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Modulation of vascular responses of guinea-pig aorta by non-endothelial nitric oxide: A minor role for the endothelium

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ABSTRACT

Acetylcholine (Ach) causes vasodilatation by nitric oxide (NO) release from the vascular endothelium. Vasoconstrictors such as α-adrenoceptor agonists (phenylephrine) or thromboxane TxA_2 mimetics (U46619) also release endothelial NO. Inhibition of nitric oxide synthase (NOS) with N^{ω}-nitro-L-arginine (L-NAME) potentiates vasoconstriction by phenylephrine and the trace amine, β-phenylethylamine (PEA), indicating underlying opposing vasodilatation. However, the roles of the endothelium and NO in vasodilator and constrictor responses of guinea-pig aorta have not been examined and are the subject of this study. Guinea-pig thoracic aorta rings were set up in aerated Krebs solution (37 °C) and isometric tension recorded. Contractions to phenylephrine were fast onset, rapidly waned and antagonised by prazosin. PEA contractions were slow onset, sustained and not antagonised by prazosin and therefore not α_1-adrenoceptor-mediated. PEA and phenylephrine contractions were enhanced by L-NAME whether endothelium was present or not. Ach produced only weak relaxation in a small proportion of endothelium intact U46619-constricted aortae, which were abolished by endothelium removal. In uncontracted aortae Ach caused small contractions, which like PEA contractions were potentiated by endothelium removal. α-Adrenoceptor agonists and trace amines release NO from non-endothelial sites causing underlying opposing vasodilatation. The endothelium plays only a minor role in vasodilator and vasoconstrictor responses of guinea-pigs aorta.

1. Introduction

The hypotensive and vasodilator effects of acetylcholine (Ach) are due to the release of nitric oxide (NO) from the vascular endothelium. Ach causes a vasodilator response in rabbit aorta pre-constricted with noradrenaline and this is converted to a contraction by removal of the endothelium, as was first demonstrated by Furchgott and Zawadski in 1980 [11]. Since then numerous studies have shown that endothelium removal can abolish the vasodilatation by Ach in rabbit [15] and normotensive [7] and spontaneously hypertensive rat aorta [2]. The vasodilator response to Ach is mediated via endothelial M_3 muscarinic receptors [2,15], whereas the endothelium-independent contraction of rabbit aorta is mediated via M_2 muscarinic receptors on smooth muscle cells [15]. That the vasodilator response to Ach is due to release of NO was demonstrated by its elimination with the nitric oxide synthase (NOS) inhibitor N^{ω}-nitro-L-arginine (L-NAME) in rat aorta [19]. Other vasodilators including bradykinin [18] and histamine [7] but not adenosine also mediate their response through the release of NO from the vascular endothelium.

It is well established that endogenous vasoconstrictors such as noradrenaline, angiotensin and thromboxane TxA_2 also promote the pharmacological release of NO from the vascular endothelium which exerts a modulating effect on the vasoconstriction [25]. Mechanical removal of the endothelium in vivo by means of a catheter, for example of a dog coronary artery, has shown potentiation of 5-hydroxytryptamine-induced vasoconstriction but not of phenylephrine [17]. Endothelial removal in rat isolated aorta also potentiates the vasoconstrictor actions of the thromboxane TxA_2 mimetic, U46619, [10] and of noradrenaline, angiotensin II and histamine [7]. Inhibition of NOS with L-NAME potentiates the responses to the selective α_1-adrenoceptor agonist, phenylephrine, in rat aorta ([24]; Taberno et al., 1996). It was assumed that the NO release is from the endothelium since there was no potentiation of phenylephrine responses in endothelium-denuded rat aorta [24]. Thus, NO release by these vasoconstrictors exerts an

Abbreviations: Ach, acetylcholine; cGMP, cyclic guanosine monophosphate; CRC, concentration-response curve; L-NAME, N^{ω}-nitro-L-arginine; NO, nitric oxide; NOS, nitric oxide synthase; PEA, β-phenylethylamine; TAAR, trace amine-associated receptor

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opposing modulating action on their vasoconstrictor activity. The role of NO and endothelium appears to differ between different blood vessels, L-NAME having no effect on phenylephrine in rat tail artery [23]. There is little information on the role of NO in the vasoconstrictor responses in the blood vessels of species other than the rat, apart from rabbit aortae, where phenylephrine vasoconstriction was potentiated by L-NAME [8]. Data for human, mouse and guinea-pig vessels is absent. Thus, we have examined the roles of NO and endothelium in the vasoconstrictor responses of the aorta of guinea-pigs to phenylephrine.

