1P INHIBITION OF ABSENCE SEIZURES IN RATS (GAERS) BY THALAMIC ADMINISTRATION OF A $GABA_{B1}$ ANTISENSE LIGAND

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Despite numerous reports indicating that functional subtypes of the metabotropic $GABA_B$ receptor exist (see Bowery et al. 2002) there are no agonists or antagonists that can reliably distinguish between possible subtypes. As we now have knowledge about the structures of the subunits that form the receptor heterodimer (see Marshall et al. 1999), it seemed appropriate to consider the use of antisense technology to define the nature of $GABA_B$ receptors associated with particular functions. In the first instance we have focussed on the genetic absence epilepsy rat from Strasbourg (GAERS) in which $GABA_B$ receptor antagonists administered bilaterally to the thalamus in vivo completely block the spontaneous spike and wave discharge activity which is manifest in these animals (Liu et al. 1992). We have chosen to use this model to develop the technique, as any reduction in the seizure activity produced by antisense administration would be immediately apparent in the awake animal. As we needed to administer the antisense continuously for prolonged periods we decided to adopt a reverse microdialysis procedure to prevent any change in tissue volume during the administration period. Male rats (GAERS) were implanted under isoflurane anaesthesia with two microdialysis probes, each equipped with a 2mm length of polyethersylfone (PES) membrane (100kDa cut-off CMA/Microdialysis), into the thalamus on either side. These were coupled via a bifurcation cannula to an Alzet osmotic minipump (flow rate 1µl h^{-1}) which was inserted subcutaneously in the same animal and contained the antisense or missense oligonucleotides or artificial cerebrospinal fluid (aCSF) as control. The choice of the PES membrane was based on in vitro transfer (70±2% n=3 ± s.e.mean) of phosphothiorate (PTO) end-capped 18-mer oligonucleotide (Whitehead et al. 2002). A bipolar twisted wire electrode was implanted into the frontal cortex at the same time and the animal was then allowed to recover. EEG recordings were taken 20-24h post surgery (day 0) and then for the following 3 days during which time the oligonucleotide (1mM 5'tACTGCACGCCGTTCTga 3'antisense $GABA_{B1}$ or 5'tCGCTCGATAGCTGCAtc3' missense) or aCSF (control) was perfusing the microdialysis probes continuously at 0.5µl h^{-1}. After 4 days the animals were sacrificed and the brain frozen rapidly in isopentane at -80°C for subsequent receptor membrane binding studies. $GABA_B$ and $GABA_A$ receptor binding on thalamic membranes was performed as previously described at a single concentration of [^{3}H]-GABA (50nM, Bowery et al. 1983). EEG activity was assessed over 30 min periods and the total duration of the seizure activity determined daily. The duration declined sequentially in the antisense-treated animals such that at day 3 the mean seizure duration time was 4.2±3.5s $30min^{-1}$ (mean±s.e.mean n=4 rats) which was significantly different ($p< 0.01$ Student unpaired T test) from the mean duration at day 0 in the same animals (428±80s) as well as from different rats administered missense, recorded at day 3(473±98s n=4). Preliminary studies on $GABA_B$ receptor binding in membranes prepared from the thalamus of antisense-treated rats showed a reduction of 63% compared with missense-treated rats. This contrasts with $GABA_A$ receptor binding in the same tissue which was the same after either treatment and not different from control. We conclude that $GABA_{B1}$ antisense oligonucleotide administered by reverse microdialysis can suppress $GABA_B$ receptor function in vivo and that this should facilitate determination of the nature of the functional receptor at different locations.

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2P ETHOSUXIMIDE HAS A POWERFUL ANTI-ABSENCE ACTION WHEN BILATERALLY INFUSED DIRECTLY INTO THE SOMATOSENSORY CORTEX (S1) OF THE GENETIC ABSENCE EPILEPSY RAT FROM STRASBOURG (GAERS)

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Ethosuximide (ETX) is used clinically for its selective effect on absence seizures. Generation of the spike and wave discharges (SWD) that characterise absence epilepsy requires functional connectivity of thalamocortical circuitry. It is widely believed that ETX exerts its therapeutic effect by reducing I_T (low voltage Ca^{2+} current) in the thalamus (Coulter et al., 1989), although more recent electrophysiological findings suggest a reduction of both the noninactivating Na^+ current and the Ca^{2+} activated K^+ current both in thalamic and cortical neurons are also involved (Crunelli and Leresche, 2002). We have recently shown that direct infusion of ETX into the ventrobasal thalamus (VB) or reticular thalamic nucleus (RTN) (Manning et al., 2002) or administration via microdialysis to a greater portion of the thalamus, did not elicit an immediate reduction in seizure activity, comparable to that observed after systemic administration. In view of recent evidence that SWD may be initiated in the somatosensory cortex (S1) (Meeren et al., 2002), the present study aimed to target the cortical component of the circuitry by directly infusing ETX into this region.

Male GAERS (384±16g) were anaesthetised with medetomidine/ketamine (0.5 & 75 mg/kg i.p. respectively) and implanted with a bipolar EEG electrode in the frontal cortex, inclined from the front at an angle of 20° from the vertical (AP, +3.2; L, -2.4; V, 2.8) and, bilaterally, with guide cannula into the somatosensory cortex (S1) (AP, -2.1; L, ±5.5; V, 4.0). The following day, the EEG signal was amplified, filtered and recorded (BioAmp ML-136, PowerLab 2/20, ADInstruments) for a 30min basal period. There were four experimental groups (n=at least 4 for each group); (a) ETX 10nmol/side infused directly into S1, (b) ETX 20nmol/side as in (a), CGP 36742, a $GABA_B$ antagonist (Manning et al, 2002), (27nmol/side) as in (a), and (d) vehicle (saline) as in (a). Following drug administration, EEG was recorded for a further 2 x 30 min (Post 1 and 2). SWD are expressed as the percentage of each 30 min period. Drug effects were assessed by one-way ANOVA, with post-hoc comparison to basal values using Dunnett's test when significant ($p<0.05$) differences were found.

Region	Conc. (nmol/side)	SWD % of basal (mean and s.e.mean)	
		Post 1	Post 2
S1	0.9% saline	88.3 ± 4.0	66.1 ± 4.6
S1	10	6.5 ± 2.2 **	8.1 ± 3.3 **
S1	20	3.4 ± 0.7 **	0.03 ± 0.01**
VB	20	80.9 ± 12.0	55.0 ± 9.7 **
RTN	20	69.1 ± 12.6	28.6 ± 9.2 **
systemic	100mg/kg	12.6 ± 7.3 **	1.8 ± 1.5 **

Table 1. Anti-absence action of bilateral infusion of ETX (10 and 20nmol/side) into the somatosensory cortex (S1) (this study), compared to infusion of 20nmol/side into the VB and RTN and systemic administration, (shaded, Manning 2002). (** $p<0.01$)

Cortical infusion of ETX at 20nmol/side and 10nmol/side (data not shown) produced an immediate and substantial cessation of seizures, comparable to that seen with systemic administration. In addition, CGP36742 also produced cessation of seizure activity (data not shown), as previously seen in the thalamus, suggesting $GABA_B$ receptor involvement within this area.

These data add further evidence to support the idea that although generalised in form, absence epilepsy is originally of focal origin and the initiation site lies within the somatosensory cortex (S1).

Further work to investigate whether other cortical areas show similar properties is underway.

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3P EVIDENCE THAT 5-HT_2 RECEPTOR AGONIST INDUCED INHIBTION OF 5-HT CELL FIRING IS MEDIATED BY CENTRAL AND NOT PERIPHERAL 5-HT_2 RECEPTORS

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Systemic administration of phenethylamine-derived 5-HT_2 agonists such as DOI ((±)-2,5-dimethoxy-4-iodoamphetamine) inhibits the firing of 5-HT neurones in the dorsal raphe nucleus (DRN) of the anaesthetised rat (Aghajanian *et al.*, 1970; Garratt *et al.*, 1991). Both 5-HT_{2A} and 5-$HT_{2B/C}$ receptor subtypes are involved in this effect, and a central mechanism is postulated (Boothman *et al.*, 2001). However, a role for peripheral 5-HT_2 receptors has also been suggested as an increase in blood pressure occurs together with the fall in 5-HT cell firing (Penington *et al.*, 1986). Here the peripheral 5-HT_2 receptor antagonist BW 501C67 (Fuller *et al.*, 1986) and the brain-penetrant 5-HT_{2A} receptor antagonist MDL 100,907, were used to test if the DOI-induced inhibition of DRN 5-HT neuronal activity is peripherally or centrally mediated.

Male Sprague-Dawley rats (240-320 g) were anaesthetised by chloral hydrate supplemented with saffan. Extracellular recordings were made using single barrel glass electrodes (filled with 2 M NaCl and 2 % pontamine sky blue; 10-16 MΩ). 5-HT neurones were identified on the basis of their firing characteristics, the inhibitory effect of 8-OH-DPAT, and the DRN location of the recording site (Boothman *et al.*, 2001). In separate experiments blood pressure was recorded from a carotid artery. Rats were injected with DOI (increasing doses of 10-100 μg kg^{-1}) or saline (0.1 ml kg^{-1}) given via a tail vein at 2 min intervals. BW 501C67 (0.1 mg kg^{-1}) or MDL 100,907 (0.2 mg kg^{-1}) was injected 5 min prior to DOI. Firing rate (n=8 rats / group) or mean arterial pressure (MAP) (n=3-5 rats / group) was measured in the final 1 min of each post-drug interval. Data were analysed using 1 or 2 way ANOVA with appropriate post hoc tests.

DOI caused a dose-related inhibition of 5-HT cell firing ($P<0.001$ versus pre-drug values), an effect commencing at 20 μg kg^{-1} and greatest (>80%) at 100 μg kg^{-1}. BW 501C67 pre-treatment (0.1 mg kg^{-1} i.v.) did not alter this effect ($P>0.05$ versus DOI alone), however it was attenuated by MDL 100,907 (0.2 mg kg^{-1}) ($P<0.001$ versus DOI alone). DOI increased MAP (80-100 μg kg^{-1}, maximum increase 10 mm Hg; $P<0.001$ versus saline) and this effect was completely blocked by pre-treatment with BW 501C67 (0.1 mg kg^{-1} i.v.) ($P<0.001$ versus DOI alone).

In summary, these data show that the DOI-induced inhibition of DRN 5-HT neurone firing was unaffected by pre-treatment with the peripheral 5-HT_2 receptor antagonist BW 501C67, but attenuated by the brain-penetrant 5-HT_{2A} receptor antagonist MDL 100,907. BW 501C67 blocked the rise in MAP caused by DOI. These data suggest that the inhibition of DRN 5-HT neurones by DOI is a result of the activation of central, and not peripheral, 5-HT_2 receptors. Investigation of the central substrate(s) underlying the effect of phenethylamines on 5-HT neuronal activity is ongoing.

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4P EVIDENCE THAT CENTRAL 5-HT_7 RECEPTORS ARE INVOLVED IN THE CONTROL OF MICTURITION IN URETHANE ANAESTHETIZED RATS

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Central 5-HT_{1A} receptors have been demonstrated to play an excitatory role in the control of the micturition reflex in anaesthetized rats (Conley *et al.*, 2001). Recently it has been suggested that central 5-HT_{1A} and 5-HT_7 receptors may interact (Thomas *et al.*, 1999). Hence, the present experiments investigate a role for 5-HT_7 receptors in the control of micturition by studying the effect of SB-269970, (R)-3-(2-(2-(4-Methylpiperidin-1-yl)-ethyl)pyrrolidine-1-sulfonyl)phenol (Lovell et al., 1999) and SB-656104 (6-((R)-2-{2-[4-(4-Chloro-phenoxy)-piperidin-1-yl]-ethyl}-pyrrolidine-1-sulfonyl)-1H-indole hydrochloride; pA_2 8.4 at the human cloned 5-HT_7 receptor, Thomas et al., 2003) , two structurally different 5-HT_7 receptor antagonists, on the micturition reflex.

Experiments were carried out in spontaneously-breathing female Sprague Dawley rats (200-270g) anaesthetized with urethane (1.2 g kg^{-1}; i.v.). Animals were maintained between 37-38 °C. "Micturition reflexes" were evoked by distension of the urinary bladder with saline infusion (0. 1 ml min^{-1}) through a cuffed cannula inserted into the bladder dome from which bladder pressure was monitored. Effects on urethra were studied as previously described (Wibberley *et al.*, 2002). Drug evoked changes, 3 min after administration, were compared with vehicle controls by unpaired Student's *t*-test and one-way ANOVA. All values are means ± s.e. mean.

SB-269970 (5 μl, i.c.v., n=5-6) 10 & 30 μg kg^{-1} significantly ($P<0.05$) increased bladder pressure threshold (7 ± 1 to 15 ± 2 and 8 ± 1 to 17 ± 2 mmHg, respectively), volume threshold (0.7 ± 0.04 to 1.0 ± 0.05 and 0.5 ± 0.07 to 1 ± 0.1 ml, respectively). Residual volume significantly increased at 30 μg kg^{-1} (0.3 ± 0.05 to 0.8 ± 0.2 ml). At 100 μg kg^{-1} there was a significant reduction in peak bladder pressure during micturition (23 ± 1 to 16 ± 1 mmHg) the increase in volume threshold and residual volume was similar to that at 30 μg kg^{-1} (0.4 ± 0.03 to 0.8 ± 0.1 ml and 0.1 ± 0.02 to 0.7 ± 0.1 ml, respectively). No significant effects were observed on reflex evoked urethral relaxation and urethral sphincter EMG. SB-269970 given intrathecally at L6-S1 (5 μl, 30 μg kg^{-1}, n=4) had no significant effect on any of the variables as did 10 μg kg^{-1} given i.v. SB-656104 (n=5) i.c.v. at 30 μg kg^{-1} had a similar effect to SB-269970 causing a significant increase in bladder pressure threshold (5 ± 0.7 to 11 ± 3 mmHg), volume threshold (0.6 ± 0.1 to 0.9 ± 0.2 ml) and residual volume (0.1 ± 0.03 to 0.6 ± 0.2 ml).

These data demonstrate that 5-HT_7 receptors located in the central nervous system control micturition in anaesthetized rats, in a similar way to that of 5-HT_{1A} receptors. However this only occurs at supraspinal levels.

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K.E Read is a BBSRC CASE student with GSK.

5P PHOSPHORYLATION OF THE HUMAN 5-HT$_{7(b)}$ RECEPTOR

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The 5-HT$_7$ receptor is expressed in both central and peripheral tissues, where for example, it modulates neuronal excitability and mediates vasodilatation, respectively (Barnes and Sharp, 1999). The amino acid sequence of the cloned 5-HT$_7$ receptor predicts seven putative transmembrane-spanning segments, a characteristic associated with the G-protein coupled receptor superfamily (Hoyer *et al.*, 1994). There are four known mammalian splice variants (5-HT$_{7(a-d)}$), which differ in their intracellular C-terminal domain (Heidmann *et al.*, 1997), all of which are positively coupled to adenylate cyclase.

Sequence analysis has identified a number of consensus sequences for post-translational modification of putative cytoplasmic domains of the 5-HT$_7$ receptor by phosphorylation. Interestingly, the number of potential phosphorylation sites differs between alternatively spliced variants. This may therefore, represent an important mechanism by which receptor function is differentially modulated. This study aimed to demonstrate phosphorylation of the large intracellular loop and the C-terminal domain of the 5-HT$_{7(b)}$ receptor (GenBank accession number NM_019860) mediated by PKA, PKC and CaMKII.cDNA sequences coding for the cytoplasmic loop (loop: Q261-T328) and C-terminal (cterm: N380-STOP) domains of h5-HT$_{7(b)}$ were cloned into the bacterial expression vector pGEX4-T3. Expression of glutathione-S-transferase (GST) fusion proteins, as well as GST alone, were induced in *E. coli* (strain BL21) in the presence of isopropyl-β-D- thiogalactosidase (IPTG: 0.5mM, 4 hours, 37°C). Purified fusion proteins, bound to glutathione agarose, were labelled with γ[^{32}P] ATP, using a standard *in vitro* kinase assay (MacDonald and Moss, 1997). Proteins were resolved by SDS-PAGE, and identified by staining with coomassie blue. Radiolabelled ([^{32}P] phosphorylated) proteins were identified by standard autoradiography of stained gels.

As expected, GST alone was detected at a relative molecular weight of 26kDa, whilst the GST-loop and GST-cterm fusion proteins were detected at ~34kDa and 32kDa, respectively. Autoradiography revealed that the C-terminal domain was phosphorylated by both PKA and CaMKII. Neither enzyme phosphorylated the intracellular loop. In addition, we have some evidence to suggest that PKC phosphorylates both the large cytoplasmic loop and the C-terminal domain. Phosphorylation of GST alone was not detected in the presence of either PKA, PKC or CaMKII.

Our current investigations focus on identifying the specific residues that are phosphorylated using phospho-amino acid analysis and site-directed mutagenesis, and also to determine the functional consequences of these post-translational modifications.

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6P PCPA MODULATES THE EXPRESSION OF THE IMMEDIATE EARLY GENE ARC INDUCED BY MDMA IN RAT BRAIN

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Arc (activity-regulated cytoskeleton-associated gene) is an effector immediate early gene whose mRNA is selectively localised in neuronal dendrites. Its expression is induced by neuronal excitation and by stimulation of 5-HT$_{2A}$ and D$_1$ receptors. MDMA (3,4-methylenedioxymethamphetamine) induces the release of monoamines, principally 5-HT and dopamine. Blockade of the 5-HT transporter inhibits the induction of 5-HT release and subsequent Arc expression by MDMA (Berger *et al.*, 1992, Aston *et al*, 2002). In this study we have investigated the effects of the tryptophan hydroxylase inhibitor para-chlorophenylalanine (PCPA) and consequent 5-HT depletion on the induction of Arc by MDMA.

Male Dark Agouti rats (190-210g) were pretreated with saline or PCPA (300mg/kg/day for 2 days then 150 mg/kg given on alternate days for 4 days). 24h after the last dose rats were injected with saline (1ml/kg, i.p.) or MDMA 6mg/kg (n=3-5 per group) and killed 2 hours later. Arc mRNA expression was analysed by in situ hybridisation histochemistry using [^{35}S]-dATP labelled oligonucleotide probe as described previously (Pei *et al.*, 2000). Statistical analysis of the results was made by one- and two-way ANOVA and Newman-Keuls post-hoc test.

MDMA induced a significant increase in Arc mRNA expression (range 216-539%, $p<0.001$) in saline pretreated rats in cingulate, orbital and insular frontal cortex, striatum and parietal cortex compared to control. Following PCPA pre-treatment, Arc expression was again increased by MDMA in these brain regions. However, in insular frontal cortex, this increase was significantly less than that in saline pretreated rats (38% lower, $p<0.05$, Fig.1), although not in orbital or cingulate cortex. In parietal cortex, MDMA induced a significantly greater increase in expression in PCPA treated (151% greater, $p<0.001$, Fig. 1) than saline treated rats. In hippocampus CA1, MDMA induced a significant increase in Arc expression in PCPA treated (+119%, $p<0.001$) but not in saline-pretreated rats.

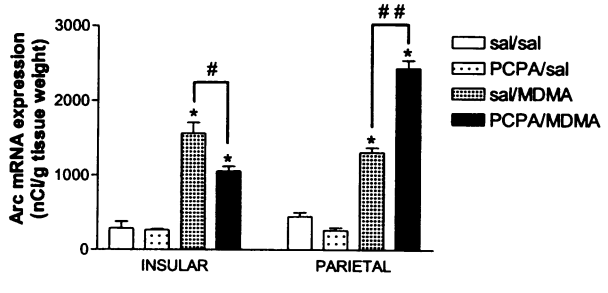

Fig. 1. Effect of PCPA pretreatment on Arc expression induced by MDMA
*$p<0.01$ compared to sal/sal controls

#$p<0.05$, # #$p<0.001$ compared to sal/MDMA group

The decrease in response to MDMA in the insular cortex following PCPA pre-treatment and its consequent depletion of 5-HT suggests that Arc induction here is predominantly mediated by 5-HT. However, the increased response to MDMA in CA1 and parietal cortex after PCPA indicates significant regional variability in the mechanism of neuronal activation by MDMA and may implicate the involvement of other neurotransmitters.

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7P EFFECT OF ACUTE AND CHRONIC NICOTINE ON LIPOGENESIS IN MOUSE ADIPOSE TISSUE

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Smoking cessation is widely believed to increase appetite and induce weight gain (Williamson *et al.*, 1991). In rodents, chronic nicotine produces hypophagia and weight loss (e.g. Winders & Grunberg, 1990). Recent studies have shown that nicotine induces uncoupling protein in white fat in obese mice (Yoshida *et al.*, 1999), suggesting a peripheral effect to increase energy expenditure. To determine whether nicotine alters adipose tissue thermogenesis we have studied acute and chronic nicotine on brown and white adipose tissue lipogenesis *in vivo*.

Adult male CBA/Ca mice (32 - 38 g) housed 4 to a cage were given nicotine or saline acutely by i.p. injection. Chronically, nicotine was added to the drinking water (0.075 mM as tartrate) to provide an average daily dose of 1.65 mg nicotine base per kg body weight over 4-5 weeks. Food and water intake were measured every 48 h (3-4 cages per treatment group). *In vivo* lipogenesis was measured using ^3H water as before (Williams *et al.*, 1999). Data are shown as means ± s.e.mean (n) from groups of mice (lipogenesis) or cages (food intake). The statistical significance of differences was measured by paired or unpaired t tests or by 2-way ANOVA .

Preliminary studies showed that acute nicotine (0.35-2.4 mg kg^{-1}) produced a dose dependent increase in brown fat lipogenesis but had no effect in white fat. The maximum increase in lipogenesis (expressed as mg atoms H h^{-1} mg $tissue^{-1}$) to 95.2 ± 19.5 (8) compared to 42.0 ± 7.1(7) in controls, was seen with 1.2 mg kg^{-1}. At doses above 2.5 mg there were signs of parasympathetic stimulation. The presence of nicotine did not affect the water intake. The effects of chronic nicotine are summarized in Table 1, shown as pre-treatment (day 0) and post-treatment (day 35) values

Table 1

	Body weight (g)	Food (g kg^{-1} day^{-1})	White fat lipogenesis	Brown fat lipogenesis
Day 0	34.1 ± 0.92	139.5 ±5.4	12.0 ± 1.4	56.2 ± 6.0
Control	(12)	(3)	(8)	(8)
Day 35	**38.7±0.9	155.7 ±7.8	13.5 ±3.9	46.8 ±5.1
Control	(12)	(3)	(6)	(6)
Day 0	36.9 ± 0.8	132.0 ±2.8	11.6 ±1.3	49.4 ± 5.6
Nicotine	(16)	(4)	(8)	(8)
Day 35	**42.6±0.9	148.8 ±7.0	13.8 ± 3.2	*70.5 ±5.6
Nicotine	(16)	(4)	(8)	(8)

$p < 0.05$,** $p < 0.01$, compared to respective day 0 control

We conclude that nicotine can stimulate brown fat lipogenesis, which may contribute to increased energy expenditure, but that tolerance occurs by 4 weeks and weight gain and food intake are not significantly affected.

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8P DOES ANANDAMIDE INCREASE MICROVASCULAR PERMEABILITY IN RAT PAWS BY RELEASING ENDOGENOUS SUBSTANCE P?

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The neuropeptide tachykinin, substance P (SP), increases microvascular permeability in a range of tissues. Endogenous SP is released from sensory neurons by electrical activation or in the presence of a variety of releasing agents including the vanilloid agonists, capsaicin and resiniferatoxin (Snijdelaar et al, 2000) and the protease activated receptor-2 agonist, trypsin (Steinhoff *et al*, 1999). The endocannabinoid, anandamide, has been shown to release the neuropeptide calcitonin gene-related peptide (Zygmunt *et al*, 2000) and, since this peptide is co-localised with SP, anandamide may also act to release SP. We have therefore assessed the effects of anandamide on microvascular permeability in paw skin of the normal (N) Wistar rat and of the GH inbred Wistar strain that is known to exhibit reduced inflammatory responsiveness to exogenous SP due to reduced numbers of NK_1 receptors (Campbell *et al*, 2000).

Rats of the GH and N strains were anaesthetised with urethane (1.25 g/kg, i.p.). The right external jugular vein was cannulated and Evans Blue dye (30mg/kg) was given i.v., followed by intraplantar injections (0.1ml) of SP, 0.5μg; resiniferatoxin, 0.3μg; trypsin, 2.5μg: or anandamide, 400μg to one hind paw and the vehicle (saline or, for anandamide only, ethanol: Tween80: saline, 1:1:8 v/v) to the other paw (n= 5 for each treatment). Thirty minutes later, the animals were exsanguinated, the paws removed, weighed and the extravasated dye extracted with formamide (3ml, 16h at 37°). Extracted dye was assayed photometrically (620nm), using standard concentrations of Evans Blue in formamide.

In N rats, all releasing stimuli gave dye extravasation values greater than those after vehicle demonstrating an increase in microvascular permeability (mean± s.e.mean ng dye/mg tissue: SP, 45.3±9.8; resininferatoxin, 10.9±1.8; trypsin 16±1.4; anandamide, 31.3±2.5:vehicles - saline, 3.2±1.1; ethanol: Tween80: saline, 13.8±1.4; $P<0.05$, Welch's *t*-test),. In GH rats, at the same doses, these stimuli induced less increase in microvascular permeability (SP 19±2.3; trypsin, 7.7±0.5: anandamide, 25.1±0.9; resiniferatoxin, 4.7±0.8 ng dye/mg tissue). Pre-treatment (30 min) of N rats with the NK1 antagonist, SR 140333 (360 nmol/kg; iv;) to block SP receptors prevented dye extravasation induced by anandamide (6.7±0.9 ng dye/mg tissue).

These results indicate that anandamide is a pro-inflammatory agent in skin *in vivo*. The interstrain difference in response to anandamide and the antagonism by SR 140333 strongly suggest that anandamide acts by releasing SP from cutaneous sensory neurons. Further analysis of the pro-inflammatory action of anandamide is warranted.

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9P A ROLE FOR NK_1 RECEPTORS IN TNFα-INDUCED NEUTROPHIL ACCUMULATION IN MOUSE SKIN

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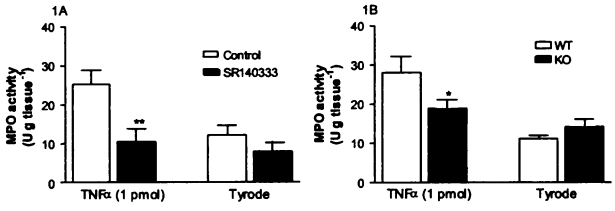
We have previously demonstrated that tachykinin NK_1 receptors play a pivotal role in mediating neutrophil accumulation in inflamed skin, but that substance P alone has no effect on neutrophil accumulation in naïve skin (Cao *et al.*, 2000). We have furthered our investigations by carrying out experiments with cytokines in order to try and learn more about the involvement of the NK_1 receptor in neutrophil accumulation.

Male and female mice (Sv129 + C57BL/6; 25-30 g), either WT or KO were anaesthetized with urethane (25% w/v; 100 μl 10 g^{-1}; i.p.) or (isoflurane:oxygen flow, 2.5%:2.5% by inhalation). The responses to intradermal injection (i.d.) of cytokines (tumor necrosis factor TNFα, IL-6 and IL-12) were assessed in the shaved dorsal skin. Plasma extravasation was measured by the extravascular accumulation of ^{125}I-albumin after 30 min and neutrophil accumulation by myeloperoxidase (MPO) assay after 4 or 8 h. Mice were killed by cervical dislocation and the skin sites were removed. Either the radioactivity was measured in skin and plasma or MPO activity evaluated. Data are mean ± s.e.mean. Statistical test was by ANOVA plus Bonferroni's modified t-test.

