49P EFFECTS OF CHRONIC VITAMIN E TREATMENT ON HYPERGLYCAEMIA-INDUCED ENDOTHELIAL DYSFUNCTION IN PORCINE AORTIC ENDOTHELIAL CELLS

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In previous studies on endothelial cells in culture we demonstrated that diabetes mellitus leads to metabolic dysfunction as characterized by reduced release of nitric oxide (Olbrich et al., 1996). Moreover, we discovered a reduced activity of the NADPH-diaphorase, a coenzyme of NO-synthase, and severe changes in endothelial cell structure and morphology, such as an increasing incidence of giant and polynuclear cells or increased cytoplasmatic and total cell area (Salameh et al., 1997) and altered intracellular calcium handling (Salameh & Dhein,1998). Since high D-glucose-derived radicals, e.g. superoxide anions, are considered to be involved in the pathogenesis of altered endothelial function, we designed this study to examine the influence of α-tocopherol, a well-known radical scavenger, on nitric-oxide release.

Therefore, we cultivated porcine aortic endothelial cells (PAEC) under normoglycemic (5mM) and hyperglycemic (20mM D-Glucose) conditions for a complete culture passage until confluency was reached (4 days). Additionally, we treated the normo- and hyperglycemic cells chronically with racemic α-tocopherol (42 µM), i.e. for the whole culture passage. After reaching confluence, we investigated the ability of the cells to release nitric oxide under basal conditions and after stimulation with ATP using the met-Hb-method (Salameh & Dhein, 1998) at 37°C. A cell free α-tocopherol (42 µM) served as control solution for calibration. As in former studies cited above, the cells were examined histomorphometrically and the activity of the NADPH-diaphorase was checked histochemically (by nitro-blue tetrazolium staining). For statistical evaluation ANOVA followed by Student's t-test (2-sided) was used.

The release of nitric oxide from PAEC was reduced by chronic hyperglycemia. (normoglycemia: 0.0037 ± 0.0005 vs. hyperglycemia: 0.0023 ± 0.0005, $p<0.05$, n=20; values given as delta extinction under basal conditions). The reduction in NO-release became even more evident after stimulation of PAEC with 1 mM ATP: normoglycemia: 0.0074±0.00061 vs. hyperglycemia: 0.0048±0.00041, $p<0.05$, n=20). Chronic treatment with α-tocopherol attenuated the reduction in NO-release. Basal NO-release was only slightly different between normoglycemic and hyperglycemic cells treated with α-tocopherol (0.0026±0.001 vs 0.0018±0.0003). The reduced NO-release after ATP-stimulation was also reversed ($p<0.05$) by α-tocopherol (0.0078 ± 0.0004 vs 0.0071 ± 0.0003, n=6). Regarding cell morphology the number of giant cells in chronic hyperglycemia was increased (normoglycemia: 7±2 vs hyperglycemia 17±3 giant cells/40mm^2, $p<0.05$, n=9). This could be prevented with α-tocopherol (normoglycemia: 7±3 vs hyperglycemia 6±3 giant cells/ 40mm^2).

Thus, chronic α-tocopherol treatment can prevent from hyperglycemic impairment of endothelial function.

Olbrich A, Rösen P, Hilgers RD & Dhein S (1996) J Cardiovasc Pharmacol 27, 187-194.
Salameh A, Zinn M & Dhein S (1997) J Cardiovasc Pharmacol 30, 182-190.
Salameh A & Dhein S (1998) Diabetes 47, 407-413.

50P CHRONIC TREATMENT WITH ACETYLSALICYLIC ACID ATTENUATES ENDOTHELIAL DYSFUNCTION INDUCED BY HIGH D-GLUCOSE CONCENTRATIONS IN PORCINE AORTIC ENDOTHELIAL CELLS

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An important problem in antidiabetic treatment is the development of diabetic angiopathy. This has been shown to be accompanied by endothelial dysfunction characterized by reduced release of nitric oxide (Olbrich et al., 1996). Moreover, reduced activity of NADPH-diaphorase, a coenzyme of NO-synthase, and severe changes in endothelial cell structure and morphology were found (Salameh et al., 1997), as well as reduced calcium signals following external stimulation (Salameh & Dhein, 1998). Since enhanced production of free radicals has been proposed as a cofactor leading to inactivation of NO and since cyclic endoperoxides from the arachidonic acid metabolism may represent a source of radicals, we wanted to focus the present study on the relevance of the cyclo-oxygenase pathway for NO-availability.

Therefore, we cultivated porcine aortic endothelial cells (PAEC) under normoglycemic (5mM) and hyperglycemic (20mM D-glucose) conditions for a complete culture passage until reaching confluence (after 4 days). Normo- and hyperglycemic experiments were carried out either in absence or presence of acetylsalicylic acid (ASA, 1µM), which was applied throughout the whole culture passage. After reaching confluency, we investigated the ability of the cells to release NO under basal conditions and after stimulation with 1 mM ATP using the met-Hb-method (Salameh & Dhein, 1998). A cell free ASA (1µM) solution served as control solution for calibration. As in former studies cited above, the cells were examined histomorphometrically and the activity of the NADPH-diaphorase was investigated histochemically (nitro-blue tetrazolium staining). For statistical evaluation ANOVA followed by Student's t-test (2 sided) was used.

Release of nitric oxide from PAEC was reduced by chronic hyperglycemia (basal NO-release was reduced (normoglycemia: 0.004±0.0005 vs hyperglycemia 0.002±0.0005 ($p<0.05$), values given as Δ absorbance). After stimulation with 1 mM ATP stimulated NO-release was also reduced in hyperglycemic cells (normoglycemia: 0.0074±0.0006 vs hyperglycemia: 0.0048±0.0004, $p<0.05$, n=21). Chronic treatment with ASA prevented nearly completely and significantly the impairment of the NO-response (basal: Normoglycemia-ASA 0.003 ± 0.0003 versus Hyperglycemia-ASA 0.0025 ± 0.0001, stimulated: Normoglycemia-ASA 0.0074 ± 0.0005 versus Hypergylcemia-ASA 0.0074 ± 0.0008, n=5). Regarding cell morpholgy we detected a significantly increased number of giant cells in chronic hyperglycemia (normoglycemia: 7±2 vs hyperglycemia: 17±3 giant cells/ 40 mm^2, $p<0.05$, n=9). This could be nearly completely prevented by ASA (Normoglycemia-ASA 6±1 vs Hyperglycemia-ASA 8±3, n=8).

Thus, we conclude that chronic treatment with ASA can prevent hyperglycemic impairment of endothelial function in PAEC and that cyclooxygenase-dependent metabolism can interfere with NO-release or inactivation.

Olbrich A, Rösen P, Hilgers RD & Dhein S (1996) J Cardiovasc Pharmacol 27, 187-194.
Salameh A, Zinn M & Dhein S (1997) J Cardiovasc Pharmacol 30, 182-190.
Salameh A & Dhein S: Diabetes (1998) 47, 407-413.

51P INFLUENCE OF THE ADP ANTAGONISTS AR-C67085 AND A2P5P ON ADP-INDUCED RESPONSES OF PLATELETS AND ISOLATED ARTERIES

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Recently, two ADP receptors (P_{2T} and $P2Y_1$) with different signal transduction pathways have been shown to be necessary for human platelets to fully respond to ADP (Gachet *et al.* 1997, Jin and Kunapuli, 1998). Besides its effect on platelets, ADP relaxes precontracted arteries via a receptor-mediated process. The ADP-antagonists AR-C67085 (2-propylthio-D-ß,γ-dichloro-methylene ATP) and A2P5P (adenosine 2'phosphate 5'phosphate) are selective antagonists at the P_{2T} and $P2Y_1$ receptors, respectively. In the present studies the inhibitory effects of these ADP-antagonists on ADP-induced platelet aggregation and vascular contractility were compared.

Platelet-rich plasma (PRP) was obtained from citrated and hirudinized venous blood from healthy volunteers. Aggregation was measured turbidimetrically in an aggregometer. In Fluo-3 loaded platelets the changes in cytosolic Ca^{2+} were measured fluorimetrically. The ADP-induced relaxant effects were studied in rat aorta and small branches of porcine pulmonary arteries by cumulative addition to precontracted vessels. Rings (2-3 mm in length) were placed in 10 ml organ baths containing Krebs-Henseleit solution. The changes in tension were measured using isometric force transducers. A passive tension of 20 mN was maintained throughout the experiments. Endothelial integrity was assessed by the relaxant response to bradykinin (10 nM) or acetylcholine (1 μM).

ADP (5 μM)-induced platelet aggregation was potently, but not completely inhibited by AR-C67085 in a concentration-dependent manner. Even at a relatively high concentration (1 μM AR-C67085) a small, transient residual reversible aggregation (15-20%) was still present, irrespective of the concentration of ADP used. The EC_{50} values in hirudinized PRP (physiological Ca^{2+} concentrations) and in citrated PRP were 13 nM and 24 nM, respectively. These results are similar to those obtained previously with other antagonists known to act at the P_{2T} receptor. In contrast, A2P5P acted as a weak competitive inhibitor of aggregation in citrated PRP (pA_2 5.05). Complete inhibition was achieved with low concentrations of ADP (e.g. 0.3 μM) and relatively high concentrations of A2P5P (30-100 μM). This was accompanied by complete inhibition of intracellular calcium mobilization.

In phenylephrine (1 μM)-precontracted aortic rings ADP (0.1-100 μM) caused an endothelium-dependent reversible relaxation (EC_{50} 0.75 μM) which was abolished in the presence of L-NAME (100 μM). Preincubation (15 min) of the aortic rings with AR-C67085 at 10 μM did not inhibit the relaxant response to ADP (EC_{50} 0.90 μM). In $PGF_{2\alpha}$ (3 μM)-precontracted porcine pulmonary arteries the ADP (0.1-100 μM)-induced relaxant response (EC_{50} 2.82 μM) was also not affected by AR-C67085 (10 μM). When the rat aorta was preincubated with A2P5P (10-30 μM) the ADP-induced cumulative relaxation curves were shifted to the right in a parallel manner. Again, A2P5P was a weak competitive antagonist (pA_2 5.07, n=5). The same results were obtained with porcine pulmonary arteries (pA_2 5.10, n=8).

These studies suggest that different receptors for ADP exist in platelets and arterial vessels. Whereas the $P2Y_1$ receptor is present on platelets and in blood vessels, the P_{2T} receptor is only available on platelets.

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52P THE EFFECTS OF OXIDISED CHYLOMICRON REMNANT-LIKE PARTICLES ON ENDOTHELIAL CELL FUNCTION IN FRESHLY ISOLATED PORCINE CORONARY ARTERY

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Hypercholesterolaemia impairs endothelium dependent vasorelaxation, an effect found to precede the occurrence of visible lesions of atherosclerosis (Cohen *et al.*, 1988). Oxidised low density lipoprotein (LDL) is known to inhibit vasorelaxation via effects on the L-arginine-nitric oxide (NO) pathway in many arteries examined *in vitro* (Myers *et al.*, 1994). Another small cholesterol rich lipoprotein particle whose vascular effects have largely been ignored is the dietary lipoprotein, the chylomicron remnant (CMR). CMRs have been shown to be taken up by the arterial wall and inhibit endothelium dependent relaxation of rat aorta (Grieve *et al.*, 1998). The aim of the present study was to examine the effects of CMR-like particles (CMR-LP) on endothelium dependent relaxation of porcine coronary arteries (PCA).

CMR-LP were synthesised using a lipid mixture containing 5% cholesterol, 70% triacylglycerol and 25% phospholipids (Diard *et al.*,1994). Apolipoprotein E was incorporated into the particles by incubation with pig plasma. The particles were oxidised by incubation with $CuSO_4$ (10 μM, 18h). PCA rings (endothelium intact) obtained from pigs (n=5 to 9) killed at an abattoir were prepared for isometric tension recording (4g resting tension) and stimulated with depolarising Krebs solution (DKS; 118 mM KCl) to test their viability. Indomethacin (10 μM) was present in the Krebs Henseleit solution throughout all experiments. Tone was raised by addition of the thromboxanemimetic U44069 (10 or 30 nM). Cumulative concentration relaxation response curves (CRC) were then obtained to bradykinin (BK; 1 nM to 1 μM), S-nitroso-N-acetylpenicillamine (SNAP; 1 nM to 0.1 mM) or pinacidil (0.1 to 30 μM) in the presence or absence of oxidised CMR-LP (ox-CMR-LP, 19 μM cholesterol in organ bath). In two further experiments, the effects of oxCMR-LP on CRC to BK were tested either in the presence of N^G-nitroarginine methyl ester (L-NAME; 300 μM) or in vessels bathed in Krebs solution containing 30 mM KCl and 3 μM nifedipine. CRC parameters were compared by a Student's *t* test.

OxCMR-LP increased the EC_{50} (15.7±2.5 vs. 23.5±2.8 nM; $P<0.05$) and reduced maximum response (91.7±3.4 vs. 81.4±5.3 %; $P<0.05$) to BK. No significant effect of the oxCMR-LP was found on the responses of the vessels to pinacidil but they increased the maximum relaxation induced by SNAP (91.3±3.8 vs. 101.6±3.02 %; $P<0.05$). CRC to BK obtained in the presence of L-NAME were not affected by oxCMR-LPs but the particles caused a significant increase in the EC_{50} value (11.1±2.3 vs. 21.3±7.6 nM; $P<0.05$) and decrease in the maximum response (103.1±7.6 vs. 90.7±5.7; $P<0.05$) to BK in vessels treated with nifedipine and 30mM KCl.

These results show that oxCMR-LP inhibited the BK-induced NO-dependent relaxation in PCA but have no effect on activation of the hyperpolarising factor pathway by BK. The observed potentiation of SNAP-induced relaxations by oxCMR-LP might be due to the inhibition of basal NO production by the vessel enhancing their sensitivity to exogenously supplied NO. These findings support the atherogenic potential of CMRs.

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53P FUNCTIONAL RESPONSES OF SUBCUTANEOUS RESISTANCE ARTERIES ISOLATED FROM PATIENTS WITH NORMAL PRESSURE GLAUCOMA

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In the absence of elevated intraocular pressure, a vascular role has been proposed in the pathogenesis of normal pressure glaucoma (NPG) due to the presence of widespread vascular disease and increased vasospastic disorders (Geijssen & Greve, 1995). Acetylcholine (ACh)-mediated vasodilatation has recently been shown to be reduced in the forearm of patients with NPG (Henry *et al.*, 1999), suggesting that this condition is one aspect of a more generalised disturbance of the vascular system. This study aimed to determine whether the functional responses of subcutaneous resistance arteries were impaired in patients with NPG.

Gluteal fat biopsies were taken under local anaesthesia from 11 patients with NPG (6 male; age 59±2 years) and 9 healthy control subjects (6 male; age 60±3 years). Arteries were dissected from the biopsies and mounted in a myograph containing physiological salt solution at 37°C, gassed with 95%O_2; 5%CO_2 and equilibrated at their optimum resting force (Mulvany & Halpern, 1977). The endothelium was removed from some arteries by rubbing the lumen with a hair. Cumulative concentration-response curves were obtained to noradrenaline (NA; 10^{-9}-$3x10^{-5}$M), 5-hydroxytryptamine (5-HT; 10^{-9}-$3x10^{-5}$M), endothelin-1 (ET-1; 10^{-11}-$3x10^{-7}$M) and K^+ (10-125mM) in both endothelium-intact and denuded arteries. Concentration-response curves were also obtained to the endothelium-dependent dilators ACh (10^{-9}-$3x10^{-5}$M) and bradykinin (BK; 10^{-10}-$3x10^{-6}$M) and the endothelium-independent dilator 3-morpholinosydnonimine (SIN-1; 10^{-8}-10^{-4}M) following sub-maximal contraction with NA. Results are mean ± s.e.mean and were compared using Student's unpaired *t*-test. Ethical approval was obtained for the study protocol and all subjects gave written informed consent.

Sensitivity and maximum contraction to K^+ and NA were similar in arteries from patients and controls and were unaffected by removal of the endothelium (Table 1). In contrast, both sensitivity and maximum contraction to 5-HT and sensitivity to ET-1 were greater in arteries from patients than in control arteries (Table 1). Endothelial removal increased sensitivity and maximum contraction to 5-HT and sensitivity to ET-1 in control arteries but did not alter these responses in arteries from patients (Table 1). Sensitivity to ACh was increased in arteries from patients (7.64±0.08) compared with controls (7.31±0.11), the maximum relaxation however, was similar in both groups (NPG, 94.83±1.48; Control, 97.69±1.29). Relaxations to BK and SIN-1 were similar in arteries from patients compared to controls.

This study has demonstrated that endothelium-dependent relaxation is not impaired in subcutaneous resistance arteries from patients with NPG, and that sensitivity to ACh is actually enhanced. The modulatory effect of the endothelium on 5-HT and ET-1-mediated contraction seen in control vessels is lost in arteries from patients. This suggests a selective defect in agonist-mediated release of endothelium-derived vasodilators in arteries from patients with NPG.

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Table 1. Maximum Contraction (E_{max}; mN/mm) and sensitivity (pD_2) values.

	Control E_{max}		Control pD_2		Glaucoma E_{max}		Glaucoma pD_2	
	Intact	Denuded	Intact	Denuded	Intact	Denuded	Intact	Denuded
NA	2.57±0.63	1.71±0.33	6.95±0.12	7.35±0.25	3.29±0.30	3.05±0.68	7.17±0.16	7.18±0.29
5-HT	0.61±0.20	1.51±0.29*	6.66±0.22*	7.42±0.10*	2.43±0.63†	2.87±0.77	7.22±0.19†	7.65±0.25
ET-1	3.03±0.63	2.19±0.58	8.58±0.13	9.49±0.38*	3.45±0.39	3.34±0.69	9.12±0.10†	9.28±0.19
K^+	2.81±0.52	2.44±0.46	1.41±0.03	1.46±0.04	2.98±0.50	2.37±0.06	1.41±0.03	1.37±0.03

Results are mean ± s.e.mean, (n=4-11). *$P \leq 0.05$ compared with intact arteries. †$P \leq 0.05$ compared with control arteries.

54P GLUCOPSYCHOSINE ENHANCES URINE FLOW RATE AND SODIUM EXCRETION IN ANAESTHETIZED RATS

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We have recently reported that the sphingolipid sphingosine-1-phosphate reduces renal blood flow and enhances urine flow rate and electrolyte excretion in anaesthetized rats (Bischoff et al. 1998). Other sphingolipids such as glucopsychosine (GPS) can mimick sphingosine-1-phosphate effects, e.g. contraction of isolated mesenteric and renal microvessels (Czyborra et al. 1999). Therefore, we have investigated the effects of GPS on renal blood flow and renal function.

Male Wistar rats (270-490 g) were anaesthetized with thiobarbital (100 mg kg^{-1}) and instrumented as previously described (Bischoff et al. 1996). The femoral artery was catheterized for blood pressure measurements by a Statham pressure transducer, the femoral vein was catheterized for volume substitution and drug infusion. Renal blood flow was determined by a electromagnetic flow sensor (Skalar) placed on the renal artery. Urine samples were collected via ureter catheters at 15 min intervals. Urine volumes were determined gravimetrically, urinary electrolyte concentrations were determined by flame photometry, serum and urinary creatinine concentrations were measured photometrically. GPS (3-30 µg kg^{-1} min^{-1}) or its vehicle bovine serum albumin (1 mg ml^{-1}) in 0.9% saline were infused for 120 min followed by a 60 min washout period (n = 6-9 per group). Statistical significance between the groups was determined by a two-way analysis of variance with $P < 0.05$ considered significant.

GPS did not significantly reduce renal blood flow (basal 6.3±0.2 ml min^{-1}) or enhance renovascular resistance (basal 17.9±0.9 mm Hg (ml min^{-1})$^{-1}$), and did not consistently affect endogenous creatinine clearance (basal 1.1±0.7 ml min^{-1}). In contrast GPS infusion significantly increased urine flow rate (basal 129±13 µl/15 min), sodium excretion (basal 13.4±2.0 µmol/15 min) and sodium concentration (basal 93±8 mmol l^{-1}). The increases were slow in onset and reached maximal values after 60-120 min after start of the infusion (e.g. increase relative to baseline of 251±91 µl/15 min, 50±14 µmol/15 min and 60±10 mmol l^{-1}, respectively, with 30 µg kg^{-1} min^{-1} GPS in the final 15 min collection period). In contrast potassium excretion (basal 39.5±2.5 µmol/15 min) was not markedly altered by GPS, and accordingly urinary potassium concentration (basal 323±18 mmol l^{-1}) decreased by 173±33 mmol l^{-1} after 120 min infusion of GPS 30 µg kg^{-1} min^{-1}. The GPS-induced alterations of urine and electrolyte excretion did not reverse within 60 min of washout vehicle infusion.

We conclude that GPS affects only tubular function in contrast to sphingosine-1-phosphate, which affects tubular function and renovascular tone (Bischoff et al. 1998). We propose that distinct sphingolipid targets may exist in the renal vasculature and tubules.

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55P EVALUATION OF THE ANTIFIBRILLATORY DRUG INTERACTIONS BETWEEN LIDOCAINE AND PROPRANOLOL IN PERFUSED RABBIT HEARTS

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We have demonstrated in several previous studies the reliability of the serial-shock method of measuring ventricular fibrillation threshold (VFT) in quantitatively assessing antifibrillatory potency of many antiarrhythmic drugs (Almotrefi & Baker,1981; Almotrefi et al., 1993). In view of this and the alarming reports of the proarrhythmic effects of several antiarrhythmic agents (Echt et al., 1991; Peters et al.,1994) it was decided to use the above technique to study the possible interactions that may occur when antiarrhythmic drugs from different classes are combined. Studies were carried out on hearts isolated from New Zealand white rabbits of either sex weighing 1.5 to 2 Kg. The details of the method used have been given previously (Almotrefi and Baker, 1981). Statistical significance was calculated by Student's paired *t* test. Perfusion with lidocaine, propranolol or combination of the two drugs produced significant, dose-dependent increase in VFT (Table 1). These results indicate that, on a dosage basis, propranolol is more potent than lidocaine in elevating VFT. They further show a significant synergistic antifibrillatory effect of the combined use of these two drugs, which may point to a higher risk of proarrhythmic action when the drugs are combined in the treatment of cardiac arrhythmias.

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Table 1. Effect of lidocaine, propranolol and their combination on (VFT) in isolated-perfused rabbit hearts.

Drug	%change in *VFT* after exposure for			%change in *VFT* after washout for		
(μmol)	15min	30min	60min	15min	30min	60min
Control	6.9±3.1	3.5±3.4	-3.5±3.3	0.6±3.8	0.9±4.4	0.3±3.4
Lidocaine						
(3.46)	35.5±2.9**	50.6±4.0***	74.2±3.5***	-4.3±4.8	-9.0±4.7	-7.3±3.6
(6.92)	51.3±3.7*	97.9±12.1*	145.1±9.4*	25.2±8.3	3.3±7.0	-1.1±3.3
(13.85)	112.5±10.8***	191.2±10.7***	218.2±10.3***	-1.9±4.6	-5.0±3.7	-9.4±2.9
Propranolol						
(0.34)	40.2±3.7**	62.4±4.2**	97.6±6.4**	48.5±8.5**	19.7±6.5	11.6±4.0
(0.68)	57.8±5.6**	104.0±8.6**	183.8±9.1***	81.9±13.0**	32.3±6.9**	16.9±4.9**
(1.35)	88.2±12.7**	204.6±16.2**	272.2±15.8**	98.5±16.7**	48.3±9.6*	28.4±10.0*
Lidocaine+Propranolol						
(3.46+0.34)	128.4±9.3***	189.3±12.5***	278.9±14.9***	47.7±9.2**	22.9±4.0**	11.0±3.1*

Values are means ± s.e.m. * $P< 0.05$, ** $P<0.005$,*** $p<0.0005$

56P ISOLATION AND CALCIUM ANTAGONIST ACTIVITY OF 3,4-DIMETHOXY BENZALDEHYDE FROM *DAUCUS CAROTA*

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Daucus carota Lin. commonly known as "carrot" is widely grown plant in different parts of the world (Nadkarni, 1976). The plant has been used traditionally in compound formulations for the relief of hypertension. Our preliminary study on the alcoholic extract (DC) of *D. carota* leaves, showed promising hypotensive activity (Gilani *et al.*, 1994). Solvent fractionation followed by vacuum liquid chromatography of DC gave various fractions, of which fraction C, on acetylation and subsequent separation afforded 3,4-dimethoxy benzaldehyde (3,4-DM). In this investigation we describe the effect of 3,4-DM on blood pressure in normotensive anaesthetized rats and its possible mode of action has been explored using isolated tissue preparations.

Adult male Sprague-Dawley rats (200-250 g) were anaesthetized with thiopentone sodium (70-90 mg/kg; i.p.). Arterial blood pressure was recorded through carotid cannulation via pressure transducer (Statham P23 AC) coupled with a Grass model 7 polygraph. Isolated tissue preparations (rabbit aorta and jejunum) were used for the study of possible mechanism of action, which were set up by the method previously used in our laboratory (Gilani, 1991). Segments (2 cm) of rabbit aorta strips and jejunum were suspended separately in a 20 ml tissue bath, filled with Kreb's solutions, aerated with a mixture of 95% O_2 and 5% CO_2 and maintained at 37°C. Intestinal responses were recorded isotonically, whereas the vascular tension was measured via force-displacement transducer (FTO3). Calcium antagonist activity was assessed by constructing the concentration-response curves of Ca^{++} in the absence and presence of test compound in a K^+-rich medium (Farre *et al.*, 1991).

Intravenous administration of 3,4-DM caused a fall in arterial blood pressure in anaesthetized rats. This effect was dose-dependent and mediated at the doses of 0.3, 1.0, 3.0 and 10.0 mg/kg causing the respective drop in blood pressure of 7.79 ± 1.26, 17.89 ± 1.87, 32.06 ± 3.24 and 45.28 ± 3.82 % of control (mean ± s.e.m.; n=4). The vasoconstrictor effect of noradrenaline (NA; 1 μg/kg) before and after the administration of 3,4-DM (3 mg/kg) was similar, whereas phentolamine (1 mg/kg), an α-adrenoceptor blocker abolished the vasoconstrictor response of NE, which rule out the possibility of α-adrenoceptors involvement. In isolated tissue preparations, 3,4-DM caused a concentration-dependent (0.1-3 mM) inhibition of spontaneous contractions of rabbit jejunum as well as K^+-induced contractions of rabbit aorta and jejunum. Furthermore, it caused a concentration-dependent (1-2 mM) rightward shift in the Ca^{++} concentration-response curves, constructed in K^+-rich medium. These data indicate that the 3,4-DM lowers blood pressure possibly through calcium channel blockade and the presence of this compound in *Daucus carota* may be responsible for the therapeutic value of this plant in hypertension.

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57P SYNERGISTIC INTERACTION OF HISTAMINE AND ADRENALINE IN PLATELET AGGREGATION INVOLVES ACTIVATION OF PHOSPHOLIPASE C AND MAP KINASE PATHWAYS

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Our recent studies have shown that various platelet agonists at low concentrations act in synergy during platelet aggregation (Shah et al. 1999). Histamine enhances platelet aggregation and also potentiates the aggregatory response of other agonists. Previous studies indicate an involvement of Ca^{2+} signalling in the synergism process in platelets but the role of other second messengers and protein kinase is unknown. This study was conducted to examine the mechanism(s) of synergistic interaction in human platelet aggregation mediated by histamine and adrenaline.

Platelet aggregation was carried out at 37°C with platelet rich plasma (PRP) having platelet counts between 2.5 and $3.0x10^8$/ml of plasma as described previously (Shah et al. 1998). The anti-aggregatory effects of inhibitors were studied by pretreatment of PRP with various inhibitors for one min followed by addition of the subthreshold concentrations of the agonists and the resulting aggregation was recorded for 5 min after challenge.

Treatment of PRP with adrenaline(0.2-20 μM) showed concentration-dependent aggregatory effects on human platelets.The simultaneous addition of subthreshold concentrations of histamine(1-2 μM) and adrenaline (0.5-2 μM) exhibited synergistic effect.The synergism between histamine and adrenaline is inhibited by pretreatment of PRP with yohimbine and diphenhydramine indicating that the effect is receptor mediated (n=16). To examine if the histamine and adrenaline mediated effects involved activation of PLC, we used PLC inhibitor (U73122). Results show that pretreatment of PRP with U73122 inhibits the synergistic effect of histamine and adrenaline with an IC_{50} of 1.2 ± 0.3 μM (SEM; n=7).

To examine if the calcium influx is involved in agonist-mediated aggregation (Heemskerk & Sage, 1994), we used Ca^{2+} channel blockers (verapamil and diltiazem) to examine their effect in aggregation. We find that the synergistic effect of histamine and adrenaline is inhibited by both verapamil and diltiazem (IC_{50}=24 μM and 38 μM, respectively). Also pretreatment of PRP with PD98059, a selective inhibitor of MAP kinase activation, leads to strong inhibitory effects (IC_{50}= 1.1 μM) on platelet aggregation induced by co-addition of subthreshold concentrations of histamine and adrenaline. The inhibitors of other signalling pathways such as genistein (25μM) an inhibitor of tyrosine kinase, chelerythrine (10μM) an inhibitor of protein kinase C, wortmannin (10μM) a PI 3-kinase inhibitor and S-Nitroso-N-acetylpenicillamine (SNAP ; 20μM) had no effect on the synergistic interaction of histamine and adrenaline. These results show that synergistic interaction of histamine and adrenaline is mediated through activation of PLC/Ca^{2+} and MAP kinase signalling pathways.

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58P PROPRANOLOL INCREASES NORADRENALINE-INDUCED PROTEIN SYNTHESIS IN VENTRICULAR CARDIOMYOCYTES OF THE ADULT RAT

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In isolated ventricular cardiomyocytes of adult rats stimulation of α_1-adrenoceptors by noradrenaline causes increases in protein synthesis. Noradrenaline, however, stimulates α_1- and β_1-adrenoceptors in the cardiomyocytes, and it has been previously shown that, in ventricular cardiomyocytes of neonatal rats, noradrenaline-induced cAMP accumulation via β-adrenoceptor stimulation was enhanced by the α_1-adrenoceptor antagonist terazosin (Barrett et al., 1993). In this study we have therefore investigated whether α_1-adrenoceptor-mediated increases in protein synthesis by noradrenaline were affected by blockade of β-adrenoceptors by propranolol.

Freshly isolated ventricular cardiomyocytes from 12 weeks old male Wistar rats were incubated for 24 hours at 37°C with noradrenaline (1 nM - 10 μM) and [^{3}H]-phenylalanine (0.4 μCi/ml) in the presence and absence of 1 μM propranolol or 1 μM prazosin, and [^{3}H]-phenylalanine incorporation was assessed, as recently described (Pönicke et al., 1997).

Noradrenaline caused a concentration-dependent increase in protein synthesis; threshold concentration was between 10 and 100 nM; increase at 10 μM was 43 ± 5 % above control (n=8); the pEC_{50}-value was 5.9 ± 0.1. The α_1-adrenoceptor antagonist prazosin (1 μM) caused a significant rightward-shift of the concentration-effect curve of noradrenaline; in the presence of prazosin 10 μM noradrenaline increased protein synthesis only by 24 ± 9 % (n=4). In contrast, in the presence of 1 μM propranolol, noradrenaline (10 nM - 10 μM) caused at each concentration a higher increase in [^{3}H]-phenylalanine incorporation than in the absence of propranolol. Increase at 10 μM noradrenaline was 57 ± 3 % (n=8, $P < 0.05$); the pEC_{50}-value for noradrenaline was 6.3 ± 0.2.

We conclude that, in ventricular cardiomyocytes from adult rats, noradrenaline-induced increases in protein synthesis via α_1-adrenoceptor stimulation is diminished by simultaneous β-adrenoceptor stimulation. Inhibition of β-adrenoceptors significantly enhances noradrenaline-induced protein synthesis.

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59P COMPARISON OF THE EFFECTS OF ENDOTHELIN 1 ON FORCE OF CONTRACTION IN RIGHT AND LEFT VENTRICLES OF THE RAT HEART

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The role and functional importance of endothelin-1 (ET-1) in cardiac tissues of several species has been intensively investigated (Rubanyi & Polokoff, 1994). However, data comparing the effects of ET-1 on both left and right ventricle of the heart are still lacking. Using classical organ bath experiments we investigated the contractile response of ventricular strips to ET-1 obtained from both ventricles of 12 weeks old male Wistar rats (n=7). Preparations, i. e. strips of 8 mm length, 2 mm width and 2 mm (left) and 1 mm (right) thickness, were placed into 10 ml organ baths containing Tyrode solution (bicarbonate-buffered, 37°C, equilibrated with 95 % O_2, 5 % CO_2), and electrically driven by square-wave pulses of double threshold at a pacing rate of 1 Hz. After an equilibration period of 60 minutes, strips were submitted to cumulative concentrations of ET-1 ranging from 10^{-11} to 10^{-6} mol/l. Subsequently, at the end of the experiments, we assessed the response to $CaCl_2$ (from 2.52 to 13.32 mmol/l) in order to determine non-receptor dependent contractile force.

