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LIST OF ABSTRACTS

Oral Communications

Guest Lecture: Salvador Moncada
Mitochondria: The new frontier in nitric oxide research

Title and Presenters **Page**

C1. Rapid activation of eNOS/Hsp90 association by dietary isoflavones involving ERK1/2 and PI(3)-Kinase/AKT 6
David J Rowlands

C2. Characteristics of the response of the iliac artery to wall shear stress in the anaesthetised pig 7
Roisin Kelly

C3. Thrombospondin-1 modulates platelet sensitivity to nitric oxide 8
Khalid Naseem

C4. Mitochondria in NO-induced cardioprotection against ischaemic damage 9
Vilma Borutaite

C5. Decreased systemic arginine/ADMA ratio in a porcine model of acute liver failure: implications for NO production and regional vascular control 10
Nathan Davies

C6. Uncovering the functions of S-nitrosothiols in plant disease resistance 11
Gary Loake

C7. Nitric oxide from neuronal nitric oxide synthase sensitises neurons to hypoxia-induced death via competitive inhibition of cytochrome oxidase 12
Guy Brown

C8. Inhibition of cytochrome c oxidase by nitric oxide 13
Peter Nicholls

C9. NO-loaded zinc ion exchanged zeolites as potential anti-bacterial agents 14
Sarah Fox

C10. y^+LAT-1 and CAT-2B respectively transport GW274150 in control and activated J774 macrophages 15
Shori Thakur

C11. Relationships between tetrahydrobiopterin (BH4) and endothelial nitric oxide synthase (eNOS): Insights from novel tetracycline (TET)-regulatable cell lines 16
Nicholas Warrick

C12. The acute sensitivity of nNOS to changes in the domain-domain interface 17
Andrew Welland

C13. Bacterial nitric oxide reductase: Cellular saviour and environmental bandit 18
Nick Watmough

C14. Detection of nitric oxide release from single neurons in the pond snail, *Lymnaea Stagnalis* 19
Bhavik A Patel

Poster Communications

Title and Authors **Page**

P.1 The chemistry of S-nitrosothiols and nitrosation 20
Butler, A.

P.2 y+LAT-1 and CAT-2B respectively transport GW274150 in control and activate J774 macrophages. 15
Thakur, S., Vyas, A., Knowles, R.G. and Baydoun, A.R.

P.3 Inhibition of induced L-arginine transport in Rat cultured aortic smooth muscle cells by dominant negative IKB-α but not by dexamethasone 21
Thakur, S., Cui, Z. and Baydoun, A.R.

P.4 SP600125 differentially regulate the inducible L-arginine nitric oxide pathway in J774 macrophages but not in rat aortic smooth muscle cells. 22
Thakur, S and Baydoun, A.R.

P.5 Differential regulation of induced nitric oxide synthesis and L-arginine transport in vascular smooth muscle cells by a JAK family member other than JAK-2 23
Sanz, A.S. and Baydoun, A.R.

P.6 Regulation of L-arginine transport and nitric oxide synthesis by phosphatases 24
Sun, L. and Baydoun, A.R.

P.7 Rapid activation of eNOS/Hsp90 association by dietary isoflavones involving ERK1/2 and PI(3)-Kinase/AKT 6
Rowlands, D.J., Siow, R.C.M. and Mann, G.E.

P.8 Hyperglycaemia: effect on NO synthase and iron transport in the endothelium 25
Rylah, O., Low, S.Y., Bruckdorfer, K.R., Kaila, S. and Srai, S.

P.9 Characteristics of the response of the iliac artery to wall shear stress in the Anaesthetised pig 7
Kelly, R.F. and Snow, H.M.

P.10 Thrombospondin-1 modulates platelet sensitivity to nitric oxide 8
Roberts, W., Riba, R. and Naseem, K.M.

P.11 Inhibition of collagen-induced platelet adhesion by nitric oxide: evidence for targeting of TxA_2 signalling. 26
Roberts, W., Riba, R. and Naseem, K.M.

P.12 Mitochondria in NO-induced cardioprotection against ischaemic damage 9
Borutaite, V. and Brown, G.

P.13 Decreased systemic arginine/ADMA ratio in a porcine model of acute liver failure: implications for NO production and regional vascular control 10
Davies, N.A., Sen, S., Hodges, S.J., Ytrebo, L.M., Mookerjee, R.P. Zou1, Z., Williams, R., Dalton, R.N., Turner, C., Revhaug, A. and Jalan, R.

P.14 Dimethylarginine (NOS inhibitor) levels predict in-patient mortality in alcoholic hepatitis 27
Mookerjee, R.P., Sen, S., Davies, N.A., Hodges, S.J., Turner, C., Dalton, R.N., Williams, R. and Jalan, R.

P.15 Characterisation of the DDAH 1 haploinsufficient mouse: A causal role of elevated ADMA in the pathogenesis of cardiovascular disease. 28
Torondel, B., Nandi, M., Stidwill, R., Rudiger, A. and Leiper, J.

P.16 Potential therapeutic benefit of novel DDAH inhibitors for the treatment of endotoxemia 29
Nandi, M., Rossiter, S., Torondel, B., Maliki, M., Smith, C., Gill, H. Stidwill, R., Leiper, J. and Vallance, P.

P.17 Uncovering the functions of S-nitrosothiols in plant disease resistance 11
Feechan, A., Kwon, E-J., Yun, B.Y., Wang, Y., Pallas, J.A. and Loake, G.J.

P.18 Inhibition of cytochrome c oxidase by nitric oxide 13
Nicholls, P., Mason, M.G., Cooper, C.E. and Wilson, M.T.

P.19 Nitric oxide inhibition of terminal oxidase in *Escherichia coli* 30
Mason, M.G., Nicholls, P., Wilson, M.T. Poole, R.K. and Cooper, C.E.

P.20 NO-loaded zinc ion exchanged zeolites as potential anti-bacterial agents 14
Fox, S., Wilkinson, T.S., Wheatley, P.S., Xiao, B., Morris, R.E., Butler, A.R., Megson, I.L. and Rossi, A.G.

P.21 Novel N-hydroxyguanidines and related compounds as NO-donors 31
Kulczynska, A., Zhang, Q., Botting, N.P., Milliken, P., Mitchell, R., Megson, I and Webb, D.J.

P.22 Relationships between tetrahydrobiopterin (BH4) and endothelial nitric oxide synthase (eNOS): Insights from novel tetracycline (TET)-regulatable cell lines 16
Warrick, N., Antoniades, C., Tatham, A.T., Crabtree, M., Cai, S., McDonald, D., Paterson, D.J. and Channon, K.M.

P.23 The acute sensitivity of nNOS to changes in the domain-domain interface 17
Welland, A.D., Miles, C.S., and Daff, S.

P.24 A Stabilised Intermediate Observed During Oxygen Activation by nNOS 32
Papale, D., Miles, C.S. and Daff, S.

P.25 Modulation of local nitric oxide effects on medium spiny neurons in the nucleus accumbens 33
Hartung, H., Tynan, P.W. and French, S.J.

P.26 Bacterial nitric oxide reductase: Cellular saviour and environmental bandit 18
Field, S.J., Thorndycroft, F.H., Matorin, A., Richardson, D.J. and Watmough, N.J.

P.27 Detection of nitric oxide release from single neurons in the pond snail, *Lymnaea Stagnalis*. 19
Bhavik A Patel

Guest Lecture: Salvador Moncada

Wolfson Institute for Biomedical Research, UCL, The Cruciform Building, Gower Street, London WC1E 6BT.

Mitochondria: The new frontier in nitric oxide research

The physiological roles of nitric oxide (NO) in the maintenance of vascular tone, in synaptic transmission and in cellular defence are firmly established; many of these actions are known to result from activation of the soluble guanylate cyclase. In 1994 it was demonstrated that NO can also affect mitochondrial function, since at physiological concentrations it inhibits cytochrome c oxidase reversibly and in competition with molecular oxygen.

Using a recently-developed technique, based on visible light spectroscopy, we have since demonstrated that endogenous NO enhances the reduction of the mitochondrial electron transport chain, thus contributing to a mechanism whereby cells maintain their oxygen consumption at low oxygen concentrations. This in turn favours the release of superoxide anion, which initiates the transcriptional activation of NF-κB as an early signalling stress response.

We have also found that inhibition of mitochondrial respiration by low concentrations of NO leads to inhibition of hypoxia-inducible factor-1α (HIF-1α) stabilisation. Furthermore, upon inhibition of mitochondrial respiration in hypoxia, oxygen is redistributed toward non-respiratory oxygen-dependent targets. This prevents the cell from registering a state of hypoxia at low oxygen concentrations, which would normally lead to a series of defensive mechanisms involving the upregulation by HIF-1α. of genes associated with, for example, glycolysis and angiogenesis.