Unlike substance P (100 pmol $site^{-1}$; 78 ± 4.9 μl g^{-1}), i.d. injection of cytokines (TNFα, IL-6 and IL-12; 0.3 - 3 pmol $site^{-1}$; n = 5) did not produce plasma extravasation in the skin of WT mice. TNFα (n = 6), but not substance P, IL-6 or IL-12 induced neutrophil accumulation in WT skin after 4 h i.d. injection. The NK_1 receptor antagonist SR140333 (120 nmol kg^{-1}, i.v., n = 9) substantially inhibited TNFα-induced neutrophil accumulation (Fig. 1A). Neutrophil accumulation in response to TNFα (1 pmol $site^{-1}$, n = 8) was also significantly reduced in KO mice after 4h i.d. injection (Fig. 1B). After 8 h, the neutrophil accumulation in response to TNFα was not significantly different from vehicle in WT mice (not shown).

Figure 1. Effect of TNFα-induced neutrophil accumulation in SR140333- (panel A) and KO groups (panel B). Mean ± s.e.mean. *P<0.05 or **P<0.01 compared to control.



These data suggest that new strategies involving regulation of endogenous tachykinins, their receptors and antagonists may be involved in the signalling of TNFα. The functional consequences in inflammatory conditions is at present unknown.

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10P THE CONTRIBUTION OF PLASMA EXTRAVSATION IN A MODEL OF MURINE JOINT INFLAMMATION

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Arthritis is a debilitating condition characterised by a chronic inflammation of the joint. In the present study, we are interested in microvascular changes that occur in the joint and have used an assay recently developed in our laboratory to investigate the relationship between microvascular plasma extravasation, blood flow and joint swelling in mice.

Female, CD1 mice (Harlan, UK; 25–30 g) were given intra-articular injections of Complete Freund's Adjuvant (CFA; 20 μg, 20 μl) and saline (contralateral joint; 20 μl) under isoflurane anaesthesia (2%; 2% O_2). Inflammation developed for 1–48 h. Mice were anaesthetised 1 h prior to the end of the experimental period and ^{125}I-albumin was administered i.v. to enable a measure of ongoing plasma extravasation. Plasma extravasation is expressed as a ratio of counts/min detected in the CFA- compared to saline-treated joint (see Table 1).

To determine whether a proportion of the ^{125}I-albumin measurement was due to increased intravascular volume/blood flow, ^{125}I-albumin was injected i.v. at the end of the time course (48 h) in some experiments. The ratio of counts/min detected in the CFA- compared to the saline-treated joint (1.18 ± 0.04) was significantly greater than 1 (one sample t test, p<0.05, n = 6), suggesting that an increase in intravascular volume/blood flow did occur. However, ^{125}I-albumin accumulation measured at 47–48 h was significantly greater (1.40 ± 0.07, p<0.05), suggesting that significant plasma extravasation takes place.

To learn more of the contribution of plasma extravasation towards joint swelling, knee diameter was determined at all time points. Results are expressed as a ratio of knee diameter of the CFA- compared to the saline-treated joint (see Table 1).

In conclusion, significant in plasma extravasation occurs in the CFA-treated mouse knee joint between 1 and 18 h and might cause the concomitant increase in joint swelling. However, the level of plasma extravasation observed thereafter (18 – 48 h) was possibly sufficient to maintain knee diameter but not to increase it further. Further experiments are required to investigate the mechanisms underlying plasma extravasation and joint swelling in the inflamed mouse knee joint.

Table 1. The time course of plasma extravasation and knee swelling in the mouse knee joint. *=p<0.05, **=p<0.01, ***=p<0.001 compared to ratio of 1 (one sample t test); #=p<0.05, ###=p<0.001 compared to at 1 h (ANOVA). Results are mean ± s.e.mean, n = 5–7.

Time (h)	Plasma extravasation	Knee swelling
1	$1.12 \pm 0.04^{*}$	1.01 ± 0.01
3	1.23 ± 0.10	1.03 ± 0.01
5	$1.29 \pm 0.07^{*}$	1.05 ± 0.02
18	$2.03 \pm 0.30^{***/\#\#\#}$	$1.09 \pm 0.02^{***/\#}$
24	$1.85 \pm 0.10^{***/\#\#\#}$	$1.12 \pm 0.01^{***/\#\#\#}$
48	$1.40 \pm 0.07^{**}$	$1.12 \pm 0.02^{***/\#\#\#}$

This work was supported by the Arthritis Research Campaign

11P ANTI-INFLAMMATORY DRUGS AND RHEUMATOID ARTHRITIS: A ROLE FOR TNF-ALPHA AND NF-KB

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Blockade of Tumor Necrosis Factor- alpha (TNF-α) release as well as inhibition of NF-kB activity represent relevant targets in rheumatoid arthritis (RA) therapy [1,2]. Anti-inflammatory drugs, which are largely used in the first stages of RA, could exert some of their effects independent of prostaglandin synthesis inhibition, *via* modulation of transcription factors such as NF-kB [3].
Here we evaluated the ability of dexamethasone (a steroid drug), indomethacin (a non selective COX inhibitor) and rofecoxib (a selective COX-2 inhibitor) to modulate *in vitro* NF-kB activity and TNF-α release from blood mononuclear cells from healthy donors. As TNF-α is released by monocytes and lymphocytes, we evaluated the effects of selected drugs on monocytes alone (M), lymphocytes alone (L) or the mixed monocyte-lymphocyte population (M+L). Cells were cultured in 6-well plates and challenged, in the presence or absence of anti-inflammatory drugs, with lipopolysaccharide (LPS) 10 ng/ml or phorbol myristate acetate (PMA) 10^{-7} –10^{-6}M. Both stimuli induced TNF-α secretion and NF-kB activation from all the cells evaluated. TNF-α release was measured by ELISA; nuclear translocation of NF-kB was evaluated by electrophoretic mobility shift assay (EMSA).
As expected, dexamethasone (10^{-11}–10^{-5}M) potently inhibited TNF-α release from LPS- or PMA-stimulated cells, with IC_{50} values in nanomolar range. The inhibition was higher in L (92 ± 4%, n= 5; IC_{50} = 0.5 nM) challenged with PMA as compared to M (82 ±3% inhibition; IC_{50} = 3.8 nM) and M+L (70 ± 2%; $p < 0.05$; IC_{50} = 10 nM). In LPS-stimulated cells, maximal inhibition afforded by dexamethasone was about 90% and IC_{50}s were very close (0.7 –1.2 nM) in the three cell populations. Indomethacin dose-dependently inhibited PMA-evoked TNF-α release in M (IC_{50} = 4.6 μM) and was less active in L and M+L, but was devoid of activity in LPS-stimulated cells. The COX-2 selective inhibitor rofecoxib did not affect PMA- or LPS-evoked TNF-α release.

In EMSA studies, dexamethasone potently inhibited (about 60% at 10^{-5} M) NF-kB nuclear translocation in both LPS- and PMA-challenged monocytes. Indomethacin failed to inhibit LPS-induced NF-kB nuclear migration, while exerted some effects (about 15% inhibition at 10^{-4}M) on PMA-challenged monocytes. On the contrary, micromolar concentrations of rofecoxib potently (> 60%) inhibited PMA-induced NF-kB activation, being less effective (about 25% inhibition) against LPS.

These results indicate that anti-inflammatory drugs differ largely on their ability to inhibit TNF-α release and NF-kB activation from human mononuclear cells, these adjunctive pharmacological property contributing to their clinical effectiveness in RA.

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12P MELANOCORTIN AGONISTS POSSESS ANTI-INFLAMMATORY EFFECTS IN MICE WITH A NON-FUNCTIONAL MC1-R (RECESSIVE YELLOW E/E)

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Out of five melanocortin receptors (MC-R) so far cloned, two of them, MC1 and MC3-R, have been proposed to be responsible for the long known anti-inflammatory actions of melanocortin peptides (e.g. adrenocorticotrophin and melanocyte-stimulating hormones, MSH) (Getting, 2002). A pharmacological and genetic approach has been here applied, taking advantage of the availability of more selective agonists and antagonists and of a mouse strain with a non-functional MC1-R, termed recessive yellow (e/e).

In vivo. Male recessive yellow (e/e) and C57 wild type mice were treated with: 1) the non-selective agonist α-MSH (10 μg s.c.); 2) the selective MC3-R agonists γ_2-MSH (30 μg s.c.) and MTII (10 μg s.c.); 3) the selective MC1-R agonist MS05 (1-100 μg s.c.) or PBS (100 μl s.c.) 30 min prior to MSU crystals (3 mg i.p.). In some cases, the mixed MC3/4-R antagonist SHU9119 (10 μg i.p.) (Fan *et al.*, 1997) was also co-administered. In all cases, PMN migration and cytokine levels in lavage fluids were then determined 6 h later.
In vitro. Peritoneal macrophages (MØ) were cultured in 6-well plates and treated with γ_2-MSH (95 μM) in the presence or absence of 9 μM SHU9119, prior to stimulation with 1 mg/ml MSU crystals. IL-1β release was measured by ELISA. For electron microscopy analysis, $5x10^6$ MØ were stained with an anti-MC3-R antibody (sc-6878, 1:200 final dilution) prior to light counterstaining with uranyl acetate and lead citrate, and examined with a JEOL 1010 electron microscope. Cyclic-AMP (cAMP) accumulation into adherent peritoneal MØ was measured by EIA (Amersham, UK). MC3-R mRNA was detected by RT-PCR using primers and procedure already described (Getting *et al.*, 1999). Data (mean ± s.e.mean) were analysed by ANOVA and Bonferroni test with $P<0.05$ taken as

MSU crystals provoked an intense PMN accumulation at 6 h with similar values being measured in both wild type and recessive yellow (e/e) mice (6.65 ± 0.37 and 5.80 ± 0.34 $x10^6$ PMN per mouse, respectively, n=6). Systemic treatment of mice with γ_2-MSH and MTII caused an approximate reduction in PMN migration by 50% in both wild type and recessive yellow (e/e) mice (n=6, $P<0.05$ in either strain). This inhibition of cell influx was associated with significant reductions in IL-1β levels as measured in cell-free lavage fluids. In recessive yellow (e/e) mice, MTII caused a reduction from 73.7 ± 8.2 pg/ml to 27.9 ± 6.1 ($P<0.05$, n=6). Treatment of these mice with γ_2-MSH reduced cytokine levels by 40% ($P<0.05$, n=6). These figures are in line with those obtained in wild type mice (Getting *et al.*, 2001). The selective MC1-R agonist MS05 failed to inhibit any of the parameters measured in either wild type or recessive yellow (e/e) mice. The anti-inflammatory actions of MTII and γ_2-MSH were absent when mice received SHU9119. *In vitro*, MSU crystal-induced IL-1β release from adherent MØ was inhibited by γ_2-MSH equally in wild type and recessive yellow (e/e) mice (~50-60%, n=3, $P<0.05$). This inhibitory action was abrogated by SHU9119. Finally, mouse peritoneal MØ expressed MC3-R mRNA and protein, as detected by RT-PCR and electron microscopy in recessive yellow (e/e) mice, with similar expression in either strain. MC3-R activation with MTII and γ_2-MSH, but not MS05, led to cAMP accumulation, an effect again sensitive to SHU9119.

In conclusion, we propose that an intact MC1-R is superfluous to bring about melanocortin peptides anti-inflammatory effects *in vitro* and *in vivo*, and rather indicate MC3-R as the relevant target for these actions.

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13P ALTERATIONS IN THE ANNEXIN 1 PATHWAY ARE ASSOCIATED WITH MODIFIED NEUTROPHIL ACTIVATION

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The existence of a functional interaction between annexin 1 (ANXA1) and the receptor for formylated peptides (FPR) on human neutrophils has recently been reported (Walther *et al.*, 2000). Using conventional gene targeting technology we have raised a mouse strain that lacks the ANXA1 gene (Hannon *et al.*, 2002). With this tool, in conjunction with the FPR KO mice recently generated (Gao *et al.*, 1999), we have investigated the role of this protein/receptor axis in two assays of neutrophil (NØ) activation.

Male or female wild type (WT), FPR KO and ANXA1 KO mice (25-30 g) were anaesthetised with 3% halothane and blood collected by cardiac puncture. Plasma membrane CD11b expression on neutrophil was measured using a whole blood protocol (200 µl aliquots) and activation with platelet activating factor (PAF; 0.3-3 µM) or formyl-peptide (fMLP; 1-10 µM). Conventional flow cytometry analysis was performed after staining with a rat anti-mouse CD11b mAb (clone 5C6; 5 $\mu g ml^{-1}$, 1h at 4°C). In some experiments an ANXA1 mimetic, peptide Ac2-26, was tested. Purified murine NØ were isolated using standard magnetic cell sorting technique (Cotter *et al.*, 2001). Isolated NØ (4 $x10^6$ ml in 25 µl) were placed on the upper chamber of a 96 well ChemoTx plate (3 µm pore; Neuroprobe Inc) with 27 µl of PAF (10-0.01 nM) or peptide Ac2-26 (30-100 µg/ml) in the lower well. NØ movement into the bottom chamber was assessed after 2 h. Data is expressed as migration index (MI) calculated vs. migration to vehicle alone. Data are mean ± s.e.mean of n experiments, and statistical differences were analysed by Mann-Whitney U test ($P<0.05$ was taken as significant).

Basal CD11b expression in blood NØ was different among the strains, with values of 54 ± 5 and 99 ± 7 mean fluorescence units (MFI) for FPR KO and WT mice, respectively (n=6, $P<0.05$). PAF increased CD11b levels in all phenotypes, with a more pronounced effect in the KO animals relative to their WT counterparts: values of 110 ± 4 and 158 ± 11 in WT and FPR KO mice, respectively (n=4; $P<0.05$ *vs.* WT). Similar findings were obtained with fMLP in the ANXA1 KO mice (not shown). On cells taken from either FPR KO or WT mice, peptide Ac2-26 (200 $\mu g ml^{-1}$) was inactive by itself, but it significantly reduced fMLP-induced CD11b up-regulation. In the chemotaxis assay, NØ taken from WT and ANXA1 KO had similar basal locomotion, however the latter cells displayed higher migration index to 1 nM PAF (MI: 3.7 ± 0.2 in ANXA1 KO *vs.* 2.3 ± 0.1 for WT; n=4, $P<0.05$). No notable difference was observed when peptide Ac2-26 (1 µM) was used as a stimulus (not shown). In contrast, cells prepared from FPR KO mice showed a reduced chemotaxis towards peptide Ac2-26 (3.2 ± 0.7 and 1.4 ± 0.1 MI, for WT and FPR KO, n=3 experiments in triplicate).

ANXA1 KO results indicate that this protein may exert a tonic inhibitory role on mouse NØ thereby down-regulating their *in vitro* activation (CD11b and chemotaxis assays). In addition, data produced with cells taken from FPR KO mice indicate that this receptor may not solely be responsible for the effects of the ANXA1-mimetic peptide Ac2-26.

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14P EXOGENOUS ANNEXIN 1 PROMOTES HUMAN NEUTROPHIL APOPTOSIS

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Annexin 1 (ANXA1) mediates part of the anti-inflammatory effects of glucocorticoids and participates in the resolution phase of inflammation. Endogenous ANXA1 is released from activated polymorphonuclear neutrophils (PMN) and acts in a auto-paracrine manner to inhibit their transmigration. (Perretti *et al.*, 1996). We have investigated here the effect of exogenous ANXA1 on human freshly isolated neutrophils.

Human PMN ($5x10^6$ ml^{-1}) were incubated with human recombinant ANXA1 for 6 h at 37°C (longer time-points being excluded for the high degree of spontaneous apoptosis). In selected experiments we used human recombinant ANXA5 or a chimæra ANXA1/5 (Lim *et al.*, 1998); or the calcium entry blocker SKF-96365 (SKF; 25 mM) or calcium-chelating agent BAPTA (25 mM). Phorbol esther (PMA) was used at 100 $ng ml^{-1}$. PMN apoptosis was tested in three ways. Firstly, using the cell-cycle assay following cell fixation in 80% cold ethanol (30 min) and staining with propidium iodide (100 $\mu g ml^{-1}$; PI) and FACS analysis. Secondly, aliquots (50 µl of $1x10^6$ cells ml^{-1} suspension) were incubated with Hoechst H33342 (10 µg/ml) for 20 min at 37°C, fixed with 10 µl of cold formaldehyde (40% w:v) and analysed by fluorescence microscopy (Olympus BH-2-RFCA microscope). Thirdly, PMN were incubated with FITC-ANXA5 (5 □l; BD-Bioscience, Oxford, UK) and PI (50 □g/ml) for 15 min prior to FACS analysis. BAD phosphorylation was monitored by immunoprecipitation and western blotting using primary anti-BAD (Cell Signalling) or anti-serin (BIOMOL). Data are mean ± s.e.mean of ≥ 3 experiments and have been analysed by ANOVA plus Bonferroni test ($P<0.05$ as significant). In four distinct experiments, ANXA1 (0.5 µM) addition to human PMN caused a higher degree of 6h apoptosis, as measured with all three assays. In control cells apoptosis ranged from 3 to 15%, whereas ANXA1 addition increased these figures to 15-50% (n=4, $P<0.05$). Furthermore, an approximate ED_{50} of 125 nM could be calculated. At variance from ANXA1, ANXA5 did not increase PMN apoptosis (9 ±0.2% *vs.* 8 ± 0.4% of untreated cells; n=3), whereas the chimæra ANXA1-5 accelerated apoptosis to an extent similar to ANXA1 (45 ± 4%, n=3; $P<0.05$ *vs.* control).

PMA- and ANXA1-dependent activation of PMN apoptosis were similar in extent (40 ± 2% and 38 ± 0.8%, respectively; n=3) and only partially additive (55 ± 1.4%, n=3) suggesting that these two compounds may share at least in part the final death signal. ANXA1-induced apoptosis was reduced by more than 50% by SKF (n=3). A similar degree of inhibition was attained with BAPTA (not shown). Finally, ANXA1-treated PMN displayed a lower % of phospho-BAD (approximately from 20-30% down to 10%, n=2 experiments).

In conclusion, exogenous ANXA1 accelerates PMN apoptosis, and this effect is likely secondary to an influx of calcium cations. In addition, the ANXA1 N-terminus, that is pharmacophore for the well-known anti-migratory actions of the protein, seems to mediate also this novel biological effect. In proved in an *in vivo* context, these findings indicate a novel mechanism by which ANXA1 can regulate PMN effectory functions.

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15P ROLE OF INTERLEUKIN-10 IN ACUTE AND CHRONIC INFLAMMATION

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Interleukin-10 (IL-10) exerts a wide spectrum of regulatory activities in the immune and inflammatory response. The aim of this study was to investigate the role of endogenous IL-10 on the modulation of the inflammatory response in mice subjected to acute and chronic inflammation (carrageenan-induced pleurisy and collagen-induced arthritis respectively).
Pleurisy and arthritis were induced in mice IL-10 wild-type (WT) or lacking of the gene for IL-10 (IL-10 knock-out, IL-10-KO). When compared to carrageenan-treated IL-10-WT mice, carrageenan treated IL-10-KO mice experienced higher rate of pleural exudation and polymorphonuclear cell migration. IL-10-KO mice experienced higher rate of clinical signs of collagen-induced arthritis (CIA) and more severe paw injury when compared to IL-10-WT mice. The degree of oxidative and nitrosative stress was significantly higher in IL-10-KO mice than in WT littermates in both models, as indicated by elevated malondialdehyde levels and formation of nitrotyrosine and poly (ADP-ribose) synthase (PARS).
The intensity and degree of staining for COX-2 and iNOS were markedly enhanced in lung tissue sections obtained from carrageenan-treated IL-10-KO mice compared to IL-10-WT mice. The degree of lung injury caused by carrageenan was anlso enhanced in IL-10-KO mice.
Levels of the pro-inflammatory cytokines (measured on exudate from carrageenan-treated mice and on plasma from collagen-treate mice) TNF-a, IL-1b and IL-6 were also greatly enhanced in IL-10-KO mice when compared in IL-10-KO mice. Taken together our results clearly demonstrate that endogenous IL-10 exerts an anti-inflammatory role during acute and chronic inflammation and tissue damage associated with carragennan.induced pleurisy and collagen-induced arthritis, possibly by regulating neutrophil recruitment, and the subsequent cytokine and oxidant generation.

16P DETECTION OF CRF_2 RECEPTORS IN THE HUMAN HEART USING [^{125}I]-ANTISAUVAGINE 30

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The endogenous ligands urocortin, urocortin II/stresscopin-related peptide and urocortin III/stresscopin all bind to the corticotrophin-releasing factor receptor 2 (CRF_2 receptor). Message RNA for the CRF_2 receptor has been detected in the heart and the receptor may therefore mediate the cardiovascular effects of the urocortins in the periphery. [^{125}I]-antisauvagine 30 is a high-affintiy radioligand that is a specific antagonist for CRF_2 receptors (Higelin *et al.*, 2001). Our aim was to conduct a detailed characterisation of [^{125}I]-antisauvagine-30 binding to native receptors and to determine if CRF_2 receptors were present in the human heart.

Radioligand binding experiments were carried out as previously described (Katugampola *et al.*, 2001). All tissue was obtained with local ethical approval. Rat brains were obtained from female Sprague-Dawley rats (300-350g), killed with CO_2. For all binding experiments, 30μm cryostat sections of non-diseased human left ventricle or rat brain were pre-incubated in hepes buffer (Hepes; 50mM, $MgCl_2$; 5mM, bovine serum albumin; 0.3%, pH 7.4). [^{125}I]-antisauvagine was incubated at 0.2nM in the kinetic and autoradiographical studies and 4pM-2nM in saturation binding experiments. Non-specific binding was determined using 1μM urocortin. Sections were washed for 10 min in 50mM Tris-HCl (pH 7.4). Bound radiolabel was detected in a γ-counter or by apposing to photographic film for 7 days. Affinity constants (K_D) and receptor desities (B_{MAX}) were calculatd using the KELL suite of programmes (Biosoft, Cambs, UK). All data were expressed as mean ± s.e.mean.

Autoradiographical studies showed that [^{125}I]-antisauvagine 30 bound predominately to the cerebral vasculature and choroid plexus of the rat brain. [^{125}I]-antisauvagine 30 binding was time-dependent, with a half time for association of 18 ± 2 min (n = 6) and a half time for dissociation of 242 ± 22 min (n = 6). [^{125}I]-antisauvagine 30 bound specifically to CRF_2 receptors, since it was not diplaceable by other, unrelated peptides including endothelin-1, apelin, angiotensin II and atrial natriuretic peptide (n = 6). The affinity of the radioligand for CRF_2 receptors in rat brain was 0.2 ± 0.03nM, with a B_{MAX} of 1.5 ± 0.62 fmol mg^{-1} (n = 6). A one site fit was preferred over a two site fit and Hill slopes were close to unity. Single site, high affinity binding was also observed in saturation assays for [^{125}I]-antisauvagine 30 in human left ventricle, with a K_D of 0.65 ± 0.22nM and a B_{MAX} of 4.7 ± 2.1 fmol mg^{-1} (n = 4 individuals). Autoradiograms revealed specific binding of the radiolabel to myocytes and small blood vessels (verified using histological staining and immunocytochemistry directed against α-actin on adjacent sections of tissue).

In conclusion, we have shown that [^{125}I]-antisauvagine 30 is a suitable tool for the study of native CRF_2 receptors. We have also demonstrated the presence of CRF_2 receptors in human heart and small blood vessels.

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17P ADENOVIRALLY-OVEREXPRESSED BETA 1- AND BETA 2-ADRENOCEPTORS ENHANCES THE CONTRACTILE RESPONSE TO CGP 12177A IN ADULT RAT CARDIOMYOCYTES

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GPCRs can alter the sensitivity of other receptors to ligands through formation of heterodimers, which may create a new ligand binding profile (Devi, 2001). This might account for the cardiostimulation seen on activation of the putative β_4-AR by the agonist CGP 12177A (CGP). Current evidence suggests that β_4-AR is a novel state of the β_1-AR (Kaumann, 2001). Recently, functional heterodimerization between β_1- and β_2-ARs has been demonstrated (Lavoie, 2002). β_1- and β_2-ARs separately overexpressed increase the responsiveness to isoproterenol (ISO) and CGP in adult rat cardiomyocytes (Lewis *et al.*, 2002[1,2]). We have examined the effect of overexpressing β_1- and β_2-ARs together on the inotropic responses to CGP in adult rat cardiomyocytes.

Single rat ventricular cardiomyocytes were isolated and infected with adenoviruses containing sequences for the human β_1- and β_2-ARs respectively (using methods previously published). Inotropic responses to CGP (in the presence of 1μM propranolol) were studied 24 hours following infection by measuring cell shortening in electrically stimulated ventricular myocytes.

Following overexpression of either β_1- or β_2-ARs alone, there was no significant difference in the pD_2 (-log EC_{50}): Control pD_2 6.35 ± 0.15 (mean ± sem, n=15); β_1-AR 6.51 ± 0.27 (n=10, p>0.57);β_2-AR 6.58 ± 0.13 (n=20, p>0.25). Following overexpression of β_1- and β_2-ARs together, there was a significant left shift in the concentration response curve to CGP (pD_2 7.31 ± 0.21, n=9, p<0.001) (Fig 1).

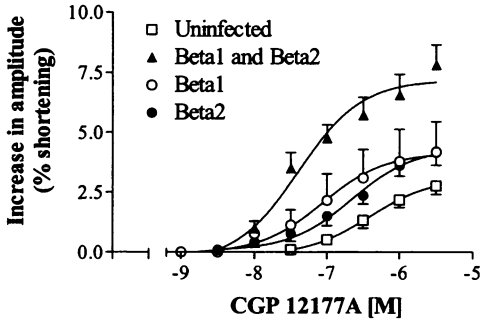

Fig 1. CRCs to CGP in control and infected myocytes

The approximate10-fold increase in sensitivity to CGP following β_1- and β_2-ARs co-overexpression compares with the lack of change in sensitivity when β_1- and β_2-ARs are overexpressed alone. This suggests the possibility of β_1- and β_2-AR heterodimerization and may provide an alternative explanation for the cardiostimulation seen with CGP.

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18P AN ANNEXIN 1 PEPTIDE REDUCES MYOCARDIAL ISCHAEMIA REPERFUSION IN THE MOUSE

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This study tested the hypothesis that the annexin 1 (ANXA1) derived N-terminal peptide Ac2-26 could exert cardio-protection in the mouse. The role for the formyl peptide receptor (FPR), recently described to interact with ANXA1-derived peptides (Walther *et al.*, 2000), was also determined.

A model used in the rat (La *et al.*, 2001) was adapted to the mouse. Male C57/BL6 (WT) or FPR-KO mice (25-30 g) (Gao *et al.*, 1999) were anaesthetised with Diazepam™ and Hypnorm™, and the jugular vein, carotid artery and trachea cannulated. A thoracotomy was achieved and ligation of the left anterior descending coronary artery (LADCA) was performed using a 6-0 suture needle. A piece of tubing was used to occlude the LADCA for 25 min, and this was followed by 60 min reperfusion. Saline (100 μl), peptide Ac 2-26 (1 mg/kg) or the FPR antagonist N-t-butoxycarbonyl-Phe-$_D$Leu-Phe-$_D$Leu-Phe (Boc2; 0.4 mg/kg) were administered at the start of the reperfusion period. Post-reperfusion, the LAD was re-occluded and Evans Blue dye injected to determine the area at risk (AAR). The heart was excised, sectioned and incubated in 2,3,5-triphenyltetrazolium chloride. The sections were then photographed and the left ventricular (LV) area, AAR and the infarct size (IS) for each tissue slice determined by computer planimetry using NIH Image software. Data (mean ± s.e.mean) of *n* mice were analysed by ANOVA and post-hoc Bonferroni test, taking a P value less than 0.05 as significant.

