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Age-related involvement of the endothelium in β-adrenoceptor-mediated relaxation of rat aorta

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Abstract

The signalling pathway involved in β-adrenoceptor relaxation was studied in aortas from rats either 8 or 54 weeks of age. The vasorelaxation produced by isoprenaline was almost completely abolished by endothelium removal in 54-week aortas, whereas in 8-week aortas, the effect was much smaller. The nitric oxide synthase inhibitor *N*-methyl-1-arginine acetate (L-NMMA) partially attenuated the isoprenaline induced relaxation to a similar extent in both age groups when the endothelium was intact, suggesting that although nitric oxide was involved, it could not explain the age-related difference. The K^+ channel inhibitor, tetraethylammonium inhibited isoprenaline vasorelaxation to a larger degree in 54-week compared to 8-week aortas indicating that K^+ channels were responsible for the age-related differences. We suggest that as the animals age, the smooth muscle cyclic AMP signalling system declines, and that this is compensated for by an alternate pathway involving the opening of K^+ channels.

Keywords: β-Adrenoceptor; Age; Endothelium; Isoprenaline; Relaxation; K^+ channel

1. Introduction

Relaxation of vascular smooth muscle by β-adrenoceptor agonists is believed to occur through activation of adenylate cyclase, the generation of cyclic AMP and the activation of cyclic AMP-dependent protein kinase. This produces a vasorelaxation by decreasing intracellular Ca^{2+} levels and decreasing the sensitivity of the contractile apparatus to Ca^{2+} through multiple actions (Murray, 1990). However, there are studies that demonstrate a poor correlation between the rise in intracellular cyclic AMP produced by the β-adrenoceptor agonist isoprenaline and the vasorelaxant response (Fermum et al., 1984) indicating that other factors may be involved. Indeed endothelium removal inhibited β-adrenoceptor-mediated relaxation in rat aorta (Kamata et al., 1989; Gray and Marshall, 1992; Delpy et al., 1996), rat hind limb (Gardiner et al., 1991), rat mesenteric arteries (Graves and Poston, 1993) and dog coronary artery (Rubanyi and Vanhoutte, 1985) implicating endothelial nitric oxide and the cyclic GMP system. Indeed, nitric oxide synthase inhibitors attenuated β-adrenoceptor-mediated relaxation in rat aorta (Gray and Marshall, 1992) and rat mesenteric artery (Graves and Poston, 1993). In contrast, Moncada et al. (1991) found that β-adrenoceptor relaxation in rat aorta was not endothelium-dependent and was not affected by nitric oxide synthase inhibition. Similarly, endothelial cell removal had no effect on β-adrenoceptor relaxation in rat aorta (Konishi and Su, 1983), rat saphenous vein (De Mey and Vanhoutte, 1982) or rat femoral artery (Konishi and Su, 1983). These inconsistencies cannot be reconciled by tissue or species differences since in rat aorta, both positive and negative findings have been reported (Konishi and Su, 1983; Kamata et al., 1989; Moncada et al., 1991; Gray and Marshall, 1992).

One factor, which varied between studies, was the size of the animals, which is directly related to age. For example, Satake et al. (1996, 1997) demonstrated that endothelium removal had a very small inhibitory effect on isoprenaline-mediated relaxation in aorta from male rats weighing 150–170 g, yet a much larger inhibitory effect

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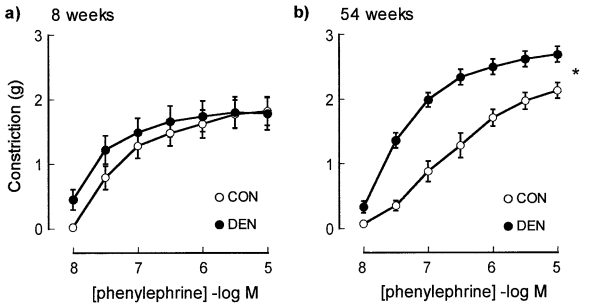

Fig. 1. The effect of endothelium removal on the constrictor response to phenylephrine (0.01–10 μM) in aorta from rats aged (a) 8 weeks and (b) 54 weeks. Phenylephrine concentration response curves were conducted in endothelium-intact (CON) or endothelium-denuded (DEN) aortic segments. Responses are expressed as grams tension developed to phenylephrine. Each point represents the mean ± S.E.M ($n = 4–10$). $^{*}P < 0.05$ significant difference between the endothelium-denuded and intact tissues in each panel (repeated-measures two-way analysis of variance).

was seen in aorta from rats weighing 200–300 g (Satake et al., 1995). Further, removal of the endothelium almost completely abolished β-adrenoceptor relaxation in aorta from male rats weighing 350–450 g (Delpy et al., 1996). We therefore decided to investigate the hypothesis that the endothelium dependence of β-adrenoceptor vasorelaxation in rat aorta varied according to the age of the rat. This was examined in two groups of rats; the first had a mean age of 8 weeks and the second had a mean age of 54 weeks.

2. Methods

2.1. Tissue preparation

The aortas were isolated from two groups of male Sprague–Dawley rats, 8 weeks of age (weighing 180–220 g) and 54 weeks of age (400–700 g), respectively. After the animals were killed by decapitation, the thoracic aorta was removed and cleaned free of fat and connective tissue and then cut into ring segments (4 mm in length). Each ring was then placed in an organ bath containing 1.0 ml of physiological salt solution (PSS) of the following composition (mM): NaCl 118, KCl 4.7, KH_2PO_4 1.03, $NaHCO_3$ 25, D-(+)-glucose 11.1, $MgSO_4$ 1.2, $CaCl_2$ 1.8, EDTA 0.067 and ascorbic acid 0.14. Where necessary, the endothelium was removed by gentle rubbing of the luminal surface with a stainless steel rod. Rings were mounted between two stainless steel hooks through the lumen with the lower hook connected to a tissue holder and the upper to an isometric force displacement transducer. Rings were washed thoroughly by replacing the PSS repeatedly and were then allowed to equilibrate for a period of 45 min under 2 g of resting tension. Supply reservoirs and organ baths were maintained at 37°C and were gassed with 95% O_2 and 5% CO_2.

