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P1 CONFORMATIONAL RELAXATION OF NNOS REDUCTASE DOMAIN BY REVERSAL OF A SINGLE CHARGE

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Mammalian neuronal Nitric Oxide Synthase (nNOS) produces NO by conversion of L-arginine to Citrulline, via a biologically unique pathway. The 83kDa reductase domain (nNOSrd) is responsible for passing electrons from NADPH, through bound FAD and FMN, onto the heme domain of nNOS. Isolated nNOSrd functions as an NADPH-dehydrogenase, and can pass electrons to external acceptors such as cytochrome *c*. Calmodulin (CaM) activates the transfer of electrons from FMN to the heme domain or to cytochrome *c* by inducing a conformational change in the reductase domain.

The structure of nNOSrd is similar to that of Cytochrome P450 oxidoreductase (CYPOR) and features a number of conserved residues including Arginine1229, which forms a salt bridge between the FAD and FMN binding domains of nNOSrd. Mutation of Arginine1229 to Glutamate creates a site of electrostatic repulsion in the domain interface abolishing the CaM-dependence of the enzyme. Pre-steady-state cytochrome *c* reduction is accelerated in the absence of CaM, up to the CaM-bound level indicating that the accessibility of the FMN to cytochrome *c* is similar in CaM-free R1229E and CaM-bound wild-type nNOSrd. Surprisingly, hydride transfer from NADPH to FAD is also accelerated by a similar magnitude. Pre-steady-state flavin reduction experiments again indicate that CaM-free R1229E reacts at a similar rate to the CaM-bound wild-type enzyme. The steady-state reduction of cytochrome *c*, however, proceeds at a slower rate than for wild-type nNOSrd and is inhibited by excess cytochrome *c*. This may result from the inhibition of the internal electron transfer from FAD to FMN.

P2 **A NOVEL HYBRID ASPIRIN-NITRIC OXIDE DONOR DRUG INHIBITS TNF-α RELEASE FROM LPS-ACTIVATED HUMAN MACROPHAGES IN VITRO**

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Non-steroidal anti-inflammatory drugs (NSAIDs) modulate inflammation primarily through inhibition of cyclo-oxygenase-mediated synthesis of pro-inflammatory prostanoids (Dannhardt & Kiefer, 2001). A common side-effect of NSAID administration is the development of gastric ulcers; hybrids of aspirin with nitric oxide (NO) donor moieties have shown some benefit in avoiding gastric injury (Cena *et al.*, 2003), but their anti-inflammatory effects have not been fully explored. Here, we set out to determine the anti-inflammatory properties of novel NO-releasing furoxan derivatives of aspirin in activated human monocyte-derived macrophages.

Peripheral venous blood was drawn from the antecubital fossa of human volunteers (non-smokers; age 20-45). Mononuclear cells were isolated from human blood using dextran sedimentation/Percoll gradients and resuspended at a concentration of 4×10^6 ml^{-1} in ISCOVES Dulbecco's modified Eagle's Medium (DMEM). Macrophages were derived from the monocytes (enriched by adherence) by culturing them in DMEM supplemented with 10% autologous serum (37°C, 7 days). On day 7, the medium in each well was changed to that containing 10 μM of either a furoxan aspirin; (3-cyanofuroxan-4-yl)methyl 2-acetoxybenzoate (B8) or (3-carbamoylfuroxan-4-yl)methyl 2-acetoxybenzoate (B7), their respective furazan NO-free counterparts; (4-cyanofurazan-3-yl)methyl 2-acetoxybenzoate (B16) or (4-carbamoylfurazan-3-yl)methyl 2-acetoxybenzoate (B15), aspirin, an existing nitroaspirin (NCX4016), a spontaneous NO donor, diethylamine diazeniumdiolate DEA/NO or dexamethasone (1 μM), with and without lipopolysaccharide (LPS;10 $ng.ml^{-1}$). Cells were then incubated at 37°C for 4 h and human TNF-α and IL-8 ELISA assays were conducted on the supernatants removed from macrophage plates.

