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S-NITROSATION OF COMPLEX I CAUSES ROS PRODUCTION BY MITOCHONDRIA

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Nitric oxide (NO) is a physiological messenger in the body but it can also be toxic to cells. Among other mechanisms, NO toxicity has been attributed to direct effects on mitochondria causing inhibition of mitochondrial respiration due to inhibition of cytochrome oxidase and production of reactive oxygen (ROS) or reactive nitrogen species (RNS). Although elevated mitochondrial ROS production is thought to be involved in pathology of oxidative damage as well as in cell signaling, the mechanisms by which mitochondrial ROS production is regulated are unclear. We were interested whether RNS can induce S-nitrosation and inhibition of complex I of the mitochondrial respiratory chain and whether such inhibition can cause production of ROS in isolated rat heart and liver mitochondria. We found that 20-30 min exposure of isolated heart or liver mitochondria to S-nitroso-N-acetylpenicillamine (SNAP) causes substantial inhibition of complex I concomitant with increase in ROS production (measured as rate of hydrogen peroxide production). SNAP-induced inhibition of complex I and stimulation of ROS generation were partially reversed by light and DTT, indicating that S-nitrosation may be involved. The inhibition of complex I and generation of ROS also occurred in the presence of PTIO, a scavenger of free NO, suggesting that the respiratory inhibition and ROS production was due to transnitrosation of the complex I. Our data suggest that S-nitrosation of complex I may cause inhibition of electron transport and stimulation of ROS production. This may be important in regulation of mitochondrial ROS production and cell death.

PEROXYNITRITE, BUT NOT OTHER NO-RELATED SPECIES, INDUCES APOPTOSIS IN HUMAN MONOCYTE-DERIVED MACROPHAGES

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The endogenous signalling molecule nitric oxide (NO) is widely recognised to induce apoptosis and necrosis at high concentration (Kim et al. 2001). However, NO reacts rapidly with superoxide to generate peroxynitrite (ONOO⁻), and with endogenous thiols to form S-nitrosothiols (RS-NO); both ONOO⁻ and RS-NO are biologically active, independent of 'pure' NO generation. The cytotoxic impact of NO will therefore depend on the nature and local concentration of the NO-related species generated in the microenvironment, as well as the nature of the target cell type. This study set out to establish the relative impact of drug-derived NO, ONOO⁻ and RS-NO on survival of human monocyte-derived macrophages.

NO-related species generated by 1,2,3,4,-oxatriazolium, 5-amino-3-(3,4-dichlorophenyl)-chloride (GEA-3162), diethylamine diazeniumdiolate (DEA/NO), (Z)-1-[2-(2-Aminoethyl)-N-(2-ammonioethyl)amino]diazene-1,1,2-diolate (DETA/NO), S-nitroso-N-valeryl-D-penicillamine (SNVP) and S-nitrosoglutathione (GSNO), were characterised using a combination of electrochemistry and electron paramagnetic resonance (EPR). Our results demonstrated that, whilst the diazeniumdiolates and S-nitrosothiols generated detectable NO in solution, GEA-3162 (300 μ M) failed to do so, except in the presence of Cu/Zn superoxide dismutase (SOD; 50-500 U.ml⁻¹), when concentrations of up to $1.6 \pm 0.1 \mu$ M NO were generated. Furthermore, neither diazeniumdiolates nor S-nitrosothiols generated significant EPR signals in the presence of Tempone-H (1 mM; 30 min incubation; 37°C), a recognised spin trap for oxidising radical species. However, a signal consistent with formation of the radical adduct 4-oxo-tempo was detected with GEA-3162 (8605 \pm 764 arbitrary units, AU); and was significantly blunted by SOD (500 U.ml⁻¹; 3346 \pm 464 AU; $p < 0.01$; $n=6$).

Apoptosis was assessed by flow cytometry in human monocyte-derived macrophages isolated from blood samples of healthy volunteers (20-40 years old; $n=12$): cells that stained positive for annexin V binding but excluded propidium iodide were identified as apoptotic, whilst necrotic cells were positive for both annexin V and propidium iodide binding. GEA-3162 (100 μ M; 24 hr) induced significant apoptosis compared to vehicle (46.8 \pm 11.9% vs 11.8 \pm 4.5; $p < 0.01$; $n=6$), whilst a higher concentration of this agent (300 μ M) induced necrosis compared to vehicle (35.0 \pm 10.0 % vs 4.7 \pm 1.0; $p < 0.01$; $n=6$). SOD (500 U/ml) significantly increased GEA-3162 (10–300 μ M) induced apoptosis ($p < 0.01$; $n=6$). The diazeniumdiolates and S-nitrosothiols (all 100 μ M and 300 μ M) had no significant effect on cell viability ($p > 0.05$ for all).

