiols were present. This suggests that, although PDI contributes positively to platelet aggregation, it does so independently of its thiol isomerase activity. These results are not in conflict with aggregation experiments in which rates of aggregation could be potentiated by either reducing or oxidizing ratios of physiologica [GSH]/[GSSG]. Undoubtedly, other platelet-surface species are susceptible to redox modification and could be responsible for observed changes in aggregation rates.

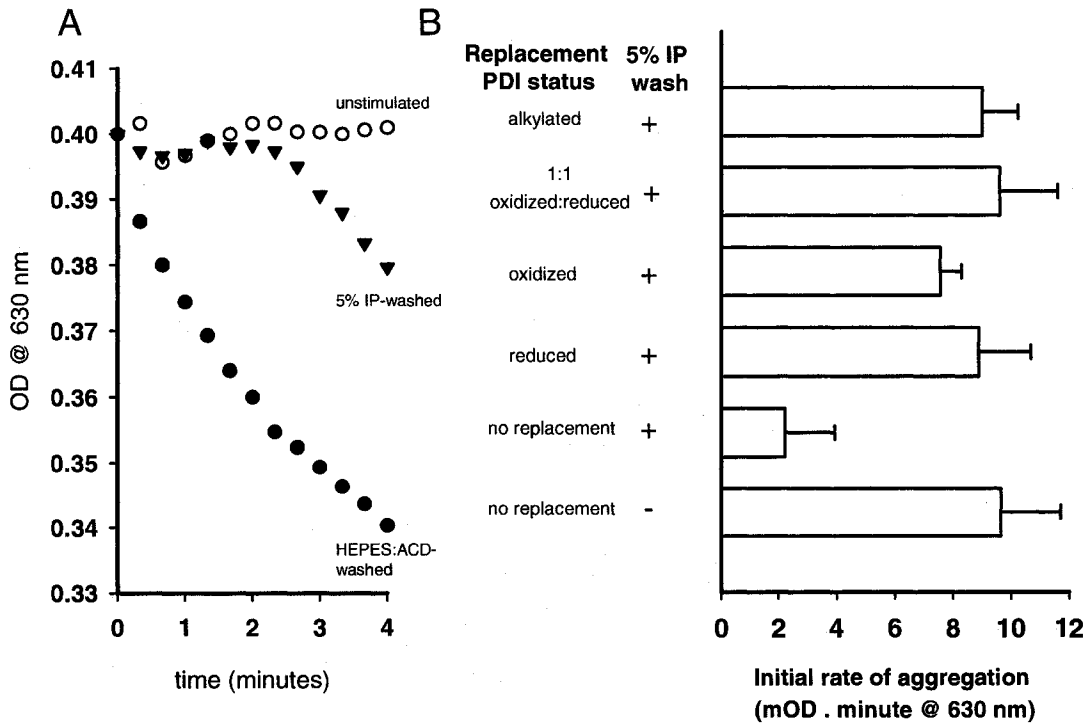


Figure 4.8 Removal and replacement of psPDI with PDI of defined redox status – effect on initial rates of aggregation

Platelets were washed with 5% isopropanol (IP) in HEPES:ACD pH 6.5 for 1 minute (▼) with gentle vortexing to assist removal of psPDI and initial rates of aggregation compared to platelets washed with buffer alone (•) and compared to unstimulated controls (○). Traces representative of n = 3 experiments are shown in (A). IP-washed platelets consistently exhibit a ~50 - 70% decrease in rates of aggregation versus buffer washed platelets ($p<0.05$). Initial rates of aggregation of IP-washed platelets can be restored by the addition of 1 μM of either fully oxidized or reduced recombinant PDI as well as by equimolar mixtures of the two and with PDI in which the active site is irreversibly blocked by alkylation. Collected data showing the average initial rates of aggregation for quadruplicate samples are shown in (B) with the error bars calculated as the standard error of the mean, representative of n = 3 experiments.

4.5 Discussion

In this study we found that ROS production from platelets of human T2D subjects was ~5-fold greater than their normal counterparts (Fig. 4.2). Measurement of platelet-surface PDI-dependent activities that require reduced active site thiols (namely denitrosation and disulfide reduction) revealed that both activities were 60%-70% lower in T2D platelets than controls indicating that psPDI was more oxidized in T2D (Fig. 4.2, 4.3). Similar to our earlier animal studies [Miersch *et al.*, 2007], platelets from T2D subjects aggregated with average initial rates that were ~1.4-fold faster than controls (Fig. 4.3). Although these observations strongly suggest oxidation of psPDI active site thiols, the question of whether psPDI oxidation state plays any significant role in potentiation of aggregation rates remained.

We attempted to test this by exposing control platelets to varying ratios of GSH/GSSG or to H_2O_2; a product of $O_2^{\bullet-}$ dismutation in the cellular milieu. As one would expect, psPDI disulfide reductase and denitrosation activities were inhibited by increasing oxidative potential as shown in Fig. 4.4A/B and 4.7.