2.2. Tissue viability assessment

Following the 45 min equilibration period, the viability of the tissues was assessed. Tissues that failed to produce a 0.5 g increase in tension to phenylephrine (0.1 μM) were rejected. The presence of the endothelium was assessed by examining the relaxation to acetylcholine (10 μM) in the presence of phenylephrine. The tissue bathing solution was then replaced repeatedly with fresh drug-free PSS until a stable baseline tension was achieved. The tension was then readjusted to 2 g.

2.3. Vasoconstrictor studies

Following assessment of tissue viability, the tissues were allowed to equilibrate for a further 45 min. Then a cumulative concentration–response curve to phenylephrine

Table 1
Size of the phenylephrine pre-constriction and the $-\log EC_{50}$ of the isoprenaline relaxation after addition of various drugs in rat isolated aorta from rats aged 8 and 54 weeks
NMMA, *N*-methyl-l-arginine acetate; GLIB, glibenclamide; ICI, ICI 118551. Values are expressed as means ± S.E.M.

	Phenylephrine constriction, g (8-week)	Isoprenaline $-\log EC_{50}$ (8-week)	*n*	Phenylephrine constriction, g (54-week)	Isoprenaline $-\log EC_{50}$ (54-week)	*n*
Control	1.37 ± 0.08	7.33 ± 0.19	28	0.98 ± 0.08[a]	6.76 ± 0.12[a]	16
Denuded	1.76 ± 0.17	6.48 ± 0.18[b]	9	2.08 ± 0.40[b]	> 5[a,b]	3
NMMA, 100 μM	1.66 ± 0.16	6.50 ± 0.29	4	1.74 ± 0.44	5.64 ± 0.46	6
GLIB, 1 μM	1.81 ± 0.05	6.76 ± 0.30	4	1.11 ± 0.27[b]	5.99 ± 0.48	5
Atenolol, 0.1 μM	1.43 ± 0.20	6.69 ± 0.36[b]	6	1.16 ± 0.15	6.50 ± 0.22	6
ICI, 0.1 μM	1.37 ± 0.13	6.28 ± 0.11[b]	7	1.10 ± 0.14	6.52 ± 0.22	6
ICI, 10 μM	1.56 ± 0.09	5.76 ± 0.12[b]	4	0.79 ± 0.07[a]	5.38 ± 0.54[b]	3

[a] $P < 0.05$ significantly different from 8-week-old animals treated with the same agent (Student's *t*-test).
[b] $P < 0.05$ significantly different from age-matched control experiments conducted in the absence of any drug (row 1; Student's *t*-test).

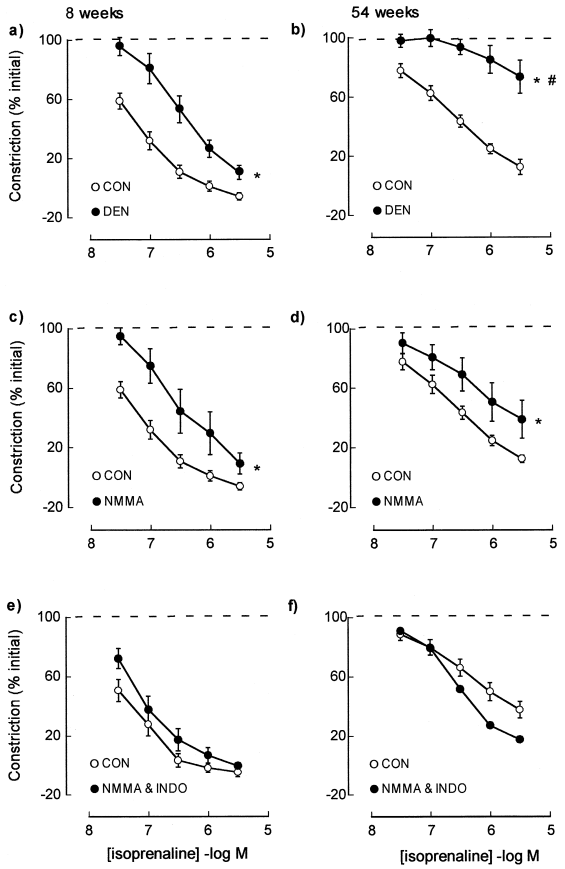

Fig. 2. The effect of endothelium removal, L-NMMA and the combination of L-NMMA and indomethacin on the relaxant effect of isoprenaline in aorta from rats aged 8 and 54 weeks. Cumulative concentration–response curves for isoprenaline (0.03–3.0 μM) were conducted in phenylephrine (0.1 μM) constricted rat aorta and responses are expressed as a percentage of the initial phenylephrine-induced constriction. Each point represents the mean ± S.E.M ($n = 3$–28). Relaxation to isoprenaline was conducted in aorta from 8-week- and 54-week-old aorta. Control experiments (CON) were conducted in endothelium intact aorta and the effect of endothelium denudation (DEN) and 100 μM L-NMMA (NMMA) or the combination of 100 μM L-NMMA and 10 μM indomethacin (INDO) was investigated. $^{*}P < 0.05$ significant difference between the control and treated tissues in each panel (repeated-measures two-way analysis of variance). $\#P < 0.05$ significant difference in treatment effects between 8-week- and 54-week-old rats (three-way analysis of variance with repeated measures).

(0.01–10 μM) was constructed with approximately 5 min between incremental drug additions.

