Potentialiation by endothelin-1 of 5-hydroxytryptamine responses in aortae from streptozotocin-diabetic rats: a role for thromboxane A₂

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1 We have previously reported maximum responses to 5-hydroxytryptamine (5-HT) are diminished in endothelium-intact and -denuded aortae from rats with streptozotocin-induced diabetes of 2-weeks duration.

2 In the present study, the thromboxane A₂/prostaglandin H₂ (TP) receptor antagonist GR32191B (1 µM) significantly reduced maximum responses to 5-HT in endothelium-intact aortae from both control and diabetic rats. In the presence of GR32191B, maximum responses to 5-HT, in endothelium-intact aortae from diabetic rats, were still significantly reduced compared to those obtained in aortae from controls.

3 GR32191B (1 µM) had no significant effect on maximum responses to 5-HT in endothelium-denuded aortae from either control or diabetic rats.

4 Interaction between 5-HT (0.1 µM–0.1 mM) and threshold concentrations of endothelin-1 (ET-1) or the thromboxane (Tx)A₂-mimetic, U46619, were examined in endothelium-intact and -denuded aortae from control and 2-week streptozotocin-diabetic rats.

5 Maximum responses to 5-HT in the presence of a threshold concentration of ET-1 (3 nM), in endothelium-intact aortae from diabetic rats, were not significantly different from those of control rats.

6 Maximum responses to 5-HT in the combined presence of ET-1 (3 nM) and GR32191B (1 µM), in endothelium-intact aortae from diabetic rats, were significantly reduced compared to those obtained in aortae from controls.

7 Maximum responses to 5-HT in the presence of ET-1 (3 nM) in endothelium-denuded aortae from diabetic rats were significantly reduced compared to those from controls.

8 Maximum responses to 5-HT in the presence of a threshold concentration of U46619 (20 or 30 nM), in endothelium-intact aortae from diabetic rats, were not significantly different from responses of controls.

9 Maximum responses to 5-HT in the presence of a threshold (5–20 nM) concentration of U46619, in endothelium-denuded aortae from diabetic rats, were not significantly different from responses of controls.

10 The results of the present study indicate that endothelial-derived TxA₂ contributes to the contractile response to 5-HT in aortae from control and diabetic rats. Endothelial-derived TxA₂ also appears to play a role in the potentiation of 5-HT responses by ET-1 in aortae from diabetic rats.

Keywords: Endothelin-1; 5-hydroxytryptamine; thromboxane A₂; diabetes; rat aortae

Introduction

It has been previously reported that addition of a threshold concentration of 5-hydroxytryptamine (5-HT) potentiates subsequent contraction induced by endothelin-1 (ET-1). However, a threshold concentration of ET-1 did not potentiate the contractile effect of 5-HT (Yang *et al.*, 1992). We have previously shown that maximum responses to 5-HT were diminished in aortae from streptozotocin (STZ)-diabetic rats of 2- and 6-weeks duration (Sikorski *et al.*, 1993; James *et al.*, 1994). The present study was undertaken to determine whether there was an interaction between 5-HT and ET-1 in aortae from STZ-diabetic rats and whether any interaction observed had an effect on the altered responsiveness previously observed to 5-HT in aortae from diabetic rats.

Methods

Male Wistar rats (270–490 g) were treated with STZ (60 mg kg⁻¹, i.v.) or vehicle (50 mM citrate buffer) under 4% halo-