Inhibition of respiration by higher concentrations of NO can initiate a variety of stress responses, including hyperpolarisation and upregulation of glucose-regulated protein 78, which protect against apoptosis. Thus, the mitochondrial actions of NO range from physiological regulation of cell respiration, through mitochondrial signalling, to the development of “metabolic hypoxia” in which oxygen is present but cannot be used for mitochondrial respiration. It is also likely that an exaggeration of some of these mechanisms underlies some pathological processes.

C.1 Rapid activation of eNOS/Hsp90 association by dietary isoflavones involving ERK1/2 and PI(3)-Kinase/AKT

(Also presented as Poster #7)

Rowlands, D.J., Siow, R.C.M. and Mann, G.E.
Cardiovascular Division, School of Biomedical & Health Sciences, King's College London, Guy's Campus, London SE1 1UL.

Estrogen protects premenopausal women against cardiovascular disease, however, hormone replacement therapy after menopause has been linked to an increased incidence of breast cancer and limited benefits on the cardiovascular system (Rossouw *et al*. 2002). We have recently hypothesised that dietary soy isoflavones such genistein, daidzein and the daidzein metabolite equol may serve as alternative estrogen receptor modulators and protect against CVD via enhanced production of endothelial-derived nitric oxide and/or EDHF (Mahn *et al*., FASEB J 19:1755-1757, 2005; Knock *et al*., Free Radic Biol Med 41:731-739, 2006; Joy *et al*., J Biol Chem 281: 27335-27345, 2006). In the present study, we report that acute treatment (0.5-2 min) of human umbilical vein endothelial cells with genistein or equol (0.01-100 nM) in Krebs-Heseleit buffer containing L-arginine (100 µM) leads to a rapid phosphorylation of eNOS-Ser^{1177} with concurrent activation of Akt and extracellular signal-regulated kinase (ERK1/2). Dissociation of caveolin-1 from eNOS and increased eNOS association with Hsp90 following acute equol treatment was further demonstrated by immunoprecipitation of eNOS. Inhibition of phosphoinositol-3-kinase/Akt or MEK1/2 activities following preincubation of cells (30 min) with LY294002 (10 µM) or U0126 (1 µM) respectively, led to a significant attenuation of eNOS phosphorylation in response to equol. Our findings provide the first direct evidence that soy-based isoflavones acutely phosphorylate eNOS in human endothelial cells via co-activation of Akt and ERK1/2 signalling pathways, resulting in enhanced eNOS dissociation from caveolin-1 and association with Hsp90. These intracellular signalling events mediate the enhanced NO generation and vascular relaxation elicited by isoflavones.

Supported by the Biotechnology and Biological Sciences Research Council

C.2 Characteristics of the response of the iliac artery to wall shear stress in the anaesthetised pig

(Also presented as Poster #9)

[1]Kelly, R.F. and Snow, H.M.
Biological Services Unit and Department of Physiology, University College Cork, Cork, Ireland, [1]Hatter Institute for Cardiology Research, 4th Floor Chris Barnard Building, Medical School Campus, University of Cape Town, Observatory 7925, Cape Town, South Africa.

The functional significance of shear stress induced vasodilatation in large conduit arteries is unclear since changes in the diameter have little effect on the resistance to blood flow. However, changes in diameter have a relatively large effect on wall shear stress which suggests that the function of flow mediated dilatation is to reduce wall shear stress. The mean and pulsatile components of shear stress vary widely throughout the arterial system and areas of low mean and high amplitude of wall shear stress are prone to the development of atheroma. In this study, using an *in vivo* model with the ability to control flow rate and amplitude of flow independently, we investigated the characteristics of the response of the iliac artery to shear stress while also looking at the effect of mean and pulsatile shear stress on arterial diameter. A possible negative feedback and protection mechanism for the flow mediated dilatation was identified whereby the shear stress-induced blood clotting mechanism is inhibited. This happens ; 1) by the reduction of shear stresses through dilatation thus inhibiting cell adhesion, or 2) release of nitric oxide (NO) by the endothelium. The results of this study confirm that increases in mean wall shear stress are an important stimulus for the release of NO by the endothelium as indicated by changes in arterial diameter and show for the first time *in vivo* that increases in the amplitude of the pulsatile component of shear stress (PSS) have a small but significant inhibitory effect on this response. They also corroborate the hypothesis that the uneven distribution of atheroma throughout the arterial system is related to the ratio of pulsatile to mean shear stress and consequent variability in the production of NO.

C.3 Thrombospondin-1 modulates platelet sensitivity to nitric oxide
(Also presented as Poster #10)

Roberts, W., Riba, R. and Naseem, K.M.
Medical Biosciences, University of Bradford, Bradford, West Yorkshire.

Adhesion and activation of blood platelets at sites of vascular damage is essential for the maintenance of haemostasis, although inappropriate activation leads to thrombosis. Excessive platelet activity is tightly regulated primarily by endothelial-derived nitric oxide (NO). NO inhibits platelet function through a well-established cGMP-dependent signalling cascade. NO diffuses into platelet and binds to soluble guanylyl cyclase (sGC), leading to elevated cGMP levels. The cyclic nucleotide directly activates PKG and indirectly activates PKA. The NO/cGMP/PKG/PKA pathway inhibits platelet function by blunting activatory signalling. However, nothing is known about how platelet activatory signalling cascades modulate the NO signalling. This critical element of the dynamic interplay between platelet signalling cascades remains undefined. TSP-1 is a homotrimeric, multidomain glycoprotein that is present in the ECM, plasma and is stored in platelets á granules. Recent evidence has demonstrated crosstalk between the TSP-1 and NO signalling cascades. Consequently, we examined the effects of TSP-1 on platelet sensitivity to NO. Preincubation of platelets with TSP-1 prevents NO-mediated inhibition of collagen-stimulated aggregation, but does not influence collagen-induced aggregation or induce aggregation itself. Furthermore, we found TSP-1 to blunt the inhibitory effects of NO platelet adhesion to immobilised collagen, such that NO-mediated inhibition of platelet adhesion was abolished in the presence of TSP-1. To examine the mechanism of action of TSP-1 we examined its influence on NO mediated VASP-phosphorylation as a marker of PKA/G activity. TSP-1 blunted the ability of NO to stimulate VASP phosphorylation in a time dependent manner. Importantly, collagen and thrombin are unable to block the effects of NO on VASP, suggesting that TSP-1-mediated signalling specifically regulates the NO pathway. These experiments indicate strongly that TSP-1 at physiologically relevant concentrations can block NO-mediated platelet function by regulating the NO signalling cascade. Thus, our data describe a potentially novel and entirely undefined role for TSP-1 in the modulation of platelets sensitivity to NO.

Funded by grants from Heart Research UK and the British Heart Foundation

C.4 Mitochondria in NO-induced cardioprotection against ischaemic damage

(Also presented as Poster #12)

Borutaite, V. and Brown, G.
Department of Biochemistry, University of Cambridge, Tennis Court Road, Cambridge, CB2 1QW.

Mitochondria are central in both life and death of cells. In healthy cardiomyocytes they produce most of the ATP required to meet the high energy demands of the beating heart, are involved in numerous metabolic processes, and generate reactive oxygen species. Mitochondria are also involved in induction of cell death. Recent evidence suggests that they are also important in integration and transmission of cellular signals in response to physiological stress (such as oxidative stress, hypoxia or ischaemia/reperfusion). Nitric oxide (NO) is an important component of cellular signalling pathways and exerts a variety of physiological effects. NO, depending on concentrations and other conditions, can be cytotoxic or cytoprotective in the heart. NO has been shown to suppress heart cell death induced by ischaemia-reperfusion and NO from iNOS is thought to be one of the main mediators of the delayed phase of ischaemic preconditioning. However, the molecular mechanism of such protection is not clear. We hypothesized that NO-mediated cardioprotection may be associated with activation of protein kinase G (PKG) inhibiting MPT opening during ischaemia and preventing the release of cytochrome c from mitochondria and subsequent apoptosis. This hypothesis was tested on Langendorff-perfused rat hearts. We found that short (3-5 min) pre-perfusion of hearts with micromolar concentrations of the NO donor DETA/NO protected hearts from ischaemia/reperfusion-induced cytochrome c release from mitochondria, subsequent respiratory inhibition and activation of caspases. These effects were prevented when hearts were treated with an inhibitor of protein kinase G (PKG). Incubation of isolated mitochondria with PKG caused changes in phosphorylation levels of mitochondrial proteins, increased mitochondrial capacity to accumulate calcium and prevented calcium-induced MPT. These findings suggest that the NO-induced protective effect against ischaemia-induced apoptosis may be mediated by PKG, phosphorylating particular protein targets in mitochondria and thus inhibiting MPT.

