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Acetylcholine-induced endothelium-dependent contractions in the SHR aorta: the Janus face of prostacyclin

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1 In the spontaneously hypertensive rat (SHR) and aging Wistar–Kyoto rats (WKY), acetylcholine releases an endothelium-derived contracting factor (EDCF) produced by endothelial cyclooxygenase-1, which stimulates thromboxane A_2 receptors (TP receptors) on vascular smooth muscle. The purpose of the present study was to identify this EDCF by measuring changes in isometric tension and the release of various prostaglandins by acetylcholine.

2 In isolated aortic rings of SHR, U 46619, prostaglandin (PG) H_2, $PGF_{2\alpha}$, PGE_2, PGD_2, prostacyclin (PGI_2) and 8-isoprostane, all activate TP receptors of the vascular smooth muscle to produce a contraction (U 46619$\gg$8-isoprostane = $PGF_{2\alpha}$ = PGH_2 > PGE_2 = PGD_2 > PGI_2). The contractions produced by PGH_2 and PGI_2 were fast and transient, mimicking endothelium-dependent contractions. PGI_2 did not relax isolated aortic rings of WKY and SHR.

3 Acetylcholine evoked the endothelium-dependent release of thromboxane A_2, $PGF_{2\alpha}$, PGE_2, PGI_2 and most likely PGH_2 ($PGI_2 \gg PGF_{2\alpha} \geqslant PGE_2 > TXA_2 >$ 8-isoprostane, PGD_2). Dazoxiben abolished the production of thromboxane A_2, but did not influence the endothelium-dependent contractions to acetylcholine.

4 The release of PGI_2 was significantly larger in the aorta of SHR than in WKY, and the former was more sensitive to the contractile effect of PGI_2 than the latter. The inhibition of PGI-synthase was associated with an increase in PGH_2 spillover and the enhancement of acetylcholine-induced endothelium-dependent contractions.

5 Thus, in the aorta of SHR and aging WKY, the endothelium-dependent contractions elicited by acetylcholine most likely involve the release of PGI_2 with a concomitant contribution of PGH_2.
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Abbreviations: COX, cyclooxygenase; EDCF, endothelium-derived contracting factor(s); NOS, nitric oxide synthase; L-NA, N^G-nitro-L-arginine; SHR, spontaneously hypertensive rat; PGH_2, prostaglandin H_2; PGI_2, prostacyclin; WKY, Wistar–Kyoto rats

Introduction

Under several pathological conditions, such as hypertension, endothelium-dependent relaxations are impaired (Vanhoutte *et al*., 2005). In the spontaneously hypertensive rat (SHR), the endothelial dysfunction is attributed to the occurrence of a concomitant endothelium-dependent contraction mediated by a yet unidentified endothelium-derived contracting factor (EDCF; Lüscher & Vanhoutte, 1986). This endothelium-dependent contraction involves the production of reactive oxygen species, the activation of endothelial cyclooxygenase (COX)-1, the diffusion of EDCF and the subsequent stimulation of thromboxane A_2 receptors (TP receptors) located on smooth muscle cells (Lüscher & Vanhoutte, 1986; Auch-Schwelk *et al*., 1990; Kato *et al*., 1990; Ge *et al*., 1995; Yang *et al*., 2002; 2003a, b; 2004).

EDCF-mediated responses are observed not only in hypertension but also in diabetes, and probably reflect the premature aging of the blood vessel wall subjected to an exaggerated oxidative stress. The information available in humans confirms that EDCF-mediated responses contribute to the blunting of endothelium-dependent vasodilatations in aged subjects and essential hypertensive patients (Vanhoutte *et al*., 2005). The identification of EDCF could therefore provide new insights into the mechanism of endothelial dysfunction and potentially reveal new therapeutic targets. Various candidates have been proposed for the identity of EDCF in the SHR; they include the endoperoxides prostaglandin H_2 (PGH_2; Ge *et al*., 1995), prostacyclin (PGI_2; Rapoport & Williams, 1996), thromboxane A_2 (Taddei & Vanhoutte, 1993) and isoprostanes (Janssen, 2002).

The purpose of the present study was to identify the EDCF released by acetylcholine from the aortic rings of SHR and Wistar–Kyoto rats (WKY).

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Methods

Experiments were performed on thoracic aortas from 1-year-old male SHR (402 ± 6 g, $n = 102$) and normotensive WKY (432 ± 8 g, $n = 52$), both from Charles River (L'Arbresles, France). The rats were anesthetized with pentobarbital sodium (50 mg kg^{-1}, intraperitoneally) and the blood pressure was measured from the carotid artery (systolic blood pressure: 185 ± 5 and 105 ± 3 mmHg, in SHR and WKY, respectively; $P < 0.05$). The aorta was then dissected free, excised and placed in cold modified Krebs–Ringer bicarbonate solution of the following composition (mM): NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2SO_4 1.2, $NaHCO_3$ 25.0, edetate calcium disodium 0.026, glucose 11.1 (control solution). In some aortas, the endothelium was removed from segments of various lengths by infusing a saponin solution (1 mg ml^{-1}, for 20 s) that was subsequently flushed with control solution. Then the aorta was cut into rings (4–5 mm in length). In some rings the isometric tension was recorded, while in others the release of prostanoids was determined.

Isometric tension recording

The rings were suspended in organ chambers (20 ml), which contained control solution (37°C) aerated with 95% O_2 and 5% CO_2, and were connected to a force transducer in order to record isometric contraction. They were stretched progressively to reach the optimal point of their length–active tension relationship (approximately 2 g). Drug incubation time was 45 min for most of the experiments. Concentration–response curves were obtained in a cumulative manner. Each ring was exposed to only one set of cumulative concentration of each given agonist. Contractile responses were expressed as a percentage of the reference contraction to KCl (60 mM), performed for each individual ring at the beginning of the experiment. Relaxations were expressed as a percentage of the maximal relaxation elicited by papaverine (100 μM) in rings contracted with phenylephrine.

Release of prostaglandins

In order to measure the release of prostanoids, rings were placed in thermostated mini-chambers containing 1 ml of control solution (37°C) aerated with 95% O_2 and 5% CO_2. The equilibration time was 1 h during which the solution was changed every 15 min. The incubation period with drugs was 20 min and acetylcholine was applied for 10 min in the presence of the drugs. Each ring was exposed once and to a single concentration of acetylcholine. Then the aortic rings were removed and the mini-chambers were freeze-clamped in liquid nitrogen and stored at −80°C for further analysis. The rings were placed in a dry hot box (60°C for 48 h) and the dry weight was measured.

The prostaglandins were measured with the following EIA kits from Cayman Chemical (Ann Arbor, MI, U.S.A.), 6-keto prostaglandin $F_{1\alpha}$, thromboxane B_2, prostaglandin E_2, prostaglandin $F_{2\alpha}$, prostaglandin D_2 MOX and 8-isoprostane. Undiluted 50 μl samples were dosed at the exception of the 6-keto prostaglandin $F_{1\alpha}$ measurement, which required a systematic 50-time dilution in control solution and some samples which were subjected to a two-time dilution for the assessment of prostaglandin E_2 and prostaglandin $F_{2\alpha}$. The various assays were performed as indicated by the manufacturer procedure booklet.

Additionally, the production of 8-isoprostane was assessed by mass spectrometry as previously described (Il'Yasova *et al*., 2004).