Trace amines such as β-phenylethylamine (PEA), tyramine and octopamine occur in the body derived from endogenous synthesis or from the diet and cause vasoconstriction and increases in blood pressure. Their accepted mechanism of action has been widely regarded as being due to an indirect sympathomimetic action releasing noradrenaline onto vascular α_1-adrenoceptors. However, recent evidence shows that they constrict blood vessels through stimulation of trace amine-associated receptors (TAARs) [3]. PEA and the α_1-adrenoceptor agonist, phenylephrine, were potentiated by L-NAME in guinea-pig endothelium-intact aortae due to removal of the opposing vasodilator actions of NO [4]. This indicated that both PEA and phenylephrine release NO probably from the endothelium in the aorta of this species. We could find only one reference to the role of endothelium in the vascular responses of this species [13]. This described endothelium-dependent relaxation of noradrenaline-precontracted aorta by Ach which was smaller than for substance P. Thus, we have examined the roles of the endothelium in the responses of guinea-pig aorta to Ach, phenylephrine and PEA and whether the potentiation of PEA and phenylephrine by L-NAME seen in endothelium-intact tissues was also seen after removal of the endothelium.

2. Methods

2.1. Guinea-pig isolated aortic rings

Male Dunkin-Hartley guinea-pigs (250-350 g) (Charles River, U.K.) were acclimatised for one week with their new surroundings before commencement of experiments. They were housed in flat bottomed cages with environmental enrichment in the form of cardboard tubes and hay and were given food and water ad-libitum. The housing conditions were: twelve hour light/dark cycles, at 50% humidity and room temperature of 20 °C ± 2 °C. Guinea-pigs were killed by cervical dislocation and exsanguination. The guidelines for the care and use of laboratory animals were followed according to the Animals (Scientific Procedures) Act 1986. The work and its reporting were undertaken according to the principles for transparent reporting and scientific rigour of preclinical research as set out in the Basel Declaration [20]. The number of animals used was 28.

The thoracic aorta was removed and cut into at least four ring sections each approximately 0.5 cm long. Fixed and mobile hangers were passed through the ring, the fixed hanger being secured in a 50 ml organ bath. The bath was filled with pre-warmed (37 °C) Krebs-bicarbonate buffer gassed with CO_2/O_2 (5%/95%) (BOC Gases, Guildford, UK). The Krebs bicarbonate buffer was made up in distilled water and had the following composition (mM): NaCl (118), $NaHCO_3$ (25), glucose (11.7), $MgSO_4.7H_2O$ (1.2), KH_2PO_4 (1.2), KCl (4.7) and $CaCl_2.2H_2O$ (2.5). Organ baths were maintained at 37 ± 0.5 °C by a circulator (type KD Grant Instruments, Cambridge, UK). A suture attached to the upper mobile hanger was connected to an isometric transducer (Dynamometer UF1, 57 g sensitivity range, Pioden Controls Ltd., Canterbury, UK). A resting tension of 1.5 g was applied to the ring and isometric tension recorded and displayed on a computer (Power Lab, Chart 5, ADInstruments, Chalgrove, Oxfordshire, UK). To check that functional endothelium was not removed by this set-up procedure, in a selection of tissues, acetylcholine (100 μM) was added to aortic rings precontracted with U46619 (0.5 or 1 μM).