The key finding of this study is that, of a number of drugs capable of generating NO or NO-related species, only the ONOO⁻ generator GEA-3162 (Taylor et al. 2004), induced apoptosis or necrosis in human macrophages. Indeed, NO concentrations generally regarded to exceed those seen in pathological conditions (μM range), failed to induce macrophage cell death, either when delivered as an acute burst (<1 hr) or over a 24 hr period. Likewise, high concentrations of RS-NO capable of transnitrosation reactions, failed to induce death pathways. We conclude that pro-apoptotic and necrotic effects of NO in human macrophages are likely to occur only via formation of ONOO⁻.

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PHENOTYPES OF VASCULAR SMOOTH MUSCLE SENSE BACTERIA DIFFERENTLY: IMPLICATIONS FOR TLRs IN VASCULAR HEALTH

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Vascular smooth muscle cells constitute the main cell type of the vessel wall. In a healthy vessel these cells have little or no secretory function. However, when vessels are inflamed, vascular smooth muscle express vasoactive genes and release large amounts of vasoactive hormones including nitric oxide (NO). The vessel wall is comprised of different phenotypes of vascular smooth muscle, of which one broad differentiation is between contractile and epithelioid. The relative role of different phenotypes of vascular smooth muscle in health and disease is not well understood. Infection of the vessel wall with bacteria is now a recognised risk factor for vascular disease and acute cardiovascular events. Thus, in the current study we have compared the ability of different phenotypes of vascular smooth muscle cells to 'sense' pathogen activated molecular patterns (PAMPs) including those associated with Gram negative *E. Coli* (which activates Toll like receptor (TLR4) and Gram positive *S. aureus* (which activates TLR2; Jimenez et al., 2005).

Rat vascular smooth muscle cells were cultured by explant in DMEM containing 10% FCS, using standard techniques. Epithelioid or contractile (spindle shaped) cells were identified and clonal cultures of epithelioid and contractile phenotypes were grown from single cell populations. For comparison, primary cultures of rat aortic smooth muscle cells (RASMs; which contain mixed populations of the different smooth muscle cell phenotypes) and J774 murine macrophages were used. Cells were incubated with bacteria or selective PAMPs for 24 or 48 hours. Medium was then removed and nitrite measured (using the Griess reaction) as an index of NOSII activity.

At 24 hours, under control culture conditions NO release by vascular smooth muscle was $<5\mu\text{M}$. *E. coli* (10^8 CFU/ml) induced NO release from primary cultures of RASMs ($15\pm 3\mu\text{M}$), epithelioid ($22\pm 1\mu\text{M}$) and contractile ($18\pm 1\mu\text{M}$) phenotypes of vascular smooth muscle. *S. aureus* (3×10^8 CFU/ml) induced NO release from RASMs ($19\pm 2\mu\text{M}$) and from the contractile phenotype ($12\pm 0.1\mu\text{M}$) of vascular smooth muscle cells, but not from the epithelioid phenotype. When TLR selective PAMPs (lipopolysaccharide, LPS from *E. Coli*, $10\mu\text{g/ml}$; PAM₃CSK4, $1\mu\text{g/ml}$; FSL-1, $1\mu\text{g/ml}$; which activate TLR4, TLR2/TLR1 and TLR2/TLR6 respectively) were added to cells, they had no effect on NO release by any of the vascular smooth muscle cells tested. J774 macrophages released NO in response to *E. Coli* ($22\pm 1\mu\text{M}$) or LPS ($28\pm 1\mu\text{M}$) but not to *S. aureus*.

These results show that bacterial PAMPs are sensed differently by vascular smooth muscle cells of different phenotypes as well as macrophages. These results have

implications for our understanding of how infection may modulate vascular inflammation.

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Funded by the British Heart Foundation and the European Union.