Both saline-treated WT and FPR-KO animal experienced similar-sized ischaemic areas of the LV, with values of 52.3 ± 1.4% (n = 6) and 56.17 ± 6.8% (n=4), respectively. Myocardial damage was expressed as IS/AAR with values of 54.3 ± 2.1% and 53.6 ± 12.6% in WT and FPR-KO mice. There was little difference between WT and KO mice also when IS was expressed relative to the LV (WT = 32.2 ± 2.0%; FPR-KO = 39.2 ± 2.9%). Peptide Ac2-26 administration did not modify ARR in either mouse type, with values of 54.2 ± 2.1% of LV (n=6) and 54.9 ± 2.8% (n=4), in WT and FPR-KO mice, respectively. However, in WT mice peptide Ac2-26 significantly attenuated the organ damage as evident from a value for IS/AAR of 34.9 ± 3.5% and IS/LV of 25.3 ± 1.2% (P<0.05, n=6). In WT mice, Boc 2 alone did not modify any of the parameters under observation (not shown, n=6) but it abrogated the protective effects exerted by the ANXA1-derived peptide (not shown).

In FPR-KO mice, peptide Ac2-26 retained its protective actions with values of 32 ± 4% and 27 ± 1% for IS/ARR and IS/LV, respectively (n=4; P<0.05 for either parameter *vs.* control).

These results suggest that the ANXA1-derived peptide Ac2-26, and possibly the parent protein as well, exerts cardio-protective actions in the mouse. Importantly, this effect appear to involve a Boc2 sensitive receptor other than the FPR.

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19P THE INFLUENCE OF ENDOGENOUSLY PRODUCED REACTIVE OXYGEN SPECIES ON THE INOTROPIC AND CHRONOTROPIC EFFECTS OF ADRENOCEPTOR- AND ET_A-RECEPTOR STIMULATION

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Reactive oxygen species (ROS) play a role in cardiovascular diseases such as congestive heart failure and hypertension. Furthermore, increasing evidence has accumulated suggesting that ROS can also be formed subsequent to the stimulation of various receptors, thus functioning as second messengers.

The objective of the present study was to elucidate the role of endogenously generated ROS in the inotropic and chronotropic response to the stimulation of α_1- and β-adrenoceptors and ET_A-receptors in isolated rat atria. For this purpose male Wistar rats (240-300g) were killed by stunning and decapitation. Right and left atria were isolated, mounted in organ baths filled with Krebs buffer solution and gassed with 100% oxygen. Left atria were paced with a field stimulator at a frequency of 3 Hz, whereas right atria were allowed to beat spontaneously. The contractile force and the frequency of beating were measured by means of a Power Lab/8s data acquisition system. After an equilibration period of 20 min, the atria were incubated with the cell-permeable superoxide dismutase- and catalase mimetic EUK-8 (400 μM), a N, N`-bis-salicyden aminoethan manganese complex (Siwik *et al.*, 1999). Thirty min later, a single dose of the α_1-adrenoceptor agonist methoxamine (300 μM), the β-adrenoceptor agonist isoprenaline (3 μM) or endothelin-1 (50 nM) was added. In addition, we investigated whether MAPK pathways are involved in ROS-induced changes of contractile force (Peters *et al.*, 2002). For this purpose, rat left atria were incubated for 45 min with the MKK^{erk}-inhibitors PD98059 (100 μM) or U0126 (10 μM) or with SB203580 (2 μM), an inhibitor of the $MAPK^{p38}$ pathway. Subsequently, hydrogen peroxide (250 μM) was administered in order to mimick the influence of endogenously generated ROS.

EUK-8 attenuated the positive inotropic effect to α_1-adrenoceptor and endothelin-1 ET_A-receptor stimulation (2.5±0.2 mN vs. 0.8±0.2 mN and 2.4±0.4 mN vs. 1.4±0.3 mN, n=6-7, $p<0.05$, respectively). In contrast, the contractile response to β-adrenoceptor stimulation remained unaffected (4.3±0.6 mN vs. 4.0±0.5 mN, n.s.). EUK-8 did not influence the chronotropic effects of α_1-adrenoceptor (-47.5±16.9 BPM vs.–38.8±14.4 BPM, n=6, n.s.), β-adrenoceptor (126.5±10.1 BPM vs. 152.3±9.4 BPM, n=6, n.s.) and ET_A-receptor (69.9±7.6 BPM vs. 54.3±9.7 BPM, n=5, n.s.) stimulation. The hydrogen peroxide-induced rise of contractile force in isolated rat left atria was abolished by the two MKK^{mek}-inhibitors PD98059 and U0126 (1.0±0.1 mN vs. –0.3±0.2 and 0.1±0.2 mN, respectively, n=5-11, $p<0.05$). The $MAPK^{p38}$-inhibitor SB203580 did not affect the increase of contractile force (0.6±0.1 mN, n=6, n.s.).

From the present study we conclude that endogenously formed ROS play a role in the inotropic responses to α_1-adrenoceptor and ET_A-receptor, but not to β-adrenoceptor stimulation in isolated rat left atria. In addition, our results suggest that ROS can activate the $MAPK^{erk}$ pathway, thereby contributing to the increase of contractile force. In contrast, ROS are not involved in receptor-dependent alterations of beating frequency.

Peters *et al.* (2002). Naunyn Schmiedebergs Arch. Pharmacol., **366**, 189-191
Siwik *et al.* (1999). Circ. Res., **85**, 147-153

20P INORGANIC NITRITE: PROTECTOR AGAINST ISCHAEMIA REPERFUSION INJURY IN THE HEART?

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Inorganic nitrite is an endogenous substance derived from the oxidation of $NO^{\cdot}$ under normal physiological conditions in the heart. However, during ischemia the acidosis that develops may be sufficient to chemically reduce the nitrite back to $NO^{\cdot}$ (Zweier *et al.* 1995). However, this process is difficult to demonstrate with currently available methods of $NO^{\cdot}$ measurement. Therefore we developed a novel $NO^{\cdot}$ trapping chamber specially designed to capture $NO^{\cdot}$ released from the isolated rat heart to determine whether $NO^{\cdot}$ is released from nitrite during ischaemia. In addition we investigated the mechanisms that might be involved in this $NO^{\cdot}$ release and assessed the effects of nitrite infusion on the damage consequent to ischaemia/reperfusion (I/R) injury.

Briefly, using air-tight $NO^{\cdot}$-trapping chambers and chemi-luminescence, $NO^{\cdot}$ production was measured from hearts of male wistar rats (290-360g) mounted in the Langendorff model and perfused with a modified Krebs solution containing L-NAME (300μM). Hearts were infused with nitrite (10-1000 μM) or saline control at low flow (0.1 ml/min) during otherwise global no flow ischaemia for 60 min. In some experiments hearts were pretreated with the xanthine oxidoreductase (XOR) inhibitors, allopurinol (100 μM) or (-)BOF 4272 (10 μM), since previous studies suggest that XOR reduces nitrite to $NO^{\cdot}$ independently of oxygen (Zhang *et al. 1998*). To investigate the effect of nitrite on I/R injury, hearts were subject to 30 min global ischaemia and 120 min reperfusion in the absence or presence of 10 or 100 μM nitrite or saline control. Left ventricular (LV) and coronary perfusion pressures were measured using an LV balloon and in-line pressure transducers. At the end of the I/R insult, to determine infarct size, hearts were cut into small pieces and incubated with nitroblue tetrazolium, which differentially stains viable and infarcted tissue.

Nitrite caused a concentration-dependent increase in $NO^{\cdot}$ production: 4.9±0.35, 8.6±1.5, 35.6±8.7 & 49.9±6.3 ppb *(mean ± S.E.M.)* at 60 min ischemia for control (n=4), 10 (n=4), 100 (n=8) & 1000 (n=7) μM nitrite respectively *($P<0.01$)* for each group compared to control (stats: 2-way ANOVA). This was apparent only during the period of ischaemia and was reversed by reperfusion. The rise of $NO^{\cdot}$ with 100 μM nitrite was inhibited by 100 μM allopurinol: 8.5±1.1 (n=4) *($P<0.01$)* and 10 μM (-)BOF 4272: 19.0±7.4 ppb (n=4) *($P<0.05$)*. Nitrite also improved LV function on reperfusion, eg the rate pressure product at 120 min: from 10584±1530 to 17733±2196 & 17134±2103, associated with comparable reductions in infarct size: from 45.3±3.2% to 14.0±2.0% and 17.8±1.5%, for control (n=6), 10 & 100 μM nitrite (both n=4) respectively *(all $P<0.001$)*. L-NAME caused no significant effects.

In conclusion $NO^{\cdot}$ produced via XOR-mediated nitrite reduction protects against I/R injury and as such may have an important protective endogenous or therapeutic role in myocardial infarction.

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21P CARDIOPROTECTIVE ACTION OF TYPE-B NATRIURETIC PEPTIDE IS NO-DEPENDENT

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Type-B natriuretic peptide (BNP) is one of a family of cardiac and vascular-derived peptides. BNP exerts a number of systemic actions through activation of a particulate guanylyl cyclase cell-surface receptor, NPR-A, and the subsequent elevation of intracellular cyclic GMP. We have recently reported that exogenous BNP confers protection in a concentration-dependent manner against acute ischaemia-reperfusion injury (D'Souza et al., 2002a). The mechanisms of this cytoprotective action are not fully elucidated although there is evidence to suggest a pivotal role of K_{ATP} channel opening (D'Souza et al., 2002b). While NPR-A is a particulate guanylyl cyclase, actions of BNP in some vascular beds have been attributed to generation of NO (Zellner et al., 1999). The present study was undertaken to identify if the NO/soluble guanylyl cyclase pathway plays any role in the cyctoprotective action of BNP in intact myocardium.

Male Sprague Dawley rats (200-300 g) were deeply anaesthetised with pentobarbitone sodium (60 mg/kg). Hearts were excised and Langendorff-perfused via the aorta at constant pressure (100 cm H_20) with Krebs-Henseleit buffer. After instrumentation and stabilisation, hearts were subjected to 35 min left coronary artery occlusion and 120 min reperfusion. Infarct size was determined as a percentage of the ischaemic risk zone by computerised planimetry of sections, double stained with Evans' blue and triphenyltetrazolium chloride. Control hearts received no treatment. BNP treated before the onset of coronary occlusion until 30 min reperfusion. To assess the involvement of NO synthase activation, L-NAME 10^{-4} M was co-perfused with BNP. To assess the involvement of soluble guanylyl cyclase, the inhibitor compound 1H-[1,2,4]oxadiazole[4,3-a]quinoxalin-1-one (ODQ) 10^{-5} M, which does not inhibit particulate guanylyl cyclase, was co-perfused with BNP. Infarct size data (I/R) and ischaemic risk zone data (R) are presented in table 1.

Table 1. Infarct size data

Treatment	n	R cm^3	I/R %
Control	8	0.39±0.04	45.0±5.5
BNP	11	0.37±0.01	23.4±2.8*
L-NAME+BNP	10	0.38±0.02	38.0±3.7
L-NAME	6	0.40±0.04	40.8±5.7
ODQ+BNP	6	0.38±0.03	41.1±5.3
ODQ	6	0.43±0.03	44.1±2.7

Data are mean ± s.e. * $P < 0.01$ versus control (1-way ANOVA)

BNP treatment significantly limited infarction during ischaemia-reperfusion. This cytoprotective effect was abolished by the non-specific NO-synthase inhibitor L-NAME or by the particulate guanylyl cyclase inhibitor ODQ. The differences in infarct size were independent of variations in ischaemic risk zone size since this was similar in all treatment groups. There were no substantial differences in coronary flow rate or left ventricular contractility between the groups (data not presented).

We conclude that the ability of BNP to limit myocardial infarction is dependent on activation of the NO/soluble guanylyl cyclase pathway. This is likely to be downstream of particulate guanylyl cyclase NPR-A activation.

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22P RELATIONSHIPS BETWEEN PRECLINICAL CARDIAC ELECTROPHYSIOLOGY DATA AND TORSADOGENIC RISK FOR 50 DRUGS: EVIDENCE FOR A PROVISIONAL SAFETY MARGIN IN DRUG DEVELOPMENT

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Since publication of the CPMP's 'Points to Consider' document on QT prolongation and torsade de pointes (TdP) in 1997 (CPMP/986/96), there has been much debate as to the relative merits of various *in vitro* and *in vivo* techniques in assessing 'torsadogenic risk' preclinically. We therefore undertook a literature survey to assess the value of preclinical electrophysiology data in predicting TdP risk.

Published preclinical data on hERG (or I_{Kr}) IC_{50}, APD_{90}, and QTc were compared against QT effects and reports of TdP in humans for 50 drugs. These data were set against the effective therapeutic plasma concentrations, unbound ($ETPC_{unbound}$). The drugs were divided into 5 categories: *1. Class Ia & III antiarrhythmics; 2. Withdrawn from market due to TdP; 3. Measurable incidence/numerous reports of TdP; 4. Isolated reports of TdP; 5. No reports of TdP.*

For *Category 1* drugs, data for hERG/I_{Kr} IC_{50}, APD_{90}, QTc *in vivo* and QTc in humans were close to the $ETPC_{unbound}$ (max) with the exception of amiodarone (a complex, atypical antiarrhythmic). This relationship was uncoupled in the other categories, with more complex relationships between the data.

For *Category 1* drugs (except amiodarone) the ratios between hERG/I_{Kr} IC_{50} and $ETPC_{unbound}$ (max) ranged from 0.1- to 31-fold. Similar ranges were obtained for drugs in *Category 2* (0.3- to 13-fold) and *Category 3* (0.03 to 35-fold). A large spread was found for *Category 4* drugs (0.05- to 35,700-fold); this category embraced an assortment of mechanisms ranging from drugs which clearly inhibit I_{Kr} currents in clinical use to others where channel block is not involved. Finally, for *Category 5* drugs, there was a clear separation (23- to 3,311-fold) between hERG/I_{Kr} activity and $ETPC_{unbound}$ values, except for verapamil (1.7-fold), which is free from QT prolongation in man; this is probably explained by its multiple interactions with cardiac ion channels.

The dataset confirms the widely-held belief that most drugs associated with TdP in humans are also associated with hERG K^+ channel block at concentrations close to or superimposable upon the free plasma concentrations found in clinical use. A 30-fold margin between C_{max} and hERG IC_{50} may suffice for drugs currently undergoing clinical evaluation, but for future drug discovery programmes pharmaceutical companies should consider increasing this margin, particularly for drugs aimed at non-debilitating diseases. However, interactions with multiple cardiac ion channels can either mitigate or exacerbate the prolongation of APD and QT that would ensue from block of I_{Kr} currents alone, and delay of repolarisation *per se* is not necessarily torsadogenic. Clearly, an integrated assessment of *in vitro* and *in vivo* data is required in order to predict the torsadogenic risk of a new candidate drug in humans.

23P NEUROKININ MEDIATED POTENTIATION OF PURINERGIC RESPONSES THE BLADDER OF THE PIG

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Neurokinin A (NKA) in the bladder dome and neck of the pig has been shown to mediate contraction via the NK2 receptor (Sellers *et al*, 2001). In the guinea pig bladder, neurokinins (Substance P) have also been shown to influence the excitatory motor response of the bladder, potentiating responses to neurogenic stimulation (Patra and Westfall, 1996). The aim of the present study was to investigate the effects of neurokinin receptor stimulation on the neurogenic responses throughout the bladder of the pig. Electrical field stimulation was used to elicit nerve-induced contraction of the smooth muscle in the pig bladder dome and neck.
The bladders from female pigs (70-90Kg) were obtained from a local abattoir. Longitudinal strips of smooth muscle were isolated from the pig bladder dome and neck (approx. 20 x 5mm). The urothelium and serosa were removed before suspension in 5ml organ baths. The tissues were bathed in Krebs-bicarbonate solution (gassed with 95% O_2/ 5% CO_2), placed under 1g of tension and maintained at 37°C. Responses to electrical field stimulation (40V, 10Hz, 0.1msec pulse width, 5-second duration, 100 second repetition) were recorded in the absence and presence of NKA (100nM). SR48968 (1μM) was used to block postjunctional NK2 recepors. Atropine (1μM) and αβmeATP (20μM) were used to determine whether there was any interaction between the NKA system and the muscarinic or P2X receptors respectively. The results are presented as mean ± s.e.m and comparisons made using a paired Student's t-test.

Electrical field stimulation produced rapid transient contractions of the bladder dome and neck (1.33 ± 0.27g and 2.57 ± 0.28g respectively, $n \geq 4$). NKA applied to the bladder neck during electrical field stimulation had no effect on the responses to field stimulation. In the dome however, NKA significantly enhanced the size of neurogenic contractions (from 1.33 ± 0.27g to 3.42 ± 0.41g, $p<0.05$, n=3) with an accompanied increase in baseline tension. The NK2 receptor antagonist SR48968 (1μM) abolished any neurogenic potentiation by NKA. αβmeATP (20μM) was able to significantly reduce the size of NKA enhanced neurogenic contractions ($p<0.05$). In the presence of atropine (1μM) NKA still potentiated responses to field stimulation (from 0.42 ± 0.17g to 2.19 ± 0.04g, $p<0.05$), which were subsequently reduced by αβMeATP (to 0.41 ± 0.10, $p<0.01$, $n \geq 3$).

These data suggest that NKA acting at postjunctional NK2 receptors in the pig bladder dome enhances the contractile response to nerve stimulation. NKA enhances responses to the purinergic but not the cholinergic component of neurogenic contraction. Such potentiation is not seen in the bladder neck region.

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Sellers *et al.* (2001). *Br. J. Pharmacol.* **135:** 242P (Suppl.)

24P INVESTIGATION OF NKA RESPONSES IN DETRUSOR MUSCLE FROM NORMAL AND NEUROGENIC BLADDERS

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In the urinary bladder the role of tachykinins in regulating function is still unclear. However it known that the NK_2 receptor subtype is solely responsible for mediation of contraction of the normal human bladder (Zeng et al., 1995). It has been suggested that tachykinins may have a role in the pathophysiology of bladder dysfunction and endogenous tachykinin release from bladder afferents is thought to induce neurogenic overactivity (Lecci et al., 1998). The present study investigates and compares the NKA-induced responses of detrusor muscle from normal and neurogenic overactive bladders.

Normal detrusor muscle was obtained from patients undergoing cystectomy for bladder cancer, whilst neurogenic overactive bladder was obtained from patients with spinal injuries. The urothelium and serosa were removed and the strips suspended in gassed Krebs at 37°C under a resting tension of 1.0g. Cumulative concentration-response curves to NKA were obtained in the absence and presence of the NK_2 receptor-selective antagonist SR 48968 (3-100nM) or the NK_3 receptor-selective antagonist SB 223412 (3-30μM) with a 30 min. incubation period. Phosphoramidon (10μM), CP99,994 (0.1μM) and meclofenamic acid (1μM) were also present.

NKA produced concentration-dependent contractions in detrusor muscle strips from both normal and neurogenic overactive bladders. Mean pEC_{50} and maximum responses were similar in both groups being 7.47±0.19 and 3.48±0.67g (n=11; 4 patients) and 7.85±0.16 and 3.00±0.46g (n=10; 3 patients) respectively. These curves were shifted potently to the right by the NK_2 receptor-selective antagonist SR 48968 producing pK_B values of 8.85±0.08 in normal detrusor (n=14) and 8.73±0.12 in neurogenic overactive detrusor (n=8). The NK_3 receptor-selective antagonist SB 223412 was less effective with pK_B values of 5.81±0.11 in normal detrusor (=12) and 5.77±0.13 in neurogenic overactive detrusor (n=11). The slopes of the Schild plot for SB 223412 were close to unity for both normal and neurogenic overactive detrusor(1.07±0.27 and 0.92±0.22 respectively). The slope for SR 48968 was low in normal detrusor being significantly different from 1 (slope = 0.50±0.06, $P<0.001$), although in neurogenic overactive detrusor the slope for SR 48968 was not significantly different from one (0.77±0.38). Maximum responses were not affected by either of these antagonists.

These data show that NKA-induced contraction of human detrusor muscle is not altered in the neurogenic overactive bladder. In addition the NK_2 receptor appears to mediate contraction in both normal and neurogenic overactive detrusor muscle.

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Zeng X., Moore K.H., Burcher E. (1995) *J. Urol.* 153, 1688-1692.

25P BLOCKADE OF NOCICEPTIN/ORPHANIN FQ – NOP RECEPTOR SIGNALLING PRODUCES ANTIDEPRESSANT-LIKE EFFECTS: PHARMACOLOGICAL AND GENETIC EVIDENCE IN THE MOUSE FORCED SWIMMING TEST

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Nociceptin/orphanin FQ (N/OFQ) is the endogenous ligand of the N/OFQ peptide receptor (NOP). This peptide/receptor system regulates several biological functions such as pain, learning and memory, fear and anxiety, feeding, and locomotor activity. Recently, it has been reported that NOP receptor antagonists induce antidepressant-like effects in the mouse forced swimming test (FST; Redrobe *et al.*, 2002). The present study was undertaken to further investigate the involvement of the NOP receptor in depression states using the novel NOP receptor antagonist, UFP-101 ([Nphe[1],Arg[14],Lys[15]] N/OFQ-NH_2, Calo' *et al.*, 2002) and NOP receptor gene knockout mice (Nishi *et al.*, 1997).

Male Swiss and CD1-C57BL/6J-129 wild-type (WT) and NOP receptor knockout (KO) mice weighting 20-25 g were used. They were housed under standard conditions (22° C, 12-h light-dark cycle) with food and water ad libitum. Each animal was used once and each group was composed by 8-12 mice. Intracerebroventricular (i.c.v.) injections (2 µl) were given, 5 min before the test, into the left ventricle, under ether anaesthesia according to Lausern & Belknap (1986). Mice were dropped into cylinders containing water at 25 ± 1 °C, for 15 min (pre-test). A test of 5 min was performed 24 h later and the immobility time measured (Porsolt *et al.*, 1977).

I.c.v. injections of UFP-101 (1-10 nmol) dose-dependently reduced the immobility time (Table 1). This effect of UFP-101was reversed by co-administration of N/OFQ, which was *per se* inactive (Table 1). In the FST, mice KO for the NOP receptor gene showed a reduced immobility time compared to their WT littermates (WT: 215 ± 10s, KO: 143 ± 12*s; *p<0.05 (Student's *t*-test)). Moreover, i.c.v. injected UFP-101 (10 nmol) significantly reduced immobility time in wild-type (saline: 210 ± 8s, UFP-101: 127 ± 18*s; *p<0.05 (Student's *t*-test)) but not in NOP receptor knockout mice (saline: 148 ± 19s, UFP-101: 138 ± 16s).

Table 1. Effects of UFP-101 alone or in combination with N/OFQ in the mouse forced swimming test.

	Control	+ N/OFQ (1 nmol)
Saline	216 ± 13	185 ± 18
UFP-101 (1 nmol)	194 ± 20	ND
UFP-101 (3 nmol)	141 ± 25*	221 ± 8#
UFP-101 (10 nmol)	91 ± 15*	159 ± 15#

Immobility time are expressed in s (mean ± s.e.mean). ND: not determined. *p<0.05 vs saline (ANOVA plus Dunnet test). #p<0.05 vs control (Student's *t*-test).

These findings obtained using a combined pharmacological and genetic approach converge indicating that block of N/OFQ-NOP receptor signalling in the brain produces antidepressant-like effects in the mouse FST. These results candidate the NOP receptor as a novel target for the development of innovative antidepressant drugs.

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26P STUDIES OF [PHE[1]ψ(CH_2-NH)GLY[2]]N/OFQ(1-13)NH_2 AT THE RECOMBINANT HUMAN NOCICEPTIN RECEPTOR USING THE ECDYSONE INDUCIBLE EXPRESSION SYSTEM

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Structure activity relationship studies of the Phe[1] pharmacophore of nociceptin/orphanin FQ (N/OFQ) yielded the pseudo-peptide [Phe[1]ψ(CH_2-NH)Gly[2]]N/OFQ(1-13)NH_2 ([F/G]) which displays tissue/assay dependent activity ranging from pure antagonist to full agonist (Calo' et al, 2000). To examine the actions of this molecule at different receptor densities in the same cellular background we have utilised the ecdysone inducible expression system to vary the expression of the human N/OFQ peptide receptor (hNOP) in Chinese hamster ovary cells ($CHO_{INDhNOP}$).

To induce expression, $CHO_{INDhNOP}$ cells (from GSK, Stevenage) were incubated for 20 hours with 0, 1, 5, 10, and 20µM Ponasterone A (P). Binding of [*leucyl*-^{3}H]N/OFQ was performed in 0.5ml of buffer containing 50mM Tris-HCl, 5mM $MgSO_4$, 10µM peptidase inhibitors (amastatin, bestatin, captopril, phosphoramidon) and varying amounts (15-350µg) of $CHO_{INDhNOP}$ membranes as described previously (Hashiba et al., 2002). ^{35}S-Guanylyl-5'-O-(γ–thio)-trisphosphate ($GTP\gamma^{35}S$) binding was performed in buffer containing 50mM Tris-HCl, 100mM NaCl, 1mM $MgCl_2$, peptidase inhibitors (as above), 150µM bacitracin, 100µM GDP, ~150pM $GTP\gamma^{35}S$ and 40µg $CHO_{INDhNOP}$ membranes (McDonald et al., 2002). Inhibition of forskolin stimulated cAMP formation was performed with whole cells in Krebs/HEPES buffer, using a competitive protein binding assay (McDonald et al, 2002).

[*leucyl*-^{3}H]N/OFQ binding increased from 24±4 fmol/mg at 1µM P to 1101±145 at 10µM P. At 20µM P there was a decrease in B_{max}. pEC_{50} values for $GTP\gamma^{35}S$ binding ranged from 7.23±0.38 to 7.72±0.06 (2µM-10µM P) for [F/G] and 8.12±0.32 to 8.60±0.07 (1µM-10µM P) for N/OFQ(1-13)NH_2 and E_{max} values (stimulation factor relative to basal) ranged from 1.51±0.15 to 3.21±0.38 (2µM-10µM P) for [F/G] and 1.28±0.03 to 6.95±1.05 (1µM-10µM) for N/OFQ(1-13)NH_2. [F/G] alone did not stimulate $GTP\gamma^{35}S$ binding at 1µM P but competitively antagonised the effects of N/OFQ(1-13)NH_2 with a pK_B of 7.62±0.08. pEC_{50} values for inhibition of cAMP formation ranged from 8.26±0.87 to 8.32±0.13 (2µM-10µM P) for [F/G] and 9.42±0.49 to 10.35±0.22 (2µM-10µM P) for N/OFQ(1-13)NH_2 and E_{max} values ranged from 19.6±4.8 to 83.2±4.0 (2µM-10µM P) for [F/G] and 40.9±2.2 to 86.0±3.7 (2µM-10µM) for N/OFQ(1-13)NH_2. At 1µM P there was no consistent inhibition with either peptide.

In the same cellular environment with the only variable being the number of receptors we show that the pharmacological profile of [F/G]N/OFQ(1-13)NH_2 can be manipulated to encompass full and partial agonism along with pure antagonism.

Supported in part by a grant from the BJA/RCA.

