Research article

Low molecular mass dinitrosyl nonheme-iron complexes up-regulate noradrenaline release in the rat tail artery

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Abstract

Background: Dinitrosyl nonheme-iron complexes can appear in cells and tissues overproducing nitric oxide. It is believed that due to their chemical nature these species may be implicated in certain pathophysiological events. We studied the possible role of low molecular mass dinitrosyl iron complexes in the control of noradrenaline release in electrically stimulated rat tail artery.

Results: A model complex, dinitrosyl-iron-thiosulfate (at 1–10 μM) produced a concentration-dependent enhancement of electrical field stimulated [3H]noradrenaline release (up to 2 fold). At the same time, dinitrosyl-iron-thiosulfate inhibited neurogenic vasoconstriction, consistent with its nitric oxide donor properties. A specific inhibitor of cyclic GMP dependent protein kinase, Rp-8pCPT-cGMPS, partially inhibited the effect of dinitrosyl-iron-thiosulfate on neurogenic vasoconstriction, but not on [3H]noradrenaline release. Another model complex, dinitrosyl-iron-cysteine (at 3 μM) elicited similar responses as dinitrosyl-iron-thiosulfate. Conventional NO and NO+ donors such as sodium nitroprusside, S-nitroso-L-cysteine or S-nitroso-glutathione (at 10 μM) had no effect on [3H]noradrenaline release, though they potently decreased electrically-induced vasoconstriction. The "false complex", iron(II)-thiosulfate showed no activity.

Conclusions: Low molecular mass iron dinitrosyl complexes can up-regulate the stimulation-evoked release of vascular [3H]noradrenaline, apparently independently of their NO donor properties. This finding may have important implications in inflammatory tissues.

Background

Dinitrosyl non-heme iron complexes (DNIC) are intrinsic nitric oxide (NO)-derived species that can appear in various NO overproducing tissues including inflammatory blood vessels [1,2]. Due to their reactivity, DNIC may have multiple biologically important targets. [3–8]. There are high molecular mass (protein-bound) and low molec-ular mass DNIC, paramagnetic and diamagnetic DNIC [9]. The low molecular mass DNIC are much more pow-erful nitrosative agents than NO or the low molecular weight S-nitrosothiols [5,9]. Depending on the micro-environment, DNIC can provide at least two types of nitro-sative modification of proteins, forming either protein-S-nitrosothiols or protein-bound dinitrosyl iron complexes

Table 1: Effects of various NO donors on stimulation evoked [3H]noradrenaline release and vasoconstriction in the rat tail arteries

Treatment	n	S_4/S_2 ratio	
		[3H]noradrenaline	Vasoconstriction
Control	7	1.08 ± 0.05	1.04 ± 0.06
DNIC-cysteine 3 μM	6	1.31 ± 0.06**	0.25 ± 0.02**
DNIC-thiosulfate 3 μM	6	1.38 ± 0.07**	0.33 ± 0.04**
Fe(II)-thiosulfate 10 μM	6	1.01 ± 0.03	0.92 ± 0.05
SNP 10 μM	7	1.04 ± 0.04	0.27 ± 0.06**
Cys-NO 10 μM	7	1.08 ± 0.06	0.23 ± 0.02**
GS-NO 10 μM	7	1.01 ± 0.03	0.27 ± 0.02**

**P < 0.01 in comparison to control arteries.

[5,9]. Under certain conditions, low molecular mass DNIC may also serve as NO donors and consequently as activators of soluble guanylyl cyclase and vasodilators [7]. Nitrosative modification of proteins can lead to a change of protein function and may have important physiological significance [8,10–12]. We have demonstrated recently, that in neuronal/neurosecretory PC 12 cells, low molecular mass DNIC specifically activate non-selective cationic channels and induce membrane depolarisation [6]. We hypothesised, that low molecular mass DNIC may also affect the process of neuromediator release, which is known to be associated with membrane depolarisation and involves an array of thiol-containing proteins which are usually sensitive to S-nitrosation [13,14]. To test this hypothesis, we used the model of the electrical field stimulated rat tail artery in which neither constitutive NO nor the NO donor SIN-1, influence the release of noradrenaline [15]. Here we demonstrate for the first time, that the two model compounds, DNIC-thiosulfate and DNIC-cysteine up-regulate noradrenaline release in the rat tail artery.

