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S-nitrosothiols affect fibrinogen structure and function.

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S-NITROSOTHIOLS AFFECT FIBRINOGEN STRUCTURE AND FUNCTION

By

Arianna Vignini

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Abstract

S-Nitrosoglutathione (50 μM) inhibited the initial rate of thrombin-catalyzed fibrinogen polymerization by ~80 %. The fact that the same concentration of S-nitrosoglutathione had no effect on thrombin-dependent hydrolysis of tosylglycylprolylarginine-*4*-nitranilide acetate suggested that the nitrosothiol was affecting fibrinogen structure. This was confirmed by circular dichroism spectroscopy where S-nitrosoglutathione and S-nitrosohomocysteine increased the α-helical content of fibrinogen by ~19 % and 11% respectively. S-carboxymethylamido derivatives of glutathione or Hcys had no effect on the fibrinogen 2^{o} structure. The S-nitrosothiol-dependent 2^{o} structural effects were reversed upon gel fitration chromatography suggesting that the effects were allosteric. Further evidence for fibrinogen-S-nitrosoglutathione interactions were obtained from S-nitrosoglutathione-dependent quenching of the intrinsic fibrinogen Trp fluorescence as well as the quenching of the S-NO circular dichroic absorbance of S-nitrosoglutathione as a function of fibrinogen concentration. These studies enabled the estimation of a K_D of ~40 μM for the fibrinogen-S-nitrosoglutathione interaction with a stoichiometry of 2:1 (S-nitrosoglutathione:fibrinogen).

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LIST OF ABBREVIATIONS

Arg	arginine
BSA	bovine serum albumin
BSANO	S-nitrosobovine serum albumin
CD	circular dichroism
cGMP	cyclic guanosin monophosphate
Chromozym TH	tosylglycylprolylarginine-4-nitranilide acetate
cNOS	constitutive nitric oxide synthase
Cys	cysteine
CysNO	S-nitrosoCysteine
DTNB	5,5'-dithiobis 2-nitrobenzoate
EDRF	endothelial derived relaxing factor
EDTA	ethylenediaminetetra-acetic-acid
FPLC	fast protein liquid chromatography
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
Gly	glycine
GSH	glutathione
GSNO	S-nitrosoglutathione
GPx	glutathione peroxidase
Hcys	homocysteine
HcysNO	S-nitrosoHomocysteine
HIV	human immunodeficiency virus
H_2O_2	hydrogen peroxide
HRV	human rhinovirus
ICE	interleukin-1β-converting enzyme
iNOS	inducible nitric oxide synthase
LPS	lipopolysaccharide
NADPH	nicotinamide adenine dinucleotide phosphate (reduced form)

NaOH	sodium hydroxide
NO	nitric oxide
NOS	nitric oxide synthase
O_2^-	superoxide anion
$ONOO^-$	peroxynitrite
PAF	platelet activating factor
PDGF	platelet-derived growth factor
Pro	proline
RES	reticuloendothelial system
$RS^{\cdot}$	thiyl radical
RSNO (SNO)	S-nitrosothiol
SDS	sodium dodecyl sulfate
sGC	soluble guanylate cyclase
SNAP	S-nitroso ampicylline
SNOAC	S-nitroso-N-acetylcysteine
TNF	tumor necrosis factor
Trp	tryptophan
Tyr	tyrosine
XO	xanthine oxidase

1.0 INTRODUCTION

In the last few years nitric oxide (NO) has been shown to be involved in many important biological events [1]. Among its diverse functions, NO has been implicated in neurotransmission [2], immune regulation [3-6], vascular smooth muscle relaxation [7-10], and inhibition of platelet aggregation [11-16].

Nitric oxide is one of the smallest known biologically active messenger molecules. It is a colourless gas, with good water solubility and contains an unpaired electron which renders it paramagnetic. NO reacts with a variety of free radicals [17] including superoxide anion ($O_2^{-\bullet}$) to form peroxynitrite ($ONOO^-$). $ONOO^-$ is a potent oxidant, which can nitrosate proteins and nucleic acids and can cause lipid peroxidation.

NO also reacts with large (serum albumin) and small molecular weight thiols (cysteine, glutathione, and homocysteine) to form S-nitrosothiols *in vivo* [18].

1.1 Generation of NO

NO can be formed as a result of two different pathways: one stems from enzymic sources while the other one stems from non-enzymic sources.

1.1.1 Enzymic sources

In eukaryotes, the enzyme responsible for NO production is nitric oxide synthase (NOS; EC 1.14.13.39). This enzyme acts in a very specific manner utilising only the terminal guanidinium nitrogen of the amino acid L-arginine [19]. NO is produced through the oxidation of L-arginine by the NADPH dependent enzyme with the concomitant production of L-citrulline [20]. This biochemical system is known as the L-

arginine/NO pathway. NOS enzymes have been classified as constitutive (cNOS) or inducible (iNOS). Three isoforms of NOS have been identified as neuronal, macrophage and endothelial types, and their sequences are extremely well conserved between different species. These isoforms are named after the tissue in which they were first purified and cloned, but have also been located elsewhere. Neuronal-type NOS is constitutively expressed in disparate cell types, including skeletal muscle [24] and airway epithelium [25]. The endothelial-type NOS is also found in myocardium [26], skeletal muscle [27], epithelial cells [28] and neurons [29] whereas most cells expressing the NOS isoform originally characterised in macrophages (iNOS), is in response to inflammatory cytokines.

1.1.2 Non-enzymic sources

Beside the enzymic pathway, NO can be generated from several chemical sources, such as S-nitrosothiols, organic nitrates, iron-nitrosyl complexes, and secondary amine/NO complex ions. All of these compounds break down to form NO both directly and indirectly.

1.2 Physiological functions of Nitric Oxide

1.2.1 NO as an activator of soluble guanylate cyclase

Guanylate cyclase can exist in the cell in two forms:

i) a soluble isoform within the cytosol;

ii) a particulate isoform, which is membrane-associated.

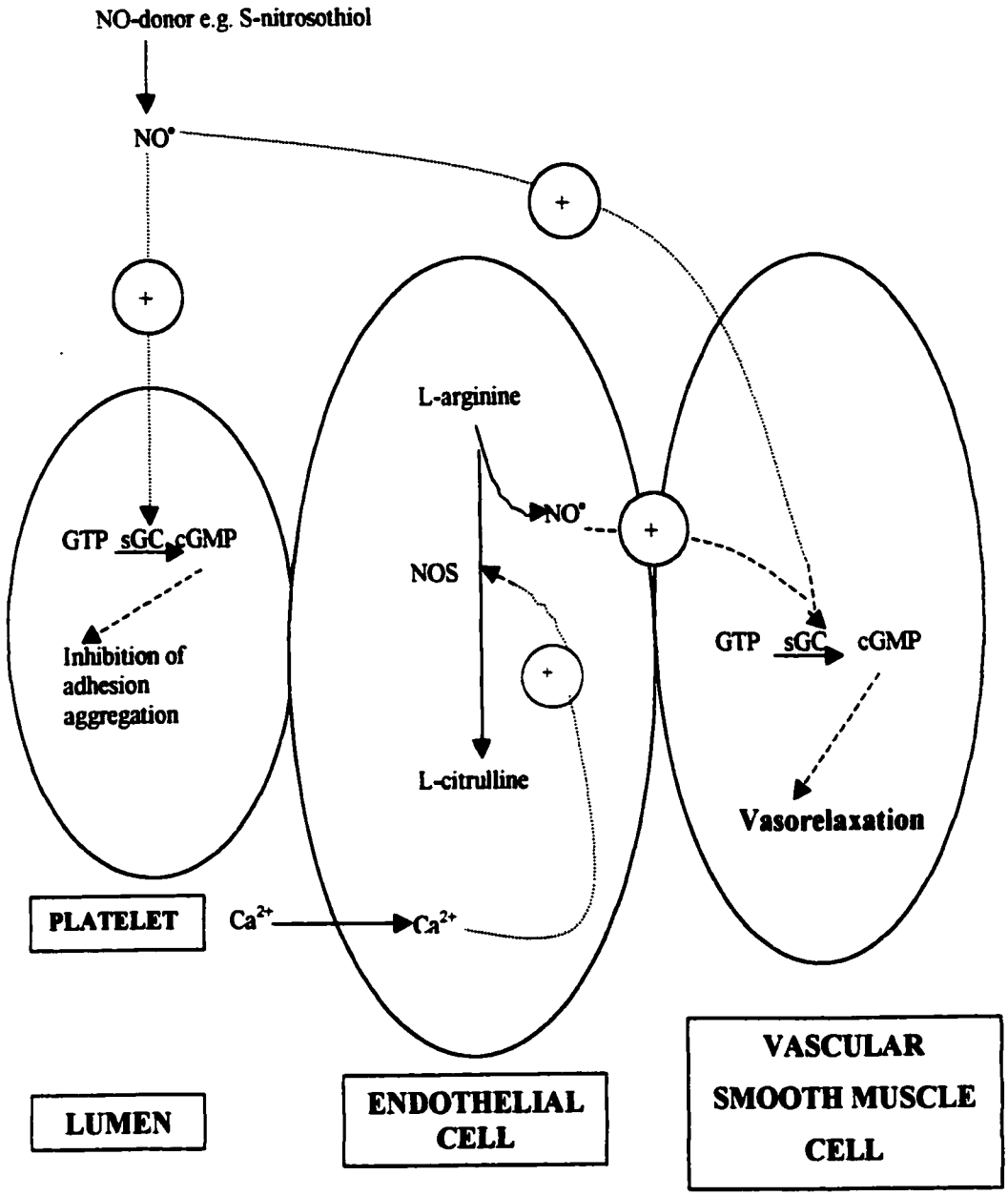
The purified soluble guanylate cyclase can be activated *in vitro* by NO-donating compounds. Once the enzyme has been activated, cGMP accumulates and in vascular smooth muscle cells it causes relaxation and hence vasodilatation [19, 27] (scheme 1).

In other cell types, the accumulation of cGMP is accompanied by different physiological effects. For example, in platelets, soluble guanylate cyclase can form cGMP after stimulation by NO derived either from the vascular endothelium or from endothelial-type NOS within the platelets themselves. This reaction leads to inhibition of platelet aggregation [12].

In the central nervous system, NO can be formed in the postsynaptic nerve cell, diffusing out and acting in one or more neighbouring neurons. In addition, NO in the brain can act upon nerve cells causing a variety of effects such as neuroprotection [28], long-term potentiation (an activity-dependent increase in synaptic strength [29]) and long-term depression (a long-lasting depression of parallel synapses following repeated excitation by climbing fibers of Purkinje cells [30]). An overproduction of NO is thought to cause neurotoxicity and certain neuro-degenerative conditions, such as Alzheimer-type dementia [31, 32]. It is also postulated that NO plays a role in the local regulation of blood supply in the brain and in the peripheral nervous system [33].

1.2.2 NO as an effector in inflammatory and immunological reactions

Both macrophages and neutrophils generate NO during inflammatory and immune reactions [4,5,35-41]. These cells have diverse roles including phagocytic and non-phagocytic destruction of foreign or damaged cells. These processes involve the production of large quantities of cytotoxic NO. In fact the formation of peroxinitrite


NO-donor e.g. S-nitrosothiol
NO°
+
+
+
L-arginine
NO°
NOS
+
L-citrulline
GTP sGC cGMP
Inhibition of adhesion aggregation
GTP sGC cGMP
Vasorelaxation
PLATELET
Ca2+
Ca2+
LUMEN
ENDOTHELIAL CELL
VASCULAR SMOOTH MUSCLE CELL

Scheme 1.

($ONOO^-$) by the rapid reaction of nitric oxide (NO) and superoxide anion ($O_2^{-\bullet}$) has a rate constant near the diffusion controlled limit ($4\text{-}7 \times 10^9 M^{-1}s^{-1}$) (scheme 2) [41]

$$NO^\bullet + O_2^{-\bullet} \longrightarrow ONOO^-$$

Scheme 2.

1.2.3 NO as a carcinogen

NO can be cytotoxic to tumour cells and at the same time it can also act as a carcinogen. This is possible because of the ability of NO to nitrosate natural secondary amines to form carcinogenic N-nitroso compounds [42].

1.3 NO-donor drugs

NO is a highly reactive, short-lived free radical specie, that cannot exist freely in large quantities in the human body. However, upon reacting with free thiols, NO can form thermally stable S-NO derivatives which mimic the physiological role of NO.

1.3.1 S-Nitrosothiols

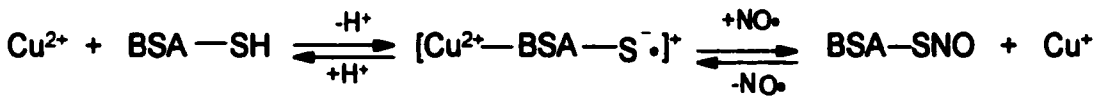
S-nitrosothiols (RSNOs) are maybe the most important class of NO-derivatives, which break down to form NO and the corresponding disulphide (RSSR) (scheme 3) [43]:

$$2RSNO \rightarrow RSSR + 2NO$$

Scheme 3.

Some of these occur naturally [i.e. S-nitrosoglutathione (GSNO), S-nitrosoCysteine (CysNO), S-nitrosoHomocysteine (HcysNO), S-nitrosobovine serum albumin (BSA-

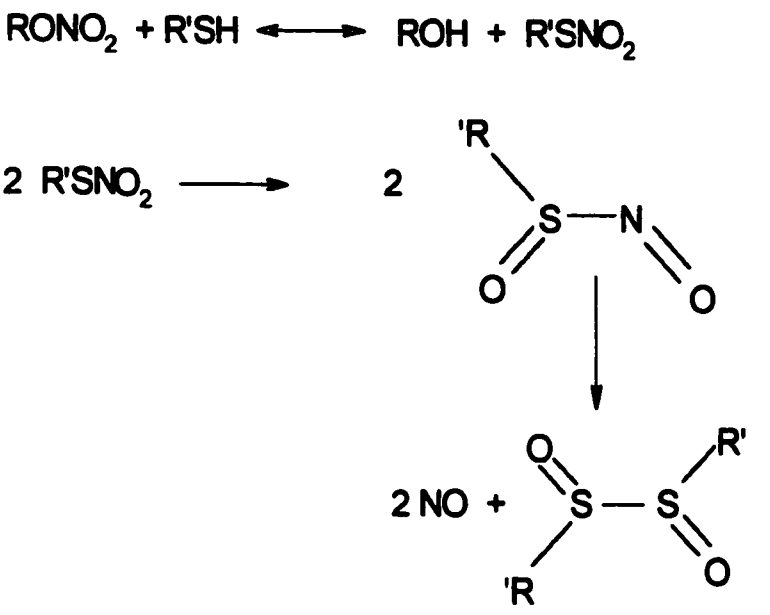
SNO)], and they may play a role in several physiological and pathophysiological processes. The stability of RSNOs is structure dependent. For example, in the mechanism for the copper-mediated formation/degradation of BSA-SNO, the sulfhydryl group of Cys-34 of BSA binds Cu^{2+} forming a copper-thiol complex, which reacts with NO to yield BSA-SNO (scheme 4). The following mechanism has been proposed that is reliant on both Cu^{2+} and BSA for the reaction with NO. This mechanism is novel as the copper-mediated reaction of NO with thiols does not directly involve NO^+ as previously suggested [44, 45]



Scheme 4.

1.3.2 Organic nitrates

Nitroglycerin has long been used as a vasodilator for the treatment of *angina pectoris*. The mechanism by which it acts is due to its breakdown with the subsequent formation of NO. This allows it to act as a potent vasodilator within the coronary and systemic blood vessels. The release of NO from organic nitrates involves both non-enzymic and enzymic sources [44]. In both cases, thiol groups are involved in the activation of organic nitrates, (scheme 5).



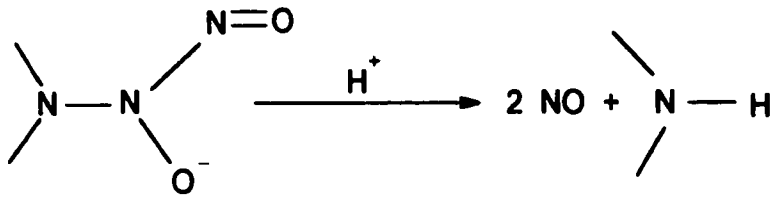
Scheme 5.

1.3.3 Iron-nitrosyl complexes

Sodium nitroprusside belongs to the class of iron-nitrosyl compounds and has clinically been used as a vasodilator. *In vitro,* sodium nitroprusside does not liberate NO spontaneously, but requires partial reduction by a one-electron transfer by light, or a variety of reducing agents such as ascorbic acid *in vivo*. Sodium nitroprusside is also a source of nitrosonium ion (NO^+), and for this reason behaves as a nitrosating electrophile. Many reports show that it can convert amines into nitrosamine and ketones into oximes, but S-nitrosation has not been detected [46].