EXPRESSION OF NITRIC OXIDE SYNTHASE IN PERIPHERAL LUNG OF COPD PATIENTS AND EFFECTS OF OXIDATIVE STRESS ON ITS ACTIVITY AND EXPRES

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Introduction: Production of nitric oxide (NO) is increased in peripheral lung in COPD, whereas it is not increased in large airways, in contrast to asthma (Brindicci C et al., ERJ 2005). However the isoform of NO synthase (NOS) which is involved in this elevated peripheral NO has not yet been identified.

Methods: Whole tissue extracts and mRNA were prepared from peripheral lung tissue obtained from patients with COPD at GOLD stages 1, 2 and 4, and non-smokers without lung function impairment and aged-matched with COPD patients (n=4-6 each). NOS isoenzyme (iNOS, eNOS, nNOS) expression was determined by Western blotting or quantitative RT-PCR. A549 cells were also used for determination of mRNA expression of eNOS, iNOS and nNOS after oxidative stress/nitrative stress, such as H₂O₂ and SIN-1, a peroxynitrite inducer. nNOS was also immunoprecipitated from cells and NOS activity were determined

Results: Levels of nNOS expression were significantly increased in COPD, especially in severe disease (nNOS/ β actin: non-smoker: 0.07 ± 0.03 ; COPD stage 1: 0.09 ± 0.02 ; COPD stage 2: 0.15 ± 0.07). iNOS expression tended to increase in COPD stage 1 and 2 compared with non-smokers, whereas no differences were seen in eNOS expression between the three groups. H₂O₂ increased nNOS, iNOS and eNOS mRNA expression, whereas nitrative stress with SIN1 stimulation predominantly increased nNOS expression in A549 cells. Immunoprecipitated nNOS is highly nitrated after oxidative stress/nitrative stress treatment, but the activity was not decreased, although iNOS and eNOS activity was decreased by nitration. This nitration on nNOS did not affect homodimerization of the enzyme detected by low-temperature SDS-PAGE/Western Blotting, although this induced a defect in the dimerization of eNOS.

Conclusions: These results suggest that nNOS is the predominant NOS isoenzyme increased in COPD, although iNOS is also increased. Oxidative/nitrative stress did not affect nNOS activity in contrast to reduction in iNOS and eNOS activity, suggesting that NO from peripheral lung of COPD is likely to be derived predominantly from nNOS.

EFFECT OF A GLUCOCORTICOID AND AN IKK-2 INHIBITOR IN A PRE-CLINICAL MODEL OF AIRWAY DISEASE: UTILITY OF EXNO AS A NON-INVASIVE BIOMARKER OF LUNG CELLULAR BURDEN.

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Currently, there is a great deal of interest in developing less invasive markers for monitoring airway inflammation to aid in the assessment of disease status and determining the effect of possible novel anti-inflammatory therapies which may take time to impact on disease pathology. Exhaled nitric oxide (exNO) has been proposed to be a reproducible, non-invasive indicator of the inflammatory status in the airway. The aim of this study was to determine the usefulness of measuring exNO as a marker of the anti-inflammatory impact of a clinically relevant glucocorticoid and an IKK-2 inhibitor (TPCA-1) in a pre-clinical rodent model of airway inflammation. Rats were given vehicle, budesonide or TPCA-1 prior to exposure to aerosolised LPS, previously shown to induce an increase in exNO and lung lumen and tissue neutrophilia/eosinophilia. Comparison of the effect of the two compounds on the various inflammatory components demonstrated certain parallels between the impact on exNO and inflammatory cell burden in the airway. Treatment with the two compounds, however, did result in differing anti-inflammatory profiles suggesting a need to characterise the impact of each type of therapy in order to understand the relevance of measuring exNO. The data in this study demonstrates the usefulness of profiling potential disease modifying therapies on exNO levels and how an effect on this non-invasive biomarker relates to effects on functional parameters such as lung cellularity. Information from studies like these, if it translates into the clinical situation, could then be used as a guide to designing and selecting appropriate dose levels in clinical studies and to determining the effectiveness of potential disease modifying therapies.