Results

Low molecular mass DNIC increase [3H]noradrenaline release

DNIC-thiosulfate (1–10 μM), in a concentration dependent manner, enhanced the electrical field stimulated [3H] noradrenaline release. This effect showed significance already at 3 μM DNIC-thiosulfate (Fig. 1). In the same arteries, DNIC-thiosulfate inhibited the stimulation-evoked vasoconstriction (Fig 1), consistent with its NO donor properties. DNIC-cysteine (at 3 μM) reproduced both these effects (Table 1). "The false complex", Fe(II)-thiosulfate (10 μM) did not affect either [3H]noradrenaline release or neurogenic vasoconstriction (Table 1). The conventional NO and NO+ donors, sodium nitroprusside (SNP), S-nitroso-L-cysteine (Cys-NO) and S-nitroso-glutathione (GS-NO) (at 10 μM) potently inhibited the neurogenic vasoconstriction, had however no effect on

[3H]noradrenaline release (Table 1). DNIC-thiosulfate did not change the basal release of [3H]noradrenaline (b_4/b_2 ratios: 0.92 and 0.93, 0.91, 0.97, 0.96 for saline and 0.3 μM, 1 μM, 3 μM, 10 μM DNIC, respectively).

Cyclic GMP-dependent protein kinase does not play a role

The membrane permeable and metabolically stable competitive inhibitor of cyclic GMP-dependent protein kinase (PKG), Rp-8pCPT-cGMPS (10 μM), did not change the electrical field stimulated [3H]noradrenaline release and vasoconstriction in control arteries (Fig. 2). Rp-8pCPT-cGMPS did not affect the augmentation of [3H]noradrenaline release caused by DNIC-thiosulfate. At the same time, Rp-8pCPT-cGMPS, partially but significantly reduced the "NO-dependent" inhibitory effect of DNIC-thiosulfate on vasoconstriction (Fig 2).

Discussion

The major finding of the present study is that DNIC-thiosulfate and DNIC-cysteine, at low micromolar concentrations, stimulate the electrical field-evoked noradrenaline release in the rat tail artery, while other NO and NO+ donors at these concentrations lack this ability.

During the past 10 years, NO has been recognised as a local neuromediator of the central and peripheral nervous system [16]. The role of NO in the process of noradrenaline release in vascular tissue is largely contradictory [17–20]. The contradiction of these results could be in part due to the involvement of different NO-related species in different experimental models. The interactions of NO with transitional metals control the reactivity of NO including nitrosation and/or nitration of different biological targets. DNIC, bound to different ligands is an example of such an NO-metal interaction [1–10]. Recently, we have shown that in neuronal/neurosecretory PC 12 cells, DNIC-thiosulfate specifically activates a depolarising cationic inward current; the mechanism of this activation is cyclic GMP-independent and involves an attachment of dinitrosyl-
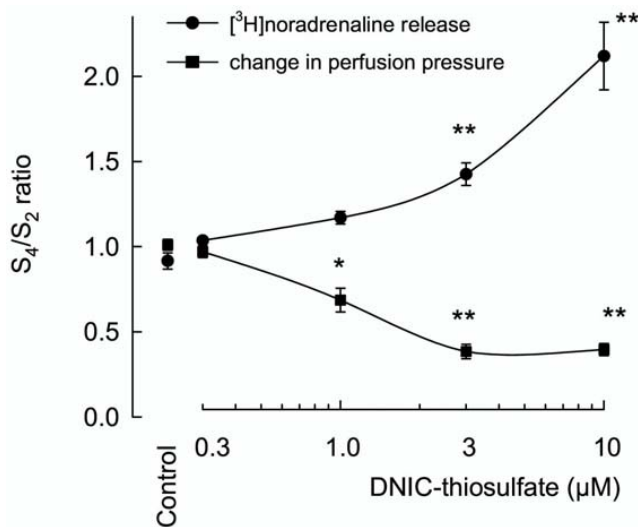
Figure 1
Effect of DNIC-thiosulfate on electrical field stimulated [³H]noradrenaline release and vasoconstriction in the rat tail artery. Four periods (S_1–S_4) of electrical-field stimulation were delivered with intervals of 16 min. DNIC-thiosulfate was added 8 min before S_3. The effect of DNIC-thiosulfate is presented as the ratio of change of [³H]noradrenaline in the perfusate or change of perfusion pressure evoked by S_4 over S_2. Each point represents the mean ± S.E.M. from 6–7 arteries. * $P < 0.05$ and ** $P < 0.01$ in comparison to control arteries.