thane anaesthesia (O₂/N₂O 2:1) as previously described (Fulton *et al.*, 1991; Hodgson & King, 1992). The animals were then housed in treatment pairs, being allowed free access to food and water at all times. Only rats displaying elevated blood glucose levels (>16 mM, Ames Minilab 1) after 2-weeks were considered to be diabetic. Control rats had normal (4–9 mM) blood glucose levels over the 2-week period. After 2 weeks, a STZ-diabetic and a control animal were killed and 5 mm rings cut from each descending thoracic aorta. Where indicated, endothelial cells were removed by gentle rubbing of the intimal surface with a thin wire. Rings were placed under 10 g resting tension in 15 ml organ baths containing Krebs solution of the following composition (mM): NaCl 118.4, KCl 4.7, CaCl₂ 2.5, NaHCO₃ 25.0, KH₂PO₄ 1.2, MgSO₄·7H₂O 1.2, glucose 11.1. Physiological solutions were maintained at 37°C, and bubbled with carbogen (5% CO₂ in O₂). After 1 h equilibration, a submaximal concentration of phenylephrine (0.3 µM) was added to the bath. At the plateau of contraction, acetylcholine (10 µM) was added and the presence of functional endothelial cells was indicated by subsequent relaxation. A threshold concentration of ET-1 (3 nM) or U46619 (20 or 30 nM) was then

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added to the organ bath and allowed to equilibrate for 5 min or until the response plateaued. A cumulative concentration-response curve to 5-HT was then obtained. 5-HT was added at intervals of between 3–10 min depending on the time taken for the previous response to reach a plateau. A similar procedure was followed for endothelium-denuded aortae using threshold concentrations of ET-1 (2 nM control; 3 nM diabetic) or U46619 (5–20 nM). Where indicated concentration-response curves to 5-HT were obtained in the presence of the thromboxane A_2 /prostaglandin H_2 (TP) receptor antagonist GR32191B, 1 μ M, a concentration previously shown to be selective for TP receptors in the rat aorta (Lumley *et al.*, 1989). GR32191B was added after the test for functional endothelium and allowed to equilibrate for 30 min before the start of the concentration-response curve. Only a single concentration-response curve to one agonist was obtained in each tissue. Contractions were recorded on a Grass polygraph (Model 79E).

After each experiment, aortic rings were oven dried (50°C) and then weighed. The order of killing STZ-diabetic and control rats was alternated between days, and the arrangement of tissues in organ baths randomized.

Drugs

The following drugs were used: acetylcholine chloride (Sigma), endothelin-1 (Auspep), GR32191B [1R-[1 α (Z),2 β ,3 β ,5 α]]-($\pm$)-7-[5-[[[1,1'-biphenyl]-4-yl] methoxy]-3-hydroxy-2-(1-piperidinyl)cyclopentyl]-4-heptenoic acid (Glaxo), 5-hydroxytryptamine creatine sulphate (Sigma), phenylephrine HCl (ICN Pharmaceutical), streptozotocin (Sigma) and U46619 ((15S)-hydroxy-11 α , 9 α -(epoxymethano) prosta-5Z, 13E-dienoic acid) (Upjohn).

Phenylephrine was dissolved in catecholamine diluent (0.312 g NaH_2PO_4 and 0.08 g ascorbic acid per litre of 0.9% (w/v) saline). Acetylcholine, 5-hydroxytryptamine and GR32191B were dissolved in distilled water. Endothelin stock was dissolved in distilled water, divided into aliquots and frozen. On the day of use it was thawed and further diluted in distilled water. U46619 stock was prepared in ethanol, which was evaporated under a stream of N_2 and redissolved in distilled water.

Statistics

Rat body weights and blood glucose levels were analysed for interactions using a two-way analysis of variance (ANOVA), and if significant, by one-way ANOVA and Tukey test (Apple Macintosh, CLR ANOVA package). Aortic ring dry weights were analysed by Student's *t* test. Multiple comparisons with a single control were analysed by a one-way ANOVA, followed by a Dunnett's *t* test (Apple Macintosh, STATVIEW package). pEC_{50} values were determined from the E_{max} of each individual curve and the geometric mean (i.e. mean of the log values) determined. Values shown are $\pm$ s.e.mean. In all cases, statistical significance is indicated by $P < 0.05$.

Results

As shown in Table 1, control rats displayed significantly increased body weights compared to their pre-injection values. Diabetic rats displayed significantly reduced body weights over the same period ($P < 0.05$, ANOVA). Blood glucose levels of diabetic rats were increased significantly, after the 2-weeks ($P < 0.05$, ANOVA). Dry weights of aortic rings from diabetic rats were significantly decreased compared to those of controls ($P < 0.05$, *t*-test).