Drugs

Acetylcholine hydrochloride, indomethacin, isoproterenol, N^G-nitro-L-arginine (L-NA), papaverine, phenylephrine, $SnCl_2$ and tranylcypromine were obtained from Sigma (La Verpillère, France). Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), PGH_2, prostaglandin E_2 (PGE_2), prostaglandin D_2 (PGD_2), prostaglandin I_2 (PGI_2), iloprost, 6-keto prostaglandin $F_{1\alpha}$ (6-keto-$PGF_{1\alpha}$), 8-isoprostane, 9α,11α-azoprosta-5Z,13E-dien-1-oic acid (U 51605), 9,11-dideoxy-9α,11α-epoxymethano prostaglandin $F_{2\alpha}$ (U 46619), N[-2-(cyclohexyloxy)-4-nitrophenyl]-methanesulfonamide (NS 398) and 2-[(1-oxopenytyl)oxy]-benzoic acid (valeryl salicylate) were purchased from Cayman Chemical Company (Ann Arbor, MI, U.S.A.). 3-[(6-amino-(4-chlorobenzensulfonyl)-2-methyl-5,6,7,8-tetrahydronapht]-1-yl)propionic acid (S 18886) and dazoxiben were synthesized at the Institut de Recherches Servier (Suresnes, France). Drug concentrations are expressed as final molar concentrations in the bath solution.

Data analysis

Data are expressed as means ± s.e.m.; n refers to the number of rats from which the aortas were taken. The ED_{20} (concentration of agonist causing a contraction representing 20% of the reference contraction to 60 mM KCl, or causing a relaxation representing 20% of the reference relaxation to 100 μM papaverine) was calculated using the Michaelis–Menten equation and nonlinear regression that included all the data points. The apparent antagonist dissociation constants were determined according to the equation $pK_b = -\log[\text{Ant}]/(\text{dose ratio} - 1)$. [Ant] represents the concentration of the antagonist and dose ratio the ED_{50} of the agonist in the presence of the antagonist divided by the ED_{50} in the absence of the antagonist. Statistical analysis was performed by two-tailed Student's t-test for control and treatment comparisons, and by ANOVA1 or ANOVA2 analysis for multiple comparisons, followed by a Newman–Keuls or a Bonferroni *post-hoc* test, respectively, where appropriate. Differences were considered to be statistically significant when P was < 0.05.

Results

Acetylcholine-induced endothelium-dependent contractions

In the presence of L-nitro-arginine, contractions in response to acetylcholine were observed in rings with, but not without, endothelium. They were transient and the maximal amplitude was observed for concentrations of acetylcholine ranging from 3 to 30 μM. They were usually smaller than the reference contraction to KCl (60 mM) (Figure 1).

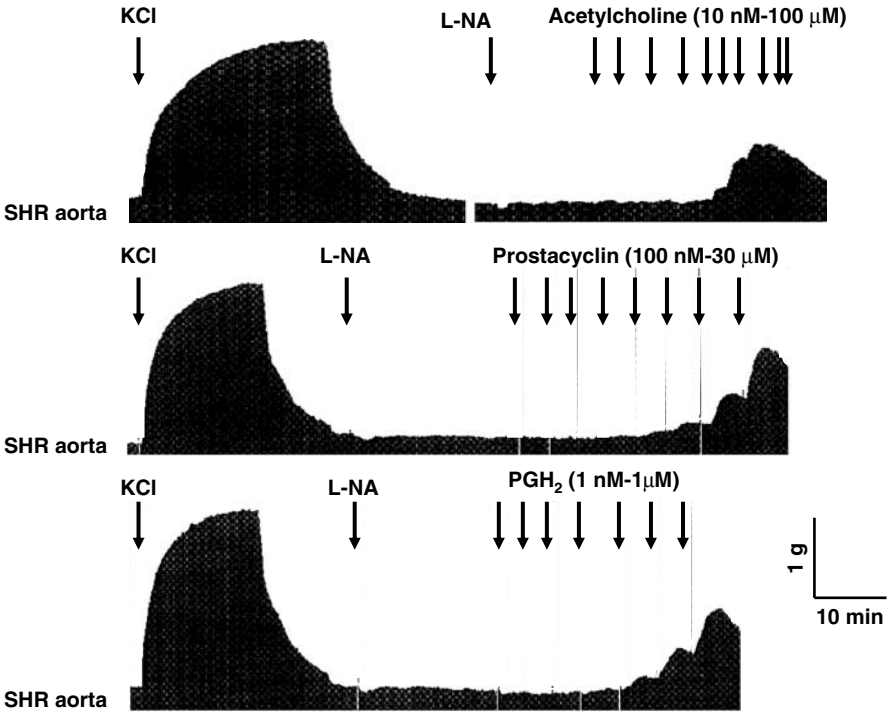

Figure 1 Tracings showing the transient contractions evoked by half-log cumulative addition of acetylcholine (10 nM–100 μM, top), PGI_2 (100 nM–30 μM, middle) and PGH_2 (1 nM–1 μM, bottom) in rings with endothelium of SHR aortas, in the presence of L-NA (100 μM). KCl (60 mM) represents the reference contraction.

Prostanoids-induced changes in tension

U 46619 (0.1 nM–1 μM), 8-isoprostane (0.1 nM–30 μM), $PGF_{2\alpha}$ (1 nM–30 μM), PGH_2 (1 nM–1 μM), PGE_2, PGD_2 and PGI_2 (10 nM–30 μM) produced concentration-dependent contractions of aortic rings of both WKY and SHR (Figure 2). However, neither iloprost nor 6-keto-$PGF_{1\alpha}$, up to 30 μM evoked a significant contraction (data not shown). In SHR rings (in the presence or absence of the endothelium), the order of potency of the agonists was U 46619$\gg$8-isoprostane = $PGF_{2\alpha}$ = PGH_2 > PGE_2 = PGD_2 > PGI_2, and was similar to that observed in aortas of the WKY: U 46619$\gg$8-isoprostane > $PGF_{2\alpha}$ = PGH_2 $\geqslant$ PGE_2 = $PGD_2$$\gg$$PGI_2$. The contractions elicited by PGI_2 and PGH_2 were transient (Figure 1), while those in response to U 46619, 8-isoprostane, PGD_2, $PGF_{2\alpha}$ and PGE_2 (data not shown) were sustained. U 46619, PGI_2 and PGH_2 were significantly more potent in the SHR than in WKY (Figure 3). The contractions in response to all the prostanoids tested were potentiated by removal of the endothelium (Table 1) or by the presence of L-NA (100 μM, data not shown).

In SHR rings without endothelium, S 18886 (0.3–30 nM) produced a rightward shift of the concentration–response curves elicited by U 46619 and 8-isoprostane. In both cases, the slope of the Schild's plot was significantly different from unity, indicating that the antagonism was not competitive. The pK_b values calculated with the lowest concentrations of S 18886 *versus* U 46619 and 8-isoprostane were similar, 9.3 and 9.6, respectively (n = 4). At the concentration of 100 nM, S 18886 virtually abolished the contractions in responses to all the other prostanoids studied, that is PGE_2, $PGF_{2\alpha}$, PGH_2 and PGI_2 (data not shown).