Figure 2
Effects of PKG inhibitor, (Rp)-8-pCPT-cGMPS on the DNIC-thiosulfate-induced vascular effects in the rat tail artery. The effect of the drugs is presented as the ratio of change of [³H]noradrenaline or change in perfusion pressure evoked by S_4 over S_2. Each point represents the mean ± S.E.M. from 6–7 arteries. * $P < 0.01$ in comparison to control arteries; # $P < 0.01$ in comparison to arteries treated with DNIC-thiosulfate alone.

iron moieties to yet unknown protein(s) of the plasma membrane [6]. To test whether DNIC could affect the neuromediator release, we here used the model of the electrically stimulated rat tail artery. An advantage of this model is that vasoactive concentrations of NO or cyclic GMP analogues have no effect on the noradrenaline release [15,21]. Thus, the potential NO-dependent effect of DNIC on noradrenaline release in this model is likely excluded. Accordingly, in the present study, the conventional NO donors, SNP, Cys-NO and GS-NO, markedly inhibited neurogenic vasoconstriction, but did not affect the release of noradrenaline. In contrast, both DNIC-thiosulfate and DNIC-cysteine were found to be quite potent stimulators of the electrically evoked noradrenaline release.

While the mechanism of the augmentation of noradrenaline release by the low molecular mass DNIC requires further investigations, it is clearly NO/cyclic GMP/PKG-independent. Because our experiments were performed in the presence of cocaine and since DNIC had no effect on the basal noradrenaline release, the possible modification of tissue noradrenaline uptake seems to be excluded. The process of neuromediator release is an extremely complex cellular action, which is known to involve several cysteine-rich proteins [13]. Thus, it is possible that, in
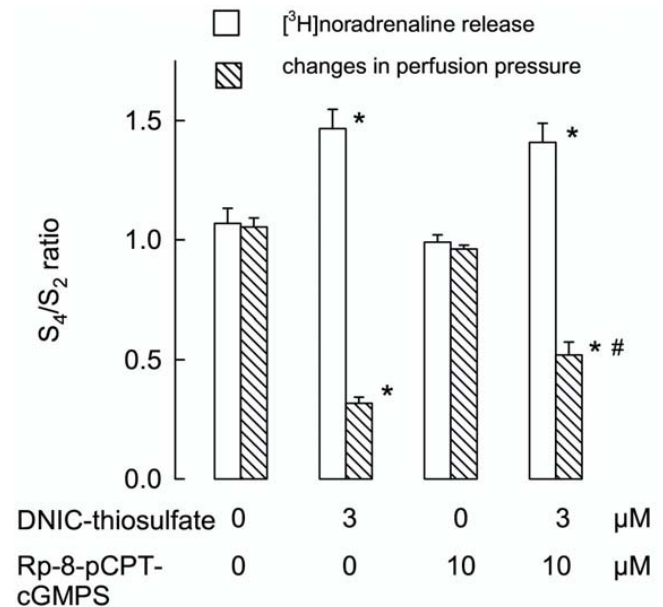
analogy with other thiol-containing proteins [10–12], the function of these proteins is sensitive to S-nitrosative modification. Since low molecular mass DNIC were shown to be much more potent S-nitrosative agents than low molecular mass S-nitrosothiols and NO [5], this scenario appears to be very likely. The potential role of oxygen radicals and lipid peroxidation in the DNIC action are less probable since the "false complex", Fe(II)-thiosulfate, which can accelerate the formation of oxygen radicals, showed no effect. Moreover, various iron-heme-NO and iron-nonheme-NO complexes proved to be strong antioxidants [22].

In this study a rather discouraging result was the lack of an apparent contractile effect of the noradrenaline released by DNIC. This could be attributed to the limitations of our experimental model, especially to the intra-luminary manner of DNIC supply and to the highly oxygenated conditions favouring oxidation of iron in DNIC and liberation of NO [23]. Thus, it is likely that in our model, NO-mediated vasodilation masked the vasoconstriction, which is expected from the DNIC-induced increase of noradrenaline release. Previously, we have observed a similar phenomenon using forskolin, which also produced

vasodilation despite a marked stimulatory effect on the release of noradrenaline [24]. Nevertheless, we believe that our findings are not only a curious pharmacological phenomenon, but also may be of pathophysiological importance. It is likely, that the loci of endogenous DNIC formation could be critical for the functional net result of DNIC action. Of note, DNIC species are not as easily diffusible as free NO, thus a concentration gradient in cells/tissues could be expected.