2.4. Vasorelaxation studies

Following assessment of tissue viability, tissues were allowed to equilibrate for a further 45 min after which time they were constricted with phenylephrine (0.1 μM). The phenylephrine constriction was well maintained over time (not shown) and for all vasorelaxant drugs, which were added cumulatively over a 1 h period, concurrent control experiments with vehicle were performed, and in each case, the vehicle did not affect the phenylephrine constriction (not shown). After the phenylephrine response had reached a stable plateau, isoprenaline (0.03–3 μM) was added in a cumulative fashion. Approximately 5 min elapsed between subsequent additions of isoprenaline. When other drugs were used, they were present from 45 min before the phenylephrine constriction was induced. The $-\log EC_{50}$ was defined as the $-\log$ of the concentration of isoprenaline to reduce the phenylephrine constriction to 50% of the initial constriction.

2.5. Drugs

Drugs were obtained from the following sources: acetylcholine perchlorate, $BaCl_2$, 4-aminopyridine, glibenclamide, indomethacin, (−)-isoprenaline bitartrate, *N*-methyl-1-arginine acetate (L-NMMA) (−)-phenylephrine hydrochloride, tetraethylammonium chloride (Sigma, St Louis, USA); L-N5-(-1-iminoethyl)ornithine HCl (L-NIO, Cayman Chemicals, Missouri, USA); apamin, iberiotoxin (Calbiochem, La Jolla, CA, USA); charybdotoxin (Auspep, Parkville, Australia); Rp-cyclic-adenosine monophosphothioate (Rp-cAMPS, Biolog Life Science Institute, Bremen,

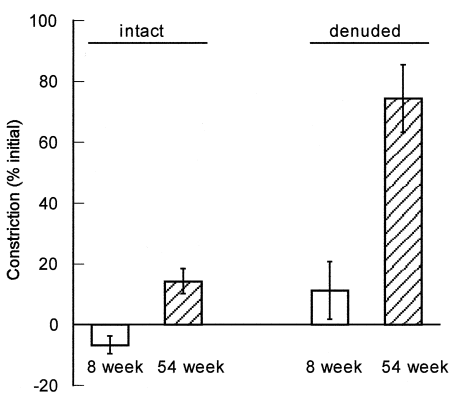

Fig. 3. The effect of endothelium removal on the relaxant effect of isoprenaline in rats aged 8 and 54 weeks using data modified from Fig. 2. Cumulative concentration–response curves for isoprenaline (0.03–3.0 μM) were conducted in phenylephrine (0.1 μM)-constricted rat aorta and the response of isoprenaline (3 μM; expressed as a percentage of the initial phenylephrine-induced constriction) is shown. Each column represents the mean ± S.E.M ($n = 3$–22). The data was taken from Fig. 2a and b, and experiments selected so that the mean phenylephrine pre-constriction was equal for both 8- and 54-week-old animals. In endothelium-intact aorta, the phenylephrine (0.1 μM) constriction was 1.21 ± 0.07 g ($n = 22$) and 1.13 ± 0.07 g ($n = 11$) in the 8 and 54 week groups respectively. In endothelium-denuded aorta, the phenylephrine constriction was 2.07 ± 0.21 g ($n = 5$) in 8 week old aorta and 2.08 ± 0.40 g ($n = 3$) in 54 week old aorta. There was no significant difference between the size of the phenylephrine (0.1 μM) constriction in 8- (open bars) or 54-week-old aorta (hatched bars) in either the endothelium-intact and endothelium-denuded treatment groups ($P > 0.05$, Student's *t*-test). The relaxation produced by isoprenaline was more markedly reduced by endothelium removal in the 54-week compared with 8-week aorta ($P < 0.05$, two-way analysis of variance).

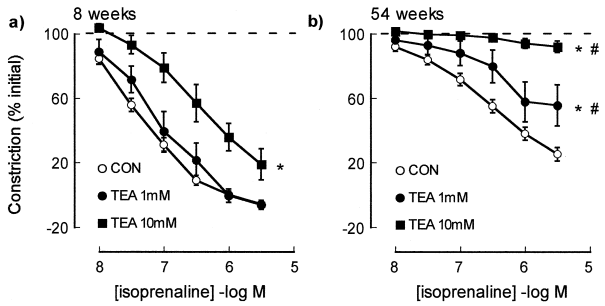

Fig. 4. The effect of tetraethylammonium on the relaxant effect of isoprenaline in aorta from rats aged 8 and 54 weeks. Cumulative concentration response curves for isoprenaline (0.03–3.0 μM) were conducted in phenylephrine (0.1 μM) constricted rat aorta and responses are expressed as a percentage of the initial phenylephrine-induced constriction. Each point represents the mean ± S.E.M ($n = 7$–47). Relaxation to isoprenaline was conducted in aorta from 8-week- and 54-week-old rats. Control experiments (CON) were conducted in endothelium-intact aorta and the effect of tetraethylammonium (TEA, 1 and 10 mM) was investigated. $^{*}P < 0.05$ significant difference between the control and treated tissues in each panel (repeated-measures two-way analysis of variance). $\#P < 0.05$ significant difference in treatment effects between 8-week- and 54-week-old rats (three-way analysis of variance).

Germany). Atenolol and (±)-1-[2,3-(dihydro-7-methyl-1*H*-iden-4-yl)oxyl-3-[(1-methylethy)amino]-2-butanol hydrochloride (ICI 118,551) were gifts from ICI Pharmaceuticals (Macclesfield, UK; now Zeneca). (±)-(R^*,R^*)-[4[2-[[2-(3-chlorophenyl)-2-hydroxyethyl]amino]propyl]phenoxy]-acetic acid sodium (BRL 37344) was a gift from Professor Roger Summers.

2.6. Statistical analysis

All values are given as the means ± S.E.M. and n indicates the number of observations. Differences between curves were analysed using a repeated-measures analysis of variance. The significant difference between the two age groups of rats and the different treatments was analysed using an analysis of variance and determination of the significance of the appropriate interaction term. The differences between $-\log EC_{50}$ values and phenylephrine constrictions were analysed using a Student's t-test. A P value of less than 0.05 was considered to be significant. GBSTAT (Dynamic Microsystems, Silver Springs, USA) statistical package was used in all calculations. Although multiple aortic rings were obtained from one rat, each ring was used for a different drug group.