In phenylephrine-contracted rings of SHR and WKY, with and without endothelium, and in the presence or not of S 18886, PGI_2 (up to 10 μM) and iloprost (up to 3 μM) did not produce a significant relaxation (data not shown). However, under the same experimental conditions, isoproterenol produced a concentration-dependent relaxation in both rings with and without endothelium (ED_{20}: 7.6 and 6.5 and maximal

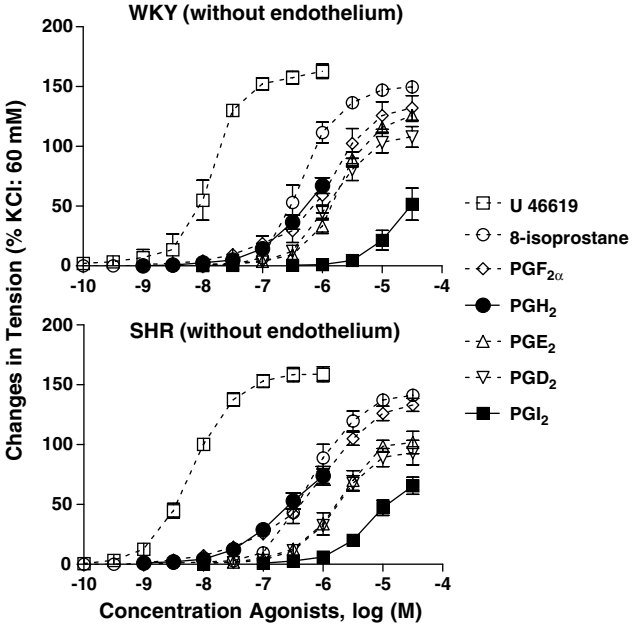

Figure 2 Concentration–response curves to various prostaglandin analog in aortic rings without endothelium of WKY (top) and SHR (bottom). Data are shown as mean ± s.e.m. of at least four different experiments.

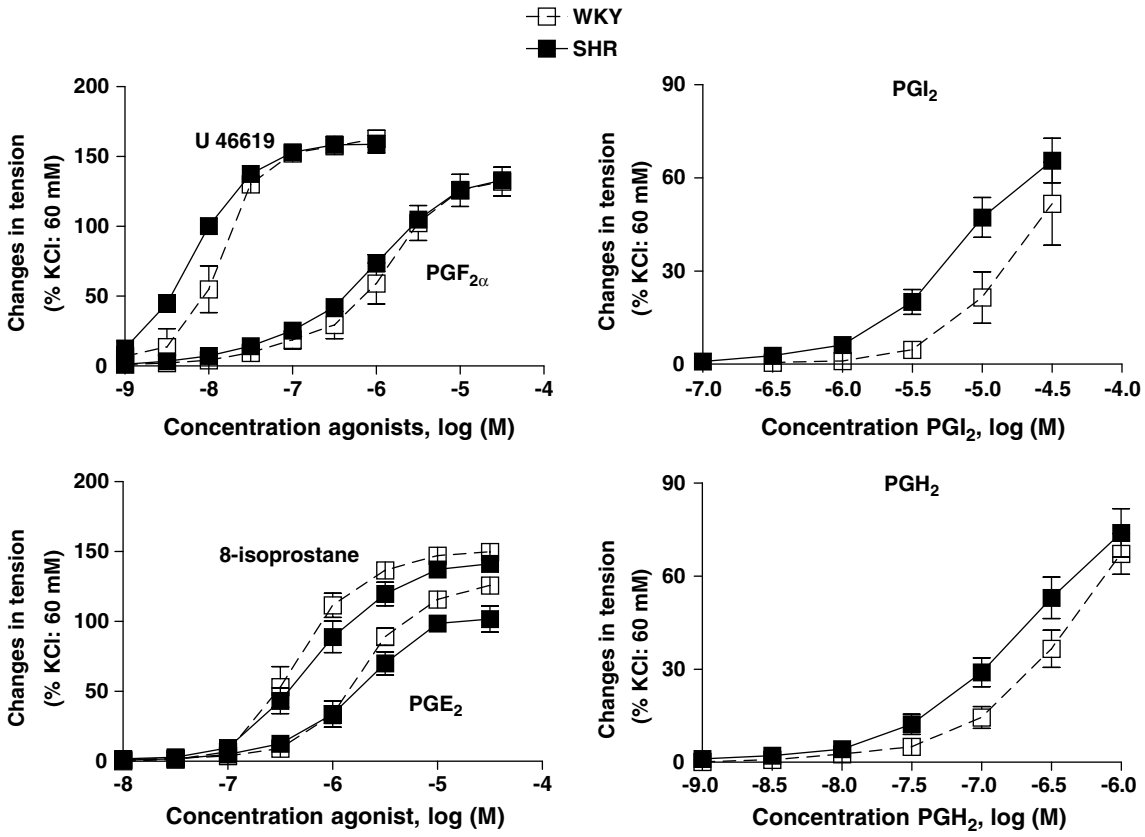

Figure 3 Concentration–response curves to U 46619 and $PGF_{2\alpha}$ (top left), 8-isoprostane and PGE_2 (bottom left) PGI_2 (top right) and PGH_2 (bottom, right) in aortic rings without endothelium of WKY and SHR. Data are shown as mean ± s.e.m. of at least four different experiments.

relaxation in % of papaverine: 72.3 ± 3.2 and 32.3 ± 3.4; $n = 5$, in SHR rings with and without endothelium, respectively).

Acetylcholine-induced release of prostaglandins

Acetylcholine (10 μM) evoked the release of 6-keto-$PGF_{1\alpha}$ (stable metabolite of PGI_2), thromboxane B_2 (stable metabolite of thromboxane A_2), PGE_2 and $PGF_{2\alpha}$ in the aorta of both WKY and SHR. This release was endothelium-dependent in both strains. The release of PGI_2 was 10–100 times larger than that of the other prostaglandins. Furthermore, in contrast to that of thromboxane A_2, PGE_2 and $PGF_{2\alpha}$, the release of PGI_2 was significantly larger in SHR than in WKY aortas (Figure 4). The release of 8-isoprostane, as assessed with the EIA kit, was within a similar range to that of thromboxane A_2, that is small but measurable ($< 200\,pg\,ml^{-1}$), and appeared both endothelium- and acetylcholine-dependent. However, these findings were not confirmed by direct measurement of 8-isoprostane by mass spectrometry (data not shown). PGD_2 levels, as assessed with the EIA kit, were low, around the threshold for detection ($< 20\,pg\,ml^{-1}$; data not shown).

Thromboxane A_2

In SHR aortic rings, dazoxiben (10 μM) abolished the acetylcholine-dependent release of thromboxane A_2 without affecting that of PGI_2 or PGE_2. However, the endothelium-dependent contractions in response to acetylcholine (presence of L-NA, 100 μM) were not affected by the presence of the thromboxane synthase inhibitor (Figure 5).

Prostacyclin

In both WKY and SHR aortas, acetylcholine induced a concentration- and endothelium-dependent release of PGI_2 and contractions that were superimposable (Figure 6). The acetylcholine-induced endothelium-dependent release of PGI_2 was dependent on the endothelial mass and half of the acetylcholine-induced endothelium-dependent release of PGI_2 occurred within 5 min following stimulation with acetylcholine (data not shown).

The acetylcholine-induced release of PGI_2 was unaffected by the presence of 100 μM L-NA (4927 ± 1882 and 3880 ± 961 $pg\,ml^{-1}\,mg\ aorta^{-1}$, $n = 6$ in the absence and presence of L-NA, respectively) or 100 nM S 18886 (3768 ± 462 and 4001 ± 430 $pg\,ml^{-1}\,mg\ aorta^{-1}$, $n = 7$ in the absence and presence of S 18886, respectively). However, the preferential cycloxygenase-2 blocker, NS 398 (1 μM), produced a partial but statistically significant inhibition of both the basal and acetylcholine-stimulated production of PGI_2, while the preferential COX-1 inhibitor, valeryl salicylate (3 mM), or the nonselective inhibitor, indomethacin (5 μM), abolished it (Figure 7).