The concentration of protein-bound DNIC (which is in equilibrium with low molecular mass DNIC) in inflammatory tissue may be as high as 100 μM [5]. Since low molecular weight DNIC have a pronounced effect on noradrenaline release already at low micromolar concentrations, the present findings may have pathophysiological implications. Recently, the tunica adventitia and its potential impact on blood vessel function attracted much attention [25]. Specifically in endotoxin-exposed rat aorta, the adventitia was recognised as the major site of inducible NO synthase expression and DNIC production [2]. Thus, in the inflammatory blood vessels, the generation of DNIC may occur in close proximity to (or even within) the sympathetic nerve endings. Therefore, one can speculate that DNIC, under these circumstances, may unexpectedly contribute to vascular contraction. Additionally, because catecholamines are known to have a trophic effect on the vascular wall [26], the DNIC-mediated increase of noradrenaline release may contribute to the thickening of inflammatory blood vessels. A recent study in patients with coronary artery disease suggests, that chelatable non-heme iron interacts with endothelial NO and thus contributes to endothelial dysfunction [27]. Whether this interaction results in the generation of DNIC-like complexes, which in turn may affect the release of catecholamines, is an intriguing question.

Conclusion

Low molecular mass iron dinitrosyl complexes augment the electrical field-stimulated release of [3H]noradrenaline in the rat tail artery. This activity discriminates iron-dinitrosyl complexes from conventional NO donors and is apparently NO independent. This finding may have important implications for inflammatory tissues with non-heme-iron dinitrosyl species formation.

Materials and Methods
Experimental protocol

A detailed description of the methodology was reported previously [15]. Briefly, male Wistar rats (12 weeks old) were killed by cervical dislocation and exsanguinated. A segment (2–2.5 cm long) of the proximal part of the ventral tail artery was dissected out and kept in oxygenated (95% O_2/5% CO_2) medium consisting of (in mM): NaCl 118, KCl 4.8, $CaCl_2$ 2.5, KH_2PO_4 0.9, $NaHCO_3$ 25, glu-

cose 11, disodium EDTA 0.03. To prevent rapid decomposition of [3H]noradrenaline, the perfusion medium was supplemented with 0.3 mM ascorbic acid. The arteries were cannulated at one end and preincubated for 1 h in 1.5 ml of medium containing 2.2 μM (-)- [3H]noradrenaline (specific activity 4.4 Ci mmol-1). The arteries were then suspended vertically (distal end upmost) between two platinum wire electrodes and perfused by means of a roller pump with the medium containing 10 μM cocaine in order to block the re-uptake of released [3H]noradrenaline. The gaps between the artery and electrodes were wide enough to allow contraction or relaxation, yet sufficiently narrow enough to stimulate efficiently the intramural nerve terminals. Each artery was subjected to six stimulation periods. Each stimulation period consisted of 24 monophasic rectangular pulses of 0.3 ms at supramaximal strength of 200 mA delivered at 0.4 Hz. The first stimulation period was applied after 96 min of perfusion and others followed at 16 min intervals. There were two initial electrical stimulation periods that were not evaluated (PS_1 and PS_2). Collection of the superfusate was started after 124 min of perfusion in 1-, 2- or 6-min fractions. The stimulation period at 128 min was termed S_1 and subsequent ones S_2–S_4. The stimulation period S_2 served as a control of the stimulation-evoked [3H]noradrenaline release and vasoconstriction (since the responses were stable thereafter). The drugs under study or their solvents were infused in the perfusion medium 8 min before S_3. Infusion took place with a syringe pump at a rate of 17 μL min-1. At the end of the perfusion period, arteries were solubilized in 1 ml Soluene-100 (Packard Instrument, Paris, France). [3H]noradrenaline in the superfusate samples and in the arteries was measured by liquid scintillation spectrometry. Under control conditions, the stimulated [3H]noradrenaline release and vasoconstriction was not changed upon the repeated electrical stimulation. However, the fractional rate of basal [3H]noradrenaline release declined with time ($b_n/b_2 < b_1/b_2$; not shown). The [3H]noradrenaline release and peak of vasoconstriction evoked by S_2 amounted to 0.169 ± 0.006 % of total tissue [3H]noradrenaline and 83.8 ± 2.6 mm Hg, respectively (n = 104; all appropriate experiments pooled).