C.5 Decreased systemic arginine/ADMA ratio in a porcine model of acute liver failure: implications for NO production and regional vascular control

(Also presented as Poster #13)

[1]Davies, N.A., [1]Sen, S., [1]Hodges, S.J., [1,2]Ytrebo, L.M., [1]Mookerjee, R.P. [1]Zou1, Z., [1]Williams, R., [3]Dalton, R.N., [3]Turner, C., [2]Revhaug, A. and [1]Jalan, R.
The UCL Institute of Hepatology, London WC1E 6HX; [2]University Hospital Northern Norway, Tromsø, Norway; [3] Guy's Hospital, Kings College, London. SE1 9RT.

Acute liver failure is associated with multi-organ dysfunction and circulatory derangement, resulting in high mortality. We have recently described a hepatic devascularized porcine model of acute liver failure (ALF) in which we observed haemodynamic perturbances and reduced nitric oxide (NO) metabolite levels. It is known that asymmetric dimethylarginine (ADMA), a potent endogenous competitive inhibitor of NO synthase, undergoes hepatic metabolism. However, ADMA is known to be increased in decompensated cirrhosis and in multi-organ failure associated with severe hepatic dysfunction. In this study, we assess the levels of systemic ADMA and arginine in order to establish a mechanism to explain our previous findings. In addition, we have assessed the plasma arginase activity, as it has been shown that hepatocytes release arginase I in response to injury. 16 female pigs were randomized into sham-operated and ALF (by total hepatic devascularization). Time 0 was at the point of hepatic artery ligation. The experiments were terminated at 6 hours. Arterial blood samples were collected at 0, 2, 4 and 6 hours. Plasma ADMA and arginine were measured by stable isotope dilution electrospray tandem mass spectrometry; Ornithine was measured by HPLC. Plasma arginase activity was measured via an *in vitro* urea production method. In arterial plasma, the arginine concentration at 6 hours was significantly reduced in ALF animals compared with the sham group (15.5 ± 6 vs. 114.1 ± 5 µmol/L; P<0.0001). The plasma ornithine level (indicative of plasma arginine consumption by arginase) was elevated in the ALF group (238 ± 23 vs. 105 ± 6 µmol/L, p><0.001). Plasma arginase activity progressively increased over 6 hours in ALF animals but did not change in the sham group [at T=6, ALF, 2.82 ± 0.6 (mM urea made/30 mins), sham, 0.89 ± 0.04. p><0.0001, two-way ANOVA]. Plasma ADMA levels were also found to be significantly increased at 6 hours in ALF compared to sham operated animals (1.98 ± .14 vs. 1.32 ± 0.05 µmol/L, p=0.004). These results provide plasma arginine:ADMA ratios of 7.8 and 86.4 for the ALF and sham groups, respectively. Our results show a dramatic decrease in arginine availability, coupled with increased levels of ADMA in this model of ALF. Hepatic devascularization precludes metabolism of ADMA whilst releasing arginase into the circulation. This combined effect of substrate depletion and increased inhibitor concentration would be expected to severely limit NO production *in vivo* with consequential effects on the micro-circulation. If these observations were extended to ALF in patients, they may suggest a beneficial role for arginine supplementation to counter the deleterious effects on NO synthesis and the resultant vascular dysfunction.

C.6 Uncovering the Functions of S-nitrosothiols in Plant Disease Resistance

(Also presented as Poster #17)

[1]Feechan, A., [1]Kwon, E-J., [1]Yun, B.Y., [1]Wang, Y., [2]Pallas, J.A. and [1]Loake, G.J.
[1]Institute of Molecular Plant Sciences, School of Biological Sciences, King's Buildings, University of Edinburgh, Mayfield Road, Edinburgh EH9 3JR; [2]Trait Research, Syngenta, Jealott's Hill, Bracknell, Berkshire RG42 6EY.

Animal S-nitrosoglutathione reductase (GSNOR) governs the extent of cellular S-nitrosylation, a key redox-based posttranslational modification. Utilising a combination of forward and reverse genetics, directed proteomics and full genome microarrays, we are uncovering the roles of S-nitrosothiols (SNOs), the products of S-nitrosylation, in plant biology. Mutations in AtGSNOR1, an Arabidopsis GSNOR, modulate the extent of cellular SNO formation in this reference plant species. Loss of AtGSNOR1 function increased SNO levels disabling multiple plant defence systems. Conversely, increased AtGSNOR1 activity reduced SNO formation enhancing protection against ordinarily virulent microbial pathogens. We demonstrate that AtGSNOR1 positively regulates at least two distinct nodes of the signalling network controlled by the plant immune system activator, salicylic acid (SA). One of these nodes has been identified as SA-binding protein 3 (SABP3), the activity of which is specifically regulated by S-nitrosylation. Collectively, our data imply SNO formation and turnover regulates multiple modes of plant disease resistance.

C.7 Nitric oxide from neuronal nitric oxide synthase sensitises neurons to hypoxia-induced death via competitive inhibition of cytochrome oxidase

Jekabsone, A. and Brown, G.C.
Department of Biochemistry, University of Cambridge, Tennis Court Road, Cambridge, CB2 1QW.

Hypoxia/ischemia is known to trigger neuronal death, but the role of neuronal nitric oxide synthase (nNOS) in this process is controversial. Nitric oxide (NO) inhibits cytochrome oxidase in competition with oxygen. We tested whether NO derived from nNOS synergizes with hypoxia to induce neuronal death by inhibiting mitochondrial cytochrome oxidase. 16 hour of mild hypoxia (2% oxygen) plus deoxyglucose (an inhibitor of glycolysis) caused extensive, excitotoxic death of neurons in rat cerebellar granule cell (CGC) cultures. Three different nNOS inhibitors halved this death, indicating a contribution of nNOS to hypoxic death. An nNOS inhibitor did not, however, block neuronal death induced either by added glutamate or by added azide (an uncompetitive inhibitor of cytochrome oxidase), indicating that nNOS acts upstream of glutamate and cytochrome oxidase. Hypoxia plus deoxyglucose induced glutamate release from pure neuronal cultures, and this release was decreased by the nNOS inhibitor. Hypoxia inhibited cytochrome oxidase activity in the cultures, but a selective nNOS inhibitor prevented this inhibition, indicating NO from nNOS was inhibiting cytochrome oxidase in competition with oxygen. Overall, these data indicate that hypoxia synergises with NO from nNOS to induce neuronal death via cytochrome oxidase inhibition. This might contribute to ischemia/stroke-induced neuronal death *in vivo*.

C.8 Inhibition of cytochrome c oxidase by nitric oxide
(Also presented as Poster #18)

Nicholls, P., Mason, M.G., Cooper, C.E. and Wilson, M.T.
Department of Biological Sciences, University of Essex, Colchester, Essex CO4 3SQ.

Mammalian cytochrome c oxidase (cytochrome aa3) is inhibited by nitric oxide (formed from NO donors such as proliNONOate) at physiologically relevant levels (10 to 100 nM). More than one inhibited species is formed. With cytochrome c in an aerobic steady state there is an immediate increase in cytochrome a reduction upon NO addition, indicating inhibition of electron transfer at the a->a3 level. Formation of the final inhibited species aa3(2+)NO, however, lags behind this initial inhibition, suggesting that NO may initially bind to a partially oxidized binuclear centre, probably to its copper atom. This behaviour can be correlated with the effects seen on flux at varying oxygen levels. At high flux and low oxygen tensions NO interacts predominantly with the fully reduced (ferrous/cuprous) centre, in competition with oxygen. However, as the oxygen tension is raised (or the consumption rate decreased) a reaction with the oxidized enzyme becomes increasingly important. The affinity (K_d) of NO for the oxygen-binding ferrous heme site is 0.2 nM. NO interaction with cupric copper results in formation of nitrite. But this process behaves kinetically as a non-competitive inhibition with an apparent NO affinity between 20 and 50 nM. This combination of competitive (heme) and non-competitive (copper) modes of binding enables NO to interact with mitochondria across the full in vivo dynamic range of oxygen tension and consumption rates.

C.9 NO-loaded zinc ion exchanged zeolites as potential anti-bacterial agents
(Also presented as Poster #20)

[1,2]Fox, S., [2]Wilkinson, T.S., [3]Wheatley, P.S., [3]Xiao, B., [3]Morris, R.E., [3]Butler, A.R., [4]Megson, I.L. and [2]Rossi, A.G.
[1] Centre for Cardiovascular Science, [2] Centre for Inflammation Research, Queen's Medical Research Institute, University of Edinburgh, EH16 4TJ; [3]School of Chemistry, University of St Andrews, KY16 9ST; [4] Free Radical Research Institute, UHI Millennium Institute, Inverness, IV2 3BL.

Transition metal ion-exchanged zeolites are excellent storage materials for nitric oxide (NO). NO is liberated from the zeolite structure on contact with an aqueous environment which makes it an ideal material for use in many biological applications where local delivery of NO is advantageous. Indeed, we have shown that these NO-loaded zeolites are powerful inhibitors of platelet aggregation (Wheatley *et al,* 2006. *JACS,* **128:**502-509) and therefore have potential as anti-thrombotic coatings on blood contacting medical devices. Here we show an application for zinc ion (Zn^{2+})-exchanged zeolites as anti-bacterial agents.