In the SHR aorta, tranylcypromine (100 μM), a putative inhibitor of the PGI-synthase did not significantly alter the acetylcholine-dependent production of either PGI_2 (acetylcholine 10 μM: 3085 ± 586 and 4116 ± 366 $pg\,ml^{-1}\,mg\ aorta^{-1}$, $n = 5$

Table 1 Prostanoids-induced contractions in aortic rings with and without endothelium of WKY and SHR

Prostaglandins	*With endothelium* $-log\ (ED_{20})$	*Without endothelium* $-log\ (ED_{20})$
U 46619		
WKY ($n=4$)	7.67	8.34*
SHR ($n=4$–20)	8.32#	8.86*#
PGH_2		
WKY ($n=5$)	6.13	6.86*
SHR ($n=5$–9)	6.16	7.24*#
$PGF_{2\alpha}$		
WKY ($n=4$)	5.59	6.80*
SHR ($n=5$)	6.29	7.15*
8-Isoprostane		
WKY ($n=6$)	6.24#	6.86*
SHR ($n=11$)	6.10	6.84*
PGE_2		
WKY ($n=5$)	5.80	6.20*#
SHR ($n=5$)	5.89	6.27*
PGD_2		
WKY ($n=5$)	5.38	6.36*
SHR ($n=5$)	5.74	6.24*
PGI_2		
WKY ($n=5$)	<4.5	5.03*
SHR ($n=8$–14)	<4.5	5.53*#

The ED_{20} is the concentration of agonist causing a contraction representing 20% of the reference contraction to KCl (60 mM); *n* indicates the number of animals from which tissues were taken. The statistical analysis was performed on the whole dose–response curves (ANOVA 2 followed by Bonferroni post-tests for paired or unpaired experiments). *Indicates a statistically significant difference between vessels with and without endothelium, while #indicates that the contractions in response to a given prostanoid were larger in the strain of rat which has been labeled. These two labelings do not necessarily indicate a statistically significant difference at the level of the ED_{20}.

in the absence and presence of tranycylpromine, respectively) or PGE_2 (data not shown). U 51605, a combined inhibitor of PGI- and thromboxane A_2 synthases, produced a concentration-dependent inhibition of PGI_2 release, which was statistically significant at each of the concentrations tested (0.5–10 μM), while the inhibition of thromboxane A_2 production was statistically significant only at the two highest concentrations (3 and 10 μM; Figure 8). By contrast, U 51605 (0.5, 1, 3 and 10 μM) produced a statistically significant increase in acetylcholine-induced release of PGE_2 and $PGF_{2\alpha}$ (Figure 9).

In the SHR aorta without endothelium, U 51605 (10 nM–10 μM) produced a concentration-dependent contraction ($-\log ED_{20}$: 7.29), which was abolished in the presence of S 18886 (100 nM; data not shown). Additionally, U 51605 (0.5–3 μM) produced a concentration-dependent noncompetitive inhibition of U 46619-induced contraction, which was statistically significant at each of the concentrations of U 51605 tested (pK_b value calculated for U 51605 at the concentration of 0.5 μM: 6.6; Figure 10).

Acetylcholine-induced endothelium-dependent contractions (10 nM–100 μM) were significantly potentiated by the presence of 0.5 μM U 51605, were not significantly affected by 1 μM U 51605 and were significantly inhibited by 3 μM U 51605 (Figure 10). In the SHR aorta with endothelium contracted with phenylephrine, U 51605, up to 3 μM, did not affect the endothelium-dependent relaxations induced by acetylcholine (data not shown).

Discussion

The present study demonstrates that, in the SHR aorta, PGI_2 qualifies as one endothelium-derived contractile factor released by acetylcholine.

In the isolated aortic rings of SHR and WKY, the various prostaglandins studied, that is, U 46619, PGH_2, $PGF_{2\alpha}$, PGE_2, PGD_2, PGI_2 as well as 8-isoprostane, all activate the TP receptors on vascular smooth muscle to cause contraction, since the contractions were blocked by the specific TP receptor antagonist, S 18886 (Simonet *et al*., 1998).

The thromboxane A_2 analog, U 46619 is by far the most potent vasoconstrictor among the various prostanoids studied. Acetylcholine induced a small but measurable endothelium-dependent release of thromboxane B_2, the stable metabolite of thromboxane A_2, which was fully and selectively blocked by the thromboxane synthase blocker, dazoxiben. Whether or not the generation of thromboxane A_2 was truly of endothelial origin or from the transcellular metabolism of PGH_2, by platelets adhering to the endothelium (Pfister *et al*., 2002) or by the smooth muscle cells themselves, is unknown. However, this production of thromboxane A_2 did not contribute to the acetylcholine-induced endothelium-dependent contraction, since an effective concentration of dazoxiben did not influence the cholinergic response (see also: Lüscher & Vanhoutte, 1986; Koga *et al*., 1989; Auch-Schwelck *et al*., 1990; Kato *et al*., 1990).

8-isoprostane (8-*epi*$PGF_{2\alpha}$) is produced from the oxidative modification of polyunsaturated fatty acids *via* a free radical-catalyzed mechanism (Morrow *et al*., 1990). Under some circumstances, 8-isoprostane could be a direct product of COX or an indirect consequence of superoxide anion production by COX-mediated metabolism (Watkins *et al*., 1999). In both WKY and SHR aortic rings, 8-isoprostane was a potent constrictor. First EIA dosages were consistent with an acetylcholine-stimulated and endothelium-dependent release of this prostanoid, supporting the hypothesis that an isoprostane could be the EDCF released by acetylcholine (Janssen, 2002). However, the results of this dosage were not confirmed by mass spectrometry analysis, suggesting that the data provided by the EIA kit should be attributed to the cross-detection of another prostaglandin(s) species. This discrepancy in the measurement of 8-isoprostane with an immunoassay kit and by mass spectrometry has been previously reported (Il'Yasova *et al*., 2004). Therefore, in the SHR aorta, 8-isoprostane is not the EDCF released by acetylcholine.

PGI_2 is generally described as an endothelium-derived vasodilator, which, by stimulating its receptor (PGI_2 receptors or IP receptors) and activating adenylate cyclase, elevates intracellular cyclic-AMP concentration and produces smooth muscle relaxation (Wise & Jones, 1996). However, in WKY and SHR, neither PGI_2 nor its stable analog iloprost was able to produce a relaxation. These results confirm earlier observations showing that IP receptor agonists cannot evoke relaxation in the aorta of these two strains of rats, at least