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27P DIFFERENCES MAY EXIST IN NOCICEPTIVE PROCESSING IN THE TRIGEMINAL AND SPINAL SYSTEMS ILLUSTRATED BY THE RESPONSE TO WIND-UP STIMULATION ASSESSED IN THE ANAESTHETISED RAT

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In this study we have investigated the ability of trains of electrical stimuli applied to the dura-mater of anaesthetised adult rats to generate wind-up of second-order neurones within the trigeminal nucleus caudalis (TNC) under physiological conditions and conditions of sensitisation.

Male, Sprague-Dawley (280-400g) rats were anaethetised with isoflurane (3-5% in oxygen), maintained under a constant infusion of 20 $mg.kg^{-1}$ per h pentobarbitone sodium and mechanically ventilated. Surgery was performed to expose the TNC and visualise the middle meningeal artery. Trains of 20 electrical stimuli at both 1 Hz and 2 Hz were applied to the dura overlying the middle meningeal artery every 3 minutes for 30 minutes to generate wind-up of TNC neurones. Recordings were made from 17 cells in the TNC. Wind up was assessed by the following calculation; Windup score = $\sum$(responses for 20 stimuli) – (1^{st} stimulus response X 20).

Five of seventeen cells exhibited an Aδ-fibre response only, 4/17 a C-fibre response only, and 8/17 both an Aδ and C-fibre component. Average response latencies and dural stimulus threshold were 14 ± 2ms, 2 ± 0.6mA, 0.3 ± 0.04ms and 45 ± 7ms, 6 ± 1.5mA, 0.8 ± 0.1ms for Aδ and C-fibres, respectively. With the exception of one cell (C-fibre only) all cells received a convergent input from the face and responses to brush and pinch of the receptive field were tested both before and after the electrical stimulation protocol. No cell tested with the 1 Hz protocol showed wind-up, however 1/7 Aδand 2/6 C- fibre mediated responses tested with the 2 Hz protocol showed a small increase in response with successive stimuli with a mean wind-up score of 31.67 ± 5.17 compared to a group protocol mean of 3.89 ± 4.00 ($P < 0.05$ Students t-test). This was not significant when compared to wind-up scores of dorsal horn neurons (typically in the range of 150-200; Martindale, unpublished observations). Responses to stimulation of the facial receptive field were not significantly different following electrical stimulation protocols, even in the 3 cells that showed a small degree of wind-up. Wind-up was also assessed in 4 cells after mustard oil application to the facial receptive field, again no wind-up was seen despite the presence of a sensitisation evidenced by an increased total response to electrical stimulation which reached a maximum of 58 ± 3 % (n = 4) above baseline, 9 minutes after mustard oil application ($P < 0.05$).

As wind-up of neurones in the TNC has been previously demonstrated in response to tooth pulp stimulation (Hamba *et al.*, 1992), it is likely that the lack of wind-up in this study reflects a different functional group of second-order neurones within the trigeminal system that specifically receive input from specialised craniofacial structures such as the dura mater.

	Aδ	C-fibre
1Hz protocol	3.9 ± 4.0	6.6 ± 3.0
2 Hz protocol	6.2 ± 6.0	10.3 ± 3.5

Table 1: mean group wind-up scores

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28P ANTI-HYPERALGESIC AND ANTI-INFLAMMATORY ACTIVITY OF A POTENT AND SELECTIVE ADENOSINE A1 RECEPTOR AGONIST 5'-DEOXY-5'-FLUORO-N-(TETRAHYDRO-PYRAN-4-YL)-ADENOSINE

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Adenosine A1 receptor agonists have been shown to inhibit mechanical and thermal nociceptive thresholds (Sawynok et al., 1986) and inhibit nociceptive processing in the spinal cord in both acute and chronic inflammatory states (Sawynok et al 1986, Reeve et al., 1995). To further investigate the potential role of A1 receptor agonists in pain we have evaluated the effects of the potent and selective A1 agonist, 5'-deoxy-5'-fluoro-N-(tetrahydro-pyran-4-yl)-adenosine EC50 relative to NECA: A1= 2.2, A2a= 268, A2b>231, A3>280) in models of inflammatory hyperalgesia in rats.

Male Random Hooded Rats (180g) were fasted overnight. the drug or vehicle (n=7 per group) were administered orally 30min prior to intraplantar (ipl) injection into the left hind paw of 100µl 2% carrageenan or 1hr prior to 100µl of 1mg/ml Freund's Complete Adjuvant (FCA). In a model of established hyperalgesia the drug was administered 2 days after FCA. The effect of the A1 agonist on carrageenan or FCA induced hyperalgesia was assessed as a decrease in weight bearing on the inflamed left hind paw (Dual channel weight averager: Clayton et al., 1997), and was determined 3 and 6 hrs respectively after the inflammatory insult. In the established model, the weight bearing and paw oedema were measured 7 hrs post dose. Paw oedema was assessed using a plethysmometer. To examine whether tolerance developed, animals were dosed twice daily with the drug for seven days followed by 100µl of 2% carrageenan ipl. The effect of the drug on the carrageenan induced hyperalgesia and paw oedema was then determined 3hrs later. The duration of action of the drug was determined by dosing ipl carrageenan at different time points post drug. The hyperalgesia and paw oedema were then determined 3 hrs post carrageenan. Data is presented as mean±s.e. mean (%inhibition) and where appropriate geometric mean and 95% confidence intervals for EC50 values were calculated.

Statistical analysis was carried out to determine whether there was a significant difference ($p<0.05$) between the vehicle treated and drug treated groups using unpaired Student's t-test. When administered 30min prior to carrageenan injection, the drug significantly inhibited carrageenan-induced decrease in weight bearing (ED50=0.04[0.01-0.06]mg/kg p.o.) and reduced the associated paw oedema (50±7.2%@0.3mg/kg p.o.). Chronic dosing with the drug (0.01-0,1mg/kg p.o. b.i.d) for 7 days followed by ipl carrageenan produced a similar dose related inhibition of the decrease in weight bearing (ED50= 0.02[0.009—0.007]mg/kg p.o.) and had significant anti-inflammatory activity (54±4.5%@0.1mg/kg p.o.) suggesting no mechanism related tolerance. The drug (0.1mg/kg p.o.) completely prevented the carrageenan induced hyperalgesia for up to 5 hrs (101±13%) post dose and was greater than 50% at 7 hrs (57±14%) post dose. At 11 hrs the anti-hyperalgesic effect was no longer significant (2±15%). The drug also exhibited prolonged and significant anti-inflammatory activity (5hrs:50±5%) but by 7 hrs the anti-inflammatory activity was no longer significant (17±5%). The drug produced a dose related inhibition of acute (ED50 0.18[0.10-0.26] mg/kg p.o.) and established (ED50 =0.5[0.2-1.1] mg/kg p.o.) FCA induced hyperalgesia but had no anti-inflammatory activity. In conclusion, the A1 agonist inhibited acute and reversed established inflammatory hyperalgesia, with a long duration of action. The A1 agonist also had some anti-inflammatory activity (carrageenan only). There was no evidence of mechanism related tolerance. The anti-hyperalgesic and anti-inflammatory action of the A1 agonist , its long duration of action and no tolerance suggest that it may have clinical utility in the treatment chronic inflammatory pain.

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29P THE EFFECT OF TRANSDERMAL APPLICATION OF A HIGHLY SELECTIVE ADENOSINE A1 RECEPTOR AGONIST IN THE CARRAGEENAN MODEL OF ACUTE INFLAMMATORY HYPERALGESIA

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Pre-clinically, adenosine A1 receptor agonists have been shown to inhibit mechanical and thermal nociceptive thresholds (Sawynok *et al.*, 1986) and have a role in modulation of both acute and inflammatory nociception in the spinal cord (Reeve et al., 1995). In this study, we have examined transdermal administration of a highly selective A1 agonist, 5'-deoxy-5'-fluoro-N-(tetrahydro-pyran-4-yl)adenosine, in the carrageenan model of acute inflammatory hyperalgesia. The transdermal preparation was formulated by pharmacy at GlaxoSmithKline by dissolving the A1 agonist in 50% propylene glycol, 20% ethanol and 30% water. Male Random Hooded rats (180-200g) were fasted overnight prior to each study. In all experiments involving transdermal application, 20µl of either the A1 agonist or vehicle was applied to the dorsal side of the left hind paw using a Gilson pipette. Non-absorbent bandage (consisting of zinc oxide plaster and steri-drape) was then immediately wrapped around the paw for 30 minutes, after which time the bandage was removed and the paw was washed using tepid water. This reduced the possibility of oral administration through licking of the hind paw, ensuring drug was taken up only by the transdermal route.

Following transdermal application of the A1 agonist (0.01-0.3mg) or vehicle, to the hind paw, the paw was washed and 100µl of 2% carrageenan was injected intraplantar into the same hind paw. The effect of the A1 agonist on carrageenan induced decrease in weight bearing (dual channel weight averager; Clayton et al., 1997) and increase in oedema (plethysmometer) on the inflamed paw was assessed 3 hours later. The effect of oral (0.03-0.1mg/kg) and transdermal (0.1-0.6mg) A1 agonist on normal slow rearing activity was measured 30 minutes after administration using Benwick Activity Monitors, which detect animal movement (Locomotor activity – LMA). This was compared to effects in the carrageenan model to allow calculation of a therapeutic index (TI). Statistical analysis was carried out to compare the difference between the drug treated group and vehicle treated group using unpaired Student's t test ($P<0.05$ considered significant). Where appropriate, ED_{50} values (50% of response observed in control animals) were calculated as geometric means with 95% confidence intervals.

Transdermal administration of the A1 agonist (0.01-0.3mg) produced a significant ($P<0.05$) and dose-related reversal of the carrageenan-induced decrease in weight bearing on the inflamed left hind paw (ED_{50} 0.02[0.01,0.03]mg). The compound also demonstrated some anti-inflammatory activity, producing a dose-related inhibition of the associated oedema (maximum effect 44% @ 0.3mg; $P<0.01$). The A1 agonist inhibited normal locomotor activity in a dose-related manner following both transdermal (ED_{50} 0.4[0.25,0.68]mg) and systemic (ED_{50} 0.1[0.07,0.22]mg/kg p.o.) administration. Transdermal administration of the A1 agonist produced a wider TI (20) than oral administration (2.5), which may be partially related to the local site of action or differences in drug distribution and metabolism, following the two routes. Pharmacokinetic studies demonstrated a difference in Cmax and AUC following the two routes of administration (data not shown), however, this was not sufficient to explain the 8 fold wider TI obtained following transdermal application. In conclusion, these studies demonstrate that transdermal application of a highly selective A1 agonist is effective, having similar efficacy to systemic administration but with a greater TI.

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Sawynok J. *et al.*, (1986). Br. J. Pharmacol. 88 923-930

30P PLAQUE-RELATED IMPAIRMENT OF NITRIC OXIDE-INDUCED RELAXATIONS IN APOLIPOPROTEIN E-DEFICIENT MICE ON A REGULAR DIET

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Endothelium-dependent relaxation in murine elastic arteries is mediated by nitric oxide (NO) via cyclic GMP-dependent pathways (Crauwels *et al.*, 2000). Although apolipoprotein E-deficient ($apoE^{-/-}$) mice are hypercholesterolemic and develop atherosclerosis, endothelium-dependent relaxation remains intact up to 6 months on a regular diet (Bonthu S. *et al.*, 1997). We investigated whether vasomotor dysfunction develops in aged $apoE^{-/-}$ mice, whether it is local or systemic (hypercholesterolemia-dependent), and the possible nature of the defect.

The thoracic aorta of anaesthetised (sodium pentobarbital, 75 mg kg^{-1}, i.p.) $apoE^{-/-}$ mice (22±0.4 months; 30±1 g; cholesterol 462±39 mg dl^{-1}; n=8) and C56BL6 mice (WT; 22±0.5 months; 33±2 g; 74±8 mg dl^{-1}; n=6) was removed and systematically sectioned (5x2 mm). Segments were mounted in organ baths (aerated Krebs-Ringer solution, 37°C, pH 7.4, indomethacin 10µM) for isometric tension recording. Relaxation was evaluated in phenylephrine-constricted rings with papaverine 100 µM and cumulative concentration response curves for acetylcholine (ACh, 3nM-10µM); 8-Br-cGMP (0.1µM-100µM); and NO donors: acidified $NaNO_2$ (fast, short NO-release, 0.1µM-100µM), spermine NONOate (long-lasting NO-release, 1nM-100µM) and nitroglycerin (GTN, NO-release after biotransformation, 1nM-10µM). Maximal responses (E_{max}) and the negative logarithm of the concentration (pD_2) resulting in 50% of E_{max} were determined and evaluated (one way ANOVA). Afterwards, segments were fixed (formaldehyde 4%, 24hours) for morphometric analysis. Data represent mean±s.e.m.

Prominent atherosclerotic plaques were seen in the proximal and the distal thoracic aorta segments of $apoE^{-/-}$ mice (TA1, TA5; intimal area: both 0.19±0.05mm^2), but not in the central segments (TA2-TA4). ACh-relaxation was impaired in $apoE^{-/-}$ mice as compared to WT mice, but only in atherosclerotic segments (TA1: E_{max} 46±11% vs. 90±4%, pD_2 5.91±0.23 vs. 6.79±0.14; $p<0.01$), and not in adjacent plaque-free segments (TA2: E_{max} 96±1% vs. 93±2%, pD_2 7.09±0.14 vs. 7.21±0.12). Endothelial function inversely correlated to plaque size (Spearman, $p<0.01$; E_{max}: r_s=-0.85, pD_2: r_s=-0.61). In atherosclerotic segments, responses to acidified $NaNO_2$ (TA1, E_{max}: 74±6% vs. 90±3) and GTN (TA1, E_{max}: 78±6% vs. 95±2%) were impaired ($p<0.05$) as compared to WT mice, and inversely correlated with plaque area ($p<0.01$, acidified $NaNO_2$: E_{max} r_s=-0.72, pD_2 r_s=-0.71; GTN: E_{max} r_s=-0.81). Relaxations to spermine NONOate were however unaltered (TA1, E_{max}: 99± 1% vs. WT 99±1%, pD_2: 6.48±0.15 vs. 6.92± 0.17). The dilator capacity of arteries with extensive plaques was not affected, as indicated by the normal responses for both cyclic GMP (TA5, E_{max}: 89± 3% vs. WT 89±3%; pD_2: 5.24±0.06 vs. 5.21±0.08) and papaverine.

In conclusion, vasomotor dysfunction in $apoE^{-/-}$ mice on a regular diet is not systemic, but plaque-related. The defect involves the NO-pathway, but is probably not situated at the smooth muscle cell level as indicated by the unaltered cyclic GMP response. The contrasts between NO-donors with different release kinetics however indicate that biodegradation of NO is enhanced in the local plaque environment.

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31P STAPHYLOCOCCUS AUREUS INDUCES NITRIC OXIDE RELEASE IN CARDIOVASCULAR TISSUE VIA A TOLL LIKE RECEPTOR 2 -INDEPENDENT PATHWAY

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The mortality of septic shock remains high (35-45%) and Gram-positive organisms are now thought to be responsible for up to 50% of cases (Geerdes *et al.*, 1992). Septic shock is typified by cardiovascular hyporesponsiveness to inotropes, possibly due in part to nitric oxide (NO) release. Toll-like receptors (TLRs) have been shown to participate in innate immune recognition of bacterial structures (Medzhitov *et al.*, 1997). Specifically, TLR2 has been shown to mediate responses to Gram-positive bacteria in immune cells (Takeuchi *et al.* 2001), but its role in cardiovascular tissue is less well understood. The aim of this study was to investigate the role of TLR2 in Gram-positive Staphylococcus aureus (S. aureus) induced NO release from the heart in an organ culture murine model.

TLR2-deficient mice (generated by gene targeting) were obtained via collaboration (Takeuchi *et al.*, 2000). C57BL/6 mice were used as controls. Adult male animals (15-31 weeks) were anaesthetised with intraperitoneal pentobarbitone and killed by cervical dislocation. Hearts were removed, cut into pieces and placed into 48 well plates with 400µL of Dulbecco's Modified Medium containing 10% fetal calf serum, penicillin (100UmL^{-1}), streptomycin (100µgmL^{-1}), amphotericin (2.5µgmL^{-1}) and non-essential amino acids.

After 2 hours equilibration, tissue was stimulated with heat killed S. aureus or left untreated. Tissue was retreated at 24 hours and incubated for a further 24 hours. NO release was indexed by measuring nitrite using the Greiss assay (Bishop-Bailey *et al.*, 1997).

S. aureus induced NO release from C57BL/6 hearts (Figure 1). Similarly, S. aureus stimulated heart tissue from TLR2-deficient mice to release NO. No statistical difference (One-way ANOVA) was seen in the ability of Gram-positive bacteria to stimulate NO from tissue from either group of animals.

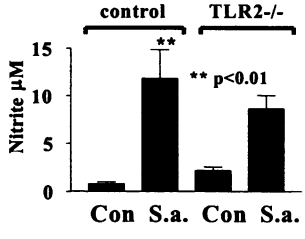

Figure 1. Effects of heat killed S. aureus on NO release in control and TLR2-deficient mice. N=8 animals. S. aureus (S.a.; 8x10^8 colony forming units /mL (cfu/mL). Con (medium alone).

In immune cells Gram-positive bacteria stimulate inflammatory mediator release via TLR2. This data suggests that TLR2 may not be involved in Gram-positive recognition by cardiovascular tissue and has implications for our understanding of the treatment of patients with sepsis.

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Geerdes HF *et al., Clin Infect Dis* 1992; 15: 991-1002
Medzhitov R *et al., Nature* 1997; 388:394-7
Takeuchi *et al., J Immunol* 2000; 165: 5392-6.
Takeuchi *et al., Int Immunopharmacol* 2001; 1: 625-35. *MRC Supported.*

32P ESCHERICHIA COLI INDUCES NITRIC OXIDE RELEASE IN CARDIOVASCULAR TISSUE VIA A TOLL LIKE RECEPTOR 4-INDEPENDENT PATHWAY

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Septic shock carries a mortality of 35-45%. The induction of nitric oxide (NO) in sepsis is associated with reduced inotropic responses, which typify the syndrome (Mitchell *et al.*, 2000). Toll-like receptors (TLRs) have been shown to participate in innate immune recognition of bacterial structures (Medzhitov *et al.*, 1997). Specifically, TLR4 has been shown to mediate responses to Gram-negative bacteria in immune cells (Takeuchi *et al.*, 2001). The role of TLR4 in cardiovascular tissue is less well understood. The aim of this study was to investigate the role of TLR4 in Gram-negative Escherichia coli (E. coli) induced nitric oxide (NO) release from the heart in a murine organ culture model.

TLR4-deficient mice, generated by gene targeting, were obtained via collaboration (Takeuchi *et al.*, 2000). C57BL/6 mice were used as controls. Adult male animals (12-29 weeks) were anaesthetised with intraperitoneal pentobarbitone and killed by cervical dislocation. Hearts were removed and ventricular tissue placed into 48 well plates with 400µL of Dulbecco's Modified Medium containing 10% fetal calf serum, penicillin (100UmL^{-1}), streptomycin (100µg/mL^{-1}), amphotericin (2.5µgmL^{-1}) and non- essential amino acids.

After 2 hours equilibration, hearts were stimulated with lipopolysaccaride from E. coli (LPS), heat killed E. coli or left untreated. Tissue was retreated at 24 hours and incubated for a further 24 hours. NO release was indexed by measuring nitrite using the Greiss assay (Bishop-Bailey *et al.*, 1997).

LPS and E. coli induced NO release from C57BL/6 hearts. (Figure 1). Similarly, LPS and E. coli stimulated heart tissue from TLR4-deficient mice to release NO. E. coli significantly increased nitrite release in both control and TLR4-/- groups (t test).

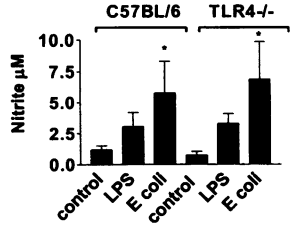

Figure 1. Effects of LPS (100µgmL^{-1}) and heat killed E. coli on NO release in control and TLR4-deficient mice. N=4 animals. E. coli 1x10^9 colony forming units /mL (cfu/mL). C (medium alone).

LPS and E. coli induced NO in both control and TLR4-deficient hearts. This suggests that, in contrast to immune responses, LPS and E. coli release NO by a TLR4-independent pathway in the cardiovascular system. This has implications for our understanding of the host response to infection.

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33P NO ROLE FOR OXYGEN-DERIVED FREE RADICALS IN ANGIOTENSIN II-MEDIATED VASOCONSTRICTION OF HUMAN CORONARY ARTERIES

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To investigate whether NAD(P)H oxidase- and/or xanthine oxidase-dependent oxygen-derived free radical production mediate angiotensin (Ang) II-induced vasoconstriction in humans, Ang II, endothelin-1 (ET-1) and NADH concentration-response curves (CRCs) were constructed in human coronary arteries (HCAs). HCAs were obtained from 19 heart valve donors (9 men, 10 women; age 18-58 years), who died of non-cardiovascular disorders.

Ang II constricted HCAs in a concentration-dependent manner (pEC_{50}: 7.73±0.07, E_{max}: 16±2.4% of the response to 100 mM K^+). NADH (100 μM), the NAD(P)H oxidase inhibitors diphenylene iodinium (DPI; 10 μM; Touyz *et al.*, 1999) and apocynin (1 mM; Hamilton *et al.*, 2002), as well as the xanthine oxidase inhibitor allopurinol (1 mM; Berry *et al.*, 2000) reduced E_{max} to 13±2.6, 9±2.4, 6±3.1, and 4±1.3, respectively. The superoxide dismutase (SOD) inhibitor diethylene thiocarbamate (DETCA; 0.1 mM) and the SOD mimetic tempol (1 mM) were without effect. Catalase (1000 U/ml) potentiated the Ang II response (pEC_{50} 7.98±0.16; E_{max} 24±7.7), suggesting that endogenous hydrogen peroxide counteracts Ang II-mediated constriction.

At the highest concentration tested (0.3 μM), ET-1 constricted HCAs to 80±5.6% of the response to 100 mM K^+. Since a maximum was not always reached, the ET-1 concentration required to obtain 30% of the response to 100 mM K^+ ($E_{30\%K+}$) was calculated, in order to obtain $pEC_{30\%}$ values. DPI, apocynin and allopurinol reduced $pEC_{30\%}$ from 7.62±0.19 (control) to 7.42±0.17, 7.21±0.13 and 7.37±0.13, whereas catalase increased $pEC_{30\%}$ to 7.96±0.13. This indicates that the effects of DPI, apocynin, allopurinol and catalase towards Ang II were mimicked in combination with ET-1.

Following preconstriction with 1 μM $PGF_{2\alpha}$, DPI, apocynin and allopurinol concentration-dependently relaxed HCAs. Relaxation amounted to 17±6.4, 106±4.1 and 90±7.1%, respectively, at the highest dose tested (0.01, 1 and 1 mM, respectively). Catalase (1000 U/ml) did not affect non-preconstricted HCAs.

Finally, NADH (0.1-100 μM) relaxed preconstricted HCAs by maximally 87±4.9%. NADH-induced relaxation in HCAs was unaffected by DETCA or the NO synthase inhibitor L-NAME (100 μM) combined with the NO scavenger hydroxocobalamin (200 μM).

In summary, the inhibitory effect of NAD(P)H and xanthine oxidase inhibitors towards Ang II-mediated vasoconstriction most likely represents physiological antagonism, independent of oxygen radicals. This conclusion is based on our observations that mimetics and inhibitors of SOD did not affect Ang II-mediated constriction, and that both endogenous hydrogen peroxide and exogenous superoxide caused vasodilatation in an NO-independent manner. Therefore, it can be concluded that Ang II-mediated vasoconstriction in HCAs is not mediated via oxygen-derived free radicals.

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34P VASCULAR NATRIURETIC PEPTIDE RECEPTORS MODULATED BY NO-CYCLIC GMP SIGNALLING

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C-type natriuretic peptide (CNP) belongs to a family of endogenous agents (including ANP and BNP) that are important in cardiovascular homeostasis. ANP and BNP are circulating mediators whereas CNP is a local mediator, stored in endothelial cells. ANP and BNP relax vascular smooth muscle by activating natriuretic peptide receptor (NPR) subtypes NPR-A (a particulate guanylate cyclase; pGC) and NPR-C (pGC-independent); CNP vasodilates by activating NPR-B (pGC-coupled) and NPR-C receptors. We demonstrated previously that nitric oxide (NO) desensitises blood vessels to ANP (Hussain *et al.*, 2001). The present study investigated the NPR subtypes involved in this interaction by comparing the effects of NO on the response of blood vessels to ANP, CNP and the selective NPR-C agonist, $cANP^{4-23}$.

Mouse (male; wild-type, WT and endothelial NO synthase knockout, eNOS KO (Huang *et al*, 1995; 25-30g) thoracic aortic rings were set up under 0.3g tension for isometric recording in Krebs solution gassed with 95%O_2/5%CO_2 at 37°C. All tissues were contracted to an approximate EC_{80} to phenylephrine (0.70±0.02 in WT and 0.76±0.02 in eNOS KO; n≥56 for both) and concentration-response curves to ANP (0.001-1μM), CNP (0.001-10μM) and $cANP^{4-23}$ (0.001-10μM) were constructed. The effect of NO/cGMP deficiency on the sensitivity of the vessels to ANP, CNP & $cANP^{4-23}$ was assessed by incubation with the NOS inhibitor N^G-nitro-L-arginine methyl ester (L-NAME; 300μM, 30min) or sGC inhibitor ODQ (5μM, 30min) or by using vessels from eNOS KO mice. The effect of NO/cGMP excess was investigated by incubating vessels with glycerol trinitrate (GTN; 30μM, 30min). pEC_{50} values were used to compare the relaxant effects of drugs. Statistical analysis was by two-way ANOVA with $P<0.05$ taken to be significantly different.

ANP and CNP were more potent on eNOS KO than on the WT aorta (pEC_{50}: 8.85±0.01 and 8.41±0.02, respectively for ANP; 7.50 ± 0.12 and 6.86 ± 0.10, respectively for CNP; $P<0.05$ for both; n≥5). In WT aorta, the potency of ANP and CNP was increased in the presence of L-NAME (pEC_{50}: 8.74±0.09 and 9.15±0.09, respectively for ANP; 6.80 ± 0.07 and 7.17 ± 0.18, respectively for CNP; $P<0.05$ for both; n≥4) or ODQ (pEC_{50}: 8.56±0.05 and 9.26±0.03, respectively for ANP; 6.87±0.1 and 7.27±0.23, respectively for CNP; $P<0.05$ for both; n≥5). Following incubation with GTN, vessels from eNOS KO mice were less sensitive to ANP and CNP (pEC_{50}: 9.24±0.05 and 8.43±0.04, respectively for ANP; 6.53±0.16 and 6.32±0.23, respectively for CNP; $P<0.05$ for both; n≥4). Incubation with excess ANP (100nM, 30min) also caused a significant decrease in the potency of CNP (pEC_{50}: 7.30±0.1 and 6.48±0.24, respectively; $P<0.05$; n≥6). Responses to ANP and CNP were attenuated by the NPR-A/NPR-B antagonist HS-142-1 (Morishita *et al*, 1991; 10μM, 30min; pEC_{50}: 7.44±0.16 and 6.80±0.33, respectively for ANP; 6.44±0.16 and 6.22±0.23, respectively for CNP; $P<0.05$ for both; n≥6). $cANP^{4-23}$ was unable to relax the tissue significantly.