In both right and left ventricular strips ET-1 caused concentration-dependent increases in force of contraction; pD_2-values for ET-1 were 7.55 ± 0.04 in right and 7.79 ± 0.05 in left ventricles. In both ventricles the selective ET_A-receptor antagonist BQ-123 antagonized ET-1 induced positive inotropic effects. However, ET-1-induced increases in force of contraction were in right (E_{max} = 0.32 ± 0.09 mN) significantly lower than in left ventricular strips (E_{max} = 1.07 ± 0.15 mN, P < 0.01). On the other hand, calcium responses did not differ between right and left ventricular strips (E_{max} = 3.9 ± 0.41 vs. 3.7 ± 0.64 mN). In order to further study the mechanism underlying the different contractile responses to ET-1 in right and left ventricles we assessed ET-receptor density (by [^{125}I]-ET-1 binding, as described by Pönicke et al., 1998) and -subtype distribution (by BQ-123 competition curves) in membranes from right and left rat ventricles. ET-receptor density was in left ventricular tissue significantly higher than in right ventricular tissue (192 ± 36 vs. 113 ± 5 fmol/mg protein, n=6, P<0.05). On the other hand, ET_A:ET_B-receptor ratio was in both ventricles approximately 80:20%.

We conclude that the receptor-independent contractility as assessed by $CaCl_2$ is not different between right and left ventricle of the rat heart, while there is a lower contractile response to ET-1 in right versus left ventricular tissue, which may be explained by the smaller ET-1 receptor density in the right ventricle.

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60P *IN VITRO* VASOCONSTRICTOR ACTIVITY OF NOVEL PEPTIDE ENDOTHELIN-1(1-31) IS DUE TO CONVERSION TO ET-1(1-21) IN HUMAN INTERNAL MAMMARY ARTERY BY AN ENZYME OTHER THAN ENDOTHELIN CONVERTING ENZYME

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Incubation of the endothelin-1 (ET-1) precursor Big ET-1 with human chymase produces a novel 31 amino acid peptide, ET-1(1-31). This is reported to have comparable vasoconstrictor activity to ET-1, without further degradation (Nakano *et al.*, 1997; Kishi *et al.*, 1998). These authors suggest that ET-1(1-31) may be physiologically important as it is present in human tissues at higher concentrations than ET-1. Our aim was to assess the importance of ET-1(1-31) as a vasoactive peptide in human arteries *in vitro*.

Human left internal mammary artery (LIMA) was obtained from 15 patients (48-79 years) receiving coronary artery bypass grafts. Rings of LIMA, denuded of endothelium, were set up for isometric tension recordings in oxygenated Krebs solution (37°C). Cumulative concentration-response curves were constructed to ET-1, ET-1(1-31) and Big ET-1 (10^{-10}-$7x10^{-7}$M). Experiments were terminated by addition of 50mM KCl and agonist responses were expressed as a percentage of this. Curves for ET-1(1-31) were also obtained in the presence of the neutral endopeptidase/ endothelin converting enzyme (ECE) inhibitor phosphoramidon (100μM) or the selective ECE-1 inhibitor PD159790 (30μM) (Ahn *et al.*, 1998) added 30 minutes earlier. Bathing medium from some experiments were analysed by radioimmunoassay for the presence of mature ET.

ET-1 contracted LIMA with an EC_{50} value of 2.81nM (1.6-5.0nM, n=12) (geometric mean with 95% CI). ET-1 was 20 fold more potent than Big ET-1, EC_{50} 58.9nM (35-99nM, n=12), which we know must be converted to ET-1 for its biological activity. We found ET-1(1-31) to have an EC_{50} of 75.8nM (46-125nM, n=12) comparable to that of Big ET-1 and less than that of ET-1 (Figure 1). In additional experiments the response to ET-1(1-31) was not affected by the presence of either phosphoramidon or PD159790.

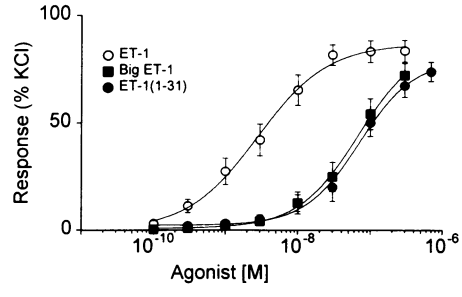

Figure 1. Cumulative concentration-response curves to ET-1, Big ET-1 and ET-1(1-31) in human LIMA.

Using a radioimmunoassay specific for mature ET that does not cross react with ET-1(1-31) we detected mature ET in the bathing medium following addition of ET-1(1-31).

Contrary to reports of direct vasoconstrictor activity of ET-1(1-31) we find that in human LIMA ET-1(1-31) is probably converted to ET-1 by an enzyme other than ECE-1. This suggests that alternative pathways for the generation of ET-1 may exist in human cardiovascular tissue.

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61P TAMOXIFEN AND 5-NITRO-2-(3-PHENYLPROPYLAMINO)-BENZOIC ACID (NPBB) REDUCE BASAL AND STIMULATED ENDOTHELIN-1 SECRETION FROM BOVINE AORTIC ENDOTHELIAL CELLS

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Chloride ion currents play an important role in the responses of vascular smooth muscle and endothelium to a variety of stimuli. Chloride channel blockers have vasodilator properties but the extent of their pharmacological effects have not been fully investigated. Moreover, recent molecular studies have shown the pattern of chloride channel gene expression in vascular smooth muscle cells differs from that in endothelial cells (Lamb *et al.*, 1999). In endothelial cells, 5-nitro-2-(3-phenylpropyl-amino)-benzoic acid (NPPB) and tamoxifen have been shown to inhibit Ca^{2+}-activated chloride currents (Nilius *et al.*, 1997). Here we have compared the effects of these agents on endothelin-1 (ET-1) secretion from cultured endothelial cells.

Bovine aortic endothelial cells (BAEC) were cultured as previously described (Corder & Barker, 1999). The effects of NPPB and tamoxifen on the secretion of ET-1 were evaluated under basal conditions and during stimulation with tumour necrosis factor-α (TNFα, 10 $ng.ml^{-1}$) or transforming growth factor-ß (TGFß, 1 $ng.ml^{-1}$). Confluent cultures (12 x 22 mm well plates) were treated for 6 h in serum free medium with the agents indicated, followed by a 1 h MTT test to ensure the absence of cytotoxic effects. ET-1 secretion was determined by radioimmunoassay (Corder & Barker, 1999). In each experiment ET-1 secretion was expressed as a % of basal release (mean basal release = 73 ± 11 $fmol.cm^{-2}.6\ h^{-1}$, n = 12). For the secretion studies results are from at least two separate experiments with triplicate determinations in each. In a separate study the effect of tamoxifen and NPPB on basal and cytokine stimulated levels of preproET-1 mRNA were examined. After 2 h treatment total RNA was harvested for estimation of preproET-1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels by RT-PCR (Corder & Barker, 1999). For each sample preproET-1 mRNA levels were normalised to the corresponding GAPDH value. Significant differences were determined by ANOVA with Fisher's test.

Compared to basal secretion TNFα and TGFß increased ET-1 secretion by 84 ± 16% and 142 ± 29% respectively (n = 8, P <0.01). NPPB (100 µM) decreased basal ET-1 secretion by 61 ± 4% and reduced the ET-1 responses to TNFα or TGFß by a similar proportion (n = 8 or 9, P <0.01). NPPB did not reduce levels of preproET-1 mRNA. Tamoxifen (10 µM) reduced basal secretion by 35 ± 3%, and TNFα or TGFß induced release by 21 ± 5% and 24 ± 3% respectively (n = 6, P <0.01). Tamoxifen (10 µM) also reduced basal levels of preproET-1 mRNA without affecting cytokine stimulated levels.

NPPB and tamoxifen reduced ET-1 secretion at concentrations reported to block chloride channel currents in endothelial cells (Nilius *et al.*, 1997). However, their actions on levels of preproET-1 mRNA indicate the mechanisms underlying the decreases in ET-1 secretion are probably different.

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62P SUPPRESSION OF MYOGENIC AND VASOCONSTRICTOR TONE IN PORCINE CEREBRAL ARTERY BY BASAL NITRIC OXIDE

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Basal nitric oxide (NO) activity exerts a tonic vasodepressor action, suppressing vasoconstrictor tone in the vasculature (Martin *et al.*, 1986, Rees *et al.*, 1989). The aim of this study was to examine the ability of basal NO to modify 5-hydroxytryptamine (5-HT) -induced contraction in the porcine cerebral artery (PCA).

Heads from adult freshly-killed Yorkshire pigs of either sex (50-75kg) were obtained from a local slaughterhouse. The brain was removed and anterior cerebral arteries were dissected out, cleaned of fat and connective tissue, and cut into rings (2.5 mm wide). Endothelium-intact (E+) and -denuded (E-) rings were then suspended in tissue baths containing oxygenated Krebs solution at 37°C for tension recording, and placed under 1 g of stretch. Data are expressed as mean ± s.e.m., n≥6 and differences determined using an unpaired t-test.

The nitric oxide synthase inhibitor, N^G-nitro-L-arginine methyl ester (L-NAME, 100 µM), produced a substantial increase in tone (1.15 ± 0.13 g) when added to E+ rings of PCA, but a much smaller increase (0.29 ± 0.12 g, P<0.01) in E- rings. This indicates that myogenic tone is present in these vessels and that basal NO exerts a profound depressive action on this tone. Despite this powerful basal NO activity, cumulative concentration-response curves to 5-HT (1 nM - 10 µM) failed to reveal the expected suppression of contraction in E+ vessels (maximum contractions: 1.48 ± 0.24 g and 1.30 ± 0.26 g; pD_2 values: 7.30 ± 0.15 and 7.72 ± 0.17 in E+ and E-, respectively, P>0.05 for both). It was possible that this lack of suppression had resulted from E+ and E- rings being mounted at different locations along their respective stretch-tension curves. To investigate this, stretch-tension curves were constructed by suspending E+ and E- rings at different levels of stretch (0 - 4 g). L-NAME (100 µM) was then added to abolish the endothelium-dependent depression of myogenic tone, and papaverine (100 µM) was subsequently added to abolish all active tone. The difference (in g) between the level of tone in the presence of L-NAME and after the application of papaverine was taken as a measure of the active myogenic tone in the vessel. This active tone was then plotted against the applied stretch and curve fits obtained. In E- rings, an applied stretch of 1 g produced 0.6 g of active tone. Extrapolation to the E+ curve fit showed that 0.6 g of active tone was achieved with 0.3 g of applied stretch. The same level of active tone (0.6 g) was established in E+ (stretch 0.3 g) and E- (stretch 1.0 g) rings and cumulative concentration-response curves to 5-HT (1 nM - 10 µM) constructed. Contractile responses were now found to be suppressed in E+ compared with E- rings (maximum contractions: 0.87 ± 0.09 g and 1.36 ± 0.16 g (P<0.05); pD_2 values: 7.20 ± 0.13 and 7.88 ± 0.10 (P<0.01) in E+ and E-, respectively).

In conclusion, the PCA generates myogenic tone in response to stretch, and this tone is powerfully suppressed in E+ vessels by high basal NO activity. E+ and E- rings set at similar levels of stretch, therefore, exhibit different levels of myogenic tone, and this can mask the ability of basal NO to suppress vasoconstrictor tone. In contrast, when E+ and E- vessels are set to the same level of active tone, the expected endothelium-dependent depression of vasoconstrictor tone is revealed. These findings have important implications for the study of blood vessels in which myogenic tone and basal NO activity are both powerful.

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63P EFFECT OF THE L-ARGININE ANALOGUE N^G-NITRO-L-ARGININE METHYL ESTER ON VASORELAXATIONS TO ATP-SENSITIVE POTASSIUM CHANNEL OPENERS IN RAT ISOLATED AORTA

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The chemical diversity of ATP-sensitive potassium channel openers (K_{ATP}COs) has suggested the involvement of multiple sites on the target membrane (Lawson, 1996). Thus, an understanding of the pharmacophore(s) of K_{ATP}COs will provide insight of the site(s) of action of these agents. Kontos & Wei (1996) suggested that L-arginine analogues antagonise the effects of pinacidil and cromakalim at the level of the K_{ATP} channel in smooth muscle cells in cat cerebral vasculature *in vivo*. We therefore studied the effects of the L-arginine analogue N^G-nitro-L-arginine methyl ester (L-NAME) on the vasorelaxations to K_{ATP}COs in rat isolated aorta.

Aortic rings, devoid of endothelium, from male Wistar rats (200-250g) were suspended under a resting tension of 2g in Kreb's bicarbonate solution (gassed with 95% O_2 & 5% CO_2 at 37°C). After 60min equilibration, cumulative concentration response curves (CRC) to pinacidil (0.05-25.6μM), cromakalim (0.05-12.8μM), diazoxide (10-160μM), aprikalim (0.01-5.12μM) or SKP-450 (0.1-51.2nM; Lee *et al.*, 1998) were constructed 30min after incubation with L-NAME (100 or 300 μM), L-N^5-(1-iminoethyl)ornithine (L-NIO 100μM) or vehicle (control) in phenylephrine(1.0μM)-contracted tissues. Responses were determined as % relaxation of the phenylephrine contraction. EC_{50} values (concentration to evoke 50% relaxation) are reported as mean ± s.e.m (n = 4-8). Concentration ratios with 95% confidence limits (CR(cl)) were determined from EC_{50} values (test:control) of paired preparations.

Concentration related relaxations, with a maximal response of 100%, were produced by aprikalim (EC_{50} 0.18±0.07μM), cromakalim (EC_{50} 0.40±0.24μM), diazoxide (EC_{50} 27.80±6.57 μM), pinacidil (EC_{50} 0.50±0.10μM) and SKP-450 (EC_{50} 4.1± 0.5nM). L-NAME (100μM) significantly displaced to the right of controls, without modifing the maximum, CRCs to aprikalim (CR(cl) 4.08 (2.21-5.95)) and pinacidil (CR(cl) 5.05 (3.56-6.54)), but not to cromakalim (EC_{50} 1.32±0.90μM), diazoxide (EC_{50} 43.72±4.42 μM) or SKP-450 (EC_{50} 7.0±2.9nM). L-NAME (300μM) did not further attenuate the relaxations to pinacidil or aprikalim. In contrast, L-NIO (100μM) failed to modify the CRCs to pinacidil (EC_{50} 0.73±0.23μM) or aprikalim relative to control.

In conclusion, L-NAME can differentiate between structurally unrelated K_{ATP}COs suggesting that pinacidil and aprikalim activate a vasorelaxant mechanism in rat aorta independent of the mechanism(s) operated by cromakalim, SKP-450 and diazoxide. The failure of L-NIO to modify the responses to pinacidil and aprikalim infers that this mechanism is not nitric oxide synthase. These observations may assist in the understanding of the site(s) at which chemically diverse K_{ATP}COs act on the channel in vascular smooth muscle cells.

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64P CHARACTERISTICS OF THE PULSE WAVEFORM DURING ALTERED NITRIC OXIDE SYNTHESIS IN THE NEW ZEALAND WHITE RABBIT

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Vasodilatation and vasoconstriction can produce significant changes in the arterial pulse waveform, particularly in the height of the dicrotic notch relative to the amplitude of the pulse wave (Klemsdal *et al*, 1994). Although organic nitrates produce such alterations, several other vasodilative and hypotensive agents such as guanethidine do not (Morikawa, 1967). In the current study, we have investigated the changes in the pulse wave form and wave velocity during altered nitric oxide (NO) synthesis since the relative height of the dicrotic notch (RhDN) may be indicative of the level of NO-dependent relaxation *in vivo*.

In the first series of experiments, a.c.-coupled infra-red photoplethysmography probes were placed over a central ear artery and a rear footpad of male New Zealand White rabbits (3-5.5 kg). Probe output was digitised and recorded for subsequent manual analysis at intervals before and during infusion of the rabbits with acetylcholine (ACh; 12μg/min i.v. for 10 min) or with N^{ω}-nitro-L-arginine methyl ester (L-NAME; 5 mg/min i.v for 10 min), an inhibitor of NO synthesis. A second series of experiments assessed the modification of responses to ACh (2 μg/kg/min i.v. for 5 min) by L-NAME infusion (10 mg/min for 15 min, starting 10 min before ACh infusion) or by zaprinast, a phosphodiesterase type V inhibitor (10 mg/kg, i.v. bolus 10 min before ACh) in animals sedated with fentanyl fluanisone (hypnorm, 0.1 ml/kg i.m.). In this case, output from a single ear probe was continuously digitised and was analysed using a computerised system. Results are presented as mean±s.e.m. and comparisons between means were made using the *Student's* t-test.

During ACh infusion, RhDN fell to 53.4±8.5% of its control value (n=6, $p<0.001$), whilst it rose by 44.3±15.0% during L-NAME infusion (n=4, $p<0.01$). In both cases, there were parallel changes in the interval between heart beats ($p<0.01$). However, neither drug affected the delay between the arrival of the systolic peak at the ear and the foot. Furthermore, using the ear traces alone, there were no changes in the interval between the systolic and diastolic peaks or in the interval between the systolic peak and the notch (all $p>0.05$). The slope of the wave just before the notch was increased by ACh ($p<0.05$) and decreased by L-NAME ($p<0.01$), and there were corresponding changes in the drop in trace height during this period ($p<0.05$). The overall amplitude of the wave, however, did not change in either case ($p>0.05$). L-NAME prevented the fall in RhDN caused by ACh (at 5 min, -22.7±5.6% to 9.8±8.6%, n=4, $p<0.05$) whilst zaprinast potentiated the response to ACh (at 5 min, -34.9±5.1% to -57.2±10.8%, n=6, $p<0.05$).

In summary, ACh produced a significant fall in the height of the dicrotic notch relative to the pulse wave amplitude *in vivo*. This fall was mediated by NO. An influence of basal NO release on RhDN was also identified. These effects did not appear to reflect alteration of either the wave speed or the intervals between various components of the waveform, but may be due to changes in the rate at which pressure falls after peak systole, in heart rate or in the magnitude of wave reflections.

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65P REGIONAL VARIATION IN RESPONSES OF RABBIT ISOLATED PULMONARY ARTERY TO NERVE STIMULATION

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It is generally accepted that noradrenaline released from sympathetic nerves is the main neuronal vasoconstrictor pathway by which pulmonary vascular tone is regulated. However, nerve-evoked responses to electrical field stimulation in different regions of the pulmonary arterial tree have not been fully investigated. The aim of this study was to examine responses to electrical field stimulation of rabbit extra and intra pulmonary arteries and in particular to investigate the role of sympathetic nerves.

Male New Zealand rabbits (1 kg) were killed by cervical dislocation. Rings (2 mm) of extra and intra pulmonary arteries were suspended in 5 ml organ baths containing Krebs-bicarbonate solution at 37°C and continuously gassed with 97% O_2 / 3% CO_2 to pH 7.4. An initial basal tension of about 20 mN was applied.

Trains of electrical field stimulation (100 pulses at 10 Hz; 0.1 ms; 60 - 100 V) evoked monophasic vasoconstrictions in the extra pulmonary artery (amplitude 7.2 ± 1.0 mN, duration 10.0 ± 1.2 minutes; mean ± s.e. mean, n = 9). In contrast, biphasic vasoconstrictions in the intra pulmonary artery were recorded. In this region there was an initial transient component (amplitude 2.1 ± 0.4 mN, n = 18) followed by a slow component (amplitude 2.1 ± 0.6 mN, n = 12). More interestingly, the second component was very long lasting (duration 22 ± 2 minutes, n = 12).

To study the role of noradrenaline in different regions the α_1 - adrenoceptor antagonist prazosin (1 μM) was used. The nerve - evoked vasoconstriction in the extra region was prazosin sensitive (83.0 ± 2.6% inhibition, n = 4) whereas prazosin had no significant effect in the intra artery (12.6 ± 8.0% inhibition, n = 6; $P > 0.05$; paired t-test).

To identify the unknown neurotransmitter(s) activating the intra pulmonary artery, the nonadrenergic cotransmitters ATP and neuropeptide Y (NPY) were investigated. The P_{2X} desensitising agent, α,β-methylene ATP (1 μM) and the NPY Y_1 receptor antagonist, BIBO3304 (1 nM; Wieland *et al.*, 1998) were applied in the presence of prazosin. Both antagonists had no significant effect on the long lasting constriction (12.2 ± 7.9%; n = 6 and 3.2 ± 3.2%, n = 5, respectively; $P > 0.05$). Surprisingly, the adrenergic neurone blocking agent, bretylium (2 μM) which caused 85% inhibition (n = 2) in the extra artery had no significant effect on the electrically evoked vasoconstriction the intra artery.

Together, these data suggest that noradrenaline released from adrenergic nerves is the predominant neurotransmitter inducing vasoconstriction in the extra pulmonary artery. Conversely, noradrenaline and possibly sympathetic nerves play a less important role in the intra pulmonary artery. The identity of the unknown neurotransmitter(s) and nerve remains to be elucidated.

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66P MONOAMINE OXIDASE INHIBITION DETERMINED IN RAT BRAIN IS UNLIKELY TO ACCOUNT FOR CARDIOVASCULAR RISKS ASSOCIATED WITH FENFLURAMINE AND PHENTERMINE

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Administration of fenfluramine (Fen) or its *d*-isomer (*d*-Fen) either alone or in combination with phentermine (Phen) in man is associated with the production of pulmonary hypertension (Abenhaim *et al.*, 1996) and heart-valve disease (Connolly *et al.*, 1997). Elevations in plasma 5-hydroxytryptamine (5HT) have been postulated as a link with these diseases (see Connolly *et al.*, 1997), but their pathobiology remains uncertain. Nevertheless, a recent report by Maher *et al.* (1999) hints that Phen may act as a monoamine oxidase inhibitor (MAOI) and that in combination with the known abilities of *d*-Fen to release 5HT (Heal *et al.*, 1998) and even of fluoxetine and other SSRIs to inhibit 5HT reuptake, their mixed use may have led to an unanticipated boost in circulating 5HT. In the absence of experimental data, this prompted us to perform direct measurements of MAOI activity for a series of anti-obesity agents and SSRIs.

MAO activities were measured in whole brain minus cerebellum of male Sprague-Dawley rats (190-200g). We adapted the method of Wurtman and Axelrod (1963) and used low substrate concentrations to permit selective assay of MAO_A (<1.0μM ^{14}C-tryptamine) and MAO_B (<1.0μM ^{14}C-ß-phenylethylamine). IC_{50} values for selective MAOIs were for harmaline (MAO_A; 7.3 nM) and lazabemide (MAO_B; 8.4 nM).

The data in Table 1 show that Phen is a very weak inhibitor of MAO_A and MAO_B with IC_{50} values in the high micromolar range. However, it is clear that both isomers of Fen and the major active metabolite of *d*-Fen, *i.e.* *d*-norFen, span Phen in terms of potencies as MAOIs. Furthermore, various SSRIs and the active metabolites of the SNRI, sibutramine (M1 and M2), are also MAOIs in the micromolar range.

On this basis, it is hard to envisage how the Fen-linked cardiovascular disorders can be attributed to MAO inhibition by Phen. Whilst Phen was more active than *d*-Fen or sibutramine, all other agents, including *d*-norFen, were at least as active as Phen itself. These include SNRIs and SSRIs which so far have not yielded such cardiovascular risks.

An alternative explanation is that, whilst Fen and *d*-Fen are known 5HT releasers, Phen also expresses this pharmacological effect *in vivo* and its effect is additive with that of *d*-Fen (Prow *et al.*, 1999). Thus, there is no reason why Phen or *d*-Fen should enhance 5HT concentrations by MAO inhibition beyond their innate ability to evoke 5HT release (Heal *et al.*, 1998; Prow *et al.*, 1999). This is in contrast to the SSRIs and M1 and M2, which do not release 5HT and where 5HT reuptake inhibition occurs at concentrations 5000- to 162000-fold less than their IC_{50} values for MAO_A inhibition (Heal *et al.*, 1998).

Table 1. MAO_AI and MAO_BI activities of anti-obesity agents and SSRIs

Treatment	n	MAO_A	MAO_B	p
		IC_{50} (μM) mean ± s.e.mean	IC_{50} (μM) mean ± s.e.mean	
Phentermine	4	142 ± 7	283 ± 23	
d-Fenfluramine	4	265 ± 16	434 ± 16	<0.001
l-Fenfluramine	5	115 ± 16	685 ± 57	<0.001
d-Norfenfluramine	4	36 ± 2	158 ± 16	<0.001
Sibutramine	4	>1000	>1000	
BTS 54354 (M1)	4	157 ± 11	41 ± 1	0.91
BTS 54505 (M2)	4	127 ± 7	34 ± 2	0.84
Fluoxetine	5	69 ± 3	22 ± 2	<0.001
Paroxetine	5	80 ± 5	16 ± 2	<0.001
Sertraline	4	31 ± 2	49 ± 4	<0.001

Data were log_{10}-transformed and subject to one-way ANOVA followed by Dunnett's test. *P* values are *versus* Phen for MAO_A inhibition.

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67P EFFECTS OF MELATONIN AND TROLOX ON ISCHAEMIA-REPERFUSION INJURY IN THE LANGENDORFF RAT ISOLATED HEART

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Several recent studies have demonstrated that the pineal hormone melatonin affords protection against damage due to ischaemic reperfusion of the stomach, brain and heart (Konturek *et al.*, 1997; Guerrero *et al.*, 1997; Tan *et al.*, 1998). The action may be due to free radical scavenger properties of melatonin described in many cell-free systems (see Reiter *et al.*, 1997 for review). In the present study, the potential protective action of melatonin was investigated using the Langendorff rat isolated perfused heart. Since it has also been reported that vitamin E protects injury caused by ischaemia-reperfusion (Nagel *et al.*, 1997), a water soluble vitamin E analogue, trolox, has been employed as an internal control.

Male adult Wistar rats (250-350 g wt) were heparinized (1000 U kg^{-1} i.p.) and anaesthetized with sodium pentobarbitone (60 $mgkg^{-1}$, i.p.). Heart was excised and cannulated via the aorta and perfused retrogradely by the Langendorff technique at a constant flow of 20 ml min^{-1} with oxgenated Krebs (95 % O_2/5 % CO_2; 37 ^oC) (Randall *et al.*, 1997). After an initial 15 min of equilibration period, hearts were subjected to an ischaemia-reperfusion protocol which consisted of 30 min aerobic perfusion, followed by 15 min homeothermic global ischaemia and 60 min aerobic reperfusion. Either melatonin (300 μM) or trolox (100 μM) was present throughout the experimental protocol. Cardiac variables (coronary perfusion pressure, CPP; left ventricular developed pressure, LVDP and heart rate, HR) were recorded. All data have been expressed as mean±s.e.mean of n experiments. Changes in the LVDP and HR within each group have been expressed as a percentage of the baseline value (measured prior to ischaemic insults). Differences within each treatment group were compared using paired Student's t-test (2-tailed) and a value of $p < 0.05$ was considered statistically significant.

After a 30 min perfusion period, there was no significant change in baseline cardiac variables (LVDP and HR) of control preparations and hearts which have been perfused with melatonin or trolox (Table 1). Neither melatonin nor trolox affected the CPP. However, in control hearts, the CPP was significant increased (Table 1).

	Control (n=6)		Melatonin (n=8)		Trolox (n=6)	
Perfusion (min)	0	30	0	30	0	30
LVDP (mmHg)	77±7	79±10	65±9	73±11	81±11	77±12
HR ($beatmin^{-1}$)	287±16	286±19	266±12	261±13	268±12	278±15
CPP (mmHg)	69±1	85±5*	80±7	89±9	110±17	127±18

Table 1 Baseline cardiac variables during 30 min perfusion. * $p < 0.05$ vs 0 min perfusion (paired Student's t-test, 2-tailed)

Following 15 min ischaemia, the LVDP of control heart was significantly reduced after 60 min reperfusion (ΔLVDP -37.1±13.6 %; $p < 0.05$) and the HR returned to near normal (ΔHR -7±4; n=6). Similar changes in the cardiac mechanical performances were observed in melatonin (ΔLVDP -66.1±8.1 %, $p < 0.01$; ΔHR -10.6±2.1%; n=8) and trolox (ΔLVDP -47.1±12.9 %, $p < 0.05$; ΔHR -17.2±12%; n=6) preparations.

Neither melatonin nor trolox afforded protection against the ischaemic reperfusion injury of the isolated heart. These negative findings may be due to the lack of antioxidant activity of the agents (at the concentrations examined), or that the cardiac dysfunction observed in the current paradigm is not entirely attributable to rapid generation of free radicals.

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68P THE EFFECT OF REMOVAL OF THE ENDOTHELIUM ON α_2-ADRENOCEPTOR-MEDIATED VASOCONSTRICTION IN PORCINE ISOLATED EAR ARTERIES

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Recent studies have demonstrated that enhanced α_2-adrenoceptor-mediated contractions can be uncovered in the porcine ear artery *in vitro* by prior pharmacological manipulation (Roberts *et al.*, 1998). The aim of this present study was to determine whether α_2-adrenoceptor-mediated vasoconstriction (both direct and enhanced) in the porcine ear artery is influenced by removal of the endothelium.

Porcine ear arteries segments were set up as before (Roberts *et al.*, 1998). Before each experiment, the tissues were contracted 3 times with 60 mM KCl. The endothelium was removed from some of the tissues by rubbing the surface of the lumen with a fine pair of forceps. Successful removal of the endothelium was indicated by the inability of the tissues to relax to 0.1 μM substance P.

The contractile response to 60 mM KCl was significantly less in endothelium-denuded segments compared to endothelium intact segments (1.5 ± 0.1 g wt in endothelium-denuded segments compared to 3.0 ± 0.2 g wt in endothelium intact segments, n=24). The small contractile response to the α_2-adrenoceptor agonist UK14304 (0.3 μM; expressed as a percentage of the 60 mM KCl response) was significantly reduced by removal of the endothelium (Table 1). Similarly, the enhanced responses to 0.3 μM UK14304 obtained after pre-contraction with the thromboxane-mimetic U46619 (0.1 μM), and relaxation back to baseline with either SNP (100-200 μM), or dbcAMP (1-3 mM) were also signifcantly reduced in endothelium-denuded tissues (Table 1). In marked contrast, enhanced responses to 0.3 μM UK14304 obtained after pre-contraction of the tissue with U46619 (0.1 μM) and relaxation with forskolin (1-2 μM) were unaffected by removal of the endothelium (Table 1).

These data demonstrate that removal of the endothelium reduces direct α_2-adrenoceptor-mediated vasoconstriction in the porcine ear artery. Furthermore, enhanced α_2-adrenoceptor-mediated responses after pre-contraction with U46619 and relaxation with either SNP or dbcAMP also appear to be dependent upon an intact endothelium. Suprisingly, however, the enhanced responses to UK14304 after relaxation with forskolin were unaffected by removal of the endothelium. The decrease in the size of the response to 60mM KCl in endothelium-denuded vessels suggests that there is also a general decrease in vasoconstriction, as well as the more specific decrease in the α_2-adrenoceptor-mediated responses

	UK14304 alone	+ U46619 + forskolin	+ U46619 + SNP	+ U46619 + dbcAMP
a	8.8 ± 2.1%	45.2 ± 14.1%	33.4 ± 5.8%	31.7 ± 8.8%
b	4.1 ± 0.9%*	45.4 ± 9.6%	14.8 ± 1.9%*	9.9 ± 2.4%*

Table 1. Responses to UK14304 (0.3 μM) in endothelium intact (**a**) and endothelium denuded (**b**) ear arteries, in the absence or presence of U46619 and forskolin, U46619 and SNP, or U46619 and dbcAMP. Results are expressed as a percentage of the contraction to 60 mM KCl and are means ± S. E. mean of 5-17 experiments. * $p<0.05$ Wilcoxon Signed Rank test vs endothelium-intact vessels.

Roberts R. E., Tomlinson A. E., Kendall D. A. *et al.*, (1998), *Br. J. Pharmacol.*, **124**, 1107-1114.
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69P ON THE ANTIOXIDANT ACTIVITY OF DIHYDROPYRIDINE-CALCIUM ANTAGONISTS

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It has recently been shown, that calcium antagonists protect cells from radical-induced damage [1 - 4]. In order to elucidate, whether this protection depends on an intracellular decrease of calcium concentration or an antioxidant activity of Dihydripyridine(DHP)-molecules, we measured the antioxidant activity of DHP calcium antagonists *in vitro* by using the „Total Antioxidant Status"-assay (TAS) by N. J. Miller and C. Rice-Evans [5].

The absorbance of the 2,2'-azinobis(-3-ethylbenzothiazone-6-sulfonate)-radical-cation ($ABTS^{+\bullet}$) was used to measure the radical-concentration and its decrease in presence of DHP-derivatives. The antioxidant capacity of the DHP-derivatives are given relative to Trolox® (Hoffman-La Roche), a better water-soluble tocopherol derivative, as TEAC (Trolox equivalent antioxidant capacity). The TEAC-values of five clinically used DHP-derivatives as measured by means of TAS are shown in table 1.