B8 had a significant inhibitory effect on TNF-α release in human monocyte-derived macrophages treated with LPS (Figure 1; $p<0.05$, 1-Way ANOVA followed by Dunnett's test; n=8-10) but not in macrophages without LPS. The effect was equivalent in magnitude to that of dexamethasone, but was not shared by DEA/NO, B7, the furazans, aspirin or NCX4016. None of the drugs studied affected IL-8 release ($p>0.05$) or induced cell death, as assessed by lactate dehydrogenase assay ($p>0.05$; n=8-10).

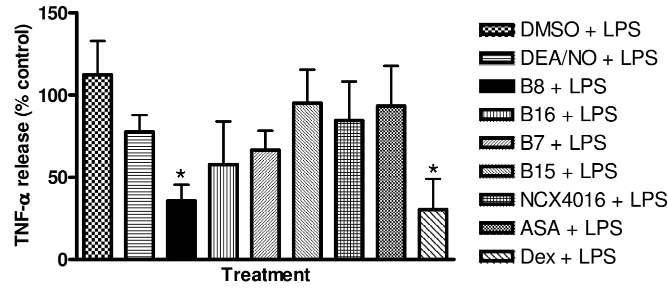

Fig.1. Effect of potential anti-inflammatory agents on LPS-induced TNF-α release in human monocyte-derived macrophages.

The lack of effects by B16 and aspirin, suggest that the inhibitory effect of B8 on TNF-α release in human monocyte-derived macrophages is NO-mediated. However, as this effect is not mimicked by the NO donor, DEA/NO, it is apparently a specific property of B8 that requires further exploration.

CENA, C. *et al.*, (2003). *J Med Chem*, 46, 747-54.
DANNHARDT, G. & KIEFER, W. (2001).. *Eur J Med Chem*, **3**6, 109-26.

P3 **A WATER-SOLUBLE CARBON MONOXIDE-RELEASING MOLECULES (CORM-3) REDUCES NEURO-INFLAMMATION IN BV-2 MICROGLIA**

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Microglial cells play a major role in regulating the neuronal inflammatory response, but their excessive stimulation is blamed for neuronal injury in various neurodegenerative diseases [1]. Interferon-γ (INFγ) is one of the most potent microglial stimuli [1] that act synergistically with other pro-inflammatory cytokines in driving multiple destructive cascades. Carbon monoxide-releasing molecules (CO-RMs) are emerging as a new class of pharmacological agents that modulate important cellular function by liberating CO in biological system. Our group has developed a water-soluble CO releaser (CORM-3) which showed remarkable cardioprotective and anti-inflammatory actions [2,3]. In the present study we examined the effect of CORM-3 on INFγ-induced inflammation in BV-2 microglial cells under normoxic and hypoxic conditions. BV-2 microglial cells were incubated with INFγ (15 ng/ml) for 24 h in normoxic conditions or subjected to 12 h hypoxia and reoxygenation (24 h) in the presence of INFγ. In normoxia, INFγ caused an increase in NO release (nitrite levels) from 0.09±0.059 to 36.6±4.5μM($p<0.001$) and an increase in TNF-α production from 162±5.7 to 405±44 pg/ml ($p<0.001$). Similarly, after hypoxia-reoxygenation (H/R) INFγ increased nitrite levels from 0.8±0.18 to74.5±1.3 μM and increased TNF-α production from 88±4 to 1989±36 pg/ml ($p<0.01$). CORM-3 (75 μM) significantly attenuated INFγ-mediated increase in nitrite levels in normoxia (from 36.6±4.5 to 5.5±0.9 μM) ($P<0.001$) and in (H/R) from 74.5±1.3 to 59.7± 1μM($p<0.001$). In addition, CORM-3 reduced TNF- α production in normoxia from 405±44 to 206±14 pg/ml ($p<0.001$), and in H/R from1989±36 to1314±157 ($p<0.001$). An inactive compound, which does not liberate CO (iCORM-3), failed to prevent the increase in inflammatory mediators mediated by INFγ suggesting that CO is responsible for the observed effects. Furthermore, blockade of endogenous heme oxygenase-derived CO by tin protoporphyrin-IX (10 μM) did not affect CORM-3 induced anti-inflammatory activity. CORM-3 did not show any cytotoxicity and appears to exert its effect at multiple levels through interaction with heme-dependent pro-inflammatory enzymes (NAD(P)H-oxidase and iNOS) and various signal transduction pathways (MAPK, P38, JNK, PI3K). These results suggest that CORM-3 could be used to modulate microglial activity in neuro-inflammatory diseases.