Zn^{2+} exchanged zeolites (NO-loaded and NO-free; 50% zeolite:PTFE binder) were incubated in 150µl of a bacterial suspension of *Psuedomonas aeruginosa* in 5% Luria bertani (LB) broth/phosphate buffered saline (PBS) at 37°C for 45 minutes. An untreated sample incubated in the same conditions acted as the control. The number of viable bacteria was estimated by plating dilutes of the bacterial suspension on LB broth agar and counting colonies approximately 16 hours later.

NO-loaded Zn^{2+} zeolites (50% zeolite:PTFE binder) can significantly reduce the number of the gram-negative bacteria, *P.aeruginosa,* in suspension after a 45 minute incubation compared to control. No significant difference from control was evident with the NO-free zeolite counterparts, suggesting the effect is NO dependent. Preliminary results with gram-positive bacteria, methicilin-sensitive *Staphylococcus aureus* (MSSA), indicate a similar effect.

These results suggest a potential role for NO-loaded Zn^{2+} zeolites as anti-bacterial agents to treat infections.

C.10 y^+-LAT-1 and CAT-2B respectively transport GW274150 in control and activated J774 macrophages

(Also presented as Poster #2)

[1]Thakur, S., [1]Vyas, A., [2]Knowles, R.G. and [1]Baydoun, A.R.
[1]School of Life Sciences, University of Hertfordshire, College Lane, Hatfield AL10 9AB; [2]GlaxoSmithKline Research and Development, Stevenage, SG1 2NY.

GW274150 has been identified as a potent inhibitor of the inducible nitric oxide synthase (iNOS) enzyme both in *in vivo* and in *in vitro* (Young *et al*. 2000, Bioorg. Med. Chem. Lett., **10**: 597-600; Alderton *et al*., 2005, Br. J. Pharmacol., **145**:301-312). We have previously demonstrated that this compound may be transported into control J774 macrophages via a broad scope carrier, characteristic of system y^+-LAT1 (Baydoun et al., 2006, Amino Acids, **31**:101-109). We now report that activation of these cells with bacterial lipopolysaccharide (LPS) results in a switch in transporters where the inducible cationic amino acid transporter (CAT)-2B becomes the critical carrier system for GW274150. Confluent monolayers of J774 macrophages were pre-treated with LPS (1 μg ml^{-1}) for 24 h in Dulbecco's modified Eagle's medium supplemented with 10 % foetal bovine serum. Transport was monitored over 1 min in Hepes-buffered Krebs solution (50μl; 37°C) containing L-[^{14}C]GW274150 (1μCiml^{-1}) and 0.1mM unlabelled compound (Baydoun *et al*., 2006, Amino Acids, **31**:101-109). Cross-inhibition studies were carried out in the presence of a 10-fold excess (1 mM) of known substrates for different amino acid transports. The Na^+-dependency of transport was determined in a modified Na^+-free Krebs buffer supplemented with choline chloride and bicarbonate. Kinetic studies were carried out using different substrate concentrations.

Activation of J774 macrophages resulted in the expected induction of iNOS expression and in NO synthesis. In addition, the control rate of GW274150 transport (2.6 pmol. μg $protein^{-1}$ min^{-1}) was significantly enhanced in cells treated with LPS (4.2 pmol. μg $protein^{-1}$ min^{-1}). This upregulation in uptake of GW274150 was further confirmed in kinetic studies, which revealed an enhanced V_{max} of 13.4 pmol. μ $protein^{-1}$ min^{-1} from a control value of 8.5 pmol. μg $protein^{-1}$ min^{-1}. The K_t remained virtually unaltered (0.24±0.01 mM in controls vs 0.26±0.02 mM in activated cells). Further characterisation revealed that the uptake process in both control and activated cells was pH insensitive, largely Na^+-independent and inhibited by L-arginine and L-lysine but unaffected by 2-methylaminoisobutyric acid, L-alanine, L-valine or ß-2-amino-bicyclo-(2,2,1)-heptane-2-carboxylic acid. More importantly, transport of L-[^{14}C]GW274150 in control but not in activated cells was markedly attenuated, and in a Na^+-dependent manner, by L-leucine, L-methionine, 6-diazo-5-oxo-L-norleucine and L-glutamine.

Our previous findings (Baydoun *et al*., 2006) together with the current data strongly suggest that control and activated J774 macrophages transport GW274150 via different carrier systems. The broad-spectrum amino acid carrier y^+-LAT1 may be the critical transporter in controls while CAT-2B may be the transporter in activated cells. Further studies looking at changes in expression of y^+-LAT1 and CAT-2B in control and activated J774 macrophages are however required and these studies are currently being carried out.

C.11 Relationships between tetrahydrobiopterin (BH4) and endothelial nitric oxide synthase (eNOS): Insights from novel tetracycline (TET)-regulatable cell lines
(Also presented as Poster #22)

Warrick, N., Antoniades, C., Tatham, A.T., Crabtree, M., Cai, S., McDonald, D., Paterson, D.J. and Channon, K.M.
Department of Cardiovascular Medicine, The Wellcome Trust Centre for Human Genetics, University of Oxford, Roosevelt Drive, Headington, Oxford, OX3 7BN.

Nitric oxide (NO) generated by endothelial nitric oxide synthase (eNOS) is a key signalling molecule in vascular homeostasis. Loss of NO bioavailability, due to reduced synthesis and increased scavenging by reactive oxygen species, including superoxide, is a cardinal feature of endothelial dysfunction in vascular disease states. Nitric oxide synthase (NOS) is regulated by the bioavailability of its cofactor, tetrahydrobiopterin (BH4). Under conditions of limited BH4 availability, eNOS becomes enzymatically uncoupled and generates superoxide. However, it is unclear whether the absolute intracellular concentration of BH4, or the ratio of BH4 to oxidised biopterins are the key determinants of eNOS coupling. The stoichiometry of BH4/eNOS interactions is not established. We aim to investigate these relationships using novel tetracycline (*TET)*-inducible cell lines with regulatable levels of intracellular BH4. A murine NIH 3T3 TET-Off cell line was modified to stably express human GTP-cyclohydrolase I (GTPCH-1), the rate limiting enzyme in BH4 synthesis (GCH^{TET} cells). GCH^{TET} cells were modified to stably express a range of eNOS-GFP fusion protein levels (eNOS^{TET} cells). These eNOS^{TET} cell lines provided an *in vitro* model system with highly regulatable levels of human GTPCH-1 mRNA and BH4 in response to doxycycline (DOX). Furthermore, we demonstrate that eNOS^{TET} cell lines have NOS enzymatic activity, through the conversion of radioactively labelled arginine to citrulline. In addition, direct measurement of NO released by eNOS^{TET} cells was achieved by spin-trapping NO with Fe(II)-diethyldithiocarbamate (Fe-DETC) and EPR spectroscopy. Finally, we demonstrate that the suppression of BH4 by culturing eNOS^{TET} cells in the presence of DOX leads to a rise in NOS-inhibitable superoxide production. This data, therefore, suggests that the suppression of BH4 levels in these eNOS^{TET} cells may lead to the uncoupling of eNOS enzymatic activity and the consequent generation of superoxide. These cell lines will provide new approaches to studying BH4-eNOS interactions in intact cells.

C.12 The acute sensitivity of nNOS to changes in the domain-domain interface
(Also presented as Poster #23)

Welland, A.D., Miles, C.S., and Daff, S.
School of Chemistry, University of Edinburgh, West Mains Road, Edinburgh EH9 3JJ.

NO production by mammalian neuronal Nitric Oxide Synthase (nNOS) is controlled by the calcium-dependent binding of calmodulin (CaM), which activates electron transfer through the enzyme. The exact mechanism of this activation is uncertain, but it is known to involve a large-scale structural rearrangement of the 83kDa reductase domain (nNOSrd). The reductase domain is itself composed of FAD- and FMN-binding domains, which are thought to become conformationally mobile on CaM binding. One of the key features of the domain-domain interface within nNOSrd is a salt-bridge involving a conserved arginine residue, Arg1229. Mutation of this residue to Glu, inducing a single charge reversal, completely abolishes the dependence on CaM. Hydride transfer from NADPH to bound FAD is accelerated 15-fold; electron transfer from FMN to external electron acceptors is enhanced 10-fold, while FAD to FMN electron transfer is inhibited by up to 100-fold. The FAD- and FMN-binding domains appear to be separated so effectively in the R1229E mutant that FAD to FMN electron transfer is now rate determining and is inhibited by the presence of the small redox protein cytochrome c. Clearly, domain interactions and interdomain motion are essential for the labile transfer of electrons through nNOS and are extremely sensitive to perturbation.