Table 1: TEAC-values of five clinically used DHP-derivatives

DHP-derivative	TEAC
Trolox®	1
Nifedipine	0,458 ± 0,019
Nitrendipine	0,621 ± 0,026
Isradipine	0,620 ± 0,039
Amlodipine	0,913 ± 0,055
Felodipine	0,938 ± 0,060

Since these DHP-derivatives differ somewhat in their chemical structure the results did not allow to define a relationship between structure and effect. Therefore we synthesised different DHP-derivates to allow analysis between the chemical structure and the antioxidant effect of the DHP-derivatives :

4-Aryl-substituted Nifedipine-derivatives and a Nitrendipine-derivative with an iso-propyl-esterfunction instead of the ethyl-esterfunction were synthesised and their TEAC-values as measured by means of TAS are shown in table 2.

Table 2: TEAC-values of the synthesised Nifedipine- and Nitrendipine-derivatives

Nifedipine-derivatives	TEAC	Nitrendipin-derivatives	TEAC
Nifedipine (Nif)	0,514 ± 0,022	3-Nitro-Nif	0,599 ± 0,182
3-Nitro-Nif	0,599 ± 0,182	Nitrendipine	0,697 ± 0,029
3-Chloro-Nif	0,829 ± 0,017	iso-propyl-Nit	0,784 ± 0,027
2,3-Dichloro-Nif	1,515 ± 0,264		
2-Chloro-Nif	1,52 ± 0,082		

The different derivatives result in distinct TEAC-values which can be related to their respective chemical structure.

From these results we conclude:

The tested DHP-derivatives show an antioxidant activity, which depends on the oxidation of the dihydropyridine-structure. Therefore the antioxidant capacity, i.e. the ability to be oxidised, depends on the electron-density of the dihydropyridine-part of the molecule.

To clarify whether similar mechanisms of antioxidant activity of DHP-derivates are given *in vivo* further experiments are needed, e.g. performing the TAS-assay with patients treated with DHP calcium antagonists.

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70P ET_B RECEPTOR-MEDIATED BRONCHOCONSTRICTION IS POTENTIATED IN LUNGS FROM CHRONICALLY HYPOXIC RATS

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Previously we have shown that ET-1-induced bronchoconstriction is mediated by the activation of ET_B receptors in normal rats lungs (Lal *et al.*, 1995). As we have recently found that chronic hypoxia (CH) increases pulmonary vascular responsiveness to ETs (Lal et al., 1999), the present study was designed to investigate the bronchial reactivity of CH rats to ET-1 and an ET_B receptor agonist sarafotoxin 6c (SX6C) in isolated lungs.

CH male Wistar rats (250-270g) were kept in a chamber in an atmosphere of 10% O_2 for 3 weeks. CH or control rats were anaesthetised with Sagatal (60 mg kg^{-1}, i.p.) heparinised (500 units; i.v), 5 min later lungs were isolated and perfused via the pulmonary artery (Krebs' solution gassed with 20% O_2, 5% CO_2, 75% N_2). Lungs were ventilated via the trachea with room air (28 strokes min^{-1}; 1ml stroke volume) and the pulmonary inflation pressure (PIP) was recorded. Bolus injections of ET-1, SX6C or carbachol were given into the pulmonary artery after 20 min stabilisation.
In a different series of experiments antagonists were added to the perfusion medium after the initial stabilisation.

CH significantly increased the ratios of right ventricular to total ventricular weight when compared with control rats (0.34 ± 0.01 vs. 0.25 ± 0.01, $p< 0.01$, n=20, Students' t-test). Haematocrit values were significantly higher in CH (59 ± 1.2 %, n= 20) compared to control (43 ± 0.8 %, n=20, $P< 0.01$) rats.

In isolated lungs basal PIP was significantly lower in CH (5 ± 0.3 mmHg) than control animals (6.4 ± 0.3 mmHg, n=20, $P< 0.05$). ET-1 (50-800 pmoles) produced dose-dependent increases in PIP in control (ED_{50} 300 ± 61 pmoles, n =6) and in CH lungs (ED_{50} 130 ± 16 pmoles, n =6). 200 pmoles of ET-1 induced increases in PIP were significantly increased in CH (3.45 ± 0.62 mmHg, n=6) compared to control lungs (1.2 ± 0.3 mmHg, n=6, $P< 0.05$).

BQ788 (10 μM) significantly inhibited ET-1-induced increases in PIP in CH lungs. 200 pmoles of ET-1-induced increases in PIP (3.45 ± 0.62 mmHg) were reduced to 1.65 ± 0.12 mmHg, $P< 0.05$, n= 6). In contrast BQ123 (10 μM) had no significant effect on ET-1-induced increases in PIP in control CH lungs (3.45 ± 0.62 mmHg) and in the presence of BQ123 (2 ± 0.43 mmHg, n=4).

SX6C (50-400 pmoles) induced increases in PIP in CH (ED_{50} 95 ± 2.6 pmoles, n=4) compared with control lungs (ED_{50} 127 ± 14.8 pmoles, n=4). The threshold dose of SX6C (50 pmoles) which induced an increase in PIP in CH lungs had no significant effect in control lungs. In addition the maximum increase in PIP caused by 200 pmoles of SX6C in CH lungs (9.8 ± 0.3 mmHg, n=4) was significantly higher than in control lungs (5.8 ± 1.1 mmHg, n=5, $P< 0.05$). In contrast, carbachol (0.3-100 nmoles) induced increases in PIP in control lungs were not altered by CH. ED_{50} values in control vs. CH lungs (11 ± 1 vs. 25 ± 5 nmoles, n=4) were not significantly different ($P> 0.05$). The maximum increase in PIP caused by carbachol in CH lungs (8 ± 2 mmHg, n=4) was similar to that in control lungs (6 ± 0.5 mmHg, n=4).

In summary, in the present experiments in CH lungs ET-1 mediated increases in PIP were significantly augmented. These responses were not affected by the ET_A receptor antagonist BQ123 but were significantly inhibited by ET_B receptor antagonist BQ788, suggesting a pure ET_B-mediated effect. In addition bronchoconstriction induced by SX6C was also potentiated whereas, responses to carbachol were unchanged. These results suggest a selective increase in bronchial reactivity to ETs caused by CH via an upregulation of ET_B receptors. This has previously only been shown in cultured airway smooth muscle cells Maxwell, *et al.* (1998).

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71P INCREASED 5-HT-STIMULATED INOSITOL PHOSPHATE GENERATION IN RESPONSE TO HYPOXIA: STUDIES WITH CULTURED HUMAN UMBILICAL ARTERY SMOOTH MUSCLE CELLS

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Hypoxia is a relative oxygen deficiency at the cellular level. Hypoxia and 5-HT are potent vasoconstrictors, both of which can be antagonised by the 5-HT_{2A}-receptor antagonist ketanserin (Van Nueten, 1985). Hypoxia can augment the vasoconstriction induced by 5-HT (Van Nueten, 1985). Here we present data that shows hypoxia increases 5-HT_{2A}-receptor stimulated inositol phosphate (IP) accumulation in human umbilical artery smooth muscle cells (HUASM).

HUASM cells were grown from artery explants by our previously described method (Hawley *et al.*, 1995). Cells were grown to confluent monolayers in 24well plates in 21% O_2 and transferred to a 3% O_2 incubator for 24hrs prior to [H^3]-IP assay. Control cells were maintained at 21% O_2. Assays were performed at 21% atmospheric O_2. HUASM cells were stimulated with 5-HT, bradykinin, carbachol or histamine before total [H^3]-IP accumulation was measured as previously described (Megson *et al.*, 1995).

5-HT stimulated [H^3]-IP accumulation was increased in HUASM cells exposed to hypoxia (3% O_2) compared to 21% O_2 controls ($p<0.0001$, ANOVA)(Figure 1a). In contrast cells stimulated with bradykinin, carbachol or histamine showed no such increase when assayed under identical conditions (Figure 1b, c and d respectively). Ketanserin inhibited 5-HT (10μM) stimulated [H^3]-IP accumulation. Apparent pKd values were calculated (Hawley *et al.*, 1995) and are similar to those reported from binding data (8.67±0.41 at 21% O_2 and 8.89±0.29 at 3% O_2) (Zifa and Fillion, 1992). These data demonstrate an O_2 sensitive increase in [H^3]-IP accumulation specific to the 5-HT_{2A}-receptor.

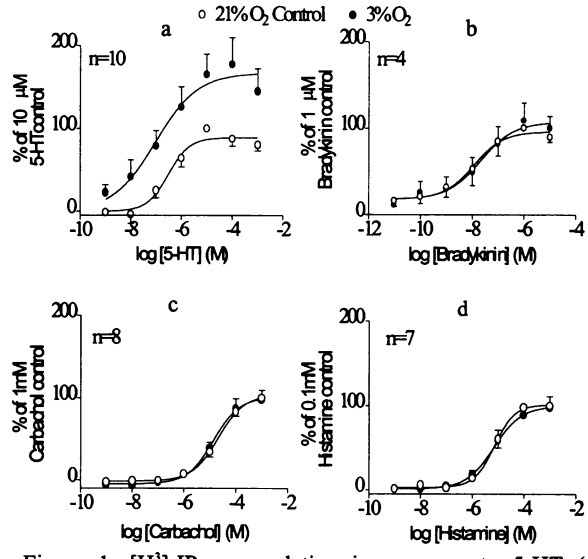

Figure 1. [H^3]-IP accumulation in response to 5-HT (a) bradykinin (b), carbachol (c) and histamine (d). Data are percentage responses relative to 10μM 5-HT, 1μM bradykinin, 1mM carbachol and 100μM histamine respectively. Data are mean±s.e.mean.

We thank the MRC and the BHF for their financial support.

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72P PRESENCE OF VASOCONSTRICTOR 5-HYDROXYTRYPTAMINE$_1$-LIKE RECEPTORS IN HUMAN HEPATIC ARTERY

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In most vascular territories, the vasoconstrictor effects of 5-hydroxytryptamine (5-HT) are mediated by the 5-HT_{2A} receptor sub-type. 5-HT_1-like receptors may also contribute to this response although, in many vascular tissues, they only become functional if the vessel has been partially pre-contracted (Choppin & O'Connor, 1995). This study aimed to determine whether 5-HT_1-like receptors contributed to 5-HT-mediated contraction of the human hepatic artery.

Hepatic arteries were collected from 16 donors (11F, 5M) during liver transplantation. Eight-sixteen rings (2mm in length) were taken from each artery and the endothelium was removed. Isometric force was measured in rings suspended in Krebs'-Henseleit solution (containing prazosin 10^{-6}M, yohimbine 10^{-6}M and indomethacin 10^{-5}M) at 37°C, bubbled with 95% O_2; 5% CO_2. Four rings were assessed in parallel after equilibration at 4g (Hadoke et al., 1998) and confirmation of contractile function with KCl (100mM). Two of these rings were partially pre-contracted (PPC) with KCl (20-35mM) and two were used as non-pre-contracted controls (NPC). One PPC and one NPC ring were incubated with either the 5-HT_{2A} receptor antagonist, ketanserin (10^{-6}M; 40 min) or the 5-HT_1-like receptor antagonist, methiothepin (10^{-7}M; 30 min). Cumulative concentration-response curves (10^{-9}-$3x10^{-5}$ M) were then obtained using either 5-HT or the 5-HT_1-like receptor agonist, 5-carboxamidotryptamine (5-CT). Finally a cumulative concentration-response curve was obtained to KCl (2.5-120mM). Results are expressed as mean ± s.e.mean for n subjects and were compared using Student's unpaired t-test. Use of human arteries was approved by the Lothian Research Ethics Committee.

5-HT and 5-CT produced similar contractions in NPC rings and the sensitivity (pD_2) to these agonists was enhanced by pre-contraction. In NPC rings, methiothepin caused a rightward shift in the response to both 5-HT and 5-CT whereas ketanserin only antagonised the response to 5-HT. Following precontraction, the effect of methiothepin on both agonists was maintained whereas ketanserin had no effect on responses to either 5-HT or 5-CT. In all cases incubation with the antagonists had no effect on maximal contractions to 5-HT or 5-CT and all vessels produced similar responses to KCl.

The present study demonstrated that 5-HT-induced vasoconstriction of non-precontrated human hepatic arteries was mediated by both 5-HT_{2A} and 5-HT_1-like receptors. The enhanced contraction obtained following partial pre-contraction, however, was mediated solely by the 5-HT_1-like receptors.

Choppin, A, O'Connor, SE (1995) *Br.J.Pharmacol*, 114, 309-14.
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Table 1. Maximum contraction (Emax, (g)) and sensitivity (pD_2) for 5-HT and 5-CT in NPC and PC arteries.

	Non-precontracted (NPC; n=9-14)						Partially Pre-contracted (PPC; n=9-16)					
	5-HT	5-HT+K	5-HT+M	5-CT	5-CT+K	5-CT+M	5-HT	5-HT+K	5-HT+M	5-CT	5-CT+K	5-CT+M
Emax	7.29±0.58	6.93±0.52	6.94±0.64	6.79±0.58	6.40±0.96	6.42±0.69	7.83±0.68	7.86±0.48	7.32±0.97	7.07±0.55	7.13±1.06	7.24±0.68
pD_2	6.46±0.06*	6.22±0.10[a]	6.10±0.08[b]	6.58±0.06*	6.54±0.09	6.12±0.09[b]	6.71±0.06	6.57±0.13	6.29±0.10[b]	6.85±0.06	6.74±0.08	6.20±0.11[c]

Values are mean±s.e.mean for (n) patients. M, methiothepin, K, ketanserin. *$P<0.05$ compared with PC arteries; [a]$P<0.05$, [b]$P<0.001$ and [c]$P<0.0001$ compared with control using students unpaired t-test.

73P A COMPARISON OF THE *IN VIVO* PHARMACOLOGY OF CLENBUTEROL ENANTIOMERS UPON SKELETAL MUSCLE TONE, VASCULAR MUSCLE AND CARDIAC CHRONOTROPIC RESPONSES

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(±) Clenbuterol is a β_2 agonist with anabolic activity which can be blocked by the β_2 antagonist ICI 118551 (Choo et al, 1992). The current study examined the potency and selectivity of (±) clenbuterol and its enantiomers against β_1 and β_2 adrenoceptor mediated responses in vivo.

Cats (3.0 - 3.8kg, male, n=11) were anaesthetised (5% halothane plus alphadalone/alphalaxone (0.98:0.38 w/v, 9.0 ± 0.5 mg kg^{-1}, I.V. and maintained with alphadalone/alphalaxone (0.3 ± 0.03 mg kg^{-1} min^{-1}, I.V)). Following a tracheotomy the animals breathed spontaneously. The animals were prepared for the measurement of soleus muscle tone, induced by electrical stimulation (7 - 13Hz) of the sciatic nerve (Bowman and Zaimis, 1958). Heart rate (β_1) and hind-limb vascular vasodilatation (β_2) were measured in beagle dogs (11-15.5kg, n = 12) treated with syrosingopine (5 mg kg^{-1}, s.c. 24 hours) before the experiment to deplete cardiac catecholamines. The animals were anaesthetised with sodium pentobarbitone (28.8 ± 1.35 mg kg^{-1} and maintained with 0.115 ± 0.005 mg kg^{-1} min^{-1} IV). The trachea was intubated and the dogs artificially ventilated. Heart rate and hind-limb vascular responses were measured as described previously (Keddie et al, 1996). All compounds were given intravenously.

All agonists resulted in a dose-dependent reduction of the tension produced during stimulation of the cat soleus muscle (Table 1). The effect of all β-agonists was abolished by ICI 118551 (0.2 mg kg^{-1}, I.V.). The β_1-selective antagonist CGP20712A (0.2 mg kg^{-1}, I.V.) was ineffective. Relative to isoprenaline, both (±)clenbuterol and (-) clenbuterol behaved as full agonists on the hind-limb (Table 1). However, due to the low potency of (+) clenbuterol it was not possible to show whether it was a full agonist.

Both enantiomers caused dose dependent reductions in hind limb perfusion pressure which were reversed by ICI 118551 but not by CGP20712A. All four β-adrenoceptor agonists increased heart rate in a dose-dependent manner and which were abolished by CGP120712A but not by ICI 118551. (±) clenbuterol, (-) clenbuterol and (+) clenbuterol had low potency at the β_1-adrenoceptor in cardiac tissue compared to isoprenaline (Table 1).

Data show that skeletal muscle effects of clenbuterol and its enantiomers was due to β_2-adrenoceptor activation. (±)clenbuterol and (-)clenbuterol were equipotent at β_2-adrenoceptors but approximately 200-fold more potent than (+)clenbuterol. It would be expected therefore the anabolic activity of (±)clenbuterol is due to the (-) enantiomer.

Table 1. Activity of clenbuterol (clen.)and its enantiomers.

Cat soleus	Isopren.	(±)clen.	(-) clen.	(+) clen.
log ED_{50}	1.92 ± 0.05 (n = 10)	2.27 ± 0.20 (n = 4)	2.33 ± 0.17 (n = 4)	4.64 ± 0.08 (n = 2)
% Isopren max	100	92.7 ± 3.5	82.3 ± 18.2	> 69.4
Dog hindlimb				
log ED_{50}	2.09 ± 0.07 (n = 12)	2.82 ± 0.11 (n = 4)	2.73 ± 0.07 (n = 4)	5.2 ± 0.15 (n = 4)
% Isopren max	100	93.5 ± 3.5	85.6 ± 7.1	> 52
Dog heart rate				
log ED_{50}	2.51± 0.05 (n = 12)	>3.81 (n = 4)	>3.72 (n = 4)	>4.18 (n = 4)
% Isopren max	100	> 35	> 15	> 3

Each value is given as the mean ± sem (units = ng kg^{-1}, iv)

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Choo, J.J. et al, (1992), *Am J Physiol.* 263: E50 - E56.
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74P THE RELATIVE ANABOLIC POTENCY OF (±) CLENBUTEROL, (-) CLENBUTEROL AND (+) CLENBUTEROL IN SLOW TWITCH-OXIDATIVE AND FAST TWITCH-GLYCOLYTIC MUSCLES OF RATS

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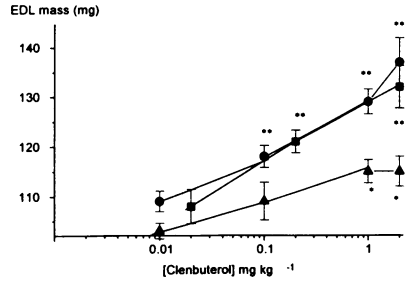
(±) Clenbuterol is a β_2 adrenoceptor agonist with anabolic activity which can be blocked by the β_2 antagonist ICI 118551 (Choo et al, 1992). Costelli et al (1995) observed that the anabolic response of fast-twitch muscle was more sensitive than that in slow twitch muscle to β_2 adrenoceptor agonists. In contrast, Maltin et al (1986) reported slow twitch muscle groups to be the most sensitive to the anabolic effects of clenbuterol. The current study examined the anabolic efficacy and relative potency of (±) clenbuterol and its enantiomers upon slow twitch-oxidative (soleus) muscle and fast twitch-glycolytic (extensor digitorum longus, EDL) muscle in a rat model.

Alderley Park Wistar rats (male, 125 - 150g body weight) were allocated randomly to be treated with either (±) clenbuterol, (-) clenbuterol, (+) clenbuterol or vehicle (water) administered by gavage daily for 14 days (0.01 to 2 mg. kg^{-1}). Food intake and body weight were measured daily. At the end of the dosing period the animals were killed by CO_2 inhalation and the EDL and soleus muscles of both limbs removed, weighed and then analysed for protein content (Lowry method).

The final body weights of the rats after treatment were: vehicle (n = 14) 271±4.4g; (±) clenbuterol (2 mg kg^{-1}, n=5) 303±6.8g (P<0.01); (-) clenbuterol (1mg kg^{-1}, n=6) 291±7.1g and (+) clenbuterol (1 mg kg^{-1}, n=6) 288±3.8g. Significant increases in EDL wet weight were evident (Fig. 1) following treatment. No increase was observed in the soleus weight. Additionally, total protein content of the EDL muscle was increased from 23.7 ± 6.6 µg in the vehicle treated rats (n = 14) to 27.5 ± 0.6 mg (P<0.001, n = 6) following 0.1 mg kg^{-1} (-) clenbuterol; to 28.9 ± 0.3 mg (P<0.001, n = 6) following 0.2 mg kg^{-1} (±) clenbuterol but was not different (22.1 ± 0.9 mg, n = 6) following 0.1 mg kg^{-1} (+) clenbuterol.

Fig. 1 Effect of racemic and enantiomeric clenbuterol on EDL muscle weight



Values represent mean ± s.e.m, for (±)clenbuterol (■), (-)clenbuterol (●) and (+)clenbuterol (▲) treated groups (*, P<0.05, **, P<0.001; ANOVA plus Student t-test compared with vehicle treated group).

These data show that, in intact animals, the fast-twitch glycolytic muscle groups were the most sensitive to the anabolic effects of β_2 adrenoceptor agonist. This is consistent with the work of Costelli et al (1995). In addition the anabolic effects of (±) clenbuterol was due largely to the activity of the (-) enantiomer.

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Maltin, C. et al, (1986), *Biosci. Rep.* 6: 811-818

75P COMPARISON OF THE ANABOLIC EFFECTS OF (±) CLENBUTEROL, (-) CLENBUTEROL AND (+) CLENBUTEROL IN DENERVATED RAT HIND-LIMB MUSCLE

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(±) Clenbuterol is a β_2 adrenoceptor agonist with anabolic activity which can be blocked by the β_2 antagonist ICI 118551 (Choo et al, 1992). The current study examined the efficacy of (±) clenbuterol and its enantiomers for anabolic effects in a rat model of muscle wasting which has many of the features of cachexia associated with injury-induced muscle atrophy in man.

Alderley Park Wistar rats (male, 26 - 29 days old) were anaesthetised (halothane 2%) and their left sciatic nerve ligated and sectioned to induce muscle wasting (Maltin et al. 1989). Following denervation, (±)clenbuterol (2 mg kg^{-1}), (-)clenbuterol (1 mg kg^{-1}) and (+)clenbuterol (1 mg kg^{-1}) were administered by gavage for 7 days. At the end of the dosing period the animals were killed by CO_2 inhalation and the extensor digitorum longus (EDL) and soleus muscles removed, weighed and their protein content measured (Lowry method). In a separate group treated similarly for four days, muscle total mRNA was prepared and run on denaturing formaldehyde gels (Sambrook et al, 1989). RNA was transferred to nylon membranes and probed with nick translated 32P-myogenin (Braun et al 1989) and MRF4 (Rohdes & Konieczny 1989). 32P-28s rRNA was used as a control.

Compared to control rats (17.7 ± 1.1g food rat^{-1} day^{-1}) reductions in food intake were recorded following the first dose of (±)clenbuterol (28%) and (-)clenbuterol (21%) but not (+)clenbuterol (2%). The final body weights were: vehicle 169±3.3g; (±)clenbuterol 157±4.6g; (-) clenbuterol 171±5.7g and (+)clenbuterol 178±6.6g. Denervation decreased the mass of the soleus and EDL muscle groups by 28% and 42% respectively. Following denervation only (-)clenbuterol increased muscle mass compared with the vehicle treated group (Table 1). Myogenin : 28s rRNA was increased from 1.14 to 2.16 following denervation. (±)clenbuterol (ratio 1.42); (-) clenbuterol (ratio 1.61), but not (+)clenbuterol (ratio 2.16) reversed this trend. In contrast, MRF4 transcription was unaltered by denervation (ratios 1.09 and 1.31) but increased by (±)clenbuterol (ratio 2.16); (-)clenbuterol (ratio 1.85), but not (+)clenbuterol (ratio 1.41).

These results show that the anabolic effects of clenbuterol, in the current model, are due to the activity of the (-)enantiomer and that the effects may be mediated by an inhibition of the transcription of myogenin and increased transcription of the growth factor MRF4.

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Choo, J.J. et al, (1992), *Am J Physiol.* 263: E50 - E56
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Rohdes, S.J. & Konieczny S.F.(1989), *Genes and Development* 3: 2050-2061
Sambrook, J. et al, (1989), *Molecular Cloning: a laboratory manual.* Section 7.43.

Table 1 Effect of racemic and enantiomeric clenbuterol on muscle from denervated rat hind limb

Treatment	EDL mass (mg)	EDL protein (mg)	Soleus mass (mg)	Soleus protein (mg)
Sham (n = 5)	58.0 ± 4.0	13.7± 2.4	54.0 ± 9.0	15.4± 0.5
Vehicle (n=8)	45.7±3.5	11.7± 1.1	31.1±3.2	7.2± 0.9
(±)Clen. (2mg.kg^{-1}, n=7)	49.7±3.5 (ns)	12.4 ± 1.0(ns)	39.0±2.6(ns)	9.3 ± 0.7(ns)
(-)Clen. (1mg.kg^{-1}, n=8)	60.6±5.5 (*)	14.7 ± 1.5(ns)	40.6±1.4 (*)	9.6 ± 0.3(*)
(+)Clen. (1mg.kg^{-1}, n=8)	50.1±2.1 (ns)	13.9 ± 1.5(ns)	37.9±3.1 (ns)	9.8 ± 0.9(ns)

Values are mean ± sem, (*, P<0.05, ANOVA plus Student t-test compared with Vehicle treated group).

76P INCREASED APOPTOSIS AND REDUCED CARDIAC VENTRICULAR WALL VOLUME IN THE POSTNATAL TRANSGENIC m(Ren-2)27 RAT

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The vasoactive peptide angiotensin II, in addition to promoting cell growth and improving contractile performance in cardiac tissue, has also been shown to induce apoptosis in cardiomyocytes via activation of surface angiotensin 1 (AT_1) receptors (Cigola *et al*, 1997). The transgenic m(Ren-2)27 (TG) rat carries the additional *Ren-2* gene (Mullins *et al*, 1990), the expression of which in the postnatal heart produces elevated local levels of angiotensin II, thus potentially affecting the cell growth/death equilibrium. This study addresses the question of ventricular modelling and development in the postnatal transgenic rat heart and the role of angiotensin II/AT_1-mediated apoptosis.

Three, male, 2- and 28-day TG and Hannover Sprague Dawley (SD) rat hearts were obtained following transcardial perfusion initially with prewash (37°C), followed by ice cold fixative (4% paraformaldehyde in 0.1M phosphate buffer). Following dehydration and paraffin wax embedding, 8µm sections containing the left ventricle were processed for either (a) haematoxylin and eosin staining prior to stereological analysis of ventricular wall volume using the Cavalieri point count method; (b) calculation of apoptotic nuclei following terminal deoxynucleotidyl transferase nick end labelling of 3'-OH ends of fragmented DNA using Fluorescein-FragEL™; or (c) double immunohistochemical labelling with antibodies to AT_1 and vimentin prior to confocal scanning laser microscopical analysis.

From Table 1 it is clear that at 28 days, the volume of the ventricular wall of the TG rat is significantly less than that of the control SD (P<0.05). Calculation of apoptotic cells within the left ventricles shows that at 28 days there were significantly more apoptotic cells in those of the TG compared with the SD (P<0.05).

Table 1. Left ventricular volume and number of apoptotic cells per unit volume in TG and control rats.

	Volume (mm^3)		Apoptotic cells/mm^3	
	SD	TG	SD	TG
2-days	3.1±0.2	3.0±0.1	3.8±0.3 x10^3	4.4±0.4 x10^3
28-days	33.3±3.4	13.8±2.1*	0.3±0.05 x10^3	1.8±0.3 x10^3*

Data expressed as mean± s.e.mean, (n=3), * = statistically significant compared with control (P<0.05) (unpaired t-test).

Identification of the AT_1 receptor by confocal microscopy showed that it was expressed at 2-days by ventricular myocytes and fibroblasts in both experimental groups, but at 28-days, predominantly cardiomyocytes displayed AT_1 immunoreactivity. These data suggest that elevated level of cell death in the TG rat heart may be a contributory factor in the decrease in ventricular muscle volume at 4-weeks. The increased level of apoptosis in the left ventricle of the neonate TG heart compared with the controls could be accounted for by *Ren-2*-derived angiotensin II acting via AT_1 receptors on cardiomyocytes.

Cigola, E. *et al.* (1997): *Exp Cell Res*, **231**, 363-371
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77P EARLY INVOLVEMENT OF VASOPRESSIN IN THE CARDIOVASCULAR SEQUELAE OF ENDOTOXAEMIA IN CONSCIOUS RATS

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In previous studies (Gardiner *et al.*, 1996) we have shown that vasopressin (AVP) may contribute to cardiovascular status 23 h after the onset of lipopolysaccharide (LPS) infusion in conscious rats, and have raised the possibility that it may be involved at earlier time points, in spite of the finding that LPS induces subsensitivity to AVP (Schaller *et al.*, 1985). Therefore, we have now compared haemodynamic responses to LPS infusion in the absence and presence of an AVP, V_1-receptor antagonist.

Experiments were carried out on conscious, unrestrained, male, Long Evans rats (350-450 g) chronically instrumented with pulsed Doppler probes to monitor changes in renal, mesenteric and hindquarters flows and intravascular catheters (all surgery was carried out under sodium methohexitone anaesthesia, 40-60 mg kg^{-1}, supplemented as needed) (Gardiner *et al.*, 1996). Animals were either given i.v. saline (0.1 ml bolus, 0.4 ml h^{-1} infusion; n = 8) beginning 1 h before LPS (*E.coli* serotype 0127 B8, Sigma) infusion (150 μg kg^{-1} h^{-1}) for 6 h, or the V_1-receptor antagonist ($d(CH_2)_5$-0-Me-Tyr-AVP; 10 μg kg^{-1} bolus; 10 μg kg^{-1} h^{-1} infusion; n = 9) before LPS (as above).

Table 1 summarises some of the results. Treatment with the AVP antagonist enhanced the integrated hypotensive effect of LPS in association with a selective enhancement of the mesenteric vasodilatation. These results are consistent with endogenous AVP acting to oppose the hypotensive and mesenteric vasodilator effects of LPS over the first 6 h of LPS infusion, and are in line with the potent mesenteric vasoconstrictor effect of AVP in conscious rats (Gardiner *et al.*, 1988). However, since recent observations indicate that AVP inhibits LPS-induced nitric oxide (NO) production through a V_1-receptor-mediated mechanism (Umino *et al.*, 1999), it is feasible that the effect of the V_1-receptor antagonist in the present study involved disinhibition of NO production.

Table 1. Resting cardiovascular variables and integrated changes (areas under or over curves (AUC_{0-6h}, AOC_{0-6h}) during infusion of LPS following pretreatment with saline (Sal) or the V1-receptor antagonist (AVPX) in conscious, Long Evans rats. HR = heart rate; BP = mean arterial blood pressure; RVC, MVC, HVC = renal, mesenteric and hindquarters vascular conductance, respectively. Values are mean ± s.e. mean; *P < 0.05 versus resting value (Friedman's test); † P < 0.05 versus corresponding saline value (Mann-Whitney U test).

		Resting	Δ (% h) AUC	Δ (% h) AOC
HR	Sal	359 ± 13	17 ± 6*	39 ± 15*
(beats min^{-1})	AVPX	371 ± 8	13 ± 6*	34 ± 9*
BP	Sal	102 ± 3	9 ± 3	27 ± 4*
(mm Hg)	AVPX	104 ± 1	2 ± 1	44 ± 6*†
RVC	Sal	64 ± 5	295 ± 43*	0
([kHz mm Hg^{-1}]10^3)	AVPX	69 ± 7	320 ± 48*	0
MVC	Sal	83 ± 5	40 ± 9*	29 ± 8
([kHz mm Hg^{-1}]10^3)	AVPX	69 ± 1	88 ± 22*†	8 ± 3
HVC	Sal	48 ± 6	66 ± 16*	70 ± 7*
([kHz mm Hg^{-1}]10^3)	AVPX	51 ± 4	98 ± 11*	41 ± 11

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Umino, T. *et al.* (1999). Am.J.Physiol., **276**, F433-F441.

78P CAROTID HAEMODYNAMIC RESPONSES TO ANGIOTENSIN II IN CONSCIOUS, HYPERTENSIVE, TRANSGENIC RATS

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Hypertensive transgenic ((mRen-2)27) rats (abbreviated to TG rats) show reduced conductances in renal, mesenteric and hindquarters vascular beds compared to the normotensive, Hannover Sprague-Dawley (SD) control strain (Gardiner *et al.*, 1995). Hypertension in TG rats is dependent on both angiotensin II (AII) and endothelin (ET) (Gardiner *et al.*, 1995), and in other models of hypertension there is evidence that there may be an increased involvement of ET in the cardiovascular effects of exogenous AII (Balakrishnan *et al.*, 1996). Therefore, the objectives of the present work were, in SD and TG rats, to compare 1) carotid haemodynamic status, 2) carotid haemodynamic responses to AII; and 3) the effects of the non-selective ET antagonist, SB 209670, on responses to AII.

All experiments were carried out on male, age-matched (3-4 months) SD and heterozygous TG rats. Under sodium methohexitone anaesthesia (40-60 mg kg^{-1} i.p., supplemented as required), a pulsed Doppler probe was implanted around the left common carotid artery and, 1-2 weeks later, catheters were positioned in the right jugular vein and distal abdominal aorta. At least 24 h after the latter procedure ascending doses of AII (3, 9, 30 and 90 μg kg^{-1} min^{-1}) were infused for 3 min with at least 10 min between infusions to allow all variables to return to baseline. Thereafter, an infusion of SB 209670 (600 μg kg^{-1} bolus; 600 μg kg^{-1} h^{-1} infusion) was begun and the infusions of AII were repeated 1 h and 6 h after the onset of treatment with SB 209670. Table 1 summarises some of the results. Under baseline conditions the hypertension in TG rats was accompanied by a reduction in carotid vascular conductance relative to SD rats. Although SB 209670 lowered blood pressure and caused carotid vasodilatation in both strains of rat, it had no significant effect on responses to AII which were similar in SD and TG rats in the absence and presence of SB 209670.