These data suggest that both the NPR-A and NPR-B -linked pGC pathways are modulated by NO/cGMP in mouse aorta; NPR-C appears unimportant in mediating responses to natriuretic peptides in this tissue. The altered responsiveness of the NPR-A/NPR-B pathways in the presence of L-NAME was similar to that observed in the eNOS KO mice, suggesting that the change in the sensitivity of these pGCs is biochemical rather than expressional. The ability of ODQ to alter sensitivity of NPR-A/NPR-B implicates cGMP in this phenomenon, rather than a direct effect of NO; this thesis is supported by the apparent cross-desensitisation between NPRs. The interactions between these endogenous dilator mechanisms may be important in the physiological regulation of vascular tone.

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35P HSP90 IS REQUIRED TO STABILIZE THE NO RECEPTOR, SOLUBLE GUANYLYL CYCLASE

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Soluble guanylyl cyclase (sGC) is the main receptor for nitric oxide (NO), mediating many of its intracellular effects. Surprisingly, very little is known about the regulation of this enzyme beyond its activation by NO. Recently, the interaction between sGC and heat shock protein 90 (Hsp90) has been reported (Venema et al., 2001). In the present work, we have studied the impact of Hsp90 on the regulation of sGC activity and protein subunit levels.

Porcine pulmonary aorta endothelial (EC) and smooth muscle (SMC) cells were isolated enzymatically by incubation of aortic inner surface with collagenase. EC grown in M199 and SMC in DMEM medium (both with 10% fetal calf serum, 100 U/mL penicillin, 100 µg/mL streptomycin). Confluent EC monolayers from the second passage and SMC of passages 6-8 were used for our experiments. cGMP accumulation was stimulated by 250 µM diethylamine NONOate or 0.5 µM calcium ionophore, A23187, for 5 min. 1 mM IBMX and 100 µM zaprinast were added to the cells 30 min before stimulation to exclude effects of phosphodiesterases. cGMP detection and protein immunoblotting was performed as described earlier (Nedvetsky et al., 2002). The data presented are means ± S.E.M. of at least three independent experiments each performed in triplicate.

When EC were treated for up to 2 h with the Hsp90 inhibitor geldanamycin (Piper, 2001; GA; 0.3µM), neither NO donor nor calcium ionophore-stimulated cGMP accumulations were affected. Long-term treatment (24h) of EC with 0.3µM GA resulted in a strong decrease of sGC subunits ($sGC\alpha_1$, 44.5± 4.8% of control, $p<0.01$; $sGC\beta_1$, 36.8±3.9% of control, $p<0.01$). This effect was not specific for EC, but also observed in SMC and PC12 cells. Another Hsp90 inhibitor, radicicol (Piper, 2001; 0.3µM, 24h), had similar effects on sGC protein levels ($sGC\alpha_1$, 40.6±12.6% of control, $p<0.01$; $sGC\beta_1$, 24.0±3.1% of control, $p<0.01$). When we followed the kinetics of both inhibitors, the half-maximal effects were observed after 4.5-5.5h for both inhibitors and both sGC subunits. Effects of Hsp90 inhibitors could not be explained by a decrease in expression of sGC, since the transcription inhibitor, 5,6-dichloro-1-β-D-ribofuranosyl-benzimidazole (100µM; 24h), produced only a slight and insignificant decrease of sGC subunits ($sGC\alpha_1$ 94.7±1.2% of control, $sGC\beta_1$ 84.1±1.5%, of control) which was significantly lower than the one produced by Hsp90 inhibitors ($p<0.05$). These data suggest that effects on gene expression are not responsible for Hsp90 inhibitor effects on sGC. Rather stability of maturated sGC protein appears to be affected. Indeed, the proteasome inhibitor, MG132 (1µM), completely prevented the GA-induced decrease in sGC.

In conclusion, our data demonstrate that Hsp90 is necessary to stabilize both sGC subunits in different types of cells. Inhibition of Hsp90 results in a rapid decrease of sGC protein levels, which is, at least in part, due to degradation along the proteasome pathway.

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36P VITAMIN C MAY MODULATE ENDOTHELIUM-DEPENDENT RELAXATION OF RABBIT AORTIC RINGS VIA DIRECT INTRACELLULAR INTERACTION WITH NITRIC OXIDE

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High concentrations of vitamin C inactivate nitric oxide (NO) through generation of reactive oxygen species. We have previously shown that vitamin C attenuates endothelium-dependent, NO mediated, relaxation of rabbit aortic rings to acetylcholine (ACh). In the present study we investigated the time-course and reversibility of effects of vitamin C on relaxation to authentic NO and ACh to determine whether these are likely to result from a predominantly intracellular or extracellular interaction with NO.

Thoracic aortic rings (2 mm) obtained from New Zealand white male rabbits (2–2.5 Kg) were mounted in organ baths containing oxygenated Krebs' solution at 37°C. Responses were recorded isometrically. Rings were constricted with phenylephrine to 80% maximum tension and then relaxed with ACh or authentic NO to obtain control relaxation responses. Rings were then washed out, incubated with vitamin C (0.1-10 mmol/L, 5-60 min) and contraction and relaxation to ACh or NO repeated. In further studies we examined whether responses to ACh and NO, after incubation with vitamin C (10 mmol/L, 60 min), were restored by washout for 15 min. Concentrations of vitamin C within aortic tissue were estimated by homogenising rings on ice with 5% metaphosphoric acid. Homogenates were centrifuged, frozen in liquid nitrogen and stored at –80°C. Subsequently samples were thawed and vitamin C concentration measured by HPLC.

Vitamin C (0.1-10 mmol/L, 15 min) produced a concentration-dependent attenuation of relaxation to authentic NO with a parallel shift to a higher concentration range of the log dose-response curve of 1.8±0.2 log units at the highest dose (n=5, $P<0.001$). Responses were restored on washout. By contrast, vitamin C produced a concentration- and time-dependent attenuation of relaxation to ACh and responses were not restored on washout. Following 15 min incubation with vitamin C (0.1 to 10 mmol/L), Emax decreased from control from 0.8±3% to 71±7%, (n=4, $P<0.001$ by ANOVA). Increasing incubation time with vitamin C (10 mmol/L) from 5 to 60 min produced progressive attenuation of relaxation to ACh (decrease in Emax from control: 45.1±7.1% at 5 min to 107.9±5.9% at 60 min, n=4, $P<0.001$ by ANOVA). After 60 min incubation with vitamin C (10 mmol/L), a 15 minute washout failed to restore responses to ACh whereas responses to authentic NO were restored. Concentration of vitamin C within tissue homogenates was 0.2±0.06 mmol/L after incubation with vehicle and 3.7±0.8 mmol/L after incubation with vitamin C (10 mmol/L) for 30 min (each n=2).

That the inhibitory effects of vitamin C on authentic NO are abolished by washout suggest that these occur in the extracellular compartment. Dependence of the inhibitory effects of vitamin C on responses to ACh on incubation time and failure of reversal after washout are consistent with intracellular inactivation of NO released by ACh. The preserved response to authentic NO after washout suggests that this occurs within the endothelial cell.

37P ENDOTHELIAL DYSFUNCTION IN A MURINE MODEL OF MILD HYPERHOMOCYSTEINAEMIA: A ROLE FOR EDHF?

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Decreased nitric oxide (NO) bioavailibility secondary to increased oxidative stress is suggested to be the link between elevated plasma homocysteine (Hcy) and endothelial dysfunction. However, most of this evidence comes from *in vitro* studies using suprapathological concentrations of Hcy. In an attempt to overcome this experimental weakness, we have used a murine model of mild hyperhomocysteinaemia (mHHcy) developed by a targeted deletion of the cystathionine β synthase (CBS, an enzyme essential in the clearance of Hcy) gene (Watanabe *et al.*, 1995). The aim of the study was to examine the role of NO in methacholine-induced relaxation in the isolated mesenteric arterial bed.

Male and female (3 month old) heterozygous (+/-) and wild-type (+/+) littermates were killed by CO_2 asphyxiation. Blood samples were taken via cardiac puncture for measurement of plasma total Hcy (tHcy) by HPLC. The mesenteric arterial bed was isolated and perfused (1ml/min) with gassed (95% O_2/5% CO_2) Krebs buffer (with indomethacin 10μM and dextran 0.01% w/v) at 37°C, as described previously for rats (McCulloch *et al.*, 1997). Following 90min equilibration, tissues were perfused for a further 90min in the absence or presence of the NO synthase inhibitor N^G-nitro-L-arginine methyl ester (L-NAME, 300μM). A cocktail of methoxamine (1-30μM) and PGF2α (1μM) was then added to increase perfusion pressure (60-100mmHg). Once a plateau was achieved, bolus doses of methacholine (100pg/10μl - 100μg/10μl) were added close-arterially to assess endothelium-dependent relaxation. The perfusion pressure was then allowed to increase and plateau again before bolus doses of sodium nitroprusside (SNP) (100pg/10μl - 100μg/10μl) were similarly applied to asses endothelium-independent relaxation. Relaxation responses are expressed as a percentage of the methoxamine/PGF2α-induced increase in pressure. All data are expressed as mean±s.e.m. ($n \geq 3$) and maximum relaxation (Rmax) responses compared by ANOVA followed by Tukey's post-hoc test. Significant differences are identified where $P<0.05$.

Plasma levels of tHcy were significantly ($P<0.001$) elevated in +/- (12.8 ± 1.2μM) compared to +/+ (6.3 ± 0.4μM) mice. Methacholine-induced relaxation was significantly ($P<0.01$) reduced in +/- compared to +/+ mice (Rmax=35.0±1.8% cf. 68.9±4.3% respectively). Conversely, tissues from both groups relaxed equally well following exposure to SNP (Rmax=88.1±5.4% for +/- and 91.5±4.7% for +/+). In the presence of L-NAME, the relaxation response to methacholine in +/- mice was almost completely inhibited (Rmax=7.3±2.7%, $P<0.05$ cf +/- in the absence of L-NAME). In +/+ mice in the presence of L-NAME, relaxation responses to methacholine were significantly ($P<0.01$) inhibited by approximately 50% (Rmax=29.5±2.7% cf +/+ in the absence of L-NAME). Again in the presence of L-NAME, tissues from both groups relaxed equally well following exposure to SNP (Rmax=103.6±4.1% for +/- and 116.0±6.5% for +/+).

These data demonstrate impaired endothelial function in the perfused mesenteric arterial bed of +/- CBS-deficient mice in the face of mHHcy. That this relaxation is almost completely inhibited by L-NAME would indicate that it is largely NO-dependent. However, in +/+ mice 50% of the endothelium-dependent relaxation is resistant to L-NAME suggesting the presence of a significant non-NO and non-prostanoid component, such as that due to endothelium-derived hyperpolarizing factor (EDHF). It is therefore possible that the endothelial dysfunction in the +/- mice is due to the loss of the EDHF-like component.

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38P PPAR-γ AGONISTS (ROSIGLITAZONE AND CIGLITAZONE) REDUCE RENAL DYSFUNCTION AND INJURY CAUSED BY ISCHAEMIA/REPERFUSION OF THE RAT KIDNEY

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Peroxisome proliferator-activated receptors (PPARs) are members of the nuclear receptor superfamily of ligand-dependent transcription factors (Evans, 1988). PPAR-γ are expressed in the kidney (Guan & Breyer, 2001) and the PPAR-γ agonist troglitazone has previously been shown to protect against non-diabetic glomerulosclerosis in the rat (Ma *et al.*, 2001). The aim of this study was to investigate the effects of different PPAR-γ agonists [rosiglitazone (ROS) and ciglitazone (CIG)] on the renal dysfunction and injury caused by renal ischaemia/reperfusion (I/R) in the anaesthetised rat.

Forty-one male Wistar rats (220-330 g) were anaesthetised using sodium thiopentone (120 mg kg^{-1} i.p.) of which 21 rats were subjected to bilateral renal ischaemia (45 min) followed by reperfusion (6 h) as described previously (Chatterjee *et al.*, 2000). ROS (3 mg kg^{-1}) and CIG (1 mg kg^{-1}) or vehicle [10 % (v v^{-1}) dimethylsulphoxide, (DMSO)] were administered twice as an i.v. bolus (2 ml kg^{-1}) 5 min before reperfusion and again after 3 h reperfusion. Twenty rats were subjected to Sham-operation and were also administered either ROS (3 mg kg^{-1}), CIG (1 mg kg^{-1}) or 10 % (v v^{-1}) DMSO. Renal dysfunction and injury were assessed by measurement of serum creatinine (sCr), fractional excretion of Na^+ (FE_{Na}) and urinary *N*-acetyl-β-D-glucosaminidase (uNAG) activity. Histological scoring (HS) of renal injury (out of a total of 300) was determined as described previously (Chatterjee *et al.*, 2000) (Table 1).

Table 1	*N*	sCr (μM)	FE_{Na} (%)	uNAG (iu L^{-1})	HS
Sham+DMSO	12	44±2	1±0	3±2	0±0
Sham+ROS	4	43±2	1±0	4±1	0±0
Sham+CIG	4	43±1	1±0	4±1	0±0
I/R+DMSO	7	176±7+	17±1+	32±9+	240±6+
I/R+ROS	7	124±18+	7±2+	14±4+	155±20+
I/R+CIG	7	140±8+	8±1+	10±1+	151±16+

Table 1: Effect of PPAR-γ agonists on biochemical and histological indicators of renal dysfunction and injury. $P<0.05$ *vs.* I/R+DMSO, $+P<0.05$ *vs.* Sham+DMSO. Data are expressed as mean±s.e.mean, analysed using one-way ANOVA followed by Dunnett's *post-hoc* test for multiple comparisons.

Renal I/R produced significant increases in sCr, FE_{Na}, uNAG and HS which were reduced significantly by the PPAR-γ agonists rosiglitazone and ciglitazone (Table 1).

These results demonstrate that adminstration of PPAR-γ agonists during reperfusion can significantly reduce the renal dysfunction and injury caused by I/R of the rat kidney. The mechanisms underlying this beneficial effect and the role of the PPAR-γ in the development of renal I/R injury warrant further investigation.

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39P ROLE OF PPAR-gamma LIGAND ROSIGLITAZONE ON THE DEVELOPMENT OF CARRAGEENAN-INDUCED LUNG INJURY

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Peroxisome proliferator activated receptors (PPARs) are members of the nuclear hormone receptor superfamily of ligand-activated transcription factors (1) that are related to retinoid, steroid and thyroid hormone receptors. The PPAR-γ receptor subtype appears to play a pivotal role in the regulation of cellular proliferation and inflammation. We postulated that rosiglitazone, a syntetic PPAR-γ specific agonist, would attenuate inflammation. In the present study we have investigated the effects of rosiglitazone in animal model of acute inflammation (carrageenan-induced pleurisy) induced by injecting 0.2 ml of a 2% carrageenan solution in the pleural cavity, between the fifth and the sixth intercostals space in anesthetised rats (sodium thiopentone, 120 mg/kg, i.p.). After 4 hours animals have been killed and exudates and lungs collected.
We report here that rosiglitazone given at 10, 30 or 100 μg/kg i.p., 30 minutes prior carrageenan injection exerts potent anti-inflammatory effect (Table 1) (e.g. inhibition of pleural exudate formation, mononuclear cells infiltration and histological injury) in vivo. Furthermore, rosiglitazone reduced: (1) the increase in the staining (immunohistochemistry) for nitrotyrosine and poly (ADP-ribose) polymerase (PARP) and (2) the expression of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) in the lungs of carrageenan treated rats.
To confirm the activity of rosiglitazone on PPAR-γ we have carried out a new set of experiments using a selective antagonist for the PPAR-γ (Bisphenol A Diglicydyl Ether) (2). This compound, given (1 mg/kg i.v.) 30 min before rosiglitazone was able to revert the beneficial effect of the PPAR-γ ligand (Table 1).

Taken together, our results clearly demonstrate that rosiglitazone treatment exerts a protective effect and part of this effect may be due to the inhibition of adhesion molecules expression and peroxynitrite-related pathways with subsequent reduction of neutrophil-mediated cellular injury.

Table 1. Effect of Rosiglitazone on carrageenan-induced inflammation, NO formation and PG production in the pleural exudate. Data are expressed as mean ± s.e. mean of 10 rats for each group. *P<0.01 versus sham. °P<0.01 versus carrageenan.

Table 1	VOLUME EXUDATE (ml)	PMNS INFILTRATION (million cells/rat)	NITRITE/ NITRATE (nmol/rat)	PGE2 (PG/rat)
SHAM + VEHICLE	0.1 ± 0.05	2 ± 0.9	9 ± 1.8	N.D
SHAM + BADGE (30 mg/kg)	0.08 ± 0.06	1.9± 0.8	11± 2.1	N.D
SHAM + ROSI (3 mg/kg)	0.09 ± 0.08	2.2 ± 0.7	11 ± 1	N.D
SHAM + ROSI (10 mg/kg)	0.1 ± 0.04	2.5± 0.95	10± 1.8	N.D
SHAM + ROSI (30 mg/kg)	0.11	2.1	12	N.D
SHAM+ROSI (30 mg/kg) + BADGE (30 mg/kg)	0.14	1.85	10.9	N.D
CAR + VEHICLE	2.00± 0.14*	64 ± 4.4*	130± 3.3*	210 ± 6.3*
CAR + BADGE (30 mg/kg)	1.74 ± 0.1°	67 ± 2.2°	127± 2.2°	200 ± 3.5°
CAR + ROSI (3 mg/kg)	1.2 ± 0.13°	33± 2.6°	100± 2.4°	150 ± 3.1°
CAR + ROSI (10 mg/kg)	1.00	23.7	76	100
CAR+ROSI (30 mg/kg)	0.23	12.8	58	75
CAR+ROSI (30 mg/kg) + BADGE (30 mg/kg)	1.83	61	119	198

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Wright,H.M. *et al.*, (2000) *J Biol Chem*, **275**, 1873-1877

40P PPAR-gamma LIGANDS ATTENUATE THE DEVELOPMENT OF INTESTINAL ISCHEMIA/REPERFUSION

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Peroxisome proliferator activated receptors (PPARs) are members of the nuclear hormone receptor superfamily of ligand-activated transcription factors (1) that are related to retinoid, steroid and thyroid hormone receptors. Activation of the peroxisome proliferator activated receptor-γ (PPAR-γ) regulates cellular proliferation and inflammation. Here we have investigated the effects of the PPAR- γ endogenous ligand cyclopentenone prostaglandin 15-deoxy $\Delta^{12,14}PGJ_2$ and a syntetic PPAR-γ specific agonist, rosiglitazone, in rats subjected to splanchnic artery occlusion (SAO) shock. SAO shock was induced by clamping both mesenteric arteries and celiac artery for 45 minutes followed by 2 hours of reperfusion. Treatment with 15d-PGJ_2 and rosiglitazone (0.3 mg/kg in 10 % v v^{-1} dimethyl sulphoxide in saline, 30 min prior to ischemia), attenuated the fall of mean arterial blood pressure and the migration of polymorphonuclear cells caused by SAO-shock. 15d-PGJ_2 and rosiglitazone also attenuated the ileum injury (histology), the increase in the tissue levels of myeloperoxidase and malondialdehyde, and significantly affected the mortality rate (Table 1). Plasma levels of the pro-inflamatory cytokines TNF-α and IL-1β were significantly reduced by the treatment with the PPAR-γ ligands. The degree of immunostaining for nitrotyrosine and for intercellular adhesion molecule-1 (ICAM-1) was markedly reduced in tissue sections obtained from SAO-shocked rats treated with 15d-PGJ_2 or rosiglitazone.
15d-PGJ_2 or rosiglitazone treatment significantly improved survival.
To confirm the activity of the ligands on PPAR-γ we have carried out a new set of experiments using a selective antagonist for the PPAR-γ (Bisphenol A Diglicydyl Ether) (2). This compound, given (1 mg/kg i.v.) 30 min before 15d-PGJ_2 or rosiglitazone was able to revert the beneficial effect of both the PPAR-γ ligands. Taken together, our results clearly demonstrate that PPAR-gamma ligands 15d-PGJ_2 and rosiglitazone treatment exerts a protective effect and part of this effect may be due to the inhibition of adhesion molecules expression and peroxynitrite-related pathways with subsequent reduction of neutrophil-mediated cellular injury.

Table 1. Effect of the PPARγ agonist on survival rate, percentage survival, and survival time in sham shocked rats or splanchnic artery occlusion (SAO) shocked rats (I/R). *P< 0.01 vs Sham; ^{0} P< 0.01 vs I/R.

	Time after reperfusion: 2 hours		
Treatment	survivors	% surviving	Survival time (min)
Sham + vehicle	10/10	100	>120
Sham + 15d-PGJ_2	10/10	100	>120
Sham + rosiglitazone	10/10	100	>120
Sham + BADGE	10/10	100	>120
Sham + BADGE + 15d-PGJ_2	10/10	100	>120
Sham + BADGE + rosiglitazone	10/10	100	>120
I/R + vehicle	4/10	36	90 ± 2.7*
I/R + 15d- PGJ_2	10/10	100	>120
I/R + rosiglitazone	10/10	100	>120
I/R + BADGE + 15d-PGJ_2	10/10	100	>120
I/R + BADGE + rosiglitazone	7/10	70	108 ± 1.5*

1) Evans, R.M. (1988) *Science,* **240**, 889-895.

2) Wright,H.M. et al., (2000) *J Biol Chem*, **275**, 1873-1877

41P ROLE OF OXIDATIVE STRESS IN THE REGULATION OF iNOS AND ARGINASE IN RAT ALVEOLAR MACROPHAGES

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L-Arginine is substrate of NO synthase and arginase, pathways of particular importance in macrophages. Arginase can limit L-arginine supply for NO synthase (Hey *et al.*, 1997) and may be involved in the development of airway hyperreactivity (Meurs *et al.*, 2000). Since oxidative stress, which is associated with acute inflammatory reactions, is known to be involved in the regulation of the expression of a number of genes, its possible role in the regulation of arginase and iNOS in rat alveolar macrophages (AM) was studied.

Rat AM were cultured for 1 to 20 h in the absence or presence of 1 µg/ml lipopolysaccharides (LPS) and/or apocynin (an inhibitor of NADPH oxidase) or other test substances as described previously (Hey *et al.*, 1995). Thereafter, arginase activity was determined or RNA was isolated for the use in RT-PCR (Mössner *et al.*, 2001; Klasen *et al.*, 2001). In addition, superoxide generation was measured by determination of the reduction of iodonitrotetrazolium violet (during 1 h incubation) to iodonitrotetrazolium (INT) formazan.

AM exposed to LPS showed a rapid increase in INT formazan formation (by 50 ± 7% after 1 h, mean ± s.e.mean, n=16), which was followed by a decline below initial values when exposed for up to 20 h (by 40 ± 5% at 20 h). Apocynin reduced basal INT formazan formation by about 25% and prevented the LPS induced increase.

Arginase activity in AM cultured in absence of LPS was 31 ± 4 mU $(10^6 \text{ cells})^{-1}$ and presence of LPS resulted in an increase by 115 ± 11%. Apocynin (500 µM) reduced basal arginase activity by 54 ± 9% and largely attenuated the LPS-mediated increase.

Table 1: Effects of LPS (1 µg/ml) and/or apocynin (500 µM) on iNOS and arginase I mRNA in rat AM.

	iNOS		Arginase I	
Culture time	5 h	20 h	5 h	20 h
Controls	100	100	100	100
Apocynin	87±16	56±10*	49±4*	41±6*
LPS	688±108*	2073±456*	325±52*	1157±257*
LPS + Apocynin	344±14#	485±381#	178±38#	256±95#

Given are means±s.e.mean of the ratios of the optical density of the PCR bands of test mRNA / ß-actin mRNA (n≥4), expressed as % of resp. control. *P<0.01 vs respective control; #P<0.01 vs resp. value with LPS alone.

In conclusion, in rat AM the expression of arginase I mRNA, like that of iNOS mRNA, is highly sensitive to oxidative stress. The inhibitory effect of apocynin on LPS-induced increase in arginase I and iNOS expression suggests that oxygen radicals may play a role as cellular signals in the transmission of the effects of LPS.

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Hey C. *et al.* (1997) *Br. J. Pharmacol.*, 121, 395-400.
Klasen, S. *et al.* (2001) *Br. J. Pharmacol.*, 132, 1349-1357.
Mössner, J. *et al.* (2001) *Pulm. Pharmacol. Ther.*, 14, 297-305.
Meurs, H. *et al.* (2000) *Br. J. Pharmacol.*, 130, 1793-1798.

42P EFFECT OF NON-STEROIDAL ANTI-INFLAMMATORY DRUGS ON MACROPHAGE ACTIVATION FOLLOWING PLATELET PHAGOCYTOSIS

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Background. Epidemiological studies have demonstrated a reduced prevalence of Alzheimer's disease among users of non-steroidal anti-inflammatory drugs (NSAIDs). Moreover, it was shown recently that indomethacin, ibuprofen and sulindac sulphide can subtly alter the γ-secretase cleaving site of the amyloid precursor protein (APP), resulting in a reduced production of the most toxic form of amyloid-β, $A\beta_{1-42}$ (Weggen *et al.*, 2001). APP and its processing have been studied almost exclusively in brain tissue in Alzheimer's disease. Recently, however, we showed for the first time that APP and Aβ are also present in macrophages around microvessels of advanced human atherosclerotic plaques (De Meyer *et al.*, 2002). A possible source for APP and Aβ in atherosclerotic plaques are platelets, which contain APP in the α-granules. Platelet phagocytosis in human atherosclerotic plaques was associated with Aβ-production and the induction of inducible nitric oxide synthase (iNOS) in macrophages, which may predispose to plaque destabilisation and rupture.

Aim. In the present study, we investigated whether iNOS induction in macrophages evoked by platelet phagocytosis can be inhibited by NSAIDs.

Methods. Interferon-γ washed primed murine J774 macrophages were incubated with human washed blood platelets for 18h with or without different NSAIDs. Nitrite was quantified in the supernatant (Griess reaction) as a measure for iNOS activity. *Results.* Indomethacin (Ind, n=6), ibuprofen (Ibu, n=4), sulindac sulphide (Sul, n=3) and meloxicam (Mel, n=3) concentration-dependently reduced nitrite production by macrophages incubated with human platelets, whereas acetylsalicylic acid (Asa, n=6) and naproxen (Nap, n=9) were inactive (Table 1). Nitrite production by macrophages exposed to lipopolysaccharide, an alternative stimulus for iNOS induction, was not altered by any NSAID.

	µM	Control	NSAID		µM	Control	NSAID
Ind	10	6.2± 0.8	6.3± 0.4	Mel	10	8.1± 0.3	8.1± 0.5
	50	4.9± 0.7	3.2± 0.3*		30	9.0± 0.6	5.6± 0.3**
	100	4.4± 0.7	1.9± 0.2*		50	9.0± 0.5	3.9± 0.1***
Ibu	100	6.3± 0.6	5.9± 0.3	Asa	50	7.9± 0.4	6.6± 1.2
	300	6.5± 0.4	4.5± 0.2**		250	8.0± 0.3	8.8± 0.6
	500	6.4± 0.4	3.8± 0.8*		500	8.5± 0.6	10.0± 1.3
Sul	1	6.6± 1.2	5.7± 0.3	Nap	100	2.0± 0.8	3.1± 1.0
	3	5.4± 0.4	4.8± 0.3		200	2.5± 1.1	3.7± 1.2
	10	5.0± 0.2	1.9±0.1***		300	2.2± 1.0	3.0± 0.9

Table 1: Effect of NSAIDs on nitrite production (mean ± s.e.m.) by macrophages after platelet phagocytosis. * P < 0.05; ** P < 0.01; *** P < 0.001 (unpaired Student's t-test).

Conclusions. Besides meloxicam, the same subset of NSAIDs reported to alter the γ-secretase cleaving site of APP reduced nitrite production (Weggen *et al.*,2001). Our results suggest that some NSAIDs, by altering Aβ production, may affect iNOS expression in macrophages during platelet phagocytosis.