1.3.4 Secondary amine/NO complex ions

These compounds are called amine NONOate [47-49] because of their formula $R_2N[N(O)NO]$. They have several interesting properties. They are stable in solid form, highly water soluble, and their NO-release rates vary according to the structure of the carrier nucleophile (R_2NH) (scheme 6). In addition, they release NO spontaneously in a pH-dependent fashion (i.e. at neutral or acidic pH).



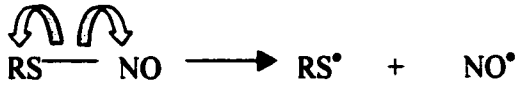
Scheme 6.

1.4 Mechanism of action of S-nitrosothiols

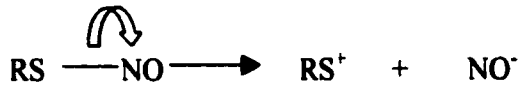
The importance of S-nitrosothiols is due to the fact that they are potentially involved in NO storage, transfer and delivery. S-nitrosothiols are found naturally *in vivo*, principally as the S-nitrosothiol of serum albumin, at a concentration of approximately 7 μM [18, 51]. High levels of GSNO have been demonstrated in human bronchial lavage fluid at 0.3 μM [50, 52]. The postulated physiological role of RSNO is to store and then release NO upon demand. The mechanism for the rapid release of RSNO-bound to NO is under investigation [18, 53].

RSNO can decompose according to both homolytic and heterolytic cleavage of the S-NO bond releasing $NO^{\cdot}$, NO^{+} and NO^{-} (scheme 7) [54]. The main biological effect of RSNO arises from homolytic cleavage of the S-NO bond with the release of NO [55].

The formation and decomposition of the low molecular weight RSNOs, such as GSNO and S-nitrosocysteine (CysNO), may be a means for the storage and transport of NO *in vivo* [56]. The breakdown of S-NO bonds can be induced by temperature [57], light [58-60], metal ions [61-65], superoxide [66, 67], and seleno compounds [68, 69].



Homolytic decomposition



Heterolytic decomposition

Scheme 7.

1.4.1 Thermodecomposition of RSNOs

Most RSNO compounds are unstable at room temperature and decompose producing disulfides and NO. Tertiary RSNOs such as S-nitroso ampicylline (SNAP), have much higher stability compared to primary alkyl RSNOs or aryl RSNOs, [57]. Thermodecomposition of RSNO can be represented as a two-step process: the first step

involves the S-NO cleavage to produce NO radical and thiyl radical (RS$^{\cdot}$), followed by the dimerization of the thiyl radical to form disulfide (RSSR) (scheme 8).

$$RS\text{-}NO \rightarrow RS^{\bullet} + NO^{\bullet}$$

$$RS^{\bullet} + RS^{\bullet} \rightarrow RS\text{-}SR$$

Scheme 8.

1.4.2 Photo decomposition of RSNO compounds

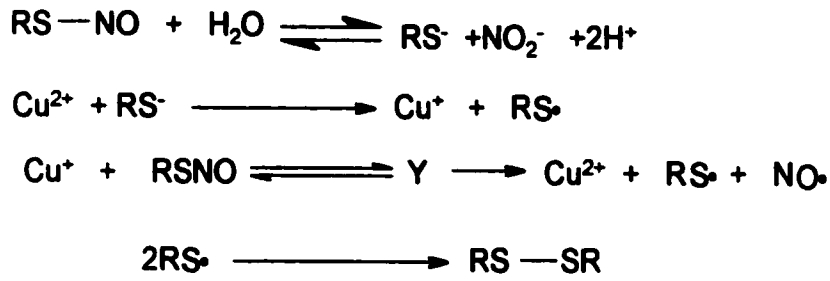
RSNOs are photosensitive, especially to UV light. Irradiation of GSNO at either 340 nm or 545 nm leads to the release of NO plus thiyl radicals [58]. The thiyl radical will react directly with GSNO to form disulfide GSSG and NO in the case of an oxygen-free environment. On the other hand, in the presence of oxygen, GSOO$^{\cdot}$ radical is first generated, which reacts with GSNO to form GSSG and NO [59].

In this way, NO can be formed not only via GSNO homolysis but also from the reactions of GSNO with GS$^{\cdot}$ and GSOO$^{\cdot}$. Studies done on leukaemia cells have revealed that the photolytic release of NO from GSNO resulted in an enhanced cytotoxic effect [60].

1.4.3 Metal ions catalysed RSNO decomposition

RSNOs decomposition in solution can be catalyzed by metal ions such as Cu^{+}, Fe^{2+}, Hg^{2+}, and Ag^{+} [61-65]. The human body contains 0.1g copper per 75 kg body weight, which is widely distributed in the blood, bone, and muscle, and can catalyze the

decomposition of RSNO. The true catalyst is Cu^{+}, which is formed after the reduction of Cu^{2+} by thiolate ion generated from hydrolysis of RSNO or free thiol. The produced Cu^{+} can then catalyze RSNO decomposition with the formation of a complex intermediate. Both Cu^{2+} and RS^{-} are generated and can be present in catalytic quantities (scheme 9).



Scheme 9.

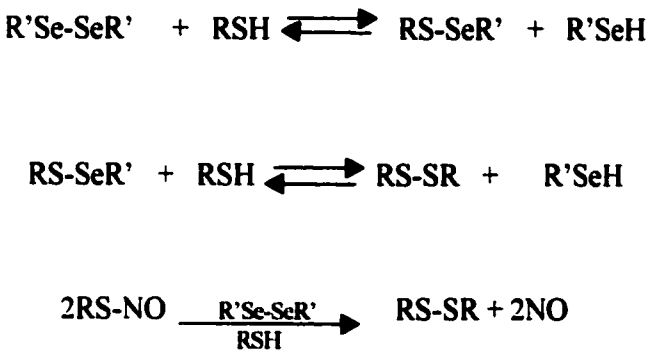
1.4.4 Superoxide catalysed RSNO decomposition.

Xanthine oxidase (XO) is a superoxide generator. In the presence of purine substrates and molecular oxygen, XO can induce CysNO and GSNO decomposition under aerobic conditions by $O_2^{-\bullet}$ dependent and independent pathways [66, 67]. GSNO decomposition is dependent on a second order reaction with $O_2^{-\bullet}$. CysNO decomposition, catalyzed by superoxide can also undergo another enzymatic pathway with the final formation of NO, suggesting the involvement of superoxide in RSNO physiology and pathophysiology.

1.4.5 Seleno compounds and glutathione peroxidase catalyzed RSNO decomposition.

It is well documented that selenium is an essential mineral in the mammalian diet, and its deficiency is closely associated with heart disease. It has been shown that glutathione

peroxidase (GPx), an essential selenium containing antioxidant enzyme, is able to potentiate the inhibition of platelet function by RSNOs [68]. GPx catalyzes the metabolism of endogenous GSNO with NO as a final product in the presence of H_2O_2. Compounds containing selenium can also catalyze the decomposition of RSNO to produce NO. The initial activation of this decomposition involves the reaction between a diselenide and a thiol, whereby the product generated from these reactions further reacts with RSNO releasing NO (scheme 10).



Scheme 10.

1.5 Kinetic and equilibrium studies of NO transfer between RSNO and thiols

All physiological events depend on the rate of the reaction. For this reason, understanding the biological roles of S-nitrosothiols require detailed information on the rates and mechanisms of the RSNO related reactions. In recent years, studies on the kinetics of the transnitrosation reaction between RSNOs and thiols have been developed. For example, in the reaction of CysNO with cysteine it has been shown that the presence of cysteine decreased rather than prolonged the lifetime of CysNO [69]. The kinetics and equilibria of NO transfer between RSNO and thiols have been investigated under different conditions [70-74]. Studies at different pHs have indicated that NO transfer

reactions are faster with increased pH [71]. When the thiol concentration is low, the addition of Cu^{2+} resulted in a substantial rate increase, indicating that Cu^{2+} takes part in the reaction. Consequently, the addition of EDTA or a specific Cu^{+} chelator completely suppresses such reactions [72]. On the other hand, when the thiol concentration is high, the reaction rate constants are unaffected by the removal of metal ions or by the specific addition of Cu^{2+} [73, 74].

1.6 Biological and physiological function of RSNOs and S-nitrosylation

NO can react with protein thiols to give the so called S-nitrosylation. S-nitrosylation is considered a very important means of modifying protein activity. BSA [75], tissue-type plasminogen activator [76], glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [77], N-methyl-D-aspartate receptor [78], oncogenic p21 [79], and transcriptional activators [80], are found to be target for this type of modification.

1.7 Apoptosis and NO/RSNO

Gene p53 is know as the tumour suppressor and it functions as a "guardian" of the genome due to its ability to induce growth arrest or apoptosis in cells with damaged DNA. After iNOS activation there is an accumulation of nitrite in the cell supernatant and this results in an accumulation of p53, preceding DNA fragmentation. If iNOS is blocked, the accumulation of p53 is down-regulated. NO plays an important role in the accumulation of p53 as proven using NO donors. The concentration of the tumour

suppressor increased in response to NO donors, whereas if NO removal decreased p53 and only a few cells enter the apopotic pathway [81].

Caspase, is a family of cysteine proteases that are able to cleave several cellular substrates. These enzymes have sequence homology with the interleukin-1β-converting enzyme (ICE). ICEs are implicated in different signals that eventually lead to cell death. Recently, it has been found that NO is able to inhibit caspase 1 and 3, resulting in the inhibition of TNFα that induces apoptosis [82]. Each caspase has been reported to contain a crucial cysteine residue in its active site [83], and NO may inhibit apoptosis by interacting with the caspase cysteine proteases [84, 85]. *In vitro*, when NO donors are incubated with these cysteine proteases, there is a decrease in the enzyme activity, due to S-nitrosylation of the essential cysteine residue [82-85]. Recent studies have shown that exogenous NO donors can be used *in vivo* as regulators in the antiapoptosis mechanism because they regulate caspase activity via S-nitrosylation [85].

1.8 Platelet neutrophil interaction

Platelets and neutrophils are both involved in the inflammatory process. The interaction of these cells may play crucial roles in vascular injury. NO is able to inhibit both the aggregation and adhesion of platelets [86], and the adhesion of neutrophils to both platelets [87] and endothelium [88] through the regulation of the expression of adhesion molecules. Platelet activity can easily be inhibited GSNO [89], and this inhibition is found to be mediated by a copper- dependent targeting systems [90]. The relationship between RSNOs and platelets has been studied to reveal that RSNO is produced by

platelet activation and that platelets are able to capture NO from GSNO directly in the plasma.

1.9 Immune defence

All the mechanisms involved in human immune defence are also associated with RSNO's biological function, including the irradication of tumours and intracellular pathogens. For example, human immunodeficiency virus (HIV)-1 protease is essential for viral replication, leading to the formation of mature infectious virions [91]. A new approach for suppressing HIV-1 replication may be the S-nitrosylation of some crucial cysteine residues of the enzyme that may possibly cause enzyme inhibition [92].

1.10 Allosterism

The word allosterism derives from the two Greek words allos that means other, and stereos that means solid or space This type of interaction happens when a ligand binds to proteins causing regulation in the process. In fact, through a variety of regulatory mechanisms an organism is able to respond to changes in its environment, maintain intra- and intercellular communication and execute an orderly program of growth and development. Regulation is exerted at every organisational level in living system, from the tight control of rates of reactions at a molecular level, to the control of expression of genetic information at a cellular level, to the control of behaviour at the organismal level. Allosteric interactions may be cooperative when the binding of one ligand at a specific site is influenced by the binding of another ligand known as an effector or modulator, at a different (allosteric) site on the protein. If the ligands are identical this is known as

homotropic effect, whereas if they are different, it's described as a *heterotropic effect*. These effects can also be termed *positive* or *negative* depending on whether the effector increases or decreases the protein's ligand-binding activity. In general, allosteric effects result from interactions among subunits of oligomeric proteins. The structure of the ligands are often quite different from the substrate.

There are different models that can describe allosteric effects, the most popular being the *symmetry model* and the *induced-fit hypothesis*. The symmetry model, or the MWC model, provides a practical rationalisation for the ligand-binding properties of many proteins. In the induced-fit hypothesis the ligand induces a conformational change in the protein which results in its increased ligand-binding affinity. The change in the conformation happens in a subunit of the protein but the influences are also seen in the neighboring subunits.

As a result the principle of the induced-fit hypothesis is that a protein's ligand-binding affinity varies with its number of bound ligands, whereas in the symmetry model this affinity depends only on the protein's quaternary state (for more detail the reader is directed to the following review [188-191]).

1.11 Fibrinogen

1.11.1 Introduction

Fibrinogen, a natural substrate for thrombin, is a plasma protein synthesised in the parenchymal cells of the liver. Thrombin is the final proteolytic enzyme of the coagulation cascade, induces fibrin clot formation by cleaving only about 1% of the mass of the fibrinogen, a remarkable transformation: the resulting thrombin-modified

molecules, denoted as fibrin monomers, aggregate spontaneously to form an insoluble polymer [93].

Fibrinogen is a glycoprotein of molecular weight 340 kD, containing about 3% carbohydrate. Fibrinogen is a slightly acidic protein with a pI of 6.3 and a solubility of about 0.2 g/L at zero ionic strength. As the ionic strength is increased, the pI shift towards acidic pH values and the solubility at neutral pH is increased.

1.11.2 Structural features of fibrinogen

1.11.2.1 Subunit chains

The molecule of fibrinogen has a chemically symmetrical structure. It is composed of three pairs of nonidentical chains (Aα, Bβ and γ), which are linked to each other by disulfide bonds to give a dimeric subunit formula $A\alpha_2B\beta_2\gamma_2$. The constituent reduced chains have molecular weights of about 64 kD, 56 kD and 47 kD, respectively [94]. These values were first estimated by ultracentrifugal analysis, but similar molecular weights of the subunit polypeptide chains were later calculated on polyacrylamide gels with sodium dodecyl sulfate (SDS) [95, 96]. The Aα-, Bβ- and γ-chains contain 610 [97, 98], 461 [99, 100] and 411 [101] residues respectively.

Fibrinogen contains four heterogeneous carbohydrate side chains, which are N-acetyl-glucosamine, mannose, galactose and sialic acid. Each Bβ- and γ-chain carries one sugar side-chain and each one is linked to asparagine residues in position 364 [102] of the Bβ-chain and 52 [103] of the γ-chain.

1.11.2.2 Heterogeneity of fibrinogen

When native fibrinogen or its reduced polypeptide chains are chromatographed on ion exchangers or agarose gels multiple peaks are found. If fibrinogen is proteolytically degraded large molecular weight fragments are produced. These fragments have altered charge and solubility properties; and they contain an Aα-chain, which has been degraded in the carboxy- terminal region [104-108].

1.11.2.3 Disulfide bridges

Disulfide bonds are crucial to the structural integrity of the fibrinogen molecule. Fibrinogen has 58 cystines which are involved in 29 disulfide bridges. The disulfide bridges link dimeric halves of the fibrinogen molecule. Seven interchain disulfide bonds connect individual subunit polypeptides within each half of the molecule. The remaining disulfide bridges (6 per each monomeric subunit set) are intrachain bonds. Cystine residues are concentrated in three clusters along each polypeptide chain. The amino-terminal disulfide knot contains all three inter- half connections, one Aα- Aα and two γ-γ bridges (fig. 1) [109]. Each subunit chain has an intrachain disulfide bridge in its carboxy-terminal portion [110, 111], while the intermediate polypeptide regions are linked by ring-like triple connections [112]. The disulfide bonds are remarkably stable to reduction under denaturing conditions.

