British Journal of Pharmacology (1996) 119, 721-729

Mechanism of 5-hydroxytryptamine-induced coronary vasodilatation assessed by direct detection of nitric oxide production in guinea-pig isolated heart

Amanda J. Ellwood & [1]Michael J. Curtis

Cardiovascular Research Laboratories, Vascular Biology Research Centre, Department of Pharmacology, Division of Biomedical Sciences, King's College, University of London, Manresa Road, London SW3 6LX

1 We assessed whether a submaximal concentration (1 μM) of 5-hydroxytryptamine (5-HT) releases nitric oxide (NO) from the coronary endothelium in guinea-pig perfused heart ($n=5$ or 6/group) by direct detection of NO in coronary effluent, and determined whether this accounts for the associated coronary dilatation. We also tested whether saponin is a selective and specific tool for examining the role of this mechanism in mediating agonist-induced coronary dilatation.

2 Continuous 5 min perfusion with 5-HT, or acetylcholine (ACh; 1 μM), substance P (1 nM) or sodium nitroprusside (SNP; 1 μM) increased coronary flow from baseline by 3.6 ± 0.2, 3.4 ± 0.2, 1.8 ± 0.1 and 4.1 ± 0.2 ml min^{-1} g^{-1}, respectively (all $P<0.05$). Coronary effluent NO content, detected by chemiluminescence, was correspondingly increased from baseline by 715 ± 85, 920 ± 136, 1019 ± 58 and 2333 ± 114 pmol min^{-1} g^{-1}, respectively (all $P<0.05$).

3 Continuous perfusion for 30 min with N^G-nitro-L-arginine methyl ester (L-NAME) 100 μM reduced basal coronary effluent NO content by 370 ± 32 pmol min^{-1} g^{-1} and coronary flow by 7.5 ± 0.5 ml min^{-1} g^{-1} (both $P<0.05$). Saponin (three cycles of 2 min of 30 μg ml^{-1} saponin perfusion interrupted by 2 min control perfusion) reduced basal coronary NO content by a similar amount (307 ± 22 pmol min^{-1} g^{-1}) but reduced basal coronary flow by only 0.6 ± 0.2 ml min^{-1} g^{-1} ($P<0.05$ versus the effect of L-NAME).

4 The increases in coronary flow in response to (5-HT), ACh and substance P were reduced (all $P<0.05$) by 100 μM L-NAME to 1.2 ± 0.3, 1.2 ± 0.4 and 0.3 ± 0.3 ml min^{-1} g^{-1}, respectively. However, the flow increase in response to SNP was not reduced; it was in fact increased slightly to 4.8 ± 0.4 ml min^{-1} g^{-1} ($P<0.05$).

5 Similarly, after treatment with saponin, the increases in coronary flow in response to 5-HT, ACh and substance P were reduced to 2.1 ± 0.3, 1.3 ± 0.3 and 0.4 ± 0.2 ml min^{-1} g^{-1}, respectively (all $P<0.05$). Again, the response to SNP was increased slightly to 4.6 ± 0.5 ml min^{-1} g^{-1} ($P<0.05$).

6 L-NAME and saponin also inhibited 5-HT, ACh and substance P-induced NO release ($P<0.05$), without affecting equivalent responses to SNP.

7 For substance P, the change in coronary flow (ΔCF) correlated with $\log_{10}$ ΔNO in the presence and absence of saponin and L-NAME; $\Delta CF = 1.2(\log \Delta NO) - 1.9$; $r=0.92$; $P<0.05$. For 5-HT the relationship was $\Delta CF = 2.2(\log \Delta NO - 2.7$; $r=0.79$; $P<0.05$, indicating that 5-HT causes a disproportionately greater increase in coronary flow per release of NO. This was taken to indicate that 5-HT relaxes coronary vasculature in part by releasing NO, but in part by additional mechanisms. ACh resembled 5-HT in this respect.

8 Saponin had no effect on cardiac systolic or diastolic contractile function assessed by the construction of Starling curves with an isochoric intraventricular balloon.

9 In conclusion, despite its minimal effect on basal coronary flow, saponin is an effective tool for revealing endothelium-dependent actions of coronary vasodilator substances and has selectivity in that it does not impair endothelium-independent vasodilatation or cardiac contractile function. 5-HT dilates guinea-pig coronary arteries largely by the release of NO from the coronary endothelium.

Keywords: 5-Hydroxytryptamine; nitric oxide; coronary flow; endothelial ablation

Introduction

5-Hydroxytryptamine (5-HT) can constrict and dilate blood vessels (Vanhoutte, 1991). The constrictor effects may be mediated by 5-$HT_{1\text{-like}}$ and 5-HT_2 receptors. Synergism with other constrictors may also occur (Chester *et al.*, 1993). In the human heart the relative involvement of 5-$HT_{1\text{-like}}$ and 5-HT_2 receptors appears to vary according to the presence and absence of endothelial injury and also between individuals (Kaumann *et al.*, 1993). In the human heart, 5-HT can elicit coronary vasodilatation via 5-$HT_{1\text{-like}}$ receptor agonism (Connor *et al.*, 1989; Chester *et al.*, 1990; Kaumann *et al.*, 1993). Data from animal studies suggest that this results from stimulation of release of the endogenous vasodilator, nitric oxide (NO), from vascular endothelial cells (Griffith *et al.*, 1984; Cocks & Angus, 1983) although, in coronary arteries, this has not been substantiated by direct detection of NO (Vanhoutte, 1991).

Several important issues relating to the coronary actions of 5-HT remain to be resolved. It has not been proven, by direct detection of NO, that 5-HT-induced coronary dilatation is mediated by endothelially-derived NO. Concomitantly, it has not been proven that following endothelial injury, loss of 5-HT induced coronary dilatation is related to impairment of NO release from the coronary endothelium. We have addressed these issues in the present study.

[1] Author for correspondence.

A related problem is the difficulty in establishing a useful animal model with which to explore endothelium-dependent and independent coronary reactivity. Endothelial injury exposes sub-endothelial receptors to putative coronary constrictor autacoids such as 5-HT. Endothelial injury may be induced in isolated vessels by mechanical means (Furchgott & Zawadzki, 1980). However, isolated vessel experiments preclude the characterization of physiological and pathophysiological coronary vascular responsiveness with an intact tissue milieu. This is particularly important in the heart since coronary artery tone is regulated by mediators released locally by cardiac tissues (myocytes and nerves) such as adenosine, nitric oxide and others (Olsson *et al.*, 1992). Thus, a model was recently developed in which endothelium ablation could be produced by saponin in the intact isolated perfused heart (Mankad *et al.*, 1991). Perfusion with saponin damages endothelium by a detergent action (Samata *et al.*, 1986; Ren *et al.*, 1993), and inhibits endothelium-dependent vasodilatation (Mankad *et al.*, 1991). Saponin therefore appears to be a useful agent to allow characterization of endothelium-dependent and independent actions of 5-HT. However, a curious anomaly has emerged in that saponin has only a small effect on basal coronary flow, yet L-NAME (N^G-nitro-L-arginine methyl ester), a NO synthase blocker (Rees *et al.*, 1990), reduces basal coronary flow to a much greater extent (Kelm & Schrader, 1988; Ellwood & Curtis, 1995). The second objective of the present studies, therefore, was to address this problem.

We achieved our objectives by comparing, in isolated perfused hearts, the actions of saponin with those of L-NAME on (i) basal coronary flow and basal NO release, and (ii) coronary flow and NO release in hearts perfused with 5-HT and two other agents which relax coronary arteries primarily via endothelium-dependent actions (acetylcholine (ACh) and substance P), and a fourth agent (sodium nitroprusside (SNP)) that does not require an intact endothelium to relax vascular smooth muscle. Our premise was that if saponin and L-NAME inhibit endothelium-dependent dilatation by inhibiting NO release then, for each agonist (5-HT, ACh and substance P – but not SNP), there should be a correlation between the change in coronary flow and the change in NO. The extent and nature of this correlation was used to characterize the role of endothelium-dependent NO release in mediating the coronary dilator action of 5-HT. Some of the findings have been presented in a preliminary form to the British Pharmacological society (Ellwood & Curtis, 1995).