Table 1. Baseline cardiovascular variables (HR, heart rate (beats min^{-1}); BP, mean blood pressure (mm Hg); CC, carotid vascular conductance ([kHz mm Hg^{-1}]10^3)) and peak responses (Δ%) to AII in SD (n = 8) and TG (n = 8) rats before and 6 h after onset of infusion of SB 209670. Values are mean ± s.e. mean; *P < 0.05 versus corresponding value in SD rats (Mann-Whitney U test); †P < 0.05 versus control baseline value (Wilcoxon test).

		Control		
			Δ(%)	
		Baseline	AII (30 μg kg^{-1} min^{-1})	AII (90 μg kg^{-1} min^{-1})
HR	SD	331 ± 4	-7 ± 2	-11 ± 2
	TG	311 ± 11	-4 ± 1	3 ± 3
BP	SD	107 ± 3	21 ± 3	34 ± 3
	TG	149 ± 5*	22 ± 1	32 ± 3
CC	SD	21 ± 3	-12 ± 6	-33 ± 3
	TG	15 ± 2*	-24 ± 3	-31 ± 5
		SB 209670 (6 h)		
HR	SD	331 ± 9	-10 ± 2	-10 ± 1
	TG	336 ± 13	-7 ± 2	-3 ± 2
BP	SD	99 ± 4†	22 ± 4	38 ± 4
	TG	132 ± 3*†	23 ± 2	37 ± 3
CC	SD	29 ± 4†	-16 ± 5	-25 ± 4
	TG	21 ± 2*†	-14 ± 7	-26 ± 11

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79P ACTIVE IMMUNISATION WITH TWO DIFFERENT CARRIER PROTEINS CONJUGATED WITH AN ANGIOTENSIN 1 (A1) ANALOGUE IN CONSCIOUS RATS: PRESSOR EFFECTS OF A1 AND ANTI-A1 ANTIBODY RESPONSE

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The efficiency of an immune response following vaccination with an immunotherapeutic comprised of a common immunogen molecule conjugated with target peptide epitopes may be difficult to predict due to potential immunosuppression attributable to the subject having been previously exposed to the immunogenic carrier protein (Herzenberg *et al.*, 1980). We have previously reported effective active immunization against exogenous AI, using a formulation comprising tetanus toxoid (TT) conjugated with AI (Gardiner *et al.*, 1998). In man, TT is a common immunogen, so there is a potential risk of immunosuppression when toxoid conjugate vaccines are evaluated in human clinical trials. In the present study, an alternative immunoconjugate carrier molecule (Keyhole Limpet Haeomcyanin, KLH) was investigated for its immunological efficiency, compared with TT.

Male, Sprague Dawley rats (initially 280-350 g: Harlan Olac: n=6 per group) were injected s.c. (0.5 ml) with saline or vaccine as follows: Group A, saline; B, 25 μg AI analogue peptide/TT; C 10 μg AI analogue peptide/KLH; 10 and D 50 μg AI analogue peptide/KLH. Aluminium hydroxide was the adjuvant in all vaccines. Rats were injected on days 0, 21 and 42. On day 62 under sodium methohexitone anaesthesia (40-60 mg kg^{-1} i.p.) catheters were implanted in the abdominal aorta and right jugular vein. Twenty four h later, conscious rats were challenged with increasing i.v. bolus doses of AI (3-60 pmol rat^{-1}) and AII (0.5-25 pmol rat^{-1}) at 15 min intervals, and mean arterial blood pressure (MAP) was monitored. For each rat, the mean changes in MAP in response to AI and AII were calculated. At the end of the experiment, animals were anaesthetized (sodium pentobarbitone 60 mg kg^{-1} i.v.) and a terminal blood sample was taken by cardiac puncture for the measurement of AI antibody response and immunoglobulin sub-typing, by ELISA.

Table 1. Effects of immunisation on anti-AI IgG titres and mean MAP responses to all AI or AII challenge doses in the same conscious rats. Significance probabilities were adjusted for the multiple MAP comparisons by Dunnett's method; * $P<0.05$ versus saline (treatment A). Values are mean ± s.e. mean.

Treatment	Antibody Titre	Change in MAP (mm Hg)	
		AI	AII
A	0	25.5 ± 7.0	24.3 ± 3.8
B	55,629 ± 35,309	15.4 ± 4.1*	22.1 ± 3.7
C	66,121 ± 32,491	13.3 ± 6.0*	15.7 ± 7.4
D	67,410 ± 18,110	13.8 ± 6.9*	19.0 ± 8.0

Table 1 summarizes some of the results demonstrating that KLH and TT conjugates had similar effects on induction of anti-AI antibodies. Both carrier protein vaccines caused a reduction of the pressor effects of AI, but had no significant influences on responses to AII. Therefore, KLH may provide a suitable alternative to TT as an immunoconjugate carrier protein with AI analogue peptide in a vaccine for the control of conditions such as hypertension.

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80P ATTENUATED CONSTRICTION TO METHOXAMINE PERFUSION AND AUGMENTED RESPONSES TO VASORELAXANTS IN ISOLATED PERFUSED MESENTERIC ARTERIAL BEDS FROM ENDOTOXAEMIC RATS

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Vasoconstrictor responses to bolus doses of noradrenaline (NA) and methoxamine (ME) are unaltered in mesenteric vascular beds removed from rats after 24h infusion of lipopolysaccharide (LPS) (Tarpey & Randall, 1998). Responses to vasorelaxant agents in mesenteric vascular beds in this model of endotoxaemia was the subject of investigation of the present study.

Under anaesthesia (sodium methohexitone, 60 mg kg^{-1} i.p., supplemented as required) intravascular catheters were implanted in male Long Evans rats (400-500g) 24h before the start of i.v. infusion of saline (0.4 ml h^{-1}) (n=5) or LPS (150μg kg^{-1} h^{-1}; E *coli* serotype 0127:B8) (n=6) (Gardiner *et al.*, 1995). After 24h, rats were anaesthetised (pentobarbitone; 44mg kg^{-1} i.v.), decapitated and the mesenteries removed and perfused at 5 ml min^{-1} with oxygenated Krebs solution (Ralevic *et al.*, 1996) containing guanethidine (5μM) for evaluation of sensory neurotransmission (not shown). After 35 min equilibration, tone was raised by ME perfusion and responses to bolus doses of the endothelium-dependent vasorelaxant adenosine 5'-triphosphate (ADP; 0.005-50nmol) and endothelium-independent relaxant sodium nitroprusside (SNP; 0.05-500nmol) determined.

Basal mesenteric perfusion pressures were similar between the saline (20.4±3.1 mmHg) and LPS (20.3±1.8 mmHg) treated groups (mean ± s.e.mean)(Student's t test). Mesenteries from rats treated with LPS achieved a significantly lower level of raised tone (43.2±5.6 mmHg) than those from saline-treated rats (61.1±2.7 mmHg) ($P<0.05$) despite a trend for requiring a higher concentration of ME (82±23μM and 41±16μM respectively). Maximal relaxations to SNP were greater in mesenteries from LPS-treated rats than from saline-treated rats (R_{max}=80.8±6.3% and 54.1±4.7%, respectively, $P < 0.01$), but the pED_{50} values (mol) were not different (8.6±0.3, LPS-treated; 8.8±0.3, saline-treated). Relaxations to ADP were not different between the two groups (LPS-treated R_{max}=61.2±7.2%, pED_{50}=10.1±0.2; saline-treated R_{max}=55.4±7.2%, pED_{50}=10.3±0.2).

These data show impaired constrictor responses to perfusion of ME in mesenteric arterial beds removed from rats after LPS treatment. This is in contrast to previous findings in the same model of endotoxaemia, showing no difference in constrictor responses to bolus doses of ME between mesenteries from rats treated with LPS and saline (Tarpey & Randall, 1998). A similar disparity between results obtained with bolus doses *versus* perfusion has previously been reported in a model of cirrhosis (Ralevic *et al.*, 1996). It is suggested that steady-state infusion of constrictors can reveal pathophysiological changes in vascular smooth muscle function that are not apparent with bolus injection. Because of inherent variability in tone and concentration of ME required to raise tone between the groups, further studies are needed to determine whether the augmented responses to endothelium-independent vasodilators are due to an increased ability of the smooth muscle to relax, or an impaired ability to contract.

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81P REGIONAL HAEMODYNAMIC EFFECTS OF THE CANNABINOID AGONIST, WIN 55212-2, IN CONSCIOUS RATS

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The proposal that the endogenous cannabinoid, anandamide, might be an endothelium-derived vasorelaxant in rats (Randall *et al.*, 1996) has stimulated renewed interest in the cardiovascular effects of cannabinoids. Recently, Niederhoffer & Szabo (1999a) reported that the CB_1-, CB_2-receptor agonist, WIN 55212-2 caused hypotension in conscious rabbits, but this appeared not to be due to a direct vasodilator action (Niederhoffer & Szabo, 1999a, b). Considering the marked species variation in cardiovascular responses to cannabinoids, we have assessed the effects of WIN 55212-2 in rats. Male, Hannover Sprague-Dawley rats (3-4 months old) were chronically instrumented with pulsed Doppler probes (renal, mesenteric and hindquarters) and intravascular catheters. All surgery was carried out under sodium methohexitone anaesthesia) (40-60 mg kg^{-1} i.p., supplemented as needed). Conscious unrestrained animals were given i.v. bolus doses of WIN 55212-2 (5, 50 and 250 μg kg^{-1}) or vehicle (20% 2-hydroxypropyl-β-cyclodextrin) under baseline conditions and, on the next day, in the presence of losartan (10 mg kg^{-1}), pentolinium (5 mg kg^{-1}, 5 mg kg^{-1} h^{-1}) and a V_1-receptor antagonist ($d(CH_2)_5$-0-Me-Tyr-AVP; 10 μg kg^{-1}, 10 μg kg^{-1} h^{-1}). Table 1 shows some of the results. In the control state, WIN 55212-2 caused a pressor effect accompanied by bradycardia, renal and mesenteric vasoconstriction and hindquarters vasodilatation. Treatment with losartan, pentolinium and the V_1-receptor antagonist caused hypotension accompanied by vasodilatation only in the hindquarters. In this state the initial pressor effect of WIN 55212-2 was reduced and a delayed hypotensive action was apparent, which was accompanied by mesenteric and hindquarters vasodilatation. However, the initial renal and mesenteric vasoconstrictor and hindquarters vasodilator effects of WIN 55212-2 were unchanged. These results indicate that potential hypotensive and vasodilator effects of WIN 55212-2 may be masked under normal conditions.

Table 1. Resting cardiovascular variables and changes in response to WIN 55212-2 in the same conscious rats (n = 9). Measurements were made under control conditions and in the presence of losartan, pentolinium and a V_1-receptor antagonist (Blocked). HR = heart rate (beats min^{-1}); BP = mean arterial blood pressure (mm Hg); RVC, MVC, HVC = renal, mesenteric and hindquarters vascular conductance, respectively ([kHz mm Hg^{-1}]10^3). Values are mean ± s.e. mean; *P < 0.05 versus resting (Friedman's test); †P < 0.05 versus control value (Wilcoxon's test).

			WIN 55212-2 (250 μg kg^{-1})	
		Resting	Δ 1 min	Δ 10 min
HR	Control	389 ± 18	-28 ± 9	-51 ± 19*
	Blocked	358 ± 15	-19 ± 7	-26 ± 13*
BP	Control	111 ± 2	20 ± 3*	10 ± 2*
	Blocked	89 ± 4†	6 ± 2*†	-6 ± 2*†
RVC	Control	89 ± 6	-22 ± 3*	-12 ± 4*
	Blocked	84 ± 7	-15 ± 1*	-2 ± 3†
MVC	Control	83 ± 7	-27 ± 6*	-17 ± 4*
	Blocked	80 ± 6	-14 ± 2*	11 ± 4*†
HVC	Control	42 ± 3	9 ± 2*	3 ± 2
	Blocked	54 ± 3†	10 ± 4*	11 ± 3*†

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82P EFFECTS OF CANNABINOID RECEPTOR LIGANDS ON SYMPATHETIC NEUROGENIC CONTRACTION OF RAT ISOLATED PERFUSED MESENTERIC ARTERIAL BED

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Cannabinoids can lower blood pressure and cause vasorelaxation by actions at specific receptors expressed throughout the cardiovascular system (Randall & Kendall, 1998). Whilst receptors for cannabinoids have been identified in the central nervous system, little is known about their role in the peripheral modulation of neurotransmission in blood vessels. This study investigates the effects of cannabinoid receptor ligands on sympathetic neurotransmission in rat isolated mesenteric arterial beds.

Male Wistar rats (250-300g) were killed by exposure to CO_2 and decapitation. Mesenteric beds were isolated and perfused with oxygenated Krebs solution at 5 ml min^{-1} (Ralevic *et al.*, 1995). After 30 min equilibration, effects of cannabinoid receptor ligands on contractile responses of the mesenteries to electrical field stimulation (EFS; 10-64Hz, 1ms, 90V, 5s) and to noradrenaline (NA; 0.5-150 nmol), were examined. A group of mesenteries was treated with capsaicin (500 nmol for 20 min), a neurotoxin selective for primary afferent nerves. F_{50} is the stimulation frequency (Hz) required to elicit a response that is half maximal.

Frequency-dependent contractions to EFS (10-64Hz) of the mesenteric arterial beds were abolished by guanethidine (5μM) and prazosin (1μM), indicating their noradrenergic sympathetic nature. A selective cannabinoid receptor agonist, CP55,940 (1μM), attenuated maximal contractions to EFS, from 57.4±4.4mmHg to 32.6±4.7mmHg (P<0.01), but had no significant effect on the F_{50} (27.7±0.9Hz and 28.3±1.0Hz with and without CP55,940, respectively) (n=6). CP55,940 increased the dose of NA (-log mol) required to elicit contractions of 50 mmHg (8.24±0.14 with, and 8.05±0.16 without CP55,940) (P<0.05; n=6; Student's *t* test). SR141716A (1μM), a selective cannabinoid CB_1 receptor antagonist, also attenuated maximal contractions to EFS, from 55±6.9 mmHg to 37.5±7.7 mmHg (P<0.01), but had no significant effect on the F_{50} (n=5). Dose-dependent contractions to NA were not significantly affected by SR141716A (1μM; n=4). However, neither the selective CB_1 receptor antagonist, LY320135 (1μM; n=4), nor the selective CB_2 receptor antagonist SR144528 (1μM; n=8) affected significantly contractions to EFS. Removal of primary afferent vasorelaxation with capsaicin augmented maximal contractions to EFS, from 46.4±7.5 mmHg to 68.8±5.9 mmHg (P<0.001), but had no significant effect on the F_{50} (n=6). After capsaicin treatment, SR141716A was still able to attenuate sympathetic contraction, reducing maximal responses to 50±6.1 mmHg (n=6).

These data show that activation of cannabinoid receptors with CP55,940 attenuates sympathetic neurotransmission in the rat mesenteric arterial bed. Attenuation of responses to NA by CP55,940 indicates a postjunctional action. Paradoxically, the cannabinoid receptor antagonist SR141716A also attenuates sympathetic neurogenic contraction of mesenteric arterial beds, but does not affect responses to NA. This prejunctional action does not involve primary afferent nerves as attenuation of responses to EFS by SR141716A was still observed after capsaicin treatment. As other selective cannabinoid CB_1 and CB_2 receptor antagonists did not mimic the effects of SR141716A, this points to a CB_1 receptor-independent action of SR141716A on perivascular sympathetic neurotransmission.

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83P SR1417161, A CANNABINOID RECEPTOR ANTAGONIST, AUGMENTS VASORELAXATION MEDIATED BY CAPSAICIN-SENSITIVE PRIMARY AFFERENTS OF RAT ISOLATED MESENTERIC ARTERIAL BEDS

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Capsaicin-sensitive primary afferent nerves in the rat mesentery mediate vasorelaxation by the release and postjunctional actions of the neuropeptide calcitonin gene-related peptide (CGRP) (Kawasaki *et al.*, 1988). Receptors for cannabinoids have been identified on the central terminals of sensory C-fibres in the spinal cord (Hohmann & Herkenham, 1998), but little is known about their role in the peripheral modulation of primary afferent neurotransmission. The present study investigates the effects of cannabinoid receptor agonists and antagonists on sensory-motor neurotransmission in the rat isolated mesenteric arterial bed.

Male Wistar rats (250-300g) were killed by exposure to CO_2 and decapitation. Mesenteric beds were isolated and perfused at 5 ml min^{-1} with oxygenated Krebs solution with guanethidine (5μM) to block sympathetic neurotransmission (Ralevic *et al.*, 1996). After 30 min equilibration, tone was raised (by 30-80mmHg) with methoxamine (ME; 5-20μM). Effects of cannabinoid receptor ligands were examined on relaxations to electrical field stimulation (EFS; 2-12Hz, 0.1ms, 60V, 30s) and the endothelium-independent relaxants CGRP (5 and 50pmol) and sodium nitroprusside (SNP; 0.005-50nmol), and the endothelium-dependent relaxant adenosine 5'-diphosphate (ADP; 0.005-5 nmol). F_{50} is the stimulation frequency (Hz) required to elicit a relaxation that is half maximal.

Frequency-dependent relaxations of the mesenteric arterial beds (2-12Hz) were augmented by SR141716A (1μM), a selective antagonist of cannabinoid CB_1 receptors. Relaxant response curves were shifted to the left; F_{50} was 5.4±0.3 Hz in the absence and 3.0±0.1 Hz in the presence of SR141716A ($P<0.01$; Student's *t* test), but there was no significant difference in maximal relaxations (64.5±5.3% and 68.1±5.5% with and without SR141716A, respectively) (n=5). Another selective CB_1 receptor antagonist, LY320135 (1μM), had no significant effect on relaxations to EFS (2-12Hz) (n=6). Dose-dependent vasorelaxations to CGRP, SNP and ADP were not significantly different in the absence and presence of SR141716A (1μM; n=5-6). Methanandamide (1μM), a stable analogue of the endogenous cannabinoid anandamide, caused relaxation such that a greater concentration of ME (30-50 μM) was required to maintain raised tone. Under these conditions, there was attenuation of the maximal relaxation to EFS, from 66.1±2.4% to 22.3±3% ($P < 0.001$), but no significant difference in the F_{50} (n=4).

These data show that primary afferent neurogenic vasorelaxation of rat mesenteric arterial beds is augmented by SR141716A, a cannabinoid receptor antagonist. As the antagonist LY320135 was without effect on sensory-motor vasorelaxation this contraindicates the involvement of a CB_1 receptor. The lack of effect of SR141716A on responses to CGRP, SNP and ADP indicates a prejunctional site of action. In light of evidence that a cannabinoid receptor agonist, methanandamide, does not also modulate the sensitivity of sensory afferent vasorelaxation it does not appear that the prejunctional action of SR141716A is mediated by block of endogenous cannabinoid tonic inhibitory modulation of primary afferent neurotransmission.

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84P CHARACTERISATION OF THE ORL_1 RECEPTOR ON ADRENERGIC NERVES IN THE ANOCOCCYGEUS

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Like the classical opioid-receptor types the ORL_1 receptor is found at peripheral sites such as the vas deferens, where nociceptin has a powerful opioid-like inhibitory effect on neuromuscular transmission (Nicholson *et al* 1997; 1998). Here we report our discovery of the probable presence of the ORL_1 receptor at another peripheral site, on sympathetic motor nerves in the anococcygeus muscle of the rat.

Anococcygeus muscles from 250-300g male rats were set up for electrical-field stimulation (5x0.5ms biphasic pulses at 10Hz, every 30s) in 3ml silanised glass organ baths containing Krebs' solution at 37°C. Ligands tested for agonist activity were added cumulatively; for experiments involving measurement of antagonists affinity, or with other treatments, a preincubation time of 20-30min was used after a 45-minute period of washout and recovery, before the next cycle of agonist addition.

The motor response to electrical field stimulation under low resting tension (isometric recording) was unaffected by up to 10μM [D-Ala^2, Me-Phe^4, Gly-ol^5]enkephalin, [D-Pen^2, D-Pen^5]enkephalin or U-69,593, confirming the absence of μ-, δ- or κ-receptors, but the addition of nociceptin produced a concentration-related inhibition up to a maximum around 75% (EC_{50} ~25nM), without affecting the response to exogenous noradrenaline. In high tone (after guanethidine 30μM) the inhibitory response was unaffected by nociceptin. In the presence of 100μM Nω-nitro-L-arginine (NOARG) the motor response was doubled in size and the efficacy of nociceptin was reduced by half. When this experiment was repeated in Krebs' solution with reduced Ca^{2+} (1.25mM) and peptidase inhibitors added (amastatin, bestatin, captopril and phosphoramidon at 30μM, Nicholson *et al.*, 1998), nociceptin now produced almost complete inhibition of the pure motor response (98% inhibition, EC_{50} 6.5nM, Table 1). Subsequent work was with the low-calcium Krebs' solution containing peptidase inhibitors and NOARG.

As in the vas deferens (Nicholson *et al.*, 1997) the effect of nociceptin on the anococcygeus was reproduced by the hexapeptide Ac-RYYRWK-NH_2 with higher potency, though reduced efficacy (Table 1). The related peptide Ac-RYYRIK-NH_2 (Berger *et al.*, 1999) had lower efficacy, and could be used as an antagonist (pA_2 9.01). As in the vas deferens, the non-selective opioid naloxone benzoylhydrazone (NalBzOH, Nicholson *et al.*, 1998) was a competitive antagonist of modest affinity, as was [Phe^{1}ψ(CH_2-NH)Gly^2]Nociceptin(1-13)NH_2 (F/GNC13) (Table 1). Nocistatin, another product of the processing of the pro-nociceptin precursor, is reported to block a behavioural response to nociceptin (Okuda-Ashitaka *et al.* 1998), but had no effect here at up to 1μM.

	pEC_{50}	Emax	pK_B/pA_2*
Nociceptin	8.4±0.1	98.3±1.2	
Ac-RYYRWK-NH_2	9.0±0.1	66.4±5.2	-
Ac-RYYRIK-NH_2	8.0±0.2	36.7±3.5	9.01*
F/GNC13	n.e.	-	7.4 (7.1-7.7)
NalBzOH	n.e.	-	6.9 (6.7-7.1)
Nocistatin (rat)	n.e.	-	n.e.

Table 1. Effects of ORL_1 receptor ligands on the rat anococcygeus
Values for pEC_{50} and Emax (% inhibition) are mean ± s.e.mean (n ≥3). Values for pK_B are determined from Schild regressions with unit slope (95% c.l.). n.e. no effect

Our results suggest the presence of an inhibitory ORL_1 receptor on adrenergic terminals in the anococcygeus, with a pharmacology like that of the receptor on sympathetic nerves in the vas deferens.

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85P β-ADRENOCEPTORS IN THE MATURATION OF AORTAE FROM NORMOTENSIVE AND PRE-HYPERTENSIVE RATS

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The vascular responsiveness to isoprenaline and β_2-adrenoceptor numbers are reduced in hypertension (Brodde & Michel, 1992) and senescence (Fleisch, 1980). The objectives of our study were to determine whether the responsiveness to isoprenaline, the affinity of isoprenaline at β_2-adrenoceptors and β_2-adrenoceptor receptor reserves were altered in the maturation of aortae from normo- and pre-hypertensive rats. The study used contractility methods with aortae isolated from male 5- and 14-week-old Wistar Kyoto rats (WKY) and Spontaneously Hypertensive rats (SHRs). Aortae were contracted with KCl, and the effects of bromoacetylalprenololmenthane (BAAM, an irreversible β-blocker; Doggrell & Surman, 1995) on the isoprenaline responses were determined. The SHRs were pre-hypertensive at 5 weeks. Maturation of WKY aortae was associated with a loss of sensitivity [isoprenaline pD_2; 5 weeks, 8.40 ± 0.04 (10): 14 weeks, 7.21 ± 0.10 (9): $P < 0.01$: Student's t-test] and maximum response to isoprenaline (% relaxation of KCl contraction; 5 weeks, 115% ± 6: 14 weeks, 88% ± 7: $P < 0.05$). Maturation of SHR aortae was also associated with a loss of sensitivity (5 weeks, 8.03 ± 0.01 (14): 14 weeks, 7.10 ± 0.11 (8): $P < 0.01$) and maximum response to isoprenaline (5 weeks, 110% ± 4: 14 weeks, 23% ± 3: $P < 0.05$). BAAM at 10^{-6} M (5- and 14-week-old WKY aorta) and 3×10^{-7} M (5- and 14-week-old SHR aorta) caused non-parallel rightward shifts of isoprenaline response curves with a depression of maximum responses. This data was used to calculate K_A values and β-adrenoceptor occupancy-response relationships. The K_A value for isoprenaline on the aortae of 5-week-old WKY was 2.11×10^{-7} M and similar values were obtained on the aortae of 14-week-old WKY and 5- and 14-week-old SHRs. The maturation of WKY and SHR aortae was associated with a loss of β-adrenoceptor reserve for isoprenaline [50% maximum response; % receptor occupancy: WKY 5 weeks; 9% ± 2 (10), 14 weeks; 43% ± 4 (4) $P < 0.01$: SHR 5 weeks; 22% ± 5 (11), 14 weeks; 44% ± 6 (9) $P < 0.01$]. 5-Week-old SHR aortae were less sensitive to isoprenaline ($P < 0.05$) and had a lesser receptor reserve for isoprenaline than age-matched WKY ($P < 0.01$). In summary, the aortae of 5-week-old SHRs are less sensitive to isoprenaline and have a lesser receptor reserve for isoprenaline than age-matched WKY. On the aortae, there is also a loss of responsiveness and receptor reserve for isoprenaline in the maturation of WKY and SHRs from 5 to 14 weeks.

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86P PERIPHERAL CHEMORECEPTOR STIMULATION ACTIVATES 5-HT NEURONS IN THE LOCUS COERULEUS OF CONSCIOUS RATS

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The observation that brief periods of hypoxia and hypercapnia enhance the discharge rate of locus coeruleus (LC) noradrenergic cells in anaesthetized animals has led to the suggestion that the activation of LC neurons may serve as an alarm system to arouse the individual in response to chemoreceptor signals (Elam et al., 1981). The afferent pathways and neurotransmitter involved in modulation of LC neurons by chemosensory stimuli are little investigated. Here we have used the push-pull superfusion technique to explore whether neurotransmitter release in the LC is changed following chemoreceptor activation in conscious rats. We have focused on 5-HT release since it is known that the 5-HT input to the LC is involved in the modulation of LC neurons by stress, pain and blood pressure changes (Singewald and Philippu, 1998).

In male Sprague-Dawley rats (250-280g), a guide cannula aiming at the LC was stereotaxically inserted under ether/ketamine anaesthesia. For recording of blood pressure and intravenous infusions of drugs, iliac artery and jugular vein were catheterized, respectively. In one group of rats peripheral chemoreceptor denervation (CD) involving transection of carotid sinus and aortic depressor nerves (Franchini and Krieger, 1993) was carried out. The other group of rats received a sham operation. Five days after surgery the LC was superfused through a push-pull cannula with artificial cerebrospinal fluid at a rate of 14 µl/min. Superfusate was collected continuously in time periods of 10 min. 5-HT was determined by HPLC with electrochemical detection (Singewald et al 1997). For stimulation of chemoreceptors, KCN was applied intravenously (Franchini and Krieger, 1993).

A bolus injection of 40 µg KCN in sham-operated rats led to brief (20 s) pressor response (59±15 mm Hg) and bradycardia (-126±12 beats/min), and enhanced the respiration rate (46±5 breaths/min) for 2 min. 5-HT release in the LC was slightly (37%) enhanced (Table 1) by this brief chemoreceptor stimulation. Intravenous infusion of KCN (15µg/min) for 10 min increased the respiration rate (39±5 breaths/min) throughout the infusion and enhanced blood pressure only slightly (6±1 mm Hg). 5-HT release in the LC was enhanced by 60% during KCN infusion. Infusion of saline (50µl/min) for 10 min did not change the respiration or release of 5-HT in the LC. A higher dose of KCN (30 µg/min for 10 min) enhanced 5-HT release for 20 min. Neither injection or infusions of KCN changed 5-HT release in CD rats, suggesting that the effect on extracellular 5-HT was due to activation of peripheral chemoreceptors.

Table 1. Effect of chemoreceptor stimulation on 5-HT release

	5-HT release in the LC (Δ%)	
	SHAM	CD
Saline (50µl/min infusion)	8±12	
KCN (40µg bolus injection)	37±11*	-7±4 †
KCN (15 µg/min infusion)	60±16*	-9±6 †
KCN (30 µg/min infusion)	60±14**	-4±8 †

Values (means ± s.e.m, N=7-11) are changes in 5-HT release in % relative to the mean of the 3 samples preceding i.v. infusion. * $p<0.05$, ** $p<0.01$ vs basal release; Friedman's test followed by Wilcoxon's test, †$p<0.05$ CD vs sham, Mann-Whitney test.

In summary, we report that peripheral chemoreceptor activation enhances 5-HT release in the LC, indicating that, in the conscious rat, serotonergic neurons are involved in the modulation of LC activity by ascending chemosensory information.

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87P COMPARISON OF [^{3}H]PRAZOSIN AND [^{3}H]TAMSULOSIN LABELLING OF WILD-TYPE AND CONSTITUTIVELY ACTIVE α_{1B} RECEPTORS

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We have recently reported that the radioligand [^{3}H]tamsulosin consistently labels fewer α_{1B}-adrenoceptors than [^{3}H]prazosin in side-by-side experiments with rat tissues or cloned rat and human receptors (Michel & Goepel 1998). Based on competition studies with noradrenaline in rat liver we had hypothesized that the difference might result from an incomplete labeling of agonist low affinity sites by [^{3}H]tamsulosin.

To test this hypothesis we have now performed saturation binding and noradrenaline competition experiments with [^{3}H]tamsulosin and [^{3}H]prazosin with wild-type (WT) hamster α_{1B}-adrenoceptors and a mutant thereof, which had been rendered constitutively active by an A293E substitution (CAM), both stably expressed in Rat-1 fibroblasts; rat liver was reinvestigated for reference purposes. Experiments were performed as previously described (Michel & Goepel 1998), and results are expressed as means ± s.e. mean of n experiments. Statistical significance of differences between [^{3}H]tamsulosin and [^{3}H]prazosin results was determined by paired, two-tailed t-tests with $p < 0.05$ considered significant.

In agreement with our previous data [^{3}H]tamsulosin labeled fewer α_{1B}-adrenoceptors in rat liver than [^{3}H]prazosin (56 ± 2 vs. 87 ± 5 fmol/mg protein, n = 6, p = 0.0036). A similar difference was observed for hamster WT (2040 ± 166 vs. 2729 ± 104 fmol/mg protein, n = 7, p = 0.0063) and CAM receptors (1578 ± 191 vs. 1838 ± 156 fmol/mg protein, n = 10, p = 0.0098). The percentage of sites labeled by [^{3}H]tamsulosin was 66 ± 5%, 75 ± 6% and 84 ± 5% of the [^{3}H]prazosin values in rat liver, WT and CAM, respectively.

Noradrenaline had biphasic competition curves for all three α_{1B}-adrenoceptor preparations. The percentage of agonist high-affinity sites in rat liver was 42 ± 7% for [^{3}H]tamsulosin and 35 ± 6% for [^{3}H]prazosin (n = 7, p = 0.4152). Corresponding numbers at the hamster WT and CAM were 31 ± 10% vs. 24 ± 5% (n = 6, p = 0.4224) and 58 ± 5% vs. 56 ± 6% (n = 6, p = 0.8058). While noradrenaline had higher affinity at CAM than at WT or rat liver α_{1B}-adrenoceptors, similar values were seen with [^{3}H]tamsulosin and [^{3}H]prazosin competition binding. The calculated affinities of noradrenaline at its high and low affinity sites were (all values as pK_i values): rat liver 7.48 ± 0.17 vs. 7.59 ± 0.37 and 5.90 ± 0.09 vs. 5.96 ± 0.08, hamster WT 7.98 ± 7.44 vs. 7.44 ± 0.19 and 5.80 ± 0.09 vs. 5.96 ± 0.06, hamster CAM 8.32 ± 0.13 vs. 8.60 ± 0.14 and 6.38 ± 0.37 vs. 6.65 ± 0.16.

We conclude that [^{3}H]tamsulosin labels consistently fewer α_{1B}-adrenoceptors than [^{3}H]prazosin. This difference is also seen with a constitutively active receptor mutant, but in contrast to our previous hypothesis cannot be explained by a differential labeling of agonist high and low affinity sites by the two radioligands.