Responses in endothelium-intact aortae

Contractile responses to 5-hydroxytryptamine 5-HT (0.1 μ M–0.1 mM) produced concentration-dependent contractile responses of endothelium-intact aortae from control and diabetic rats (Figure 1a). Maximum responses to 5-HT of aortae from diabetic rats were significantly less than those of control rats (Figure 1a and Table 2, $P < 0.05$, ANOVA, previously published; James *et al.*, 1994). Aortae from diabetic rats were significantly less sensitive to 5-HT than aortae from control rats (indicated by pEC_{50} values, $P < 0.05$, ANOVA, Table 2).

Maximum responses to 5-HT in the presence of GR32191B (1 μ M) of aortae from diabetic rats were significantly attenuated compared to those from controls (Figure 1a and Table 2, $P < 0.05$, ANOVA). Maximum responses to 5-HT in the presence of GR32191B, of aortae from control and diabetic rats, were significantly reduced compared to those obtained in the absence of GR32191B (Figure 1a and Table 2, $P < 0.05$, ANOVA).

Contractile responses to 5-hydroxytryptamine in the presence of endothelin-1 ET-1 (3 nM) produced small contractile responses in aortae from control (0.43 ± 0.11 g) and diabetic (0.53 ± 0.16 g) rats. In the presence of ET-1, maximum responses to 5-HT were not significantly different between aortae from control and diabetic rats (Figure 1b and Table 2). However, maximum responses of aortae, in the presence of ET-1, from control and diabetic rats were significantly attenuated and potentiated, respectively, compared to the corresponding responses obtained in the absence of ET-1 (Figure 1b versus 1a; $P < 0.05$, ANOVA; Table 2).

Maximum responses to 5-HT, in the combined presence of ET-1 (3 nM) and GR32191B (1 μ M), in aortae from diabetic rats were significantly reduced compared to responses of controls (Figure 1c and Table 2, $P < 0.05$, ANOVA). Maximum responses to 5-HT in the combined presence of ET-1 (3 nM) and GR32191B (1 μ M), in aortae from control rats were significantly reduced compared to responses obtained to 5-HT alone (Figure 1c versus 1a and Table 2, $P < 0.05$, ANOVA). Aortae, in the combined presence of ET-1 and GR32191B, from diabetic rats were significantly less sensitive to 5-HT than aortae from control rats (indicated by pEC_{50} values, $P < 0.05$, ANOVA, Table 2).

Table 1 Body weights, blood glucose levels and aortic ring dry weights of control and streptozotocin (STZ)-diabetic rats

	(n)	Body weight (g)		Blood glucose (mm)		Aortic weight (mg)	
		Initial	Final	Initial	Final	With E	Without E
Control	40–48	336 $\pm$ 5	384 $\pm$ 7*	5.1 $\pm$ 0.2	6.6 $\pm$ 0.2	1.85 $\pm$ 0.04	1.95 $\pm$ 0.06
STZ-diabetic	44–50	333 $\pm$ 5	300 $\pm$ 4**	5.5 $\pm$ 0.2	21.0 $\pm$ 0.4**	1.74 $\pm$ 0.03*	1.73 $\pm$ 0.06*

Initial measurements were made at the time of STZ or vehicle injection, and final measurements made 2 weeks later. E = endothelial cells.

* $P < 0.05$, significantly different from corresponding control group, *t* test or ANOVA

** $P < 0.05$, significantly different from initial value in same treatment group, *t* test or ANOVA

Values shown are $\pm$ s.e.mean.