2.7. Animal statement

The investigation conforms with the Australian code of practice for the care and use of animals for scientific purposes published by the National Health and Medical Research Council.

3. Results

3.1. The effect of age on phenylephrine constriction

There were several differences between the 8 and 54-week-old rat aortas. Firstly, the $-\log EC_{50}$ values for phenylephrine in endothelium-intact aortic rings were significantly different ($P < 0.05$, Student's t-test) between 8-week- (7.45 ± 0.08) and 54-week-old rats (6.83 ± 0.09), although the maxima were not significantly different (see Fig. 1). Endothelium removal (Fig. 1) increased the maximum constriction produced by phenylephrine in 54-week-old aortas, but had no significant effect on the maximum constriction developed in 8-week-old aortas (Fig. 1a and b). This indicates a greater underlying endothelium-dependent inhibition of phenylephrine constriction in aortas from 54-week-old rats. These age-related differences were also seen in other experiments where phenylephrine (0.1 μM) was used to pre-constrict the aortas before addition of isoprenaline (see Table 1).

3.2. The effect of endothelium removal on isoprenaline-mediated relaxation

In endothelium-intact aortas, the vasorelaxant action of isoprenaline (0.03–3.0 μM) was significantly greater in

Table 2
Size of the phenylephrine pre-constriction and the $-\log EC_{50}$ of the isoprenaline relaxation after addition of tetraethylammonium in rat-isolated aorta from rats aged 8 and 54 weeks
TEA, tetraethylammonium. Values are expressed as means ± S.E.M.

	Phenylephrine constriction, g (8-week)	Isoprenaline $-\log EC_{50}$ (8-week)	n	Phenylephrine constriction, g (54-week)	Isoprenaline $-\log EC_{50}$ (54-week)	n
Control	1.38 ± 0.06	7.42 ± 0.09	47	1.24 ± 0.09	6.28 ± 0.16[a]	34
TEA, 1 mM	1.50 ± 0.18	7.12 ± 0.22	7	1.38 ± 0.21	5.35 ± 0.42[a,b]	7
TEA, 10 mM	1.87 ± 0.10[b]	6.28 ± 0.25[b]	10	2.16 ± 0.08[a,b]	> 5[a,b]	10

[a] $P < 0.05$ significantly different from 8-week-old animals treated with the same agent (Student's t-test).
[b] $P < 0.05$ significantly different from age-matched control experiments conducted in the absence of any drug (row 1; Student's t-test).

the 8-week-old aortas compared with the 54-week-old aortas ($P < 0.05$ two-way analysis of variance with repeated measures; compare Fig. 2a and b; Table 1). Removal of the endothelium reduced isoprenaline relaxation in 8-week-, and to a greater extent, in 54-week-old aortas ($P < 0.05$, three-way analysis of variance with repeated measures; Fig. 2). The residual endothelium-independent relaxation in 8-week-old aortas ($-\log EC_{50}$ 6.94 ± 0.31, $n = 6$) was significantly inhibited by the protein kinase A inhibitor Rp-cAMPS (0.5 mM, $-\log EC_{50} < 5$, $n = 7$, $P < 0.05$, Student's t-test).

The size of the phenylephrine pre-constrictions varied between the two age groups of rats (see Table 1), and this may affect the interpretation. We sought to analyse this in two ways. Firstly, we assessed the relationship between the size of the phenylephrine constriction and the extent of the isoprenaline relaxation in endothelium intact and endothelium denuded aortas. This was done using the data from the experiments in Fig. 2a and b at the highest concentration of isoprenaline used (3 μM). In the endothelium-denuded 8- and 54-week combined group, there was no significant relationship between the size of the phenylephrine constriction (y) and the isoprenaline vasorelaxation (x) ($y = 0.0039x + 1.71$; $r^2 = 0.054$) as was the case in the endothelium-intact combined group ($y = 0.0011x + 1.22$; $r^2 = 0.002$). We also matched the pre-constrictions by stepwise deletion of upper and lower experiments from each age group such that the mean pre-constriction was equal for both 8- and 54-week-old animals for aortas that were endothelium intact. A similar procedure was used to compare endothelium-denuded aortas. The mean values of the selected constrictions are shown in Fig. 3. Using this modified data, where the 8- and 54-week pre-constrictions were matched, the relaxation produced by isoprenaline was more markedly reduced by endothelium removal in the older animals ($P < 0.05$, three-way analysis of variance; Fig. 3), which agrees with the unmodified data (Fig. 2).

3.3. The effect of nitric oxide synthase inhibition on isoprenaline-mediated relaxation

In 8-week-old aortas, the nitric oxide synthase inhibitor L-NMMA (100 μM) inhibited the isoprenaline relaxation to a similar extent as endothelium removal (Fig. 2). However, in 54-week-old aortas, although L-NMMA reduced the response to isoprenaline, this was significantly less than the effect of endothelium removal ($P < 0.05$, three-way analysis of variance with repeated measures; Fig. 2). This suggests that there were endothelium-dependent factors additional to nitric oxide contributing to the isoprenaline relaxant effect in 54-week aortas. Indeed, the phenylephrine constriction was also enhanced by denudation in 54-week aortas, but not in 8-week aortas, whereas L-NMMA had no significant effect on phenylephrine constriction at either age (Table 1), again suggesting an additional endothelial factor in the older aortas. When the nitric oxide synthase inhibitor L-NIO (100 μM) was used in