Methods

All experiments were performed in accordance with the United Kingdom Home Office 'Guide on the Operation of the Animals (Scientific Procedures) Act 1986'.

Male Dunkin Hartley guinea-pigs (Charles River, Kent, U.K., 300 – 350 g) were terminally anaesthetized with sodium pentobarbitone (60 mg kg^{-1}, i.p.) and heparinized (sodium heparin, 250 i.u., i.p.). A rapid thoracotomy was performed and the hearts quickly excised and placed in ice-cold modified Krebs solution containing (in mM): glucose 11.1, $CaCl_2$ 1.4, NaCl 118.5, $NaHCO_3$ 25.0, $MgSO_4$ 1.2, $NaHPO_4$ 1.2 and KCl 4.0. The calcium concentration, lower than that in true Krebs solution, reflects more closely free blood calcium in a variety of species (Galinanes & Hearse, 1990). The hearts were cannulated via the aorta (Langendorff mode) within 90 s of removal. Hearts were perfused under constant pressure (100 cmH_2O) perfusion with modified Krebs solution gassed with 95% O_2 and 5% CO_2, pH 7.4 and delivered at 37°C. All solutions were made with 'ultra pure' water (reverse osmosis, Milli RO-50, Millipore, Watford, U.K.) and vacuum filtered through 5 micron filter paper (Millipore), to remove particulate coronary spasmogens. To eliminate any effect that change in heart rate may have on coronary flow, hearts were paced at 275 beats min^{-1}, pulse width 0.5 ms, 1 pulse s^{-1} (Harvard student stimulator, Edenbridge, U.K.), via two stainless steel electrodes placed in the left ventricle. Heart rate was verified from a unipolar electrogram recorded from the left ventricle and displayed on a Grass polygraph (model RPS 7C8, Quincy, U.S.A.).

Coronary flow measurement

Coronary flow was measured by timed collection of coronary effluent over 30 s which was then weighed (1 ml = 1 g), by a Ohaus balance (Cambridge, U.K.), accurate to 1 mg (Rees & Curtis, 1993). Three consecutive measurements were taken during the final 90 s of drug perfusion and the mean was calculated. Coronary flow and change in coronary flow (ΔCF) were expressed as + or − ml min^{-1} g^{-1} wet weight of the ventricle. Individual ΔCF values were less variable than individual crude values of coronary flow. For example, in one heart a basal flow of 11.31 ml min^{-1} g^{-1}, was increased to 13.83 ml min^{-1} g^{-1} by an agonist, whereas in another heart, the same agonist increased basal flow from a lower basal value of 9.39 to 11.85 ml min^{-1} g^{-1}, yet ΔCF was almost identical in the two hearts (2.52 and 2.46 ml min^{-1} g^{-1}, respectively). Thus, calculation and use of ΔCF values reduced group s.e.mean values and allowed better precision for comparing differences between groups.

Following cannulation, the hearts were allowed to stabilize for 30 min or until 3 consecutive coronary flow measurements were within 0.4 ml min^{-1} g^{-1} of one another. The study protocol was then commenced. Hearts were excluded from the protocol if the coronary flow was found to be less than 8 ml min^{-1} g^{-1} at the beginning of the study protocol. All drugs were delivered continuously for a set time period (5 min). Hearts ($n = 5/6$, 4 groups) received one of the following agonists: 1 μM 5-HT (a submaximal concentration, determined from a separate group of hearts which received a continuous perfusion of 5-HT at 5 min increments from 0.001 to 10 μM), 1 μM ACh, 1 μM SNP or 1 nM substance P. This was followed by 15 min control (drug-free) perfusion, after which the response to the agonist was reassessed. The hearts were then perfused with either 100 μM L-NAME for 30 min, or 30 μg ml^{-1} saponin, delivered in 3 cycles (2 min of saponin followed by 2 min vehicle, repeated 3 times) followed by 15 min of control perfusion, then the effects of 1 μM 5-HT, 1 μM ACh, 1 μM SNP or 1 nM substance P were reassessed. The choice of saponin concentration was based on that used by Mankad *et al.* (1991). In preliminary studies we found that the same schedule, but with higher concentrations of saponin (50 or 60 μg ml^{-1}) caused cardiac contracture and irreversible ventricular fibrillation (indication of the production of vascular muscle and cardiac injury).

The response to a full concentration range of 5-HT following a saponin protocol was carried out in initial studies to assess the best concentration of 5-HT to use subsequently. However, full concentration-response studies were not practicable as the initial protocol required approximately 160 min to complete in each heart. Moreover, the possibility of desensitization following repeated administration of agonists, especially 5-HT (Ben-Harai *et al.*, 1991) necessitated minimization of the number of times each heart was exposed (we chose three times for each agonist). Although this precludes the estimation of pEC_{50} values, it improves the reliability of the data.

ACh and substance P were incorporated into the protocol to allow comparison of the effects with two other agents that dilate vascular smooth muscle in part by releasing NO. SNP was used as it dilates smooth muscle independently of endogenous NO release; its effects would not be expected to be reduced by L-NAME or saponin in contrast to the effects of ACh, substance P or 5-HT. Moreover, its use provided a test for whether saponin, when used, possessed selective actions on the coronary endothelium since inhibition of the actions of SNP by saponin would indicate that saponin had impaired the intrinsic ability of the coronary vasculature to relax (i.e., had caused smooth muscle damage).

Measurement of NO release by chemiluminescence

NO release into the coronary effluent was measured by the method described by Menon *et al.* (1989), with a Sievers NO analyser (NOA) model number 270B, (Dyson Instruments, Hetton, U.K.). This method is based on a gas phase chemiluminescence reaction between NO and ozone:

$$NO + O_3 \rightarrow NO_2 + O_2 + v$$

The chemiluminescence was detected by a photomultiplier. The output was recorded and processed by a Mac Lab computer (model 2E, AD instruments Hastings, U.K.).

A 1 ml aliquot of coronary effluent collected during the final minute of drug perfusion was decanted into a plastic eppendorf tube (BDH Laboratory supplies, Dagenham, U.K.) and immediately frozen in liquid nitrogen and stored at −20°C. Subsequently, the samples were thawed and analyzed. The NO in the coronary effluent undergoes rapid autoxidation to nitrite (Kelm & Schrader, 1990). Therefore the samples were injected into a purge vessel containing an acidified reducing solution (1% potassium iodide and glacial acetic acid) to liberate NO. An inert gas (helium) was used to purge the NO under reduced pressure into the nitric oxide analyzer. NO content of the samples was calculated by using a standard curve derived from reducing sodium nitrite in the purge vessel. Modified Krebs solution was used to make up the standards to allow for any contaminant nitrite present in the chemical constituents of the perfusion solution. NO content was expressed as pmol min^{-1} g^{-1} wet weight of perfused cardiac tissue (Pabla & Curtis, 1995). In preliminary studies we found that the agonists used produce a peak release of NO during 3.5 to 5 min after the start of administration. Therefore all NO data values presented refer to release during this 90 s period.