Table 2 Maximum and pEC₅₀ values for aortae from control (C) and streptozotocin (STZ)-treated rats with (+) and without (–) endothelial cells (E)

		C+E	STZ+E	C–E	STZ–E
5-HT	Maximum (g)	3.06 ± 0.17	1.30 ± 0.07*	4.14 ± 0.24	2.60 ± 0.16*
	pEC ₅₀ (-log M)	5.52 ± 0.10	5.23 ± 0.05*	5.90 ± 0.20	5.42 ± 0.12
5-HT + ET-1	Maximum (g)	1.97 ± 0.31†	2.04 ± 0.28†	3.30 ± 0.21	2.13 ± 0.09*
	pEC ₅₀ (-log M)	5.46 ± 0.13	5.29 ± 0.17	6.80 ± 0.33	6.20 ± 0.30
5-HT + ET-1 + GR32191	Maximum (g)	1.88 ± 0.09†	0.89 ± 0.13*	NT	NT
	pEC ₅₀ (-log M)	5.94 ± 0.06	5.54 ± 0.07*	NT	NT
5-HT + GR32191	Maximum (g)	2.30 ± 0.16†	0.57 ± 0.17*†	3.73 ± 0.20	2.03 ± 0.39*
	pEC ₅₀ (-log M)	5.57 ± 0.10	5.01 ± 0.24	6.47 ± 0.28	5.52 ± 0.11*
5-HT + U46619	Maximum (g)	3.08 ± 0.39	2.18 ± 0.43	3.23 ± 0.28	3.23 ± 0.14
	pEC ₅₀ (-log M)	6.51 ± 0.30†	6.01 ± 0.34†	ND	6.42 ± 0.10*†

* $P < 0.05$, significantly different from corresponding control group, ANOVA.

† $P < 0.05$, significantly different from 5-HT alone from the same treatment group, ANOVA.

NT = not tested; ND = not determined. Values shown are $\pm$ s.e.mean.

Figure 1 Responses of rat aortic rings, with endothelium, to 5-hydroxytryptamine (5-HT, $n = 5-6$). (a) 5-HT alone (diabetic, $\square$; control, $\circ$) or in the presence of GR32191B ($1 \mu\text{M}$) (diabetic, $\blacksquare$; control, $\bullet$). (b) In the presence of endothelin-1 (ET-1, 3 nM) (diabetic, $\square$; control, $\circ$). (c) In the presence of ET-1 (3 nM) and GR32191B ($1 \mu\text{M}$) (diabetic, $\square$; control, $\circ$).

Contractile responses to 5-hydroxytryptamine in the presence of U46619 U46619 (20 nM control, 30 nM diabetic) produced small contractile responses in aortae from control ($0.60 \pm 0.30 \text{ g}$) and diabetic ($0.34 \pm 0.22 \text{ g}$) rats. In the presence of U46619, maximum responses to 5-HT were not significantly different between aortae from control and diabetic rats (Figure 2 and Table 2). There was a significant increase in sensitivity to 5-HT in the presence of U46619 of aortae from control and diabetic rats compared to responses

Figure 2 Responses of rat aortic rings, with endothelium, to 5-hydroxytryptamine (5-HT, $n = 5-6$) in the presence of U46619 (20 or 30 nM) (diabetic, $\square$; control, $\circ$).

obtained to 5-HT alone (indicated by pEC₅₀ values, $P < 0.05$, ANOVA, Table 2).

Responses in endothelium-denuded aortae

5-HT ($0.1 \mu\text{M}$ – 0.1 mM) produced concentration-dependent contractile responses in endothelium-denuded aortae from control and diabetic rats (Figure 3a). Maximum responses to 5-HT of aortae from diabetic rats were significantly less than responses from control rats (Figure 3a and Table 2, $P < 0.05$, ANOVA; previously published; James *et al.*, 1994). Maximum responses to 5-HT, in the presence of GR32191B ($1 \mu\text{M}$), of aortae from diabetic rats were still significantly attenuated compared to 5-HT responses, in the presence of GR32191B, from controls (Figure 3a and Table 2, $P < 0.05$, ANOVA). In the presence of GR32191B ($1 \mu\text{M}$), aortae from diabetic rats were significantly less sensitive to 5-HT than aortae from controls (indicated by pEC₅₀ values, $P < 0.05$, ANOVA, Table 2).