C.13 Bacterial nitric oxide reductase: Cellular saviour and environmental bandit
(Also presented as Poster #26)

Field, S.J., Thorndycroft, F.H., Matorin, A., Richardson, D.J. and Watmough, N.J.
Centre for Metalloprotein Spectroscopy and Biology, School of Biological Sciences, University of East Anglia, Norwich, NR4 7TJ.

That part of the global nitrogen cycle known as denitrification requires four specialist enzyme activities to catalyze the sequential conversion of nitrite ions to dinitrogen: $NO_3^- \rightarrow NO_2^- \rightarrow$ Nitric Oxide (NO) $\rightarrow N_2O \rightarrow N_2$. However under aerobic conditions most denitrifying bacteria produce the potent greenhouse gas N_2O rather than dinitrogen to the extent that in global terms >70% of anthropogenic N_2O emissions result from agricultural soil management. The respiratory Nitric Oxide Reductase (NOR), which catalyses the key reaction in this pathway, $2NO + 2H^+ + 2e^- \rightarrow N_2O + H_2O$ has another important biological role. Since NOR links NO detoxification to energy conservation it is used by a number of clinically relevant bacteria, to evade the NO mediated response to colonization by potential host cells. NOR is a close relative of the protonmotive heme-copper oxidases which reduce O_2 to H_2O. Like the heme-copper oxidases NOR, which is not electrogenic, has a dinuclear center at its active site, but one in which a heme is coupled to non-heme iron (Fe_B) not copper. We have described in detail the redox properties of NOR and based on the results propose that reductive coupling of NO to form N_2O takes place at Fe_B. Current work in our laboratory is aimed at understanding how NOR controls the temporal and spatial arrival at its active site of its three co-substrates, to allow productive reaction, without generating inhibitory species such as Fe(II)-NO heme in the dinuclear centre.

References:

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C.14 Detection of Nitric Oxide Release from Single Neurons in the Pond Snail, *Lymnaea Stagnalis*

(Also presented as Poster #27)

Patel., B.A.

PFSU, Department of Bioengineering, Chemical Engineering Building, Imperial College London, Prince Consort Road, South Kensington Campus, London, SW7 2AZ.

Nitric oxide (NO) plays a number of important physiological roles in the central nervous system (CNS). NO is thought to play a role in modulation of neurotransmitter function by affecting the release of monoamines and amino acids neurotransmitters which are believed to be involved in learning and memory formation m) pigmented neurones allow this type of analysis to be carried out at the level of the single identified neurone. However at present there has been limited work showing the release of nitric oxide from neurones. We have utilised the CNS of the pond snail Lymnaea stagnalis in order to record NO release. The large (~80 For real-time NO measurements we have utilised a p-Eugenol/Nafion coated 30 µm carbon fibre microelectrode with amperometric detection. The electrode has a limit of detection of approximately 3 nM and a sensitivity of 9.5 nA µM-1. The sensor was very selective against a variety of neurochemical interferences such as ascorbic acid, uric acid and catecholamines and secondary oxidation products such as nitrite. Nitric oxide release was measured from the cell bodies of two neurons, the cerebral giant cell (CGC) and the B2 buccal motor neuron, in the intact but isolated CNS. NO release was stimulated from selected Lymnaea neurons using a high Ca2+/ high K+ ringer and the ability of L-NAME, and arginine to affect release examined. However, electrical stimulation did not cause release of detectable levels of NO. The greater implications into how levels of nitric oxide effect signalling during ageing will be studied.

P.1 The chemistry of S-nitrosothiols and nitrosation

Lyn Williams (Presented by Anthony Butler)
Bute Medical School and Centre for Biomolecular Sciences, University of St. Andrews, Fife KY16 9ST, UK.

For many years S-nitrosothiols were laboratory curiosities but, with the coming of the nitric oxide story, they assume an unexpected significance. Much of the work on the chemistry of these compounds was undertaken by Lyn Williams who, until he retired five years ago was at the University of Durham. In August of this year he died following an accident which left him quadriplegic. This poster will summarise his work on the chemistry of S-nitrosothiols and nitrosation reactions and assess his contribution to the nitric oxide story.

P.3 Inhibition of induced L-arginine transport in Rat cultured aortic smooth muscle cells by dominant negative IKB-α but not by dexamethasone

Thakur, S., Cui, Z. and Baydoun, A.R.
School of Life Sciences, University of Hertfordshire, College Lane, Hatfield AL10

Induction of iNOS in rat aortic smooth muscle cell (RASMC) is accompanied by an increase in transport of L-arginine. Although both processes are dependent on *de-novo* protein synthesis, the induction of iNOS can be selectively blocked by dexamethasone (Wileman, *et al.*, 1995. *Br.J.Pharmacol.*, **116**: 3243-3250) which acts, at least in part, by preventing NF-kB binding to the iNOS promoter (Kleinert *et al.*, 1996, *Mol. Pharmacol.*, **49**: 15-21). Whilst this indicates that stimulation of L-arginine transport may be independent of NF-κB, there is no direct evidence in support. To confirm this hypothesis we have explored the effects of select inhibitors of NF-κB together with dominant negative IκB on the induction of L-arginine transport and iNOS expression in RASMC.

Confluent monolayers of RASMCs were activated for 24 h with LPS (100 μg ml^{-1}) and IFN-γ (50 U ml^{-1}) in the absence and presence of either dexamethasone (1-10 μM), MG132 (a proteasome inhibitor; 0.03 μM - 3 μM), BAY 11-7082 (an inhibitor of IκB phosphorylation; 0.3 μM - 30μM) or caffeic acid phenyl ethyl ester (CAPE; an inhibitor of p65 NFκB translocation; 1.0 μM - 100μM). In parallel studies, partially confluent (~60 %) monolayers of RASMCs were transfected for 6 h with a dominant negative IκB-α construct prior to activation with LPS (100 μg ml^{-1}) and IFN-γ (50 U ml^{-1}). Nitric oxide production was determined using the Griess assay. L-arginine transport was assessed as described previously (Wileman, *et al.*, 1995. *Br.J.Pharmacol.*, **116**: 3243-3250). Activation of NF-κB was monitored by the electrophoretic mobility-shift assay (EMSA) and changes in levels of dominant IκB-α or endogenous IκB-α were determined by western blotting.

Induced NO synthesis and L-arginine transport were both inhibited by MG132, which at 3 μM reduced accumulated nitrite levels by 80±8 % and L-arginine transport by 52±15 % respectively ($p<0.01$; n=6). CAPE was also effective in attenuating both processes, reducing NO production by 55±11 % and transport by 50±6 % at 100μM ($p<0.05$; n=6). BAY 11-7082 was less potent against induced L-arginine transport, reducing the latter by 10±33 % ($p>0.05$) whilst inhibiting NO production by 45±12 % at 10 μM ($p<0.01$; n=6). In contrast, dexamethasone blocked iNOS expression and NO production but had no significant effect on L-arginine transport even when used at concentrations of up to 10 μM. EMSA confirmed that NF-κB was activated in RASMCs by LPS and IFN-γ in a time-dependent manner, peaking at 2 h. This activation was unaffected by dexamethasone but either blocked or significantly inhibited by the maximum concentrations of each of the other drugs used above. Following transfection, levels of dominant negative IκB-α were significantly increased whilst endogenous IκB-α protein expression was markedly attenuated. More importantly, stimulated L-arginine uptake and iNOS expression were completely abolished in transfected cells.

These findings indicate that NF-κB is essential, at least in part, for the induction by LPS and IFN-γ of both iNOS and transport of L-arginine in RASMC. Moreover, our data indicate that dexamethasone may act via mechanisms that are independent of NF-κB in exerting its inhibitory action on iNOS induction. These mechanisms, including the destabilisation of iNOS mRNA and or induction of protein degradation are currently being investigated.

P.4 SP600125 differentially regulate the inducible L-arginine nitric oxide pathway in J774 macrophages but not in rat aortic smooth muscle cells

Thakur, S. and Baydoun, A.R.
School of Life Sciences, University of Hertfordshire, College Lane, Hatfield AL10 9AB.