2.2. Experimental protocols

After 1 h equilibration, a cumulative concentration-response curve (CRC) for β-phenylethylamine (PEA) or phenylephrine was obtained by addition of half logarithmic increments in concentration, each successive concentration being added after the peak effect was reached for the preceding concentration. The contraction to each concentration was allowed to develop fully which could take up to 15 min in the case of PEA. After the maximum effect, the tissue was washed and after approximately 15 min a second wash to restore baseline. A second CRC was then constructed in the presence of L-NAME (100 μM), which was left in contact with the tissue for 15 min before commencing the second CRC. At the end of each experiment, isotonic KCl (60 mM) was routinely added.

Relaxation responses for single doses of Ach (100 μM) or CRCs for sodium nitroprusside were obtained after preconstriction of the aorta to a plateau with U46619 (0.5 or 1 μM). Sodium nitroprusside was added cumulatively in half logarithmic increments until the maximum relaxation.

2.3. Endothelial removal

Endothelium was removed in some tissues before setting up by gently rolling the aortic ring around the fixed hanger several times before inserting the mobile hanger. In other experiments the endothelium was removed after construction of the first CRC for the contractile responses to Ach or phenylephrine. In these, the tension was restored to baseline by washing following the contractions and the tissue was raised from the organ bath. The tension was slackened off so that the aortic ring could be rotated around the hangars several times to remove endothelium. The tissue was returned to the bath and the resting tension restored to 1.5 g. After a short equilibration period (10 min), the second CRC was constructed.

2.4. Analysis of results

Contractions at the plateau response to each concentration of agonist were measured from the baseline before the CRC. These were then expressed as a percentage of the contraction to KCl in each experiment, to normalize each response to the maximum contractility of each tissue. The mean responses (± S.E.M.) were then plotted. Time courses for approximately 50% responses to PEA and phenylephrine were plotted by measuring the increase in tension from the previous dose maximum every 15 s for the first minute, every 30 s for the next 4 min and then every minute. These were plotted as a percentage of the maximum contraction to that concentration of agonist and the mean (± S.E.M.) plotted against time. n values are the number of guinea-pigs providing aortae. Maximum responses before and after inhibitors and at individual concentrations in the same tissue were compared by paired Student's *t*-tests, whereas responses in different tissues were compared by Student's unpaired *t*-tests. EC_{40} values were calculated as the molar concentration required to produce 40% of the maximum response to KCl. This was to ensure that values were obtained for all tissues as not all reached 50% of the KCl maximum contraction. These were converted to the $-\log EC_{40}$ values and the mean values (± S.E.M.) calculated. They were compared by Student's paired or unpaired *t*-tests. Differences were considered significant when $P < .05$.

2.5. Drugs used

N^{ω}-nitro-L-arginine methyl ester hydrochloride (L-NAME), prazosin hydrochloride, (−)-phenylephrine hydrochloride, β-phenylethylamine hydrochloride (PEA) and U46619 (9,11-Dideoxy-11α,9α-epoxymethanoprostaglandin F2α) were obtained from Sigma-Aldrich (Poole, Dorset, UK). All chemicals for the Krebs-bicarbonate buffer were of analytical grade and were obtained from Fisher Scientific,