INTERLEUKIN-6 INDUCES THE INDUCIBLE NITRIC OXIDE SYNTHASE AT THE TRANSCRIPTIONAL LEVEL

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Interleukin 6 (IL-6) is an inflammatory cytokine with a wide range of functions. For example, in the heart it has been associated with the incidence of cardiovascular events, but as a pleiotropic cytokine, it can have positive as well as negative effects. Inducible nitric oxide synthase (iNOS or NOSII) is also expressed as part of the inflammatory response, producing high levels of nitric oxide (NO) which has been involved in cell fate and apoptosis. NOSII expression is induced in most cell types by various external stimuli such as bacterial lipopolysaccharides (LPS) and cytokines. It has been shown that NOSII is a mediator of late preconditioning during hypoxia of the heart, but the link between NOSII induction and the cytokines involved in this effect is unknown. We show that IL-6 induces the human NOSII gene and potentiates stimulation by other cytokines, mainly IL-1beta and IFN-gamma, increasing the cytokine mix (IL-1beta, IFN-gamma and TNF-alpha) activation two fold. This increase in mRNA accumulation is due to an activation of the hNOSII promoter, as tested with a luciferase construct. As a consequence, there is an increase in NO output in the stimulated cells. The transcriptional activation of NOSII by IL-6 is independent of NFkB but can be completely inhibited by AG490, a specific inhibitor of Jak2, implicating this enzyme and the STAT transcription factors in this regulation. Using previously described deleted constructs of the hNOSII promoter, we localised the site for IL-6 activation to the region between -1998 and -5018, which is well known to be implicated in activation by inflammatory stimuli.

NITRIC OXIDE SUPPRESSES CYCLOOXYGENASE BIOACTIVITY IN THE VASCULATURE AND PLATELETS.

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COX inhibition is associated with increased risk of cardiovascular events. The mechanisms that underlie susceptibility to adverse events by NSAIDs are unknown. Here we show that hypertensive and pro-thrombotic activities of the COX-2 selective inhibitor celecoxib are revealed in mice only after *in vivo* inhibition of NO generation. The non-selective inhibitor indomethacin was hypertensive but anti-thrombotic when NO was absent. *In vitro* myography of aortic rings confirmed that vasoconstriction required inhibition of both NOS and COX-2. Urinary COX metabolite levels were unchanged by NOS inhibition excluding direct effects of NO on COX turnover. With washed human platelets, inhibition of aggregation to U46619 was over 1,000 times more sensitive to NO than aggregation to thrombin (+ indomethacin). This further supports the idea that NO primarily inhibits COX signaling downstream of thromboxane, and not by altering COX turnover directly. The data suggest that the susceptibility to vascular side-effects of NSAIDs will be greatest in patients with impaired vascular function associated with decreased NO bioavailability, and that strategies aimed at preserving NO should be important considerations for their continued clinical use.

REGULATION OF PLATELET NITRIC OXIDE SYNTHASE ACTIVITY BY TYROSINE PHOSPHORYLATION.

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Platelet NOS has putatively been identified as the endothelial NOS (eNOS) isoform. eNOS in endothelial cells is regulated by co-ordinated signalling mechanisms utilising multiple protein kinases and phosphatases. However, the regulation of platelet NOS is poorly understood. Here, we demonstrate the potential importance of tyrosine phosphorylation in the regulation of platelet NOS.

Platelet activation with thrombin (0.05, 0.1, 0.5U/ml) stimulated NO synthesis as evidenced by increased cGMP and the conversion of L-[3H]arginine to L-[3H]citrulline. Enzyme activity was independent of aggregation, but required elevated [Ca²⁺]_i. Phosphorylation of eNOS at serine1179 (serine1177 human sequence) in endothelial cells by PKB is associated with increased enzyme activity. Using immunoprecipitation and immunoblotting techniques, we found no phosphorylation at serine1179 on platelet NOS under basal conditions or following stimulation with collagen, thrombin and collagen related peptide. In contrast to our observations with ser1179, platelet NOS is tyrosine phosphorylated under basal conditions. Interestingly, total phosphorylation did not change after thrombin-stimulation (0.05, 0.1 and 0.5U/ml) despite increased enzyme activity (2.44±0.26, 5.77±1.63, 6.49±1.58 fold compared to basal p<0.05).

To determine the influence of tyrosine phosphorylation on enzyme activity, we treated platelet NOS with a recombinant protein tyrosine phosphatase. Dephosphorylation of platelet NOS, increased basal enzyme activity by 1.5-fold (±0.61) as evidenced by accumulation of L-[3H]citrulline. Thrombin stimulation of platelets increased enzyme activity by 4-fold (±1.05) above basal. However, if the enzyme was dephosphorylated, thrombin induced a 6-fold (±1.5) increase above basal. The potential importance of dephosphorylation was strengthened by our findings that the tyrosine phosphatase SHP-1 is associated with platelet NOS basally. Increased amounts of SHP-1 associated with NOS after thrombin activation, while a SHP-1 inhibitor, PTP-1 (50µM), caused a 2-fold decrease in thrombin-induced NOS activation.