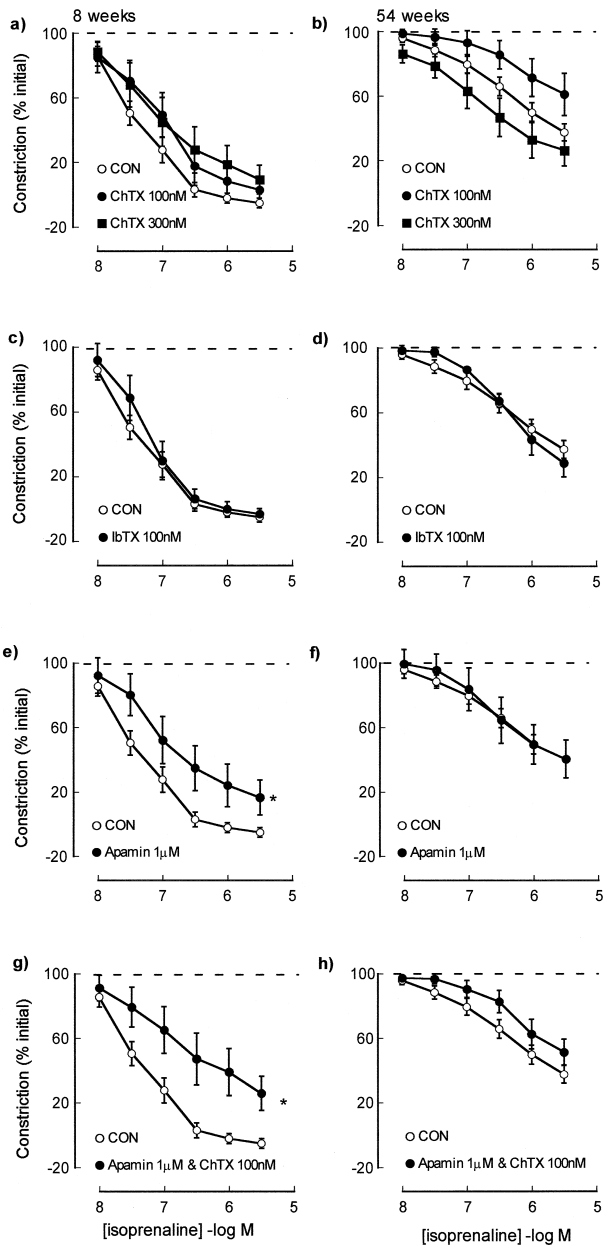

Fig. 5. The effect of charybdotoxin, iberiotoxin and apamin on the relaxant effect of isoprenaline in aorta from rats aged 8 and 54 weeks. Cumulative concentration–response curves for isoprenaline (0.03–3.0 μM) were conducted in phenylephrine (0.1 μM) constricted rat aorta and responses are expressed as a percentage of the initial phenylephrine-induced constriction. Each point represents the mean ± S.E.M ($n = 4$–28). Relaxation to isoprenaline was conducted in aorta from 8-week- and 54-week-old rats. Control experiments were conducted in endothelium intact aorta (CON) and the effect of charybdotoxin (ChTX 100 and 300 nM), 100 nM iberiotoxin (IbTX), 1 μM apamin and the combination apamin and ChTX 100 nM was investigated. $^*P < 0.05$ significant difference between the control and treated tissues in each panel (repeated-measures two-way analysis of variance).

54-week-old aorta, the phenylephrine(0.1 μM) constriction was 1.77 ± 0.17 g, $n = 12$, which was not significantly different ($P < 0.05$, Student's *t*-test) to that in the presence of L-NMMA (1.74 ± 0.44 g, $n = 6$, Table 1). When the combination L-NMMA (100 μM) and indomethacin (10 μM) was used, there was no significant effect on isoprenaline relaxation in either 8- or 54-week aortas ($P > 0.05$, two-way analysis of variance, Fig. 2).

3.4. The effect of K^+ channel inhibitors on isoprenaline-mediated relaxation

In endothelium-intact 8-week-old aortas, tetraethylammonium, an inhibitor of K^+ channels had either no effect (1 mM) or a small effect (10 mM) on the vasorelaxant response to isoprenaline (Fig. 4a; Table 2). In contrast in 54-week-old aortas, tetraethylammonium (1 and 10 mM) had a greater inhibitory effect on the relaxation produced by isoprenaline ($P < 0.05$, three-way analysis of variance; Fig. 4b; Table 2). However, neither charybdotoxin (100 or 300 nM) nor iberiotoxin (100 nM), both large conductance Ca^{2+}-activated K^+ channel inhibitors (BK_{Ca}) affected the relaxation produced by isoprenaline in aortas from either 8- or 54-week-old rats (Fig. 5; Table 3). Furthermore, apamin (1 μM) a small conductance Ca^{2+} activated K^+ channel blocker (SK_{Ca}) had a tendency to attenuate the isoprenaline relaxation in 8-week- but not 54-week-old aortas ($P < 0.05$, two-way analysis of variance; Fig. 5; Table 3). The combination of apamin and charybdotoxin had similar effects to apamin alone, but the effect in 8-week aorta was statistically significant ($P < 0.05$, two-way analysis of variance; Fig. 5). The relaxation response to isoprenaline was not affected by the ATP-sensitive K^+ channel (K_{ATP}) inhibitor glibenclamide (1 μM) in 8-week-old aortas (Fig. 6a; Table 1), but glibenclamide produced a small inhibition in 54-week-old aortas ($P < 0.05$, two-way analysis of variance; Fig. 6b; Table 1). 4-Aminopyridine (1 mM), an inhibitor of delayed rectifier K^+ channels (K_v), had no effect on isoprenaline-mediated relaxation in aortas from 8-week-old rats, but a small potentiation of the isoprenaline mediated relaxation ($P < 0.05$, two-way analysis of variance; Fig. 6; Table 2) was seen in aortas from 54-week-old rats (Fig. 6; Table 2). The presence of Ba^{2+} (30 μM) produced a small but statistically significant attenuation of isoprenaline mediated relaxation at 8 weeks, but had no effect at 54 weeks (Fig. 6). The phenylephrine constriction was not affected by tetraethylammonium (1 mM), charybdotoxin, iberiotoxin, apamin, 4-aminopyridine or Ba^{2+}, however, was enhanced by tetraethylammonium (10 mM) in 8- and 54-week-old aortas and by glibenclamide (1 μM) in 54- but not 8-week-old aortas (Tables 1–3).