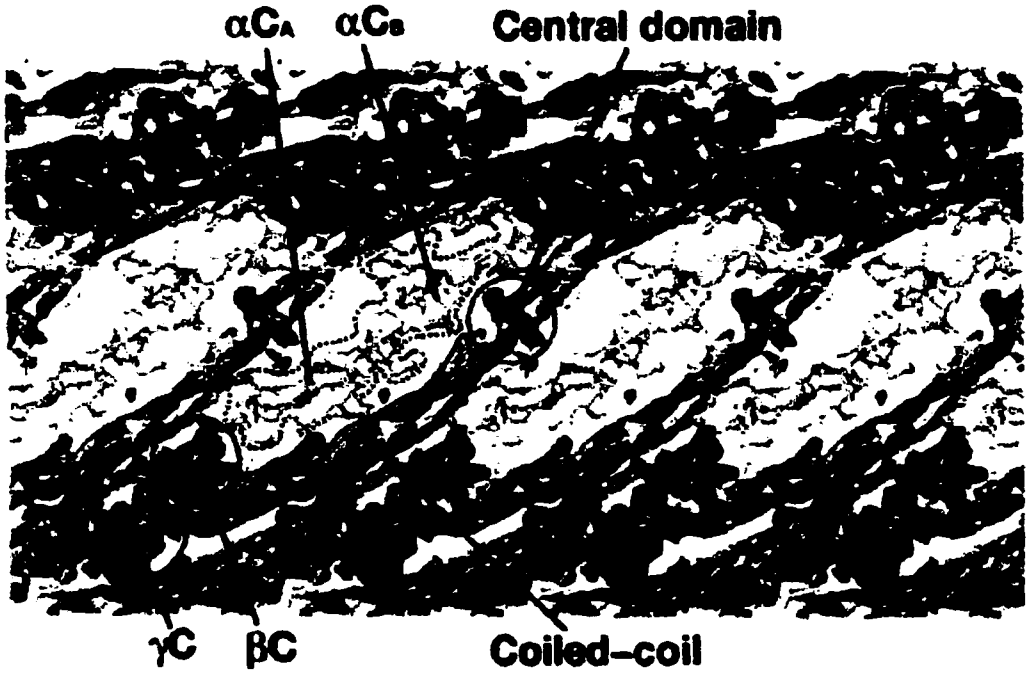


Fig. 1- Section of an electron density map showing native chicken fibrinogen mainframe backbone density (blue) and disordered regions of αC domains (gray) [113].

1.11.2.4 Amino acids composition and sequence

The α, β, and γ chains of the fibrinogen protein contains some homologous sequences. If the cysteine residues of the fibrinogen chains are aligned, a striking similarity is observed. It has been suggested that each chain is composed mostly of α-helices and forms a three stranded "coiled coil" [114]. Moreover, if the complete sequences of the three polypeptide chains are aligned, a striking homology between Bβ-and γ-chains can be seen in several regions [115-117]. It was determined that 33% of homology mainly belongs to cysteine, tryptophan and glycine residues (See table 1 for more details).

Aα-Chain

1 ADSGEGDFLAEGGGVRGPRVVERHQSACKDSDWPFCSDEDWNYKCPSGCR
51 MKGLIDEVNQDFTNRINKLKNSLFEYQKNNKDSHSLTTNIMEILRGDFSS
101 ANNRDNTYNRVSEDLRSRIEVLKRKVIEKVQHIQLLQQKNVRAQLVDMKRL
151 EVDIDIKIRSCRGSCSRALAREVDLKDYEDQQKQLEQVIAKDLLPSRDRQ
201 HLPLIKMKPVPDLVPGNFKSQLQKVPPEWKALTDMPQMRMELERPGGNEI
251 TRGGSTSYGTGSETESPRNPSSAGSWNSGSSGPGSTGNRNPGSSGTGGTA
301 TWKPGSSGPGSTGSWNSGSSGTGSTGNQNPGSPRPGSTGTWNPGSSERGS
351 AGHWTSESSVSGSTGQWHSESGSFRPDSPGSGNARPNNPDWGTFEEVSGN
401 VSPGTRREYHTEKLVTSKGDKELRTGKEKVTSGSTTTTRRSCSKTVTKTV
451 IGPDGHKEVTKEVVTSEDGSDCPEAMDLGTLSGIGTLDGFRHRHPDEAAF
501 FDTASTGKTFPGFFSPMLGEFVSETESRGSESGIFTNTKESSSHHPGIAE
551 FPSRGKSSSYSKQFTSSTSYNRGDSTFESKSYKMADEAGSEADHEGTHST
601 KRGHAKSRPVRGIHTSPLGKPSLSP
602

Bβ-Chain

1 ZGVNDNEEGFFSARGHRPLDKKREEAPSLRPAPPPISGGGYRARPAKAAA
51 TQKKVERKAPDAGGCLHADPDLGVLCPTGCQLQEALLQQERPIRNSVDEL
101 NNNVEAVSQTSSSSFQYMYLLKDLWQKRQKQVKDNENVVNEYSSELEKHQ
151 LYIDETVNSNIPTNLRVLRSILENLRSKIQKLESDVSAQMEYCRTPCTVS
201 CNIPVVSGKECEEIIRKGGETSEMYLIQPDSSVKPYRVYCDMNTENGGWT
251 VIQNRQDGSVDFGRKWDPYKQGFGNVATNTDGKNYCGLPGEYWLGNDKIS
301 QLTRMGPTELLIEMEDWKGDKVKAHYGGFTVQNEANKYQISVNKYRGTAG
351 NALMDGASQLMGENRTMTIHNGMFFSTYDRDNDGWLTSDPRKQCSKEDGG
401 GWWYNRCHAANPNGRYYWGGQYTWDMAKHGTDDGVVWMNWKGSWYSMRKM
451 SMKIRPFFPQQ

γ-Chain

1 YVATRDNCCILDERFGSYCPTTCGIADFLSTYQTKVDKDLQSLEDILHQV
51 ENKTSEVKQLIKAIQLTYNPDESSKPNMIDAATLKSRKMLEEIMKYEASI
101 LTHDSSIRYLQEIYNSNNQKIVNLKEKVAQLEAQCQEPCKDTVQIHDITG
151 KDCQDIANKGAKQSGLYFIKPLKANQQFLVYCEIDGSGNGWTVFQKRLDG
201 SVDFKKNWIQYKEGFGHLSPTGTTEFWLGNEKIHLISTQSAIPYALRVEL
251 EDWNGRTSTADYAMFKVGPEADKYRLTYAYFAGGDAGDAFDGFDFGDDPS
301 DKFFTSHNGMQFSTWDNDNDKFEGNCAEQDGSGWWMNKCHAGHLNGVYYQ
351 GGTYSKASTPNGYDNGIIWATWKTRWYSMKKTTMKIIPFNRLTIGEGQQH
401 HLGGAKQAGDV

Table 1: Consensus primary structure for Human fibrinogen [118]. C=cysteine; Y=tyrosine; W=tryptophan

1.11.2.5 Domains in the fibrinogen molecule

After digestion of fibrinogen with proteases such as plasmin, two main core fragments are recovered: a small fragment that corresponds to the central domain (fragment E) and a set of larger fragments that correspond to the end portion (fragments D). The two fragments of D represent half of the mass of the whole fibrinogen while fragment E makes up only 15%. The remaining portion of fibrinogen is characterized by the C-terminal two-thirds of the α-chains, which are removed after treatment with proteases [119, 120]. The two outer D domains are connected by a "coiled-coil" region to the central E domain (fig. 2). The E region contains the "knobs" that are the regions exposed after thrombin removes the fibrinopeptides from the α-chain N-termini located in a central position. These "knobs" interact with the "holes" that are located on the fragment D in the distal domain.

Fibrinogen is a dimeric protein whose oligomerization proceeds in both directions with the formation of a two-molecule thick protofibril and a half-molecule spread out partly covered. When the fibrin clot is proteolysed, fragment E and crosslinked D ("double D" or "D-dimer") are formed as well as a very unstable complex known as "D_2E" [121].

Fragment D has a length of 130-Å and is formed by the three stranded coiled-coil region and a double-back fourth helical of α-chain, and continues with the carboxyl halves of the β- and γ- chains. While the β domain is folded back close to the coiled-coil, the γ domain extends on to a distal position [122].

The importance of fragment E is due to the fact that its central region, after the removal of the fibrinopeptides A and B, orders the noncovalent assembly of fibrin units into a half-staggered arrangement [123].

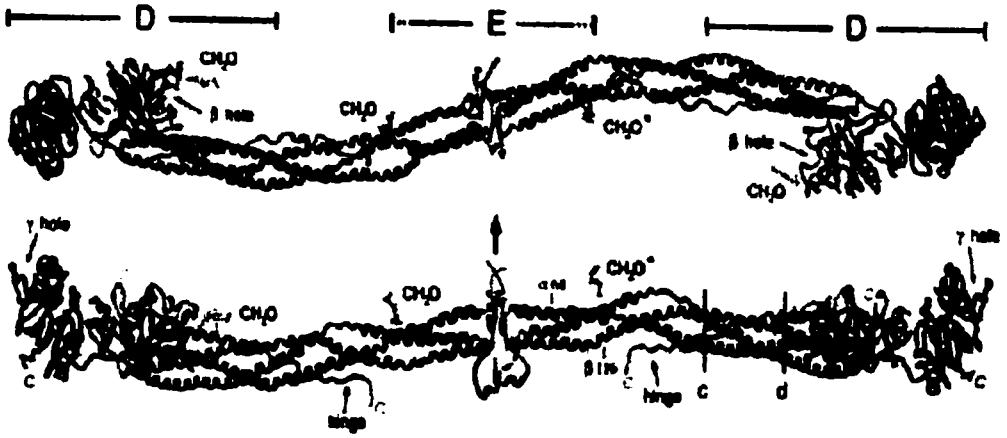


Fig.2: Modified bovine fibrinogen showing the sigmoidal shape of the coil-coil axis. Aα-chain is in blue, Bβ-chain is in green, γ-chain is in red, the antiparallel portion of the Aα-chain is shown in light blue.

1.11.2.6 Structural model proposed for the fibrinogen molecule

The most recent publication on fibrinogen (April 2000) depicts the crystal structure of native chicken fibrinogen at 5.5-Å resolution [113]. The overall shape is sigmoidal the flexible αC domains are not considered and the full length measure 460-Å. The central domain has the shape of an oblate disk defined by sets of radial spokes on each side of the covalent dimer. The amino-terminal segments of the α- and β-chains, which constitute the fibrinopeptides A and B respectively, are not included in the map and it has been suggested that these regions must be highly flexible. The flexible αC domains show a very weak trace and it is impossible to attribute individual chains, or even to assign particular molecules to the αC domains. An interaction may occur between αC domains of adjacent molecules, rather than within the same molecule.

The crystal structure of modified fibrinogen at 4-Å resolution [124] shoves the axis of the α-helical coiled-coil rod having a sigmoidal shape with a length of 450-Å. This finding indicates that the curvature appears to be an intrinsic feature of fibrinogen's coiled coil. In the modified fibrinogen there is the removal of part of the flexible C-terminal region of the Aα chain and some of the Bβ chain (about 15% of the molecule).

Both structures fail to show any significant structural differences in the main frame region (fig. 3). The sequence similarities are stronger in the globular regions than in the coiled coils, but even in the coiled coil region, the three-dimensional structures are virtually indistinguishable.

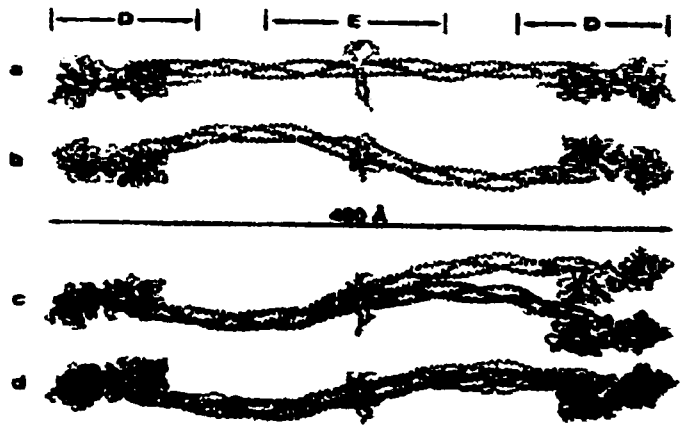


Fig. 3- Mainframe αC backbone structures of full-length chicken fibrinogen. A) top view; b) side view; c) demonstration of pseudosimmetry of fibrinogen dimer; d) superposition of chicken model (green) and the modified bovine fibrinogen model (red).

1.11.3 Conversion of fibrinogen to fibrin

1.11.3.1 Activation of fibrinogen by removal of fibrinopeptides

Only about 1% of the mass of the fibrinogen is cleaved by thrombin to initiate the polymerization into a fibrin clot. Both Aα- and Bβ-peptide chains are attacked by thrombin at two sets of Arg-Gly bonds, with the release of the fibrinopeptides A and B, respectively. Moreover, a slower reaction cleaves another peptide bond in the α-chain, which results in the liberation of the tripeptide Gly-Pro-Arg. Both peptides A and B are acidic by nature and carry three negative charges. Therefore, the amino-terminal domain that corresponds to the central nodule of intact fibrinogen is negatively charged but a net positive charge results after the removal of two peptides A and two peptides B carrying collectively 12 negative charges. Electrostatic attraction forces between amino-terminal and carboxy-terminal domains of interacting fibrin molecules are the main forces that play an important role in the process of fibrinogen polymerization. [125].

Surprisingly the removal of one fibrinopeptide A is sufficient to aggregate with another fibrinogen molecule. In contrast when thrombin cleaves peptide B, the process happens much slower than the release of peptide A.

Once the fibrinopeptides are released, subsequently reduce negative charges on fibrinogen molecules, resulting in the non-specific neutralization of repulsive electrostatic forces between the molecules. As a consequent, definite contact sites of intact fibrinogen become exposed. It has been demonstrated that the amino-terminal disulfide knot activated by thrombin has high binding affinity for the carboxy terminal of fragment D [126]. As shown in fig. 4, there is a set of "complementary" polymerization sites in different parts of the fibrinogen molecule, with some of them exposed in the native form

and others becoming unblocked or activated only after thrombin removes the fibrinopeptides. Recent analysis has focused its attention on identifying contact sites in fibrin monomers that take part in the polymerization reaction. Two complementary binding sites in the carboxy-terminus of the γ-chain and in the amino-terminal sequence of the fibrin α-chain have been described [127], and the existence of two extra polymerization sites has been suggested [128] (fig. 5).

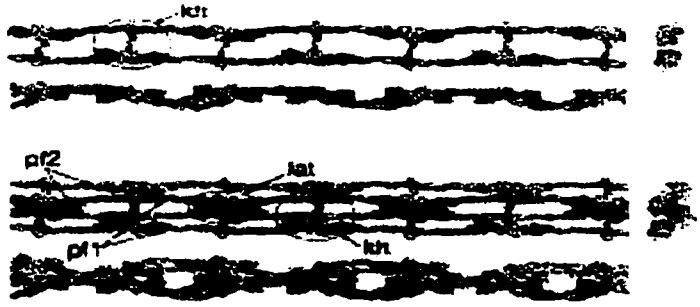


Fig. 4- Views of a single protofibril formed by knob-hole (kh) interactions. Light green, γ-chains; blue, β-chains; red, α-chains. End-on view is shown at the right side. PF1 and PF2 represent the lateral association (lat) of two protofibrils.

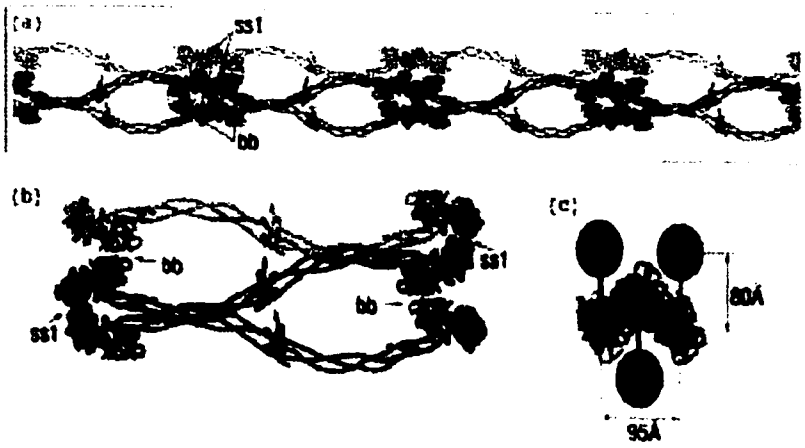


Fig.5- a) Three protofibrils associated by lateral involvement of γC domains; b) the γC associations (denoted ss1) are depicted as blue to red and red to green. The βC associations (denoted bb) follow the same pattern except red to green and blue to red. c) End-on view of three associated protofibrils. The solid colored discs denote the molecular units of the protofibrils not included in a and b; the colored vertical bars denote knob-hole interactions.

1.11.3.2 Interaction of fibrinogen with other molecules and platelets

Fibrinogen appears to be a very "sticky" protein and is known to interact with many synthetic polymers that are used for artificial prosthetic devices. If fibrinogen is adsorbed it may attract platelets and stimulate thrombus formation. Fibrinogen is able to interact with other proteins such as fibronectin. In this case, the binding site appears to be located in the carboxy-terminal portion of the fibrinogen Aα-chain [129]. As mentioned previously, fibrinogen carries a net negative charge, but it can form insoluble precipitates

with acidic polysaccharides, such as dextran sulfate and cellulose sulfate [130]. Fibrinogen has a heterogeneous distribution of charges in the molecule.