Contractility studies

In order to assess whether the actions of saponin are restricted to the coronary vasculature (as intended) or extend to the myocardium, the contractile function of guinea-pig hearts ($n=24$) was assessed. Impairment of contractile function would indicate that the saponin protocol (as described above) does not produce injury restricted to the vasculature. The hearts were randomly assigned to three groups, time control (drug-free solution), saponin protocol, or 5-HT (1 μM). We examined the actions of 5-HT in order to assess whether a positive inotropic action could be elicited since, although the hearts were not required to do any external work during the studies on coronary flow, an increase in inotropic activity could conceivably increase ATP utilization, and this might affect coronary flow (Olsson *et al.*, 1992). The hearts were prepared as above, except that a saline filled balloon made of domestic wrapping film was inserted into the left ventricle as previously described (Curtis *et al.*, 1986). The balloon was connected to a pressure transducer linked to a computer (MAC LAB, model 2E). The position of the balloon in the left ventricle was checked by slow inflation. This involved slowly injecting 0.05 ml of saline (0.9%, used to prevent growth of algae inside the balloon during long term use). This produced a sustained increase in systolic pressure. Saline was then withdrawn from the balloon until systolic pressure was zero. A Starling curve was then constructed by adding 0.02 ml increments of saline to the balloon at 1 min intervals until the systolic pressure plateaued (at about 0.3 ml added volume). The hearts were then perfused for 10 min at zero balloon volume, following which a second Starling curve was constructed. Hearts then received either Krebs alone, or 5-HT (1 μM) for 10 min, or the saponin protocol (as above). A final Starling curve was then constructed.

Drugs

ACh hydrochloride, 5-HT hydrochloride, substance P, SNP, L-NAME and saponin, were all obtained from Sigma Chemicals (Poole, U.K.). All drug solutions were made up as stock solutions of 1 mM in de-ionised water and stored at 4°C until used.

Statistics

Hearts were used as their own controls. This allows the use of the paired t test for statistical analysis. Other comparisons were made by use of an unpaired t test, modified by Dunnett's correction for multiple comparisons where appropriate. All data are expressed as mean ± s.e.mean. A P value <0.05 was considered to be statistically significant.

Results

Coronary flow and NO release with time during drug-free perfusion

In time matched controls, there was a small decline in coronary flow over time which was matched by a similar decline in NO release. At time zero (equivalent to just before the agonist protocol was begun in the experiment proper) the mean coronary flow and NO were 11.84±0.13 ml min^{-1} g^{-1} and 715±43 pmol min^{-1} g^{-1}, respectively. After a further 60 min (equivalent to the end of the experiment proper) coronary flow was 11.05±0.3 ml min^{-1} g^{-1} and NO was 655±46 pmol min^{-1} g^{-1}.

Effect of saponin and L-NAME on basal coronary flow and NO release

A 30 min period of perfusion with L-NAME (100 μM) caused a significant vasoconstriction ($P<0.05$), coronary flow falling from 11.4±0.4 to 7.5±0.5 ml min^{-1} g^{-1} (−33±1%) which was accompanied by a significant reduction in basal NO release, from 720±22 to 350±27 pmol min^{-1} g^{-1} (−51±4%, $P<0.05$, $n=21$). The saponin protocol caused only a small reduction in basal coronary flow (i.e., flow in the absence of added agonist) compared to the reduction caused by L-NAME. Values before and after saponin treatment were 11.2±0.3 ml min^{-1} g^{-1} and 10.6±0.3 ml min^{-1} g^{-1}, respectively (−6±1%, $n=24$, $P<0.05$). This was accompanied by a significant reduction in basal NO release in saponin treated

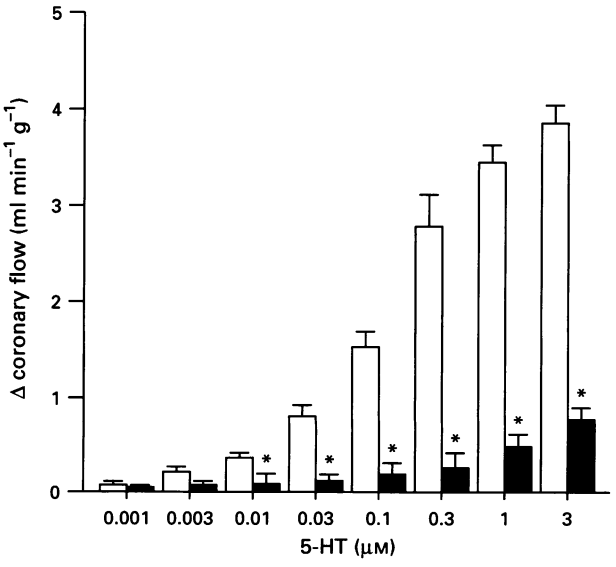

Figure 1 Changes in coronary flow (ΔCF ml min^{-1} g^{-1}) in response to 5-HT (0.001 to 3 μM). Values were recorded during the final 90 s of a 5 min exposure to 5-HT, in the absence (open columns) or presence of saponin pretreatment (solid columns). Data are mean ± s.e.mean, $n=5$ or 6. *$P<0.05$ versus no saponin.

hearts, from 710±22 to 403±22 pmol min^{-1} g^{-1} (−43±3%, $P<0.05$, $n=24$).

Effect of 5-HT, ACh, substance P and SNP on coronary flow and NO release

In initial experiments, 5-HT perfusion produced a concentration-dependent increase in coronary flow over the concentration range used (0.001 to 3 μM, Figure 1). However, this was not reproducible when the concentration-response relationship was re-examined 60 min later (data not shown), so only a single concentration of 5-HT was used in the experiment proper. In addition, a separate group of hearts was used to examine the concentration-response relationship to 5-HT following a saponin protocol. The vasodilator response to all concentrations of 5-HT was inhibited, e.g. at 1 μM 5-HT from 3.6±0.2 to 0.5±0.3 ml min^{-1} g^{-1} ($P<0.05$), but saponin did not unmask any 5-HT-induced coronary constriction (Figure 1).

Each agonist caused a significant increase in coronary flow ($P<0.05$) (Figure 2). The vasodilator responses to 5-HT, substance P and SNP were reproducible when examined for a second time, 15 min after the first exposure to drug, although the response to ACh was slightly enhanced e.g., from 3.5±0.1 to 4.1±0.2 ml min^{-1} g^{-1} ($P<0.05$). Thus, the single-concentration exposure protocol effectively avoided tachyphylaxis to the response to 5-HT and the other agonists that would have complicated interpretation of the effects of saponin and L-NAME.

Effects of saponin and L-NAME on agonist-induced changes in coronary flow and NO

The vasodilator responses to 1 μM ACh, 1 μM 5-HT and 1 nM substance P (Figure 2) were accompanied by release of NO into the coronary effluent (Figure 3, $P<0.05$). Neither saponin nor L-NAME had any effect on the vasodilator response to SNP (Figure 2), nor on the ability of SNP to elevate coronary effluent NO concentration (Figure 3). The responses to ACh and 5-HT were greatly attenuated by L-NAME. Moreover, despite its negligible effect on basal coronary flow, saponin had equivalent effects to L-NAME on agonist-induced vasodilatation and NO release. Nevertheless, there remained a small but measurable residual vasodilatation in response to both ACh and 5-HT after either saponin or L-NAME treatment. This contrasted with the vasodilator response to substance P which was almost completely abolished by both saponin and L-NAME. Despite these small differences in the inhibition of agonist-stimulated increases in coronary flow, substance P-, ACh- and 5-HT-stimulated increases in NO release were inhibited by saponin and by L-NAME treatment, each to a similar extent. The residual NO release was less than 100 pmol min^{-1} g^{-1} for all three agonists following saponin or L-NAME.

Relationship between NO release and coronary flow

For each of the agonists, there was a linear correlation ($P<0.01$) between log ΔNO and increase in coronary flow (ΔCF) in the presence and absence of saponin and L-NAME. For the individual agonists, the best correlation occurred with substance P: ΔCF = 1.2(log ΔNO)-1.9, $r=0.92$, $P<0.05$, (Figure 4a). The correlation for ACh was ΔCF = 1.4(log ΔNO)-1.3, $r=0.79$, $P<0.05$ (Figure 4b), and for 5-HT was: ΔCF = 2.2(log ΔNO)-2.7, $r=0.79$, $P<0.05$ (Figure 4c). These relationships demonstrate that for 5-HT there was a disproportionate increase in coronary flow in relation to the amount of NO released. In the case of SNP, the amount of NO released was greatly disproportional to the degree of vasodilatation produced, (compare Figure 2 with Figure 3). It was not appropriate to regress the relationship between ΔNO and ΔCF during perfusion with SNP since all values clustered around a point regardless of whether the hearts had been pretreated with saponin or L-NAME or neither.