Studies were carried out to investigate the role of the Jun N-terminal kinase (JNK) pathway in the induction of iNOS and of L-arginine transport in rat cultured aortic smooth muscle cells (RASMCs) and J774 macrophages using the JNK-specific inhibitor, SP600125. RASMCs or J774 cells were respectively activated with bacterial lipopolysaccharide (LPS; 100 μg ml^{-1}) plus interferon-γ (IFN-γ; 50 U ml^{-1}) or LPS alone (1 μg ml^{-1}) in the absence and presence of SP600125 (0.1 - 10 μM). Nitric oxide production was determined by the Griess assay and L-arginine transport was assessed as described (Baydoun *et al.*, 1999. *Biochem.J*. **344**: 265-272). Expression of iNOS was monitored by western blotting. Activation of AP-1 was monitored by the electrophoretic mobility-shift assay (EMSA) using oligonucleotide containing an AP-1 consensus sequence. Induced NO synthesis and L-arginine transport were not significantly altered in RASMCs treated with SP600125 (0.1-10 μM). Western blot analysis showed that iNOS expression was not altered even in the presence of the highest concentration (10 μM) of drug used. EMSAs confirmed that AP-1 was activated in RASMCs by LPS and IFN-γ in a time-dependent manner, peaking at 2 h. This activation was inhibited in a concentration-dependent manner by SP610025 which at 10 μM reduced AP-1-DNA binding back to non-activated control levels. In contrast to its lack of effect in RASMCs, SP600125 caused marked concentration-dependent inhibition of NO production in J774 macrophages, virtually abolishing accumulated nitrite levels at 10 μM. Interestingly, transport of L-arginine remained unaltered in both control and activated cells. These data indicate that the JNK signaling pathway is essential for the induction of iNOS but only in J774 macrophages. Moreover, although iNOS and L-arginine transport are induced in parallel, the induction of transporter activity is not regulated by the JNKs, thus indicating selective and differential regulation of these two processes.

P.5 Differential regulation of induced nitric oxide synthesis and L-arginine transport in vascular smooth muscle cells by a JAK family member other than JAK-2

Sanz, A.S. and Baydoun, A.R.
School of Life Sciences, University of Hertfordshire, College Lane, Hatfield, AL10 9AB.

In vascular smooth muscle cells, the expression of iNOS appears to be critically dependent on the presence of interferon-γ (IFN-γ) (Baydoun *et al*., 1999. *Biochem J*. **344:** 265-272) suggesting that activation of a JAK family member may be critical for this process to occur. However, studies utilising the JAK-2 selective inhibitor AG490 have proved controversial and inconclusive (Cruz *et al*., 1999. *Am. J. Physiol*., **277:** C1050-C1057; Marrero *et al*., 1998. *Biochem. Biophys. Res. Commun*., **252:** 508-512). Thus, it is still unclear as to what role the JAKs play in the induction of iNOS. Moreover, there is little data available on the regulation of L-arginine transport by this family of kinases. We have therefore examined the critical requirement of the JAKs for the induction of both iNOS and L-arginine transport in rat cultured aortic smooth muscle cells (RASMC) using the new inhibitor of the JAK family of proteins referred to JAK inhibitor I. This compound, unlike AG490, is reported to block JAK-1, JAK-2, JAK-3 and Tyk-2 (Thompson *et al*., 2002. *Bioorg. Med. Chem. Lett*. **12**, 1219-1223).

Confluent monolayers of RASMCs were pre-treated with increasing concentration of either AG490 (1-100 μM) or JAK inhibitor I (0.01- 10 μM) for 30 minutes prior to activation with 100 μg ml^{-1} LPS plus 50U ml^{-1} IFN-γ for 24 h. Changes in nitrite production, iNOS expression and L-arginine transport were monitored as described previously (Baydoun *et al*., 1999. *Biochem. J*., **344,** 265-272). In parallel studies, the effects of both AG490 and JAK inhibitor 1 on phosphorylation of STAT-1 was investigated by western blotting using a phosphospecific anti-STAT-1 antibody.

AG490 failed to cause any statistically significant change in either nitrite production or L-arginine transport at non cytotoxic concentrations of up to 100μM. In contrast, JAK inhibitor I caused marked reductions in accumulated nitrite levels, inhibiting the latter by >80% at 3μM. These changes were reflected in a parallel decrease in iNOS protein expression which was virtually abolished at 10 μM. Interestingly, L-arginine transport was unaffected by JAK inhibitor 1 despite the critical requirement of IFN-γ for its induction. Western blotting for changes in $tyrosine^{701}$ phosphorylation of STAT-1 revealed a time-dependent phosphorylation of the latter which was apparent 3 min after activation of cells. This reached a peak at 10-15 min and was sustained over 120 h following exposure to LPS and IFN-γ. More importantly, AG490 did not block STAT-1 phosphorylation while JAK inhibitor 1 completely abolished expression of the phosphoprotein at 10 μM.

These results suggest that activation of JAK-2 may not be critical for the induction of iNOS in RASMCs. However, the inhibitions caused by JAK inhibitor I strongly indicate that one of the other JAK family members, which remain to be identified, may be involved. Moreover, the selective inhibition of NO production but not of L-arginine transport indicates for the first time that these two pathways may be differentially regulated by the JAK/STAT pathway.

P.6 Regulation of L-arginine transport and nitric oxide synthesis by phosphatases

Sun, L. and Baydoun, A.R.
School of Life Sciences, University of Hertfordshire, College Lane, Hatfield, AL10 9AB.

Exposure of vascular cells to pro-inflammatory mediators including LPS and cytokines results in the parallel induction of the enzyme nitric oxide synthase (iNOS) and in L-arginine transport. The consequence of these inductions is the overproduction of the highly labile but extremely potent vasodilator molecule, nitric oxide (NO) which has been implicated in various inflammatory diseases including septic shock, inflammatory bowel disease and arthritis. Considerable effort has focused on unravelling the signal transduction mechanisms associated with the induction of both iNOS and cationic amino acid transporters (CATs) with the conclusion that several protein kinases, including the mitogen activated kinases (particularly p38), may be critical. Relatively little is however known about the potential post-translational modification of these proteins and the consequence this might have on their cellular localization or function. Emerging evidence suggest that both iNOS and CATs possess a consensus site for phosphorylation by cAMP dependent kinases and by protein kinase C (PKC). Thus the possibility exists that the localisation and function of these proteins (particularly CATs) may be regulated following phosphorylation or dephosphorylation. Preliminary studies have therefore been carried out examining the effects of select phosphatase inhibitors on the induction of the L-arginine-NO pathway in rat cultured aortic smooth muscle cells (RASMCs) exposed to LPS (100 $\mu g\ ml^{-1}$) and IFN-γ (100 U ml^{-1}) or in J774 macrophages treated with LPS (1 $\mu g\ ml^{-1}$) alone. Nitric oxide production was determined using the standard Griess assay and uptake of L-arginine was monitored using L-[^{3}H]arginine as described previously (Baydoun *et al*., 1999. *Biochem. J*., **344,** 265-272). In parallel studies, iNOS protein expression was detected by western blotting using a specific monoclonal anti-iNOS antibody. In these studies, the specific serine-theronine phosphatase inhibitor okadaic acid (OA) potentiated NO production and L-[^{3}H]arginine uptake in a concentration-dependent manner in RASMC but not in the J774 macrophage cell line. Interestingly, the increases in NO production were not accompanied by any significant change in iNOS protein expression as detected by western blotting. In contrast to OA, the tyrosin-specific phosphatase inhibitor sodium orthovanadate (SO) failed to show any significant effects on NO production, L-[^{3}H]arginine transport or iNOS expression. These findings suggest that serine-theronine specific but not tyrosin-specific phosphatase may be involved in regulating iNOS and CAT function. The effect is however cell-type specific and may have important implications in the regulation of these processes under inflammatory conditions.

P.8 Hyperglycaemia: effect on NO synthase and iron transport in the endothelium

Rylah, O., Low, S.Y., Bruckdorfer, K.R., Kaila, S. and Srai, S.
Department of Biochemistry and Molecular Biology, Hampstead Campus, University College London, Rowland Hill Street, London, NW3 2PF.

Oxidative stress is a key factor in promoting endothelial dysfunction; a major feature of metabolic and cardiovascular disorders. Nitric oxide (NO) is an important regulator of cell function, especially in the vasculature. A decrease in its bioavailability, as found under conditions of oxidative stress, has important implications for both glucose and iron metabolism since both the expression of iron transporters and glucose uptake are affected by NO. Thus, we investigated the inter-relationship between glucose, iron and NO in the microvascular endothelium. Cell lysates were prepared from cultured human microvascular endothelial cells which had been incubated with the NO donor DETA NONOate (0.01 mM), haemin (iron loading, 0.1 mM) and deferroxamine (iron chelation, 0.1 mM) in the presence and absence of hyperglycaemia (25 mM glucose). Hyperglycaemia was used to promote oxidative stress. The protein expression of endothelial NO synthase (NOS) and the iron transporters DMT-1 (divalent metal ion transporter 1) and Ireg-1 (iron regulatory transporter 1) in these lysates was investigated using Western blotting. Hyperglycaemia up-regulated the iron transport proteins Ireg-1 and DMT-1, whereas in the presence of NO, there was decreased DMT-1 but not Ireg-1 expression. Iron overload, in the presence of hyperglycaemia decreased DMT-1 expression but conversely increased Ireg-1. Furthermore, NOS expression was unaffected by NO or iron overload but was increased by iron chelation in euglycaemic conditions. However, in the presence of hyperglycaemia there was increased NOS expression which was inhibited by iron overload. In conclusion, the availability of NO appears to be an important regulator of iron homeostasis in the endothelium. In conditions of hyperglycaemia-induced oxidative stress, there is likely to be a decrease in bioavailable NO which could lead to dysregulation of iron homeostasis. Furthermore, control of iron homeostasis by NO may be a key factor in preventing endothelial dysfunction as seen in cardiovascular disease.