De Meyer *et al.* Circ. Res. (2002) 90, 1197-1204
Weggen *et al.* Nature (2001) 414, 212-216

43P HISTAMINE INDUCES SHAPE CHANGE AND ACTIN POLYMERISATION IN HUMAN EOSINOPHILS VIA THE NOVEL H_4 RECEPTOR

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Eosinophils are major effector cells in the immune system with a beneficial role in immune defence against parasitic helminths but they can also be damaging as part of the inflammatory process of allergic diseases such as asthma (Arm and Lee 1992). The pro-inflammatory mediator histamine is released from mast cells at the site of inflammation and its actions through existing receptors H_1, H_2 and H_3 are well characterised. A histamine H_4 receptor was recently discovered, the function of which is unknown (Oda et al 2000). The H_4 receptor is expressed on human eosinophils and may therefore represent a novel therapeutic target for the treatment of allergic disease. The aim of this study was to determine the function of the H_4 receptor on eosinophils.

Polymorphonuclear leukocytes (PMNL) and eosinophils (purity >95%) were prepared from human peripheral blood for assays of eosinophil shape change (Sabroe et al., 1999) and actin polymerisation (Dichmann et al., 2001), both measured by flow cytometry. Data are expressed as mean ± s.e.mean percent increases in either forward light scatter (FSC) or fluorescence compared to unstimulated cells (n=5 donors).

The eosinophils, but not the neutrophils, in the PMNL cell preparation responded to histamine (0.004-2μM) with an increase in FSC detected in the shape change assay. This eosinophil shape change response to histamine was concentration-dependent with a maximal response at 1μM histamine (47 ± 5% increase in FSC compared to unstimulated cells). This histamine-induced eosinophil shape change was completely abolished by thioperamide (10μM), a H_3/H_4 receptor antagonist but was not inhibited by pyrilamine and cimetidine (10μM), H_1 and H_2 receptor antagonists, respectively. The H_3 receptor antagonists clobenpropit and clozapine (0.004-2μM) that are also H_4 receptor agonists both induced eosinophil shape change (37 ± 4% and 33 ± 7% maximum increases in FSC respectively), which was inhibited by thioperamide (10μM). The H_3/H_4 receptor agonists imetit, R-α-methyl histamine and N-α-methyl histamine (0.004-2μM) also induced eosinophil shape change (50 ± 2%, 37 ± 3% and 21 ± 3% maximum increases, respectively). Furthermore, pertussis toxin (1μg/ml) completely inhibited shape change responses induced by histamine showing that these effects are mediated by coupling to a $G_{i/o}$ protein. In addition, histamine (0.015-100μM) induced actin polymerisation in eosinophils (54 ± 6% increase in fluorescence at 10μM), which was inhibited by thioperamide (10μM) but not by pyrilamine or cimetidine (10μM). However histamine (0.1-3μM) did not induce chemotaxis or chemokinesis of human eosinophils.

We conclude that histamine stimulates shape change and actin polymerisation of human eosinophils via the novel H_4 receptor. This study demonstrates that the H_4 receptor is involved in eosinophil function and therefore may provide a novel target for allergic disease therapy.

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44P CIGARETTE SMOKE EXTRACT (CSE) STIMULATES HUMAN THP-1 MONOCYTES DIRECTLY AND SYNERGISES WITH IL-1β TO RELEASE IL-8

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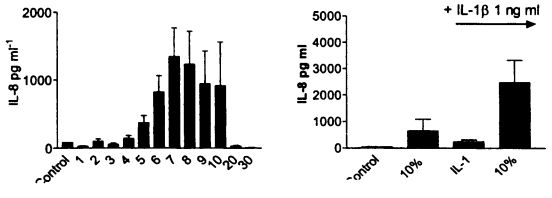
COPD is a leading cause of morbidity and mortality in Europe and the US (ranked 3rd and 4th respectively). The main risk factors for COPD are smoking and occupational exposure to particulate matter. COPD is characterised by the recruitment of inflammatory cells, into the lung (Jeffery, 1998) with macrophages constituting 95-98%of this population and thought to play a key role in the pathology of COPD (Shapiro, 1999). Cigarette smoke directly activates lung macrophages and so can be used as a model to study events and drugs relevant to COPD. However, such models remain poorly characterised. Here we have compared the ability of different inflammatory insults with that of cigarette smoke to stimulate the release of IL-8 from a human monocyte/macrophage cell line (THP-1).

For 100% solutions, cigarette smoke was drawn from four cigarettes (full strength Marlboro) through 100mls of RPMI 1640 to produce our strongest smoke solution. The 'strength' of each smoke extract made was assessed by measuring nitrite levels using the Greiss reaction (Bishop-Bailey et al., 1997). LPS is a known contaminate of cigarette smoke and stimulates our cells to release IL-8 at a threshold concentration of 10μg ml^{-1}. LPS content of smoke extract was measured using the limulus test, in accordance with the manufactures instructions (Sigma, UK) and found to be undetectable in our 100% solutions (<100pg ml^{-1}; from 2 separate extracts).

Cigarette smoke solutions stimulated THP-1 cells to release IL-8,at 24hrs, in a bell-shaped concentration dependent fashion (Figure a; n=9). IL-1 β(1ng ml^{-1}) also stimulated THP-1 cells to similar levels of IL-8 at 24hrs (Figure b; n=9; *=p<0.05, *one-way ANOVA*). When IL-1β (1ng ml^{-1}) and cigarette smoke solution (10%) were added simultaneously to cells a synergistic release of IL-8 was observed at 24hrs (Figure b; n=9). Cell viability at 10% cigarette smoke solution was not significantly decreased.

Figure a **Figure b**



Here we show that cigarette smoke directly activates, and then at higher concentrations, inhibits the release of IL-8 from THP-1 monocytes. We also show a powerful synergy between cigarette smoke and IL-1β in stimulating these cells. These observations suggest that this model may be of potential use in the search for new therapeutic agents to treat COPD.

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45P IN VIVO EFFECTS OF SAR 943, A RAPAMYCIN ANALOGUE, IN A MURINE MODEL OF AIRWAY INFLAMMATION AND REMODELING

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No current therapy is considered to be satisfactory for severe asthma and alternative approaches are required for what is still a major unmet medical need. In this study we compared the effect of a rapamycin derivative, SAR 943 (32-deoxorapamycin) with the clinically used steroid, budesonide, using a murine model of lung inflammation and remodelling.

All the procedures have been previously described by this laboratory (Trifilieff *et al.*, 2000). On day 0 actively sensitised female BALB/c mice (5 weeks old) were exposed to an aerosol of ovalbumin (OVA) or phosphate buffered saline, (PBS). Airway reactivity to methacholine (0.1 M) was measured on day 1. Bronchoalveolar lavage was performed for cytokine levels (day 1), differential cell counts (day 2) and cellular fibronectin levels (day 2) determination. On day 7, fixed lungs were embedded in paraffin and epithelial cell proliferation and mucus hypersecretory phenotype assessed.

Animals (n = 6-10) were dosed intranasally, under halothane/oxygen/nitrous oxide anaesthesia, with either test compound or vehicle (50 µl of 2% dimethyl sulfoxide in PBS) 1 h before and 24 h after the aerosol challenge. Statistical significance ($P < 0.05$) was determined using a Kruskal Wallis test with Bonferroni correction for multiple comparisons. Data are expressed as mean ± s. e. mean.. OVA challenge induced a significant lung inflammation (IL-5 and IL-4 production, infiltration of eosinophils, neutrophils and lymphocytes) and remodelling (cellular fibronectin production, lung epithelial cell proliferation and mucus hypersecretory phenotype as well as hyperreactivity to methacholine). SAR 943 and budesonide, were equipotent in inhibiting all these parameters (Table 1).

In conclusion, SAR 943 is as effective as budesonide in inhibiting both lung inflammation and remodelling in a murine model of asthma. Hence, this class of compound could offer beneficial effects in patients with severe asthma.

Trifilieff *et al.*, 2000, *J. Am. Physiol.*, 279, L1120-L1128.

Table 1: Effect of SAR 943 and budesonide (Bud) on OVA-induced lung inflammation and remodelling.

	IL-5 pg ml^{-1}	IL-4 pg ml^{-1}	Eosinophils 10^3 cell ml^{-1}	Neutrophils 10^3 cell ml^{-1}	Lymphocytes 10^3 cell ml^{-1}	Fibronectin ng ml^{-1}	Epithelium proliferation % of cells	Epithelium mucus % of cells	Airway reactivity Penh
PBS	0 ± 0*	5 ± 1*	0 ± 0*	0 ± 0*	0.6 ± 0.4*	6 ± 1*	11 ± 1*	3 ± 1*	0.7 ± 0.1*
OVA	48 ± 4	194 ± 10	74 ± 17	15 ± 2	11 ± 2	58 ± 2	28 ± 2	69 ± 2	2.3 ± 0.1
SAR 943 (0.1)	36 ± 3	124 ± 12	50 ± 15	14 ± 1	6.3 ± 1.2	49 ± 3	23 ± 2	61 ± 3	1.6 ± 0.1
SAR 943 (1)	18 ± 1*	78 ± 7*	17 ±3*	8.8 ± 1.2	3.6 ± 0.7*	26 ± 2*	16 ± 1*	29 ± 6*	1.1 ± 0.1*
Bud (1)	21 ± 2*	88 ± 4*	19 ± 6*	15 ± 3	4.2 ± 0.7*	43 ± 3	14 ± 1*	35 ± 8*	1.3 ± 0.1*
Bud (3)	7 ± 1*	18 ± 2*	2.6 ± 0.9*	2.6 ± 0.3*	2.1 ± 0.5*	11 ± 2*	11 ± 1*	15 ± 3*	0.7 ± 0.1*

(Doses in mg kg^{-1}). * $P < 0.05$ when compared with OVA-challenged animals

46P FURTHER DEFINITION OF THE MECHANISM OF THE CONTRACTILE RESPONSE TO ADENOSINE ON LUNG PARENCHYMAL STRIPS FROM ACTIVELY SENSITISED, ALLERGEN CHALLENGED, BROWN NORWAY RATS

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Parenchymal strips removed from actively sensitised (AS) Brown Norway (BN) rats challenged with allergen show hyperresponsiveness to adenosine (Ado) (Hannon et al., (2001). The augmented response is mast cell mediated and a preliminary pharmacological analysis suggested involvement of a receptor which could not be classified as any of the known Ado receptor subtypes (Hannon et al., 2002). We have now explored the possibility of an intracellular site of action for Ado using NBTI, a blocker of facilitated Ado transport. We have also evaluated the effects of four recognised Ado receptor antagonists on the responses to Ado to define further the pharmacology of this atypical receptor.

Parenchymal strips were prepared from the lungs of AS, BN rats challenged i.t. with ovalbumin (0.3 mg kg^{-1}) 3 h prior to death (Hannon et al., 2002). Only one agonist response was generated per strip. and the sensitivity to 5-HT was routinely measured. NBTI and the antagonists were incubated with the tissue for 30 min prior to challenge with Ado.

Ado (0.1-1 mM) induced concentration-related contractile responses (Figure 1). NBTI (100 nM) shifted the concentration-response curve to Ado to the left by 3-fold but had no effect on responses to 5-HT (0.1-100 µM) or NECA (0.01-0.1 mM). The response to Ado (1 mM) was blocked concentration-dependently by 8-SPT, CGS 15943, ZM 241385 and XAC (Figure 1). However, blockade was seen only at relatively high concentrations and in the case of CGS 15943 and ZM 241385 appeared to plateau at ca. 3-fold blockade.

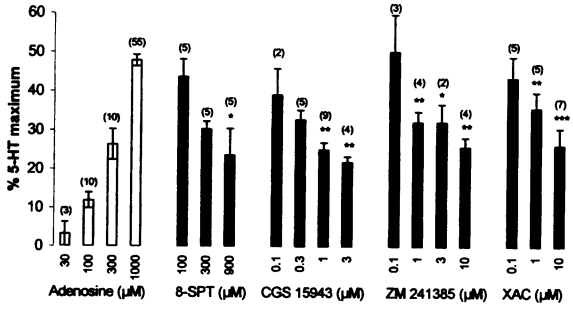

Figure 1 Concentration-response curve to Ado and the effects of Ado receptor antagonists on responses to Ado (1 mM) on lung parenchymal strips from AS, OA challenged BN rats. Responses represent means ± s. e. mean of the numbers of experiments shown above the columns. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ that the value differs significantly from its paired control value (Student's t-test).

Thus, Ado does not require to enter the cell by facilitated transport to induce contraction of the parenchymal strip. Although blockade is seen with all the antagonists, comparison of the concentrations required with their affinities for the rat ado receptor subtypes (Fredholm et al., 2001; Fozard et al., this meeting) suggests that the receptor mediating the contractile response to ado cannot be categorised as one of the four recognised adenosine receptor subtypes.

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47P ILOPROST INHIBITS U46619-INDUCED SUPEROXIDE FORMATION AND NADPH OXIDASE EXPRESSION IN CULTURED PORCINE PULMONARY ARTERY VASCULAR SMOOTH MUSCLE CELLS

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In adult respiratory distress syndrome (ARDS) platelets adhere to the pulmonary vasculature and release thromboxane A_2 (TXA_2) (Jeremy et al, 1994). Other vasoconstrictors upregulate NADPH oxidase expression, a major source of O_2^- which augments ARDS (Chabot et al, 1998). The objective of this study was to determine whether TXA_2 (± endotoxin [LPS] and cytokines) promote O_2^- formation and the expression of NADPH oxidase in pig pulmonary artery vascular smooth muscle cells (PAVSMCs). The effect of endotoxin and cytokines on TXA_2 and prostacyclin (PGI_2) formation was also studied, as well as effects of the PGI_2 analogue, iloprost.

PAVSMCs were incubated with U46619 (± LPS, TNFα or IL-1α: all ± iloprost) and O_2^- measured using the reduction of ferricytochrome-c and the expression of NAPDH oxidase using Western blotting. Segments of pig pulmonary artery were incubated with LPS, TNF α, IL-α for 16h and TXA_2 and PGI_2 formation measured with ELISA.

U46619 stimulated the formation of O_2^- by PAVSMC, an effect augmented by LPS, TNFα and IL-1α (table 1) and inhibited by diphenyleneiodonium chloride, indicating that the source of O_2^- was NADPH oxidase. Iloprost also inhibited these effects (table 1). Analysis of the expression of NADPH oxidase using Western blotting confirmed these observations. LPS, TNFα and IL1α augmented TXA_2 formation but inhibited that of PGI_2 (Table 2).

Table 1. Effect of U46619 (U4; 10 nM) ± LPS (10 μg / ml), TNF α (10 ng / ml) or IL-1α (10 ng / ml) and ± iloprost (100 ng/ml) on O_2^- formation by cultured PAVSMCs (μmoles / mg protein / h [mean ± SEM, n =6]) following a 16 h incubation.
* p< 0.05 comparing controls vs treated without iloprost and **p < 0.05 comparing iloprost vs control groups (student's t test).

	Control	U4	U4+TNFα	U4+IL1α	U4+ LPS
- iloprost:	10.3±1.6	20.2±1.9*	28.9±1.6*	33.0 ±1.7*	26.5±1.4*
+ iloprost:	8.9±1.6	12.6±1.8**	13.9±1.4**	16.1 ±1.3**	13.7±1.3**

Table 2. Effect of LPS (10 μg / ml), TNF α (10 ng / ml) and IL-1α (10 ng / ml) on the formation of TXA_2 (as TXB_2) and PGI_2 (as 6-keto-$PGF_{1\alpha}$) [pg / mg tissue / min] by PAVSMCs (mean ± SEM, n =6). *p < 0.05 controls vs treated groups (student's t test)

	Control	LPS	TNF α	IL-1α
TXB_2	11 ± 1.4	19 ± 1.6*	24 ± 1.4*	22 ± 1.9*
6-keto-$PGF_{1\alpha}$	199 ± 0.6	94 ± 0.8*	90 ± 0.7*	65 ± 3*

These data demonstrate that endotoxin and cytokines markedly alter the ratio of TXA_2:PGI_2 formation in PAVSMCs. Since U44619 upregulates NAPDH oxidase and O_2^- formation, an effect inhibited by iloprost but augmented by LPS and cytokines, it is suggested that: 1) the differential alterations of TXA_2 and PGI_2 formation play an aetiological role in ARDS and 2) the therapeutic impact of TXA_2 receptor antagonists and PGI_2 in ARDS may be augmented by the pharmacological inhibition of O_2^- formation.

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48P NITROASPIRINS AND SIN-1, BUT NOT ASPIRIN, INHIBIT THE EXPRESSION OF ENDOTOXIN- AND CYTOKINE-INDUCED NAPDH OXIDASE IN VASCULAR SMOOTH MUSCLE CELLS FROM PIG PULMONARY ARTERIES

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Adult respiratory distress syndrome (ARDS) is a difficult condition to treat, conventional NSAIDs being ineffective. Superoxide (O_2^-) formation, which promotes inflammation and the negation of NO bioactivity, is central to ARDS (Chabot et al, 1998). NO has been used to treat ARDS (Stuart-Smith & Jeremy, 2001). The inhibition of O_2^- formation and the administration of NO may be a means of treating ARDS. In order to test this proposal, the effects of nitro-aspirin (NCX 4016 and NCX 4050 [Del Soldato et al, 1999) on O_2^- formation and NADPH oxidase expression by pig pulmonary artery vascular smooth muscle cells (PAVSMCS) compared to the effects of the NO donor, SIN-1 and aspirin (ASA) alone was investigated.

Cultured PAVSMCs were incubated with LPS, TNF α, IL-1α (± NO-ASA, SIN–1 or ASA) for 16h and O_2^- release measured using the reduction of ferricytochrome-c. In parallel, the expression of $gp91^{phox}$, an active catalytic subunit of NAPDH oxidase, was assessed using Western blotting.

LPS, TNF α and IL-1α all stimulated the formation of O_2^- in PAVSMCs (table 1), an effect inhibited by diphenylene--iodonium chloride (DPI), indicating that the source of O_2^- was NADPH oxidase. SIN-1, NCX 4016 and NCX 4050, but not ASA alone, inhibited the formation of O_2^- in PAVSMCs (table 1) as well as the expression of $gp91^{phox}$. The guanylyl cyclase inhibitor, ODQ, completely reversed these inhibitory effects of NCX 4106, NCX 4050 and SIN-1.

Table 1. Effect of NCX 4016 (100nM), NCX 4050 (100nM), SIN-1 (1μM) and ASA (10μM) on O_2^- formation induced by LPS (10 μg / ml), TNF α (10 ng / ml) or IL-1α (10 ng/ml) in cultured PAVSMCs (μmoles / mg protein / hour [mean ± SEM, n = 6]) following a 16 hour incubation. *p < 0.05 treated vs controls (student's t test)

	Control	LPS	TNF α	IL-1α
Control (vehicle)	10.1 ± 0.8	15.4 ± 0.6	20.7 ± 0.9	20.9 ± 0.7
NCX 4016	7.8 ± 0.6	11.0 ± 0.8*	11.3 ± 0.7*	11.2 ± 0.8*
NCX 4050	8.3 ± 0.4	10.2 ± 0.5*	11.7 ± 1.0*	11.8 ± 0.7*
SIN-1	8.0 ± 0.9	8.4 ± 0.6*	12.3 ± 0.7*	11.3 ± 0.4*
ASA	9.1 ± 0.5	14.5 ± 1.0	18.5 ± 1.2	9.8 ± 0.7

These data demonstrate that LPS and cytokines promote the formation of O_2^- in PAVSMCs through the upregulation of NADPH oxidase, which in turn is down-regulated by NO. The negation of NO by O_2^- may precipitate a self- perpetuating cascade that augments the progress of ARDS. The potent inhibitory effects of NO-ASA appear to be mediated by the NO-releasing capacity and not through the ASA moiety. NO-ASA may be useful in treating not only ARDS but also pathologies associated with oxidative stress.

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49P INDUCTION OF HEME OXYGENASE-1 IN THE PORCINE LUNG AFTER BIRTH

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The increase in pulmonary blood flow at birth results in profound physiological changes including vascular remodelling and the maturation of the endothelium-dependent relaxant response (Haworth, 1995). When such adaptive processes fail persistent pulmonary hypertension of the newborn (PPHN) may occur. Carbon monoxide (CO), produced endogenously from heme catabolism by heme oxygenase (HO), is reported to cause vasodilation in a number of vascular beds and influence vascular remodelling. Thus, we have investigated the expression and function of HO in the porcine lung in the adaptive period after birth.

Lungs were harvested from fetal, newborn (NB), 1-, 3- and 14-day-old piglets. Western blotting was performed for the inducible form of HO (HO-1), and the constitutive form (HO-2). Rings of intrapulmonary artery (IPA) from 14-day-old pigs were mounted in organ baths under 1g of tension and, after a 1 hour equilibration period, precontracted to ~75% maximum (due to KCl, 125mM) with the thromboxane mimetic U46619 ($1x10^{-8}$-$1x10^{-7}$M). Preparations were then incubated in the presence or absence of the nitric oxide synthase inhibitor, L-NAME ($1x10^{-4}$M) or the inhibitors of HO activity, chromium mesoporphyrin (ChMP, $5x10^{-6}$M) and tin protoporphyrin (SnPP: $1x10^{-5}$M). Experiments were also carried out in the presence of the NaOH vehicle. After a 30-minute equilibration period, cumulative concentration response curves were constructed to acetylcholine (ACh: $1x10^{-9}$-$1x10^{-4}$M).

Levels of HO-1 were low or undetectable in lung tissue from fetal animals but increased after birth (Fig. A), n=3.

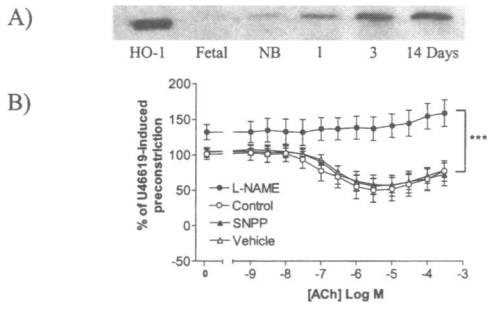

Figure A) Representative blot showing the increase in HO-1 protein in porcine lung after birth. B) Effect of SnPP and L-NAME on tone of preconstricted porcine IPA rings from 14-day old pigs and on dilator responses to ACh, mean ± s.e.m., n=8 from n=4 animals. L-NAME vs. control: ***P<0.0001, Two-way ANOVA.

HO-2 protein was expressed in the fetal lung tissue and levels remained unchanged across the age groups (data not shown, n=3). Where L-NAME caused an increase in the tone of porcine IPA ring preparations, SnPP and vehicle had no effect (Fig. B) and ChMP actually reduced tone (vehicle vs. ChMP: 105.4±7.7 vs. 59.4±10.4 % of U46619-induced tone, P<0.001 Mann-Whitney, n=8 from n=4 animals). L-NAME, but not SnPP, ChMP or vehicle inhibited ACh-induced relaxation.

HO-1 is induced in the porcine lung after birth. This upregulation does not appear to play a role in the regulation of vascular tone and the increase in pulmonary blood flow that occurs following the transition from placental- to air-breathing. The role of HO-1 induction on the remodelling of the pulmonary vasculature after birth remains to be elucidated.

Haworth S.G. (1995). *Exp. Physiol.* **80,** 843-53.

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50P HEME OXYGENASE-1 DEFICIENT MICE DO NOT APPEAR TO HAVE PULMONARY HYPERTENSION

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Heme oxygenase (HO) catalyses the conversion of heme to carbon monoxide (CO), free iron and biliverdin. There are two major forms of HO, HO-2 is expressed constitutively whilst HO-1 is induced by oxidative stress. CO is known to cause vasodilatation in many vascular beds. At the current meeting we show that HO-1, but not HO-2, is upregulated in the porcine lung during the transition from placental- to air-breathing and that its activity does not impact on vasomotor responses. The HO/CO pathway also has anti-proliferative and anti-apoptotic actions. Indeed, HO-1 expression protects against the development of hypoxia-induced pulmonary hypertension (Minamino *et al.*, 2001). Thus, in the current study we have used genetically modified mice lacking the HO-1 gene (Duckers *et al.*, 2001), to investigate the role of this enzyme in pulmonary vessel remodelling and hypertension.

Lungs were harvested from 1-, 4-, 10-, 14-day-old and male adult mice. Western blotting was performed for HO-1 and HO-2. In other experiments, lungs from adult (24±4 weeks) male wild type (C57BL/6J) and adult (25.5±4 weeks) male HO-$1^{-/-}$ mice were inflated and fixed in 10% formal saline overnight. Tissue was transferred to 70% alcohol prior to embedding in paraffin wax for immunohistochemistry. Sections (4μM) were stained for smooth muscle α-actin. External and internal pulmonary artery diameters were measured and %medial wall thickness calculated. All data is given as mean ± s.e.m.

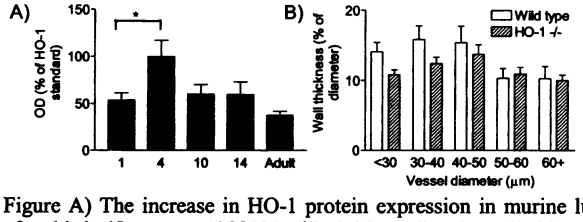

Figure A) The increase in HO-1 protein expression in murine lung after birth (One-way ANOVA: *P<0.05, n=4). B) Wall thickness (expressed as a % of external vessel wall diameter) of pulmonary arteries from wild type (n=4) vs. HO-$1^{-/-}$ (n=6) mice.

The weight ratio of right ventricle (RV) to left ventricle (LV) + septum (S)(RV/LV+S) was also calculated.

HO-1 was upregulated in the murine lung after birth (Fig. A). HO-2 expression was present at comparable levels in all age groups (data not shown, n=4). In adult mice, medial wall thickness (Fig. B) and RV/LV+S (0.26±0.04 vs. 0.25±0.01) were not significantly different (Two-way ANOVA and Mann-Whitney respectively) in wild type vs. HO-$1^{-/-}$ animals.

HO-1, but not HO-2, is upregulated in the lung after birth. Pulmonary hypertension is associated with structural changes in the pulmonary vasculature and the right ventricle. However in adult mice lacking the HO-1 gene we found no evidence of pulmonary hypertension as assessed by pulmonary arterial wall thickness or the weight ratio of RV to LV+S. A role for HO-1 in the adaptation of the pulmonary arteries after birth is currently the subject of investigation.

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51P HEME OXYGENASE IS EXPRESSED IN HUMAN PULMONARY ARTERY SMOOTH MUSCLE WHERE CARBON MONOXIDE HAS AN ANTI-PROLIFERATIVE ROLE

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Heme oxygenase (HO) catalyses the conversion of heme to carbon monoxide (CO), free iron and biliverdin. HO exists in 2 major isoforms. HO-2 is the constitutive form of the enzyme and HO-1 is the inducible form, expressed at sites of inflammation and oxidative stress. Little is known about the role of HO/CO in the pulmonary circulation. Thus, we have investigated the expression of HO in human pulmonary artery smooth muscle cells (PASMCs) and studied the effects of a carbon monoxide-releasing molecule or CO-RM (Motterlini *et al.*, 2002) on the proliferation of these cells in culture.