After thrombin attacks fibrinogen to generate fibrin monomers, thrombin then binds to the insoluble fibrin. Thrombin that is bound to fibrin has been shown to be partially protected from inactivation by antithrombins [131, 132] suggesting this binding is a potential defence against formation of the thrombus.

After an injury in the vessel wall fibrinogen is needed for the interaction between platelets in the blood and the wall itself. *In vitro*, it has been shown that washed platelets aggregated by ADP is dependent on the presence of fibrinogen because it binds to specific receptors in the platelets membranes. This binding requires divalent ions and occurs after ADP induces a change in platelet shape. Platelet receptors recognise a binding site on fibrinogen that is located in the γ-chain [133], apparently in the carboxy-terminal region of the γ-chain [134]. It has also been suggested that the fibrinogen Aα-chain takes part in the binding to platelets [129].

1.11.4 Synthesis and turn over of fibrinogen

Fibrinogen is synthesized in the hepatic parenchimal cells, normally produced at about 30 mg/kg/day. In a cell free system, the three different polypeptide chains are synthesized from three distinct mRNAs separately [135-138], and this suggests that fibrinogen is not synthesized as a large molecule, which is then processed into the three separate polypeptides chains. Furthermore, each polypeptide chain is synthesized as a longer precursor than the one that is ultimately secreted [133, 134]. The supplementary amino-terminal part of the chain appears to be specific in different species, and this suggests the

presence of the "signal" peptides implicated in the transport of the polypeptides from the cytosolic side of the rough endoplasmic reticulum to the cysternal side.

Glycosylation of the subunit chain is also another important event and it has been studied *in vitro*. This evidence indicates that Aα-chains are not glycosylated while both Bβ- and γ-chains are glycosylated at their asparagine residues. The γ-chain receives its carbohydrate as a cotranslational event that starts very early; since even nascent γ-chains that are incomplete are glycosylated. On the other hand, the Bβ-chain appears to be glycosylated only after the polypeptide termination or immediately following release into the cisternal space of the rough endoplasmic reticulum. The polypeptide chains are already assembled in the rough endoplasmic reticulum as a dimeric molecule ($A\alpha_2B\beta_2\gamma_2$) by disulphide bonds.

Synthesis of fibrinogen is higher during infection and inflammation. It has been demonstrated that a number of non-specific agents causing tissue injury or inflammation can stimulate hepatocytes to produce fibrinogen and other hepatic proteins directed towards different external spots at the same time [139]. The mechanism of this response is called "acute-phase reaction" and it has been utilized to experimentally induce an increase in the rate of fibrinogen synthesis.

The reticuloendothelial system (RES) and the kidneys are the sites where fibrinogen and its derivatives are resolved and eliminated [140].

1.12 Thrombin

Thrombin belongs to the family of serine proteases that consists of two disulfide-linked chains: in humans, a 36-residue A chain and a 259-residue B chain. B chain in the

thrombin enzyme is homologous and has similar specificity to trypsin. Thrombin is highly specific in cleaving only certain Arg-X, and less frequently Lys-X bonds with a preference for a Pro preceding the Arg or Lys [141].

Thrombin is not a normal constituent of the circulating blood, and is generated by the catalytic cleavage of its plasma precursor, prothrombin (factor II), by the activated Stuart factor (X_a). This is the final step of one or both of the two convergent chains of reactions called the intrinsic and extrinsic pathways of coagulation. The transformation from the zymogen form to the active enzyme, requires the presence of an activated cofactor, factor V_a, released from factor V by thrombin itself, and whose binding to prothrombin accelerates the activity of factor X_a in a non-enzymatic fashion.

Thrombin is a glycoprotein where three important sites have been identified on its surface:

i) the catalytic site that confers its serine protease activity,

ii) the exosite one responsible for the binding of the substrate (fibrinogen or thrombin receptor)

iii) the exosite two responsible for the binding of antithrombin III and inactivation of thrombin [142].

The earliest identified function of thrombin is the cleavage of fibrinogen into fibrin monomers and the activation of the fibrin-stabilizing factor (factor XIII) and protein C. The clot consists of a network of insoluble fibrin fibres that trap blood cells and serum. Thrombin has the properties of activating factor XIII to act as a transaminase and form covalent links between the carboxyl and amino groups of two different fibrin monomers, enhancing the strength of the clot.

Thrombin is more than a simple plasma enzyme; it also has properties that allow it to stimulate platelets and cause them to expand aggregates and release components of the alpha and dense granules [143, 144]. Thrombin also has many effects on other cells such as in the development of LPS-induced liver injury, where it alters the synthesis, expression and release of proteins from endothelial cells [145, 146]. This increases the production of the platelet-derived growth factor (PDGF), factor XIII, tPA, PAI, platelet activating factor (PAF), modification of the interactions between endothelial cells and the underlying matrix or between endothelial cells, and the expression of adhesion glycoproteins to the cell surface, therefore increasing the binding of inflammatory cells to the endothelium. Thrombin is a potent chemotaxin; it induces chemotaxis in neutrophils [147]. In addition, thrombin can alter the production of cytokines and arachidonic acid metabolites in macrophages [148], and releases inflammatory components [149, 150]. However, little is known about thrombin activity in the liver resident macrophages and its effect on hepatic parenchimal cells [151].

Only in the last few years has the mechanism by which thrombin activates platelets and other cells been discovered, and only in 1991 was a thrombin platelet receptor cloned [152], revealing a new mechanism for receptor activation. After binding to its receptor, thrombin cleaves the amino-terminal sequence that binds to the receptor and activates it. The sequence that is capable of self-activation was named the tethered ligand, and now various peptides similar to this sequence have been synthesised [153]. Their ability to activate the thrombin receptor has been demonstrated in various cell types other than platelets [154-156].

Another important characteristic of thrombin is its ability to proteolytically inactivate other thrombin molecules. Clot formation is therefore self-limiting, a safeguard that helps prevent blood clots from propagating away from the site of an injury.

1.13 Aim of the study

The aim of the following study was to describe the effects on fibrinogen structure and function upon incubation with S-nitrosothiols such as GSNO and HcysNO.

A decrease in fibrinogen polymerization was first observed after incubation of the fibrinogen/thrombin solution with GSNO. This effect can be due to either modification on fibrinogen or in thrombin.

Thrombin assays were performed through the hydrolysis of the pseudosubstrate Chromozym TH (tosylglycylprolylarginine-4-nitranilide acetate) and no difference in the initial rate of the reaction $\pm$ GSNO were observed.

Once it was confirmed that thrombin is not affected by GSNO, the attention was focused on fibrinogen. CD measurements were done and the secondary structure content was calculated. The idea that the S-nitrosothiols may interact with fibrinogen seemed plausible as conformational changes were found. This effect was reversible, as the fibrinogen CD spectrum was similar to the native spectrum subsequent to separation of GSNO from fibrinogen by FPLC.

Fluorescence studies of free amino acids (L-tyrosine and L-tryptophan) and fibrinogen incubated with GSNO and GSH were performed. A quench in fluorescence intensity was noted when GSNO was present in solution.

S-nitrosothiols are photolabile compounds. Following radiation, the S-NO bond can be cleaved with the release of NO. The fibrinogen/GSNO sample was irradiated @340 nm, and the CD spectra before and after were taken. There were no significant changes.

The NO-meter was used to quantify the NO released in the sample, but no NO was detected, even when the electrode was placed inside the cuvette in the CD chamber.

All results obtained are detailed in the following sections.

2.0 MATERIALS AND METHODS

2.1 Materials and sample preparation

Bovine *fibrinogen*, fraction I, type I-S, bovine *thrombin*, *DTNB* (*5,5'*-dithiobis *2*-nitrobenzoate) and *Iodoacetamide* were purchased from Sigma-Aldrich.

Chromozym TH (tosylglycylprolylarginine-*4*-nitranilide acetate) was purchased from Roche Molecular Biochemicals Canada.

Fibrinogen was first dissolved in a very small amount of 50 mmol/L glycine-NaOH buffer (pH=8.5). Fibrinogen concentration was determined by extrapolation of the value from a BSA standard curve. Its extinction coefficient in solution is 15.1-15.5 [100].

Fibrinogen and enzyme were dissolved in 5 mM PBS buffer (NaCl 0.137M, KCl 2.7mM, Na_2HPO_4 10 mM, KH_2PO_4 1.76 mM pH=7.4) when required.

Fibrinogen concentration used for CD spectroscopy was 0.1 mg/mL (3.03×10^{-7}M).

Synthesis of GSNO: Glutathione (Sigma) is dissolved in ice cold 0.5 M HCl. Equimolar sodium nitrite was added and the reaction was carried out in the dark at 4 °C for 40 min. The pH of the reaction mixture was adjusted to 7.4, and crystallized by the slow addition of cold acetone.

HcysNO was synthesised by the same procedure.

2.2 Kinetics of fibrinogen polymerization

Kinetic measurements of fibrinogen polymerization were carried out using an Agilent 8453 UV-Visible Spectrophotometer with a Peltier temperature controller (25 °C). Fibrinogen was prepared as mentioned above and the final concentration was 0.09

mg/mL (2.64×10^{-4} M), thrombin was dissolved in PBS buffer and its final concentration was 0.045 mg/mL (4.14×10^{-8} M). The turbidity of the solution was measured at 660 nm for 100 sec with an integration time of 1 sec. The blank used was PBS buffer. In the case of kinetics in the presence of GSNO, the fibrinogen sample was incubated with GSNO for 10 minutes in the dark. Every curve is the result of 10 different scans.

2.3 Thrombin assay

The activity of thrombin was determined with the colorimetric pseudo-substrate Chromozym TH. Thrombin (4.14×10^{-8} M), either preincubated with 50 μM GSNO for 10 minutes or not, was added to a 0.2 mM solution of Chromozym TH in PBS (25 °C). The absorbance (405 nm) was measured as a function of time in the spectrometer described above. The blank hydrolysis rate was subtracted from the enzymatic rate.

2.4 Circular dichroism measurements

Circular dichroism (CD) spectroscopy was used to determine the changes in the secondary structure of the protein in the presence of GSNO and HcysNO and GSH and Hcys. The solvent used was the PBS buffer. The final concentration of GSNO, GSH, HcysNO and Hcys were 25, 30 and 50 μM. Briefly:

Fibrinogen (0.3 μM) in PBS buffer were placed in a 1 cm quartz cuvette, the CD spectra were measured over the wavelength region of 190 nm to 260 nm (protein 2° structure) or 500 nm to 600 nm (RS-NO environment) in an AVIV CD spectrometer model 62A DS. Each spectrum is an average of 10 different scans obtained by collecting data 1 nm intervals with an integration time of 10 s, at a constant temperature of 25 °C.

The secondary structure composition was determined with the aid of the CDNN program Version 2 to deconvolution software (http://bioinformatik.biochemtech.uni-halle.de/cdnn).

2.5 Free SH content

The free thiol content of the samples were determined colorimetrically with the aid of DTNB (412 nm and Σ=13600 $M^{-1}cm^{-1}$) final concentration 50 μM in PBS buffer.

2.6 Blockage of free thiols with Iodoacetamide

GSH or Hcys (5 mmol) were dissolved in Tris-Acetate buffer (50mM, pH 8.5). To this solution 5 mmol iodoactamide was added. The mixture was stirred at room temperature for 2h. The solutions were then diluted appropriately in PBS and used without further purification.

2.7 Fluorescence measurements

Tyrosine and tryptophan were dissolved in PBS buffer as previously mentioned. 3 mL of tyrosine at a final concentration of 15 μM, and 3 mL of tryptophan at a final concentration of 10 μM were placed in a 1 cm cuvette at a temperature of 25 °C.

The titration with different concentrations of GSNO and GSH was performed over a range of 10-350 μM and the fluorescence intensity was measured with the aid of a Spex Fluorolog 1680

The excitation wavelength used for tyrosine was 275 nm and 290 nm for tryptophan while the emission wavelength were 303 nm and 348 nm respectively.

The absorbance of the solution at the excitation λ did not exceed 0.1. Fractional quenching at a given [GSNO] was estimated by dividing this value by the maximal quenching obtained at the largest [GSNO]. This data was then fitted to a saturation function ($\Delta F/\Delta F_{tot}$= [GSNO]/ (K_D + [GSNO]) in order to estimate the K_D.

2.8 NO measurements

NO measurements were carried out using a NO-sensitive electrochemical probe, ISO-NO *2*, WPI.

In the case of NO measurements inside the CD spectrometer, the electrode was inserted into the cuvette containing the GSNO/fibrinogen solution in the sampling chamber.

2.9 FPLC

Fibrinogen incubated with GSNO was passed through a G-25 column using PBS buffer as eluant and ethanol 20% to clean the column in a Biologic system (Bio Rad).

3.0 RESULTS

3.1 GSNO effects on thrombin catalyzed fibrinogen polymerization

Thrombin is a serine protease that catalyses the transformation of fibrinogen into fibrin by removing the small polar peptides, the fibrinopeptides A and B, from the parent molecule. The release of these fibrinopeptides reduces the excess of negative charges on the fibrinogen molecule and as a result, definite contact sites which appear to be masked in intact fibrinogen, become exposed.

This process can be monitored *in vitro* by measuring the increase in turbidity at 660 nm of fibrinogen solution in the presence of catalytic amounts of thrombin. As can be seen in Fig. 6 A (white fill bar) the initial rate of fibrinogen polymerization (4.9 E-4/s) decreased by 2.7-fold (grey fill bar) and 4.7-fold (black fill bar) in the presence of 20 μM and 50 μM GSNO respectively.

Following this initial experiment, the effect can either be on the fibrinogen or on thrombin.

3.2 Effects of GSNO on thrombin

Thrombin, plasmin, trypsin are all serine proteases, they are endopeptidases, enzymes that can cleave peptide bonds at internal positions in the polypeptide chains. Most of these proteolytic enzymes will also hydrolyse synthetic compounds whose structures are analogous in certain ways to the polypeptide substrate. The thrombin activity was measured with the protease pseudosubstrate Chromozym TH that can be easily cleaved by the enzyme; thrombin was incubated with 50 μM GSNO for 30 min in the dark.

Fig 6 A

Kinetic of fibrinogen polymerization

Legend

Kinetic measurements of fibrinogen polymerization carried out using an Agilent 8453 UV-Visible Spectrophotometer with a Peltier temperature controller (25 °C). Fibrinogen at a final concentration of 2.64×10^{-4} M was incubated with thrombin at a final concentration of 4.14×10^{-8} M. The turbidity of the solution was measured at 660 nm for 100 sec with an integration time of 1 sec. Every curve is the result of 10 different scans.
White fill bar represent native fibrinogen incubated with thrombin; gray fill bar represents fibrinogen incubated with thrombin and GSNO 20 μM; black fill bar represents fibrinogen incubated with thrombin and GSNO 50 μM.
Error bars represent standard deviation (n=10)

Fig 6 B

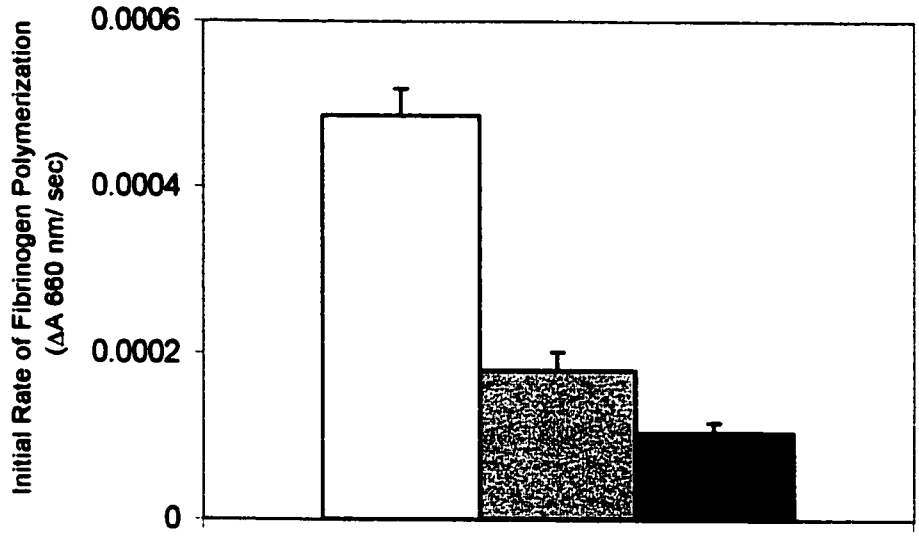
Kinetic of Chromozym TH hydrolysis by thrombin

Legend

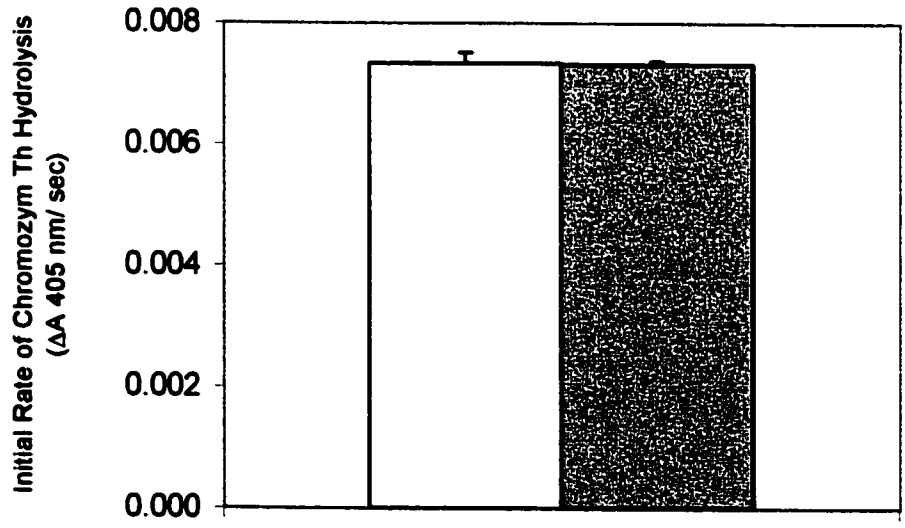
The activity of thrombin was determined with the colorimetric pseudo substrate Chromozym TH. Thrombin (4.14×10^{-8} M), either preincubated with 50 μM GSNO (gray fill bar) for 10 minutes or not (white fill bar), was added to a 0.2 mM solution of Chromozym TH in PBS (25 °C). The absorbance (405 nm) was measured as a function of time in the spectrometer described above.
Error bars represent standard deviation (n=10)

Figure 6

A


Initial Rate of Fibrinogen Polymerization
(ΔA 660 nm/ sec)
0.0006
0.0004
0.0002
0

B


Initial Rate of Chromozym Th Hydrolysis
(ΔA 405 nm/ sec)
0.008
0.006
0.004
0.002
0.000

The results indicated that thrombin is not affected by GSNO, as the initial rates of Chromozym TH hydrolysis are indistinguishable ± GSNO (fig. 6 B).