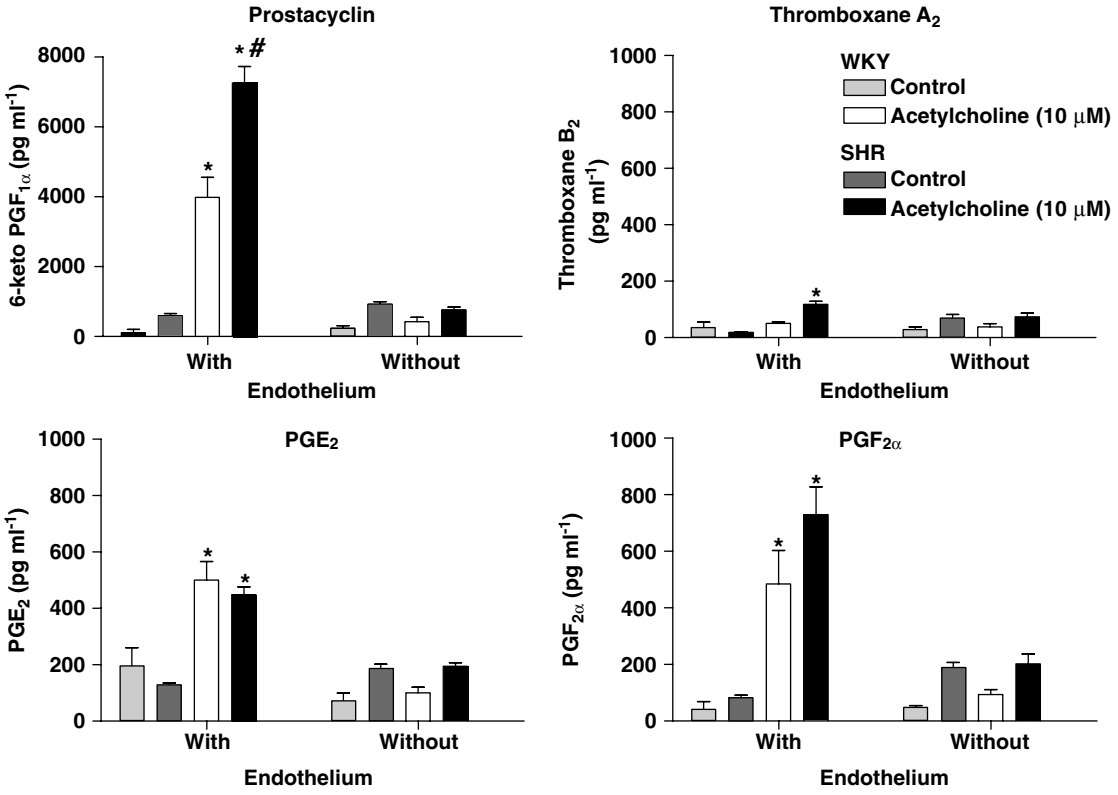

Figure 4 Basal and acetylcholine-dependent release of prostaglandins (PGI_2, PGE_2, thromboxane A_2 and $PGF_{2\alpha}$) in aortic rings, with and without endothelium, of WKY and SHR. Data are shown as mean ± s.e.m. of at least four different experiments. The * indicates a significant effect of acetylcholine and # a significant difference between WKY and SHR.

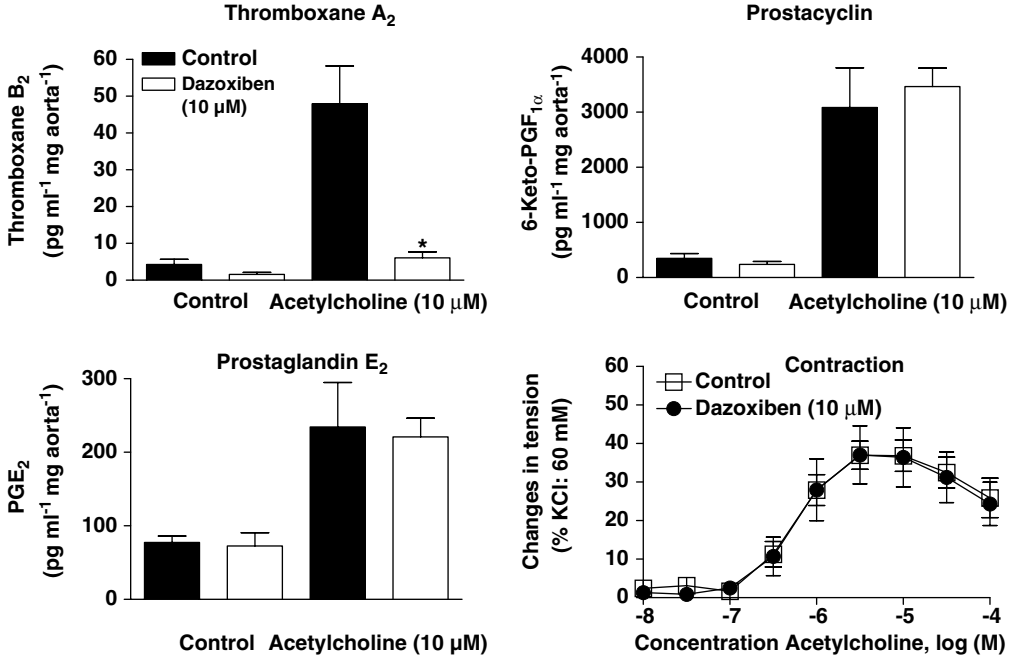

Figure 5 Effects of dazoxiben (10 μM) on the basal and acetylcholine-dependent release of thromboxane A_2 (a), PGI_2 (b) and PGE_2 (c), as well as the acetylcholine-induced endothelium-dependent contraction in the aortic rings with endothelium of SHR (d). Contractile experiments were performed in the presence of L-NA (100 μM). Data are shown as mean ± s.e.m. of at least five different experiments. The * indicates a significant effect of dazoxiben.

when older than 15 weeks (Levy, 1980; Rapoport & Williams, 1996). In both WKY and SHR, the IP receptor gene expression decreases with age and, at any given age, is systematically less expressed in SHR than in WKY (Numaguchi *et al*., 1999). The present study supports the hypothesis of a dysfunction linked to the IP receptor itself, since the aorta

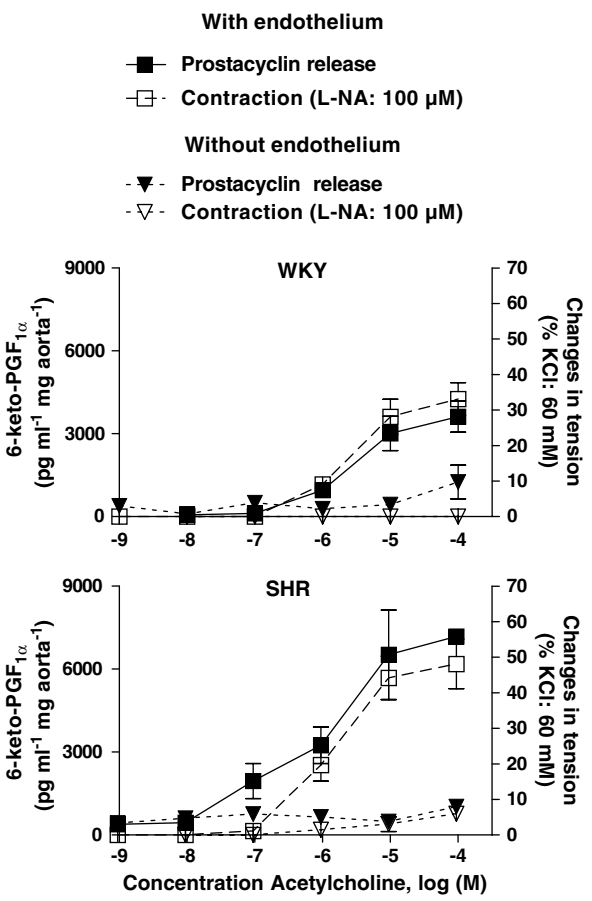

Figure 6 PGI_2 release (left *Y*-axis) and acetylcholine-induced contraction (right *Y*-axis) in aortic rings with and without the endothelium of WKY (top) and SHR (bottom). Contractions were obtained in the presence of L-NA (100 μM). Data are shown as mean $\pm$ s.e.m. of at least three different experiments.

from both strains did relax in response to isoproterenol, a β-adrenoceptor agonist which also evokes cyclic-AMP-dependent relaxation.

PGI_2 evokes contractions in various vascular preparations including human coronary arteries (Pomerantz *et al*., 1978; Davis *et al*., 1980; Zhao *et al*., 1996). In smooth muscle cells of the guinea-pig carotid artery, PGI_2, but not iloprost, induces TP receptor-dependent depolarization and firing of action potentials (Corriu *et al*., 2001). The present study confirms that in the aorta of both WKY and SHR, PGI_2 produces also contractions *via* the activation of TP receptors (Levy, 1980; Williams *et al*., 1994; Zhao *et al*., 1996), and shows that it is more potent in SHR than in WKY.