Solutions and drugs

The drugs used were cocaine hydrochloride (Cooperation Pharmaceutique Française, Nancy, France), (-)-noradrenaline hydrochloride, SNP (Sigma, St Louis, USA), Rp-8pCPT-cGMPS, sodium salt (Biolog, Bremen, Germany). (-)- [Ring-2,5,6-3H]noradrenaline (specific activity 55.5 Ci mmol-1, New England Nuclear, Dreieich, Germany) was diluted with unlabelled (-)-noradrenaline hydrochloride in order to obtain a specific activity of 4.4 Ci mmol-1. Monomeric forms of DNIC-thiosulfate and DNIC-cysteine (Fe:ligand molar ratio, 1:20) were synthesised in

a Thumberg vessel as described previously [23]. Briefly, the solutions of FeSO$_4$ 7H$_2$O (2 mM) and corresponding ligand-compound (40 mM) were treated separately with NO gas (300 mm Hg; 5 min) in oxygen free conditions, then mixed and evaporated (1 min). The solutions of DNIC-thiosulfate and DNIC-cysteine were tested by electron paramagnetic resonance spectroscopy, then frozen and stored in liquid nitrogen. Fe(II)-thiosulfate complex was prepared by dissolving FeSO$_4$ 7H$_2$O and Na$_2$S$_2$O$_3$ (molar ratio, 1:20) in oxygen free water just before use. Cys-NO and GS-NO were synthesised as 50 mM solutions in a Thumberg vessel [23] and stored in liquid nitrogen.

Data analysis

The effects of drugs on the stimulation-evoked [^{3}H]noradrenaline release and vasoconstriction were estimated as the ratio S$_4$/S$_2$. Results are given as mean ± S.E.M. of n experiments. The Mann-Whitney test was used for comparison of mean values. Bonferroni's correction was used for multiple comparisons to a single control. A *P* value of < 0.05 was regarded as significant.

This investigation conforms to authorisation 02816 given by the French government, Department of Agriculture and has been conducted in conformity with the Recommendations from the Declaration of Helsinki and the Guiding Principles in the Care and Use of Animals.