The decrease in fibrin clot formation must be then due to fibrinogen.

3.3 GSNO effects on fibrinogen secondary structure

To demonstrate the dependence of changing fibrinogen polymerization on its structure, the far-UV CD spectra of fibrinogen as a function of [GSNO] and [HcysNO], the other S-nitrosothiols of choice, were analysed. The final concentrations of 25, 30 and 50 μM were used.

Both the RSNOs increased the –ve ellipticity at ~ 207 nm and decreased the +ve ellipticity at 190 nm (fig. 7 A-B). Upon deconvolution, the GSNO/HcysNO-dependent change in the CD spectrum corresponded to a ~ 20% increase in helicity coming at expense of ~ 10% loss in β-sheet and ~ 5% decrease in random coil content (fig.8 A-B-C and 9 A-B-C).

This change appears to be RSNO specific as S-methylamidoGSH (fig. 10 A) or S-methylamidoHcys (fig. 10 B) failed to alter the CD spectrum of fibrinogen.

The S-methylamidoGSH and methylamidoHcys were formed upon incubation of fibrinogen with iodoacetamide in order to protect its 29 disulfide bonds.

Results and data are also summarized in tables 2, 3, 4 and 5.

Fig 7 A

Circular dichroism spectra of fibrinogen incubated with different [GSNO]

Legend

CD spectroscopy used to determine the changes in the secondary structure of fibrinogen in the presence of GSNO. 3 mL of fibrinogen at a final concentration of 3.03×10^{-7}M were placed in a 1 cm quartz cuvette, the CD spectra were measured over the wavelength region of 190-260 nm in a AVIV CD spectrometer model 62A DS. Each spectrum was the result of at least 10 different scans obtained by collecting data at 1 nm intervals with an integration time of 10 s, at a constant temperature of 25 °C. ◆ CD spectra of native fibrinogen: CD spectra of fibrinogen incubated with 25 μM of GSNO; ▲ CD spectra of fibrinogen incubated with 30 μM of GSNO: ■ CD spectra fibrinogen incubated with 50 μM of GSNO

Fig 7 B

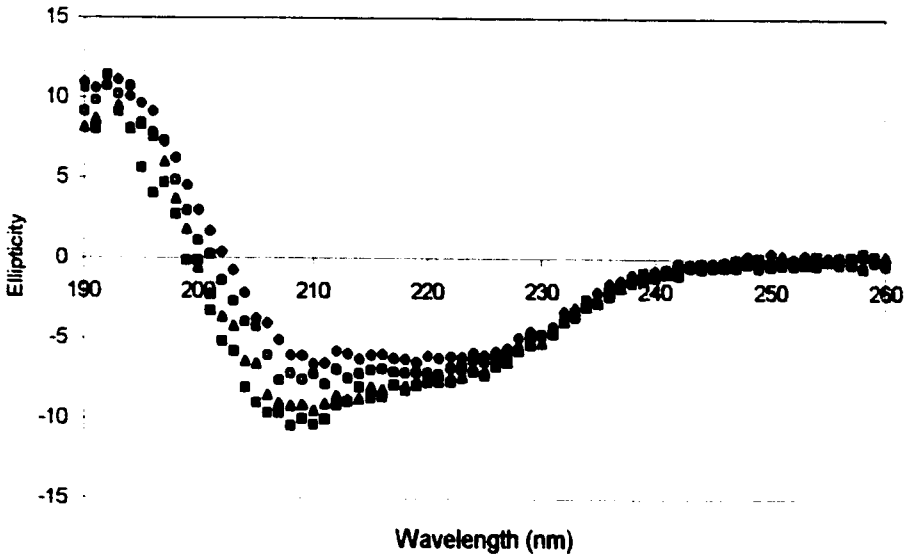
Circular dichroism spectra of fibrinogen incubated with different [HcysNO]

Legend

CD spectroscopy used to determine the changes in the secondary structure of fibrinogen in the presence of GSNO. 3 mL of fibrinogen at a final concentration of 3.03×10^{-7}M were placed in a 1 cm quartz cuvette, the CD spectra were measured over the wavelength region of 190-260 nm in a AVIV CD spectrometer model 62A DS. Each spectrum was the result of at least 10 different scans obtained by collecting data at 1 nm intervals with an integration time of 10 s, at a constant temperature of 25 °C. ◆ CD spectra of native fibrinogen: CD spectra of fibrinogen incubated with 25 μM of HcysNO; ▲ CD spectra of fibrinogen incubated with 30 μM of HcysNO: ■ CD spectra fibrinogen incubated with 50 μM of HcysNO.

Figure 7

A


15
10
5
0
-5
-10
-15
Ellipticity
190
200
210
220
230
240
250
260
Wavelength (nm)

B

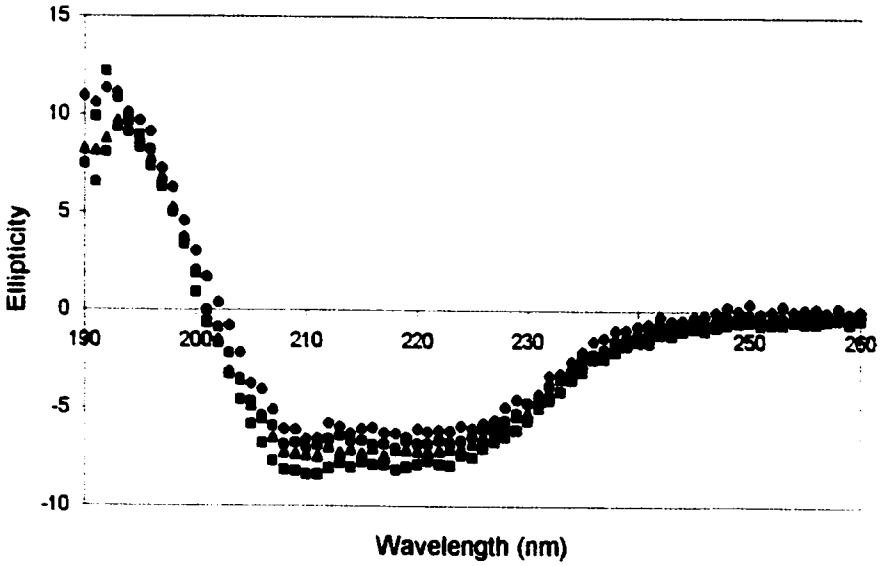

15
10
5
0
-5
-10
Ellipticity
190
200
210
220
230
250
260
Wavelength (nm)

Fig 8 A, B, C

Secondary structure composition of fibrinogen incubated with different [GSNO] and [GSH]

Legend

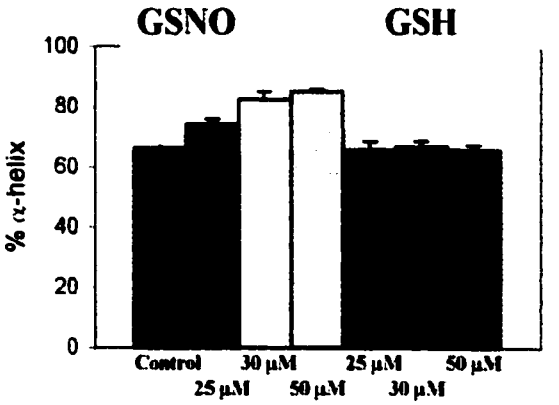
Secondary structure composition of fibrinogen incubated with different [GSNO] and [GSH] determined with the aid of the CDNN program Version 2 to deconvolution software (http://bioinformatik.biochemtech.uni-halle.de/cdnn). In the case of fibrinogen incubated with GSH, Iodoacetamide was used to protect the disulfide bonds in the fibrinogen. A 1 mM solution of iodoacetamide was added to the fibrinogen treated with the GSH at a final concentration of 3.4 μM, this caused the formation of S-methylamidoGSH.

Error bars represent standard deviation (n=10)

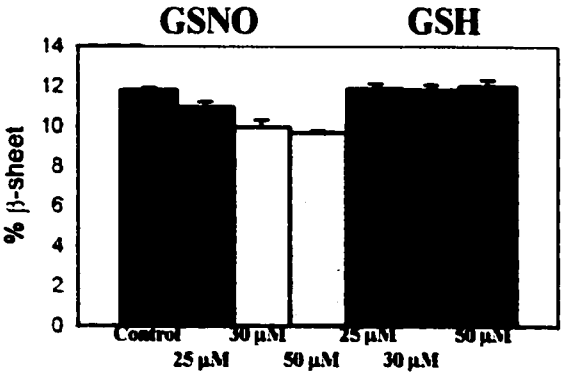
A) α-helix content of fibrinogen incubated with 25-30-50 μM of GSNO compared to the incubation of fibrinogen with 25-30-50 μM of GSH
B) β-sheet content of fibrinogen incubated with 25-30-50 μM of GSNO compared to the incubation of fibrinogen with 25-30-50 μM of GSH
C) random coil content of fibrinogen incubated with 25-30-50 μM of GSNO compared to the incubation of fibrinogen with 25-30-50 μM of GSH.

Figure 8

A


GSNO
GSH
% α-helix
100
80
60
40
20
0
Control
30 µM
25 µM
50 µM
25 µM
50 µM
30 µM

B


GSNO
GSH
% β-sheet
14
12
10
8
6
4
2
0
Control
30 µM
25 µM
50 µM
25 µM
50 µM
30 µM

C

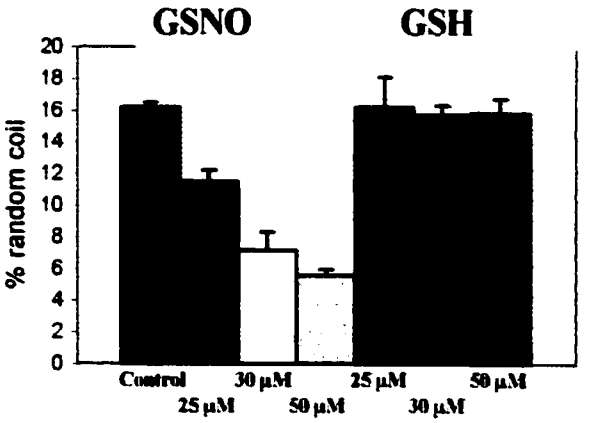

GSNO
GSH
% random coil
20
18
16
14
12
10
8
6
4
2
0
Control
30 µM
25 µM
50 µM
25 µM
50 µM
30 µM

Fig 9 A, B, C

Secondary structure composition of fibrinogen incubated with different [HcysNO] and [Hcys]

Legend

Secondary structure composition of fibrinogen incubated with different [HcysNO] and [Hcys] determined with the aid of the CDNN program Version 2 to deconvolution software (http://bioinformatik.biochemtech.uni-halle.de/cdnn). In the case of fibrinogen incubated with Hcys, iodoacetamide was used to protect the disulfide bonds in the fibrinogen. A 1 mM solution of iodoacetamide was added to the fibrinogen treated with Hcys at a final concentration of 3.4 μM, this caused the formation of S-methylamidoHcys.

Error bars represent standard deviation (n=10)

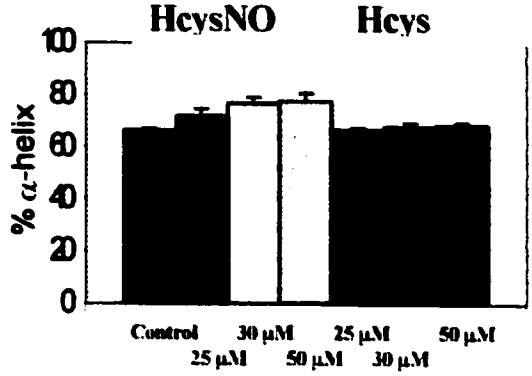
A) α-helix content of fibrinogen incubated with 25-30-50 μM of HcysNO compared to the incubation of fibrinogen with 25-30-50 μM of Hcys

B) β-sheet content of fibrinogen incubated with 25-30-50 μM of HcysNO compared to the incubation of fibrinogen with 25-30-50 μM of Hcys

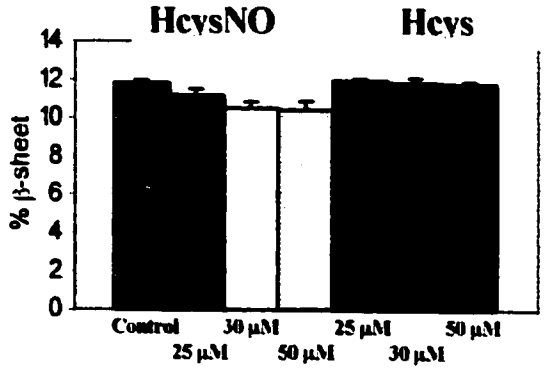
C) random coil content of fibrinogen incubated with 25-30-50 μM of HcysNO compared to the incubation of fibrinogen with 25-30-50 μM of Hcys

Figure 9

A


HcysNO
Hcys
% α-helix
100
80
60
40
20
0
Control
30 μM
25 μM
50 μM
25 μM
50 μM
30 μM

B


HcysNO
Hcys
% β-sheet
14
12
10
8
6
4
2
0
Control
30 μM
25 μM
50 μM
25 μM
50 μM
30 μM

C

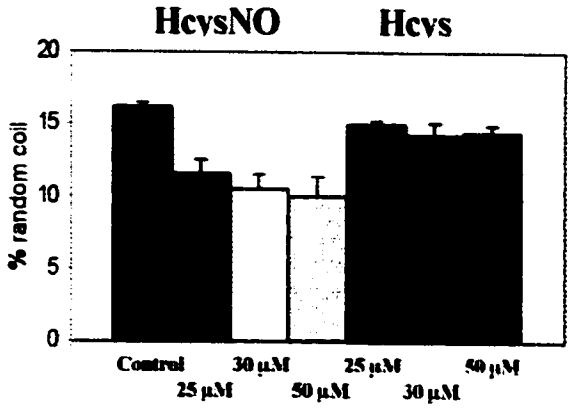

HcysNO
Hcys
% random coil
20
15
10
5
0
Control
30 μM
25 μM
50 μM
25 μM
50 μM
30 μM

Fig 10 A

Circular dichroism spectra of fibrinogen incubated with different [GSH]

Legend

CD spectroscopy used to determine the changes in the secondary structure of fibrinogen in the presence of GSNO. 3 mL of fibrinogen at final concentration of 3.03×10^{-7}M were placed in a 1 cm quartz cuvette, the CD spectra were measured over the wavelength region of 190-260 nm in a AVIV CD spectrometer model 62A DS. Each spectrum was the result of at least 10 different scans obtained by collecting data at 1 nm intervals with an integration time of 10 s, at a constant temperature of 25 °C. Iodoacetamide was used to protect the disulfide bonds in the fibrinogen. A 1 mM solution of iodoacetamide was added to the fibrinogen treated with GSH at a final concentration of 3.4 μM, this caused the formation of S-methylamidoGSH.