Acetylcholine produced a concentration-dependent release of PGI_2. This release was fully endothelium-dependent and was markedly larger than the release of any of the other prostaglandins (i.e. thromboxane A_2, PGE_2, $PGF_{2\alpha}$ or PGD_2). This observation is consistent with previous report indicating that in most blood vessels PGI_2 is the principal metabolite of arachidonic acid, the endothelial cells being the predominant site of its synthesis (Moncada *et al*., 1976). The release of PGI_2 was significantly larger in SHR than in WKY aortas (about two-fold) at any given concentration of acetylcholine tested. The acetylcholine-induced endothelium-dependent contraction and endothelium-dependent release of PGI_2 were superimposable for both SHR and WKY. The time course of PGI_2 release was rapid and compatible with the time course of endothelium-dependent contractions. Furthermore, the endothelium-independent contraction elicited by exogenously added PGI_2 mimicked the endothelium-dependent contractions elicited by acetylcholine. These contractions were in both cases transient of small magnitude and virtually abolished by the presence of a functional NO-synthase. By contrast, contractions in response to U46619, 8-isoprostane, PGE_2, $PGF_{2\alpha}$ or PGD_2 were sustained and slowly developing. Furthermore, if the endothelium-derived NO, a potent functional antagonist, produces a marked rightward shift of the concentration–response curves of these prostaglandins, it virtually abolishes the contractions to PGI_2 and the endothelium-dependent contractions to acetylcholine. Therefore, the release of PGI_2 could explain the endothelium-dependent contractions in response to acetylcholine. In both cases, the transient nature of the contraction can be due to the rapid degradation of PGI_2 into its inactive metabolite 6-keto-$PGF_{1\alpha}$. Furthermore, the release of a weak agonist of the TP receptor, such as PGI_2, may explain the relatively small amplitude of the endothelium-dependent contractions in response to acetylcholine.

The release of PGI_2 was not affected by the inhibition of NO-synthase, consistent with an exclusive role of NO as a functional antagonist of the EDCF response (Yang *et al*., 2004), or by S 18886, the TP receptor antagonist, in agreement with the observation that the activation of the TP receptor mediates the effect of EDCF on smooth muscle cells, but does not contribute to the endothelial production of the factor (Yang *et al*., 2003b). The pattern of inhibition of PGI_2 release by COX inhibitors was identical to that of the endothelium-dependent contractions, that is, partial inhibition by the preferential COX-2 inhibitor, NS 398, and complete inhibition either by the preferential COX-1 inhibitor, VAS, or the nonselective inhibitor, indomethacin (Yang *et al*., 2002). Whether the partial inhibition by the COX-2 inhibitor should be attributed to a contribution of this enzyme, which is expressed in the endothelium of aging and hypertensive rats (Heymes *et al*., 2000; Alvarez *et al*., 2005), or to an ancillary effect of NS 398 on COX-1 remains to be determined. Taken into conjunction, these results substantiate the suggestion of Rapoport and Williams (1996) that PGI_2 is one of the EDCFs released by acetylcholine in the aorta of SHR and WKY.

However, Kato *et al*. (1990) as well as Ge *et al*. (1995) have proposed that PGH_2 must be the EDCF released by acetylcholine. This proposal was based on the indirect measurement of PGH_2 release and on the observation that the endoperoxide was a more potent contracting agent in SHR than in WKY. The present study confirms the latter observation and shows that the transient contractile response mimics the acetylcholine-induced endothelium-dependent contractions.

PGH_2 is an unstable prostaglandin which is spontaneously or enzymatically transformed in the more stable isomer PGE_2 and in the presence of mild reducing agents, such as $SnCl_2$, is converted to $PGF_{2\alpha}$ (Hamberg *et al*., 1974; Ge *et al*., 1995; Camacho *et al*., 1998). The amount of PGH_2 can be estimated, theoretically, from the difference in $PGF_{2\alpha}$ production in the absence and presence of $SnCl_2$. In the present study, the presence of $SnCl_2$ (1 mM) produced unspecific effects and did not allow a proper quantification of prostaglandin release or a proper recording of changes in isometric tension (unpublished observations). In endothelial cells, if the constitutive presence

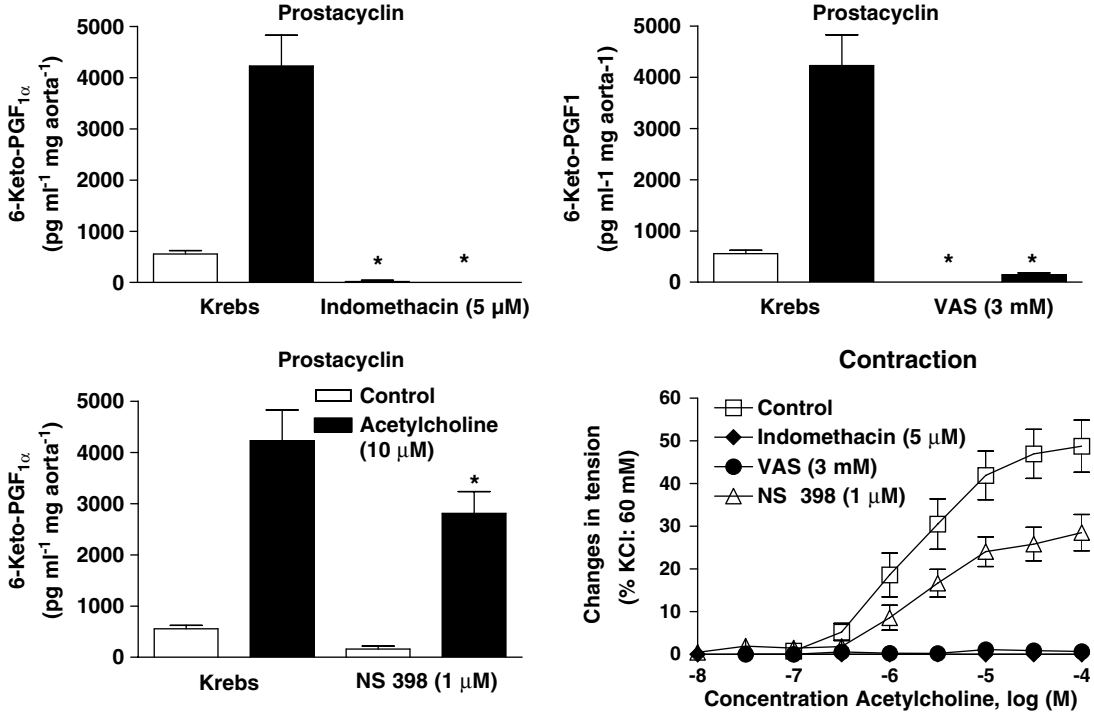

Figure 7 Effects of the COX inhibitors indomethacin (5 μM, top left), valeryl salycylate (3 mM, top right) and NS 398 (1 μM, bottom left), on the basal and acetylcholine-dependent release of PGI_2, as well as on the acetylcholine-induced endothelium-dependent contractions (bottom right) in rings with endothelium of SHR aortas. Data are shown as mean $\pm$ s.e.m. of at least six different experiments. The * indicates a significant effect of an inhibitor.