3.5. The effect of selective β-adrenoceptor antagonists on isoprenaline-mediated relaxation

The β_1-adrenoceptor-selective antagonist atenolol (0.1 μM), slightly but significantly attenuated the relaxant response to isoprenaline in endothelium-intact 8-week-old aortas (Fig. 7), but had no significant effect in 54-week-old aortas (Fig. 7). The β_2-selective antagonist ICI 118551 (0.1 and 10 μM) significantly attenuated the relaxant response to isoprenaline in endothelium-intact aortas from 8-week-old aortas (Fig. 7; Table 1). However, the relaxant response to isoprenaline in 54-week-old aorta was unaffected by 0.1 μM ICI 118551, but was inhibited by 10 μM ICI 118551 (Fig. 7; Table 1). The inhibitory effect of ICI 118551 was qualitatively different between 8-week-old

Table 3
Size of the phenylephrine pre-constriction and the $-\log EC_{50}$ of the isoprenaline relaxation after addition of various drugs in rat isolated aorta from rats aged 8 and 54 weeks
ChTX, charybdotoxin; IbTX, iberiotoxin; INDO, indomethacin; NMMA, *N*-methyl-l-arginine acetate; 4-AP, 4-aminopyridine. Values are expressed as mean ± S.E.M.

	Phenylephrine constriction, g (8-week)	Isoprenaline $-\log EC_{50}$ (8-week)	*n*	Phenylephrine constriction, g (54-week)	Isoprenaline $-\log EC_{50}$ (54-week)	*n*
Control	1.25 ± 0.45	7.38 ± 0.45	19	1.48 ± 0.13	5.98 ± 0.19[a]	18
ChTX, 100 nM	1.26 ± 0.09	7.09 ± 0.29	11	1.53 ± 0.12	5.51 ± 0.28[a]	8
ChTX, 300 nM	1.31 ± 0.38	6.88 ± 0.32	7	1.77 ± 0.33	6.37 ± 0.42	6
IbTX, 100 nM	1.37 ± 0.11	7.18 ± 0.17	4	1.43 ± 0.27	5.89 ± 0.27[a]	5
Apamin, 1 μM	1.36 ± 0.16	6.83 ± 0.38	8	1.15 ± 0.11	5.96 ± 0.42[a]	6
Apamin, 1 μM and ChTX, 100 nM	1.78 ± 0.10	6.37 ± 0.44	8	1.76 ± 0.14	5.60 ± 0.37	6
4-AP, 1 mM	1.61 ± 0.31	6.21 ± 0.53	6	0.93 ± 0.19[b]	6.41 ± 0.37[b]	8
INDO, 10 μM and NMMA, 100 μM	2.11 ± 0.13	7.13 ± 0.16	7	1.75 ± 0.25	5.62 ± 0.39	7
Ba^{2+}, 30 μM	1.87 ± 0.31	6.64 ± 0.40	4	1.69 ± 0.23	5.79 ± 0.45	5

[a] $P < 0.05$ significantly different from 8-week-old animals treated with the same agent (Student's *t*-test).
[b] $P < 0.05$ significantly different from age-matched control experiments conducted in the absence of any drug (row 1; Student's *t*-test).

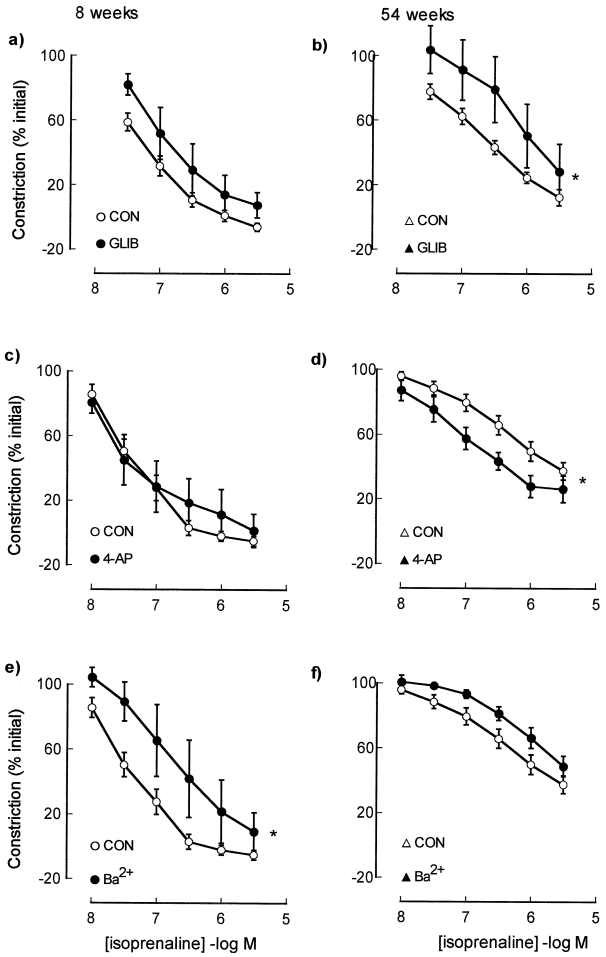

Fig. 6. The effect of glibenclamide, 4-aminopyridine and Ba^{2+} on the relaxant effect of isoprenaline in aorta from rats aged 8 and 54 weeks. Cumulative concentration–response curves for isoprenaline (0.03–3.0 μM) were conducted in phenylephrine (0.1 μM)-constricted rat aorta and responses are expressed as a percentage of the initial phenylephrine-induced constriction. Each point represents the mean ± S.E.M ($n = 4$–28). Relaxation to isoprenaline was conducted in aorta from 8-week- and 54-week-old rats. Control experiments were conducted in endothelium intact aorta (CON) and the effect of 1 μM glibenclamide (GLIB) 1 mM 4-aminopyridine (4-AP) and 30 μM Ba^{2+} were investigated. $^{*}P < 0.05$ significant difference between the control and treated tissues in each panel (repeated-measures two-way analysis of variance).

aortas compared to 54-week-old aortas in that the shapes of the inhibition curves were different (Fig. 7). In both 8- and 54-week-old aortas, neither atenolol nor ICI 118551 affected the constrictor effect of phenylephrine (Table 1). Finally, we tested the dilator effect of the β_3-adrenoceptor-selective agonist BRL 37344 and in concentrations up to 10 μM, it had no relaxant effect in either 8- or 54-week aortas (not shown).