◆ CD spectra of native fibrinogen; CD spectra of fibrinogen incubated with 25 μM of GSH; ▲ CD spectra of fibrinogen incubated with 30 μM of GSH: ■ CD spectra of fibrinogen incubated with 50 μM of GSH

Fig 10 B

Circular dichroism spectra of fibrinogen incubated with different [Hcys]

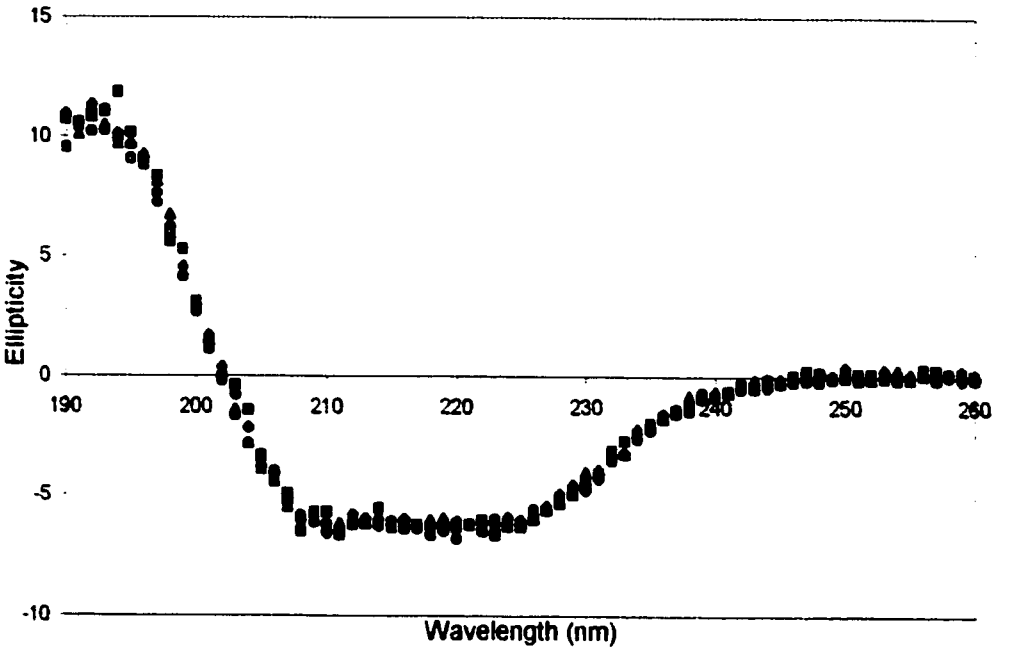
Legend

CD spectroscopy used to determine the changes in the secondary structure of fibrinogen in the presence of GSNO. 3 mL of fibrinogen at final concentration of 3.03×10^{-7}M were placed in a 1 cm quartz cuvette, the CD spectra were measured over the wavelength region of 190-260 nm in a AVIV CD spectrometer model 62A DS. Each spectrum was the result of at least 10 different scans obtained by collecting data at 1 nm intervals with an integration time of 10 s, at a constant temperature of 25 °C. Iodoacetamide was used to protect the disulphide bonds in the fibrinogen. A 1 mM solution of iodoacetamide was added to the fibrinogen treated with Hcys at a final concentration of 3.4 μM, this caused the formation of S-methylamidoHcys.

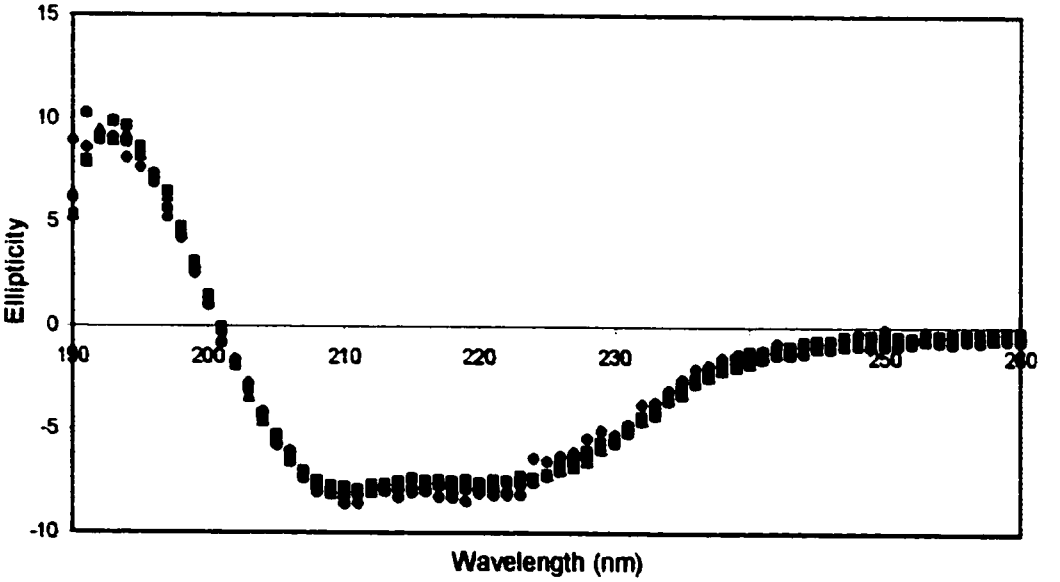
◆ CD spectra of native fibrinogen: CD spectra of fibrinogen incubated with 25 μM of Hcys; ▲ CD spectra of fibrinogen incubated with 30 μM of Hcys: ■ CD spectra of fibrinogen incubated with 50 μM of Hcys

Figure 10

A


15
10
5
0
-5
-10
Ellipticity
190
200
210
220
230
240
250
260
Wavelength (nm)

B


15
10
5
0
-5
-10
Ellipticity
190
200
210
220
230
250
Wavelength (nm)

	α-helix	β-sheet	random coil
Fibrinogen	66.2 ± 0.7	11.8 ± 0.1	16.17 ± 0.306
Fibrinogen +GSNO 25μM	74.1 ± 1.7	10.93 ± 0.252	11.47 ± 0.751
Fibrinogen +GSNO 30μM	82.2 ± 2.651	9.97 ± 0.351	7.13 ± 1.17
Fibrinogen +GSNO 50μM	85.07 ± 0.67	9.67 ± 0.115	5.6 ± 0.361

Table 2: data related to the secondary structure contents of fibrinogen incubated with GSNO.

	α-helix	β-sheet	random coil
Fibrinogen	66.2 ± 0.7	11.8 ± 0.1	16.17 ± 0.306
Fibrinogen +GSH 25μM	65.6 ± 2.821	11.87 ± 0.252	16.17 ± 1.904
Fibrinogen +GSH 30μM	66.63 ± 1.858	11.8 ± 0.265	15.7 ± 0.608
Fibrinogen +GSH 50μM	65.67 ± 1.45	12 ± 0.3	15.833 ± 0.924

Table 3: data related to the secondary structure contents of fibrinogen incubated with GSH.

	α-helix	β-sheet	random coil
Fibrinogen	66.2 ± 0.7	11.8 ± 0.1	16.17 ± 0.306
Fibrinogen +HcysNO 25μM	71.8± 2.535	11.17± 0.33	11.55 ± 1.00
Fibrinogen + HcysNO 30μM	76.74 ± 2.27	10.5 ± 0.324	10.52 ± 1.028
Fibrinogen + HcysNO 50μM	77.18 ± 3.1	10.4 ± 0.45	9.96 ± 1.415

Table 4: data related to the secondary structure contents of fibrinogen incubated with HcysNO.

	α-helix	β-sheet	random coil
Fibrinogen	66.2 ± 0.7	11.8 ± 0.1	16.17 ± 0.306
Fibrinogen +Hcys 25μM	66.35 ± 0.714	11.925 ± 0.1	14.95 ± 0.238
Fibrinogen + Hcys 30μM	67.45 ± 1.5	11.825 ± 0.25	14.225 ± 0.87
Fibrinogen + Hcys 50μM	67.9 ± 1.16	11.725 ± 0.15	14.425 ± 0.5

Table 5: data related to the secondary structure contents of fibrinogen incubated with Hcys.

3.4 FPLC

These experiments were performed to determine whether fibrinogen:RSNO interactions were reversible.

A G-25 column through an FPLC system (Bio-Logic) was used to separate fibrinogen from GSNO.

The major problem was to standardise the protein control versus the one treated with GSNO by appropriate dilution.

The results (fig. 11) were surprising, because the fibrinogen CD spectrum was very similar to the native spectrum subsequent to separation of GSNO from fibrinogen by FPLC.

3.5 Detection of NO production during the CD experiments and in general during incubation of fibrinogen with RSNO

The S-NO bond is photolabile. Although the experiments using S-nitrosothiols were carried out in the dark, it is possible that the observed conformational changes might result from nascent NO released during the CD experiments and reacted with protein functional groups. This aspect was tested by inserting an NO-specific electrode into the cuvette containing the GSNO/fibrinogen solution in the sampling chamber of the CD spectrometer.

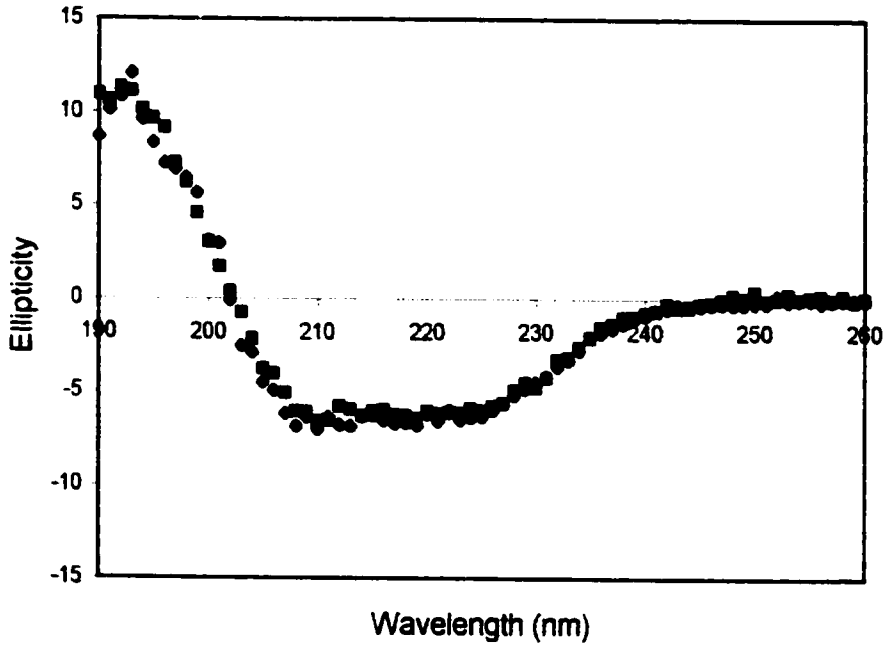
Fig 11

CD spectra of fibrinogen incubated with GSNO and passed through a G-25 column in an FPLC system

Legend

FPLC was used to verify whether the bound between fibrinogen and GSNO is reversible or not. Fibrinogen incubated with GSNO was passed through a G-25 column using PBS buffer as eluent and ethanol 20% to clean the column. CD spectra were measured before and after in the same system mentioned above. ◆ CD spectrum of fibrinogen incubated with 50 μM of GSNO; ■ CD spectrum of the same fibrinogen incubated with GSNO after run through the G-25 column.

Figure 11


15
10
5
0
-5
-10
-15
Ellipticity
190
200
210
220
230
240
250
260
Wavelength (nm)

As shown in fig 12 there was no detectable NO produced during the CD experiment.

The specific NO-electrode was also used to detect any possible presence of NO when fibrinogen was incubated with GSNO. NO was measured in a solution composed of GSNO in PBS buffer until a plateau was reached, fibrinogen was then added and the electrode was notable to detect any presence of NO in the sample. This was sufficient proof that the addition of the two compounds together did not cause any NO release.

3.6 GSNO/fibrinogen irradiation @ 340 nm

As mentioned before, S-nitrosothiols are photosensitive compounds and upon irradiation the S-NO bound can be cleaved with consequent and possible reaction of the NO with secondary amines, those in the peptide backbone.

A fibrinogen/GSNO solution was run in the CD spectrometer, the sample was irradiated at 340 nm for 5 minutes and the CD of this sample was taken. This experiment supported evidence that there are no differences between the two spectra (fig. 13), NO most likely does not interact with the fibrinogen structure.

The interaction between the fibrinogen and the RSNO is unlikely to involve any covalent bound. Fibrinogen has no free –SH group. All the cysteine residues are involved in the formation of the 29 disulfide bonds that stabilize and give peculiar characteristic to the protein. Nitrosation or S-nitrosylation is unlikely involved. GSNO therefore might allosterically interact with fibrinogen through tyrosine and tryptophan. These amino acid residues are crucial in fibrinogen molecule.

Fig 12

NO detection through a specific NO electrode

Legend

Spectrum of the NO-specific electrode inserted into the cuvette containing the GSNO/fibrinogen solution in the sampling chamber of the CD spectrometer.
1nmol=100 units

Figure 12

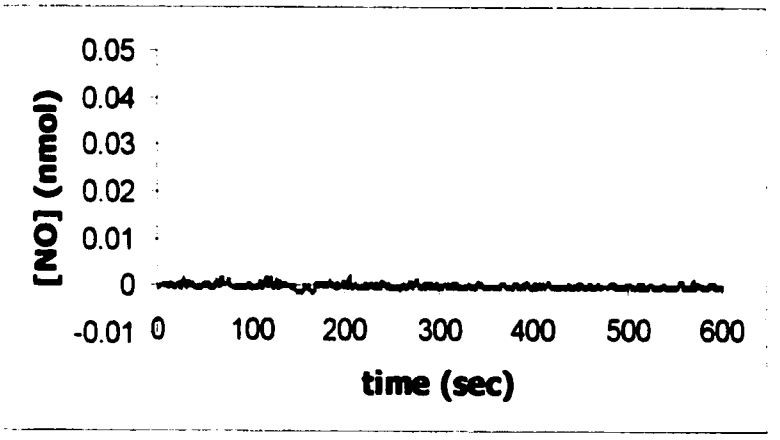

0.05
0.04
0.03
0.02
0.01
0
-0.01
[NO] (nmol)
0
100
200
300
400
500
600
time (sec)

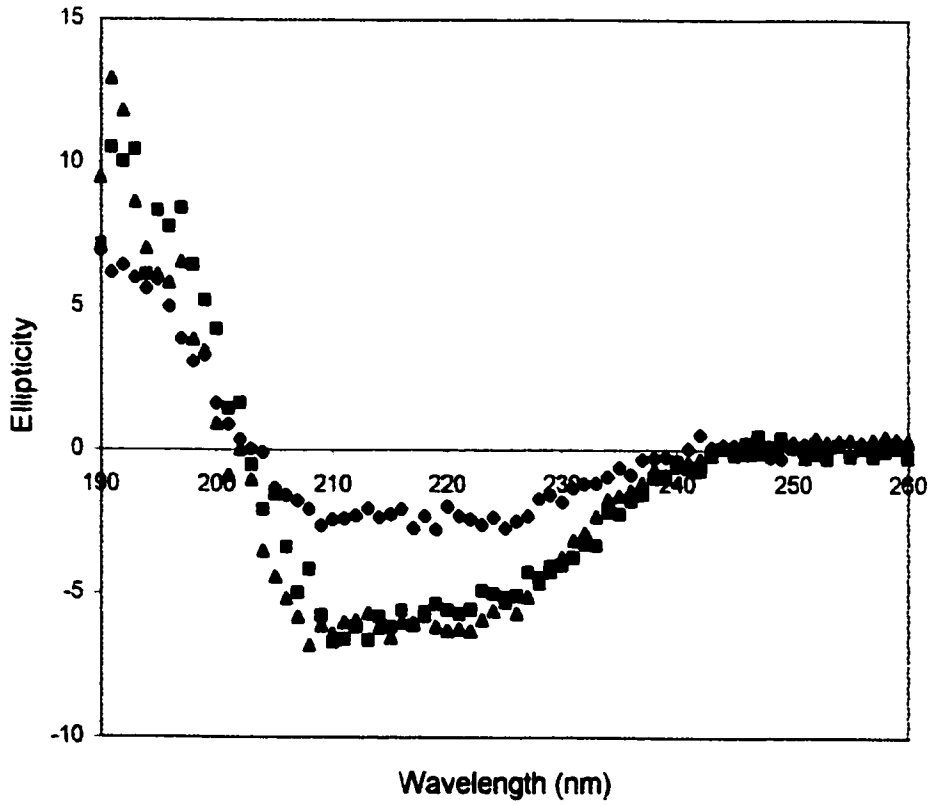
Fig 13

GSNO/fibrinogen irradiation @ 340 nm

Legend

◆ CD spectrum of native fibrinogen; ■ CD spectrum of fibrinogen incubated with GSNO 50 μM; ▲ CD spectrum of fibrinogen incubated with GSNO 50 μM after irradiation at 340 nm for 5 minutes

Figure 13


15
10
5
0
-5
-10
Ellipticity
190
200
210
220
230
240
250
260
Wavelength (nm)

3.7 GSNO perturbs L-tyr and L-trp fluorescence of free amino acids and fibrinogen

To test our hypothesis on allosteric interactions, we performed fluorescence quenching experiments. Pure solutions of amino acids tyrosine and tryptophan in PBS buffer as well as fibrinogen, were prepared, titrated with different concentrations of GSNO and GSH as control, and the fluorescence was studied.