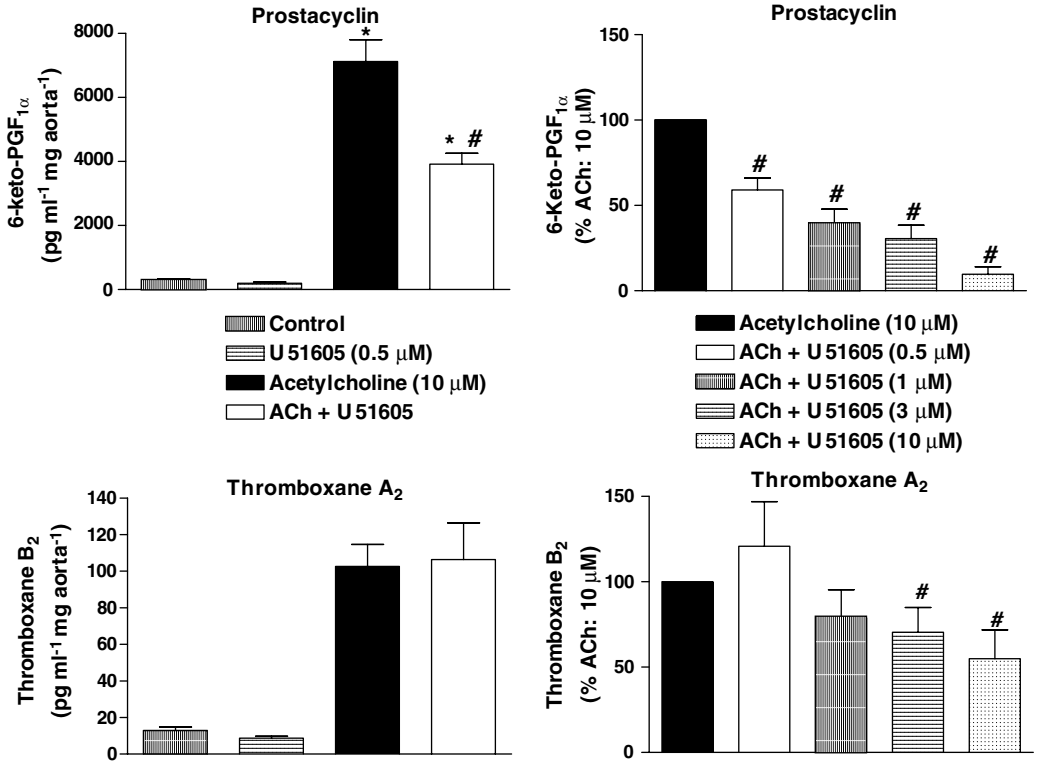

Figure 8 Effects of U 51605 at 0.5 μM (left) and at 1, 3 and 10 μM (right) on basal and acetylcholine-stimulated release of PGI_2 (top) and thromboxane A_2 (bottom) in isolated aortic rings with endothelium of SHR. Data are shown as mean $\pm$ s.e.m. of at least five different experiments. As the experiments involving the various concentrations of U 51605 were not contemporary, the data shown in the graphs situated on the right-hand side are expressed in percentage of the control acetylcholine response. The * indicates a significant effect of acetylcholine and # a significant effect of U 51605.

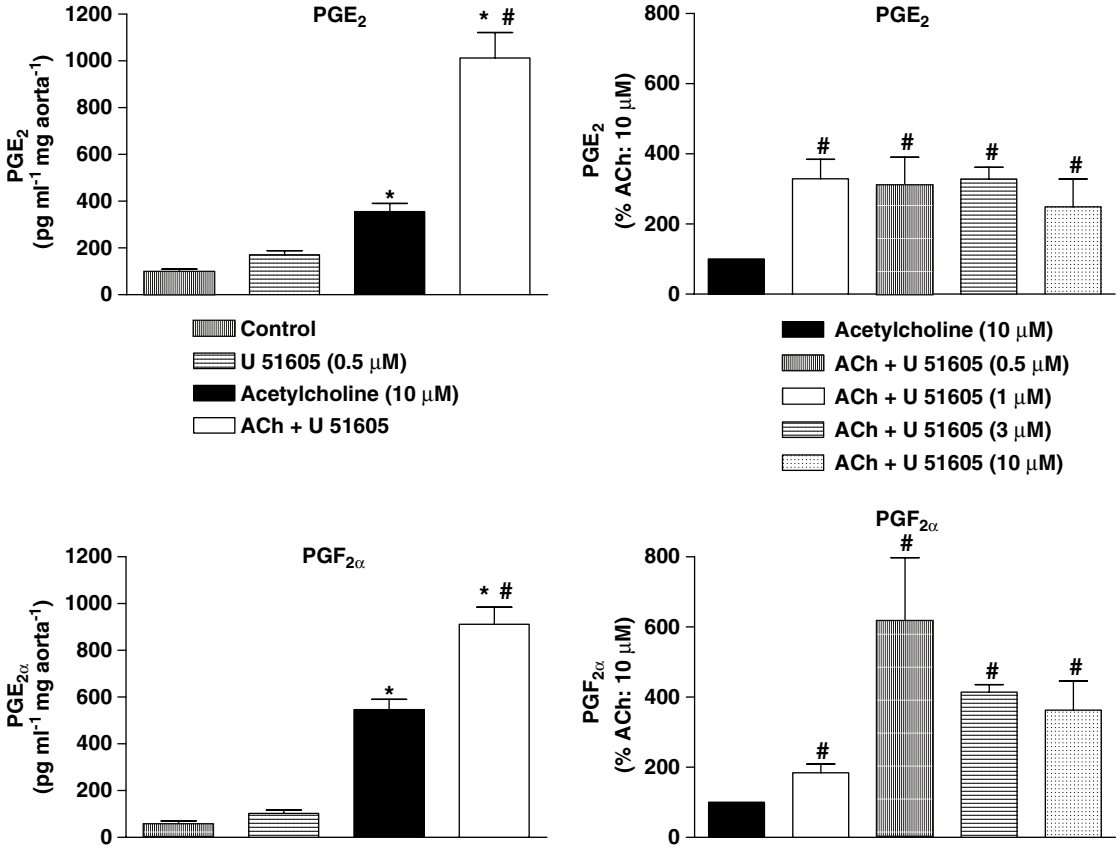

Figure 9 Effects of U 51605 at 0.5 μM (left) and at 1, 3 and 10 μM (right) on basal and acetylcholine-stimulated PGE_2 (top) and $PGF_{2\alpha}$ (bottom) release in aortic rings with endothelium of SHR. Data are shown as mean $\pm$ s.e.m. of at least five different experiments. As the experiments involving the various concentrations of U 51605 were not contemporary, the data shown in the graphs situated on the right-hand side are expressed in percentage of the control acetylcholine response. The * indicates a significant effect of acetylcholine and # a significant effect of U 51605.

of the soluble PGE-synthase associated with COX-1 is debatable, the parallel induction of the membrane-bound form of PGE-synthase with COX-2 is well documented (Soler *et al*., 2000; Murakami *et al*., 2002). Since the induction of COX-2 has been suggested in the aorta of SHR and aging WKY (Heymes *et al*., 2000; Alvarez *et al*., 2005), the production of PGE_2 might be of enzymatic origin. However, it cannot be excluded that the rate of formation of PGH_2 exceeds its metabolism. If one assumes that, under the present experimental conditions, the entire production of PGE_2 is a perfect surrogate for the endothelial production of PGH_2, this production is roughly 30 times less than that of PGI_2. Although PGH_2 is approximately 30 times more potent than PGI_2 in producing contraction, it is difficult to attribute an exclusive role to the endoperoxide in acetylcholine-induced endothelium-dependent contractions.

In the spontaneous hypertensive rat, the augmented responsiveness to endoperoxides, unlike the overexpression of COX-1, is already present in the aorta of prehypertensive animals (Iwama *et al*., 1992; Jameson *et al*., 1993; Ge *et al*., 1999). Thus, this hyper responsiveness may constitute a genetic platform for the disease. By contrast, the overexpression of COX probably reflects an adjustment to the chronic hypertensive process, resulting in premature aging of the endothelial cells. This interpretation is reinforced by the observations that endothelium-dependent contractions appear also in arteries of aging normotensive animals (Koga *et al*., 1989; Fujii *et al*., 1999; Heymes *et al*., 2000). The resulting massive increase in PGI_2 production is associated with the disappearance of functional IP receptors leading to relaxation. Taken in conjunction, these observations suggest that both PGI_2 and PGH_2 can contribute to the acetylcholine-induced endothelium-dependent contractions.