Acknowledgements

References

1. Henry Y, Lepoivre M, Drapier JC, Ducrocq C, Boucher JL, Guissani A: **EPR characterization of molecular targets for NO in mammalian cells and organelles.** *FASEB J* 1993, **7:**1124-1134
2. Kleschyov AL, Muller B, Stoeckel ME, Stoclet JC: **Adventitia-derived nitric oxide in rat aortas exposed to endotoxin: cell origin and functional consequences.** *Am J Physiol heart circ physiol* 2000, **279:**H2743-H2751
3. Rogers PA, Ding H: **L-cysteine-mediated destabilization of dinitrosyl iron complexes in proteins.** *J Biol Chem* 2001, **276:**30980-30986
4. Bergamini S, Rota C, Canali R, Staffieri M, Daneri F, Bini A, Giovannini F, Tomasi A, Iannone A: **N-acetylcysteine inhibits in vivo nitric oxide production by inducible nitric oxide synthase.** *Nitric Oxide* 2001, **5:**349-60
5. Boese M, Mordvintcev PI, Vanin AF, Busse R, Mülsch A: **S-nitrosation of serum albumin by dinitrosyl-iron complex.** *J Biol Chem* 1995, **270:**29244-29249
6. Giannone G, Takeda K, Kleschyov AL: **Novel activation of non-selective cationic channels by dinitrosyl-thiosulfate in PC12 cells.** *J Physiol* 2000, **529:**735-745
7. Mülsch A, Mordvintcev P, Vanin AF, Busse R: **The potent vasodilating and guanylyl cyclase activating dinitrosyl-iron(II) complex is stored in a protein-bound form in vascular tissue and is released by thiols.** *FEBS Lett* 1991, **294:**252-256
8. Stoclet JC, Troncy E, Muller B, Brua C, Kleschyov AL: **Molecular mechanisms underlying the role of nitric oxide in the cardiovascular system.** *Exp Opin Invest Drugs* 1998, **7:**1769-1779
9. Vanin AF, Kleschyov AL: **EPR detection and biological implications of nitrosyl non-heme iron complexes.** *In: Nitric Oxide in Allograft Rejection and Antitumor Defence* 199849-82
10. Stamler JS, Toone EJ, Lipton SA, Sucher NJ: **signals: translocation, regulation, and a consensus motif.** *Neuron* 1997, **18:**691-696
11. Gow AJ, Stamler JS: **Reactions between nitric oxide and haemoglobin under physiological conditions.** *Nature* 1998, **391:**169-73
12. Bauer PM, Buga GM, Fukuto JM, Pegg AE, Ignarro LJ: **Nitric oxide inhibits ornithine decarboxylase via S-nitrosylation of cysteine 360 in the active site of the enzyme.** *J Biol Chem* 2001, **276:**34458-34464
13. Buchner E, Gundersen CB: **The DnaJ-like cysteine string protein and exocytotic neurotransmitter release.** *TINS* 1997, **20:**223-227
14. Manalis RS, Cooper GP: **Evoked transmitter release increased by inorganic mercury at frog neuromuscular junction.** *Nature* 1975, **257:**690-691
15. Bucher B, Ouedraogo S, Tschöpl M, Paya D, Stoclet JC: **Role of the L-arginine-NO pathway and cyclic GMP in electrical field-induced noradrenaline release and vasoconstriction in the rat tail artery.** *Br J Pharmacol* 1992, **107:**976-982
16. Bredt DS, Snyder SH: **Nitric oxide: a physiologic messenger.** *Annu Rev Biochem* 1994, **63:**175-195
17. Egi Y, Matsumura Y, Murata S, Umekawa T, Hisaki K, Takaoka M, Morimoto S: **The effects of N^G-nitro-L-arginine, a nitric oxide synthase inhibitor, on norepinephrine overflow and antidiuresis induced by stimulation of renal nerves in anesthetized dogs.** *J Pharmacol Exp Ther* 1994, **269:**529-535
18. Toda N, Yoshida K, Okamura T: **Analysis of the potentiating action of NG-nitro-L-arginine on the contraction of the dog temporal artery elicited by transmural stimulation of noradrenergic nerves.** *Naunyn-Schmiedebergs Arch Pharmacol* 1991, **343:**221-224
19. Schwarz P, Diem R, Dun NJ, Forstermann U: **Endogenous and exogenous nitric oxide inhibits norepinephrine release from rat heart sympathetic nerves.** *Circ Res* 1995, **77:**841-848
20. Ohlmann P, Martinez MC, Bucher B, Andriantsitohaina R, Muller B, Schneider F, Stoclet JC: **Effect of lipopolysaccharide treatment on neurogenic contraction and noradrenaline release in rat arteries.** *Fundam Clin Pharmacol* 2000, **14:**593-600
21. Ouedraogo S, Tschöpl M, Stoclet JC, Bucher B: **Effects of cyclic GMP and analogues on neurogenic transmission in the rat tail artery.** *Br J Pharmacol* 1994, **112:**867-872
22. Kanner J, Harel S, Granit R: **Nitric oxide as an antioxidant.** *Arch Biochem Biophys* 1991, **289:**130-136
23. Vanin AF, Malenkova IV, Serezhenkov VA: **Iron catalyzes both decomposition and synthesis of S-nitrosothiols: optical and electron paramagnetic resonance studies.** *Nitric Oxide* 1997, **1:**191-203
24. Bucher B, Pain L, Stoclet JC, Illes P: **Role of cyclic AMP in the prejunctional alpha-2 adrenoceptor modulation of noradrenaline release from the rat tail artery.** *Naunyn Schmiedeberg's Arch Pharmacol* 1990, **342:**640-649
25. Gutterman DD: **Adventitia-dependent influences on vascular function.** *Am J Physiol* 1999, **277:**H1265-H1272
26. Zhang H, Faber JE: **Trophic effect of norepinephrine on arterial intima-media and adventitia is augmented by injury and mediated by different alfa-1-adrenoceptor subtypes.** *Circ Res* 2001, **89:**815-822
27. Duffy SJ, Biegelsen ES, Holbrook M, Russell JD, Gokce N, Keaney JF Jr, Vita JA: **Iron chelation improves endothelial function in patients with coronary artery disease.** *Circulation* 2001, **103:**2799-804