Michel, M.C. & Goepel, M. (1998) *Eur. J. Pharmacol.* 342: 85-92

88P A NOVEL PEPTIDE TOXIN FROM THE INDIAN RED SCORPION BLOCKS THE VOLTAGE-GATED CLONED POTASSIUM CHANNEL, hKv1.6

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BT-2 is a novel polypeptide which has been isolated from the venom of the Indian red scorpion, *Mesobuthus tamulus*. The toxin contains 35 amino acids and is cross-linked by three disulphide bridges with similar homology to the charybdotoxin family. Two isomers of the toxin have been identified differing by one amino acid at residue 6. One isomer contains a valine residue and has a molecular weight of 3946, with the other having an isoleucine (MW 3960). The toxin was identified in a dendrotoxin binding screen and its pharmacology investigated on the dendrotoxin-sensitive voltage-gated K^+ channel, hKv1.6.

RNA encoding the kKv1.6 K^+ channel was expressed by microinjection into Chinese hamster ovary cells. The cells were sub-cultivated according to standard methods and plated 8-12 hours prior to injection. Whole cell patch-clamp recording was carried out on previously injected cells at between 4-8 hours post-injection. A standard voltage step protocol was used to evoke currents for testing, consisting of step every 10s from a holding potential of -60mV to +30mV lasting 500ms. Both isomers of BT-2 were tested at a concentration of 500nM. All experiments were performed at room temperature, 20-22°C.

The Val-6 isomer was found to rapidly inhibit both the peak and the steady-state current by 18.9±1.0% and 37.1±1.1% (n=7 cells), respectively (figure 1), with the inhibition being fully reversed upon washout. This difference in the inhibition of the peak and steady-state current may reflect some state-dependent mechanism. The current-voltage relationship showed that the Val-6 isomer did not alter the activation voltage of the channel. The application of the Ile-6 isomer caused only a small inhibitory effect on the peak and steady-state current, 4.6±1.1% and 4.1±1.0% (n=5 cells), respectively (figure 1).

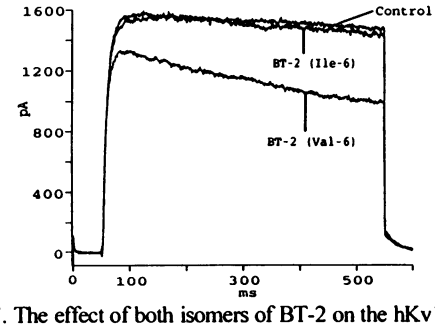

Figure 1. The effect of both isomers of BT-2 on the hKv1.6 current recorded in the same cell. Currents evoked with a voltage-step (500 ms duration) from holding potential of -60mV to + 30mV.

This is the first study to demonstrate the inhibition of the cloned potassium channel, hKv1.6, by a particular form of the novel scorpion toxin, BT-2.

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89P EFFECT OF ELECTRODE MATERIAL ON THE INHIBITION OF NON-ADRENERGIC, NON-CHOLINERGIC RESPONSES OF ISOLATED GUINEA-PIG TAENIA CAECI BY N^{ω}-NITRO-L-ARGININE (NOARG)

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Non-adrenergic, non-cholinergic (NANC) electrical field stimulation of the Guinea pig taenia caeci *in vitro* produces relaxations which are inhibited in a highly repeatable manner by the nitric oxide synthase (NOS) inhibitors such as N^{ω}-nitro-L-arginine (NOARG) and N^{ω}-nitro-L-arginine methyl ester (NAME), and reversed by L-arginine (Piotrowski *et al*, 1993, Piotrowski *et al*, 1994, Piotrowski *et al*, 1996). Although there is some inter-laboratory consensus with such observations, they are not a universal finding. For example, Williams & Parsons, (1995) have reported a lack of inhibition by NOARG of NANC relaxations in the taenia caeci. In order to try to determine the basis for the observed differences, the effects of different electrode materials were examined.

Segments of taenia caeci approximately 2cm in length were removed from freshly killed guinea pigs of either sex (310-590g) and set up in 10ml isolated tissue baths maintained at 37°C and supplied with Kreb's solution containing atropine (10μM) and guanethidine (10μM) and bubbled with 95%O_2/5%CO_2. Supramaximal electrical field stimulation, using 1ms rectangular pulses, was applied for the duration of the experiment in 20Hz trains lasting 10s and repeated every 90s via parallel electrodes made either from stainless steel (Harvard Instruments type 10-1000), or modified by replacement with other metals (Goodfellow Metals). The electrode geometry was not altered by the modifications. Mechanical activity of the preparations was recorded isotonically using a resting tension of 0.5g.

Table 1: Effects of NOARG followed by L-arginine added cumulatively on NANC relaxations of Guinea pig taenia caeci. Responses are expressed as means±sem of control relaxations from 4-6 experiments.

Electrode material	NOARG (10μM)	L-Arg (1mM)
Stainless steel (SS)	82.6±2.2% *	97.9±2.4% n.s.
Silver	86.1±4.1% *	97.1±5.2% n.s.
Gold	94.0±1.8% n.s.	100.7±7.4% n.s.
Palladium	97.9±4.8% n.s.	101.9±7.0% n.s.
Platinum	99.5±1.8% n.s.	102.5±3.9% n.s.
SS+CPA (1μM)	100.4±10.2% n.s.	101.8±13.3% n.s.

*=$p<0.05$, n.s.=not significant, compared to pre-drug controls, Student's t-test; CPA=chloroplatinic acid (BDH). This substance is produced during hydrolysis of saline solutions using platinum electrodes.

The results of this study suggest that the electrode materials, particularly platinum, employed in studies of nitrergic responses may have a significant bearing on experimental outcome. A substantial body of published work may consequently need to be reevaluated.

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90P ROLE OF PIGMENTATION IN THE ATYPICAL BEHAVIOUR OF DARIFENACIN IN DOG CILIARY SMOOTH MUSCLE

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The ciliary smooth muscle of the bovine (Honkanen *et al.*, 1990), canine (McIntyre and Quinn, 1995) and human (Woldemussie *et al.*, 1993) eye express muscarinic M_3 receptors. Recent functional pharmacological characterization of contractile muscarinic receptors in dog ciliary muscle (DCM, brown eye) revealed, however, that the antagonism produced by darifenacin, a selective muscarinic M_3 receptor antagonist, was inconsistent with the singular involvement of M_3 muscarinic receptors (Choppin *et al.*, 1999). Melanin-rich pigmentation of ocular tissues can potentially cause antagonist sequestration leading to underestimation of antagonist concentration in the receptor compartment (Salazar *et al.*, 1976). In the present study, we compared the effects of darifenacin in DCM from pale blue and brown colored eyes in order to assess the role of pigmentation in the atypical behavior of darifenacin.

Tissues from beagle or mongrel dogs (both sexes, 5-10kg), having a different color pigment in each eye, were used in the present study using methodology similar to that described by Choppin *et al.* (1998). Cumulative concentration-response curves to (+)-cis-dioxolane (1nM - 300μM) were established in the absence and presence of darifenacin (90 min equilibration). Antagonist affinities (pK_B) were determined using the Gaddum equation (Gaddum, 1943). Each dog served as its own control. Quantitative determination of the melanin contents in DCM from brown and blue eyes was performed using the method described by Aravind Menon *et al.* (1992).

(+)-Cis-dioxolane induced concentration-dependent contractions of DCM (pEC_{50} = 7.0±0.1, n=17 and 6.8±0.1, n=20, in brown and blue-eyed DCM, respectively). In the brown eye, the antagonism produced by darifenacin exhibited two phases: a darifenacin-resistant (pK_B<6) and a darifenacin-sensitive (pK_B>8; possibly M_3-mediated) component (Figure 1). In the ciliary muscle from the blue eye, however, darifenacin produced surmountable, competitive antagonism of the responses to (+)-cis-dioxolane (pK_B=8.76±0.07, Figure 1).

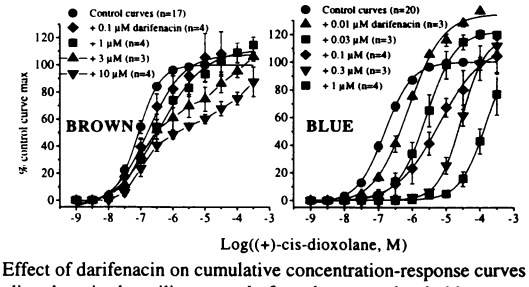

Figure 1: Effect of darifenacin on cumulative concentration-response curves to (+)-cis-dioxolane in dog ciliary muscle from brown and pale-blue eyes. Contractile effects are normalized to the control. The values shown are means±s.e.mean, n=3-4.

The histopathology experiments highlighted the presence of melanine within the smooth muscle layers of the brown eye but not in the blue eye. The melanin content in the brown-eyed DCM was marginally, but not statistically ($p > 0.05$), greater than that in the blue-eyed DCM (25.5±3.1 and 16.7±5.8 $\mu g.mg^{-1}$ of tissue, respectively). Incubation of blue-eyed DCM with melanin (5, 10 or 50 $\mu g.mg^{-1}$ of tissue for 1.5 or 18 h) did not alter the competitive behavior of darifenacin in this tissue.

In conclusion, the data suggest that M_3 muscarinic receptors mediate contractile effects in the blue-eyed DCM. The anomalous behavior of darifenacin in brown-eyed DCM appears to be unrelated to melanin content and may suggest the involvement of multiple, possibly novel, muscarinic receptor subtypes.

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91P THE CHARACTERISATION OF α_1-ADRENOCEPTORS IN MURINE LIVER USING RADIOLIGAND BINDING AND TRANSGENIC MICE

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It is now believed that there are three native α_1-adrenoceptor (α_1-AR) subtypes (α_{1A}, α_{1B}, α_{1D}) which correspond to three recombinant subtypes (α_{1a}, α_{1b}, α_{1d}) (Hieble *et al*, 1995). Identification of native subtypes remains difficult but might be aided by using genetically altered mice which have had their α_{1b}-AR gene deleted through targeted gene disruption (Cavalli *et al*, 1997). Our aim was to determine the α_1-AR subtypes present in the murine liver through the use of radioligand binding and transgenic mice.

Saturation binding studies were performed with C57-Black (male, 30-45g) wildtype (WT) and knockout (KO) liver membrane preparations incubated with ^{3}H-prazosin (0.025nM-5nM) in a Tris-HCl buffer (pH 7.4) and were terminated after 30 minutes at 22°C using a Brandell cell harvester. Competition assays were performed with liver membranes and recombinant cells expressing α_{1b}-AR's using selective α_1-AR ligands, A86641, L765,314 (Patane *et al*, 1998)and BMY7378 (α_{1a}, α_{1b} and α_{1d} respectively). Non specific binding was measured in the presence of 10μM phentolamine.

^{3}H-Prazosin identified high affinity binding sites for both the WT and KO liver. WT membranes revealed an affinity (K_D) of 0.3±0.08nM and a maximal receptor number (B_{max}) of 50±3.1fmol/mg. KO membranes had a lower K_D of 0.14±0.04nM and a reduced B_{max} of 30±2fmol/mg. The pK_i values obtained for competition studies in WT and KO liver are shown in Table 1 along with the values obtained for recombinant α_{1b}-cells. In each case, competition curves were monophasic, except for A86641 in KO liver, where a biphasic curve was observed.

Table 1:- pK_i Values obtained in WT and KO liver compared with values obtained in recombinant α_{1b}-cells.

	WT liver	KO liver	α_{1b}-cells
A86641	5.8	9.4, 6.5	6.1
L765314	7.6	7.1	7.6
BMY7378	6.5	5.8	6.8

The WT liver appears to contain a single population of α_{1b}-AR. The KO liver, which might have been expected to contain no α_1-AR's, has total specific α_1-AR binding equivalent to 60% of the WT. Absence of α_{1b}-AR protein from the KO has already been illustrated (Cavalli *et al*,1997) and the low pK_i value for BMY7378 rules out the α_{1b}- and α_{1d}-ARs, respectively, as being responsible for the observed binding. By elimination, therefore the α_1-AR expressed in the KO appears to be of the α_{1A} subtype which is supported by the affinity data from L765,314 and BMY7378. However the reason for the biphasic curve to A86641 remains to be explained.

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We would like to thank Merck & Co., Pennsylvania for their generous donation of the L765, 314 compound.

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92P MODULATION BY NITRIC OXIDE (NO) OF ACETYLCHOLINE (ACh) RELEASE FROM GUINEA-PIG TRACHEA

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NO-synthase inhibitors enhance nerve mediated cholinergic contractions of guinea-pig trachea but not responses to exogenous ACh which suggests that endogenous NO inhibits ACh release. However, biochemical experiments failed to detect an effect of NO-synthase inhibitors on field stimulation evoked release of ACh (Brave et al., 1991). The aim of the present study was to investigate the effects of the NO donor S-nitroso-N-acetylpenicillamine (SNAP) on ACh release from the guinea-pig isolated trachea.

Experiments were performed essentially as described by Kilbinger et al. (1991). Briefly, epithelium-free trachea strips were incubated with [^{3}H]choline (5 μCi/ml) in a 2 ml organ bath and subsequently superfused with a physiological salt solution. The strips were stimulated twice 30 min apart (S1, S2; 20 Hz, 600 pulses applied in trains of 100 pulses every 30 sec). SNAP (100 and 300 μM) added 20 min before S2 caused a small but significant increase in the electrically evoked [^{3}H]ACh release (120 ± 4 % and 124 ± 10 % of control, Fig. 1). Resting outflow of [^{3}H]ACh was not affected by SNAP.

In another series of experiments the role of capsaicin-sensitive neurones in the facilitatory effect of SNAP was tested. Capsaicin desensitization was carried out by superfusing the strips (80 min before S1) with a solution containing 3 μM capsaicin for 40 min followed by a superfusion with capsaicin-free solution. This treatment selectively blocks neurally-mediated non-cholinergic contractions of guinea-pig trachea (Ellis & Undem, 1990). After capsaicin pre-treatment SNAP (100 and 300 μM) significantly inhibited the electrically evoked release of [^{3}H]ACh to 74 ± 4 % and 78 ± 2 % of the control value (Fig. 1). A similar inhibition by SNAP was seen when the preparations were superfused with the NK2 receptor antagonist SR 48968 (Martin et al., 1992) instead of capsaicin. In the presence of 300 nM SR 48968 (added to the medium 80 min before S2) SNAP (100 μM) reduced the evoked release of [^{3}H]ACh to 82 ± 5 % (n=5) of the control value (100 ± 4 %, n=5, $p< 0.02$).

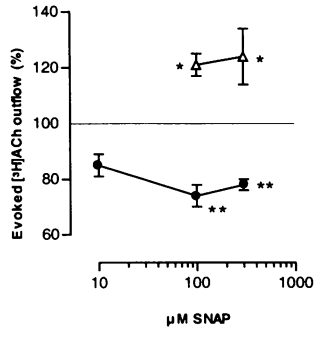

Figure 1

The effects of SNAP on stimulation evoked release of [^{3}H]ACh from guinea-pig trachea; [Δ] no pretreatment; [•] capsaicin pretreatment. Mean ± s.e.mean (n=4-7). Significance of difference from control values (Δ : 100 ± 4%, n=7; • : 100 ± 4 %, n=9): * p<0,05, ** p<0,01.

The results suggest that exogenous NO facilitates the release of endogenous tachykinin(s) from afferent neurones in the guinea-pig trachea. The tachykinin(s) in turn stimulate(s) NK2 receptors at cholinergic neurones which leads to an increase in ACh release and facilitation of cholinergic neurotransmission as shown previously (Hall et al., 1989). After depletion of tachykinins by capsaicin pre-treatment, or after NK2 receptor blockade exogenous NO inhibited the evoked release of ACh.

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93P A STUDY OF THE EFFECT OF IBOGAINE AGAINST NEUROGENIC CONTRACTIONS OF THE RAT VAS DEFERENS AND GUINEA-PIG ISOLATED DETRUSOR MUSCLE

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Ibogaine, a putative anti-addictive alkaloid found in *Tabernanthe iboga*, has been reported to recognize μ-opioid binding sites, but the functional significance of this interaction is unclear (Sweetman *et al.*, 1995; Pablo and Mash 1998). During the course of experiments designed to determine the effect of ibogaine at prejunctional μ-opioid receptors on the rat vas deferens we noted that purinergic contractions were enhanced. We have, therefore, extended the study to investigate the effect of ibogaine against purinergic and cholinergic responses of the guinea-pig detrusor muscle.

Male Dunkin Hartley guinea-pigs (500-900g) and male Hooded Lister rats (200g) were killed by exsanguination followed by decapitation. The prostatic end of the rat vas deferens and 10x2 mm strips of the guinea-pig detrusor muscle were placed in an isolated organ bath, containing Krebs-Henseleit solution and 1 g wt and 2 g wt resting tension applied, respectively. After 30 min equilibration preparations were electrically stimulated (0.3 ms, 200 mA; single pulses, 0.1Hz for vas deferens; 10Hz, 2s trains every 5 min for the detrusor) until responses were constant. In the rat vas deferens the effect of DAMGO, a selective μ-opioid receptor agonist, and UK-14304, a selective α_2-adrenoceptor agonist, was examined in the presence and absence of ibogaine. In the detrusor muscle the effect of ibogaine against neurogenic responses was examined in the absence and presence of 0.1μM atropine or 300μM suramin. The agonist pD_2 (negative logarithm of the concentration producing 50% of the maximum response) was determined. Responses have been expressed as a percentage of the control neurogenic responses and are shown as the mean ± s.e.mean. Differences between mean values were considered significant if $p < 0.05$ (Student t-test).

In the rat vas deferens, DAMGO inhibited neurogenic responses in a concentration-dependent manner (pD_2 - 6.71±0.17, n=6), an effect significantly reduced in the presence 30 μM ibogaine (pD_2- 6.10±0.15,n=6) and abolished by 1μM naloxone (n=4). Similarly, UK-14304-induced inhibition of neurogenic contractions (pD_2 - 9.49±0.13,n=6) was significantly impaired by the presence of 30μM ibogaine (pD_2 - 8.86±0.08, n=6). Ibogaine (30μM) significantly increased neurogenic contractions (88.4±14.1%, n=10), while suramin (300μM), a P2x purinoceptor inhibitor, abolished responses.

Atropine (0.1μM) and suramin (300μM) reduced neurogenic contractions of the guinea-pig isolated detrusor (Table 1), while a combination of both agents abolished responses (n=5). Ibogaine significantly enhanced the control and purinergic component of neurogenic responses, but not the cholinergic component (Table 1).

Table 1: The effect of ibogaine (10μM) against neurogenic contractions of the guinea-pig isolated detrusor (n=5).

	Control	Atropine	Suramin
Vehicle	100	43.7±4.3	68.6±11.1
Ibogaine	132.8±11.3*	82.9±6.8*	72.7±11.4

* - Significantly different from the vehicle response.

Ibogaine antagonized the effect of DAMGO and UK-14304 on the rat vas deferens, suggesting a non-specific action on responses to prejunctional receptors. On the other hand, the enhancement of purinergic responses in both preparations is novel and appears to be selective. Further experiments with antiaddictive analogues of ibogaine are warranted to establish the significance of this observation.

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94P INHIBITION OF NITRIC OXIDE RELEASE IN RAW 264.7 CELLS BY CANNABINOIDS

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Jeon *et al.* (1996) have found Δ^9-tetrahydrocannabinol (THC) to produce a dose-related inhibition of nitric oxide production, iNOS transcription and cyclic AMP production in RAW 264.7 cells, a macrophage cell line that expresses cannabinoid CB_2 but not CB_1 receptors. In this study we have investigated whether these findings extend to palmitoylethanolamide (PEA) or to the cannabinoid receptor agonist, (+)-WIN55212, using the (–)-enantiomer of WIN55212 and pertussis toxin (PTX) to elucidate the involvement of cannabinoid receptors (Pertwee, 1997).

RAW 264.7 cells were grown in RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 units ml^{-1} penicillin and 100 μg ml^{-1} streptomycin. They were plated at $5x10^5$ cells ml^{-1} and nitric oxide (NO) release stimulated with lipopolysaccaride (LPS; 0.2-200 ng ml^{-1}). Cannabinoids were added in ethanol, either with the LPS (single dose experiments), or 30 min prior to LPS (LPS concentration-response experiments). Incubations were for 24 hours. Supernatants were mixed with an equal volume of Griess reagent and nitrite production was measured by an absorbance reading at 550 nm, using $NaNO_2$ to generate a standard curve. In experiments with single concentrations of LPS, NO release was normalised to 100%. For LPS concentration-response experiments, NO release was calculated as a percentage of the maximum. EC_{50} and E_{max} values were calculated using Prism (GraphPad Software, San Diego). Statistical significance was determined using a one-sample t-test ($P<0.05$). Results are expressed as means ± s.e.mean or as means with 95% confidence limits.

(+)-WIN55212 significantly inhibited NO production by 31.9± 6.4% at 10 μM (200 ng ml^{-1} LPS; n=7) and by 26.3±7.4% and 64.7±6.7% at 1 μM and 10 μM respectively (20 ng ml^{-1} LPS; n=6). PEA significantly inhibited NO production by 15.9±4.9% and 31.7±5.6% at 1 μM and 10 μM respectively (200 ng ml^{-1} LPS; n=6) and by 20.2±7.2% and 39.1±7.7% at 1 μM and 10 μM respectively (20 ng ml^{-1} LPS; n=6). (+)-WIN55212 produced a parallel rightward shift in the LPS concentration-response curve. The EC_{50} values for LPS were 18.6 ng ml^{-1} (12.6-27.5) in the presence of vehicle and 95.1 ng ml^{-1} (38.2-236.9) in the presence of 10 μM (+)-WIN55212 (n=10). The E_{max} values for LPS were unchanged: 109% (99.4-118.7) with vehicle and 86.6% (70.7-102.5) with 10 μM (+)-WIN55212. (–)-WIN-55212 had no effect on the LPS concentration-response curve (n=5). EC_{50} and E_{max} values were 25.8 ng ml^{-1} (12.4-53.9) and 97.6% (85.9-109.4) respectively with vehicle and 18.4 ng ml^{-1} (9.5-35.4) and 83.2% (75.4-90.9) respectively with 10 μM (–)-WIN55212. PTX pretreatment (100 ng ml^{-1} for 24 hours) abolished the inhibitory effect of (+)-WIN-55212. Thus, EC_{50} values for LPS were 40.0 ng ml^{-1} (25.1-62.7) with vehicle and 42.7 ng ml^{-1} (17.6-83.2) with 10 μM (+)-WIN55212 (n=3). PEA did not produce a rightward shift in the LPS concentration-response curve. However, it did reduce the E_{max} of LPS from a vehicle control value of 100% (95.5-106.5) to 86.4% (76.6-95.1) at 1 μM PEA and 45.1% (35.8-54.5) at 10 μM PEA (n=6). The inhibition produced by PEA was unaffected by PTX pretreatment, the E_{max} of LPS after PTX being 108.1% (81.8-134.3) for vehicle and 48.3% (27.0-69.7) for 10 μM PEA (n=3).

These results provide further evidence that cannabinoids inhibit NO production in macrophages, albeit at micromolar concentrations. The effect of WIN55212 is both stereoselective and PTX-sensitive. PEA is effective in a similar concentration range to WIN55212 but is not PTX sensitive. This study points to differences in the mechanisms of action of (+)-WIN55212 and PEA which require further investigation.

Supported by Wellcome Trust grant 047980 and by Pfizer.

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95P MODULATION OF RHODAMINE-123 UPTAKE IN CANNABINOID-TREATED RAW264 CELLS

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The majority of cancer deaths can be attributed either to the cancer's intrinsic resistance to chemotherapeutic agents or to the development of resistance following a period of initial sensitivity. Experiments on cultured cells have shown that drug resistance often correlates with the presence of one or more of the P-glycoproteins or multidrug resistance (MDR) proteins (Stein, 1997). Both of these classes of protein are membrane-bound transporters that actively export a wide range of compounds. The substrates for these molecules inlcude many chemotherapeutic agents e.g. colchicine and daunomycin.

A number of compounds have been shown to inhibit the action of the MDR proteins and P-glycoproteins *in vitro*, reversing drug resistance (Tsuruo *et al.*, 1981). The most notable of these is verapamil, which has undergone clinical trials in combination therapy. These probably proved unsuccessful because the concentrations of verapamil that were attainable *in vivo* were far lower than those shown to reverse drug resistance *in vitro* (Milroy, 1993).

In the course of our studies on the ability of cannabinoids to induce apoptosis in RAW264 cells, a murine monocyte/macrophage cell line, we have found that treatment with a number of cannabinoid receptor ligands leads to an increase in the cellular uptake of the fluorescent dye rhodamine-123. This dye can be used to measure mitochondrial membrane potential, which is known to decrease in the early stages of apoptosis. The dye is also known to be a substrate of P-glycoproteins and, as such, its uptake has been used as a measure of P-glycoprotein expression and also drug resistance. Uptake of the dye has been shown to be increased by reversers of multidrug resistance (Altenberg *et al.*, 1994).

RAW264 cells (passages 3-16), maintained in DMEM + 10% FCS, were cultured in 24-well plates (Falcon), $2x10^6$ cells/ml and treated with a range of cannabinoids (0-30μM), for 2 hours in the presence of 2μg/ml rhodamine-123. Following treatment, the cells were washed repeatedly in HBSS and retained dye was extracted from the cells by the addition of acetic acid/ethanol/water(1:49:50). Uptake of the dye was measured on a fluorescent plate reader (excitation/emission filters, 485/530nM). The compounds studied included Δ^9-tetrahydrocannabinol (THC), CP 55940, methanandamide, palmitoylethanolamide and oleamide.

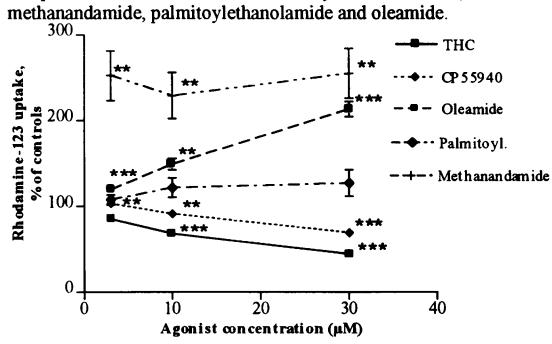

*Fig. 1:Rhodamine uptake in RAW264 cell treated with cannabinoids, expressed as % of untreated control values (mean ± SEM for 5 determinations). Results were compared statistically to untreated controls using Mann Whitney U tests with Bonferroni's correction (**P<0.05, ***P<0.01)*

The significant increases in rhodamine uptake observed in both the methanandamide and oleamide treated cells suggests that these compounds, which are either endognous or analogues of endogenous compounds, may act as P-glycoprotein inhibitors and as such may prove to reverse multidrug resistance. Using this assay 10μM verapamil was found to enhance rhodamine uptake by 216%±13.4 compared to control. The decreases observed in THC and CP 55940 treated cells are likely due to the induction of apoptosis that we have observed to occur by other means.

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96P EFFECT OF PROPRANOLOL ON OVALBUMIN-INDUCED CONTRACTION OF LUNG FROM SENSITISED GUINEA-PIGS

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It is well established that β-adrenoceptor antagonists such as propranolol can cause an exacerbation of asthma which in some cases may prove fatal (Graft *et al.* 1992). There have been many explanations offered for this phenomenon and we now propose the adverse effect is due to inverse agonism at constitutively activated β_2-adrenoceptors on lung mast cells, a hypothesis supported by the finding that propranolol enhanced histamine and interleukin-5 generation from anti-IgE challenged human lung fragments (Remy *et al.* 1996).

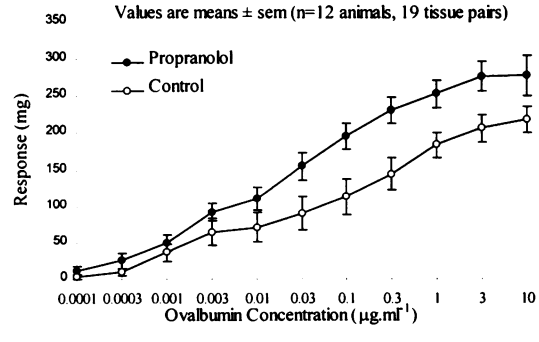
Vanda *et al.* (1992) have shown that propranolol can induce hyperreactivity to ovalbumin in parenchymal strips from sensitised guinea pigs. This study has been repeated using a paired design that involved cutting a single parenchymal strip in half. One half was used as control, the other received treatment. Each tissue was set up in a 10 ml organ bath which contained Krebs solution plus indomethacin at 32 μM, gassed with 5% CO_2 in O_2 and maintained at 37°C. After 45 minutes incubation with propranolol (1 μM) or vehicle and using isometric measurement of the contractile response, a cumulative dose response curve to ovalbumin was constructed.

The dose response curves obtained (figure 1) were similar to those reported by Vanda *et al.* (1992). Using an analysis of variance based approach the ovalbumin response was found to be significantly greater ($p<0.01$) in the propranolol treated tissue at the following ovalbumin concentrations:-
0.03 $\mu g.ml^{-1}$, 0.1 $\mu g.ml^{-1}$, 0.3 $\mu g.ml^{-1}$ and 1.0 $\mu g.ml^{-1}$.

In 10 experiments propranolol showed a marked increase in ovalbumin response (>40% at 3 of the above concentrations). The mean control curve from these experiments was right shifted by over a log unit when compared to the mean control curve of the remaining 9 experiments where propranolol had little/no effect on response (<25% increase at 3 of the above concentrations). Such a finding is not inconsistent with our hypothesis. Observation of the effect of an inverse agonist at the β_2-adrenoceptor would necessitate constitutive activation of these receptors. If spontaneously activated β_2-adrenoceptors did exist on mast cells then their presence may well be manifested as a reduced response to an allergic stimulus leading to a right shifted control curve to allergen.

The present study reproduces previous findings that propranolol can potentiate an allergic response in lung tissue and goes further in demonstrating that the propranolol effect may be linked to the magnitude of the control response to allergen. These results suggest that propranolol may cause bronchoconstriction because it is an inverse agonist at β_2-adrenoceptors on lung mast cells. The most convincing evidence for this hypothesis would be the blockage of the propranolol effect with a neutral antagonist.

Figure 1: Effect of propranolol on ovalbumin response in parenchyma from sensitised male albino Dunkin-Hartley guinea pigs (650-1100 g). Values are means ± sem (n=12 animals, 19 tissue pairs)



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97P A NEW *IN VITRO* MODEL TO STUDY THE RESPONSE TO ALLERGEN IN DOG NASAL EPITHELIUM

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There are few *in vitro* models in which the response to allergen of the nasal epithelium may be studied. A new *in vitro* model is described using dog nasal epithelium mounted in Ussing chambers in which the effects of indomethacin on an allergic response have been investigated.

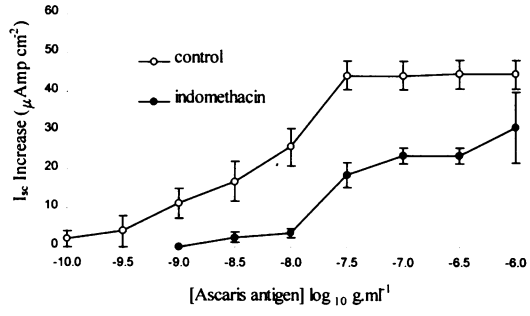
Three male beagle dogs (10-15 kg) were orally infected with *Ascaris suum* eggs in the larvae stage (1000-2000 per kg of body weight). The dogs were then sacrificed and the nasal turbinates removed. After dissection, thin sheets of epithelium were mounted in small reservoir Ussing chambers (4 mm diameter, holding 0.5 ml volume). Both sides of the tissue were bathed in 10 mls Krebs solution whilst being maintained at 37°C and continually gassed with 5% CO_2 in O_2. One dog could provide up to sixteen pieces of nasal epithelium but the tissue was delicate and susceptible to deterioration over time.

The Ussing chamber technique was used to measure short circuit current (I_{sc}) in the dog nasal epithelium. The contribution of Na^+ absorption to I_{sc} was removed by adding 100μM amiloride, leaving Cl^- secretion as the dominant active transport pathway. After the control and treated tissues were incubated with vehicle and 1μM indomethacin, respectively, for 30 minutes, cumulative concentration-effect(E/[A]) curves to ascaris antigen were constructed in 0.5 log unit increments, the responses being expressed as increase in I_{sc} above baseline. All drugs were added simultaneously to both apical and basolateral sides of the tissues. Upon reaching a maximum response to ascaris antigen a high concentration, 30μM, of ATP, a standard activator of Cl^- secretion, was added to establish tissue viability.

Although the response to ascaris antigen varied widely, even within a single dog, a definite concentration-dependent increase in I_{sc} was elicited. The antigen response was reduced by 1 μM indomethacin (figure 1). A similar effect of indomethacin has been reported in dog tracheal epithelium (Lazarus *et al.* 1986) which suggests that cyclo-oxygenase products play a significant role in the changes in ion movement that occur following an antigen challenge in the nose as well as the lung of the dog.

This *in vitro* model may be of value in investigating the allergic response in dog nasal epithelium.