1. Popko B, Corbin JG, Baerwald KD, Dupree J, Garcia AM. The effects of interferon-gamma on the central nervous system. *Mol. Neurobiol.* 1997;14:19-35.

2. Clark JE, Naughton P, Shurey S, Green CJ, Johnson TR, Mann BE, Foresti R and Motterlini R. Cardioprotective actions by a water-soluble carbon monoxide-releasing molecule. *Cir. Res.* 2003;93:e2-e8.

3. Sawle P, Foresti R, Mann BE, Johnson TR, Green CJ, Motterlini R. Carbon monoxide-releasing molecules (CO-RMs) attenuate the inflammatory response elicited by lipopolysaccharide in RAW264.7 murine macrophages. *Br. J. Pharmacol.* 2005;145:800-10.

P4 NITRIC OXIDE-LOADED Co^{2+} AND Zn^{2+}-EXCHANGED ZEOLITES HAVE POWERFUL ANTI-AGGREGATORY PROPERTIES IN HUMAN PLATELETS

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Endothelium derived nitric oxide (NO) is a potent inhibitor of platelet activation (Radomski *et al* 1987). Polymers incorporating NO donating substances (e.g. diazeniumdiolates) have shown potential as antithrombotic surface coatings (Frost *et al* 2005), but have limited NO capacities. Zeolites are nanoporous solids that might offer an alternative to polymer-based antithrombotic coatings because they can act as high capacity storage materials for gases, including NO. The chemical characteristics of zeolites are easily manipulated by altering the specific cation, the porosity of the zeolite and the binding material in which it is embedded. In this study, we investigated the NO release properties of Zn^{2+} and Co^{2+} cation-exchanged zeolites in human plasma, and determined the extent and duration of their antiplatelet activity.

NO-loaded Zn^{2+} and Co^{2+}-exchanged zeolites of various compositions (10%-75%) in polytetrafluoroethylene binder (PTFE; pressed into 3 mm dia. discs) were found to generate substantial NO for ~60 min upon immersion in platelet-rich plasma (PRP; 1 ml, 37°C; n=6). The NO-release profile was dependent on both the exchanged cation (Zn^{2+} or Co^{2+}) and the composition of zeolite:PTFE binder (see Fig. 1).

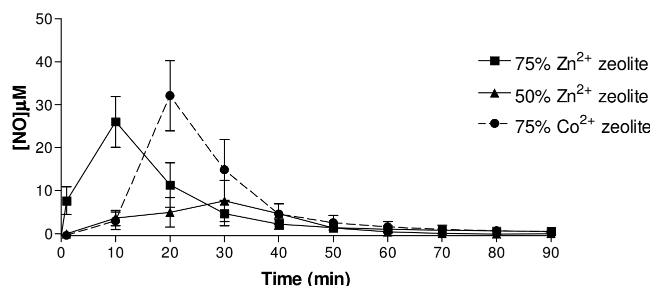

Fig. 1: NO generation from Zn^{2+} and Co^{2+}-exchanged zeolites (50-75%: PTFE) in PRP.

Turbidometric platelet aggregometry was used to determine the ability of NO-loaded zeolites to inhibit collagen induced platelet aggregation in PRP *in vitro*. Discs (3 mm dia.) containing NO-loaded Zn^{2+} and Co^{2+} ion-exchanged zeolites (10-75% in PTFE binder) were suspended in PRP (500 µl; 37ºC; 1-80 min) before stimulation with collagen (2.5µl.ml^{-1}). Zn^{2+} exchanged zeolite (≥50%) and Co^{2+} exchanged zeolite (75% only) significantly inhibited platelet aggregation throughout the incubation period (80 min;

p<0.01; n=6-8); NO-free counterparts failed to alter platelet aggregation over the same period, indicating the essential role of NO in the effect.