Contractile responses to 5-hydroxytryptamine in the presence of endothelin-1 Threshold concentrations of ET-1 produced small contractile responses in aortae from control (2 nM ; $0.24 \pm 0.14 \text{ g}$) and diabetic (3 nM ; $0.51 \pm 0.30 \text{ g}$) rats. In the presence of threshold concentrations of ET-1, maximum responses to 5-HT of aortae from diabetic rats were still significantly less than those obtained in aortae from control rats (Figure 3b and Table 2, $P < 0.05$, ANOVA).

Contractile responses to 5-hydroxytryptamine in the presence of U46619 Threshold concentrations of U46619 (5 – 20 nM) produced small contractile responses in aortae from control ($0.08 \pm 0.01 \text{ g}$) and diabetic ($0.08 \pm 0.01 \text{ g}$) rats. In the presence of threshold concentrations of U46619, maximum

Figure 3 Responses of rat aortic rings, without endothelium, to 5-hydroxytryptamine (5-HT, $n = 4-6$). (a) 5-HT alone (diabetic, □; control, ○) or in the presence of GR32191B (1 μ M) (diabetic, ■; control, ●). (b) 5-HT in the presence of U46619 (5-20 nM) (diabetic, □; control, ○) or endothelin-1 (ET-1, 2 or 3 nM) (diabetic, ■; control, ●).

responses to 5-HT of aortae from diabetic rats were not significantly different from those obtained from control rats (Figure 3b and Table 2). In the presence of U46619, there was an increase in sensitivity to 5-HT in aortae from control and diabetic rats compared to responses obtained to 5-HT alone (indicated by pEC_{50} values, $P < 0.05$, ANOVA, Table 2).

Discussion

We have previously reported that maximum contractile responses to 5-HT of aortic rings from 2-week STZ-diabetic rats are attenuated compared to those obtained in control rats (Sikorski *et al.*, 1993; James *et al.*, 1994). It has been widely reported that TxA_2 levels are significantly increased during diabetes and that aortic rings from STZ-diabetic rats synthesize decreased amounts of prostacyclin (for a review see Hodgson *et al.*, 1992). The results of the present study suggest that endothelial-derived TxA_2 contributes to the contractile response to 5-HT in rat aortae. It also appears that TxA_2 is involved in the potentiation of 5-HT responses, by ET-1, in endothelium-intact aortae from diabetic rats.

In the present study, the potent and selective TP receptor antagonist GR32191B (Lumley *et al.*, 1989; Humphrey *et al.*, 1990) attenuated the maximum contractile responses produced by 5-HT in endothelium-intact aortae from both control and diabetic rats suggesting that contraction produced by 5-HT is mediated in part by TxA_2 , or possibly its precursor PGH_2 , acting at TP receptors. In addition, GR32191B did not affect the maximum contractile responses to 5-HT in endothelium-denuded aortae suggesting that PGH_2/TxA_2 from the endothelium contributes to the contractile response of 5-HT. Indeed, the vasoconstrictor effects of 5-HT on placental vessels have been shown to be due, at least in part,

to the stimulation of endogenous TxA_2 production (Howarth *et al.*, 1993).

As previously mentioned, it has been reported that threshold concentrations of 5-HT potentiate subsequent contraction induced by submaximal concentrations of ET-1 in rat aorta, but that the opposite does not occur (Yang *et al.*, 1992). This potentiation was inhibited by the addition of either the cyclo-oxygenase inhibitor, indomethacin or the TP receptor antagonist, SQ 29548. However, these workers did notice a reduction (which was not significant) in maximum responses to 5-HT in the presence of ET-1. Nakayama *et al.*, (1991) have shown that although concentration-response curves to 5-HT, in the pig coronary artery, are potentiated by ET-1 (0.1 nM), they were significantly attenuated when the concentration of ET-1 was increased 100-fold (i.e. to 10 nM). The potentiating effect of ET-1 (0.1 nM) occurred without a significant increase in intracellular calcium. This inhibitory effect of higher concentrations of ET-1 (i.e. 3–10 nM) on subsequent responses to 5-HT has also been observed in the endothelium-denuded perfused rabbit ear artery (Wong-Dusting *et al.*, 1991). A similar reduction, in 5-HT responses in the presence of ET-1, was observed in the present study with endothelium-intact aortae from control but not diabetic rats. In fact, ET-1 significantly potentiated contractile responses to 5-HT in endothelium-intact aortae from diabetic rats.