Our findings provide the first evidence that platelet NOS may be regulated by a novel mechanism involving tyrosine phosphorylation and dephosphorylation.

This work was funded by the British Heart Foundation and the School of Life Sciences, University of Bradford.

ROLE OF PROTEIN DISULPHIDE ISOMERASE IN DELIVERY OF VARIOUS REDOX FORMS OF NITRIC OXIDE INTO PLATELETS

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Introduction:

Recent evidence indicates that cell surface protein disulphide isomerase (PDI) mediates the denitrosation of S-nitrosothiols (Sliskovic *et al.*, 2005) and the intracellular delivery of nitric oxide (NO) from this class of NO donor compounds (Zai *et al.*, 1999; Ramachandran *et al.*, 2001). It is not clear, however, whether this activity of PDI is only relevant to S-nitrosothiols or, more generally, to NO in all its redox forms. It has also been reported that soluble guanylate cyclase is activated only by the free radical NO[•] and not by other redox forms (Dierks and Burstyn, 1996). The aim of our study, therefore, was [1] to investigate the role of PDI in mediating the effects on platelets of donors of nitrosonium (NO⁺), nitric oxide radical (NO[•]) and nitroxyl (NO⁻) and [2] to determine whether donors of these redox forms are able to activate platelet soluble guanylate cyclase.

Methods:

Washed human platelets were incubated with GSNO (an S-nitrosothiol), DEANO and Angeli's Salt to deliver NO⁺, NO[•] and NO⁻ respectively, and the entry of reactive NO species (NO_x) was monitored by both DAF-FM fluorescence and cyclic GMP accumulation. The role of PDI was investigated by pre-treating platelets for 30 minutes with increasing concentrations of known PDI inhibitors (bacitracin, phenylarsine oxide [PAO], and the anti-PDI antibody RL90) prior to the addition of NO donor.

Results:

An increase in DAF-FM fluorescence was observed following treatment of platelets with donors of all NO redox forms, and in all cases this was inhibited in a dose-dependent manner by bacitracin (0.05-5 mM), PAO (1-100 μM) and RL90 (0.2-20 μg/ml). DEANO and Angeli's Salt (both 1 μM) provoked an approximately 15 fold increase in intra-platelet cyclic GMP, whereas for a similar response 10 μM GSNO was required. Bacitracin (0.05-5 mM) inhibited cyclic GMP accumulation in response to all three NO donors. PAO and RL90 could not be used in this system because of interference detected in control experiments using YC-1, an NO-independent stimulator of soluble guanylate cyclase.

Conclusion:

We conclude that the role of PDI in transmitting NO_x into platelets is not restricted to S-nitrosothiols only, but is relevant to the delivery of all redox forms of NO. In addition, our observation of a cyclic GMP response to all NO donors implies either that all redox forms of NO are able to activate soluble guanylate cyclase, or that platelets have the

capacity to convert NO^+ and NO^- to free radical NO^\cdot . The mechanism(s) involved in such reactions remain uncertain.

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ESSENTIAL REQUIREMENT FOR SPHINGOSINE KINASE ACTIVITY IN eNOS-DEPENDENT NO RELEASE AND VASORELAXATION