P.11 Inhibition of collagen-induced platelet adhesion by nitric oxide: evidence for targeting of TxA_2 signalling.

Roberts, W., Riba, R. and Naseem, K.M.
Medical Biosciences, University of Bradford, Bradford, West Yorkshire. UK.

Nitric oxide (NO) inhibits platelet adhesion to areas of vascular damage by mechanisms that remain poorly defined. The aim of the present study was to determine the pathways targeted by NO which lead to inhibition of platelet adhesion to collagen under static conditions. Collagen induced a time and concentration-dependent increase in adhesion that was partially, but not completely, dependent on the release of thromboxane A_2 (TxA_2) and adenosine diphosphate (ADP). S-nitrosoglutathione (GSNO, 0.1-100mM), caused a prolonged concentration-dependent inhibition of adhesion to collagen. However, the inhibitory effects of NO were lost if platelets were incubated with apyrase and indomethacin, suggesting that NO inhibited adhesion by abrogating the effects of secondary mediators. The temporal relationship between platelet-derived TxA_2 and ADP in adherent platelets is unclear. Under our experimental conditions dense granule secretion from platelets adhering to collagen was TxA_2-dependent. NO inhibited adhesion induced dense granule secretion, but not TxA_2 generation. Since TxA_2 was upstream of ADP release our data suggested that NO blocked dense granule secretion induced by TxA_2. Indeed using the thromboxane mimetic U46619 we found NO to inhibit TxA_2-induced signalling leading to secretion. The soluble guanylyl cyclase inhibitor ODQ (1H-[1,2,4]Oxadiazolo[4,3-a]quinoxalin-1-one), completely blocked protein kinase G activation, but only partially blocked the inhibitory effects of NO on both adhesion and secretion, suggesting that NO-mediated effects were both cGMP-dependent and independent. In conclusion, our results demonstrate that NO inhibits platelet adhesion to collagen by blocking TxA_2 induced ADP release. The data suggests that NO modulates adhesion by reducing the availability of platelet derived ADP and has little effect on adhesion to collagen in the absence of secondary mediators.

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P.14 Dimethylarginine (NOS inhibitor) levels predict in-patient mortality in alcoholic hepatitis

[1]Mookerjee, R.P., [1]Sen, S., [1]Davies, N.A., [1]Hodges, S.J., [2]Turner, C., [2]Dalton, R.N., [1]Williams, R. and [1]Jalan, R.
[1]Institute of Hepatology, University College London, WC1E 6HX. [2]Guys Hospital Medical School, London. SE1 9RT.

Acute alcoholic hepatitis (AH) has a high 28 day mortality compared to alcoholic cirrhosis alone. Asymmetric dimethylarginine (ADMA) has been linked with hepatic dysfunction and poor outcome in critical care patients. In this study, we investigated whether ADMA, and its isomer symmetric dimethylarginine (SDMA), could be used to predict in-patient mortality in AH.

Plasma, (48 hrs post admission) from 52 supportively managed decompensated alcoholic cirrhotic patients (bilirubin > 85μmol/L; increasing ascites and creatinine <150 μmol/L), was analyzed for ADMA and SDMA (measured using fragmentation specific stable isotope dilution electrospray mass spectrometry-mass spectrometry). Patients were characterised by: histology, inflammatory indices: (CRP; WCC; SIRS, IL-6 and 8, TNFα and TNF receptors) into those with inflammation (AH) and those with cirrhosis alone.

14/ 27 AH patients were alive after 28 days compared with 23/ 25 patients with only cirrhosis. Renal dysfunction (creatinine > 150 μmol/L; creatinine clearance < 35 mls/hr) occurred in all AH deaths and in 1/ 2 cirrhotic deaths. Plasma ADMA was significantly higher in AH patients who died compared to survivors: (0.85±0.09 vs. 0.59± 0.03 μmol/L; $p<0.01$) and also higher in the AH group compared with subjects without inflammation (0.73±0.06 vs. 0.45±0.02, $p<0.001$). Plasma SDMA levels were markedly higher in AH patients who died: (1.9± 0.4 vs. 0.7 ± 0.1 μmol/L; $p<0.01$) and higher in all AH patients compared to cirrhotics: (1.5± 0.2 vs. 0.44± 0.03 μmol/L; $p<0.001$). Area under ROC curves (AUC) was used to determine the predictive value for ADMA, SDMA, Child-Pugh score, MELD, Discriminant Function >32, and a combined dimethylarginine score ("DAS"- ADMA + SDMA), respectively: AUC: 0.74, 0.78, 0.64, 0.59, 0.65 and 0.84. With a cut-off value of 1.5, sensitivity of the DAS score was 79%, with a specificity of 77% and a positive predictive value of 77.8%. The DAS score also proved the strongest predictor of 28 day mortality amongst the whole cohort of decompensated cirrhotics, compared to MELD and C-P: AUC: 0.89.

We confirm the observation of high 28 day mortality in AH, especially from end-organ dysfunction. Current predictors of outcome in AH, such as Discriminant Function, are largely used to determine intervention. In this study, we demonstrate the superior predictive value of dimethylarginines, which are independent of clinical assessment variables such as ascites and encephalopathy, and nutritionally dependent markers such as creatinine, used by other survival scoring systems. An early assessment of DAS may direct need for intervention, and be used to monitor the subsequent effects, in patients with AH.

P.15 Characterisation of the DDAH 1 haploinsufficient mouse: A causal role of elevated ADMA in the pathogenesis of cardiovascular disease.

Torondel, B., Nandi, M., Stidwill, R., Rudiger, A. and Leiper, J.
Division of Medicine, UCL, 5, University Street London WC1 E6JJ.

An association exists between elevated levels of the endogenous nitric oxide synthase (NOS) inhibitor asymmetric dimethylarginine (ADMA) and a number of cardiovascular diseases. However, to date, no studies have shown a causal relationship between elevated ADMA and cardiovascular pathology. Dimethylarginine dimethylaminohydrolase (DDAH) catalyses the hydrolysis of ADMA in vivo. In order to test the hypothesis that elevated ADMA levels are causal in the pathogenesis of cardiovascular disease we generated mice deficient in DDAH1 and studied their systemic and pulmonary circulations both in vivo and ex vivo. Mice were generated by targeted deletion of exon 1 of the DDAH1 gene. Homozygous deletion of DDAH1 was lethal in utero whilst heterozygous DDAH1 haploinsufficient mice were viable. DDAH1 protein, DDAH activity and ADMA levels in tissue and plasma were measured in both DDAH1 +/- mice and their wild type littermates. The vascular reactivity of isolated aortas and pulmonary arteries and the effects of 1mM L-arginine on acetylcholine induced relaxations were also assessed and in addition to histologicical examination of the pulmonary vasculature. *In vivo* studies were conducted in minimally anesthetized DDAH1+/- and wild type littermates. Mean arterial and right ventricular pressures were measured using a fluid filled catheter inserted into the right carotid artery or right ventricle respectively and cardiac output, heart rate and systemic vascular resistance were simultaneously measured using echocardiography. Heterozygous deletion of DDAH1 resulted in reduced DDAH1 protein and activity and elevated tissue and plasma ADMA levels. Endothelial dysfunction was observed in both systemic and pulmonary vessels from DDAH1 +/- mice ex vivo an effect that was fully reversible by L-arginine. *In vivo*, mean arterial blood pressure and systemic vascular resistance were increased whilst cardiac output and heart rate were decreased. Furthermore increased right ventricular pressures together with increased muscularisation of resistance arteries in the pulmonary vasculature were observed. This study has demonstrated that in DDAH1 +/- mice, elevated ADMA causes endothelial dysfunction and results in increases in both mean arterial and pulmonary pressures indicating that elevated ADMA may be causal in the pathogenesis of cardiovascular disease.

P.16 Potential therapeutic benefit of novel DDAH inhibitors for the treatment of endotoxemia

Nandi, M., Rossiter, S., Torondel, B., Maliki, M., Smith, C., Gill, H. Stidwill, R., Leiper, J. and Vallance, P.
Division of Medicine, UCL, 5, University Street London WC1 E6JJ.