Our results suggest that Co^{2+} and Zn^{2+}-exchanged zeolites are high capacity storage materials of NO with powerful antiplatelet activity *in vitro*. The NO release profiles and antiplatelet effects were dependent on the specific cation exchanged and on the relative proportion of zeolite:PTFE binder. These zeolites will act as prototypes for the development of zeolites with optimal NO release capacities lasting for considerably longer durations.

Frost M.C. *et al* (2005) *Biomaterials* 26, 1685 -1693
Radomski *et al* (1987) *The Lancet* 330 (8567), 1057-1058

P5 VON WILLEBRAND FACTOR INDUCES PLATELET NITRIC OXIDE ACTIVATION THROUGHT INTERACTION WITH GPIb-IX-V

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Activation of platelet nitric oxide synthase (NOS) upon collagen stimulation is mediated by signalling from GPVI (Riba *et al.*, 2005). However, the initial contact between platelets and collagen under shear relies on the earlier interaction between platelet and von Willebrnad factor (vWF) immobilized in the subendothelial matrix (Farndale *et al.*, 2004). vWF signalling in platelets is mediated primarily by its interaction with GPIb-IX-V. However, vWF is also able to bind to activated $\alpha_{IIb}\beta_3$ (Schmugge *et al.*, 2003). The aims of this study were: (i) to analyse the ability of vWF-platelet interaction to induce NOS activation; (ii) to dissect the contribution of GPIb-IX-V and $\alpha_{IIb}\beta_3$ to induce synthesis of NO; and (iii) to investigate the mechanisms involved in NOS activation induced by vWF.

:Platelets were treated with ristocetin/vWF in the presence or absence of GPIb-IX-V blocking antibody (6D1) and the $\alpha_{IIb}\beta_3$ blocking peptide (RGDS). Activation of NOS was analysed by measuring accumulation of cGMP as a marker of NO bioavailability and by direct measurement of [^{3}H] L-citrulline production.

vWF induced a concentration-dependent increase in cGMP formation, where significant differences were found with vWF (30µg/ml) compared to basal (274 ± 20.2 vs 193 ± 24.3fmol/10^8platelets, $p<0.01$). Furthermore, we demonstrated that NO synthesis in vWF-stimulated platelets was mediated by activation of GPIb-IX-V and was independent of $\alpha_{IIb}\beta_3$, since RGDS did not cause any change in vWF-induced cGMP accumulation. In addition, no significant differences in [^{3}H] L-citrulline formation were found in vWF-stimulated platelets in the absence or presence of RGDS (10.86 ± 0.6 and 11.6 ± 0.6 fmol/mg, respectively), but the GpIb blocking antibody Mab6D1 reduced [^{3}H] L-citrulline back to basal (9.92 ± 0.5fmol/mg, $p<0.05$). Although vWF-GPIb-IX-V interaction was necessary, NO synthesis required release of calcium, ADP and TxA_2. To further characterize the mechanisms of vWF-induced NOS activation, we investigated the role of Src kinases, PI(3)kinase/PKB and PKC. These enzymes are known to be activated in vWF-stimulated platelets and to play a role in collagen-induced platelet NOS activation (Riba *et al.*, 2005). Inhibition of Src kinases with PP2(20µM) induced a decrease in cGMP accumulation but did not reach statistical significance. Preincubation of platelets with wortmannin, a PI(3)kinase inhibitor, significantly reduced vWF-induced cGMP formation (201 ± 24.2fmol compared to 285.9 ± 47.3fmol/10^8platelets, $p\leq0.05$). In contrast, inhibition of PKC had no effect on vWF-induced cGMP accumulation.

Our results show that vWF activates platelet NOS through interaction with GPIb-IX-V and requires release of intracellular calcium, secondary mediators and activation of PI(3)kinase/PKB pathway.