It is commonly accepted that aggregation is influenced by dynamic shuffling of thiol and disulfide bonds at the platelet surface [Essex *et al.*, 2004b, Robinson *et al.*, 2006] and that psPDI plays a critical role in these events [Lahav *et al.*, 2002]. Although it is known that PDI exhibits redox-dependence [Wilkinson *et al.*, 2004] and that redox conditions within the vasculature are known to fluctuate [Go *et al.*, 2005], there have been no reports that systematically evaluate the role of psPDI-redox status under varying conditions. These studies are likely hampered by the use of conventional aggregometers in which the number of samples that can be simultaneously measured is limited.

Thus, we assessed the initial rates of platelet aggregation in the presence of either 100 μM GSH or GSSG and varying ratios of the two similar to that found under normal and pathological conditions. Aggregation of platelet samples was monitored by optical density at 630 nm in a microplate reader. This method has been validated in comparison to aggregation by convential aggregometers [Krause *et al.,* 2001] and has the advantage of being able to run a number of replicates simultaneously. Using this methodology we observed, as seen in Fig. 4.5A, that under varying ratios of GSH/GSSG that initial rates of aggregation were maximal under both the most reducing and most oxidizing conditions used and a minimum at ~2:1 ratio of GSH/GSSG. Importantly, these results are in general accord with prior observations that platelet aggregation is potentiated by the addition of either GSH [Essex *et al.,* 2003] or GSSG [Essex *et al.,* 2004a] suggesting, in an alternate but not exclusive interpretation, that aggregation at either extreme is potentiated in a PDI-dependent fashion. Further, we confirm under these conditions that the redox status of the PDI active site is altered and that enzyme thiols are progressively lost with increasing oxidative potential as seen in Fig. 4.5B.

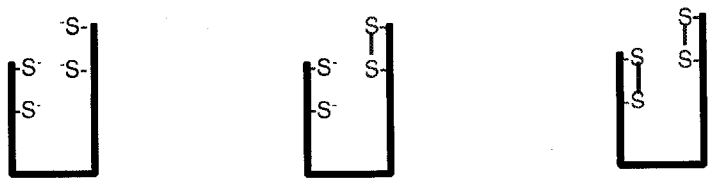
In order to further demonstrate that the observed changes in psPDI-dependent activities in diabetics were the result of the action of H_2O_2 on PDI thiols, we performed *in vitro* experiments in the presence and absence of purified PDI inhibitor - bacitracin [Robinson *et al.,* 2006, Mandel *et al.,* 1993]. When platelets were exposed to H_2O_2 in the presence of purified bacitracin, no substantial effect on psPDI activities or on aggregation profiles was observed (Fig. 4.6B, 4.7), an indication that bacitracin blocked access to the PDI active site thiols. In the absence of bacitracin, treatment of platelets with 25 μM H_2O_2, inhibited PDI denitrosation activity by ~35% and potentiated initial

rates of aggregation by ~2-fold (Fig. 4.6B, 4.7). Importantly, platelets treated with bacitracin alone showed no evidence of inhibition of either aggregation or denitrosation, suggesting that wash and dilution steps were effective in removing inhibitor.

Lastly, we attempted to directly assess the role of PDI redox status by isopropanol-assisted removal and replacement of psPDI. Western blots of platelets have shown that PDI is liberated from the cell-surface in an isopropanol-dependent manner with 1-5% alcohol versus buffer alone (data not shown). Platelets washed in this manner exhibit substantially inhibited initial rates of aggregation presumably due to the loss of psPDI that can be restored by the addition of purified enzyme to the reaction mixture (Fig. 4.8). Although it is likely that platelets washed with isopropanol also release other proteins, the restoration of aggregation by exogenous PDI suggests that PDI is perhaps one of the most critical components lost from the cell surface.

Surprisingly though, our experiments demonstrate that aggregation is restored, not only as expected with reduced and oxidized PDI, but also in the presence of either a 1:1 mixture of reduced:oxidized enzyme (attempting to mimic the ½ oxidized state) or enzyme in which all thiols had been covalently blocked by iodoacetamide. These studies indicate our hypothesis, that elevated NOX activity in T2D oxidizes psPDI active site thiols, thereby giving rise to elevated rates of aggregation, may be, only in part, correct.