In order to determine more precisely the contribution of PGI_2 in the endothelium-dependent contractions evoked by acetylcholine, it was attempted to inhibit PGI synthase. The monoamine oxidase inhibitor and antidepressant tranylcypromine, at the concentration used in the present study, is often presented as a nonspecific inhibitor of this enzyme (Garcia-Cohen *et al*., 2000). However, under the present experimental conditions, tranylcypromine did not affect the production of PGI_2 or PGE_2, confirming previous observations in the same artery (Rapoport & Williams, 1996), and precluding its utilization. U 51605 is a stable analog of PGH_2 and a partial agonist at TP receptors (Huzoor-Akbar *et al*., 1985; Mukhopadhyay *et al*., 1985), properties which were confirmed in the present study. U 51605 produced a concentration-dependent inhibition of PGI_2 release and for the two highest concentrations tested also that of thromboxane A_2, indicating that this compound is a preferential inhibitor of PGI synthase (Gorman *et al*., 1977; 1979). Since dazoxiben, the specific thromboxane synthase inhibitor, did not affect the acetylcholine-induced

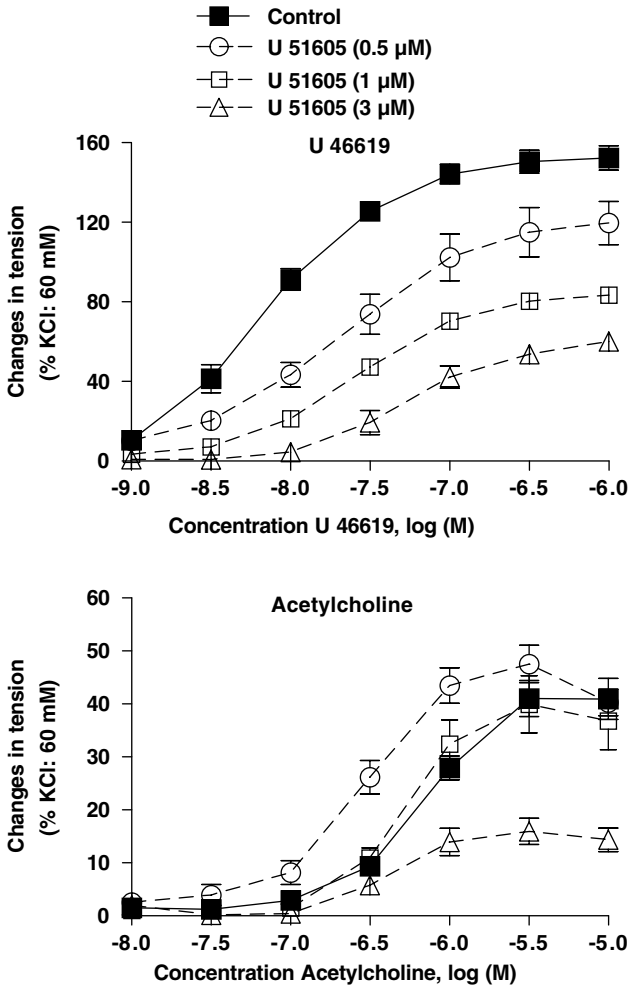

Figure 10 Effects of U 51605 in SHR isolated aortic rings. Concentration-dependent inhibition of U 46619-induced contraction in isolated aortic rings without endothelium by U 51605 (0.5, 1 and 3 μM; top). Effects of U 51605 (0.5, 1 and 3 μM) on the endothelium-dependent contractions to acetylcholine (presence of L-NA: 100 μM, bottom). Data are shown as mean$\pm$s.e.m. of at least five different experiments.

endothelium-dependent contractions, this compound could be considered as a useful tool to investigate the role of protacyclin in these contractions. Importantly, U 51605 did not interact with the endothelial muscarinic receptor since this drug did not affect the endothelium-dependent relaxation in response to acetylcholine.

U 51605 at 0.5 μM, the lowest concentration tested, significant blocked the TP receptor and produced a 50% inhibition of PGI_2 release. Paradoxically, the inhibition of the release of PGI_2, the putative EDCF and the concomitant blockade of TP receptors were associated with an enhancement of the endothelium-dependent contractions to acetylcholine. This paradox is only apparent since the inhibition of PGI_2 release was compensated by a major increase in PGE_2 and $PGF_{2\alpha}$ production. In endothelial cells, the inhibition of PGI synthase consistently leads to an increase in PGE_2 production (Zou *et al*., 1999; Bachschmid *et al*., 2003). Again, whether or not the release of PGE_2 and $PGF_{2\alpha}$ could be solely attributable to the nonenzymatic transformation of PGH_2 or is partially linked to the activation of endothelial PG synthases and/or the transcellular metabolism of PGH_2 by the underlying smooth muscle cells is unknown.

U 51605 at 1 μM did not potentiate, and at 3 μM inhibited the acetylcholine-induced endothelium-dependent contractions, most likely because of the overwhelming antagonistic properties of this compound toward TP receptors. Additionally, the fact that U 51605 does not produce a concentration-dependent increase in PGE_2 and $PGF_{2\alpha}$ production while the inhibition of PGI_2 release was concentration-dependent may indicate other nonspecific properties of U 51605.

In conclusion, the endothelium-dependent contractions elicited by acetylcholine in the aorta of SHR and aging WKY most likely involve at least in part the release of PGI_2. This conclusion is based on the following: (a) in WKY and SHR, PGI_2 is a contracting but not a relaxing factor; (b) PGI_2 is a more potent contracting agent in SHR than in WKY; (c) the contractions evoked by PGI_2 mimic the endothelium-dependent contractions produced by acetylcholine both in terms of duration and amplitude; (d) the PGI_2 and the endothelium-dependent contractions both involve activation of TP receptors; (e) PGI_2 is the most abundant prostaglandin released by acetylcholine and is of endothelial origin; (f) the release of PGI_2 is two times larger in SHR than in WKY; (g) the time course of the release of PGI_2 is compatible with the time course of the observed endothelium-dependent contractions; (h) the release of PGI_2 correlates with the amplitude of the endothelium-dependent contractions over the full concentration range of acetylcholine in both WKY and SHR; (i) the endothelium-dependent contractions and the release of PGI_2 are affected similarly by COX inhibitors; and (j) the inhibition of PGI_2 synthesis enhances the acetylcholine-induced endothelium-dependent contractions. Paradoxically, this observation also supports the hypothesis that PGI_2 contributes to endothelium-dependent contractions, since the inhibition of PGI-synthase may enhance PGH_2 spillover, a more potent TP receptor agonist than PGI_2 itself. This hypothesis that PGI_2 is an EDCF is in agreement with a recent study suggesting that PGI_2 is the main factor accounting for endothelial dysfunction in the SHR aorta (Blanco-Rivero *et al*., 2005).

PGH_2 most likely contributes also to the endothelium-dependent contractions evoked by acetylcholine. Most of the arguments developed above for a contribution of PGI_2 apply also for PGH_2. Nevertheless, although it is difficult to quantify exactly the extent of PGH_2 production, the amount released in response to acetylcholine is 30–100 times less than that of PGI_2. However, under conditions when the PGI-synthase activity is inhibited, either pharmacologically (U 51605) or under pathological conditions (for instance, following the peroxynitrate-dependent tyrosine nitration of the enzyme; Zou *et al*., 2002), the contribution of PGH_2 will increase and so will the amplitude of the endothelium-dependent contractions.

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