In our experiments, Tyr at a final concentration of 15 μM, after the addition of GSNO at 0.6 times the tyrosine concentration, 6 times, 10 times and so on, a decrease in fluorescence intensity was noticed. When the above procedure was repeated using GSH as control, no changes were seen in the fluorescence intensity. The fit of the fractional quenching to a saturation function resulted in an estimated K_D that was 70 μM. In fig. 14 A-B-C the results are shown. These emission spectra show that the extent of the loss of fluorescence is proportional to the concentration of GSNO added to the sample.

This is direct evidence that GSNO, but not GSH, is interacting with tyrosine.

The same procedure was used for tryptophan, but its final concentration was lowered to 10 μM, because the fluorescence intensity was too high. The results were comparable, i.e. quenching in fluorescence with increasing [GSNO], no loss in fluorescence when GSH was added. This is direct evidence that GSNO, but not GSH is interacting with tryptophan. The fit of the quench data to a saturation function resulted in an estimated K_D that was 75 μM. In fig. 15 the results are shown.

The intrinsic Trp fluorescence spectrum was monitored as a function of [GSNO]. The fibrinogen fluorescence was quenched in a saturable manner (Fig. 16 A-B) indicating that the process was not due to collisional quenching. The fit of the quench data to a

saturation function resulted an in estimated K_D of 43 μM. In an effort to estimate the stoichiometry of GSNO/fibrinogen interactions, 100 μM fibrinogen was titrated with [GSNO]. Since this is above the estimated K_D, the fluorescence quenching should represent stoichiometric binding. Under these conditions the quenching profile was characterized by two slopes which when extrapolated gave an approximate ratio of 2:1 (GSNO/fibrinogen) (Fig. 16 C).

3.8 Fibrinogen perturbs the S-NO CD spectrum

Any possible interactions between fibrinogen and GSNO were further characterised by titrating GSNO with fibrinogen, and monitoring the CD spectra between 400 and 600 nm. The CD absorption spectrum (550 nm) of RSNOs is spectrally well separated from that resulting from protein secondary structure [157].

In order to estimate the affinity of RSNO/aromatic side chain interactions by an independent technique, the CD spectrum at a constant amount of GSNO was monitored at 550 nm as a function of [fibrinogen]. The CD spectral maximum decreased in a saturable manner with an estimated K_D of ~ 40 μM. (fig. 17).

Fig 14 A, B, C

GSNO perturbs L-Tyr fluorescence of free amino acid in a saturable fashion

Legend

Tyrosine was dissolved in PBS buffer. 3mL of tyrosine at a final concentration of 15 μM, was placed in a 1 cm cuvette at a temperature of 25 °C.

The titration with different concentrations of GSNO and GSH was performed over a range of 10-350 μM and the fluorescence intensity was measured with the aid of a Spex Fluorolog 1680. The excitation wavelength was 275 nm while the emission wavelength was 303 nm. The fluorescence intensity was taken 10 nm after the excitation wavelength to avoid the light scattering. The absorbance of the solution at 290 nm did not exceed 0.1. Fractional quenching at a given [GSNO] was estimated by dividing each single quench by the maximal quenching obtained at the largest [GSNO]

A) Tyrosine (15 μM) titrated with different [GSNO], ranging from 10 to 300 μM.

— tyrosine, — 10 μM GSNO, —50 μM GSNO, — 100 μM GSNO, — 150 μM GSNO, 200 μM GSNO, —250 μM GSNO, — 300 μM GSNO.

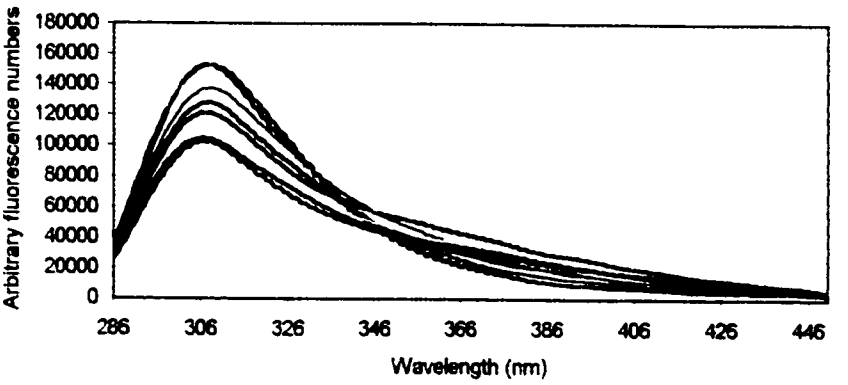
B) Tyrosine (15 μM) titrated with different [GSH] as control

— tyrosine, — 10 μM GSH, — 50 μM GSH, — 100 μM GSH, — 150 μM GSH, 200 μM GSH, —250 μM GSH, — 300 μM GSH.

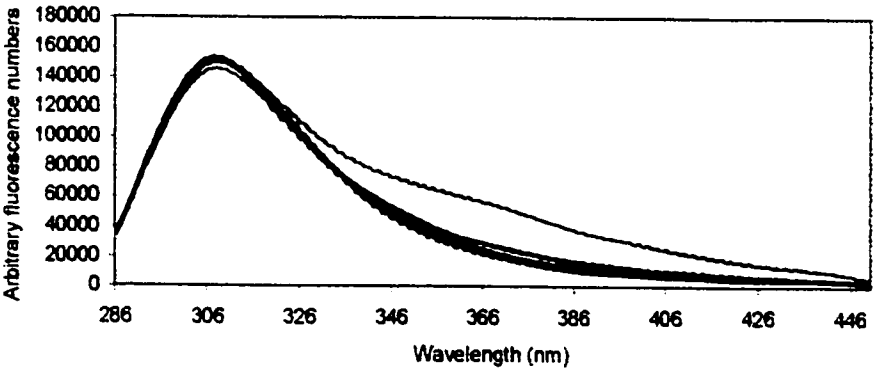
C) The fit of the fractional quenching to a saturation function

Figure 14

A



B



C

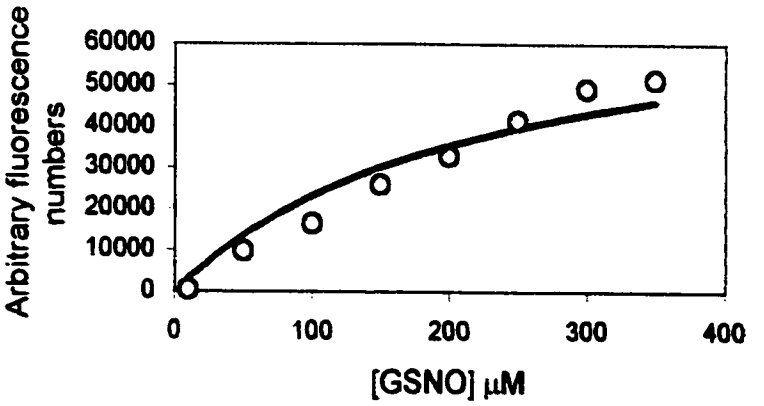


Fig 15 A-B-C

GSNO perturbs L-Trp fluorescence of free amino acid in a saturable fashion

Legend

Tryptophan was dissolved in PBS buffer. 3mL of tryptophan at a final concentration of 10 μM was placed in a 1 cm cuvette at a temperature of 25 °C. The titration with different concentrations of GSNO and GSH was performed over a range of 10-300 μM and the fluorescence intensity was measured with the aid of a Spex Fluorolog 1680.

The excitation wavelength used was 280 nm while the emission wavelength was 348 nm. The fluorescence intensity was taken 10 nm after the excitation wavelength to avoid the light scattering. The absorbance of the solution at 290 nm did not exceed 0.1. Fractional quenching at a given [GSNO] was estimated by dividing each single quench by the maximal quenching obtained at the largest [GSNO].

A) Tryptophan (10 μM) titrated with different [GSNO]

— tryptophan, — 10 μM GSNO, 50 μM GSNO, — 100 μM GSNO, — 200 μM GSNO, — 300 μM GSNO.

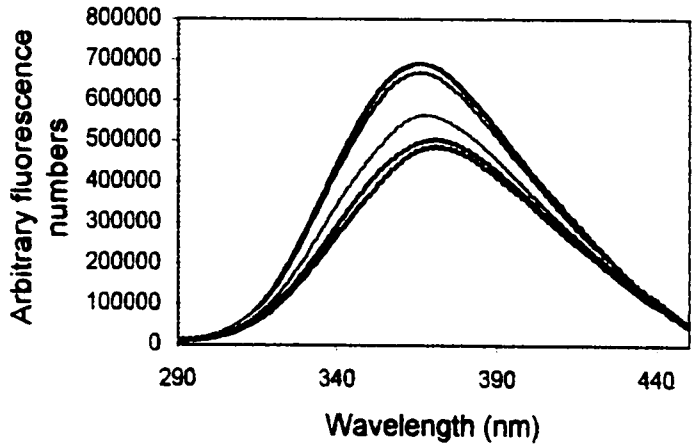
B) Tryptophan (10 μM) titrated with different [GSH] as control

— tryptophan, — 10 μM GSH, 50 μM GSH, — 100 μM GSH, — 200 μM GSH, — 300 μM GSH

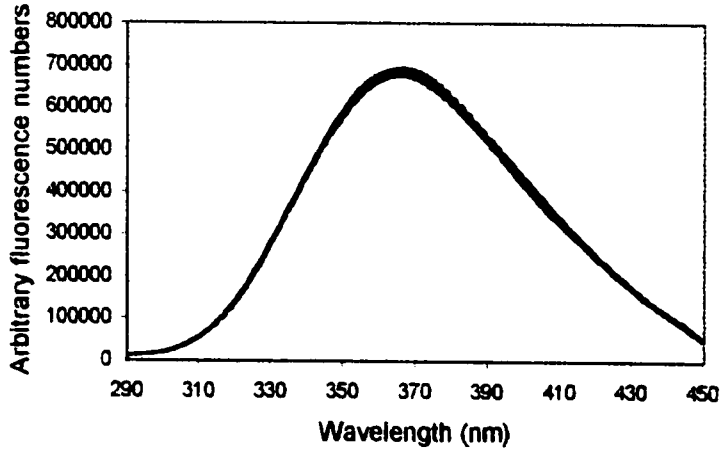
C) The fit of the fractional quenching to a saturation function

Figure 15

A



B



C

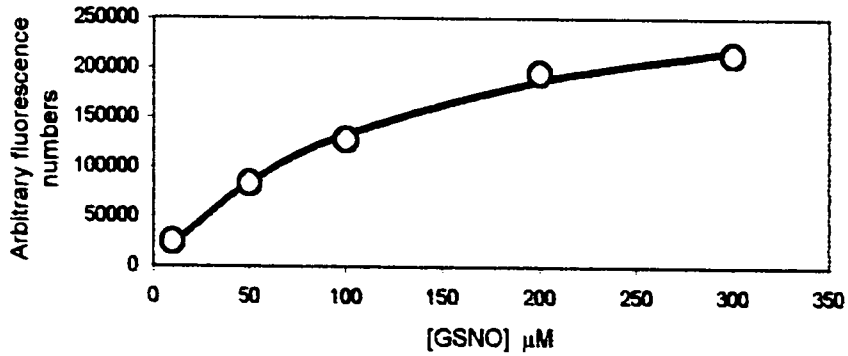


Fig 16 A-B-C

GSNO perturbs fibrinogen fluorescence in a saturable fashion

Legend

Fibrinogen was dissolved in PBS buffer. 3 mL of fibrinogen were placed in a 1 cm cuvette at a temperature of 25 °C. The titration with different concentrations of GSNO was performed over a range of 10-300 μM and the fluorescence intensity was measured with the aid of a Spex Fluorolog 1680. The excitation wavelength used was 280 nm while the emission wavelength was around 350 nm. The fluorescence intensity was taken 10 nm after the excitation wavelength to avoid the light scattering. The absorbance of the solution at 290 nm did not exceed 0.1. Fractional quenching at a given [GSNO] was estimated by dividing this value by the maximal quenching obtained at the largest [GSNO]. Fibrinogen fluorescence was quenched in a saturable manner indicating that the process is not due to collisional quenching. This data was then fitted to a saturation function ($\Delta F/\Delta F_{tot}$= [GSNO]/ (K_D + [GSNO]) in order to estimate the K_D that resulted ~ 43 μM. In an effort to estimate stoichiometry of GSNO/fibrinogen interactions 100 μM fibrinogen was titrated with [GSNO]. Since this is above the estimated K_D, the fluorescence quenching should represent stoichiometric binding. Under these conditions the quenching profile was characterised by two slopes which, when extrapolated, gave an approximate ratio 2:1 (GSNO/fibrinogen)

A) Fibrinogen titrated with different [GSNO].

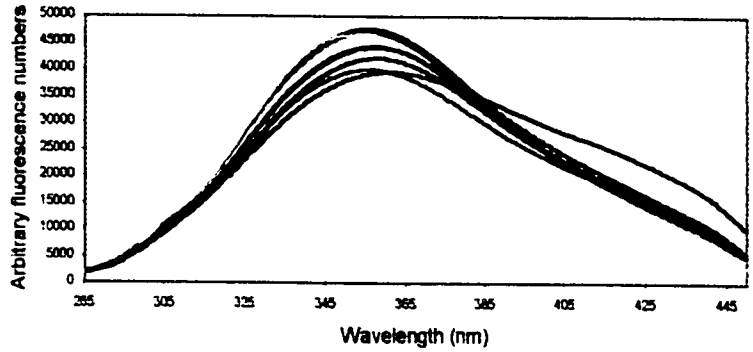
— fibrinogen, — 5 μM GSNO, 10 μM GSNO, — 20 μM GSNO, — 50 μM GSNO, — 80 μM GSNO, — 100 μM GSNO, — 150 μM GSNO.

B) The fit of the fractional quenching to a saturation function

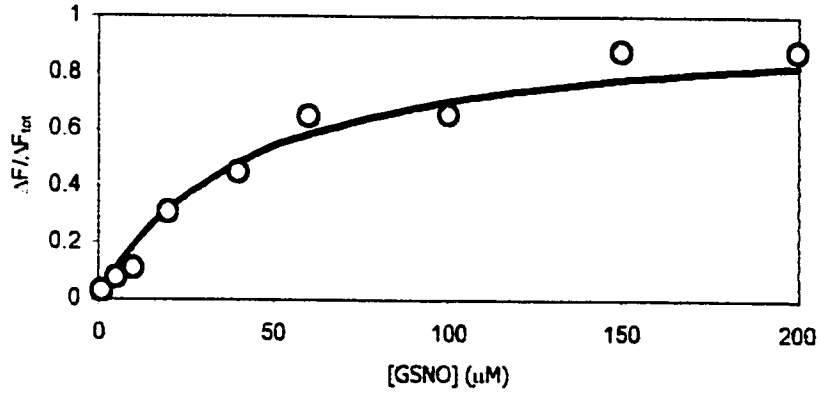
C) stoichiometry of GSNO/fibrinogen interactions.