Farndale, R. W., Sixma, J. J., Barnes, M. J., and de Groot, P. G. (2004). The role of collagen in thrombosis and hemostasis. J Thromb Haemost 2, 561-573.
Riba, R., Sharifi, M., Farndale, R. W., and Naseem, K. M. (2005). Regulation of platelet guanylyl cyclase by collagen: evidence that Glycoprotein VI mediates platelet nitric oxide synthesis in response to collagen. Thromb Haemost 94, 395-403.

Schmugge, M., Rand, M. L., and Freedman, J. (2003). Platelets and von Willebrand factor. Transfusion and Apheresis Science 28, 269-277.

P6 ARE iNOS AND ET-1 INDUCED AND CO-LOCALISED IN DAMAGED SMOOTH MUSCLE CELLS OF SAPHENOUS VEIN HARVESTED FOR CABG?

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Previously we reported [1] that the conventional harvesting of saphenous veins (SV) for coronary artery bypass graft surgery (CABG) induced structural damage to venous smooth muscle cells (SMCs). This coincided with the rapid induction of immunoreactive iNOS in these cells. No distortion to SMCs and no iNOS were observed in grafts harvested with the less-invasive 'no-touch' method [1, 2].

To study the effects of harvesting techniques on the expression of iNOS and ET-1 in SV used as grafts in patients undergoing CABG.

Patients undergoing CABG received either SV grafts harvested conventionally or SV grafts harvested using the 'no-touch' technique. On the time of implantation, samples of the grafts were obtained for ultrastructural and immunocytochemical examination - for the detection of immunoreactivity for ET-1 at the electron microscopic level.

Immunoreactivity for ET-1 was induced in conventionally harvested SV grafts, where it was detected at the electron microscopic level in structurally damaged venous SMCs. Damaged cells displayed electron transparent 'vacuoles' of various shapes. Immunoreactivity for ET-1 was frequently clustered at various locations in the cytoplasm. The distribution of immunoreactivity for ET-1 was similar to the distribution of immunoreactivity for iNOS as previously described in damaged SMCs of conventional SV grafts for CABG [1].

The conventional harvesting of SV for CABG induces both iNOS and ET-1 in structurally distorted venous SMCs. Thus, the possibility exists that both iNOS and ET-1 are co-induced and co-localised in the structurally distorted/damaged SMCs of SV by harvesting procedures. We currently examine this using a more direct approach.

Loesch A, Dashwood MR, Souza DSR (2004) 'Damaged' smooth muscle cells of saphenous vein during harvesting for coronary artery bypass graft (CABG) are immunoreactive for iNOS: ultrastructural findings. Abstracts: 5th NO UK Forum.

Souza DSR (1996) A new no-touch preparation technique. Technical notes. Scand J *Thor Cardiovasc Surg* 30:41-44.

P7 NITRIC OXIDE DEPENDENT AND INDEPENDENT VASCULAR EFFECTS OF IGF-1 IN HUMAN SAPHENOUS VEIN

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In healthy normotensive rats IGF-1 enhances nitric oxide (NO) production and causes vasodilation via activation of the PI3 kinase/Akt pathway. However this pathway may be defective and IGF-1 mediated vasodilation attenuated in hypertensive rats (McCallum *et al* 2004). Vasodilatory responses to IGF-1 have also been reported to be attenuated in animals with hypercholesterolemia (Hasdai *et al* 1999). We postulated that vasodilation to IGF-1 in human conduit vessels may be influenced by cardiovascular status.

Contractile responses to phenylephrine (PE 0.01-10μmol/l) were studied in organ chambers in rings of saphenous vein from patients with severe coronary artery disease (CAD) undergoing coronary artery bypass graft surgery and from control subjects with no documented cardiovascular disease undergoing surgery for removal of varicous veins. Paired rings from each subject were studied in the presence of IGF-1 (0.1 μmol/l) or vehicle. The effects of high potassium (K^+ 30mmol/l), nitric oxide synthase inhibition (L-NAME 0.1 mmol/l) or P13 kinase inhibition (LY294002 3 μmol/l) on PE mediated contraction were also examined. The area under the PE concentration response curve (AUC) was calculated for each ring and the AUC in the presence of IGF-1 and vehicle compared by t-test. Rings from 5-7 subjects were studied for each treatment and results expressed as mean±SEM.