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Sphingosine-1-phosphate (S1P) is a bioactive sphingolipid that acts both as an extracellular ligand for endothelial differentiation gene receptor family and as an intracellular messenger. Cellular levels of S1P are low and tightly regulated by sphingosine kinase (SPK). Recent studies have suggested that eNOS pathway may function as a downstream target for the biological effects receptor-mediated of S1P. Here we have studied the possible interplay among eNOS, S1P and hsp90 whose recruitment is critical for eNOS activation by using isolated rat aortic rings. Thoracic aorta was rapidly excised from male Wistar rats (Charles River, 200-250 g) sacrificed by exsanguination and cleaned from fat. Rings of 2-3 mm width were cut and placed in organ baths filled with oxygenated Krebs solution at 37°C and connected to an isometric transducer under resting tension of 0.5 g. S1P (10 nM-50 µM) causes an endothelium-dependent vasorelaxation in rat aorta (max relaxation 72±14.4%, n=6) which is PTX sensitive (max relaxation 23±1.2%, n=6 p<0.001), inhibited by L-NAME (max relaxation 20±2.9%, n=6 p<0.001) and mainly dependent on hsp90. When rat aorta rings were incubated with the SPKI inhibitor DL-threo-dihydrosphingosine (DTD) there was a concentration dependent reduction of Ach-induced vasorelaxation (10 nM-50µM) (from max relaxation 87±2.3% to 32±7.6%, n=6 p<0.001) implying a consistent contribution of sphingolipid pathway in Ach-induced vasorelaxation, through sphingosine release and phosphorylation. Co-immunoprecipitation experiments consistently showed increased association of hsp90 with eNOS following exposure of cells to S1P as well to BK or calcium ionophore A-23187. Interestingly, as opposite to A-23187, BK and S1P effect were significantly inhibited by pre-treatment with the SPK inhibitor DTD. In conclusion our data demonstrate that exists an interplay between eNOS, S1P and hsp90 where coupling to hsp90 plays a major role as confirmed by functional and molecular studies.

NO SUPPRESSES MYOGENIC TONE IN RAT MIDDLE CEREBRAL ARTERY BY ACTIVATING BK_{Ca} CHANNELS

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The rat middle cerebral artery exhibits a strong myogenic response. This myogenic response is normally suppressed by a basal release of NO as inhibition of nitric oxide synthase (NOS) causes both vasoconstriction and smooth muscle depolarization associated with oscillations in tension and membrane potential (vasomotion; McNeish *et al.*, 2005). We wished to characterise the cellular mechanisms which underpin the vasoconstrictor effect of inhibitors of NOS by simultaneously measuring smooth muscle cell (SMC) membrane potential and tension or SMC [Ca²⁺] and tension

Male wistar rats (200-300g) were killed by cervical dislocation and exsanguination. The brain was removed and placed immediately in ice-cold krebs solution. Segments of the middle cerebral artery (length, ~2mm; diameter, ~150 µm) were mounted in krebs solution (or mops solution for imaging experiments) in a mulveny-halpern myograph. Smooth muscle cell membrane potential (e_m) was recorded with sharp glass microelectrodes (tip resistances of 80-120 mΩ) filled with 2m kcl. Changes in smc [Ca²⁺] were recorded on a confocal microscope (olympus fv-300) with vessels that had been pre-incubated with a Ca²⁺-sensitive fluorescent dye (fluo-4am). Data are mean ± s.e.mean of 4 or more animals. Statistical comparisons were made using one-way anova with bonferroni's post-test.

Inhibition of NOS with L-NAME (100 µM) caused SMC depolarization associated with constriction and the development of oscillations in E_m and tension. L-NAME also caused an increase in SMC [Ca²⁺] and synchronisation of Ca²⁺ waves, that appeared to be temporally linked to changes in tension and E_m . The effect of L-NAME was mimicked by the guanylyl cyclase blocker, ODQ (10µM) and the BK_{Ca} blocker, iberiotoxin (100nM). Relaxation and hyperpolarization elicited by the NO donor DEA-NONOate (300 nM) was inhibited by iberiotoxin and ODQ but not by glibenclamide (10 µM), apamin (50 nM) or TRAM-34 (1 µM). L-NAME-induced constriction could be fully reversed by nifedipine (1µM, block of L-type Ca²⁺ channels), which also abolished oscillations in SMC [Ca²⁺]. In the presence of L-NAME, niflumic acid (100 µM, blockade of calcium activated chloride channels; Cl_{Ca}) relaxed and hyperpolarised middle cerebral arteries but had little effect on SMC [Ca²⁺], a structurally distinct Cl_{Ca} blocker, DIDS (300 µM), failed to elicit relaxation or hyperpolarization.

In rat middle cerebral arteries the basal NO synthase activity results in suppression of myogenic tone. Inhibition of NOS causes vasoconstriction and depolarization involving the entry of Ca²⁺ through L-type Ca²⁺ channels. The vasomotion appears to be underpinned by oscillations in membrane potential and SMC [Ca²⁺]. The peak of

oscillations in E_m (circa -25mV) and the effect of a blocker of Cl_{Ca} , niflumic acid, suggest that a chloride conductance may be involved in the L-NAME induced constriction and vasomotion; however a structurally distinct blocker of Cl_{Ca} , DIDS, had no effect. Blockade of BK_{Ca} mimics the effects of L-NAME suggesting that NO normally suppresses myogenic tone by activation of SMC BK_{Ca} channels.