Effect of saponin and 5-HT on cardiac contractility

Balloon inflation generated a typical Starling curve (Figure 5a). There was a time-dependent run down in cardiac contractility in control hearts, with a downward displacement of developed pressure throughout the whole Starling curve. With 0.02 ml added volume the developed pressure (systolic pressure minus end diastolic pressure) was 66±6 mmHg in the first

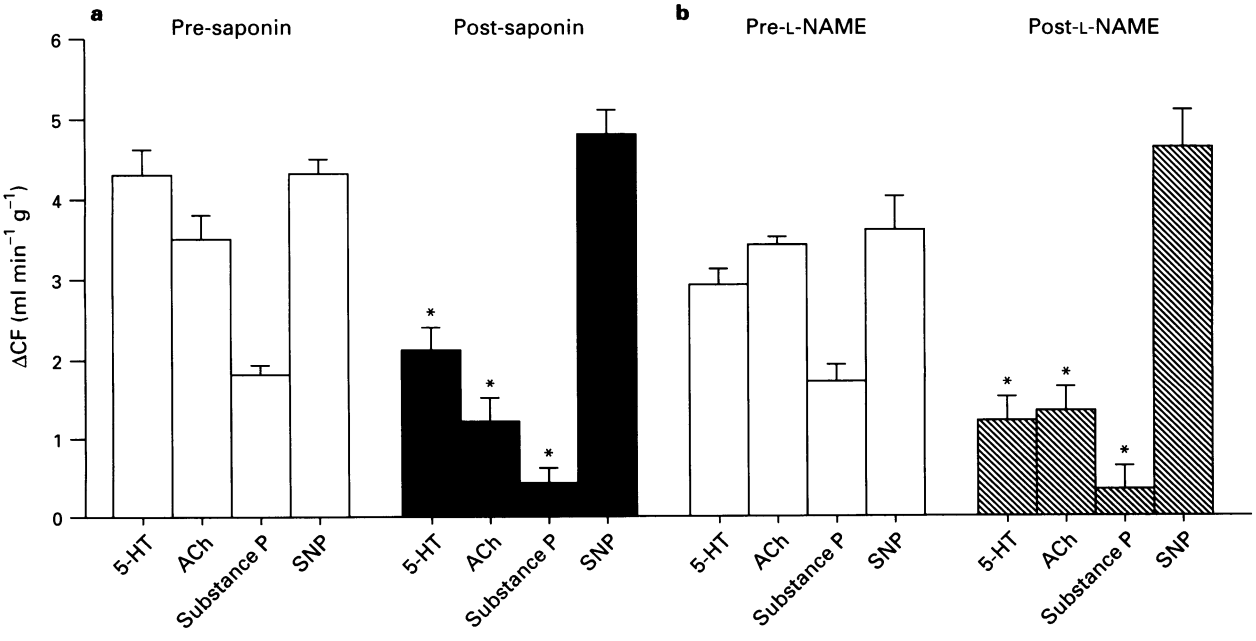

Figure 2 Changes in coronary flow (ΔCF, ml min^{-1} g^{-1}) in response to 5-HT (1 μM), acetylcholine (ACh, 1 μM), substance P (1 nM) and sodium nitroprusside (SNP, 1 μM). Values were recorded during the final 90 s of a 5 min exposure to the agonists before (open columns) and after treatment with (a) saponin (solid columns) or (b) L-NAME (cross-hatched columns). Data are mean±s.e.mean, $n=5$ or 6. *$P<0.05$ versus pre-saponin or pre-L-NAME values.

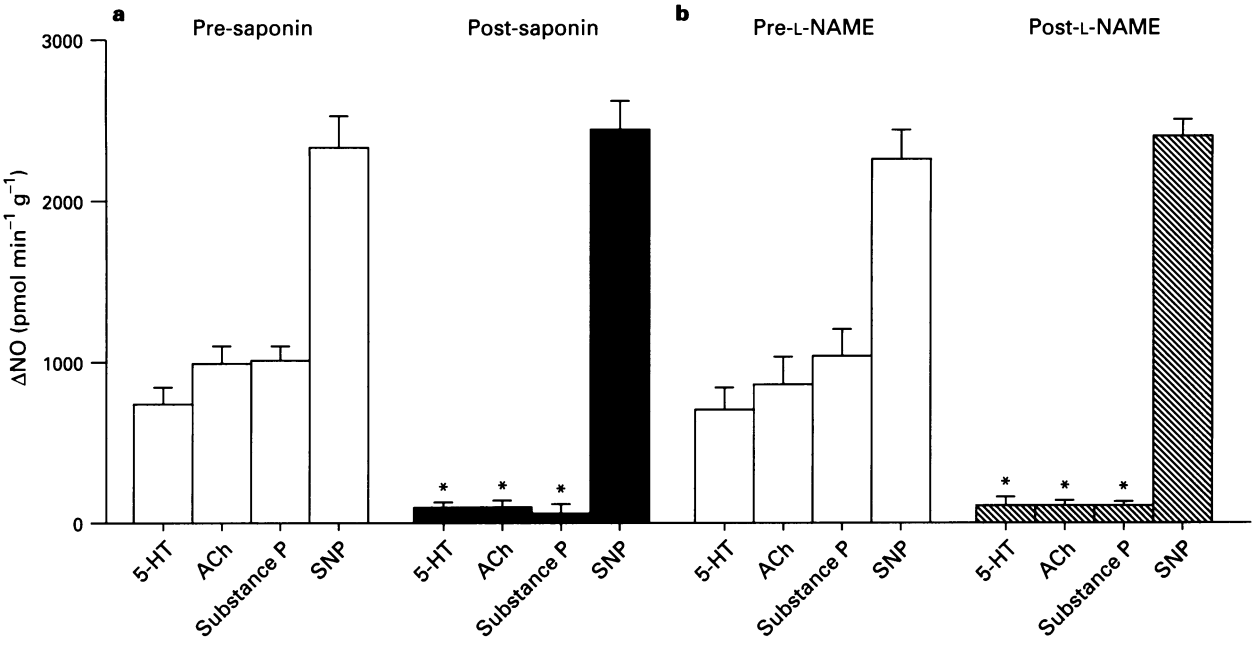

Figure 3 Changes in coronary NO content (ΔNO, pmol $min^{-1} g^{-1}$) in response to 5-HT (1 μM), acetylcholine (ACh, 1 μM), substance P (1 nM) and sodium nitroprusside (SNP, 1 μM). Values were recorded during the final 90 s of a 5 min exposure to the agonists, before (open columns) and after treatment with (a) saponin (solid columns) or (b) L-NAME (cross-hatched columns). Data are mean ± s.e.mean, $n = 5$ or 6. $^*P < 0.05$ versus pre-saponin or pre-L-NAME values.

Starling curve (time = 0 min), and 40 ± 5 mmHg in the third Starling curve (time = 60 min, $P < 0.05$). At the highest added volume of 0.3 ml the developed pressure in the first Starling curve was 127 ± 7 mmHg compared to 102 ± 5 mmHg in the third Starling curve ($P < 0.05$). For clarity only the second (time = 30 min) and the third Starling curves are shown for control, saponin-treated and 5-HT-treated groups (Figure 5a,b and c).

To account for the time-dependent run down in the developed pressure in the control group, and in the pre-drug Starling curves of the 5-HT and saponin treated groups (Figure 5b and c), a comparison was made between the change in developed pressure (ΔDVP) in the second Starling curve (time matched control) and the third curve (control, saponin or 5-HT) of the three groups. There was no difference between ΔDVP in saponin-treated hearts and ΔDVP in time matched control hearts (Figure 6). This indicates that the concentration of saponin used in this study had no effect on cardiac contractility. 5-HT had a positive inotropic effect. The ΔDVP in 5-HT-treated hearts was significantly different to the ΔDVP in time matched control hearts ($P < 0.05$, Figure 5). Diastolic function, as assessed from end diastolic pressure values was not affected by saponin or 5-HT (data not shown), indicating that saponin (and 5-HT) did not affect myocardial relaxation (lusitropy).