Pulmonary artery was cultured as described previously (Wort *et al.*, 2001). Confluent PASMCs were treated for 24 h with culture medium alone (5% fetal calf serum, FCS) or medium containing lipopolysaccharide (LPS, 1μg/ml) or sodium nitroprusside (SNP, 1mM) or hemoglobin (1mg/ml). Western blotting was performed to determine the levels of HO-1 and HO-2 protein. In other experiments, cells were incubated for 24 hours with serum-free culture medium or medium containing 3% FCS in the presence or absence of increasing concentrations of either SNP (1-100μM) or the CO-RM, tricarbonyldichlororuthenium (II) dimer (1-100μM). Methyl-[^{3}H]thymidine was then added to cells for a further 6 hours. DNA incorporating labelled thymidine was harvested and radioactivity measured as an index of proliferation.

Under basal conditions PASMCs did not express HO-1. SNP and hemoglobin, but not LPS, induced HO-1 protein (Fig. A).

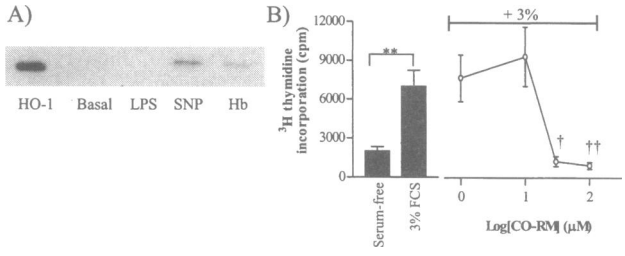

Figure A) Representative Western blot for HO-1 in human PASMCs treated with LPS, SNP or hemoglobin (Hb). B) Effect of the CO-RM, tricarbonyldichlororuthenium (II) dimer on PASMC proliferation. Incorporation of ^{3}H was expressed in counts per minute (cpm), mean ± s.e.m., n=9. **P<0.01 Mann-Whitney. †P<0.05, ††P<0.01 Kruskal-Wallis (vs. 3% FCS), post-test Dunn's.

Under basal conditions PASMCs expressed HO-2 (data not shown, n=3); levels were unchanged by treatments. Proliferation was inhibited by SNP (data not shown) and tricarbonyldichlororuthenium (II) dimer (Fig. B).

Here we show that PASMCs in culture express HO-2 under basal conditions and HO-1 under conditions of oxidative stress. Furthermore we demonstrate that a CO-RM potently and effectively inhibits proliferation of these cells. Pulmonary hypertension is characterised by chronic and progressive remodelling of pulmonary vessels. Our data suggests that agents that manipulate the HO-CO pathway may have novel therapeutic potential in the treatment of this disease.

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52P METABOLISM OF ASYMMETRIC DIMETHYLARGININES IS REGULATED IN THE LUNG DEVELOPMENTALLY AND WITH PULMONARY HYPERTENSION INDUCED BY HYPOBARIC HYPOXIA

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Nitric oxide (NO) plays an important part in lowering pulmonary vascular resistance after birth and in persistent pulmonary hypertension of the newborn (PPHN) NO mediated dilation is dysfunctional[1]. The endogenous nitric oxide synthase inhibitor, asymmetric dimethylarginine (ADMA) circulates in plasma and its concentrations are elevated in certain cardiovascular diseases, including pulmonary hypertension. ADMA is metabolised by the enzyme dimethylarginine dimethylaminohydrolase (DDAH), the activity of which regulates ADMA concentrations and provides a mechanism for modulating NOS *in vivo*. We investigated the changes in expression and activity of the two isoforms of DDAH in lungs from newborn piglets both during normal development and in PPHN.

The cardiac lobe was taken from normal Large White piglets (n=57) in the following age groups: fetal (1 week pre-term), newborn (at 5min), 1 day (12-24 h), 3 days, and juvenile/adult (14 days-3 months). Animals were killed with an overdose of pentabarbitone (100mg/kg) the cardiac lobe was either snap frozen and stored at -70°C until further use, or taken fresh for use in the enzyme activity assay. For immunohistochemistry, lung sections were taken from the hilar region of the lung and then fixed and embedded in wax as previously described. To induce pulmonary hypertension, newborn pigs were placed in a hypobaric chamber for 3 days with a continuous supply of modified cows milk (n=12). The internal temperature was maintained at 29°C and the air pressure maintained at 50.8kPa. Animals placed in these chambers developed pulmonary hypertension with right ventricular hypertrophy and have a systemic arterial oxygen saturation of 71±5% due to right left shunting through persistent fetal channels.

Immunoreactivity for DDAHI and DDAHII was present in both large and small airways and in the pulmonary vasculature. Very little difference was observed in distribution throughout the lung, although DDAHI was expressed more strongly in bronchial smooth muscle and nerves. Expression of DDAHI protein did not significantly change with age. DDAHII protein was expressed at a very low level in samples from both the fetal and newborn age groups, significantly increasing at 1 day of age (n=4,p<0.05, one-way ANOVA). At 3 days of age DDAHII protein levels had decreased to those found at birth and this level was maintained with age. When compared with their age matched controls, protein expression of DDAHI did not change in the hypertensive animals. However the expression of DDAHII decreased by 85±3% with pulmonary hypertension (n=4, p<0.05, unpaired t-test). DDAH activity decreased 80±7% in the hypertensive animals when compared to their age matched controls (n=4, p<0.05, unpaired t-test).
These results indicate that each DDAH isoform is differentially regulated during both lung development and PPHN. Suppression of DDAHII expression may be a mechanism underlying PPHN.

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53P EFFECT OF DEXRAZOXANE ON THE CELL CYCLE AND INTRACELLULAR ATP LEVELS IN K562 CELLS

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The bisdioxopiperazine razoxane and its enantiomer, dexrazoxane have a multiplicity of biological and clinical effects. They have shown promise in cardioprotection, as inhibitors of angiogenesis and in the treatment of various diverse conditions such as psoriasis and cancer, where dexrazoxane has been shown to delay the emergence of drug resistance (Hellmann, 1999; Sargent *et al*, 2001). It is reasonable to propose that there must be a mechanism(s) of action which is common to these wide-ranging effects. The aim of this study was to elucidate possible common actions such as a deceleration of cellular proliferation, by studying the effect of dexrazoxane on 1) the cell cycle and 2) intracellular ATP levels.

The effect of dexrazoxane on the cell cycle of the human leukaemia line K562, was assessed using propidium iodide staining and flow cytometry after cells were incubated in dexrazoxane for 2 – 96 hours. Expression of the cell cycle control gene, *CDK1*, which is involved in the G2/M transition was also measured in these cells using immunofluorescence with the SC54 monoclonal antibody. Intracellular ADP/ATP ratios after incubation in dexrazoxane were assessed using the ApoGlow kit according to manufacturer's instructions (BioWhittaker, Wokingham). All experiments were carried out on at least 2 occasions and results are expressed as mean values ± s.e.m.

Incubation in IC_{50} levels of dexrazoxane (70μM) led to G2/M arrest within 8 hours. At 24 hours 35 ± 6% (n=3) of cells had tetraploid or higher DNA content, suggesting DNA re-replication. As DNA re- replication can be caused by alterations in *CDK1* (Itzhaki *et al*, 1997), CDK1 protein expression was measured after 24 hours incubation in 70μM dexrazoxane and levels were reduced to 45 ± 15% (n=3) of that found for control cells. *CDK1* is known to be transcribed in a cell-cycle dependent manner and is involved in entry into M-phase whilst preventing unscheduled entry into S-phase (Itzhaki *et al*, 1997). Our results, therefore, suggest that these cells are undergoing two or more S-phases without intervening mitoses, so delaying cellular proliferation.

ADP/ATP ratios were measured in K562 cells which had been incubated in dexrazoxane. After 24 hours, there was a 48 ± 12 % (n=3) reduction in this ratio compared to control suggesting a block in ATP hydrolysis. The block appeared to be concentration dependent (Table 1).

Table 1. Effect of increasing concentrations of DXRz on intracellular ADP/ATP ratio.

DXRz μM	*0*	*9*	*18*	*35*	*70*
ADP/ATP ratio	0.429	0.405	0.374	0.341	0.303

Our results suggest that both alterations in *CDK1* and/or hydrolysis of ATP by dexrazoxane may be affecting cellular proliferation so leading to the multifaceted action of this interesting agent. Further studies of these observations are warranted to establish relationships between these factors.

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54P ACETYLCHOLINE-INDUCED CONTRACTION OF THE RAT ISOLATED ILEUM INVOLVES ACTIVATION OF L-TYPE CALCIUM CHANNELS AND INOSITOL TRISPHOSPHATE RECEPTORS

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Acetylcholine (ACh) causes contraction of longitudinal muscle in the rat ileum via activation of M_3 receptors (Brown *et al.*, 1980) which trigger $InsP_3$ mobilisation and Ca^{2+} release (Caulfield, 1993). The aim of this study was to determine the sources of Ca^{2+} mobilised during ACh-induced tissue contraction in absence and presence of the M_3 receptor antagonist 4-DAMP (4-diphenylacetoxy-N-methyl piperidine methiodide (Brown, *et al.*,1980), the L-type Ca^{2+} channel blocker verapamil (Lee & Tsien, 1983), a membrane-penetrable modulator of IP_3-induced Ca^{2+} release, 2-amino-ethoxydiphenylborate (2-APB) (Maruyama *et al.*, 1997), the SERCA ATP-ase inhibitor BHQ (*tert*-butylbenzohydro-quinone (Wictome *et al.*, 1992) and low Ca^{2+}.

Ileal segments (4 sections taken 15-30 cm from the ileo-caecal junction) were obtained from male Wistar rats (220 – 250g) and mounted in 10ml organ baths containing Tyrode's solution at 35°C (gassed with 95% O_2 : 5% CO_2) under a tension of 1g. Tissues were equilibrated for 20 min before non-cumulative dose response curves to ACh were obtained using a 3 min cycle with 30 s contact time using an isotonic transducer. In Ca^{2+}-free solutions, Ca^{2+} was replaced isosmotically with Mg^{2+}. Antagonists were added to the buffer and tissues equilibrated for 20 min before retesting the effects of ACh. Data reported are mean ± s.e. and statistical significance was determined using Student's *t*-test of pair differences.

ACh (10 nM – 1 μM) caused dose-dependent contraction with an EC_{50} value of 220 ± 30 nM (n = 12). Inclusion of 4-DAMP (10 nM) in the buffer increased the ACh EC_{50} value to 1.3 ± 0.3 μM ($P<0.01$, n = 12), while at a dose of 100 nM the antagonist reduced the maximum response to applied ACh by 95 ± 5 % (n = 8; $P<0.0001$). The maximum response caused by ACh was reduced by 65 ± 8% (n = 6; $P<0.001$) and 100% (n = 6; $P<0.0001$) in the presence of 10 μM and 20 μM verapamil respectively. Incubation of tissues in low Ca^{2+} saline reduced the effects of ACh by 94 ± 3% (n = 6, $P<0.0001$). Although 2-APB (1μM & 10 μM) increased the maximum response of ACh by 67 ± 17 % (n = 6, $P<0.05$) and 58 ± 20% (n = 6, $P<0.05$) respectively, 50 μM 2-APB reduced the effects of the agonist by 67 ± 12% (n = 6; $P<0.05$). BHQ (1 nM) increased the ACh maximum response by 51 ± 16% (n = 8, $P<0.05$) whereas, simultaneous incubation in BHQ (1 nM) and verapamil (10 μM) reduced the effects of the agonist by 66 ± 9% (n = 8, $P<0.001$).

These data suggest that activated M_3 receptors cause ingress of Ca^{2+} through L-type Ca^{2+} channels. However, the effects of 2-APB show that ACh-induced tissue contraction also involves release of Ca^{2+} from $InsP_3$-activated stores. Inhibition of BHQ-induced potentiation by verapamil suggests that these stores are replenished by entry of Ca^{2+} through L-type channels.

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55P CELL SURFACE EXPRESSION OF OXYTOCIN RECEPTORS ON BOTH CONTRACTILE AND SECRETORY PHENOTYPES OF PRIMARY HUMAN UMBILICAL AND AORTIC VASCULAR SMOOTH MUSCLE CELLS

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There is some evidence to suggest oxytocin has effects on human vascular smooth muscle cells (VSMC) (Strålin *et al.*, 2001). The aim of this study was to investigate cell surface expression of oxytocin receptors (OTRs) on human umbilical artery (Um) and aortic (Ao) VSMC. The highly-selective OTR antagonist [^{125}I]OTA was used as tracer in radioligand binding studies on primary cells.

VSMC were cultured in a secretory phenotype for up to 10 passages in the presence of 5% serum, epidermal growth factor and fibroblast growth factor-2. These cells divided rapidly and expressed low levels of SMC α-actin. In the presence of low serum (0.25 %) and the absence of growth factors, the cells ceased to divide, expressed high levels of SMC α-actin and converted to a contractile phenotype in 6 days (Rainger *et al.*, 2001).

Whole cell binding experiments were performed as described by Hawtin *et al.*, (2002). Contractile cells were seeded in 12-well plates at a density of 10^4 cells per well, and secretory cells were seeded at a density of 5 x 10^3 cells per well. Cells were washed with PBS, followed by the addition of binding buffer containing [^{125}I]OTA (20-80 pM). Competition binding curves were determined for the peptide antagonist OTA and the non-peptide antagonist L-371,257 (10^{-11}-10^{-6}M). K_i values were calculated from the IC_{50} values using the Cheng-Prusoff equation (Table 1) (mean ± s.e.m).

Table 1. Pharmacological Characteristics of OTRs Expressed by hVSMC

VSMC	Ligand	K_i nM
Um-secretory	OTA	0.29 ± 0.1 (n=3)
Um-contractile	OTA	0.35 ± 0.1 (n=3)
Um-secretory	L-371,257	2.1 ± 0.2 (n=6)
Um-contractile	L-371,257	2.6 ± 0.4 (n=4)
Ao-secretory	OTA	2.8 ± 1.5 (n=3)
Ao-contractile	OTA	2.8 ± 1.4 (n=3)
Ao-secretory	L-371,257	1.9 ± 0.5 (n=3)
Ao-contractile	L-371,257	3.0 ± 1.1 (n=3)

B_{max} values (fmol.mg protein^{-1}): Um-secretory, 15 ± 2 (n=9); Um-contractile, 36 ± 5 (n=9); Ao-secretory, 17 ± 4 (n=7); Ao-contractile, 94 ± 7 (n=7).

In conclusion, OTRs are expressed on both Um-VSMC and Ao-VSMC. There are significantly more OTRs on the surface of contractile VSMC compared to their secretory counterparts (P<0.01, Student's unpaired t-test).

This work was supported by the British Heart Foundation grant P/G 99053.

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56P MATRIX METALLOPROTEINASE (MMP) ACTIVITY IN AORTA FROM SPONTANEOUSLY HYPERTENSIVE HEART FAILED RATS: EFFECT OF CHRONIC NON-SELECTIVE ENDOTHELIN RECEPTOR ANTAGONISM

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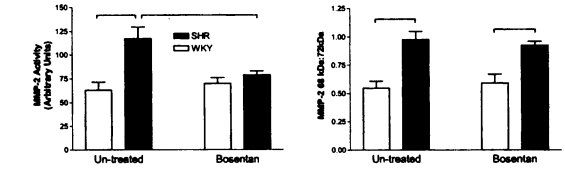
Progression to decompensated heart failure is associated with ventricular remodelling and an increase in cardiac MMP activity (Spinale *et al.*, 2000). Little is known about the role of the endothelin (ET) system on changes in vascular MMP activity associated with heart failure. We investigated (i) alterations in vascular MMP activity in Spontaneously Hypertensive rats (SHR) during transition from compensated hypertrophy to decompensated heart failure, and (ii) potential modulation of MMP following chronic treatment with the mixed ET_A/ET_B receptor antagonist, bosentan.

Male SHR (n=11) and control Wistar Kyoto rats (WKY; n=11) were randomly assigned to receive either placebo or bosentan (100 mg/kg/day). Treatment commenced prior to onset of heart failure and was maintained for a period of x months, when animals (18mo) were sacrificed under terminal anaesthesia. Aortas were ground to a powder in liquid N_2, protein extracted in a RIPA buffer (50mM Tris, 150mM NaCl, 1% NP-40, 0.1% SDS, 2mM EDTA, protease cocktail, pH 7.5) and separated by SDS-PAGE supplemented with gelatin (0.1%). Gels were washed (2.5% Triton X-100), incubated (18h) at 37°C in substrate buffer (Tris-HCl 50 mM, $CaCl_2$ 10mM and $NaCl_2$ 50 mM), and stained (0.05 % coomassie blue), prior to analysis (Syngene Gene Tools) for MMP activity (band intensity). Data were analysed by 2-way ANOVA with *post hoc* analysis (Bonferroni) and expressed as mean±s.e.m.; P<0.05 indicates statistical significance.

Figure 1 A B



MMP-2 activity in aorta from SHR and normotensive WKY rats and the effect of chronic administration of bosentan. Horizontal bars indicate statistical significance (P<0.05)

MMP-2 activity was 1.86 fold higher (P<0.05) in SHR compared with WKY controls. Chronic administration of bosentan decreased MMP-2 activity by 33% in the SHR group (P<0.05), but had no effect on WKY rats (Fig. 1A). The ratio of active:pro MMP-2 was higher (1.79 fold) in SHR compared with WKY rats (P<0.01). This ratio was, however, unaffected by treatment with bosentan (1.56 fold; Fig. 1B), reflecting generalised attenuation of both active and pro MMP-2 activity in treated SHRs.

In conclusion, MMP-2 activity and the pro:active MMP-2 ratio are increased in aortic tissue of decompensated SHR rats suggesting active vascular remodelling. Chronic treatment with bosentan, attenuates this response through a generalised reduction in MMP-2 activity.

Spinale FG, Coker ML, Bond BR, Zellner JL. *Cardiovasc Res* 2000; **46**: 225-238.

Acknowledgements: Dr M. Clozelle (Actellion) for the gift of Bosentan

57P *IN VIVO* IMAGING OF ENZYME CONVERSION OF [^{18}F]-BIG ET-1 TO [^{18}F]-ET-1 AND INHIBITION OF ENZYME ACTIVITY USING PHOSPHORAMIDON - A POSITRON EMISSION TOMOGRAPHY STUDY

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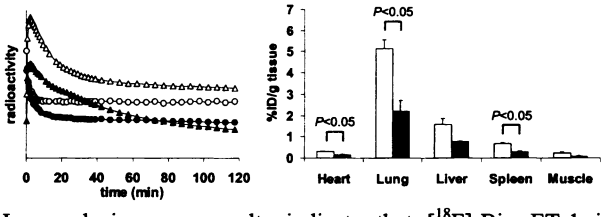
Endothelin-1 (ET-1) is a multifunctional peptide in humans and alteration in the ET receptor system has been linked to a number of vascular diseases. ET-1 is produced from its precursor peptide Big ET-1 by the action of endothelin-converting enzymes (ECE). We have previously showed that ET receptors can be imaged *in vivo* using positron emission tomography (PET) and [^{18}F]-ET-1 (Johnström *et al.*, 2002). The aim of this work was to investigate the use of [^{18}F]-Big ET-1 to image ECE activity *in vivo* and whether [^{18}F]-Big ET-1 conversion to [^{18}F]-ET-1 could be blocked with the ECE/NEP inhibitor phosphoramidon.

[^{18}F]-Big ET-1 kinetics and distribution was studied using PET (microPET, Concord Microsystems). [^{18}F]-Big ET-1 was injected intravenously into anaesthetised (isofluorane) Sprague-Dawley rats, with (n=3) or without (n=3) pre-treatment with phosphoramidon 10 mg/kg. At the end of acquisition the animal was killed and organs were removed for analysis of uptake of radioactivity in the tissue. From the acquired PET data images were reconstructed showing the distribution of [^{18}F]-Big ET-1. Regions-of-interest for various organs were drawn in the PET images and time-radioactivity curves were constructed. Ex vivo data were compared using Student's two-tailed *t*-test and differences were considered significance at $P<0.05$.

In lung an initial decrease of radioactivity was observed, most likely reflecting blood flow, which then levelled out to reach equilibrium, suggesting binding to ET receptors. In liver the radioactivity peaked within the first couple of minutes and then slowly decreased with a terminal $t_{½}$ on the order of 10 h (Figure 1). Pre-treatment with phosphoramidon reduced uptake in tissue (Figure 2) in accordance with inhibition of ECE activity and formation of ET-1. In liver a change in distribution kinetics was observed (Figure 1).

Figure 1. Distribution kinetics [^{18}F]-Big ET-1 in lung (○) and liver (△) microPET. Filled symbols: rat pre-treated with phosphoramidon.

Figure 2. Ex vivo distribution of radioactivity in various organs (mean±s.e.mean). Filled bars: rat pre-treated with phosphoramidon.



In conclusion, our results indicate that [^{18}F]-Big ET-1 is converted to [^{18}F]-ET-1 *in vivo* with subsequent binding to ET receptors. Furthermore, these data suggest that [^{18}F]-Big ET-1 has a potential to monitor efficacy of novel ECE inhibitors *in vivo* with PET.

Johnström P. *et al.* (2002). *Clinical Science.* **103** (Suppl. 48), 4S-8S.

Supported by grants from BHF and MRC

58P PRE- AND POSTJUNCTIONAL INHIBITORY ACTIONS OF THE AT_1-RECEPTOR ANTAGONISTS EPROSARTAN AND CANDESARAN IN THE ISOLATED RABBIT THORACIC AORTA

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Several studies have shown that angiotensin II (Ang II) enhances electrical field stimulation (EFS)-evoked sympathetic neurotransmission by acting on pre-synaptic AT_1-receptors. The objective of the present study was to compare the sympatholytic potency of eprosartan and candesartan and their ability to inhibit direct vasoconstriction by Ang II. To investigate the sympatholytic potency of eprosartan and candesartan we studied their effects on Ang II-enhanced EFS-evoked [^{3}H]-noradrenaline release. To investigate the potency of the selective AT_1-receptor blockers regarding inhibition of direct vasoconstrictor response by Ang II we evaluated their effects on concentration-response curves (CRC) elicited by Ang II. Experiments were performed in isolated rabbit thoracic aorta preparations (NZW, male, 2000-2800g). Ang II (10 *n*M) enhanced the EFS-evoked sympathetic outflow by a factor 2.01 ± 0.11 ($p<0.05$). The angiotensin II-induced enhancement could be concentration-dependently inhibited by both eprosartan (pIC_{50} 8.02 ± 0.16) and candesartan (pIC_{50} 10.76 ± 0.13). Ang II (1 *n*M - 0.3 μM) caused a concentration-dependent increase in contractile force of rabbit thoracic aortic rings (E_{max} 20.62 ± 2.24 mN, pD_2 8.16 ± 0.04). Eprosartan inhibited Ang II induced contractions in a competitive manner (pA_2 8.90 ± 0.11), thus resulting in a rightward displacement of the CRC for Ang II. In contrast, candesartan inhibited Ang II-induced contractions in a non-competitive manner, thereby reducing E_{max} without causing a rightward shift of the CRC for Ang II (pD_2 10.80 ± 0.13). We conclude that the facilitating effect of Ang II on sympathetic transmission is mediated by presynaptic AT_1 receptors. Furthermore, eprosartan appears to be less potent regarding sympatho-inhibition compared to candesartan. This difference in potency also holds for the inhibition of direct vasoconstriction elicited by Ang II. In addition, concentrations needed to achieve inhibition of direct vasoconstriction are in the same range as sympatho-inhibitory concentrations.

59P VASOPRESSIN-INDUCED FACILITATION OF ADRENERGIC RESPONSES IN THE RAT MESENTERIC ARTERY IS V1-RECEPTOR DEPENDANT

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Objective: To analyse the possible involvement of the V_1- and V_2-receptor in vasopressin (AVP)-induced facilitation of the sympathetic nervous system. Furthermore, we aimed to determine whether the site of facilitation by AVP is located pre- or post-synaptically.

Methods: We used the mesenteric artery of male Wistar rats in an organ-bath setup for isometric recordings. To investigate the influence of blockade of both pre- and post-synaptic V_1 and possibly also V_2-receptors, we studied the effects of AVP and desmopressin (a selective V_2-agonist) on electrical field stimulation (EFS)-induced vasoconstriction, in the presence or absence of SR 49059 (V_1-antagonist) and SR 121463 (V_2-antagonist). To investigate the role of the post-synaptic AVP-receptors in AVP induced vasoconstriction, we studied the effect of either SR 49059 or SR 121463. Furthermore, we investigated the direct effect of desmopressin (0.03-300 nM) alone, and after precontraction with noradrenaline (NA) 10 □M. In addition, the effect of AVP on postsynaptic α-adrenoceptor mediated responses was studied by exposing the vessels to NA (0.01-10 μM) in the presence or absence of SR 49059 (V_1-antagonist). The results of the NA experiments are expressed as a fraction of the preceding potassium (120 mM)-induced contraction. All data are expressed as mean ± SEM.

Results: Electrical field stimulation (1-16 Hz) caused a frequency-dependent increase in contractile force. At frequencies of 2 and 4 Hz a sub-pressor concentration of AVP (0.3 nM) increased the stimulation-induced (SI) vasoconstrictor responses by a factor 3.7 ± 0.8, and 3.1 ± 0.4, respectively (n=12, $p<0.05$ compared to control). At a frequency of 2 Hz the enhancement was completely antagonized by SR 49059 10 nM to a factor of 0.9 ± 0.2 (n=6, $p<0.05$ compared to AVP alone). Conversely the V_2-antagonist SR 121463 (1 nM) did not influence the AVP induced facilitation of SI contractions (the factor of increased contraction: 3.21 ± 0.6 (n=6)). Also at the highest concentration of SR 121463 (10 nM) the SI contractions remained comparable to AVP, but the significance was lost in comparison to control (factor of increased contraction 2.2 ± 0.6 (n=7, $p>0.05$ SR 121463 compared to control)). The highly selective V_2-agonist desmopressin (0.03 nM-300 nM) did not induce any vasoconstriction, nor vasorelaxation and it did not influence the SI-induced contractions. Contractile responses to exogenous NA 100 nM 0.02 ± 0.01 (n=7) were enhanced in the presence of AVP 0.3 nM 0.16 ± 0.05 (n=8, $p<0.05$) and this effect was completely antagonised by SR 49059 0.02 ± 0.02 (n=6).

Conclusion: The facilitating effect of AVP on noradrenergic neurotransmission is at least partly mediated by post-synaptically located V_1-receptors. By contrast the V_2-receptors appear to be not involved in AVP induced facilitation of the sympathetic nervous system.

60P FARNESOID X RECEPTOR (FXR) AS A NOVEL TARGET IN VASCULAR SMOOTH MUSCLE CELLS

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FXR is a recently identified bile acid-activated nuclear receptor, whose synthetic ligands have been demonstrated to inhibit the growth of a number of cancer cell lines (Niesor *et al.*, 2001). Here we show that FXR is expressed in human vascular smooth muscle cells (VSMC), and that synthetic FXR ligands inhibit VSMC growth.

Immuno-histochemical analysis (Walsh *et al.*, 2002) of FXR (goat anti-FXR 1:50; Santa Cruz) in human vessels, was performed on a human cardiovascular tissue array (Ambion). For *in vitro* experiments, human VSMC were grown from saphenous vein as previously described (Bishop-Bailey *et al.*, 1998). FXR expression *in vitro* was measured by RT-PCR (Niesor *et al.*, 2001), and by immunoflorescence (Bishop-Bailey *et al.*, 1999) using goat anti-FXR (1:50) and as a control goat anti-FXR + synthetic blocking peptide (1:10) pre-absorbed for 1h before use. VSMC in culture were incubated with the synthetic FXR ligands (gifts from Dr Eric Niesor, ILEX Oncology, Inc., Geneva) SR45023A (SR45; 0.3-100μM), and SR9213 (SR92; 1-100μM) for 48h. Cell viability was measured by the MTT assay (Bishop-Bailey *et al.*, 1998).