The hypothesis that endothelial-derived eicosanoids may be involved in the altered responses of vascular smooth muscle during diabetes is not new. Tesfamariam *et al.* (1991) have suggested that altered receptor-mediated endothelium-dependent relaxation in diabetes was due to increased production of vasoconstrictor prostanoids by the endothelium as a consequence of protein kinase C (PKC) activation. Larkins & Dunlop (1992) and Ruderman *et al.* (1992) have suggested that the activation of PKC may lead to increased release of vasoconstrictor prostanoids. In the present study the TP receptor antagonist, GR32191B, prevented the potentiating effect of ET-1 in endothelium-intact aortae from diabetic rats suggesting that this potentiation was mediated by a vasoconstrictor cyclo-oxygenase product (i.e. PGH_2 or TxA_2). Importantly, the enhancement of 5-HT responses could be mimicked by the TxA_2 -mimetic, U46619, further implicating TxA_2 involvement in the interaction between 5-HT and ET-1. Interestingly, the potentiating effect of ET-1 on 5-HT responses was observed only in endothelium-intact aortae from diabetic rats. However, the addition of exogenous TxA_2 (i.e. U46619) resulted in potentiation of 5-HT responses in both intact- and denuded- aortae, thus providing further support for the role of the endothelium in the interaction between 5-HT and ET-1.

5-HT (Nakaki *et al.*, 1985), ET-1 (Highsmith *et al.*, 1992) and TxA_2 (Dorn & Becker, 1993) produce their contractile effects on rat aorta by similar mechanisms, that is, the stimulation of phospholipase C and a subsequent increase in inositol-1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) levels. In turn DAG stimulates PKC. Prolonged activation of PKC may lead to the activation of negative feedback pathways which result in the inactivation of cell-surface receptors, the inhibition of hydrolysis of phosphatidyl 4,5 bisphosphate and activation of Ca^{2+} pumps which reduce intracellular Ca^{2+} (Nishizuka, 1984). This negative feedback has been suggested as the mechanism responsible for the ET-1-induced inhibition of 5-HT responses previously observed by other workers (Nakayama *et al.*, 1991; Wong-Dusting *et al.*, 1991). A similar negative feedback pathway, induced by the interaction of exogenous ET-1 and endogenous TxA_2 , may explain the inhibition of responses to 5-HT observed in the present study in endothelium-intact aortae from control rats. However, this mechanism does not appear to have been activated in aortae from diabetic rats as potentiation of 5-HT responses was observed rather than inhibition. This indicates that there may be an abnormality in this pathway, in vascular smooth muscle, during diabetes.

The interactions observed in endothelium-denuded aortae displayed an additional component to those observed in endothelium-intact aortae. As well as 'normalizing' maximum responses to 5-HT, the TxA_2 -mimetic, U46619, potentiated the responses to lower concentrations of 5-HT, in aortae from both control and diabetic rats. This potentiation could be due to U46619 changing the response threshold for contraction to 5-HT by mobilizing intracellular calcium (Young *et al.*, 1986; Flavahan & Vanhoutte, 1988). This effect may be masked in endothelium-intact aortae by the release of endothelium-derived relaxing factors such as nitric oxide and prostacyclin.

The results of the present study indicate that TxA_2 , released from the endothelium, contributes to the contractile response of 5-HT in aortae from control and diabetic rats. Endothelial-derived TxA_2 also appears to play a role in the potentiation of 5-HT responses by ET-1 in aortae from diabetic rats.

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