Discussion

In the present study we have explored the mechanism by which 5-HT dilates intact coronary vasculature, and how this is affected by coronary endothelial ablation. By comparing the actions of 5-HT with three other coronary vasodilators and measuring the release of NO into coronary effluent we sought to establish the role played by NO in mediating the actions of 5-HT, and examine how this role is affected by endothelial ablation.

Does 5-HT relax coronary vasculature by releasing NO?

Our first objective was to test whether 5-HT relaxes coronary vasculature by releasing NO from the coronary endothelium. In order to do this we compared the actions of 5-HT with two agonists known to be endothelium-dependent vasodilators, namely, ACh and substance P (Furchgott, 1983). All three agonists produced an increase in coronary flow which was accompanied by an increase in NO release measured by chemiluminescence. Although it has been suggested that 5-HT dilates coronary vessels, at least in part by the release of NO, this is the first direct evidence which demonstrates this to be the case. However, although 5-HT increases coronary flow and coronary NO content this is not sufficient evidence to prove that the latter is the sole cause of the former. For several reasons explained below, it appears that dilatation produced by 5-HT is not exclusively mediated by NO release. In order to explore this we investigated the effects of NO synthase blockade with L-NAME and endothelial ablation with saponin.

Is saponin a specific and selective tool for ablating coronary endothelium?

We used saponin as a tool to assess the role of the endothelium-dependent NO release in mediating the actions of 5-HT. Saponin is known to damage vascular endothelium (Shirasaki & Su, 1985; Samata *et al.*, 1986; Wiest *et al.*, 1989). However, insufficient damage would allow residual NO release to complicate the analysis of the action of a putative NO-releasing vasodilator such as 5-HT, whereas excessive exposure to saponin might be sufficient to damage the underlying smooth muscle or the myocardium, giving a false positive when assessing responses to 5-HT. We discounted the latter possibility by showing that saponin had no effect on cardiac contractility, assessed from Starling curves, and did not impair the ability of SNP to cause coronary vasodilatation. However, the effects of saponin on coronary flow and NO release were complex and the possibility of inadequate endothelial ablation was further considered, due to the much smaller reduction in coronary flow caused by saponin compared to L-NAME.

Following treatment with saponin there was a reduction in basal release of NO, although this was less than the reduction seen following 30 min perfusion with L-NAME. Although this difference may be due to the ability of L-NAME but not saponin to inhibit NO release from sources additional to endothelium, such as cardiac nerve fibres (Klimaschewski *et al.*,

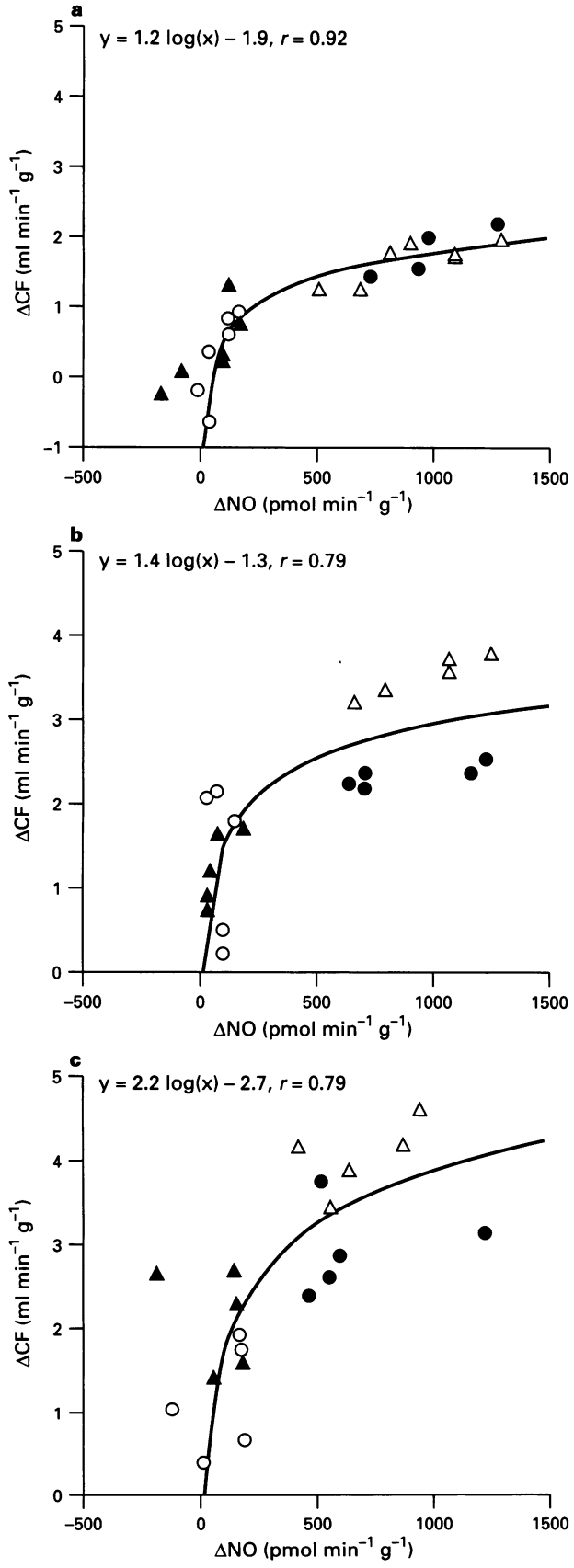

Figure 4 Regression analysis of change in coronary flow from baseline (ΔCF) versus change in NO from baseline (ΔNO) for control (pre-saponin, △, pre-L-NAME, ●), 30 μg ml^{-1} saponin-treated (▲) and 100 μM L-NAME treated (○) hearts in response to (a) substance P, (b) ACh and (c) 5-HT. Data points represent values for individual hearts measured 5 min after administration of each agonist.