Figure 1: Effect of indomethacin (1μM) on ascaris antigen response in dog nasal epithelium. Values are means ± sem (n=3 animals, 12 tissues for control, 5 tissues for treated)



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98P COMPARISON OF NSAIDs AND NO-NSAIDs ON PGE_2 AND GMCSF PRODUCTION BY HUMAN SYNOVIOCYTES

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The therapeutic use of non-steroidal anti-inflammatory drugs (NSAIDs) is limited by their gastric toxicity. In response, NO-NSAIDs have been developed by the coupling of a nitrobutylester moiety to standard NSAIDs. Unlike their parent compounds, these modified NSAIDs have little gastric toxicity (Wallace *et al*, 1994). As for standard NSAIDs, NO-NSAIDS inhibit activity of both cyclo-oxygenase (COX) -1 and COX-2 (Wallace & Cirino 1994). In cytokine-treated synoviocytes inhibition of COX-2 leads to an increase in granulocyte-macrophage colony-stimulating factor (GMCSF) production due to a reduction in PGE_2 formation (Breese et al, 1999). This may contribute to the inflammatory process, for GMCSF appears increased in synovial fluid. Therefore we have compared the effects of two conventional NSAIDs and their NO derivatives on cytokine-induced PGE_2 and GMCSF production.

Synovium was obtained from patients undergoing routine surgery. Explants of synoviocytes were cultured in DMEM fortified with 2mM glutamine and 20% fetal calf serum, and incubated at 37°C and 5% CO_2. Explanted synoviocytes were identified by morphology and cultured to confluency. Cells were treated with a cytokine mixture of tumour necrosis factor-α, interleukin-1β and interleukin-6 (all 10ng/ml) in the presence of naproxen, aspirin, their respective NO-derivatives (10^{-6}-10^{-3}M) or vehicle (DMSO) for 24hr.

PGE_2 production was measured by radioimmunoassay and GMSCF was determined by specific sandwich ELISA.

DRUG (n=4-5)	IC_{50} PGE_2 (μM)	EC_{50} GMCSF (μM)
Aspirin	2.8	78
NO-Aspirin	36**	>1000
Naproxen	3.7	21
NO-Naproxen	11.5 ***	63

Table 1. *Effects of NSAIDs and NO-NSAIDs on PGE_2 and GMCSF production by cytokine-treated human synoviocytes.* (* P<0.05: unpaired t-test between NSAIDs and NO-NSAIDs).

With the following rank order of potency, aspirin > naproxen > NO-naproxen > NO-aspirin inhibited PGE_2 production by cytokine stimulated synoviocytes (table 1). In the same experiments, naproxen, NO-naproxen and aspirin increased GMCSF production (table 1). By contrast, NO-aspirin, even at a concentration where PGE_2 was completely blocked (1mM), did not increase GMCSF production. In conclusion, NO-substituted NSAIDs inhibit COX activity in human synoviocytes, an effect that is associated with increase in GMCSF production by NO-naproxen but not NO-aspirin. These observations may have implications for the therapeutic potential of nitrobutylester modified NSAIDs.

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99P EFFECTS OF THE SELECTIVE CYCLOOXYGENASE-2 INHIBITOR NS398 ON PROSTANOID RELEASE FROM THE STOMACH AND OTHER RAT ORGANS - COMPARISON WITH NON-SELECTIVE COX-INHIBITORS

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Selective inhibitors of the cyclooxygenase 2 (COX-2) have been shown to be potent anti-inflammatory drugs that spare the gastrointestinal tract and the kidney. This is based on the assumption that the isoform responsible for normal organ function is COX-1, whereas COX-2 is solely responsible for inflammatory processes. Recently, this view has been challenged, because (i) COX-2 selective agents produced less anti-inflammatory effects in different animal models as compared to non-selective COX-inhibitors and (ii) COX-2 selective agents inhibited gastric ulcer healing in rats. To further characterize organ specific effects of non-steroidal anti-inflammatory drugs (NSAIDs) we have investigated the eicosanoid release from different rat organs ex vivo after oral administration of COX-2 selective (NS398), COX-2 preferential (diclofenac, meloxicam) and non-selective COX-inhibitors (ketorolac).

Prostanoid and leukotriene release from tissue fragments of the stomach, kidney, lung and brain were determined after incubation of tissue fragments ex vivo in Tyrode solution for 10 min at 37°C.

Ketorolac (0.1, 0.3 and 0.9 mg/kg) inhibited prostanoid release from all organs most potently and led to a significant increase of leukotriene release from the lung at the highest dose (0.9mg/kg) tested.

The effects of diclofenac and meloxicam (1, 3 and 9 mg/kg each) were very similar for all organs tested. At 9 mg/kg prostanoid release from gastric mucosa was inhibited by 79.1±11.4% and 87.6±7.7% by diclofenac and meloxicam, respectively. NS398 (1, 3 and 9 mg/kg) did not significantly reduce prostanoid release from brain, lung and kidney as compared to vehicle. However, at 9mg/kg NS398 prostanoid release from gastric mucosa was inhibited by 86.9±12.7% ($p<0.001$). At this dose NS398 has been previously shown to be COX-2 selective.

COX-2 seems to contribute to prostanoid production in the rat stomach and may play an important role in cytoprotective mechanisms.

100P ABSENCE OF HISTAMINE H_3 RECEPTORS IN RAT ILEUM AND HUMAN COLON

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The histamine H_3 receptor was first reported in the rat cerebral cortex, where it was found to regulate histamine synthesis and release (Arrang *et al.*, 1983). Since then, various studies have shown the receptor to modulate the release of several neurotransmitters in many systems.

The aim of this study was to investigate the potential influence of the H_3 receptor on neuronal activity in the rat ileum and human colon and compare the findings with those reported in the guinea-pig ileum, a tissue often used for investigating putative agonists and antagonists at H_3 receptors (Leurs *et al.*, 1991).

Ileal segments obtained from Hooded Wistar rats of either sex were set up according to the method of Coupar & Liu (1996) in order to measure both longitudinal and circular muscle responses. Tissues were stimulated in one of two ways: (i) using 8 s trains of 1 ms duration at 20 Hz, 3 min apart at a supramaximal voltage (Coupar & Liu, 1996); (ii) application of single pulses, 0.1 ms pulse duration at 5-10 V every minute (Menkveld & Timmerman, 1990), which resulted in twitch contractile responses only in the longitudinal muscle.

Specimens of human colon which were free from any macroscopically visible lesions were obtained from the operating theatre as soon as possible after surgical removal for cancer of the colon. These were set up and stimulated in similar method to that reported by Bennett & Stockley (1975)

All experiments were performed in Krebs-Henseleit buffer in the presence of mepyramine (1 μM), to inhibit any H_1 receptor activation by histamine or its more potent analogue (R)α-methylhistamine. The agonists were added non-cumulatively for 1-2 min. Tissues were initially incubated with antagonists for 30 min before retesting agonists.

Cholinergic nerve stimulation of either the longitudinal or circular muscle of the rat ileum was not significantly affected ($P > 0.05$) by either histamine (0.1 – 3 μM; 96±7 (mean±sem) and 81± 8% of control, respectively) or (R)α-methylhistamine (0.1 – 3 μM; 99±6 and 92±5% of control, respectively; n=3-4). Cholinergic nerve stimulation of the human colon was similarly unaffected by histamine or (R)α-methylhistamine (80-90±9% of control responses; n=3). The contractile responses to substance P (1 μM) in the rat ileal longitudinal and circular muscle was also unaffected ($P > 0.05$) by histamine (10 μM; 92±7 and 99±14% of control respectively) or (R)α-methylhistamine (10 μM; 104±22 and 107±23% of control respectively; n=3). Similar findings were seen in the human colon. The contractile responses to nicotine (0.1 μM) were also unaffected in either tissue by histamine or its analogue ($P > 0.05$).

These findings suggest that H_3 receptors have no role in the modulation of neuronal function in the rat or human intestine unlike in the guinea-pig.

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101P TIME COURSE OF TNBS-INDUCED COLITIS IN THE MOUSE

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Inflammatory bowel disease (IBD) is a chronic condition characterised by recurrent intestinal tissue injury mediated by the host's immune response. One animal model of IBD involves administration of an enema containing the hapten, trinitrobenzene sulphonic acid (TNBS) (e.g. Elson *et al.*, 1996). Whilst several studies have identified mRNA for different pro-inflammatory mediators (e.g. Neurath *et al.*, 1995), few of these have measured mediator production. In the present study, we have developed an *ex vivo* assay to measure mediators released from mouse colon. We have also examined the time course of mediator release from mouse colon *ex vivo* at different time points following administration of a TNBS enema. Female BALB/c mice (20-25g) were anaesthetised with hypnorm (10%) i.p. and an enema (75µl) containing either saline, ethanol (50%) or ethanol (50%) + TNBS (50mgkg^{-1}) was administered. At subsequent time points (2,4,6 & 12 days), mice were killed by cervical dislocation and their colons removed. The colons were washed and cut in half longitudinally. One half was incubated in Krebs' at 37°C for 15min + calcium ionophore (A23187, 3µM), the other half was incubated with vehicle control (DMSO). The Krebs' solution was then stored at -70°C prior to the assay. ELISA assays were performed to measure LTB_4, PGE_2, IL-1β and TNF-α released from colon. A significant increase in IL-1β and LTB_4 release from TNBS vs. ethanol dosed mice was observed 2 days post TNBS dosing (Table 1). These increases were not seen at later time points. No differences in TNF-α or PGE_2 were seen between mice treated with ethanol and TNBS at any time point (data not shown), however TNF-α levels increased in all groups of animals at day 6 and 12 compared with days 2 and 4. In addition, A23187 was unable to stimulate the production of any mediator. Since A23187 was unable to stimulate mediator production, data from vehicle and A23187-stimulated tissues was combined (Table 1).

Table 1. IL-1β and LTB_4 released from mouse colon (pg/mg colon)

Days post administration of an enema of saline, ethanol or TNBS

	2	4	6	12
IL-1β Saline	0.11 ± 0.04	0.03 ± 0.02	0.35 ± 0.15	0.20 ± 0.05
IL-1β Ethanol	0.19 ± 0.05	0.10 ± 0.04	0.26 ± 0.23	0.29 ± 0.10
IL-1β TNBS	1.34 ± 0.48*	0.03 ± 0.02	0.23 ± 0.23	0.09 ± 0.09
LTB_4 Saline	2.56 ± 1.38	0.25 ± 0.08	1.01 ± 0.37	1.01 ± 0.16
LTB_4 Ethanol	1.07 ± 0.39	0.32 ± 0.04	2.38 ± 0.56	0.43 ± 0.20
LTB_4 TNBS	5.08 ± 1.91*	0.23 ±0.07	0.88± 0.33	0.44 ±0.08

Values are expressed as mean ± s.e.m (n=9-10 per group); *P<0.05 vs. ethanol (unpaired t test) for each mediator

In conclusion we have developed an *ex vivo* assay to measure mediator levels released from mouse colon. We have also established that a single enema of TNBS produces an acute inflammatory response characterised by increased production of Il-1β and LTB_4 2 days post dosing. However, this inflammatory response does not appear to be sustained at later time points.

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102P RESPONSES TO Y RECEPTOR AGONISTS ARE INSENSITIVE TO HALOPERIDOL PRETREATMENT IN HUMAN AND MOUSE COLONIC MUCOSAE *IN VITRO*

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Peptide YY (PYY) and neuropeptide Y (NPY) are potent antisecretory peptides in the mammalian gastrointestinal (GI) tract which activate Y receptors in mucosal preparations from various mammalian species (Cox, 1998). In addition to these established Y-selective effects, there is some evidence for the involvement of proposed σ sites in NPY and PYY responses, specifically in mouse isolated jejunum (Rivière *et al.*, 1993) and PYY inhibition of prostaglandin-induced intestinal secretion in human volunteers (Rozé *et al.*, 1997). Here we examine whether haloperidol, a drug with affinity for σ sites, has any effect upon antisecretory responses to either PYY, human pancreatic polypeptide (hPP) dopamine (DA) or the α_2-agonist, UK 14,304 in mouse and human colon mucosae *in vitro*.

Human colonic tissue was obtained from the margins of specimens removed during surgical resection for colonic cancer (with the approval of St Thomas' Hospital Research Ethical Committee). Muscle layers were removed from human and mouse (male or female 129 SV) descending colon by dissection and resulting mucosal preparations (0.6 cm^2 and 0.2 cm^2 areas, respectively) were voltage-clamped at 0 mV (Cox *et al.*, 1988). Changes in short-circuit current (I_{sc}) were recorded in response to basolateral addition of PYY (either 5 nM in murine or 30 nM in human tissue, both are near their respective EC_{50} values), hPP (30 nM, to human tissue only) or DA (1 µM) followed by UK 14,304 (1 µM), in control (0.1% ethanol) and haloperidol (1 µM) pretreated mucosae. All mouse colon preparations were pre-stimulated with vasoactive intestinal polypeptide (VIP, 30 nM). The means ± 1 s.e.m. from each data group were compared using an unpaired Student's *t*-test.

VIP stimulated prolonged elevations in I_{sc} in mouse colon (98.0 ± 6.0 µA.cm^{-2}, n=18) while basal I_{sc} in human tissue was 79.7 ± 6.4 µA.cm^{-2} (n=34). Haloperidol reduced human colon basal I_{sc} (-10.1 ± 1.5 µA.cm^{-2}, n=17) but this was not significantly different from vehicle controls (-6.5 ± 1.3 µA.cm^{-2}, n=17, P = 0.1) while in mouse colon, responses were -16.8 ± 3.8 µA.cm^{-2} (n=10) and -5.9 ± 1.2 µA.cm^{-2} (n=8, $P < 0.05$) respectively. Subsequent responses to DA were attenuated by haloperidol in each tissue (P = 0.19 in human colon, -6.3 ± 2.1 to -2.7 ± 1.1 µA.cm^{-2}, n=4; and P = 0.01 in mouse colon, -6.3 ± 0.5 to -1.3 ± 1.3 µA.cm^{-2}, n=4). PYY responses were unchanged in human tissue (controls, -16.5 ± 5.3 µA.cm^{-2}, n=6; with haloperidol, -22.0 ± 6.0 µA.cm^{-2}, n=6, P = 0.5) and mouse colon (controls, -9.4 ± 1.2 µA.cm^{-2}, n=4; plus haloperidol, -11.7 ± 2.9 µA.cm^{-2}, n=6, P = 0.6). Antisecretory responses to hPP in human colon were also not altered by haloperidol (-8.7 ± 1.8 µA.cm^{-2}, n=6) compared with controls (-8.7 ± 2.5 µA.cm^{-2}, n=6, P = 1.0). Additionally, UK 14,304 responses following each of the agonists above were unchanged by pretreatment with the neuroleptic ($P > 0.4$ throughout).

We conclude that the I_{sc} responses to PYY, hPP and UK 14,304 in human colon and, PYY and UK 14,304 responses in mouse colon are not sensitive to haloperidol at a concentration that does inhibit DA responses in both tissues (significantly in mouse colon). Thus *in vitro* mucosal responses to Y agonists and the α_2-agonist, UK 14,304 do not involve either DA or endogenous activator(s) of σ sites.

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103P CANNABINOID CB_1 RECEPTORS MEDIATE INHIBITION OF NEURONALLY-EVOKED CONTRACTIONS OF ISOLATED URINARY BLADDER FROM THE RAT AND MOUSE, BUT NOT HUMAN, PIG, DOG OR MONKEY

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Several cannabinoid agonists concentration-dependently inhibit electrically-evoked contractions in mouse bladder *via* a pre-junctional mechanism (Pertwee & Fernando, 1996). To date, no evidence has been reported to confirm a role of cannabinoid receptors in the urinary bladder of other mammals.

This study investigated the effects of standard cannabinoid ligands (Pertwee & Fernando, 1996) and JWH 015 (1-propyl-2-methyl-3-(1-naphthoyl)indole) and SR 144528 (N-[(1S)-endo-1,3,3-trimethyl bicyclo [2,2,1] heptan-2-yl]-5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)-pyrazole-3-carboxamide) on electrically-evoked contractions in mouse and rat bladders for the purpose of receptor classification. In addition, the effects of WIN 55212-2 on frequency-response curves in mouse, rat, dog, pig, primate and human bladder strips were investigated to determine whether cannabinoid receptors inhibit electrically-evoked bladder contractions in these species.

Using established methods for construction of concentration - effect curves (Pertwee & Fernando, 1996), bladder strips were stimulated for 2 min following 30 min incubation with ligand(s) (0.5msec pulses, 0.5sec trains, 0.1 trains per sec, 12 or 8 Hz, 8 or 7 volts for mouse and rat tissues respectively). In other experiments, frequency-response curves were generated after 1hr incubation with WIN 55212-2 (3μM) or vehicle by stimulation for 0.5sec once every min (0.5msec pulses, 1–128 Hz).

The agonist and antagonist potencies in Tables 1 and 2 are consistent with CB_1 receptor interaction in the mouse bladder. Furthermore they identify a cannabinoid receptor in rat bladder which is pharmacologically similar to the mouse CB_1 receptor except in its affinity for the CB_2-selective antagonist SR 144528. This may result from species differences in receptor pharmacology, or heterogeneity in the receptor population.

In contrast to its effect in the bladder of rodents, WIN 55212-2 (3μM) does not affect electrically-evoked contractions in dog, pig, monkey or human bladder tissues (n = 4 - 6). These data suggest that CB_1 receptors do not modulate neuronally-mediated responses in bladders isolated from these animals.

Table 1 Agonist potencies (pEC_{50}) and intrinsic activities (α, % inhibition of twitch height) for inhibition of electrically-evoked bladder contraction. Mean ± s.e.mean of 4-6 replicates.

agonist	*mouse bladder*		*rat bladder*	
	pEC_{50}	α	pEC_{50}	α
CP 55,940	7.65 ± 0.1	79.3 ± 3.4	7.69 ± 0.2	32.3 ± 10.5
WIN 55212	7.67 ± 0.2	78.7 ± 3.8	7.48 ± 0.3	41.5 ± 9.2
HU 210	7.83 ± 0.1	54.7 ± 9.7	-	9.3 ± 0.7
JWH 015	6.72 ± 0.1	80.5 ± 3.3	6.76 ± 0.2	26.8 ± 4.5
Anandamide	6.05 ± 0.2	66.0 ± 8.6	-	5.3 ± 1.1

Table 2 Antagonist affinities (pK_B) in the mouse and rat bladders determined from competition with WIN 55212-2. Mean ± s.e.mean of 4-6 replicates

antagonist	*mouse bladder*	*rat bladder*
SR 141716A	8.66 ± 0.13	8.42 ± 0.41
SR 144528	< 6.5	8.02 ± 0.41
AM 630	6.16 ± 0.08	6.08 ± 0.33

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104P THE ROLE OF M2-MUSCARINIC RECEPTORS IN CONTRACTION OF THE PIG URINARY BLADDER

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In urinary bladder, the density of M_2-muscarnic receptors is greater than that of the M_3-subtype, the M_2:M_3 ratio being about 9:1 and 3:1 in the rat and human bladder respectively (Wang et al., 1995). However, several studies have identified the M_3-receptor as the sole muscarinic receptor subtype responsible for contraction of the bladder to muscarinic agonists *in vitro*. Recently, an M_2-mediated contraction (re-contraction) to muscarinic stimulation has been demonstrated in the rat bladder following M_3-receptor inactivation and elevation of cAMP levels (Hedge et al., 1997). This study examines whether a similar role for M_2-receptors can be demonstrated in the dome of the pig bladder.

In radioligand binding studies, competition experiments with [^{3}H]QNB were used to determine the ratio of M_2:M_3 receptors in the pig bladder (Goepel et al., 1998). In functional experiments, pig detrusor strips (dome region) were set up in gassed Krebs solution at 37°C and concentration-response curves (CRCs) obtained to carbachol in the absence and presence of the antagonists 4-DAMP (M_3-selective, 3-30nM) and methoctramine (M_2-selective, 0.3-3μM). Similar experiments were performed on tissues following selective M_3-inactivation (incubation of tissues with 40μM 4-DAMP mustard for 60min in the presence of 1μM methoctramine to "protect" M_2-receptors), precontraction with 50mM KCl and relaxation with isoprenaline (30μM). Parallel control experiments were used to correct for time-dependent changes in tissue sensitivity. Antagonist affinity (pK_B values) and Schild plots were constructed from shifts of CRCs to carbachol.

In competition binding, displacement of [^{3}H]QNB by 4-DAMP best fitted a 2-site model (Hill slope=0.44±0.11), the ratio of high affinty (M_3, pK_i=9.7) and low affinity (M_2, pK_i=7.4) sites suggesting a predominant (60%) population of M_2-receptors (n=6).

On normal detrusor muscle strips *in vitro*, 4-DAMP and methoctramine caused parallel rightward shifts of CRCs without affecting maximum responses and yielded mean (±sem) pK_B values of 9.4±0.1 (n=12) and 5.9±0.1 (n=18) respectively. Schild slopes were 0.94±0.12 for 4-DAMP and 0.89±0.15 for methoctramine.

In tissues where the M_3-receptors had been inactivated and cAMP levels elevated, 4-DAMP was less potent, the pK_B value being 8.7±0.1 (n=27), significantly lower ($P<0.05$) than in the normal tissues. In contrast, methoctramine was more potent after M_3-inactivation, the apparent pK_B value increasing significantly ($P<0.01$) to 7.0±0.1 (n=18). Maximum responses were reduced by all concentrations of methoctramine ($P<0.05$) but the Schild slopes were 1.17±0.09 for 4-DAMP and 0.91±0.20 for methoctramine.

These *in vitro* data suggest that in the pig bladder, where the muscarinic receptor population appears to be similar to that of human, the M_3-receptor subtype mediates contraction of the normal detrusor muscle. However an involvement of M_2-receptors in contraction may be observed following pharmacological manipulation of the receptor population.

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105P FP-RECEPTOR HETEROGENEITY IN THE ISOLATED MYOMETRIUM FROM PREGNANT AND NON-PREGNANT DONORS

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Studies from this laboratory have demonstrated the presence of TP-receptors on the human pregnant myometrium (P) and the human umbilical artery (HUA) (Abbas *et al.*, 1997; Senior *et al.*, 1993). The aim of this study was to investigate further the TP-receptor population on the human P myometrium and HUA by comparing the effects of the TP mimetics I-BOP and U-46619 (Coleman, 1991).

Samples of human myometrium were obtained from P patients undergoing Caesarean section (patients at term but not in labour). Samples of umbilical cord were obtained from full term pregnancies. All tissue was obtained from consenting donors. Strips of the myometrium were set up for superfusion (2ml min^{-1}) with oxygenated (95% O_2/5% CO_2) Krebs solution containing 2.79μM indomethacin as previously described by Senior *et al.*, 1991. After equilibration of the tissue, agonists were injected directly into the superfusate as bolus doses. When used the TP-receptor antagonist, BAYu3405 (McKenniff *et al.*, 1991), was included in the superfusate (10^{-7}M) for 30 minutes prior to agonist dosing. As the profile of the spontaneous activity changed throughout the course of the experiments, comparisons were made between preparations in a non-paired manner. Variations in myogenic activity have been normalised and taken into account (Senior *et al.*, 1991). Briefly, the responses to agonist (T) were expressed relative to the intrinsic spontaneous background activity (B) as a T/B ratio. Excitatory potencies were given as ED_1 values (T/B ratio equal to 1) which were expressed as geometric means (nmol), n=5 in all cases. Rings of umbilical artery were suspended in Krebs solution containing indomethacin (2.79μM) in a 10ml organ bath and oxygenated with 2.5% O_2/ 8% CO_2/ balance N_2 as described previously (Amin *et al.*, 1995). Dose-response curves were constructed in a cummulative manner. The data was analysed by one-way ANOVA with Dunnett's t-test.

In P myometrium U-46619 and I-BOP both elicited bell-shaped dose response curves, with a decline in the responses occurring after 10nmol and 1nmol, respectively. I-BOP was shown to be significantly more potent ($P<0.001$) in the P human myometrium than U-46619. The TP-antagonist BAYu3405 (10^{-7}M) was shown to elicit a rightward shift in the dose response to U-46619 with the bell-shape effect being abolished. I-BOP was not antagonised by BAYu3405 in the P human myometrium. I-BOP and U-46619 both elicited potent constrictor effects in the HUA. BAYu3405 (10^{-7}M) elicited a rightward shift in the dose response to U-46619. In contrast I-BOP was unaffected by BAYu3405.

Table 1. ED_1 (nmol) and EC_{50} (M) values for I-BOP and U-46619 calculated in the P myometrium and HUA respectively.

	ED_1 (95% C.L.)	EC_{50} (95% C.L.)
U-46619	0.23(0.1-0.6)	8×10^{-8}(8.0×10^{-9}-2×10^{-7})
U-46619 + BAYu3405	1.5(1.0-3.0)	9×10^{-7}(8.5×10^{-8}-8×10^{-6})
I-BOP	0.005(0.001-0.01)	5×10^{-8}(2.5×10^{-8}-3×10^{-7})
I-BOP + BAYu3405	0.004(0.002-0.009)	5×10^{-8}(2.8×10^{-8}-3.2×10^{-7})

The inability of BAYu3405 to antagonise the effects of I-BOP may implicate a heterogeneous population of TP-receptors in P myometrium and HUA.

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106P THE EFFECT OF THE EP_2 AGONIST, BUTAPROST, ON THE CONTRACTILE RESPONSE TO OXYTOCIN ON ISOLATED HUMAN MYOMETRIUM FROM PREGNANT DONORS

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Previous studies in our laboratory have shown the presence of the EP_2 receptor on human myometrium from pregnant donors, which mediates an inhibition of myogenic activity (Senior *et al.*, 1993). Oxytocin receptors have been located on the human myometrium (Fuchs *et al.*, 1982) and oxytocin itself has a putative role as a uterotonin. This preliminary study examines the stimulatory effect of oxytocin in the presence of the EP_2 receptor agonist butaprost (Gardiner, 1986) on isolated human myometrium taken from pregnant donors.

Samples of human myometrium were obtained from pregnant donors (at term but not in labour) during Caesarean section (all patients gave written consent). Myometrial strips were set up for superfusion at 2ml min^{-1} with oxygenated (95% O_2 / 5% CO_2) Krebs containing 1μM indomethacin, as described by Senior *et al.*, (1993). To establish a concentration which inhibits myometrial activity butaprost was infused at a rate of 0.02ml min^{-1} and a concentration effect curve was constructed by infusing different concentrations of butaprost over a 15 minute period and measuring the time, in minutes, for recovery of normal myogenic activity. Oxytocin (10 fmol – 0.1 nmol) was injected directly into the flow of the superfusate as a bolus dose and a dose response curve was constructed. The dose response curve to oxytocin was repeated in the presence of 10^{-6} M butaprost. As the profile of spontaneous activity changed throughout the course of the experiments, comparisons were made between preparations in a non paired manner. Due to variations in myogenic activity, results have been normalised to take into account the level of background activity, thus the results have been expressed as T/B ratios of 'test' (T) agonist responses as a ratio of background (B) contractions. Responses were analysed by one-way ANOVA followed by a *post-hoc* Dunnett's T-test (n=5 in all cases).

Butaprost inhibited myogenic activity and the time toresumption of normal myogenic activity, after removal of butaprost from the superfusate, increased in a dose dependent manner (Table 1).

Table 1:- Time taken for normal spontaneous activity to resume after cessation of infusion of varying concentrations of butaprost

Butaprost conc (M)	10^{-9}	10^{-8}	10^{-7}	10^{-6}	10^{-5}
Recovery time (mins)	0	5	21	75	>120
SEM	0	3.5	9.7	22	30

Oxytocin was a potent contractile agonist eliciting dose related responses (Table 2). In the presence of 10^{-6}M butaprost, the responses to oxytocin were significantly attenuated ($P < 0.001$) up to 50 pmol.

Table 2:- Response of oxytocin alone and in the presence of 10^{-6}M butaprost

Oxytocin dose (pmol)	0.01	0.1	1	10	100
Response (T/B)	0	0	1.52	1.94	2.87
SEM	0	0	0.13	0.04	0.3
Response of 10^{-6}M butaprost (T/B)	0	0	0	0.05	2.48
SEM	0	0	0	0.04	0.59

The results of this study indicate that the selective EP_2 receptor agonist, butaprost, can inhibit myometrial activity. The presence of butaprost in the superfusate caused a significant reduction of the oxytocin response. This effect is currently subject to further investigation.

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107P INSULIN AS A FEEDBACK REGULATOR OF GLUCAGON-LIKE PEPTIDE-1 (GLP-1) RELEASE?

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GLP-1 is an enteroendocrine peptide that accounts for up to 60% of the pancreatic insulin released after an oral glucose load. A wide range of endogenous and exogenous secretagogues cause GLP-1 release from L cells, which are mainly located in the small intestine. One of the most intriguing, but poorly studied potential endocrine feedback regulators of GLP-1 release is insulin. An inhibitory effect of insulin on L cell secretion might explain elevated GLP-1 levels in type 1 and type 2 diabetics, as well as in insulin-deficient rodents (Matsuyama et al., 1975; Orskov et al., 1991; Kreymann et al., 1988).

In the present studies we investigated the existence of insulin receptors on L cells using the permanent GLUTag cell line and a primary culture of fetal rat intestinal cells (FRIC). GLUTag cells, derived from intestinal endocrine tumors arising in the large bowel in proglucagon-simian virus 40 large T antigen transgenic mice, were grown in DMEM (low glucose) containing 10% (vol/vol) FBS in a humidified 10% CO_2 atmosphere. Intestines from a 20- to 21-day-old litter of fetal Wistar rats were dispersed enzymatically with collagenase (4 mg/ml), hyaluronidase (5 mg/ml) and DNase-I (0.5 mg/ml). The cells were allowed to recover in DMEM (high glucose) containing 5% (vol/vol) FBS, 50 IU/ml penicillin and 50 µg/ml streptomycin for 48 h in a humidified 5% CO_2 atmosphere.

By performing immunoprecipitation with an insulin receptor antibody (AB-3, Clone 29B4) the presence of insulin receptors on the cell surface of GLUTag cells was demonstrated (n=4). Positive labeling of the cytoplasm of all formalin-fixed GLUTag cells was observed by immuno-fluorescence microscopy using an insulin receptor substrate-1 (IRS-1) antibody (1:25 in 0.3% Triton X-100/PBS, pH 7.4); Fluorescence-intensity was equally distributed throughout the preparation, suggesting equal levels of IRS-1 expression (n=6). On a molecular level expression of insulin receptor mRNA was shown by means of RT-PCR using primers that correspond to nt1996-2017 and nt2259-2278 of mouse insulin receptor beta subunit cDNA (n=4). Preliminary experiments with FRIC cultures were performed employing double-labeling immuno-fluorescence microscopy with a GLP-1 (7-37) antiserum (1:100 in 0.3% Triton X-100/PBS, pH 7.4) and an IRS-1 antibody (1:25 in 0.3% Triton X-100/PBS, pH 7.4). These studies indicated the presence of insulin receptors on GLP-1 positive intestinal L cells (n=4). It also became evident that L cells comprise less than 1% of the total cell population.

In conclusion, the present studies demonstrate the existence of insulin receptors on L cells using GLUTag cells and FRIC cultures as models. However, the functional correlation between insulin receptors and GLP-1 inhibition in GLUTag cells and FRIC cultures still has to be investigated.

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GLUTag cells were obtained from D. Drucker, 1149336 Ontario Inc./ University of Toronto

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108P A COMPARISON ON THE CONCENTRATIONS OF CERTAIN PESTICIDES AND POLYCHLORINATED HYDROCARBONS IN BONE MARROW AND FAT TISSUE

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Morgan et al. compared the total tissue concentration of dichlorodiphenyl-trichloroethane (DDT), dichlorodiphenyldichloro-ethylene (DDE), and dieldrin with the lipid contents of five tissues (blood, kidney, liver, brain and fat tissue) in order to study the enrichment of pesticides with increasing lipid content. The close correlation between DDT and DDE concentrations and the tissue fat content (in the loaritmic plot) sowed an evident proportionality of both substances. On the other hand, whole body scintigraphy of incorporated ^{14}C hexachlorobiphenyl showed a strong enrichment of the higher chlorinated component in bone marrow of mice (Brandt,1977). This observation is of importance because there are PCB-induced lesions in bone marrow and peripheral blood components. An even higher concentration of PCB was observed in the bone marrow of primates.

Chlorinated hydrocarbon (CHC) and polychlorinated biphenyl (PCB) concentrations in the bone marrow of 29 adults were determined, referred to lipid content to allow comparison with literature values for adipose tissue of 20 adults in the Federal Republic of Germany.

Clorinated hydrocarbon (CC) and polychlorinated biphenyl (PCB) concentrations in the bone marrow of 29 adults were determined from samples collected between 1985 and 1995 and compared with values in adipose tissue of 20 adults examined in Germany. Prior informed consent was obtained in all cases. The samples were analyzed for alpa- and beta HCH, HCB, the DDT-metabolites, p,p-DDT and p,p-DDE and the major PCB-coneners as described elsewhere (Niessen,1984; Scheele, 1996; Scheele,1992) referred to as lipid content. These values were compared with the results in fat tissue obtained by Dmochewitz et al. using the same methods.