Figure 16

A



B



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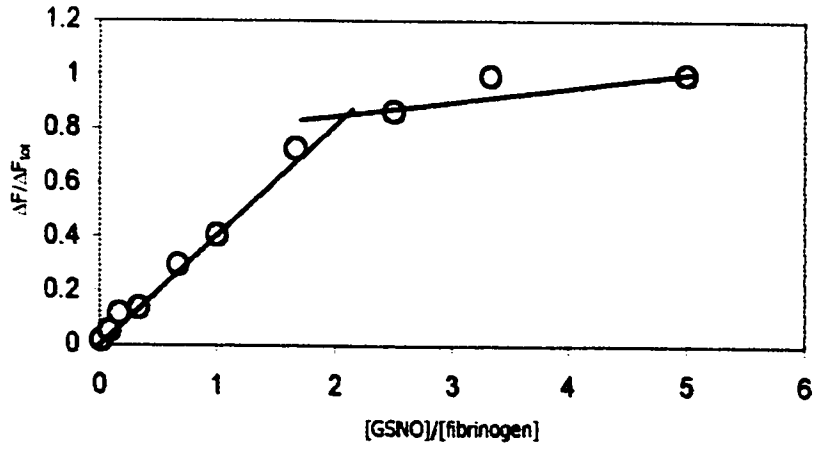


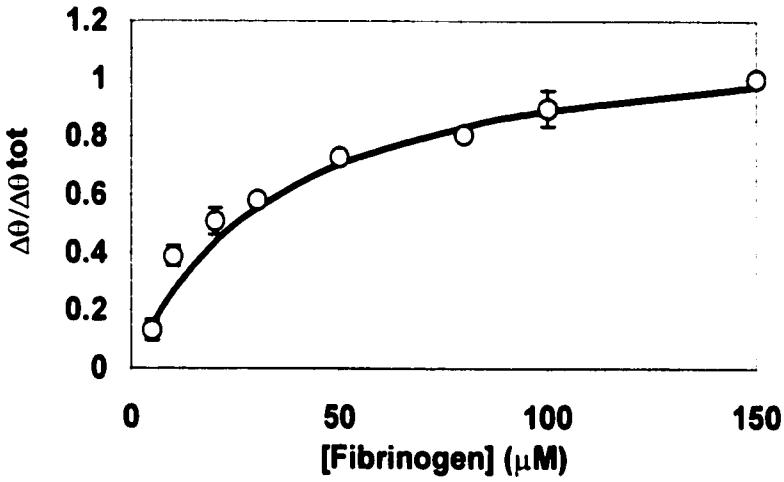
Fig 17

Fibrinogen perturbs the S-NO CD spectrum

Legend

CD spectrum at a constant [GSNO] monitored at 550 nm as a function of [fibrinogen]. 3 mL of GSNO (final concentration 60 μM) were placed in a 1 cm quartz cuvette, the CD spectra were measured over the wavelength region of 500-600 nm in the same CD spectrometer mentioned before. Each spectrum was the result of at least 10 scans obtained by collecting data at 1 nm intervals with an integration time of 10 s, at a constant temperature of 25 °C. Fibrinogen concentration used was over a range of 5-70 μM. The CD spectral maximum decreased in a saturable manner with an estimated K_D of ~ 40 μM. Error bars represent standard deviation (n=10)

Figure 17



4.0 DISCUSSION

There have been several reports on RSNOs-dependent inhibition of blood clot formation and platelet aggregation [158-161]. The plasma factor XIII (F XIII) is a transglutaminase, which catalyzes the cross-linking of fibrin monomers during blood coagulation. Catani et al., [162] established that high concentration of RSNOs have inhibitory effects on that factor and therefore on the blood clot formation.

Low-molecular weight thiols, such as cysteine and glutathione, are capable of forming S-nitrosothiols adducts with vasodilatory and antiplatelet properties. Protein thiols can also interact in the presence of NO and/or Endothelium Derived Relaxing Factor (EDRF) to form uniquely stable S-nitrosoproteins, which may also have potent antiplatelet effects [158]. The antiplatelet action of S-nitroso-proteins is associated with the stimulation of the guanylyl cyclase with a significant decrease in fibrinogen binding to platelets. S-nitroso-proteins undergo thiol-nitrosothiol exchange with low-molecular-weight thiols to form low-molecular-weight S-nitrosothiols, and they also interact directly with the platelet surface.

Fibrinogen binding is essential for platelet aggregation regardless of any agonists involved, and its inhibition appears to be the critical mechanism by which platelet function is impaired by agents such as organic nitrates. Therefore, the inhibition of platelets by nitrates may offer an additional mechanism to thrombotic processes [159].

S-nitrosothiol S-nitroso-N-acetylcysteine (SNOAC) significantly inhibits the binding of fibrinogen to activated platelets [160]. This inhibition occurs in a dose-dependent fashion and is preceded by a rapid marked increase in platelet cGMP which is also dose

dependent, and by inhibition of the intracellular calcium flux usually induced by the exposure of platelets to a variety of agonists. The primary effect of SNOAC leads to a marked decrease in the maximum number of fibrinogen molecules bound to the platelets, as well as an increase in the K_D of the binding equilibrium between platelet and fibrinogen.

In all of these cases, the mechanism of inhibtion has been attributed to either the NO-mimicking activity of RSNOs in triggering the NO/cGMP pathway, or has involved the transnitrosation or S-thiolation of an essential free thiol on the proteins participating in thrombosis or platelet activation.

The activity of several enzymes not involved in hemostasis, namely creatine kinase [163], papain [164] protein tyrosine phosphatases [165], and human rhinovirus 3C protease [166], have also been shown to be attenuated by RSNO-dependent transnitrosation or *S*-thiolation.

Creatine kinase is reversibly inhibited by incubation with S-nitrosothiols via S-thiolation and S-nitrosation with the relative importance of this reaction in biological system dependent on both the environment of the protein thiol and on the chemical nature of the S-nitrosothiol [163]. At the same time, S-nitrosothiols can directly attack the highly reactive thiolate in the papaine's active site (Cys (25)) to form a mixed disulfide between the inactivator and the enzyme; this process lead to inactivation of papaine [164]. Even in the protein tyrosine phosphatase low molecular weight S-nitrosothiols but also S-nitrosylated proteins could serve as good inactivators through an S-nitrosylation process. Moreover this finding points to a possibility that the endogenous S-nitrosylated albumin [165] may function as an inhibitor for a variety of cysteine dependent enzymes.

Different S-nitrosothiols are able to inactivate human rhinovirus (HRV) 3C and the inhibition activity is time and concentration dependent [166].

In the present study, GSNO was observed to inhibit thrombin-catalyzed fibrinogen polymerization. Thrombin did not appear to be affected since the initial rates of Chromozym TH hydrolysis were unchanged after a 15 min exposure to GSNO. This suggested that the GSNO inhibited polymerization via its actions on fibrinogen. This was puzzling as all of the thiols in fibrinogen are involved in disulfide bridges. Before proceeding further we ensured that the GSNO samples did not contain any free thiols (i.e. unreacted GSH). The amount of free thiol was determined by DTNB titrations according to a colorimetric assay and these were blocked by iodoacetamide. In fact, it is well documented in the literature that a thiol-disulfide exchange may happen, with the formation of a mixed disulfide. *S*-methylamidoGSH or *S*-methylamidoHCys did not perturb fibrinogen 2° structure as determined by CD. On the other hand, both HCysNO and GSNO increased the ellipticity, increased the –ve ellipticity at 207 nm and decreased the +ve ellipticity at ~190 nm which translated to ~ 15% increase in α-helix content at 50 μM GSNO (or HCysNO). Higher RSNO concentrations could not be utilized owing to the absorptivity of the solutions.

We choose the far-UV CD spectra because they are dominated by the contribution of the peptide bonds, although some side chains may also contribute. CD spectrum of a protein is highly sensitive to the conformation of peptide bonds, therefore it can be used to monitor the secondary structure of proteins [167].

However, it should be stressed that the far-UV CD of the peptide bond essentially reflects the dihedral angles (fig. 18). This means that individual bonds with dihedral

angles close to those occurring in a certain type of secondary structure will show the spectrum of that particular structure, irrespective of whether an identifiable length of this structure is present.

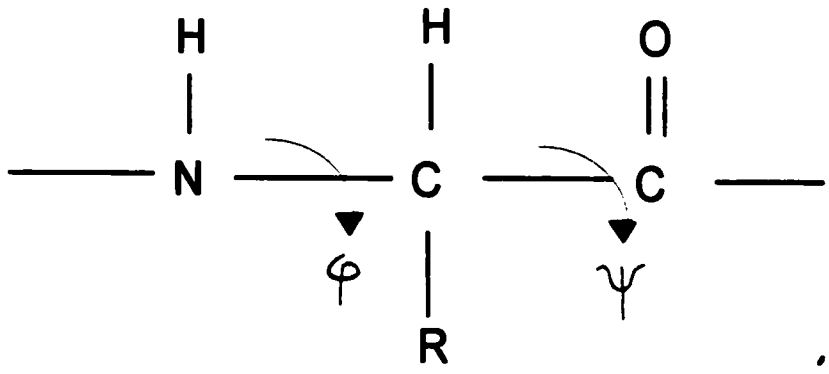


Fig 18- The dihedral angle

The increased [θ] at 207 nm indicates an increased α-helix content. Fragment E and the coiled-coil portion of fragment D, as reported in the studies by Azpiazu and Chapman [168], are particularly rich in α-helix segments. Our CD results showed an increase in α-helix content, and this may be due to the fragment E and the coiled-coil portion of fragment D

RSNOs also have a strong CD absorbance at 550 nm [157]. When the S-NO CD signal was monitored as a function of [fibrinogen] the 550nm absorbance decreased in a saturable manner with an estimated apparent K_D of ~40 μM.

The RSNO-induced structural perturbations were reversible since the CD spectra of fibrinogen solutions exposed to GSNO were identical to the native protein upon removal of the GSNO by gel filtration chromatography.

These observations point to GSNO and HCysNO altering fibrinogen structure without a chemical reaction. In other words RSNOs induce a conformational change in the protein by binding at specific site(s). We hypothesize that these RSNO-binding sites might be regions of the protein that are disordered and rich in aromatic side chains.

The requirement for the GSNO-binding domains being located in unstructured regions of the protein is suggested by the fact that GSNO increases the α-helix content of the protein, thus suggesting that it interacts in regions with little or no structure and induces α-helix formation. The recent crystal structure data [113, 124] of fibrinogen indicate that the protein consists of a ~ 400 Å long sigmoidal coiled coil that connects globular C-terminal domains with a central domain consisting of the N-terminal regions, the α_2 β_2 and γ_2 subunits. Despite a large degree of structure, there are several domains in the protein that are unstructured. By far, the most unstructured of these is the C-terminal end of the α subunit termed αC (A and B). In the crystal structure data of chicken fibrinogen [113], αCA and αCB appears disordered in the electron density map, this despite the fact that the chicken protein is shorter by 119 residues and lacks the 10 × 13 repeated sequences [118, 119] that add additional floppiness to the bovine and human proteins. These αC-domains which have been termed "free swimming appendages" interfere with crystal formation and had to be proteolyzed in the structure published by Brown et al., [124].

The second predicted requirement for the GSNO binding site is that it be rich in aromatic residues. This conclusion is based on the intrinsic Trp quenching data which is attributed to energy transfer between Trp residues (ex 290 nm em 330 nm) of the protein and the S-NO moiety of GSNO (acceptor-absorbance maxima 343 nm, 543 nm).

The fact that this quenching was saturable and occurred at a concentration much lower than expected for collisional quenching, suggests that GSNO interacts at specific sites on fibrinogen. We have previously observed that the fluorescence of N-dansylhomocysteine was quenched upon its S-nitrosation [169]. This quenching was attributed to an energy transfer between the dansyl moiety donor (ex 340 nm, em 540 nm) and RS-NO (acceptor, absorbance 543 nm). In order for this occur the RS-NO must be *syn* to the dansyl ring. Therefore, the αC-domains are ideal GSNO binding sites both from the point of lacking structure and being Trp-rich as there are 12 Trp between residues 291 and 610.

Certain conformational features of fibrinogen are essential for maintenance of fibrinogen's integrity as the substrate of thrombin [170]. The involvement of the N-terminal disulfide knot in determining the biological specificity of fibrinogen has also been demonstrated [171]. The N-terminal ends of all three pairs of fibrinogen chains are joined together by disulfide bonds in the central region of the molecule.

During fibrinogen digestion, two major fragments are recovered: the E fragment (45 kD) corresponding to part of the central domain and the coiled-coil, and the D fragment (100 kD), which includes the whole distal domain and a portion of the coiled-coil [172].

Medved [173] reported that the α-helix probably exists in the 390-550 residue section of the α chain. The C-terminal parts of the Aα chains, the αC domain, plays an important role in fibrin assembly and other biological processes [174, 175]. Their importance is

evident from the fact that congenital defects in this region including single amino acid substitutions in fibrinogen Caracas II and Dusart [174, 175] or deletion of the Aα 461-610 region in fibrinogen Marburg [176], are associated with a family history of recurrent thrombosis. The αC domains are also known to interact with thrombospondin [177] and integrin-type platelet and endothelial cell receptors [178]. In addition, these regions control activation of factor XIII [179] and subsequently serve as its substrate, becoming cross-linked to each other and to fibronectin, α_2-antiplasmin, thrombospondin and von-Willebrand factor [180-182].

Some early investigations have shown that the absence of the αC domain influences fibrin polymerization. Weisel and Papsun [183] observed that addition of thrombin to early fragment X preparation yields clots that are mechanically less stable and appear to consist of fibers that are substantially less branched than those of normal clots. It was proposed that the αC domain is necessary for the branching of fibres to form a three dimensional gel. Early results showed that the αC domains accelerate the formation of protofibrils, fibers and the fibrin network, especially in dilute solutions, but do not substantially influence the structure of intermediates or the final clot [184]. However, later experiments clearly indicated that the αC domains not only accelerated the fibrin polymerization process but also influenced the final clot structure and were not necessary for clot formation [185]. Although the importance of the αC domain is well established, not much is known about its structure.

Recently, Matsuka et al., [186] proposed that the C-terminal part of the Aα chain comprises an autonomous functionally active, flexible region that plays a key role in the αC polymer formation and stabilization of fibrin clots with factor XIIIa by expressing

two fragments: αC 45 K (Aα 221-610) and a truncated version, αC 30 K (Aα 368-610), in E. coli. Localisation of functionally important regions of fibrinogen αC domain has been hampered by the difficulty of isolating intact homogeneous fragments containing this domain, primarily because of its vulnerability to proteolysis [187].

6.0 CONCLUSION

Blood clotting is a complex reaction requiring a large number of interacting components, most of which circulate in the plasma. The final event of this reaction is the conversion of a soluble plasma protein, fibrinogen into an insoluble network which seals the ruptured blood vessel. The limited cleavage by thrombin of small negatively charged polypeptides, fibrinopeptides A and B results in exposure of polymerization sites that bind to the complementary structure located in both terminal nodules of the fibrin(ogen) molecule, thus giving rise to overlapping fibrillar aggregates.

Coronary artery disease (CAD) is almost always the result of atherosclerosis- hardening of the arteries. CAD is the most common type of hearth disease and the leading cause of death in the United States and in many other countries. Platelets have a small role in some atherosclerotic lesions, but play a major role in the formation of the thrombi. It is usually a mural and occlusive thrombus that leads to a Myocardial Infarction (MI). Some hemostatic factors such as fibrinogen, factor VII, factor VIII, antithrombin III, and others, have been investigated and have shown to have a reasonable association with ischemic heart disease (IHD).

Of these various thrombogenic factors, the most convincing independent risk factor for Cardiac vascular disease (CVD) has been plasma fibrinogen. Possible mechanism for the role of fibrinogen as a thrombogenic risk factor include its role as a precursor of fibrin and subsequent thrombosis and its effect on increases in blood viscosity, which in turn affects the blood flow hemodynamic characteristics that can lead to thrombosis.

The results of our study show that small molecular weight RSNOs can drastically alter fibrinogen structure without chemically reacting with the protein. The functional consequence of these interactions is the *in vitro* inhibition of thrombin catalyzed fibrin polymerization.

These S-nitrosothiols may be used as therapeutical agents to treat a wide variety of diseases, they can be used as anticlotting agents with minimal patient risk since GSNO is not toxic and does not show adverse effects on one's health.

Secondly we demonstrated that RSNOs can potentially regulate essential physiological processes via allosteric as well as chemical interactions with their targets. This is the first time that an S-nitrothiol has been shown to have allosteric effects on proteins. These RSNO-dependent allosteric interactions are predicted to occur in aromatic amino acid rich regions of the α chains converting them to more ordered α-helices.

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PUBBLICATIONS

1. M. Tannous, R. A. Rabini, A. Vignini, N. Moretti,, P. Fumelli, B. Zielinsky, L.Mazzanti, B.Mutus. Evidence for iNOS-dependent peroxynitrite production in diabetic platelets. Diabetologia (1999) 49: 539-544
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ABSTRACTS

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