During the onset of sepsis, the induction of inducible nitric oxide synthase (iNOS) and the subsequent generation of large amounts of nitric oxide (NO) is thought to be partly responsible for the marked hypotension ultimately leading to inadequate organ perfusion and cardiovascular collapse. A potential therapeutic target in sepsis is inhibition of excess NO generation, although to date, direct NOS inhibition has proved unsuccessful. Asymmetric dimethylarginine (ADMA) is an endogenously occurring competitive inhibitor of NOS. ADMA is subject to hydrolysis catalysed by the enzyme dimethylarginine dimethylaminohydrolase (DDAH). We therefore hypothesised that pharmacological inhibition of DDAH would raise endogenous ADMA levels and potentially inhibit the excess NO production observed during sepsis (endotoxemia). Novel inhibitors of DDAH were synthesised, co-crystallised with the enzyme and characterised using *in vitro* enzyme assays and *in vitro* and *in vivo* rat models of endotoxemia. Co-crystallisation revealed the binding mode of the novel inhibitor, L-291. Isolated enzyme studies demonstrated that L-291 caused a concentration dependent inhibition of DDAH activity whilst having no direct effect on NOS activity. *In vitro* functional studies using isolated rat aorta demonstrated that L-291 reversed the iNOS mediated dilatation produced by LPS treatment by 16% ± 4.4 at 100uM and 25%± 4.1 at 200uM. In anaesthetised rats, a bolus dose of LPS induced iNOS mediated vasodilatation resulting in a fall in blood pressure. Administration of L-291 in LPS treated rats elevated circulating ADMA levels when compared with saline control (2.48 ± 0.14uM vs. 1.47±0.21uM) and significantly attenuated this fall in blood pressure. We have generated a novel selective DDAH inhibitor, which elevates circulating ADMA levels *in vivo*. In a rat model of acute endotoxemia DDAH inhibition attenuates iNOS mediated hypotension and stabilises blood pressure.

P.19 Nitric oxide inhibition of terminal oxidase in *Escherichia coli*

Mason, M.G., Nicholls, P., Wilson, M.T., Poole, R.K. and Cooper, C.E.
Department of Biological Sciences, Essex University, Colchester, Essex CO4 3SQ.

Eukaryotic cytochrome *c* oxidase (cytochrome aa_3) is inhibited by nitric oxide (NO), which binds at the heme/copper binuclear centre, competes with oxygen at high turnover but inhibits non-competitively at low turnover (Mason *et al*., 2006. *Proc Natl Acad Sci U S A,* **103:** 708-13). Are these phenomena general or an adaptive pattern peculiar to the eukaryotic enzyme? *E. coli* expresses two terminal quinol oxidases. A cytochrome *bo* oxidase, structurally similar to eukaryotic cytochrome aa_3, is expressed in air, but under hypoxic conditions a cytochrome *bd* oxidase predominates. The oxygen uptake kinetics and NO sensitivities of the two quinol oxidases were therefore studied with membrane preparations from *E. coli* cytochrome bo^+bd^- and bd^+bo^- mutants.

Using high resolution respirometry with NADH as substrate, the *bo* oxidase showed a K_M (O_2) of 6 µM, whereas the kinetically higher affinity *bd* oxidase showed a K_M (O_2) of 0.27 µM. NO inhibited both types of quinol oxidase. Under equivalent conditions (TN ≈ 250 $e^- s^{-1}$ reaction centre^{-1} and 100µM O_2) the IC_{50} (50% inhibition by NO) values for *bo* and *bd* were 65 nM and 200 nM respectively. The inhibition of both enzymes was strictly competitive with O_2 at high turnover. The calculated K_d values for NO binding to reduced enzyme were then 0.55 nM for *bd* and 4.4 nM for *bo*. The lower NO sensitivity of the *bd* oxidase thus reflects its ‘affinity’ for O_2 despite tight NO binding. The NO complex of the *bd* oxidase also dissociates more rapidly than that of the *bo* enzyme (off rates of 0.16 and 0.03 sec^{-1} respectively). Under normoxic conditions the *bd* oxidase is thus less NO-sensitive than the *bo* oxidase and recovers from inhibition more rapidly, a possible functional advantage for this enzyme during environmental challenge by NO-generators such as nitrite. Unlike the eukaryotic cytochrome aa_3, neither bacterial enzyme has so far shown a non-competitive component in its pattern of response to NO. Further experiments under low turnover conditions will however be needed to confirm this.

P.21 Novel N-hydroxyguanidines and related compounds as NO-donors

[1]Kulczynska, A., [1]Zhang, Q., [1]Botting, N.P., [2]Milliken, P., [2]Mitchell, R., [2]Megson, I and [2]Webb, D.J.
[1]The University of St Andrews; [2]University of Edinburgh.

There is still a need for active NO donor drugs that are tissue selective to allow the delivery of the NO to the desired site of action. NO is endogenously produced from a two-step oxidation of L-arginine catalysed by nitric oxide synthase (NOS). The intermediate in the oxidation is N-hydroxyarginine (NHA). As a mimic to the natural process, analogues of NHA have been synthesised and screened as NOS substrates, such as N-homohydroxyarginine, N-norhydroxyarginine and many non-amino acid derived N-hydroguanidines (NHG). Although several NHGs were found to be NOS substrates, the potential of this type of compound as NO-donors in different tissues remains largely unexplored. In this work, we have synthesised a series of novel N-hydroxyguanidines as their HCl salts by nucleophilic addition of hydroxylamine to the corresponding cyanamides which were obtained from the condensation of primary or secondary amines with cyanogen bromide. Their vasodilatation activity has been tested by *in vitro* myography of rat aortic ring. A number of other compounds containing functional groups similar to the hydroxyguanidine group were also synthesised and tested as NO donors. These include hydroxamic acids, formamidines, amidoximes and oximes.

P.24 A stabilised intermediate observed during oxygen activation by nNOS

Papale, D., Miles, C.S. and Daff, S.
School of Chemistry, University of Edinburgh, West Mains Road, Edinburgh EH9 3JJ.

The mechanism of NO synthesis by mammalian NOS enzymes is a two-step process. L-arginine is first converted to N-hydroxy-L-arginine (NOHA), which is then converted to NO and citrulline. Both steps are thought to proceed via similar mechanisms in which the cofactor H4B activates heme-bound dioxygen by donating a single electron. Conversely, the guanidinium group of substrate stabilises bound dioxygen in the active site by hydrogen bonding. Events following the transfer of an electron from H4B to dioxygen are poorly understood; in particular, the mechanism of oxygen-atom insertion into the substrate is unclear. By analogy with cytochromes P450, a high-valent heme-iron oxo species (compound 1) may be formed. Alternatively, direct reaction between a heme-peroxy intermediate and substrate may occur. It may be that the two steps of NO synthesis occur via different mechanisms. Our attempts to modulate the oxygen-activation process in nNOS by site-directed mutagenesis have resulted in a mutant which binds substrate (L-Arg) 10-fold more tightly than the wild-type enzyme, perturbing the energetics of catalysis. Kinetics studies show that H4B still transfers an electron to heme-bound dioxygen at the same rate in the mutant, but that a stabilised reactive intermediate is then observed. Instead of rapidly forming product, the intermediate decays at a rate of 1.2 s-1. The visible spectrum of the intermediate resembles a high-valent heme iron oxo species and may be a glimpse of the oxygen insertion process.

P.25 Modulation of local nitric oxide effects on medium spiny neurons in the nucleus accumbens

Hartung, H., Tynan, P.W. and French, S.J.
Department of Pharmacology, Mansfield Road, Oxford, OX1 3QT, UK

Nitric oxide (NO) has been shown to be an important neuromodulator in the dorsal striatum as well as in other areas of the brain. The nucleus accumbens is the major part of the ventral striatum, whose function has been described as a motivation-action interface, and which plays a key role in addiction. In addition, due to its convergent innervation from brain areas associated with the limbic system, the nucleus accumbens function and dysfunction is of relevance to schizophrenia. The aim of our experiments is to explore whether pharmacological manipulation of NO can alter the firing rate and patterns of accumbens medium spiny neurons (MSN), and whether this effect is of particular importance for the processing of inputs from the prefrontal cortex (PFC). We therefore electrically stimulated the PFC of rats, and in the juxtacellular position, extracellularly recorded the firing response of single units in the accumbens to this stimulation. We then examined whether microiontophoresis of drugs that modifies the NO release, or availability, potentially alters the firing rate and pattern of the same single unit under conditions of PFC stimulation. Finally, we labelled the same MSN unit by passing a current down the neurobiotin containing recording electrode. Following perfusion with paraformaldehyde and glutaraldehyde the brain was sectioned and the neurobiotin revealed with an avidin-biotin reaction and diamethylbenzadine. The labelled neuron was examined for further characterization in the light and electron microscope. The results of this study suggest that neurons in the nucleus accumbens respond to PFC stimulation and that this response is further modulated by pharmacological manipulation of NO release or action. The recorded neurons filled with neurobiotin were identified as medium spiny neurons. The labelled neurons were traced in their entirety including their full dendritic tree and local axon arborisation and distal axonal targets.