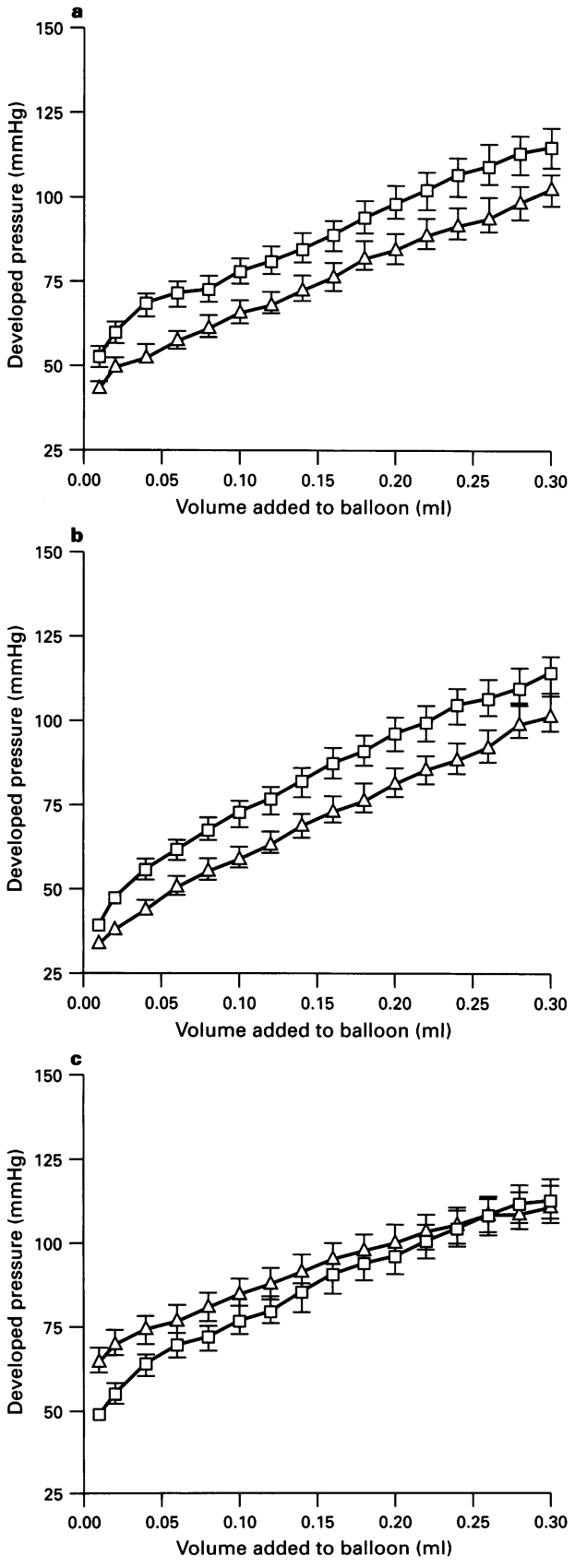

Figure 5 The change in cardiac contractility (expressed as developed pressure) with time in (a) control perfused hearts (Krebs only) at perfusion time = 30 min (□) and perfusion time = 60 min (△), (b) saponin-perfused hearts at perfusion time = 30 min (time matched control, □) and perfusion time = 60 min (30 μg ml^{-1} saponin, △) and (c) 5-HT perfused hearts at perfusion time = 30 min (time matched control, □) and perfusion time = 60 min (1 μM 5-HT, △). Data shown are means and vertical lines indicate s.e.mean.

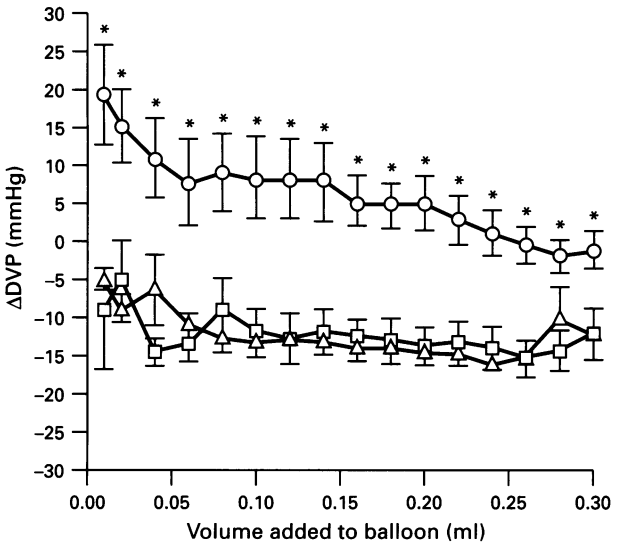

Figure 6 The effects of 30 μg ml^{-1} saponin (△) and 1 μM 5-HT(○) on cardiac contractility compared to control (□). Data are expressed as change in developed pressure (ΔDVP) at 30 min perfusion time minus equivalent values after 60 min perfusion. *$P<0.05$ versus control values; vertical lines indicate s.e.mean.

1992), it may indicate that saponin did not completely ablate the coronary endothelium, with residual release of NO from the portion of the endothelium that remained intact. However, this appears unlikely, since saponin almost completely abolished vasodilatation and NO release in response to substance P. Substance P, at the concentration we used, is regarded as having a relatively selective ability to relax vascular smooth muscle by releasing NO (Enokibori *et al.*, 1994; Kilpatrick & Cocks, 1994). This confirms that saponin did induce substantial endothelial damage and that the damage was selective.

If both saponin and L-NAME inhibit agonist-induced coronary vasodilatation by reducing endothelium-dependent NO release, then both agents ought to possess quantitatively similar actions on agonist-induced changes in coronary flow and NO release. This was found to be the case. The actions of saponin and L-NAME on agonist-induced changes in coronary flow were almost identical (Figure 1) as were their effects on NO release (Figure 2).

Thus, the saponin protocol used fulfilled the criteria necessary for it to qualify as an effective (specific and selective) tool for ablating endothelium- (and NO-) dependent coronary vasodilatation. Moreover, its actions and the actions of L-NAME on responses to 5-HT confirmed that the coronary dilator effects of 5-HT were mediated primarily by the release of NO from the coronary endothelium.

Actions of saponin and L-NAME on basal versus stimulated coronary flow and NO changes

Saponin caused a small but significant reduction in baseline coronary flow whereas L-NAME caused a large reduction in baseline coronary flow. A failure of saponin to ablate the coronary endothelium can be ruled out as an explanation for this, on the basis of the data discussed above. The concentration of saponin used (30 μg ml^{-1}) has been found by others to have no effect on baseline coronary flow (Mankad *et al.*, 1991), although a higher concentration (50 μg ml^{-1}) has been shown to reduce coronary flow in potassium arrested perfused hearts (Wiest *et al.*, 1989). One possible explanation for our findings is that L-NAME can reduce basal coronary flow by a combination of inhibition of endothelium-dependent NO release and some additional (NO-independent) mechanism.

NO synthase inhibitors have been shown consistently to increase vascular tone in isolated tissues (Rees *et al.*, 1989), perfused organs (Mankad *et al.*, 1991; Smith *et al.*, 1992) and *in vivo* (Benyo *et al.*, 1991). However, unpublished findings from our laboratory have demonstrated that coronary vasoconstriction to 100 μM L-NAME occurs before there is significant inhibition of NO release in rat isolated hearts. This supports the suggestion that the concentration of L-NAME chosen in the present study may have additional actions which are independent of the inhibition of NO synthase. In further support of this, Lippton *et al.* (1992) have shown that L-NAME can cause a significant vasoconstriction at concentrations below those sufficient to inhibit NO-dependent vasodilation. It was not our objective to establish the identity of these mechanisms, although further studies may be of future interest. In this connection, it has been suggested previously that L-NAME can act as a muscarinic antagonist (Buxton *et al.*, 1993).

Does 5-HT cause vasodilatation exclusively by releasing NO?

Although 5-HT clearly releases NO and this contributes substantially to the associated coronary vasodilatation, this may not be the only mechanism by which 5-HT dilated coronary arteries. It has been recently shown in rabbit external jugular vein that an endothelial 5-HT receptor exists which mediates vasodilatation independently of NO release (Browning ,*et al.*, 1995). Furthermore, 5-HT receptors which mediate vasodilatation are also present in vascular smooth muscle (Browning *et al.*, 1995). In the present study we compared the profile of flow and NO changes in response to 5-HT, before and after endothelial ablation and NO synthase inhibition, with the profile of changes caused by ACh, substance P and SNP. The lack of effect of saponin on the response to SNP shows that saponin had no direct effect on the coronary vasculature smooth muscle. This, and the lack of effect of L-NAME on the response to SNP, confirmed that neither L-NAME nor saponin affected the intrinsic ability of the coronary vasculature to relax. Thus the changes in NO and vascular reactivity in response to 5-HT following saponin and L-NAME cannot be attributed to altered vascular reactivity.

At the concentration used (1 nM), substance P dilates vessels almost exclusively via the release of NO (Enokibori *et al.*, 1994; Kilpatrick & Cocks, 1994) and responses to substance P are more sensitive to blockade by L-NAME than responses to ACh (Rees *et al.*, 1989). Consistent with this, the present study provided evidence for a cause and effect relationship between ΔNO and ΔCF in response to substance P, since the increase in coronary flow and NO were each completely abolished by both saponin and L-NAME. This contrasts with the response to 5-HT where there was a small residual increase in coronary flow despite the almost complete inhibition of stimulated NO release in hearts treated with saponin or L-NAME. Regression analysis showed that there was proportionately greater vasodilatation for the amount of NO released in response to 5-HT compared with substance P. If it is accepted that substance P at the concentration used dilates coronary arteries exclusively by releasing NO, as the present and published data suggests, this confirms that mechanisms in addition to NO release are involved in the vasodilator response to 5-HT in guinea-pig hearts. The existence of a residual response to 5-HT after saponin pretreatment and the disproportionate increase in flow in relation to ΔNO, even when the endothelium had not been ablated, means that 5-HT does not cause coronary vasodilatation exclusively by releasing NO, regardless of whether the endothelium is intact or denuded.