The concentrations of 5 of the 7 investigated substances were lower in bone marrow than in fat tissue. The concentration of hexachloro-benzene (HCB) was 8-fold, the PCBsum was 6-fold, the concentration of p,p'-dichlorodiphenyl-trichloroethane (p,p'-DDT) was 3- fold lower in bone marrow and the concentration of p,p'-dichlorodiphenyl-dichloroethylene (p,p'-DDE) was 77% of the concentration in fat tissue. While the concentration of beta-hexachlorocyclohexane (beta-HCH) was 41% of adipose tissue, alpha-hexachlorocyclohexane (alpha-HCH) and dieldrin on the other hand were increased 10 and 19-fold, respectively.

All CHC and the PCB-sum except alpha HCH and dieldrin were found to be higher in fat tissue than in the bone marrow, with HCB, PCB-sum, and p,p DDT being eight-fold, six-fold, and three-fold increased respectively. Additional studies on age-related concentrations of CHC and PCB in different tissues are necessary to resolve this aspect.

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109P DIFFERENCES IN TOXICITY OF TWO STRUCTURAL ISOMERS OF DICHLOROPROPANOL CAN BE PARTIALLY EXPLAINED BY DIFFERENTIAL METABOLISM

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Previous *in vivo* and *in vitro* studies have shown that hepatotoxicity of dichloropropanol (DCP) in rats is mediated predominantly by cytochrome P4502E1 (CYP2E1), which is also an important isozyme in humans (Hammond and Fry, 1997). 1,3-Dichloropropan-2-ol (1,3-DCP) is more toxic than its structural isomer 2,3-dichloropropan-1-ol (2,3-DCP) and the CYP2E1-mediated glutathione (GSH) depletion is greater. The aim of this work was to investigate how observed differences in toxicity and GSH depletion are related to differences in reactive metabolite generation.

Microsomes and hepatocytes were prepared from male Wistar rats (180-200g) treated with isoniazid (0.1%w/v in drinking water for 10 days) to induce CYP2E1. Reactive metabolite generation was measured in a microsomal GSH depletion assay (Garle and Fry, 1989) with 30-1000μM DCP. GSH depletion and loss of lactate dehydrogenase (LDH) from cultures after exposure for 2 and 24h respectively, over the same concentration range, were determined in 24h hepatocyte cultures (Hammond and Fry, 1997). As appropriate, cultures were incubated for 30 min with 200μM cyanamide to inhibit aldehyde dehydrogenase, prior to exposure to dichloropropanol (Cederbaum and Dicker, 1981). Values presented are mean ± S.E.M. (n = 6).

In the microsomal assay both isomers depleted GSH in a dose-dependent manner. Maximum depletion and apparent Km values were determined by non-linear regression. Although mean Km values were not statistically different, Table 1, maximum depletion by 2,3-DCP was 1.5-fold greater than by 1,3-DCP. This indicates differences in the reactivity of the metabolites generated from the isomers rather than differences in affinity for CYP2E1.

Table 1. Kinetic parameters of microsomal GSH depletion

isomer	apparent Km μM	maximum depletion nmolGSH/30min/mg
2,3-DCP	135 ± 22	121 ± 7
1,3-DCP	112 ± 25	79 ± 6*

* $p < 0.001$ v 2,3-DCP, unpaired t-test.

In hepatocyte cultures both GSH depletion and loss of LDH were dose-dependent. However in cultures 1,3-DCP had greater effects on GSH depletion and loss of LDH than 2,3-DCP: table 2. Pre-incubation of cultures with cyanamide increased the toxicity of 2,3-DCP but not 1,3-DCP, indicating that the reactive metabolite of 2,3-DCP, but not 1,3-DCP is likely to be an aldehyde, consistent with structure and predicted metabolism.

Table 2. Cellular GSH depletion and loss of LDH

DCP isomer	nmol GSH depleted/2hr/mg at 300μM DCP		concentration in μM that reduces LDH by 50%	
	control	cyanamide	control	cyanamide
2,3-	8.8 ± 2.1	21.8 ± 2.4*	418 ± 33	229 ± 47*
1,3-	20.6 ± 4.3†	24.5 ± 2.8	69 ± 20†	69 ± 17

* $p < 0.05$ cyanamide v control, † $p < 0.05$ 1,3-DCP control v 2,3-DCP control ANOVA/Bonferroni.

Despite the greater reactivity of its metabolite with GSH, 2,3-DCP is less toxic in cells than its structural isomer, and this seems to be due, at least in part, to detoxification of this metabolite.

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Hammond, A.H. and Fry, J.R. (1997) *Toxicology* 118: 171-179.

110P USE OF A GLUTATHIONE DEPLETION ASSAY FOR THE DETECTION OF NEOANTIGEN-FORMING AND REDOX-CYCLING COMPOUNDS

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Measurement of glutathione (GSH) depletion in a liver microsomal incubation system has previously been shown to be a useful screen for detection of reactive metabolites of cytotoxic drugs (e.g.paracetamol), Garle and Fry., 1989. It is also recognised that a number of xenobiotics initiate toxicity through either an immunological mechanism involving neo-antigen formation by the covalent binding of reactive metabolites to tissue protein (procainamide, phenobarbitone), or via redox cycling mechanisms (diquat, nitrofurantoin). This study illustrates the ability of the glutathione depletion assay to identify such toxins, and compares results obtained with microsomal preparations from rat and five human livers.

Liver microsomes were prepared from male Wistar rats (150-170g) killed by cervical dislocation, and surgical material obtained following liver resections in human patients (H1-H5). The depletion of GSH, added to an incubation containing liver microsomes, cofactors and xenobiotic, was measured as described by Garle and Fry., 1989. The initial level of GSH was 200μM. Depletion induced by coloured compounds was measured by the method of Hissin and Hilf., 1976. Protein content was determined by the method of Lowry et al., 1951. The xenobiotics tested were paracetamol (P), procainamide (PR), phenobarbitone (PB), diquat (D), and nitrofurantoin (N).

All compounds elicited a concentration-dependent GSH depletion in incubations with rat liver microsomes. These responses were abolished if cofactors for NADPH generation were omitted. The results obtained with 1mM concentrations are presented in Table 1. A rank order in magnitude of effect similar to that in rat liver microsomes was obtained in incubations employing human liver microsomes. Similar levels of depletion were obtained with the two preparations with the exception of nitrofurantoin, which was higher with human liver microsomes. There was considerable inter-subject variation in the extent of GSH reactivity recorded with human liver microsomes. The results indicate that toxic metabolites that invoke neo-antigen formation and redox cycling may be detected by a liver microsomal GSH depletion assay previously described for detection of reactive metabolites that invoke toxicity through covalent binding. As such, this assay may be useful for detection of toxic compounds during early stages of drug development.

Table 1: GSH depletion in nmol/mg protein/30 minutes for 1mM concentrations of the drug. Results are expressed as the mean ± SEM of 4 experiments. NT (not tested), A (no measurable effect).

Sample	P	PR	PB	D	N
Rat	11.1±2.0	8.8±1.4	5.9±0.4	21.3±1.7	34.2±0.0
H1	29.3±1.6	16.6±3.5	A	15.9±0.6	49.6±0.0
H2	7.8±0.4	7.2±1.4	4.0±1.6	32.4±1.0	67.9±0.7
H3	16.6±1.7	14.6±2.9	A	71.1±0.4	79.8±2.9
H4	25.8±5.9	4.8±1.8	A	64.9±2.2	114.4±3.1
H5	NT	15.2±2.9	A	47.5±1.7	105.0±1.5

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111P EFFECTS OF WORTMANNIN ON ANTIGEN-INDUCED AIRWAY INFLAMMATION AND BRONCHOCONSTRICTOR RESPONSIVENESS IN ACTIVELY SENSITISED BROWN NORWAY RATS

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Phosphatidylinositol 3 (PI3)-kinase is activated in mast cells following cross-linking of the high affinity receptor for IgE, FcεRI (Yano *et al.*, 1993; Marquardt *et al.*, 1996; Bhattacharyya *et al.*, 1998). Mast cell degranulation *in vitro* can be abolished by low concentrations of wortmannin, a potent and selective inhibitor of PI3-kinase (Cardenas *et al.*, 1998). We report here the effect of wortmannin on antigen-induced airway inflammation and bronchoconstrictor responsiveness in actively sensitised Brown Norway (BN) rats.

BN rats weighing 200-300g were used. For sensitisation, ovalbumin (OA, 0.02mgml^{-1}) was mixed with aluminium hydroxide (20mgml^{-1}) and injected (0.5ml per animal s.c.) coincidentally with *Acullulare pertussis adsorbat vaccine* (Acel-P Lederle; 0.2ml per animal i.p., diluted 1:4 with saline 0.9%) on days 1, 15 and 22. On day 29 animals were anaesthetised (Pentothal 70mgkg^{-1} i.p.) and set-up for measurement of airway resistance (R_L) and cardiovascular parameters (Hannon *et al.*, 1995). In separate groups of animals total leukocyte numbers and differential cell counts in bronchoalveolar lavage (BAL) fluid were obtained using an automated cell analysing system (Cobas Helios, Axon Lab, Switzerland). Eosinophil peroxidase (EPO) activity and protein concentrations in BAL fluid were determined using standard photometric assays.

Intratracheal (i.t.) instillation of OA (3-60mgkg^{-1}) induced dose-related bronchoconstrictor responses which peaked at 5min and resolved within 15min. Pretreatment with wortmannin (10 & 100μgkg^{-1}, i.t., 1h prior to OA-challenge) induced a dose-dependent inhibition of the bronchospasm induced by OA, 60mgkg^{-1} i.t. (65 and 87% respectively, $p<0.001$). The ED_{50} was approximately 5μgkg^{-1}.

Aerosol challenge with OA (3.2mgml^{-1} for 60min) led to an inflammatory response in the airways of sensitised BN rats when assessed by changes in the BAL fluid leukocyte numbers, EPO activity and protein concentration measured 48h post challenge. Wortmannin (10 & 100μgkg^{-1}, given i.t. 1h prior to and 24h post OA exposure), produced a dose-dependent reduction in all parameters (ED_{50} 3-5μgkg^{-1} i.t.) (Figure 1).

Bronchoconstrictor responses to adenosine (0.3 & 1mgkg^{-1}, i.v.) elicited 3h post OA-challenge (0.3mgkg^{-1} i.t.), which are mast cell dependent (Hannon, *et al.*, 1999), were markedly and dose-dependently reduced by wortmannin (10 & 100μgkg^{-1}, i.t.) given 1h prior to OA-challenge (71 and 93% respectively, $p<0.001$). Responses to methacholine (3 & 10μgkg^{-1}, i.v.), and 5-HT (3-30μgkg^{-1}, i.v.), were also reduced following wortmannin but to a markedly lesser extent than those to adenosine.

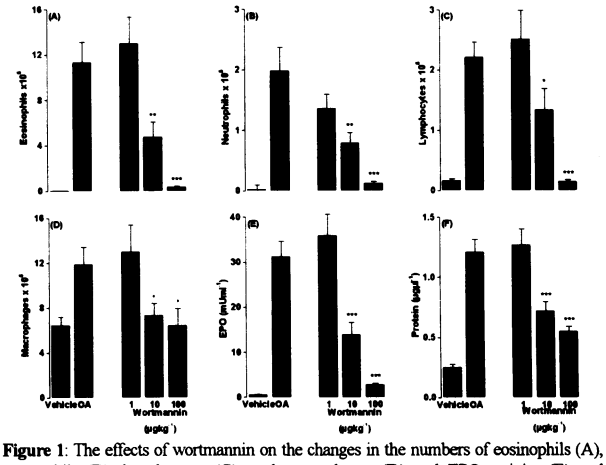

Figure 1: The effects of wortmannin on the changes in the numbers of eosinophils (A), neutrophils (B), lymphocytes (C), and macrophages (D) and EPO activity (E) and protein concentration (F) measured in BAL fluid of sensitised BN rats 48h post OA challenge. *p<0.05, **p<0.01, ***p<0.001 indicates significant difference between animals challenged with OA with and without wortmannin treatment.

The results show wortmannin to be a potent inhibitor of both the immediate-type allergic response and the late-phase pulmonary inflammation induced by OA-challenge in sensitised, BN rats. The data suggest the mast cell as one likely cellular target. Inhibition of PI3-kinase is the presumed mechanistic basis for the observed effects of wortmannin.

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112P TRANSFORMING GROWTH FACTOR β1 STIMULATED IL-8 RELEASE, CYCLOOXYGENASE-2 EXPRESSION AND PROSTAGLANDIN E_2 RELEASE IN HUMAN AIRWAY SMOOTH MUSCLE CELLS

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Recent evidence suggests that TGF-β (especially TGF-β1) may play a key role in tissue inflammation in airway inflammatory disorders such as asthma (Border & Ruoslahti, 1992). We have recently shown that human airway smooth muscle (ASM) may be an important source of IL-8, a potent neutrophil and eosinophil chemoattractant, and that the release of prostanoids may play a role in the generation of IL-8 by inflammatory stimuli (Pang & Knox, 1998). In this study we tested the ability of TGF-β1 to stimulate IL-8 release, COX-2 expression and PGE_2 generation in human ASM cells. Additionally, we explored the role of COX products and COX-2 induction in TGF-β1 induced IL-8 release.

Confluent primary cultures of human ASM cells at passage 3 were studied. Cells were growth arrested for 24 h in serum-free DMEM before use. IL-8 was assayed by ELISA, COX expression was identified by Western blotting, and PGE_2 release was measured by RIA. Incubation of the cells with 10 ng/ml TGF-β1 over a 24 h period stimulated IL-8 release, PGE_2 release, and COX-2 induction in a time-dependent manner; with the maximum IL-8 release at 16 h (133.7 ± 16.9 pg/ml, n=6, $P<0.001$), maximum PGE_2 release at 16 h (201.7 ± 30.4 pg/ml, n=6, $P<0.001$), and maximum COX-2 induction at 4, 8, and 16 h time points. TGF-β1 (0.01–10 ng/ml) also enhanced IL-8 release, PGE_2 release, and COX-2 induction in a concentration- dependent manner. Pre-treatment for 1 h with transcription inhibitor actinomycin-D (1 μM) and steroid dexamethasone (1 μM) prior to incubation with TGF-β1 (10 ng/ml) for 16 h abolished TGF-β1 stimulated IL-8 release (n=6, $P<0.001$). Pre-treatment for 1 h with the non-selective COX inhibitor indomethacin (Indo, 10 μM) and the selective COX-2 inhibitor NS-398 (10 μM) prior to incubation with TGF-β1 (10 ng/ml) for 16 h inhibited TGF-β1 induced PGE_2 release (TGF-β1, 112.8 ± 19.2 pg/ml; TGF-β1+Indo, 32.5 ± 5.4 pg/ml, n=6, $P<0.01$; TGF-β1+NS-398, 54.9 ± 7.5 pg/ml, n=6, $P<0.05$) but had no effect on TGF-β1 stimulated IL-8 release.

These results show for the first time that TGF-β1 stimulates IL-8 release, COX-2 induction and PGE_2 generation from human ASM. Unlike previous studies where prostanoid generation was a pre-requisite for bradykinin induced IL-8 release (Pang & Knox, 1998), COX-2 induction and PGE_2 production are not critical for TGF-β1 induced IL-8 release. This suggests that TGF-β1 uses different signalling pathways to release IL-8 compared to bradykinin. Collectively, these findings suggest that human ASM cell may be an important target cell for TGF-β1 in asthma thereby generating cytokines and prostanoids which contribute to the airway inflammatory response.

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113P INVOLVEMENT OF PROTEIN KINASE A AND PROTEIN KINASE C IN BRADYKININ-STIMULATED CYCLOOXYGENASE-2 INDUCTION IN HUMAN AIRWAY SMOOTH MUSCLE CELLS

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We have previously reported that BK stimulates arachidonic acid (AA) release, prostanoid (mainly PGE_2) generation and the induction of the inducible cyclooxygenase (COX) isoenzyme COX-2 via B_2 receptors in human airway smooth muscle (ASM) cells (Pang & Knox, 1997). However, the post-receptor signal transduction has not been fully explored. Since AA and PGE_2 (and other cyclic AMP stimulants) have been shown to induce COX-2 expression via the activation of protein kinase C (PKC) and the cAMP-dependent protein kinase A (PKA) respectively in other cell systems, the aim of this study was to investigate whether the activation of PKC and PKA was involved in BK stimulated COX-2 induction.

Confluent primary cultures of human ASM cells at passage 3 were used. The cells were serum starved for 24 h before use. AA release was measured as PGE_2 generation. PGE_2 content was assayed by RIA and COX-2 induction was analysed by Western blotting. Incubation of the cells with 10 μM BK over a 24 h period stimulated PGE_2 release and COX-2 induction in a time-dependent manner, with the maximum PGE_2 accumulation at 16 h (2.35±0.20 ng/mg protein) and the maximum COX-2 induction at 4 and 8 h time point. Pre-treatment for 30 min with the $cPLA_2$ inhibitor $AACOCF_3$ (1 μM) or the non-selective COX inhibitor indomethacin (ind, 1 μM) abolished BK stimulated PGE_2 release (n=6, $P< 0.001$), but markedly enhanced BK stimulated COX-2 induction at almost all time points. However, neither the inhibitors nor the exogenous AA and PGE_2 (0.1-10 μM, 4 and 24 h) caused COX-2 induction on their own. Pre-treatment with PGE_2 and the direct adenylyl cyclase activator forskolin (both 10 μM) strongly suppressed BK mediated COX-2 induction whereas AA was not effective. KT-5720, a PKA inhibitor, enhanced both BK stimulated PGE_2 release (BK 3.31±0.12, BK+KT-5720 10 μM, 11.09±0.89 ng/mg protein, n=6, $P<0.001$) and COX-2 induction but had no effect on its own. PKC inhibitors bisindolylmaleimide I (Bis) and calphostin C (Cal, both 10 μM) inhibited the PGE_2 release (BK 6.36±0.83, BK+Bis 2.27±0.37 BK+Cal 2.72±0.40 ng/mg protein, n=6, $P<0.001$) and COX-2 induction whereas the PKC activator PMA (10 μM) caused PGE_2 release and COX-2 induction in a time-dependent manner, with the maximum PGE_2 accumulation at 16 h (28.6±2.43 ng/mg protein) and the maximum COX-2 induction at 4 and 8 h time point.

Our results suggest that the activation of PKC (unlikely to be mediated by AA) is involved in BK stimulated COX-2 induction in human ASM cells and the released prostanoids may exert a negative feedback on the COX-2 induction via the activation of PKA by a cAMP-dependent mechanism.

Reference:
Pang L. H. & Knox A. J. (1997) *Am. J. Physiol.-Lung Cell. Mol. Physiol.* 17, L1132-L1140.

114P ANTIPROLIFERATIVE EFFECTS OF NITRIC OXIDE AND ATRIAL NATRIURETIC PEPTIDE IN HUMAN CULTURED AIRWAY SMOOTH MUSCLE CELLS

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Airway smooth muscle (ASM) hypertrophy and hyperplasia are important determinants of bronchial responsiveness in asthma and agents which interfere with these processes may prevent airway remodelling. We tested the hypothesis that activators of soluble and particulate guanylyl cyclases would inhibit cultured human airway smooth muscle cells (HASMC) proliferation. HASMC proliferation was assessed by [^{3}H]-thymidine incorporation, MTT assay and cell counting. We report that the nitric oxide (NO) donor (S-nitroso-N-acetyl penicillamine [SNAP, 10^{-6} to 10^{-4} M] and human atrial natriuretic peptide (ANP_{1-28}, 10^{-8} to 10^{-6} M), which activate soluble and particulate guanylyl cyclases respectively, inhibited serum and thrombin-induced proliferation of cultured HASMC. 10% Serum-induced proliferation was inhibited by 62±6.1% and 50±3.4% by 10^{-4} M SNAP and 10^{-6} M ANP respectively (both n=6, $p<0.001$). 1 u.ml^{-1} thrombin-induced proliferation was abolished by 10^{-4} M SNAP and inhibited by 33±6.8% by10^{-6} M ANP. The antimitogenic effect of SNAP was reversed by haemoglobin (Hb, 10^{-5} M), a NO scavenger, suggesting that NO donation was involved. 10^{-4} M SNAP inhibited 10% serum-induced proliferation by 88±1.5% and 6±0.4% in the absence and presence of Hb respectively (n=6, $p<0.001$). Similarly, 10^{-4} M SNAP inhibited 1 u.ml^{-1} thrombin-induced proliferation by 63±7.5% in the absence of Hb while it had no effect in the presence of Hb (n=6, $p<0.05$). The antiproliferative effects of SNAP was potentiated by zaprinast (cGMP-specific phosphodiesterase inhibitor) parallel to an increase in cGMP accumulation. 10^{-4} M SNAP inhibited 10% serum-induced proliferation by 73±4.5% and 85±3.1% and 1 u.ml^{-1} thrombin-induced proliferation by 66±2.1% and 80±3.1% in absence and presence of 10^{-6} M zaprinast respectively (n=6, p=0.05 and <0.01 respectively). 10^{-4} M SNAP-induced cGMP accumulation was 4.6±0.2 and 9.9±0.5 pmol/mg protein in absence and presence of 10^{-6} M zaprinast respectively (n=4, $p<0.001$). Similarly, the antimitogenic effect of ANP was enhanced by zaprinast. In addition, the cell-permeable cGMP analogue, 8-bromo cGMP (10^{-6} to 10^{-3} M), inhibited both serum and thrombin-induced proliferation in a dose-dependent manner. 10^{-3} M 8-bromo cGMP inhibited 10% serum-induced proliferation by 35±7.0% and 1 u.ml^{-1} thrombin-induced proliferation by 69±1.5% (n=6, $p<0.001$ for both). The fact that the antimitogenic effects of SNAP and ANP are potentiated by selective inhibition of cGMP-specific PDE and mimicked by 8-bromo cGMP suggests that cGMP-dependent mechanisms were involved. However, ANP_{1-28} produced a smaller antiproliferative effect than SNAP in contrast to their abilities to elevate cGMP and rat $ANP_{104-126}$, which binds selectively to ANP-c receptors without elevating cGMP, had a small antiproliferative effect suggesting that cGMP-independent mechanisms were also involved.
These results provide evidence for a novel antiproliferative effect of NO and ANP in HASMC mediated through cGMP-dependent and cGMP-independent mechanisms.

115P A PRO-OXIDANT CHALLENGE *IN VIVO* IS PRO-DIABETIC IN THE INSULIN-RESISTANT OBESE ZUCKER RAT

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The obese Zucker rat exhibits many features of syndrome X (Reaven, 1995), including impaired glucose tolerance, hyperinsulinaemia and oxidant stress relative to insulin sensitive, lean Zucker rats (Laight *et al.*, 1998a). Oxidant stress is also present in type II diabetics and contributes to insulin resistance (Paolisso *et al.*, 1994). Since vitamin E improves insulin action in the obese Zucker rat *in vivo* (Laight *et al.*, 1998b), we have now tested the hypothesis that a pro-oxidant challenge can deteriorate insulin action further and promote diabetes in the insulin resistant obese Zucker rat.

Male 12-week old Zucker rats were treated daily for 7 days with the redox cycling compound hydroquinone (HQ, 50 mg kg^{-1} i.p.) together with the glutathione-depleting agent L-buthionine sulfoximine (BSO, 50 mg kg^{-1} i.p.) (Laight *et al.*, 1999). Fasted animals were then anaesthetised with thiopentone sodium (120 mg kg^{-1} i.p.) to allow an i.v. glucose tolerance test. D-glucose (0.5 g kg^{-1} i.v.) was injected at t=0 min and venous blood samples drawn in EDTA anticoagulant at t=1, 3, 6, 12 and 24 min. Glucose tolerance and glucose-stimulated insulinaemia were assessed as area under the curve (AUC) between t=0 and t=24 min. Data are mean±s.e. mean and were analysed by one way analysis of variance followed by Bonferroni's test.

The body weights of 13-week old obese Zucker rats were not significantly affected by pro-oxidant treatment (control 432.7±10.7 g; HQ+BSO 413.5±10.8 g; n=5-6) and were greater (P<0.01) than corresponding lean Zucker rat weights (control 270.0±11.1 g; HQ+BSO 260.5±4.6 g; n=6). Fasting glycaemia was similar in obese and lean Zucker rats while obese animals showed a fasting hyperinsulinaemia (Figure 1). HQ+BSO treatment in the obese Zucker rat significantly increased both fasting glycaemia and insulinaemia (Figure 1). Furthermore, 4 out of 6 obese animals treated with HQ+BSO tested positive for glycosuria (>5 % wv^{-1}) after 1 week. Intravenous glucose tolerance was impaired in obese relative to lean Zucker rats and was not significantly altered by HQ+BSO treatment (Figure 2). However, i.v. glucose-stimulated insulinaemia, which was greater in obese relative to lean animals, was further markedly elevated by HQ+BSO treatment in the obese Zucker rat (Figure 2)

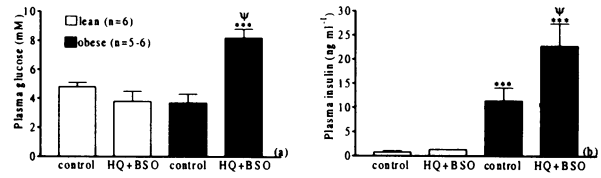

Figure 1. Fasting glycaemia (a) and insulinaemia (b) in Zucker rats. ***P<0.01 vs corresponding lean group; ψ P<0.05 vs obese control.

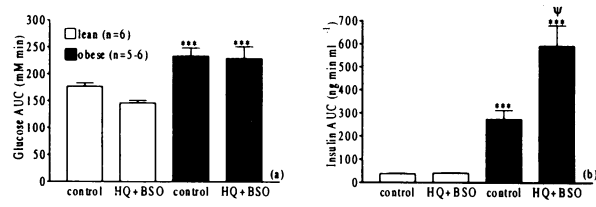

Figure 2. Glucose tolerance (a) and insulinaemia (b) in Zucker rats. ***P<0.01 vs corresponding lean group; ψ P<0.05 vs obese control.

Our data suggest a pro-oxidant-mediated worsening of *in vivo* insulin resistance in the obese Zucker rat. Furthermore, the development of fasting hyperglycaemia and glycosuria, marks the transition from impaired glucose tolerance to diabetes. We therefore demonstrate for the first time, a vulnerability to a deterioration in insulin action in an established insulin resistant state following a pro-oxidative insult. This may now provide a new model of the progression of syndrome X to type II diabetes in man.

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116P THE THIAZOLIDINEDIONE, MCC-555, PREVENTS NITRIC OXIDE SYNTHASE INDUCTION IN THE PANCREAS OF THE ZUCKER DIABETIC FATTY RAT

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The Zucker Diabetic Fatty (ZDF) rat is a model of human diabetes which exhibits impaired insulin sensitivity, hyperphagia and overt diabetes. Over time, the young pre-diabetic ZDF rat exhibits a gradual loss in β-cell function leading to a reduction in insulin secretion, the mechanism of which is not fully understood. There are suggestions that the loss in β-cell function may involve the accumulation of excess nitric oxide (NO) (Unger, 1997). We have examined whether the thiazolidinedione, MCC-555 (Ishii *et al.*, 1996; Pickavance *et al.*, 1998), can prevent the loss in β-cell function when administered to young pre-diabetic ZDF rats and whether there is evidence for increased NO accumulation in the pancreas. Male 6-week-old pre-diabetic ZDF rats (240 g; *n*=9) were treated with MCC-555 (10 mg/kg in 0.5% carboxymethyl-cellulose vehicle, p.o) for 28 days. Pre-diabetic ZDF and non-diabetic ZDF controls (200 g) were administered vehicle alone (5 ml/kg; *n*=9/group). Rats were anaesthetised with pentobarbitone (30 mg/kg, i.p.) and then killed by cardiac exsanguination. Pancreata were dissected and divided into two portions, one half being snap-frozen in liquid nitrogen and the other fixed in buffered formalin solution and then wax-embedded. Nitric oxide synthase (NOS) activity and nitrate/nitrite concentrations were measured in the frozen pancreas using previously published protocols (Widdowson *et al.*, 1996). β-Cell damage was examined in haematoxylin/eosin-stained sections of the fixed wax-embedded pancreatic tissue. Vehicle-treated diabetic ZDF rats exhibited elevated pancreatic NOS activity (mean±S.E.M.: 1439±124 *vs* 864±113 fmol/mg protein in non-diabetic controls; $p<0.01$; Student's *t*-test), which was normalized by MCC-555 treatment (860±93 fmol/mg protein). Correspondingly, raised nitrate/nitrite levels in vehicle-treated diabetic ZDF rats (6.31±0.79 *vs* 2.82±0.50 nmol/mg protein in non-diabetic controls; $p<0.01$) were also reduced by MCC-555 treatment (4.01±0.68 nmol/mg protein). A microscopic examination of pancreatic β-cell islets of vehicle- and MCC-555-treated diabetic ZDF rats showed marked hypertrophy of the islets with increased fibrosis in both groups, as compared to non-diabetic controls. However, whereas one or two apoptotic cells were observed in islets from vehicle-treated diabetic ZDF rats, no apoptotic cells were observed in islets from MCC-555-treated diabetic rats, in common with islets from non-diabetic controls. In conclusion, we have shown that treatment of pre-diabetic ZDF rats with MCC-555 partially protects pancreatic β-cells from apoptosis and cellular damage that may involve the excess production of NO.

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117P THE EFFECT OF OREXIN-A AND OREXIN-B ON THE MODULATION OF NEUROENDOCRINE MARKERS

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Measurement of neuroendocrine responses to a novel drug challenge provides a valid means of assessing receptor function (Cowen *et al.*, 1993). In the present study this methodology is applied to the elucidation of the function of orexin-A and orexin-B, novel neuropeptides that are derived from the same precursor, prepro-orexin, shown to be present in high density in the hypothalamus (Sakurai *et al.*, 1998, De Lecea *et al.*, 1998). The effects of orexin-A and orexin-B on plasma prolactin, growth hormone, thyroid stimulating hormone (TSH) and corticosterone were determined.

Male Sprague Dawley rats (350-450g) previously implanted with i.c.v. cannulae (placement verified using drinking response to angiotensin II) were used. The rats were pretreated with the novel ligands orexin-A at 0.3, 1, 3, 10 and 30 ug i.c.v and orexin-B at 3, 10 and 30 ug i.c.v. After 40 min rats were killed by rapid decapitation and the trunk blood collected into prechilled EDTA tubes. The blood samples were then centrifuged at 3000rpm for 15 min and the plasma harvested and stored at -70°C for subsequent assay of the neuroendocrine markers using suitable radioimmunoassays (RIAs). Data were analysed using one way analysis of variance followed by least significance difference t-test, using Statistica ™ software. Significance was taken at the $p<0.05$ level. Data are shown as mean ± s.e.m.

Orexin-A caused a significant ($p< 0.05$) decrease at 1, 3, 10 and 30 ug i.c.v. in both prolactin (control = 4.8 ± 1.4 ng/ml, at 1, 3 and 10 ug below limit of detection) and growth hormone plasma levels (control = 29.8 ± 12.5 ng/ml, e.g. 30ug = 1.72 ± 0.29 ng/ml).Orexin-B also caused a significant ($p< 0.05$) decrease in plasma prolactin levels at 10 and 30 ug i.c.v (control = 4.8 ± 1.4 ng/ml, at 30ug = 0.04 ± 0.5 ng/ml), whilst having no effect on growth hormone plasma levels (control = 23 ± 12 ng/ml, at 30 ug = 25.75 ± 13.5 ng/ml). Orexin-A elicited a dose dependent increase in corticosterone plasma levels (control = 44 ± 9 ng/ml, at 30ug = 140.5 ± 21.1 ng/ml) significant ($p< 0.05$) at 10 and 30ug i.c.v. In contrast orexin-B had no effect on plasma corticosterone levels (control = 44 ± 9 ng/ml, at 30ug = 69 ± 10 ng/ml). Orexin-A had no effect on TSH plasma levels (control = 6.8 ± 1.74, at 10ug = 7.6 ± 1.32 ng/ml) whilst orexin-B caused an increase in plasma TSH levels (control = 6.8 ± 1.7 ng/ml, 3ug = 12.95 ± 1.9 ng/ml) reaching significance ($p< 0.05$) at 3ug i.c.v.

These data demonstrate that orexin-A and orexin-B modulate neuroendocrine function. This agrees with other findings where orexin-A has been demonstrated to modulate input controlling neuroendocrine neurones in the arcuate nucleus in hypothalamic slices *in vitro* (van den Pol *et al.*, 1998) and the release of luteinizing hormone-releasing hormone (Pu *et al.*, 1998).