4. Discussion

The current study examined the signalling pathways associated with isoprenaline-induced relaxation of aortas isolated from rats of different ages. The reason for the study was to examine the hypothesis that inconsistent results in the literature regarding the requirement for an intact endothelium for β-adrenoceptor relaxation (see Introduction) was because there were age-dependent changes in the β-adrenoceptor signalling pathways. We used aortas from 8-week-old and 54-week-old rats and found a far greater diminution in the effect of isoprenaline after endothelium removal in the older animals, supporting our hypothesis of age dependence. Although age is often not reported in studies, there is indirect evidence supporting the hypothesis since studies using heavier rats (> 350 g: 100% dependent Gray and Marshall, 1992; 66% dependent Delpy et al., 1996) show a greater endothelium dependence of β-adrenoceptor relaxation than studies using smaller rats (< 350 g: 50% dependent Kamata et al., 1989; 0% dependent Konishi and Su, 1983; and 0% dependent Moncada et al., 1991). Furthermore, in one laboratory, Satake et al. (1996, 1997) demonstrated that endothelium removal had a very small inhibitory effect on isoprenaline-mediated relaxation in aorta from male rats weighing 150–170 g, yet a much larger inhibitory effect was seen in aortas from rats weighing 200–300 g (Satake et al., 1995).

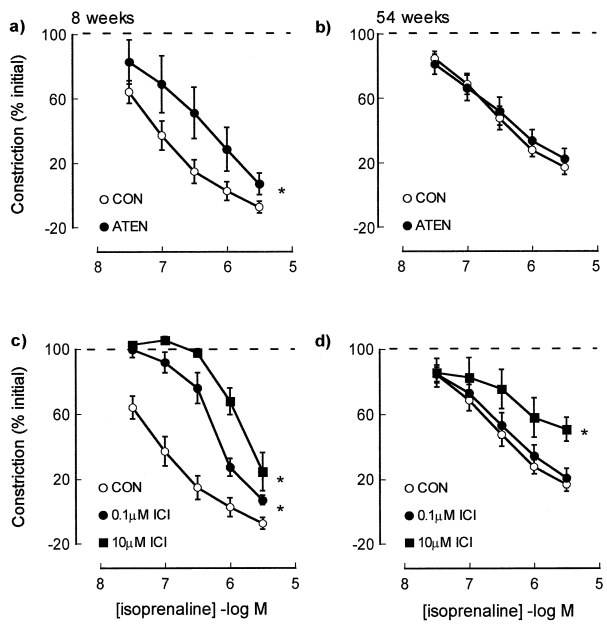

Fig. 7. The effect of atenolol and ICI 118551 on the relaxant effect of isoprenaline in aorta from rats aged 8 and 54 weeks. Cumulative concentration–response curves for isoprenaline (0.03–3.0 μM) were conducted in phenylephrine (0.1 μM) constricted rat aorta and responses are expressed as a percentage of the initial phenylephrine-induced constriction. Each point represents the mean ± S.E.M ($n = 3$–28). Relaxation to isoprenaline was conducted in aorta from 8-week- and 54-week-old rats. Control experiments (CON) were conducted in endothelium-intact aorta and the effect of 0.1 μM atenolol (ATEN), and ICI 118551 (ICI, 0.1 and 10 μM) was investigated. $^{*}P < 0.05$ significant difference between the control and treated tissues in each panel (repeated-measures two-way analysis of variance).

The constrictor effect of phenylephrine was potentiated to a greater extent by endothelial removal in 54-week compared to 8-week aortas, which suggests that there was a greater tonic inhibitory effect of the endothelium in the older rats. Similarly, endothelial removal increased the constrictor response to noradrenaline to a greater extent in aortas from 86-week- compared to 12-week-old female rats (Hashimoto et al., 1998). There are also age-related parallels with angiotensin II in rat aortas where endothelium removal potentiated angiotensin II constriction more in 25-week-old aortas when compared to 6-week-old aorta (Wakabayashi et al., 1990). This effect of the endothelium may be because the endothelium produces a basal release of nitric oxide or that the constrictor agent induces the release of nitric oxide to attenuate constrictor influences (see Moncada et al., 1991). It should be noted however that L-NMMA did not significantly increase phenylephrine constriction in older rats although the results were highly variable. It may be that the L-NMMA concentration was not sufficiently high to cause inhibition although the concentration used in the present study caused maximum inhibition of endothelium dependent acetylcholine relaxation in rat aorta (Rees et al., 1990). This study (Rees et al., 1990) also noted that L-NIO, another nitric oxide synthase inhibitor, potentiated phenylephrine constrictions to a greater extent than L-NMMA. When this was tested in the present study, the constrictor effect of phenylephrine in the presence of L-NIO was not different to that with L-NMMA. It may be that the endothelial factor, which suppresses phenylephrine constriction, is not nitric oxide.

Since endothelium removal altered the constriction produced by phenylephrine to a greater extent in older rats, it is possible that this could have confounded our interpretation of the effects of the endothelium in the vasorelaxation produced by isoprenaline in the two age groups. However, there was no significant relationship between the size of the phenylephrine pre-constriction and the isoprenaline relaxation. Secondly, it was possible to achieve similar pre-constrictions by deleting experiments from each experimental series to contrive a mean pre-constriction, which was equal for both 8- and 54-week-old animals. In this case, the relaxation produced by isoprenaline was more markedly reduced by endothelium removal in the 54-week animals when compared to the 8-week ones, similar to the unmodified data.