FXR was detected in VSMC in human vessels by immuno-histochemisty, and in VSMC *in vitro* at the level of mRNA by RT-PCR, and protein by immunoflorescence (figure 1A). 48h incubations of SR45 and SR92 induced concentration-dependent inhibitions of VSMC viability (figure 1B). SR45 was more potent than SR92 in accordance with its ability to activate FXR mediated responses (Niesor *et al.*, 2001).

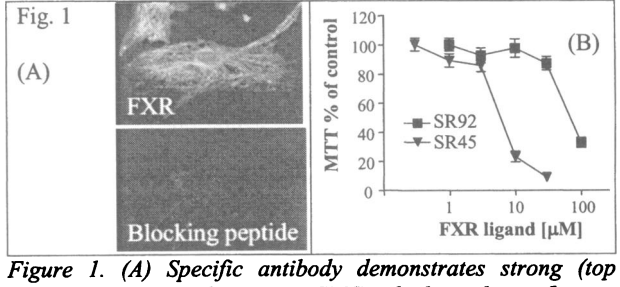

Figure 1. (A) Specific antibody demonstrates strong (top panel) expression of FXR in VSMC, which is absent (bottom panel) when primary antibody is pre-absorbed with blocking peptide (1:10). (B) Cell viability (MTT assay) of VSMCs incubated with SR45 or SR92 for 48h. Cells were initially serum starved (24h), and experiments were performed in serum free conditions. Data is mean± S.E.M for n=15 from 5 experiments.

Here we show for the first time that FXR is expressed in human VSMC, and that FXR ligands inhibit VSMC proliferation. FXR may therefore represent a novel therapeutic target for vascular diseases.

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Bishop-Bailey, D. *et al.*, (1998). *Arterio. Thromb. Vasc. Biol.* **18**,1655-61
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61P THE ROLE OF SEROTONERGIC AND BETA-ADRENERGIC RECEPTORS IN NEBIVOLOL-INDUCED VASORELAXATION

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Nebivolol is a highly selective β_1 adrenoceptor blocker with additional vasodilating properties. Although it has been shown that the nebivolol-induced vasorelaxation is NO- and cGMP-dependent, the receptor that mediates these actions remains subject to debate. Serotonergic as well as beta-adrenergic pathways have been suggested to be involved (Kakoki *et al.*, 1999; Gosgnach *et al.*, 2001). Therefore, functional experiments, investigating the receptor involved in nebivolol-induced vasorelaxation, were performed in an organ bath model.

Aortic rings, isolated from male Wistar Kyoto rats (240-260g), were exposed to cumulative concentrations of nebivolol (100 nM-10μM). One way analysis of variance (ANOVA), followed by the Dunnet post test for comparison versus control, or the Student's t-test were performed for statistical evaluation of the results. In concentrations of 3 μM and higher, nebivolol produced a concentration-dependent vasodilatation. In the highest concentration applied (10 μM), this resulted in a vascular tone of 55.2 ± 7.8% of the applied precontraction. In similar concentrations, metoprolol did not cause vasodilatation. The vasodilator effect of nebivolol, in concentrations of 3 and 10 μM, was significantly blocked by exposure to 100 μM of the NO synthase inhibitor L-NNA (102.3 ± 2.3% vs. 84.6 ± 4.2% and 99.1 ± 2.2% vs. 55.2 ± 7.8%, respectively) ($P<0.05$; n=6-13). Mechanical removal of endothelium had the same effect (98.0 ± 0.9% vs. 84.6 ± 4.2% and 84.62 ± 1.8% vs. 55.2 ± 7.8%, respectively) ($P<0.05$, n=6-13). In order to investigate whether serotonergic pathways play a role in the observed nebivolol-induced vasorelaxation,
the vessel rings were exposed to the specific 5-HT_{1A} receptor antagonist NAN-190 and to the 5-$HT_{1/2}$ receptor antagonist methysergide. Neither NAN-190 (1 μM), nor methysergide (1 μM), significantly affected nebivolol-induced vasorelaxation,
resulting in a relative vascular tone of 46.6 ± 6.7% and 57.7 ± 10.7% respectively, at a concentration of 10 μM nebivolol (N.S.; n=6-13). In addition, the involvement of beta-adrenergic pathways was investigated. Specific β_2 adrenoceptor blockade with butoxamine (50 μM) did not influence the vasodilatory effect of nebivolol, resulting in a relative vascular tone of 53.8 ± 4.6% (N.S.; n=6-13). However, the addition of 1 μM of the selective β_3 adrenoceptor antagonist S-(-)-cyanopindolol significantly affected the response to 10 μM nebivolol and prevented nebivolol-induced vasorelaxation (82.0 ± 4.5% vs. 55.2 ± 7.8%; $P<0.05$; n=6-13).The specific β_3 agonist BRL 37344 was applied to confirm that β_3 adrenoceptor stimulation leads to NO-dependent vasorelaxation in the rat thoracic aorta. Indeed, BRL 37344 produced vasodilatation that was significantly blocked by L-NNA. In a concentration of 1 μM BRL 37344, L-NNA (100 μM) increased vascular tone from 81.6 ± 5.5% to 98.8 ± 0.5% ($P<0.05$; n=6) and in the maximal concentration of BRL 37344 applied (10 μM), from 45.1 ± 9.0% to 83.9 ± 6.2% ($P<0.05$; n=6). β_3 adrenoceptor blockade completely inhibited BRL 37344-induced vasorelaxation: 97.5 ± 0.8% (S-(-)-cyanopindolol) vs. 81.6 ± 5.5% (control) in a concentration of 1 μM BRL 37344 and 94.7 ± 1.7% (S-(-)-cyanopindolol) vs. 45.1 ± 9.0% (control) in a concentration of 10 μM BRL 37344 ($P<0.05$; n=6). Based upon the data presented in this functional study we conclude that, in the rat aorta, nebivolol-induced NO-dependent vasorelaxation is, at least in part, caused by a β_3 adrenoceptor agonistic effect.

Kakoki *et al.* (1999). Hypertension, **33**, 467-71
Gosgnach *et al.* (2001). J. Cardiovasc. Pharmacol., **38**, 191-99

62P IMPAIRMENT OF ENDOTHELIAL FUNCTION IN THE RAT AORTA AFTER EXPOSURE TO REACTIVE OXYGEN SPECIES: PROTECTIVE EFFECT OF THE BETA-BLOCKER NEBIVOLOL

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In addition to its direct negative inotropic and vasodilator effects, nebivolol has been associated with reduced levels of oxidative stress. Janssen *et al.* (1999, 2001) demonstrated that nebivolol prevents against hydroxyl radical (·OH)-induced injury in right ventricular rabbit cardiac trabeculae and in demembranised muscles from explanted human hearts, respectively. In urine samples of healthy human volunteers, it was shown that nebivolol significantly decreased levels of 8-iso-PGF_{2a}, a marker of oxidative stress (Troost *et al.*, 2000). Oxidative stress is associated with impairment of endothelium-dependent vasorelaxation, possibly caused by a loss of nitric oxide (·NO) bioactivity in the vessel wall. Endothelial dysfunction, as a result of enhanced levels of reactive oxygen species (ROS), plays an important role in the pathogenesis and progression of cardiovascular diseases such as hypertension and atherosclerosis (Dhalla *et al.*, 2000). In this study, we investigated the effect of nebivolol on ROS-induced endothelial damage and compared it to the effect of metoprolol, a selective β_1 receptor antagonist supposedly without antioxidative properties.

Experiments were performed in the rat aorta, isolated from male Wistar Kyoto rats (240-260g). Endothelial function was assessed during the priming procedure, by exposing the vessels to a precontraction with phenylephrine (1 μM) followed by a single dose of metacholine (1 μM). After a 10 min incubation with either 100 nM nebivolol, 300 nM nebivolol (subeffective with regard to vasorelaxation), 300 nM metoprolol or none of the above (control), ROS were generated by means of electrolysis of the organ bath medium.

Ten minutes after electrolysis (15 s; 15 mA) or no electrolysis (15 s; 0 mA), endothelial function was again determined by means of addition of 1 μM phenylephrine followed by 1 μM metacholine. The endothelium-dependent response in all groups was calculated as percentage of the first one, during the priming procedure. When electrolysis of the organ bath medium was applied, endothelium-dependent vasorelaxation was significantly impaired, which resulted in a relative relaxation of 13.6 ± 4.2% (electrolysis) versus 98.5 ± 16.2% (no electrolysis; $P<0.05$; n=4-7). Interestingly, the presence of nebivolol protected endothelial function from ROS-induced damage in a concentration-dependent manner. When electrolysis was applied in the presence of 100 and 300 μM nebivolol, metacholine-induced vasorelexation amounted to 28.2 ± 3.5% and 65.0 ± 6.3%, respectively (n=4-7, $P<0.05$). Incubation with the β_1 adrenoceptor antagonist metoprolol (300 nM) did not protect from ROS-induced loss of endothelial function (12.0 ± 2.9%; n=4-7; N.S.).

The present study clearly demonstrates, that nebivolol alleviates ROS-induced impairment of endothelium-dependent vasorelaxation. The results invite further clinical research on the antioxidative and endothelium-protective effects of nebivolol.

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Troost *et al.* (2000). Br. J. Clin. Pharmacol., **50**, 377-79
Dhalla *et al.* (2000). J. Hypertens., **18(6)**, 655-73.

63P ARACHIDONIC ACID INDUCED CONTRACTIONS IN HUMAN PULMONARY VEINS

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Arachidonic acid (AA) is metabolised by cyclooxygenase (COX) to prostaglandins and thromboxane A_2. These prostanoids activate selective receptors (DP, EP_{1-4}, FP, TP or IP) to regulate the vascular smooth muscle tone. Contractions of the human pulmonary artery (HPA) are induced by the activation of TP- and EP_3- receptors (Qian *et al.*, 1994) while in vein (HPV) by TP- and EP_1- receptors (Walch *et al.*, 2001).

The aim of this study was to examine the effect of AA on HPV and HPA tone. The response to exogenous AA was measured in isolated vascular preparations under basal or pre-contracted tone (norepinephine: NE; 10μM).

Vessels were obtained from patients undergoing thoracotomy, cut as rings and set up in organ baths with Tyrode's solution. Changes in force were recorded using isometric transducers and physiographs. The effect of either AA (100μM) or cumulative concentrations of AA (10 and 100μM) were measured at different times after challenge. These stimulations were produced in absence or presence of the endothelium, the COX inhibitor (indomethacin, 1.7μM; 30 min) or the EP_1 antagonists (AH6809, 10μM or SC19220, 100μM; 30 min).

NE contractions were: 1.51±0.15g and 1.68±0.30g in HPA and HPV (n=6), respectively. AA (100μM) induced a peak contraction in the HPV but not in HPA, an effect which was independent of smooth muscle tone (Table 1). This peak contraction was significantly reduced after treatment of the HPV with the antagonists (Table 1), indomethacin (n=5) or in absence of the endothelium (n=7) (data not shown).

Table 1. Effect of AA stimulation on HPA and HPV.

Vessels and treatments	AA 10μM t=15 min	AA 100μM t=72 s	AA 100μM t=15 min
pre-contracted vessels			
HPA (n=6)	-0.30±0.10	+0.11±0.05*	-0.35±0.04
HPV (n=6)	-0.45±0.24	+0.85±0.32	-0.84±0.31
basal tone			
HPA (n=14)	NP	+0.03±0.02*	-0.15±0.03*
HPV (n=16)	NP	+0.55±0.16	-0.49±0.12
HPV (n=4) + AH6809	NP	+0.08±0.08*	-0.14±0.04
HPV (n=4) + SC19220	NP	+0.02±0.02*	-0.15±0.08

Each value (means±s.e.mean) indicates the change in force in comparison with the previous tone. Contractions (+) and relaxations (-) are expressed in g and n = number of lungs used. NP, not performed and t = time. * indicates $P<0.05$ versus the appropriate untreated HPV (Student's t-Test).

These results suggest an endothelial release of a prostanoid in the HPV that induced a peak contraction by activation of the EP_1 receptor present on the vascular smooth muscle.

Qian *et al.*, (1994) *Br. J. Pharmacol.*, 113, 369-374.
Walch *et al.*, (2001) *Br. J. Pharmacol.*, 134,1671-1678.

64P HAEMODYNAMIC EFFECTS AND FATE OF ASYMMETRIC DIMETHYLARGININE IN HUMANS; INSIGHTS INTO PATHOPHYSIOLOGY OF THE ADMA/DDAH PATHWAY

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Plasma levels of an endogenous NOS inhibitor, asymmetric dimethylarginine (ADMA), are elevated in chronic renal failure, hypertension, and chronic heart failure (Vallance *et al.*, 1992; Usui *et al.*, 1998). Despite these observations the cardiovascular effects of a systemic increase in ADMA in humans have not been studied.

In a randomised, double-blind, placebo-controlled trial in 12 healthy male volunteers, we compared the effects of intravenous low dose ADMA (3mg/kg) and placebo on heart rate, blood pressure, cardiac output and systemic vascular resistance (SVR) at rest and during exercise. We also tested the hypothesis that ADMA is metabolised in humans by dimethylarginine dimethylaminohydrolase (DDAH) enzymes. Overall statistical significance was tested using repeated-measures ANOVA; data from individual timepoints were tested further by the Student's paired *t* test.

Low dose ADMA reduced heart rate by 9.2±1.4% from 58.9±2.0 bpm ($P<0.001$), and cardiac output by 14.8±1.2% from 4.4±0.3 L/min ($P<0.001$). ADMA also increased mean blood pressure by 6.0±1.2% from 88.6±3.4mmHg ($P<0.005$), and SVR by 23.7±2.1% from 1639.0±91.6 dynes.s.cm^{-5} ($P<0.001$). Handgrip exercise increased cardiac output in control subjects by 96.8±23.3% but in subjects given ADMA cardiac output increased by only 35.3±10.6% during exercise ($P<0.05$). DDAHs metabolise ADMA to citrulline and dimethylamine (DMA). Urinary DMA:creatinine ratios significantly increased from 1.26±0.32 to 2.73±0.59 following ADMA injection ($P<0.01$). We use this data to estimate that humans generate approximately 300μmol of ADMA per day, of which approximately 250μmol is metabolised by DDAHs.

This study defines the cardiovascular effects of a systemic increase in ADMA in humans. These are similar to changes seen in diseases associated with ADMA accumulation, suggesting that ADMA could directly contribute to their pathogenesis. Finally, our data also indicate that ADMA is metabolised by DDAHs extensively in humans in vivo.

65P TETRAHYDROBIOPTERIN DEFICIENT MOUSE (HPH-1) DISPLAYS PULMONARY HYPERTENSIVE PHENOTYPE.

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GTP-cyclohydrolase 1 (GTP-CH1) catalyses the first and rate limiting step for the *de novo* production of tetrahydrobiopterin (BH_4), an essential cofactor for nitric oxide synthase (NOS) (Nichol *et al.* 1985). A deficiency of BH_4 may lead to reduced NOS activity (Gross and Levi, 1992), at least *in vitro,* but little is known about the functional consequences of BH_4 deficiency in blood vessels *in vivo.* Pulmonary hypertension caused by functional and structural changes in the pulmonary vascular wall. Structurally, this disease is characterised by right ventricular hypertrophy, increased wall area of resistance vessels and muscularisation of normally non-muscular vessels. The hyperphenylalaninemic mutant mouse (HPH-1) displays a 90% deficiency of GTP-CH1 and has been extensively characterised for it's neurological phenotype.

The heart and lung were dissected from 12 week old, 25-30g weight, HPH-1 and C57BL/6 (wild type) mice (mixed male/female). To assess right ventricular hypertrophy, atria were removed at the plane of the atrial- ventricular valves, and the right ventricle (RV) dissected from the left ventricle plus septum (LV+S). The RV and LV+S were subsequently weighed and the RV:LV+S ratio calculated. To measure wall thickness of resistance vessels from HPH-1 and C57BL/6 mice, 4μm lung sections were cut from paraffin blocks and subsequently immunostained for alpha smooth muscle actin. Light microscopy was used to identify and digitise resistance vessels at the level of alveolar ducts, respiratory bronchioli and terminal bronchioli, and wall area measured using Open Lab Software v 3.04. Additionally, extension of muscle into arteries was assessed by identifying arteries at the level of the alveoli using a light microscope (x40) and classifying as non-muscular or muscular.

HPH-1 mice had right ventricular hypertrophy when compared with the C57BL/6 wild type mice (ratio RV:LV+S: 0.511 ± 0.05 HPH-1; 0.240 ± 0.02 C57BL/6, mean±sem, p=0.0015 two tailed unpaired t-test, n=6). Additionally there was increased muscle in the resistance arteries of HPH-1 mice compared with C57BL/6 (n=6) (Table 1) and extension of muscle into the normally non-muscularised smaller and more peripheral vessels (n=6) (Table 2).

Table 1 Mean±sem percentage wall area of resistance arteries.

	Terminal	Respiratory	Alveolar
HPH-1	26.9 ± 1.90	23.3 ± 1.07	20.07* ± 1.05
C57BL/6	20.85 ± 1.48	18.7 ± 1.37	13.02 ± 0.62

*p=0.0021 two tailed non parametric T-test

Table 2 Mean ± sem percentage of muscularised and non muscularised arteries at level of alveoli.

	% Non muscularised	% Muscularised
HPH-1	27.5* ± 2.50	72.5* ± 3.31
C57BL/6	67.5 ± 1.06	32.5 ± 1.35

* p = <0.05 two tailed non parametric T-test

The HPH-1 mouse mutant exhibits the phenotype for pulmonary hypertension when compared to the C57BL/6 wild type. These results suggest that BH_4 deficiency alters nitric oxide pathways and vascular function *in vivo.*

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66P CHANGES IN BLOOD FLOW IN THE RAT PANCREAS DURING EXOCRINE STIMULATION AT SUBMAXIMAL AND SUPRAMAXIMAL LEVELS

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Cholecystokinin (CCK) is an endogenous stimulator of the exocrine pancreas. Hyperstimulation with the CCK analogue, caerulein (pGlu-Gln-[Met^3]-CCK-8, sulfated), is a method for the experimental induction of acute interstitial-oedematous pancreatitis in rats. We have investigated the role of CCK receptor subtypes in changes in pancreatic blood flow, measured by the hydrogen gas clearance method (Livingstone *et al.*, 1989) during caerulein-induced submaximal or supra-maximal exocrine stimulation in barbiturate-anaesthetized female Sprague-Dawley rats, 200-250g).

During a 1 h i.v. infusion of a low dose of caerulein (0.4 nmol kg^{-1} h^{-1}), which did not induce any signs of inflammation, pancreatic blood flow increased by 26 ± 8% over basal values (78 ± 12 μl min^{-1} 100^{-1} mg^{-1}; n = 6) and returned to baseline values within 60 min after the end of the infusion. This effect was abolished (P<0.05) by the CCK_2 antagonist PD135158 (Hughes *et al.*, 1990; 2 μmol kg^{-1}, i.v.), while the CCK_1 antagonist PD140548 (Boden *et al.*, 1993; compound 30p) was without effect. Rat gastrin-17-I (2.4 nmol kg^{-1} h^{-1}) increased blood flow to an extent (30 ± 7%) similar to that of caerulein. This effect was also inhibited by PD135158 (P<0.05). Amylase secretion into the biliopancreatic duct was stimulated by caerulein (1360 ± 206 u h^{-1}; n = 10) and by gastrin (1132 ± 491 u h^{-1}; n = 6). Both effects were abolished by the CCK_1 antagonist lorglumide (1 μmol kg^{-1}, s.c.; P<0.05), but were unaffected by the CCK_2 antagonist YF476 (Semple *et al.*, 1997) (1 μmol kg^{-1}, s.c.).

During pancreatitis induced by high doses of caerulein (3× 25 nmol kg^{-1} i.p. at 1 h intervals), a significant (P<0.05) increase in blood flow (62 ± 27%, n = 9) was observed only while the inflammatory oedema had not yet developed, *i.e.* at the first measurement at 20 min. At later time points (50–170 min), blood flow reverted to basal levels unless the oedema formation was prevented by the kinin B_2 receptor antagonist icatibant (300 nmol kg^{-1}, s.c.). The effects on blood flow were abolished by PD135158 (2 μmol kg^{-1}, i.v.; P<0.05), but remained unaffected by PD140548. Conversely, lorglumide (30–300 nmol kg^{-1}, s.c.), but not YF476 (1 μmol kg^{-1}, s.c.), prevented the induction of pancreatitis, as indicated by the significant (P<0.05) inhibition of the pancreatic oedema (9.6 ± 0.6 g g^{-1} dry wt; n = 7) and of extravasation of the protein marker Evans blue (612 ± 25 μg g^{-1} dry wt; n = 7).

In conclusion, caerulein-induced exocrine stimulation of the pancreas, both at submaximal and at supramaximal levels, is due to CCK_1 receptors, whereas CCK_2 receptors mediate increases in blood flow. We are currently investigating whether selective CCK_1 agonists could be used for investigations of acute pancreatitis without having direct effects on the microcirculation. (Austrian National Bank, grant 9314).

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67P ROLE OF CYCLOOXYGENASE (COX)-2 IN THE ENHANCED RENAL VASOCONSTRICTOR EFFECT OF ARACHIDONIC ACID (AA) IN THE DIABETIC RAT

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AA elicits COX-dependent vasoconstriction mediated by stimulation of TP receptors in the isolated perfused kidney of the rat. Before the recognition of multiple COX isoforms, we reported enhanced renal vasoconstrictor responsiveness to AA in the diabetic rat (Quilley and McGiff, 1990). As COX-2 expression is reportedly increased in the diabetic rat kidney (Komers et al., 2001), we investigated the contribution of COX-2 to the enhanced renal vasoconstrictor responses to AA.

Male Wistar rats (150-175g) made diabetic with streptozotocin (70mg/kg iv) were studied 4-8 weeks later. Kidneys from diabetic or vehicle-treated control rats were perfused with oxygenated Krebs' buffer (37°C) at constant flow to obtain perfusion pressures (PP) between 60-90 mmHg and responses to AA determined as described previously (Quilley and McGiff, 1990). Indomethacin and nimesulide, where used, were added to the buffer at the beginning of the perfusion. In some cases, 1 minute samples of the perfusate were collected immediately before and immediately after the administration of AA for estimations of prostaglandins by ELISA.

1,3 and 10μg AA increased PP by 85±37, 186±6 and 161±29mmHg in diabetic rat kidneys (n=5) compared to 3±1, 17±8 and 74±18mmHg, respectively, in the control rat kidneys (n=5) ($p<0.05$). The increased vasoconstrictor response to AA in diabetic rat kidneys was associated with greater release of prostaglandins, eg, release of 6-ketoPGF$_{1\alpha}$ from diabetic rat kidneys increased by 3.37±0.59ng/min after 1μg AA compared to 1.46±0.50ng/minfor the control group. Inhibition of COX with indomethacin (10μM) abolished the vasoconstrictor effect of AA in both groups (n=3) and prevented the increased release of 6-ketoPGF$_{1\alpha}$. In contrast, the COX-2 inhibitor, nimesulide (5μM) was without effect on the renal vasoconstrictor effect of AA in control rats (n=5) but reduced the effect of 1μg and 3μg AA by 82% and 42%, respectively, in the diabetic rat (n=5). Nimesulide did not affect basal release of 6-ketoPGF$_{1\alpha}$ in either group but reduced that stimulated by 1μg AA to 0.64+0.06ng/min and 1.50+0.48ng/min. in the control and diabetic groups, respectively.

These results indicate that COX-2-derived prostanoids contribute to the vasoconstrictor response to AA in the diabetic, but not in the control rat kidney and are consistent with Western blot analysis showing a 3-fold increase in renal COX-2 protein expression in the diabetic rat. It is unlikely that 20-HETE, a cytochrome P450-derived AA metabolite and a potent renal vasoconstrictor, contributes to vasoconstrictor effect of AA as 20-HETE release, measured by GC-MS, was greatly reduced in the diabetic (0.34±0.06ng/min) compared to the control (2.00±0.66ng/min). Further, 20-HETE was not increased in response to AA.

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68P EFFECTS OF THE CANNABINOID (CB_1) RECEPTOR ANTAGONIST, AM 251, ON THE REGIONAL HAEMODYNAMIC RESPONSES TO LIPOPOLYSACCHARIDE INFUSION IN CONSCIOUS SPRAGUE-DAWLEY RATS

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In anaesthetised rats, the hypotensive response to a bolus dose of LPS has been shown to be blocked by the CB_1-receptor antagonist, SR 141716A, leading to the suggestion that endogenous cannabinoids underlie a 'novel paracrine mechanism of vasodilatation in endotoxic shock' (Varga *et al.*, 1998), although no measurements of peripheral vascular tone were provided. We have now measured the regional vascular effects of LPS infusion in conscious rats, in the absence and presence of the CB_1-receptor antagonist, AM 251.
Under anaesthesia (fentanyl and medetomidine, 300 mg kg^{-1} of each i.p.), male Sprague-Dawley rats (380-440g) had pulsed Doppler flow probes and, subsequently, intravascular catheters implanted in a two-stage procedure, separated by at least 14 days. On the day following catheterisation, measurements of heart rate (HR), mean arterial blood pressure (BP) and renal (R), mesenteric (M), and hindquarters (H) vascular conductance (VC) were made in conscious, freely-moving animals. Rats were given AM 251 (3 mg kg^{-1} i.v., n=8) or the vehicle (5% propylene glycol, 2% Tween 80 in saline, n=10) 30 min before, and at 6h and 24h during infusion of LPS (*E Coli* 0127: B8, 150 μg kg^{-1} h^{-1} i.v.). Resting cardiovascular variables were not different prior to administration of vehicle or AM 251 (HR 311 ± 14, 333± 11 beats min^{-1}; BP 98 ± 2, 107± 2 mmHg, RVC 81 ± 8, 87 ± 7 (kHz mmHg^{-1}) 10^3, MVC 116 ± 10, 96 ± 11 (kHz mmHg^{-1}) 10^3, HVC 36 ± 3, 37 ± 2 (kHz mmHg^{-1}) 10^3, respectively). Some of the effects of LPS infusion are shown in Table 1.

Table 1. Changes in HR (beats min^{-1}), BP (mmHg) and VC (%), 1.25 and 24h after onset of infusion of LPS in rats treated with vehicle (Veh, n=10) or AM 251 (AM, n=8). Values are mean ± s.e. mean; * P ≤ 0.05 vs baseline (Friedman's test), # P ≤ 0.05 vs vehicle (Mann-Whitney test).

Time		HR	BP	RVC	MVC	HVC
1.25 h	Veh	+114±14*	-17±4*	+44±9*	+16±9	+125±12*
	AM	+29±13*#	-13±4*	+30±4*#	-1±7	+90±9*#
24 h	Veh	+86±13*	+22±4*	+40±12*	-26±6*	+43±10*
	AM	+48±5*#	+16±6*	+21±8*	-35±7*	+14±9*#

In both groups, a marked hypotensive response occurred 1.25h after the onset of LPS infusion. Pretreatment with AM 251 did not significantly affect the fall in BP, but attenuated the accompanying increases in HR, RVC and HVC. At 24h after the onset of LPS infusion, BP was similarly elevated in both groups, but in those pretreated with AM 251, there were smaller rises in HR and HVC. We have previously shown that the hindquarters vasodilator response to synthetic cannabinoids is mediated by β_2-adrenoceptors, and inhibited by AM 251, suggesting that CB_1-receptors may stimulate catecholamine release (Gardiner *et al.*, 2002). The present results, showing a clear effect of AM 251 on the HR and HVC responses to LPS, are more consistent with an effect of cannabinoids on catecholamine release and/or action rather than directly on vascular tone.

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