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118P INVOLVEMENT OF HYPOTHYROIDISM IN THE REDUCTION IN ISCHAEMIA-REPERFUSION ARRHYTHMIAS IN HEARTS FROM STREPTOZOTOCIN DIABETIC RATS

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Previously we showed marked similarities between the effects of streptozotocin (STZ)-induced diabetes and methimazole-induced hypothyroidism in reducing ischaemia-reperfusion-induced arrhythmias in subsequently isolated and perfused hearts (Zhang et al., 1999). STZ-induced diabetic rats showed marked reductions in serum concentrations of free thyroxine (FT_4) and triiodothyronine (FT_3). The present work was undertaken to test further the hypothesis that the protection afforded against ischaemia-reperfusion induced arrhythmias by STZ diabetes is mediated by the diabetes-induced hypothyroidism. Male Sprague-Dawley (200-220 g) were made diabetic using STZ (60 mg/kg, *i.p.*). The diabetic rats were divided into three groups of 12, which received daily injection of saline (control), insulin (protamine zinc insulin, 10 IU $kg^{-1}day^{-1}$, *s.c.*) or triiodothyronine (T_3, 10 μg $kg^{-1}day^{-1}$, *s.c.*) from 72 hours after induction of diabetes until the end of 8 weeks after STZ-injection. The hearts were isolated, perfused (Langendorff mode, Wu et al., 1994) and subjected to 30-minute occlusion (left main coronary artery, MLCA) followed by 30-minute reperfusion. Cardiac arrhythmias were determined during both occlusion and reperfusion. Statistical analysis used Fisher's Exact test or ANOVA plus Tukey's test. The serum free thyroxine (FT_4) concentration was markedly decreased in the control diabetic group (pmol/l, control 28.4±1.4; STZ 15.6±1.5 $P<0.001$) and these concentrations were restored to control values by insulin administration (STZ + insulin 27.4±1.7). Insulin or T_3 administration also largely reversed the signs of hypothyroidism in the STZ-diabetic rats, namely the reduced rectal temperature and the prolonged QT interval measured from the electrocardiogram (QT interval, msec; control 70.5±2.7; diabetic 86.3±2.8 ($P<0.01$ vs control); diabetic + T_3 69.2±3.5 ($P<0.01$ vs diabetic); diabetic + insulin 75±2.7 ($P<0.05$ vs diabetic)). Serum glucose concentrations were restored by insulin but not by T_3 (mM, diabetic control 32.4±1.5; + insulin 8.1±0.8; + T_3 29.3±1.4). There were significant negative correlations between serum glucose and FT_4 and FT_3 concentrations in diabetic rats (e.g. for FT_3 $n = 12$ $r = -0.79$ $P<0.01$). In controls the incidence of ventricular fibrillation (VF) and sustained VF after MLCA occlusion were 77.8 and 66.7% respectively, these being reduced to zero in control diabetic rats ($P<0.01$). Treatment of diabetic rats with T_3 restored the incidence of VF to 50% ($P<0.05$ vs diabetic) and sustained VF to 33% (n.s.). Treatment of diabetic rats with insulin similarly restored the incidence of VF to 57% ($P<0.05$ vs diabetic) and sustained VF to 43%. These data support the hypothesis that the reduced incidence of cardiac arrhythmias is secondary to the hypothyroidism induced by the experimental diabetes.

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119P LEPTIN RELEASE FOLLOWING HIGH-ENERGY DIET FEEDING AND PRIOR TO INCREASED ADIPOSITY PREDICTS THE FINAL DEGREE OF OBESITY IN RATS

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Rodents given palatable food vary greatly in the degree to which they overeat and become obese. This individual susceptibility to dietary obesity is unexplained, but must be due to differences in the ability of palatable food to over-ride the homeostatic mechanisms that control food intake and body weight. Leptin normally acts on the hypothalamus to inhibit feeding and stimulate thermogenesis (Halaas *et al.*, 1995). The melanocortin-4 receptor (MC4-R) system is also implicated in the inhibition of feeding (Fan *et al.*, 1997) and may partly mediate leptin's central hypophagic actions (Schwartz *et al.*, 1997). In this study we have investigated the relative importance of these satiety signals, prior to the development of obesity, in determining individual susceptibility to hyperphagia and obesity in rats. Male Wistar rats fed a high-energy diet for 2 weeks consumed significantly more calories than controls fed a standard pellet diet (diet fed = 505 ± 11; controls = 413 ± 11 KJ/day; P<0.01; Student's t-test; n=8 per group). When plasma analytes were measured at 1 week, using commercially available kits (Harrold *et al.*, 1999), high-energy diet fed rats were found to have increased plasma leptin concentrations, (diet fed = 4.07 ± 0.19; controls = 3.02 ± 0.26 ng/ml; P<0.01) prior to increased body weight and adipose tissue mass. A significant reduction in MC4-R density in specific hypothalamic nuclei, as determined by autoradiography with [^{125}I]NDP-MSH (Harrold *et al.*, 1999) was also recorded. After a further 7 weeks of high-energy diet feeding the rats became significantly obese (diet fed = 480 ± 9; controls = 419 ± 14 g; P<0.01). These animals were retrospectively stratified into 'high-weight gain' (range 316-409 g; n=10) and 'low-weight gain' (range 196-288 g; n=8) groups according to whether or not weight gain exceeded that in chow-fed controls (range 183-286 g; n=9). All diet fed rats were hyperphagic, hyperleptinaemic and hyperinsulinaemic and had significantly raised fat-pad masses compared with controls, but each of these measures was significantly greater in the high-weight than in the low-weight gain group. When plasma leptin levels after 1 week were related retrospectively to weight gain at 8 weeks a negative correlation with the final degree of adiposity in diet fed rats (r^2 = 0.38; P<0.001) was identified, demonstrating that an early leptin response to high-energy food intake is associated with the ability to attenuate obesity. MC4-R were down-regulated in the same hypothalamic nuclei of all diet-fed rats, with this binding being consistently lower in the low-weight gain group than in the high-weight gainers (e.g. hypothalamic ventromedial nucleus (VMH) = 0.45 ± 0.06 vs 0.54 ± 0.07 fmol/mg tissue; controls = 0.76 ± 0.04 fmol/mg tissue; P<0.01). Furthermore, the down-regulation of MC4-R in the VMH in response to increased melanocortin synaptic activity in diet fed rats was also negatively correlated to the early leptin response (r^2 = 0.30; P<0.05), suggesting that this is a key site of leptin action. These data show that the magnitude of the early leptin response to high-energy food predicts the degree of protection against ensuing obesity, possibly by stimulating neurons that project selectively to MC4-R in the VMH.

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120P THE HUMAN 23kDa PHOSPHATIDYL ETHANOLAMINE BINDING PROTEIN: A NOVEL MODULATOR OF μ-OPIOID RECEPTOR COUPLING AND DESENSITISATION

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In 1990 Grandy *et al.* isolated a 23kDa-PEBP (phosphatidyl ethanolamine binding protein) by using a morphine affinity column assay, suggesting an association with the μ-opioid receptor (MOR). In addition, several studies in the field of PEBP research indicate a possible role of 23kDa-PEBP in signal transduction. Nevertheless the function of this protein is still unknown.

Opioid receptors are known to couple via Gi Go-protein to inwardly rectifying potassium channels (KIR), leading to a change in K^+ conductance of the membrane. To study the interaction of the human 23kDa-PEBP (h23kDa-PEBP) and the human MORl (hMORI), we performed two electrode voltage clamp experiments on *Xenopus* oocytes expressing inwardly rectifying potassium channels (KIR3.1, KIR3.4), h23kDa-PEBP and hMOR1. Oocytes were injected with *in vitro* transcribed cRNA of KIR (0.5ng [KIR3.1], 0.5ng [KIR3.4]), hMOR1 (lng) and h23kDa-PEBP (Sng). 3-7 days after injection, oocytes were voltage-clamped at 80mV and tested for morphine-induced activation of KIR-mediated currents. KIR-mediated currents (I_{bas}; I_{act}) were measured after superfusion of oocytes with 16mM KCl_2-solution (I_{bas}) and 16mM KCl_2-solution containing 1 μM morphine (I_{act}).

Coexpression of h23kDa-PEBP in oocytes induced both a significant increase in morphine-induced KIR-mediated current (hMOR1: I_{act}=553nA, SEM=41.5nA; hMORl+h23kDa-PEBP: I_{act}=897.7nA, SEM=57.8nA;n=5 ;p<0.01, unpaired t-test) and KIR-mediated basal current (hMOR1: Ibas=426.2nA, SEM=41.7; hMOR1 +23kDA-PEBP: I_{bas}=576.5nA, SEM=34.1nA; n=5; p<0.05, unpaired t-test). In the case of morphine, this effect was dose-dependent, reaching maximum at a concentration of 1μM morphine without changing EC_{50} significantly (EC_{50} [hMOR]: 5.4 nM±1.52) and naloxone blockable (l μM). No stimulatory effect on KIR-mediated currents was detected in oocytes expressing KIRs and h23kDa-PEBP only, indicating that the stimulatory effect is restricted to the presence of hMOR1. A comparable h23kDaPEBP-mediated stimulation of KIR current (I_{act}) could be measured after injection of *E.coli* extracts containing recombinant expressed h23kDa-PEBP into oocytes expressing hMOR1 and KIRs (hMOR1: I_{act}=42.3nA, SEM=6.2nA; hMORl+h23kDa-PEBP: I_{act}=64.5nA SEM=57.9nA; n=4 ;p<0.05, unpaired t-test)

Chronic superfusion of oocytes with 1μM morphine for 5 min results in desensitization of the μ-opioid receptor, characterized by a time-dependent decrease in I_{act} (47.2 %, SEM= 3.2%). Chronic superfusion of oocytes coexpressing h23kDa-PEBP with 1pM morphine for 5 min resulted in a loss of the stimulatory effect of h23kDa-PEBP on I_{act} after 5 min compared to control oocytes (hMOR1: I_{act}: 275.6nA; SEM=80.4nA; hMORl+h23kDa-PEBP: I_{act}=398.7nA SEM=53.72nA; n=5; p>0.05, unpaired t-test).

These data show that the h23kDa-PEBP plays an important role in regulating μ-opioid receptor coupling to KIR. Since the mechanism of stimulation is still unknown, further studies will focus on specific sites of interaction between h23kDa and the signal transduction pathway of the μ opioid receptor.

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121P MUTATION SCREENING OF THE HUMAN MUSCARINIC M_2 RECEPTOR GENE IN MALTESE ASTHMATIC PATIENTS

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Muscarinic M_2 receptors are important in the regulation of airway tone. Disregulation of muscarinic M_2 receptor function has been suggested to underlie bronchial hyperresponsiveness (Ten Berge *et al.*, 1995) and hence potentially to be an important mechanism in determining the risk of an individual developing asthma. We have therefore screened the muscarinic M_2 receptor gene for the presence of polymorphisms which could account for inter-individual variation in responses.

Ten overlapping fragments spanning the entire coding region of the muscarinic M_2 receptor gene, together with part of the 5' and 3' untranslated region (UTR) (-174 to +459) were generated by the polymerase chain reaction (PCR) and used for single stranded conformation polymorphism (SSCP) analysis. SSCP was performed at 5°C and at 15°C using precast gels (Pharmacia GeneGel Excel Kit®) and a Pharmacia MultiPhor II® electrophoresis unit. Gels were viewed by silver staining. For each PCR fragment of interest, DNA from 46 random cord blood samples and 46 physician diagnosed asthmatic samples were used as a template for analysis. Direct sequencing of PCR products was used to confirm the presence of polymorphisms where suggested by SSCP.

In marked contrast to the human β_2-adrenoceptor gene, the coding region for the human muscarinic M_2 receptor gene is highly conserved. We identified a single conservative polymorphism (bp 1197, T→C, Thr→Thr) in one individual from the random cord blood population. No other polymorphisms were detected in the coding region of the gene. Analysis of the 3' UTR region showed an additional A at bp 1793; however this was present in 10 sequenced samples, suggesting the published sequence (Accession number M16404 – Genbank DNA sequence database, Bonner *et al*, 1987) is incorrect. BsmI restriction digest on a further 29 samples suggested the presence of this A insertion in all samples. This introduces a previously unrecognised C-Rel/NFκB transcription factor consensus sequence into the 3' UTR. An additional polymorphism was found at bp 1696 (T→A). This was common, (allelic frequency=65%, n=20), but does not alter transcription factor recognition sites.

These data suggest that polymorphic variation within the human muscarinic M_2 receptor gene coding region is unlikely to account for inter-individual variability in response to muscarinic M_2 receptor agents. The potential functional significance of the 3' UTR polymorphism (bp 1696) remaines to be determined.

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122P EXPRESSION OF HOMOLOGUES OF THE TRANSIENT RECEPTOR POTENTIAL (TRP) GENE IN PRIMARY CULTURED HUMAN AIRWAY SMOOTH MUSCLE CELLS

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Human airway smooth muscle cells respond to agonists such as histamine with an initial rise in intracellular calcium dependent upon release from intracellular stores followed by a sustained elevation dependent upon entry across the cell membrane (Murray & Kotlikoff. 1991). The pathway for calcium entry has not been defined at a molecular level. Recently a number of mammalian homologues of drosophila, transient receptor potential (TRP) genes have been identified: expression studies suggest that human TRP (HTRP) homologues may account for calcium entry through non-voltage dependent channels (Hofmann *et al.*, 1999) in recombinant cell systems.

In order to identify the HTRP genes expressed in cultured human airway smooth muscle we used RT-PCR with specific primers for HTRP 1–7. mRNA was extracted from 162cc flasks of monolayers of cultured cells using the Micro-Fast Track kit from Invitrogen. 1μg of cDNA was used as a template for RT-PCR using random hexamers and Reverse Transcriptase (Gibco BRL). PCR conditions were initially optimised for each HTRP product. Human genomic DNA was used as a positive control and products subjected to electrophoresis on 1% agarose gels.

Human airway smooth muscle cells expressed HTRP 1,2,3,4 and 6. Further characterisation of the RT-PCR product for HTRP 3 was performed due to the difference in product size obtained with genomic DNA. Direct sequencing demonstrated the presence of an intron of >1kB at bp2195 of the published HTRP 3 sequence (Zhu *et al.*, 1996), accounting for the disparity in RT-PCR product compared with the products obtained from genomic DNA. The identity of RT-PCR products HTRP 1 and 2 was also confirmed by direct sequencing. The partial sequence obtained was identical to the published sequence except for an A→G base change at position 1755 of HTRP 1 (Zhu *et al.*, 1995).

These data demonstrate that the putative calcium entry channels HTRP 3 and 6 are expressed in cultured human airway smooth muscle cells. Expression of HTRP 1,2 (a pseudogene) and 4 was also observed, but no expression was seen of HTRP 5 or the brain specific homologue HTRP 7.

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123P DIFFERENTIAL EXPRESSION OF HUMAN β_2-ADRENOCEPTOR PROMOTER CONSTRUCTS IN COS-7, BEAS-2B AND HUMAN CULTURED AIRWAY SMOOTH MUSCLE CELLS

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We have previously demonstrated that expression in COS-7 cells of luciferase constructs under the control of the human β_2-adrenoceptor (β_2AR) 5'-flanking region is driven by a region contained within the first 379 base pairs (bp) immediately 5' to the start codon of the receptor (Scott *et al.*, 1999). However, COS-7 cells do not constitutively express β_2ARs and the aim of the studies described here was to ascertain if the control of human β_2AR gene expression is different in cells expressing human β_2ARs constitutively. For these experiments we used a human airway epithelial cell line (BEAS-2B) and primary cultures of human airway smooth muscle (HASM) cells, both of which constitutively express the human β_2AR.

The activity of a range of deletion reporter gene constructs containing 1486, 1244, 1018, 744, 549, 379 or 274 bp of 5'-flanking region of the human β_2AR gene, subcloned into the promoter-less luciferase vector pGL3 Enhancer (Promega), was assessed in transient transfection assays in 24 well plates. Results were corrected for variations in transfection efficiency using a co-transfected *Renilla* luciferase plasmid (pRL.CMV, Promega). 250ng firefly luciferase and 12.5ng *Renilla* luciferase were transfected per well using the cationic lipid Transfast (Promega) at a charge ratio of Transfast:DNA of 1:1. Cells were harvested 48h post-transfection and firefly and *Renilla* luciferase activity was measured using the Dual Luciferase Reporter Assay System.

Whereas in COS-7 cells the majority of activity resided within a 379bp fragment immediately upstream of the β_2AR start codon, in BEAS-2B and HASM cells there was a marked further increase in activity in the region from –380 to –549bp: BEAS-2B; 2.31 $\pm$ 0.14 fold (n=8, $p<0.0001$), HASM; 2.19 $\pm$ 0.56 fold (n=9, $p<0.05$) *c.f.* COS-7 cells; 1.00 $\pm$ 0.04 fold (n=12). In addition, we examined the role of the 5'-leader peptide beta upstream peptide (BUP), which is thought to be involved in the translational inhibition of β_2AR expression (Parola & Kobilka, 1994) using a construct in which the start codon for BUP was mutated (ATG→CTT) to prevent its expression. Mutation of the BUP start codon resulted in an increase in expression of p0.38β_2AR-LUC in all cell types: COS-7; 1.54 $\pm$ 0.04 fold (n=10, $p<0.0001$), BEAS-2B; 2.69 $\pm$ 0.24 fold (n=4, $p<0.01$), HASM; 2.24 $\pm$ 1.17 fold (n=7). All data were analysed using paired t tests.

These data demonstrate that in two cell populations constitutively expressing the human β_2AR (BEAS-2B and cultured HASM cells) control of human β_2AR gene expression appears to be different than in COS-7 cells, which do not express this receptor. A number of potential transcriptional regulatory motifs within this region, including several Sp1 and AP-2 sites (Scott *et al.*, 1999), could account for this differential expression. Inactivation of the short open reading frame for BUP resulted in increased expression of reporter gene constructs in all cell types studied suggesting a role for BUP in control of human β_2AR gene expression.

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124P INHIBITION OF K_{ATP} CHANNEL ACTIVITY BY KETOCONAZOLE IN CRI-G1 INSULIN-SECRETING CELLS

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The imidazole antifungal clotrimazole has been shown to inhibit K_{ATP} channel activity in the mouse pancreatic β cell. Surprisingly this compound appears to inhibit insulin secretion in these cells by virtue of its ability to also inhibit voltage-gated channels (Welker and Drews, 1997). In view of these observations, we have examined the ability of the related antifungal ketoconazole to modulate both voltage-gated and ATP sensitive currents in the CRI-G1 insulin secreting cell line.

In inside-out patches bathed in symmetrical 140mM KCl, bath application of ketoconazole reversibly inhibited K_{ATP} channel activity in a concentration dependent manner. For example 1μM ketoconazole produced 38.2 ± 3.4% (n=3) inhibition, 10μM a 82.5 ± 3.7% (n=16), and 50μM complete inhibition (n=3) of channel open-state probability (P_o). Similarly bath applied ketoconazole also inhibited K_{ATP} channel activity in outside-out patches. These actions of ketoconazole were independent of membrane potential over the range +60 to -60mV.

Under whole-cell voltage clamp conditions K_{ATP} channel currents were evoked from CRI G1 cells bathed in physiological saline which contained (in mM) NaCl 135.0, KCl 5.0, $CaCl_2$ 1.0, $MgCl_2$ 1.0, HEPES 10.0, whilst the electrode solution contained (in mM) KCl 140.0, $MgCl_2$ 1.0, $CaCl_2$ 2.0, EGTA 10.0, HEPES 10.0. At a holding potential of -70mV, the K_{ATP} channel current evoked from ± 10mV pulses was reversibly inhibited by ketoconazole with an IC_{50} of 5.2 ± 0.8μM and an associated Hill coefficient of 1.2 ± 0.2 (n=10).

To determine the specificity of action of ketoconazole, the effects of 50μM ketoconazole were examined on voltage-gated currents in CRI-G1 cells. To study Ca^{2+} and Na^+ currents the pipette solution contained (in mM) CsCl 140.0, $CaCl_2$ 2.0, $MgCl_2$ 1.0, K-EGTA 10.0, HEPES 10.0. whilst 300nM TTX and 10mM $CaCl_2$ were added to the bath solution to study Ca^{2+} currents and 1mM $CdCl_2$, 20mM TEA and 4mM 4- aminopyridine were added to the bath solution to study Na^+ currents. Under these conditions, ketoconazole had little effect on either inward current at all potentials tested (n=4).

To study the outward K^+ currents 300nM TTX and 1mM $CdCl_2$ were added to the bath solution and the electrode solution contained (in mM) KCl 140.0, $MgCl_2$ 2.5, $CaCl_2$ 2.0, EGTA 10.0, ATP 2.0, HEPES 10.0. In contrast to the lack of effect of ketoconazole on the voltage-gated inward currents, 50μM ketoconazole produced 27.5 ± 4.5 % (n=5) inhibition of the delayed outward K^+ current (activated by a voltage step from -80 to +60mV) although no change in the activation threshold of the currents was observed.

In conclusion we have demonstrated that ketoconazole is a potent inhibitor of K_{ATP} channel currents in the CRI-G1 cell line but in contrast to clotrimazole, has a weaker inhibitory effect on voltage-gated channels in these cells.

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125P FUNCTIONAL CHARACTERISATION OF OKADAIC ACID RESISTANCE IN HIT CELLS

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Okadaic acid (OA) is a fairly selective inhibitor of serine/threonine phosphatases 1 and 2A with IC50 values of 15 - 50 nM and 0.5 - 1 nM, respectively (Honkanen *et al.*, 1994). In HIT cells, an insulin-secreting hamster B-cell line, OA time- and concentration-dependently led to apoptotic cell death (Krautheim *et al.*, 1999). Prolonged incubation of HIT cells with OA concentrations stepwise increased from 5 to 100 nM permitted the selection of HIT cells which were finally resistant against apoptosis induction by 100 nM OA (HIT100R).

HIT and HIT100R cells were tested for differences in the apoptosis inducing potency of OA and cantharidic acid (CA), a PP1 and PP2A inhibitor which is structurally unrelated to OA (Honkanen, 1993). In Western Blots a release of cytochrome c from mitochondria into the cytosol was detectable in HIT cells after 24 h treatment with 50 nM OA and higher, while in HIT100R cells only a slight increase could be shown after 24 h with 500 nM OA. In contrast with 10 µM CA a release of cytochrome c was already detectable after 12 h in both cell lines. Translocation of cytochrome c into the cytosol in combination with an additional cytosolic factor is believed to activate a DEVD-cleaving caspase (Mancini *et al.*, 1998). An increase of DEVDase activity, indicating the activation of caspases 3, 6 and/or 7 (Cohen 1997), following OA or CA treatment could be shown to accompany cytochrome c release (table 1).

These differences between the effects of OA and CA are presumably due to the mechanism of resistance that developed in HIT100R cells. A frequent cause of resistance against apoptosis induction is an increased expression of the P-glycoprotein as mediator of the multidrug resistance (MDR) phenotype (Bagetto, 1997). In Northern Blot analysis an increased expression of the pgp1 gene in HIT100R cells could be shown.

P-glycoprotein mediated transport can be quantified with the fluorescent MDR substrate rhodamine123. When HIT and HIT100R cells were incubated for 3 h with 6.57 µM rhodamine123 intracellular accumulation in HIT100R cells was significantly reduced to 1135.35 ± 97.06 nM from 1837.46 ± 153.57 nM in HIT cells (mean ± SEM, n=3, $p<0.05$, Student's unpaired t-test). Viability assays with MDR substrates and MDR modulators (verapamil and reserpine) also supported the role of the MDR phenotype for OA resistance of HIT100R cells.

Thus, the resistance of HIT100R cells against OA seems to depend on the MDR phenotype. It can be overcome by higher concentrations of OA, suggesting that accumulation of a certain intracellular amount of OA is necessary to induce apoptotic cell death.

Table 1: DEVDase activity in U/mg protein (1 U = 1 pmole substrate conversion per minute, mean ± SEM, n=5-6).

	control	10 µM CA		100 nM OA	500 nM OA
		12 h	24 h	24 h	24 h
HIT	9 ± 2	17 ± 2	81 ± 15	95 ± 24	228 ± 60
HIT100R	2 ± 1	59 ± 17	80 ± 22	7 ± 2	69 ± 15

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126P NEUROKININ-3 RECEPTORS ARE EXPRESSED ON GABAERGIC INTERNEURONS AND EVOKE GABA RELEASE IN THE MOUSE STRIATUM

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Within the mammalian striatum, tachykinin receptors are known to perform an important modulatory role in the activity of cholinergic interneurons (Bell *et al.*, 1998; Preston *et al.*, 1998) and a less well characterised role within GABAergic interneurons (Kawaguchi, 1997). In view of this, in the present study we have investigated the ability of known tachykinin agonists and antagonists to modulate GABA release from mouse striatal slices using an in vitro superfusion technique. Additionally, we have correlated the expression of these receptors in GABAergic interneurons.

[^{3}H]GABA release: Brains were removed from 14 to 24 day-old C57-BL/6 mice (both sexes) and 300 µm striatal slices were prepared in physiological saline which comprised (mM): 125·0 NaCl, 25·0 $NaHCO_3$, 10·0 glucose, 2·5 KCl, 1·25 NaH_2PO_4, 2·0 $CaCl_2$ and 1·0 $MgCl_2$, bubbled with a 95 % O_2-5 % CO_2 gas mixture. The slices were incubated in the presence of 0·5 µM [^{3}H]GABA for 1 h at 37°C, placed in perfusion chambers, and perfused at 0·5 ml min^{-1} at 37°C with physiological saline containing 1 mM nipecotic acid and 0.1 mM aminooxy acetic acid. 1 µM atropine was also included in the saline to prevent possible indirect GABA release via muscarinic receptor activation. Release of [^{3}H]GABA was induced by the inclusion of drugs for 2 min in the perfusion buffer. Antagonists were added for 10 mins before, and during, the evoked release of [^{3}H]GABA. 1 ml perfusate samples were collected and the radioactivity counted. *In situ hybridisation:* [^{35}S]-labelled probes complementary to mRNA encoding preprosomatostatin, nitric oxide synthase (NOS) and neurokinin receptor subtypes were hybridised to alternate, adjacent 5 µm striatal coronal sections. Sections were exposed to photoemulsion for 10 weeks, developed and finally stained with cresyl violet.

Significant [^{3}H]GABA release was observed in a concentration-dependent manner when the slices were challenged with the NK-3 agonist senktide (EC_{50} = 920 ± 15 pM, n=4). When pre-incubated with the selective NK-3 receptor antagonist SR142801 (100 nM), a 74.5 ± 17.3% reduction from the control senktide response was seen (n=4, $p<0.01$). The response was also shown to be TTX sensitive (reduction of 61.9 ± 23.6 %, n=3, $p<0.05$). The NK-1 and NK-2 receptor agonists [sar^9,met$(O_2)^{11}$]-substance P and [β-ala^8]NKA_{4-10} respectively had no effect on [^{3}H]GABA release at concentrations up to 50 nM (n=4).

To exclude the possibility that the observed responses were through indirect pathways involving nitric oxide or nicotinic acetylcholine receptors (nAChR), slices were pre-incubated with specific inhibitors. 100 µM N^G-Monomethyl-L-Arginine (NOS inhibitor) did not have any significant effect on senktide-induced [^{3}H]GABA release (n=4), indicating that NOS is not involved in release. Similarly, when slices when perfused with the nAChR antagonist, hexamethonium (100 µM), GABA release was not modulated (n=3).

Thin adjacent section *in situ* hybridistaion revealed NK-3 receptors co-expressed with both NOS and preprosomastatin which are biochemical markers of one class of GABAergic interneuron (Kawaguchi *et al.*, 1995). In conclusion, these findings suggest that tachykinins are able to modulate GABA release within the striatum at least in part via interaction with NOS/somatostatin neurons.

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127P INTRACELLULAR CALCIUM MOBILIZATION FOLLOWING ACTIVATION OF AN ENDOGENOUS MUSCARINIC RECEPTOR IN HUMAN EMBRYONIC KIDNEY (HEK 293) CELLS

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HEK 293 cells are a commonly used cell line for the heterologous expression of receptors and study of signalling molecules. Interest in muscarinic receptors, and in the development of selective ligands, stems from their possible therapeutic potential in, for example, Parkinson's disease, and disorders of cardiac and bladder function (Caulfield & Birdsall, 1998). Here we demonstrate the presence of an endogenous muscarinic receptor in these cells, coupled to mobilization of intracellular calcium.

HEK 293 cells (passage 27-34) which had been stably transfected with recombinant human H_1 histamine receptor DNA (Presland & Hill, 1998) were grown in 75 cm^2 cell culture flasks as described previously (Cooper *et al.*, 1997). Confluent monolayers, detached using phosphate-buffered saline/EDTA, were loaded with the calcium sensitive fluorescent dye FURA-2/AM for measurement of intracellular calcium concentration, $[Ca^{2+}]_i$, as previously described (Iredale & Dickenson, 1995). Cells were stimulated with carbachol (100 nM - 1 mM), in order to determine the presence and magnitude of the $Ca^{2+}{}_i$ signal. Cells were also incubated with 100 nM antagonist for 30 min prior to carbachol exposure.

Carbachol caused a concentration-dependent increase in 340/380 nm ratio over basal. Table 1 shows $logEC_{50}$ values and maximal responses (percentage of the 1 mM carbachol response). In the presence of 4-DAMP, 1 mM carbachol elicited a small increase in 340/380 nm ratio but lower concentrations showed no change, hence a $logEC_{50}$ could not be calculated. In the presence of 4-DAMP, 100 μM histamine elicited a substantial calcium mobilization.

Drug Treatment	$logEC_{50}$	Maximal response
Carbachol	-4.67 ± 0.04	100
Carbachol + 4-DAMP	>-3	(14.3 ± 0.2)†
Carbachol + methoctramine	-4.47 ± 0.21	95.0 ± 10.3
Carbachol + pirenzepine	-4.47 ± 0.12	60.4 ± 3.2

Table 1 – $LogEC_{50}$ values & maximal responses (expressed as % of 1 mM carbachol response, mean ± s.e.m. n=3). †Response to 1 mM carbachol + 4-DAMP as % of the control 1 mM carbachol response, rather than the maximal response to carbachol + 4-DAMP.

One way ANOVA (with a Bonferroni *post-hoc* test) showed there to be no significant differences in $logEC_{50}$ values for carbachol, methoctramine and pirenzepine. Analysis of the response to 1 mM carbachol in the presence of pirenzepine or 4-DAMP showed a significant ($P<0.05$) difference compared to control values.

Here, we have demonstrated a concentration-dependent increase in $[Ca^{2+}]_i$ following stimulation with carbachol in HEK 293 cells. The $logEC_{50}$ was unaffected by methoctramine and pirenzepine, but the signal was almost totally abolished by 4-DAMP. When considered alongside the antagonist affinity constants (Caulfield & Birdsall, 1998), these data suggest the presence of an endogenous M_3 muscarinic receptor in these HEK 293 cells. The pirenzepine data suggest a smaller M_1 receptor population or a non-competitive effect of pirenzepine on M_3 receptors.

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128P DISTRIBUTION OF VOLTAGE-GATED SODIUM CHANNEL SUBUNITS IN THE RAT CNS: A COMPARATIVE STUDY

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Rat brain voltage gated Na^+ channels have been shown to be composed of three glycoprotein subunits, a pore-forming α subunit and two smaller auxiliary subunits β1 and β2 (Messner & Catterall, 1985). Although several studies have previously documented the distribution of these individual subunits in the rat CNS, no comparative studies have previously been performed. Consequently in the present study, the distribution of mRNAs encoding the major CNS Na^+ channel α subunits, I, II and VI, and the auxiliary subunits β1 and β2, has been examined in the adult rat brain by semi-quantitative *in situ* hybridisation histochemistry.

Adult male Wistar rats (200-300g) were killed by cervical dislocation followed by decapitation. The whole brain was rapidly dissected out and snap-frozen in isopentane (5 seconds) cooled on dry-ice. 10μm thick sections were cut at -20°C, and fixed in 4% paraformaldehyde. Hybridisation buffer containing 45bp long radiolabelled oligoprobes, diluted to a concentration of 3000cpm/μl was added onto each slide, and hybridised in a humid atmosphere at 42°C overnight. Excess non-specifically bound probe was removed using stringency washes of 1×SSC at room temperature and 55°C, dehydrated in ethanol, air dried, and apposed to X-ray film for 7 days. Data was quantified by measuring the relative optical density of defined brain areas using an MCID image analyser, version M4 (Imaging Research Corp. Ontario, Canada). Following *in situ* hybridisation, slides of interest were coated with liquid emulsion, for 8 weeks, developed, and stained in Cresyl Violet.

αI mRNA exhibited a relatively ubiquitous distribution pattern, being expressed in the majority of brain areas examined, including the hippocampus, cortex, olfactory system, septum, basal ganglia, thalamus, cerebellum and brainstem, to varying degrees. αII mRNA was also widely expressed, but only at moderate levels in the basal ganglia, thalamic and midbrain areas, whilst almost no expression was detected in the brainstem nuclei. Relatively high levels of αVI expression was detected throughout the cerebral cortex, hippocampus, olfactory bulb and cerebellum, whilst moderate levels of αVI mRNA was also observed in all other brain areas and nuclei examined.

The voltage gated Na^+ channel subunits β1 and β2 demonstrated very different patterns of distribution. β1 subunit expression were detected in layers 4 and 5 of cortex, hippocampus, thalamus, and certain brainstem nuclei, with low levels found in the midbrain. Interestingly, no β1 mRNA expression was observed in the areas of the basal ganglia and septum. However, moderate levels of β2 mRNA were detected in virtually all brain areas examined.

In conclusion, we have performed a detailed quantitative analysis of all known CNS voltage gated Na^+ channel subunits. These data indicate that each subunit has a unique and discrete expression pattern. Of particular interest is the regionally specific distribution of the β1 subunit, which may be taken to infer that not all CNS voltage gated Na^+ channels contain this subunit.

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