Supported by the British Heart Foundation
McNeish *et al.* (2005) *Stroke*. 36, 1526-1532

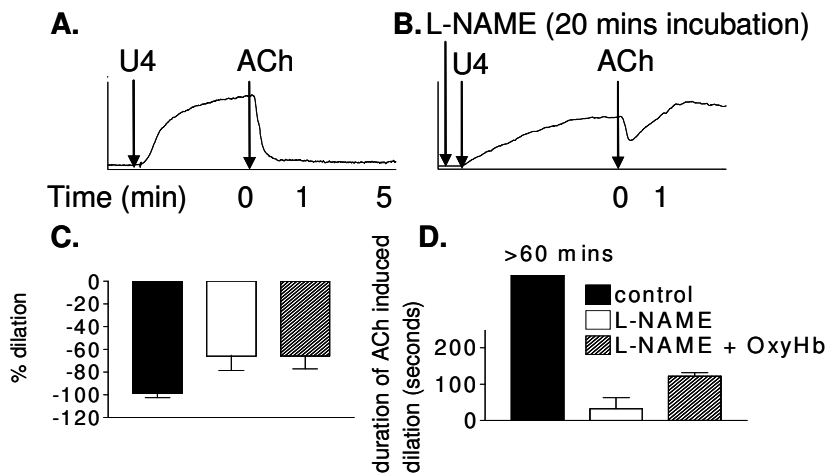
TEMPORAL RELATIONSHIP BETWEEN ENDOTHELIUM DERIVED NITRIC OXIDE AND 'EDHF'

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The identity of Endothelium Derived Hyperpolarizing Factor (EDHF) is yet to be resolved conclusively. However, it is increasingly stated that EDHF is the major factor involved in vasodilation in small arteries, and that nitric oxide has little contribution to the overall vascular tone in these vessels (Coleman *et al*, 2004). In the current study we have compared the effects of NO synthase inhibition on amplitude and duration of endothelium dependent dilator response in rat second order mesenteric vessels.

Male Wistar rats ($200 \pm 15.4\text{g}$) were killed by lethal exposure to CO_2 . Second order mesenteric arteries were mounted in isometric wire myographs, normalized and endothelium confirmed as described previously (Harrington and Mitchell, 2004). Arteries were pre-contracted with EC_{80} U46619 ($3 \times 10^{-7}\text{M}$) and dilated with $3 \times 10^{-6}\text{M}$ Acetylcholine (figure A). Both the level of vasodilation and the time taken to re-contract to pre-dilatory levels were recorded. Some arteries were incubated with 10^{-4}M L-NAME or L-NAME plus oxygenated hemoglobin (OxyHb) for 20 minutes prior to the addition of acetylcholine. Acetylcholine induced a sustained vasodilator response which lasted for more than 1 hour. When vessels were pre-treated with L-NAME the amplitude of acetylcholine induced dilation was modestly reduced (Figure B and C) however the duration of the dilation was dramatically inhibited (Figure B and D). Similar results were obtained when OxyHb was included together with the L-NAME.



Acetylcholine (ACh) induces sustained dilation of U46619 (U4) contracted vessels. (B) Pretreatment with L-NAME or L-NAME plus OxyHb reduced the duration of ACh induced dilation (B and D) with only modest effects on

amplitude (C), (n=4)

When amplitude of response was considered the contribution of NO to the dilator effects of acetylcholine was modest, however when duration of response was considered, more than 95% of acetylcholine induced endothelium dependent dilation could be attributed to the release of NO. This data challenges the notion that in small mesenteric arteries of the rat, NO is a redundant pathway (in favor of EDHF). The transitory dilatory response which remains when all NO is blocked (by the combination of L-NAME plus OxyHb) is however, likely to be EDHF (please see Harrington *et al* 2005, this meeting).

Coleman *et al* (2004) Clin Ex Pharm Phys 31; 641-649

Harrington LS and Mitchell JA (2004) Br J Pharmacol 143: 611-617