There have been several suggestions as to the endothelial factors involved in β-adrenoceptor vasorelaxation. The first is that activation of β-adrenoceptors induces the release of nitric oxide from the vascular endothelium (Graves and Poston, 1993). The second hypothesis is that basal nitric oxide stimulates cyclic GMP production and that this inhibits cyclic AMP degradation (Delpy et al., 1996). Both of these hypotheses require that the β-adrenoceptor relaxation be blocked by nitric oxide synthase inhibitors as has been reported (e.g. Graves and Poston, 1993; Delpy et al., 1996). We also observed this, but the attenuation of isoprenaline relaxation by the nitric oxide synthase inhibitor L-NMMA was small and the magnitude of the inhibition was the same for both 8-week and 54-week aortas, thus, it cannot explain the age-related differences of the present study. Another endothelium dependent vasodilator is prostacyclin, a cyclo-oxygenase metabolite of arachidonic acid (see Busse et al., 1993), however, the combination of the cyclo-oxygenase inhibitor indomethacin with L-NMMA did not affect the isoprenaline relaxation. This points to an endothelial factor other than prostacyclin and nitric being involved.

An endothelium-derived hyperpolarizing factor (EDHF) has been proposed to be released by various agents to relax smooth muscle (see Félétou and Vanhoutte, 1996). Some studies suggest that EDHF activation hyperpolarizes vascular smooth muscle through Ca^{2+}-activated K^+ channels (see Félétou and Vanhoutte, 1996), which are also involved in β-adrenoceptor responses (Scornik et al., 1993), and, thus, an EDHF may be the endothelial factor responsible for age-related changes in isoprenaline relaxation. If an EDHF is to explain the age-related effects of the endothelial involvement in isoprenaline-mediated relaxation, then isoprenaline relaxation in older animals should be less apparent with K^+ pre-constriction compared to phenylephrine pre-constriction. Indeed, whereas we observed isoprenaline relaxation in aorta at 54 weeks in phenylephrine constricted aorta, others have found either no relaxation or a small relaxation to isoprenaline beyond 24 weeks in K^+ constricted aorta (O'Donnell and Wanstall, 1984; Hyland et al., 1987; Sawyer and Docherty, 1987; Chapman et al., 1999). Furthermore, the age-dependent reduction in isoprenaline-mediated relaxation in aorta was greater in K^+ than in phenylephrine-constricted aortas (Borkowski et al., 1992; Chapman et al., 1999). These findings support the view that the signalling pathways involved in isoprenaline-mediated relaxation switch towards an increased role of K^+ channels in older rats.

In the present study, tetraethylammonium, a K^+ channel blocker with some selectivity towards BK_{Ca} (Brayden, 1996), produced a greater inhibition of isoprenaline relaxation in 54-week compared to 8-week aortas supporting the switching of the signalling pathway towards an increased role of K^+ channels in the older aortas. However, the concentrations we used (1 and 10 mM) may not be entirely selective for the BK_{Ca} channel (see Brayden, 1996). Indeed, both charybdotoxin and iberiotoxin, which are more selective BK_{Ca} blockers, did not affect isoprenaline relaxation in either age group even though the concentrations used were well in excess of their reported IC_{50} for BK_{Ca} (see Brayden, 1996). This suggests that other types of K^+ channels are involved. A range of other inhibitors against other K^+ channels: apamin (SK_{Ca}), glibenclamide (K_{ATP}), 4-aminopyridine (K_v), Ba^{2+} (K_{IR}) (see Brayden, 1996) showed neither a marked blockade of the isoprenaline effect nor an age dependence of blockade, which raises the question of which channel is involved.

The above discussion has concentrated on an enhanced endothelium-dependent pathway in older aortas. However, it should also be noted that there is also a decreased non-endothelium-dependent effect in these aortas. This non-endothelium-dependent effect involves cyclic AMP as evidenced by the inhibitory effect of Rp-cAMPS in the present study. Other studies have also shown a decreased cyclic AMP relaxation with age. Indeed, relaxation mediated by adenosine and parathyroid hormone receptor agonists as well as the G-protein activator cholera toxin, which are all known to occur through the adenylate cyclase–cyclic AMP signalling pathway, is impaired in older animals (Deisher et al., 1989; Ishikawa et al., 1995; Chapman et al., 1999). Furthermore, relaxation produced by the cyclic AMP analogue dibutyryl cyclic AMP is reduced in older animals (Tsujimoto et al., 1986). It also has been observed that cyclic AMP generation in response to β-adrenoceptor stimulation is reduced in older blood vessels (Deisher et al., 1989). It may be that the enhanced endothelial effect is to compensate for this.

Age-related signal transduction switching could relate to different populations of β-adrenoceptors being involved in the isoprenaline-mediated vasorelaxation. In agreement with previous studies (O'Donnell and Wanstall, 1984; Satake et al., 1997), in the present study, the predominant receptor involved in isoprenaline-mediated relaxation is the β_2-adrenoceptor subtype, with a possible smaller role for β_1-adrenoceptors. However, there appeared to be no marked differences between the two age groups except that in the 54-week group, the β_2-adrenoceptor-selective antagonist ICI 118551, produced non-parallel shifts in the isoprenaline concentration–response curves, and at lower concentrations of ICI 118551, there was some resistance to blockade. The explanation of these effects is unclear, and it is not indicative of an enhanced β_3-adrenoceptor effect as the β_3-adrenoceptor-selective agonist BRL 37344 had no vasorelaxant effect.

In summary, the signalling pathways involved in β-adrenoceptor vasorelaxation are multifaceted. They include a nitric oxide-dependent pathway, which does not vary with age of the animal. Secondly, there are endothelium-independent effects, which involve cyclic AMP, and this appears to decline with age. Finally, there appears to be an endothelium-dependent pathway, which involves tetraethylammonium-sensitive K^+ channels, and this increases with age. The precise coupling mechanisms remain to be resolved, but these observations may answer some of the discrepancies in the literature associated with β-adrenoceptor vasorelaxation.

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