IGF-1 attenuated contractile responses to PE in both control and CAD patients. In controls this effect of IGF-1 was abolished by co-incubation with either L-NAME or LY294002. In CAD patients attenuation of responses to PE by IGF-1 was still observed in the presence of L-NAME and was not affected by high K^+. However in the presence of L-NAME + 30mmol/l K^+ no difference was observed between responses to PE in the presence and absence of IGF-1.

	Control Patients			CAD Patients		
Co-incubation	Vehicle	IGF-1	p	Vehicle	IGF-1	p
Vehicle	1.57±0.07	1.20±0.13	0.013	2.11±0.10	1.63±0.11	0.003
L-NAME	1.84±0.25	1.73±0.13	0.616	1.84±0.30	1.53±0.27	0.024
LY294002	1.24±0.11	1.15±0.09	0.249	-	-	-
K^+	-	-	-	1.89±0.31	1.54±0.28	0.017
L-NAME + K^+	-	-	-	1.32±0.16	1.27±0.18	0.621

Table 1 Phenylephrine mediated constriction expressed as AUC (gm tension)

These results suggest that in saphenous vein from control subjects IGF-1 attenuates vasoconstriction to PE via increased NO production through the P13 kinase/Akt pathway. In subjects with CAD IGF-1 mediated attenuation of constriction to PE is still observed but both NO and K^+ channel pathways are involved.

McCallum RW, Hamilton CA, Graham D, Jardine E, Connell JMC, Dominiczak AF. *J Hypertens* 2005; 23: 351-358.

Hasdai D, Nielsen MF, Rizza RA, Holmes DR Jr, Richardson DM, Cohen P, Lerman A. *Hypertension* 1999; 34: 89-95.

P8 ARE S-NITROSOTHIOLS EDRFS IN THE PULMONARY CIRCULATION?

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There is considerable evidence that the S-nitrosothiol, L-S-nitrosocysteine (L-SNC), is a non-prostanoid endothelium-derived relaxing factor (EDRF). The aim of this study was to provide evidence that an S-nitrosothiol such as L-SNC is an EDRF in the pulmonary circulation of conscious rats. Methods: Male Wistar rats (~250g) were anesthetized with pentobarbital (50 mg/kg ip) and instrumented for the measurement of pulmonary artery (PAP) and mean arterial (MAP) pressure, pulmonary blood flow velocity (PBF) and cardiac output (CO, both via pulsed miniaturized Doppler flow probes). Pulmonary vascular resistance (PVR) and total peripheral resistance (TPR) were determined by the formulae, PVR = PAP ÷ PBF, and TPR = MAP ÷ CO, respectively. Rats were allowed 7-10 days to recover before use. All drugs were given as bolus injections via the pulmonary artery. Data are expressed as mean ± SEM (n≥5 for all groups). Results: The endothelium-dependent agonist, acetylcholine (0.1, 0.25, 0.5 µg/kg PA), elicited dose-dependent falls in PVR (-15 ± 3%, -28 ± 5% and -36 ± 5%, respectively, $P < 0.05$ for all). The NO-donor, MAHMA NONOate (5, 10, 20 nmol/kg PA) did not affect PVR (+1 ± 2%, +0 ± 2% and -1 ± 3, respectively, $P > 0.05$ for all) whereas the higher dose reduced MAP (12 ± 3%, $P < 0.05$) and TPR (22 ± 3%, $P < 0.05$). L-SNC (5-20 nmol/kg) elicited dose-dependent falls in PVR (-8 ± 2%, -20 ± 4% and -34 ± 5%, respectively, $P < 0.05$ for all). In contrast, D-SNC (5-20 nmol/kg PA) elicited minor falls in PVR (-1 ± 1%, -2 ± 3% and -7 ± 2%, respectively, $P < 0.05$ for last response only). Our results suggest that endothelium-dependent agonists may release an EDRF other than NO in the pulmonary circulation of conscious rats. Although not definitive, these results support evidence that this EDRF may be an S-nitrosothiol that activates stereoselective recognition sites.