PDI has two active sites in its a and a' domains which face each other in the recently solved crystal structure [Tian *et al.,* 2006]. These two active sites can be in one of 3 redox forms reduced, ½-reduced and oxidized as seen in Scheme I:



Scheme I

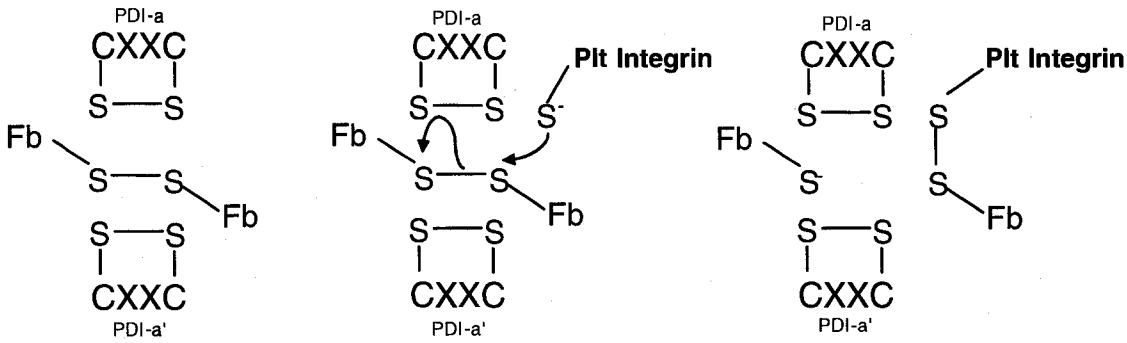
PDI is thought to be involved in mixed disulfide formation between fibrinogen (or collagen) and their specific free thiol-containing integrins [Lahav *et al.*, 2003, Essex *et al.*, 2003] as shown in Scheme I:

Platelet-integrin S-H + fibrinogen S-S

→platelet-integrin-S-S-fibrinogen (aggregation)

Scheme I

A reduced and ½ reduced PDI active site may catalyze both the reduction of the fibrinogen disulfide, as well as the integrin-fibrinogen mixed disulfide and the formation of integrin-fibrinogen mixed disulfide. However, we suggest that a quiescent, fully oxidized PDI can essentially act as a chaperone facilitating the formation of the integrin-fibrinogen mixed disulfide by simply binding the fibrinogen in the right conformation for nucleophilic attack of the integrin-SH thus permitting the facile one step formation of the integrin-fibrinogen mixed disulfide (Scheme II).



Scheme II

In light of these observations, oxidation of psPDI active site thiols certainly impedes the reduction of S-nitrosothiols and disulfides however; the ability of PDI to restore platelet aggregation, independent of its oxidoreductase activity, suggests that its chaperone activity may instead be responsible for its role in assisting in integrin-ligand disulfide bond formation. Indeed, investigations have shown 1) that the αIIbβ3 integrin bears intrinsic thiol-isomerase [O'Neill, *et al.*, 2000] and 2) that the ability of PDI to potentiate tissue factor coagulant function is also independent of its oxidoreductase activity [Versteeg *et al.*, 2007].

5.1 Conclusion

Understanding platelet function and dynamics under the spectrum of vascular conditions to which they are exposed bears significant importance in mitigating thrombotic disorders which pre-dispose individuals to risks associated with cardiovascular dysfunction.

This thesis has examined the role of protein disulfide isomerase at the exofacial surface of the platelet plasma membrane, as well as features of vascular relevance which influence its activity in animal models bearing characteristics of a pre-diabetic state, human normal and diabetic subjects and *in vitro*. The findings herein, show that the thiol-disulfide status of platelet-surface PDI is susceptible to changes in vascular redox and contributes to a pro-aggregatory state, in which platelets lose their ability to metabolize S-nitrosothiols required for generation of platelet anti-aggregant cGMP. Importantly, lost denitrosative activity and platelet hyperaggregability could be restored in animal models by treatment with statins which are thought to inhibit the excessive generation of NAD(P)H oxidase-derived ROS from various vascular sources, including platelets and endothelial cells, via a pleiotropic mechanism. Interestingly, despite several lines of evidence which implicate PDI both as critical in platelet-surface, thiol-disulfide dynamics and as a target of oxidant species, we have shown that, although PDI contributes positively to platelet aggregation, that it does so independently of its oxidoreductase activity.

This thesis further examined the dynamics of nitric oxide to gain insight into factors which influence its signaling properties. As a unique bioactive radical, signals transduced by nitric oxide are governed by localized generation, diffusion and reactivity

rather than interaction with cell-surface receptors. We have questioned the assumption that nitric oxide diffuses freely through biological membranes, irregardless of structure or composition, thus bearing no significant impact on its subsequent signaling properties.

Indeed we have found that cholesterol incorporated into model bilayers substantially alters the diffusion of nitric oxide. In cellular systems, it was further found that cholesterol alters the equilibrium of •NO-signaling, measured either by its interaction with guanylate cyclase or through subsequent nitrosative reactions measured by DAF-2T fluorescence. Thus it appears that the ordering effect of cholesterol in biological membranes may fine-tune the spatial reactivity of nitric oxide and subsequent signaling targets such that the reactive equilibrium of •NO is shifted from intracellular nitrosylation of haem targets such as guanylate cyclase to the nitrosation of extracellular nucleophiles. This represents a novel observation and paradigm that may have relevance to pathological conditions in which cholesterol metabolism is disturbed. Whether these observations are directly due to changes in diffusion, altered reactivity, physical interactions or a combination therein remains open to debate.

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