Actions of ACh and SNP

Although the actions of ACh and SNP *per se* did not represent a major focus of the present study, certain observations require consideration.

Data for ACh were qualitatively and quantitatively similar to data for 5-HT. Following treatment with saponin and L-NAME a residual vasodilator response to ACh was present despite almost complete inhibition of ACh-stimulated NO release. This is broadly consistent with data from Ward and Angus (1993).

Possible explanations for the residual vasodilator response to ACh include endothelium-dependent actions, such as release of an endothelium-dependent hyperpolarizing factor (Komura *et al.*, 1991) or prostanoids (Ignarro *et al.*, 1987), and endothelium-independent actions (Xie & Triggle, 1994), including activation of calcium-dependent potassium channels leading to hyperpolarization (Tare *et al.*, 1990; Chen & Cheung, 1992; Garland & McPherson, 1992; Lefroy *et al.*, 1993; Waldron & Garland, 1994; Plane *et al.*, 1995).

The vasodilator response to the endothelium-independent vasodilator SNP was enhanced by both saponin and L-NAME. On the basis of similar findings (Shirasaki & Su, 1985; Smith *et al.*, 1992), it has been suggested (Moncada *et al.*, 1991) that this effect may be due to a specific supersensitivity to the action of nitrovasodilators caused by the inhibition of basal NO release.

Effects of saponin and 5-HT on contractile function

Cardiac contractile function was measured in part to assess whether the effects of saponin were restricted to actions on the coronary vascular endothelium. Saponin had no effect on systolic or diastolic function, so any possibility that actions on responses to 5-HT, ACh and substance P resulted from changes in contractile function can be discounted. 5-HT itself had a small but significant positive inotropic action. It is conceivable that by increasing systolic function, 5-HT may cause the ventricle to utilize more ATP, leading to coronary vasodilatation via the action of the ATP metabolite, adenosine (Olsson *et al.*, 1992). It is therefore possible that part of the coronary vasodilatation produced by 5-HT that is disproportionate to NO release may be mediated by this mechanism. However, the differences in metabolic demand that exist between the loaded heart (balloon containing) and the unloaded heart may render this mechanism unimportant in the unloaded heart (i.e., the preparation we used for examining coronary flow changes). Moreover, the small effects of 5-HT on contractile function could be revealed only when time-dependent run-down had been taken into account, so the likelihood that the effects were sufficiently substantial to influence coronary autoregulation is not great.

Limitations of the present study

The model described herein may be utilized for examining the mechanism controlling 5-HT- (and other agonist-) induced coronary dilatation and its loss following endothelial ablation. However, despite evidence that 5-HT may constrict coronary arteries after endothelium damage in man (Chester *et al.*, 1990; McFadden *et al.*, 1991), we were unable to demonstrate coronary constriction in response to 5-HT in the guinea-pig heart even after the administration of saponin and L-NAME. This means that the model is not suitable for examining the mechanism responsible for 5-HT-induced coronary vasospasm. Clearly the inhibition by saponin and L-NAME of the coronary dilator and NO-releasing effects of 5-HT, ACh and substance P precludes the possibility that coronary constriction in response to 5-HT was masked by endothelium-dependent NO-dependent vasodilatation, so it would appear that either the receptors that mediate 5-HT-induced coronary constriction are not present in guinea-pig coronary vasculature, or that 5-HT-induced coronary constriction can be masked by an uncharacterized endothelium-independent (NO-independent) coronary vasodilator mechanism. Further work will be necessary to establish which of these possibilities is correct.

Conclusion

We have shown for the first time direct evidence (by the measurement of NO release and the inhibition of NO release by saponin and L-NAME) that 5-HT does indeed cause coronary vasodilatation by the release of NO. However, it is apparent that this is probably only one of several mechanisms which may operate in mediating the coronary actions of 5-HT in the guinea-pig isolated heart. It was not the objective of the present study to identify the nature of all these mechanisms, only the possibility of their existence and their importance relative to NO release in our chosen model. Their existence was demonstrated but their importance is evidently small relative to endothelium-dependent NO release. A saponin perfusion protocol was shown to represent a specific method for selectively ablating the coronary endothelium, despite the lack of its effect on basal coronary flow. This method may therefore be utilized in further studies to characterize the coronary actions and mechanisms of action of 5-HT. The ability of L-NAME to reduce basal coronary flow contrasts with the lack of effect of saponin, despite the ability of both L-NAME and saponin to inhibit agonist-stimulated NO release to a similar extent. This implies that L-NAME may reduce basal coronary flow by an NO-independent mechanism, the nature of which remains to be determined.

The authors thank the B.B.S.R.C., and Pfizer U.K. (Sandwich, Kent) for financial support of this project. A.J.E. is a B.B.S.R.C.-CASE Award student.

References

BEN-HARAI, R.R., DALTON, B.A., OSMAN, R. & MAAYANI, S. (1991). Kinetic characterization of 5-hydroxytryptamine receptor desensitisation in isolated guinea-pig trachea and rabbit aorta. *J. Pharmacol. Exp. Ther.*, **257**, 416–424.

BENYO, Z., KISS, G., SZABO, C., CSAKI, C. & KOVACH, A.G.B. (1991). Importance of basal nitric oxide synthesis in regulation of myocardial blood flow. *Cardiovasc. Res.*, **25**, 700–703.

BROWNING, C., GILES, H. & MARTIN, G.R. (1995). The use of phenoxybenzamine to discriminate 5-HT receptors mediating endothelium-dependent and -independent vasorelaxation. *Br. J. Pharmacol.*, **116**, 262P.

BUXTON, I.L.O., CHEEK, D.J., ECKMAN, D., WESTFALL, D.P., SANDERS, K.M. & KEEF, K.D. (1993). N^G-nitro L-arginine methyl ester and other alkyl esters of arginine are muscarinic receptor antagonists. *Circ. Res.*, **72**, 387–395.

CHEN, G. & CHEUNG, D.W. (1992). Characterisation of acetylcholine-induced membrane hyperpolarisation in endothelial cells. *Circ. Res.*, **70**, 257–263.

CHESTER, A.H., ALLEN, S.P., TADJKARIMI, S. & YACOUB, M.H. (1993). Interaction between thromboxane A_2 and 5-hydroxytryptamine receptor subtypes in human coronary arteries. *Circulation*, **87**, 874–880.

COCKS, T.M. & ANGUS, J.A. (1983). Endothelium-dependent relaxation of coronary arteries by nonadrenaline and serotonin. *Nature*, **305**, 627–630.

CONNOR, H.C., FENIUK, W. & HUMPHREY, P.P.A. (1989). 5-Hydroxytryptamine contracts human coronary arteries predominantly via 5-HT_2 receptor activation. *Eur. J. Pharmacol.*, **161**, 91–94.

CURTIS, M.J., MACLEOD, B.A., TABRIZCHI, R. & WALKER, M.J.A. (1986). An improved perfusion apparatus for small animal hearts. *J. Pharmacol. Methods*, **15**, 87–94.

ELLWOOD, A.J. & CURTIS, M.J. (1995). Effect of saponin versus L-NAME on the relationship between coronary flow and nitric oxide production under basal and agonist-stimulated conditions in guinea-pig isolated heart. *Br. J. Pharmacol.*, **116**, 62P.

ENOKIBORI, M., OKAMURA, T. & TODA, N. (1994). Mechanism of underlying substance P-induced relaxation in dog isolated superficial temporal arteries. *Br. J. Pharmacol.*, **111**, 77–82.

FURCHGOTT, R.F. (1983). Role of the endothelium in responses of vascular smooth muscle. *Circ. Res.*, **53**, 557–573.

FURCHGOTT, R.F. & ZAWADZKI, J.V. (1980). The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature*, **286**, 373–376.

GALINANES, M. & HEARSE, D.J. (1990). Species differences in susceptibility to ischemic injury and response to myocardial protection. *Cardioscience*, **2**, 127–142.

GARLAND, C.J. & MCPHERSON, G.A. (1992). Evidence that nitric oxide does not mediate hyperpolarisation and relaxation to acetylcholine in the rat small mesenteric artery. *Br. J. Pharmacol.*, **105**, 429–435.

GRIFFITH, T.M., HENDERSON, A.H., EDWARDS, D.H. & LEWIS, M.J. (1984). Coronary constriction: role of endothelium. *J. Physiol.*, **351**, 13–24.

IGNARRO, L.J., BYRNS, R.F., BUGA, G.M. & WOODS, K.S. (1987). Mechanisms of endothelium-dependent vascular smooth muscle relaxation elicited by bradykinin and VIP. *Am. J. Physiol.*, **253**, H1074–H1082.

KAUMANN, A.J., PARSONS, A.A. & BROWN, A.M. (1993). Human arterial constrictor serotonin receptors. *Cardiovasc. Res.*, **27**, 2094–2103.

KELM, M. & SCHRADER, J. (1988). Nitric oxide release from isolated guinea-pig heart. *Eur. J. Pharmacol.*, **155**, 317–321.

KELM, M. & SCHRADER, J. (1990). Control of coronary vascular tone by nitric oxide. *Circ. Res.*, **66**, 1561–1575.

KILPATRICK, E.V. & COCKS, T.M. (1994). Evidence for differential roles of nitric oxide (NO) and hyperpolarisation in endothelium-dependent relaxation of pig coronary artery. *Br. J. Pharmacol.*, **112**, 557–565.

KLIMASCHEWSKI, L., KUMMER, W., MAYER, B., COURAUD, J.Y., PREISSLER, U., PHILIPPIN, B. & HEYN, C. (1992). Nitric oxide synthase in cardiac nerve fibres and neurones of rat and guinea-pig heart. *Circ. Res.*, **71**, 1533–1537.

KOMURA, T., LAMPING, K.G., EASTMAN, C.L., HARRISON, D.G., MARCUS, M.L. & DELLSPERGER, K.C. (1991). Effect of an arginine analogue on acetylcholine-induced coronary vascular dilatation in dogs. *Am. J. Physiol.*, **261**, H2001–H2007.

LEFROY, D.C., CRAKE, T., UREN, N.G., DAVIES, G.J. & MASERI, A. (1993). Effect of inhibition of nitric oxide synthase on epicardial coronary artery caliber and coronary blood flow in humans. *Circulation*, **88**, 43–54.

LIPPTON, H.L., QINGZHONG, H. & HYMAN, A. (1992). L-NAME enhances pulmonary vasoconstriction without inhibiting EDRF-dependent vasodilatation. *Am. J. Physiol.*, **73**, 2432–2439.

MANKAD, P.S., CHESTER, A.H. & YACOUB, M.H. (1991). 5-Hydroxytryptamine mediates endothelium-dependent coronary vasodilatation in the isolated rat heart by the release of nitric oxide. *Cardiovasc. Res.*, **25**, 244–248.

MCFADDEN, E.P., CLARKE, J.G., DAVIES, G.J., KASKI, J.C., HAIDER, A.W. & MASERI, A. (1991). Effect of intracoronary serotonin on coronary vessels in patients with stable angina and patients with variant angina. *New Engl. J. Med.*, **324**, 648–654.

MENON, M.K., WOLF, A., ZEHETGRUBER, M. & BING, R.J. (1989). An improved chemiluminescence assay suggests non nitric oxide-mediated action of lysophosphatidylcholine and acetylcholine. *Proc. Soc. Exp. Med. Biol.*, **191**, 316–319.

MONCADA, S., REES, D.D., SCHULZ, R. & PALMER, R.M.J. (1991). Development and mechanism of a specific super sensitivity to nitrovasodilators after the inhibition of vascular nitric oxide synthesis *in vivo*. *Proc. Natl. Acad. Sci. U.S.A.*, **88**, 2166–2170.

OLSSON, R.A., BURINGER, R. & SPAAN, J.A.E. (1992). Coronary circulation. In *The Heart and Cardiovascular System*: 2nd edition ed. Fozard, H.A., Haber, T., Jennings, R.B., Kutz, A.M. & Morgan, H.E. pp. 1393–1426. New York: Raven Press.

PABLA, R. & CURTIS, M.J. (1995). Effects of nitric oxide modulation on cardiac arrhythmias in the rat isolated heart. *Circ. Res.*, **77**, 984–992.

PLANE, F., PEARSON, T. & GARLAND, C.J. (1995). Multiple pathways of underlying endothelial-dependent relaxation in the rabbit isolated femoral artery. *Br. J. Pharmacol.*, **115**, 31–38.

REES, D.D., PALMER, R.M.J., HODSON, H.F. & MONCADA, S. (1989). A specific inhibitor of nitric oxide formation from L-arginine attenuates endothelium-dependent relaxation. *Br. J. Pharmacol.*, **96**, 418–424.

REES, D.D., PALMER, R.M.J., SCHULZ, R., HODSON, H.F. & MONCADA, S. (1990). Characterisation of the three inhibitors of endothelial nitric oxide synthase *in vitro* and *in vivo*. *Br. J. Pharmacol.*, **101**, 746–752.

REES, S.A. & CURTIS, M.J. (1993). Selective IK blockade as an anti-arrhythmic mechanism in ischaemic heart disease: Effects of UK 66-914 in the rat and rabbit. *Br. J. Pharmacol.*, **108**, 139–145.

REN, L.M., NAKANE, T. & CHIBA, S. (1993). Muscarinic receptor subtypes mediating vasodilation and vasoconstriction in isolated, perfused simian coronary arteries. *J. Cardiovasc. Pharmacol.*, **22**, 841–846.

SAMATA, K., KIMURA, T., SATOH, S. & WATANABE, H. (1986). Chemical removal of the endothelium by saponin in the isolated dog femoral artery. *Eur. J. Pharmacol.*, **128**, 85–91.

SHIRASAKI, Y. & SU, C. (1985). Endothelium removal augments vasodilation by sodium nitroprusside and sodium nitrite. *Eur. J. Pharmacol.*, **114**, 93–96.

SMITH, R.E.A., PALMER, R.M.J., BUCKNALL, C.A. & MONCADA, S. (1992). Role of nitric oxide synthesis in the regulation of coronary vascular tone in the isolated perfused rabbit heart. *Cardiovasc. Res.*, **26**, 508–512.

TARE, M., PARKINGTON, H.C., COLEMAN, H.A., NEILD, T.O. & DUSTING, G.J. (1990). Hyperpolarisation and relaxation of arterial smooth muscle caused by nitric oxide derived from endothelium. *Nature*, **346**, 69–71.

VANHOUTTE, P.M. (1991). Platelet-derived serotonin, the endothelium and cardiovascular disease. *J. Cardiovasc. Pharmacol.*, **17** (supp 5), S6–S12.

WALDRON, G.J. & GARLAND, C.J. (1994). Contribution of both nitric oxide and a change in membrane potential in acetylcholine-induced relaxation in rat small mesenteric artery. *Br. J. Pharmacol.*, **112**, 831–836.

WARD, J.E. & ANGUS, J.A. (1993). Acute and chronic inhibition of nitric oxide synthase in conscious rabbits: Role of nitric oxide in the control of vascular tone. *J. Cardiovasc. Pharmacol.*, **21**, 804–814.

WIEST, E., TRACH, V. & DAMMGEN, J. (1989). Removal of endothelial function in coronary resistance vessels by saponin. *Basic. Res. Cardiol.*, **84**, 469–478.

XIE, H. & TRIGGLE, C.R. (1994). Endothelium-independent relaxations to acetylcholine and A23187 in human umbilical artery. *J. Vasc. Res.*, **31**, 92–105.

(Received June 20, 1996
Accepted July 26, 1996)