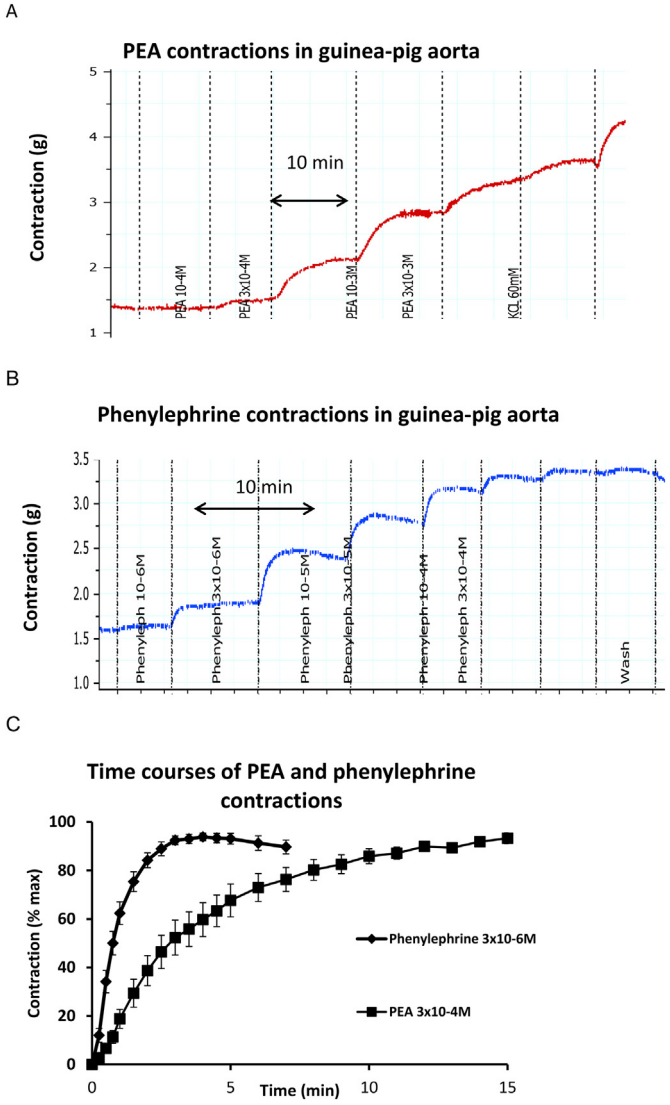

Fig. 1. Contractions of guinea-pig aorta to β-phenylethylamine (PEA) and phenylephrine. A. Typical cumulative concentration-response curve for PEA with KCl (60 mM) added at the maximum dose. B. Typical cumulative concentration-response curve for phenylephrine. C. Mean time courses for contractions to 3×10^{-4}M of PEA ($n = 14$), which was the 40.0 ± 6.8%KCl response, and to 3×10^{-6}M of phenylephrine ($n = 13$) which was the 55.5 ± 6.6%KCl response. Increases in tension from the previous dose maximum were measured and expressed as a percentage of the maximum contraction to that concentration of agonist and the mean (± SEM) plotted against time.

Leicestershire, UK. PEA, L-NAME and phenylephrine were dissolved in distilled water. Prazosin hydrochloride was dissolved in dimethylsulfoxide (DMSO):distilled water (1:10) and further diluted 1 in 10 with DMSO:water (1:10).

3. Results

3.1. Effects of phenylephrine and PEA on guinea-pig aorta

Phenylephrine and β-phenylethylamine (PEA) caused concentration-related contractions of guinea-pig aorta (Fig. 1). The mean -logEC$_{40}$ values were 5.87 ± 0.18 ($n = 11$) and 3.56 ± 0.09 ($n = 22$), respectively. The contractions to phenylephrine were rapid in onset and then waned while the amine was still present in the bath. The time course for the response to 3×10^{-6}M of phenylephrine (55.5 ± 6.6% KCl), showed a peak at 3.9 ± 0.4 min ($n = 13$). In contrast, PEA contractions were slow in onset and sustained and the mean peak for the 40.0 ± 6.8% response (3×10^{-4}M) occurred at 13.6 ± 1.3 min ($n = 14$), which was significantly longer than for phenylephrine (Fig. 1C).

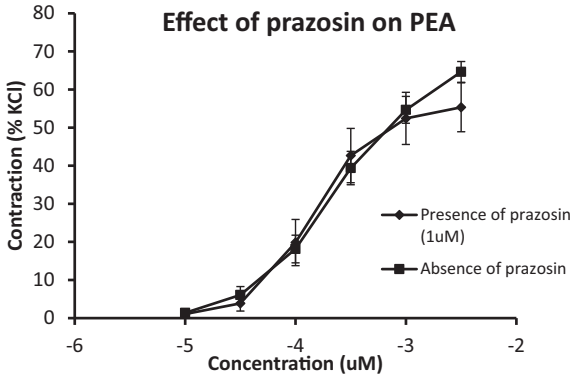

Fig. 2. Effect of prazosin (1 μM) on the contractions of guinea-pig aorta to β-phenylethylamine (PEA). Mean concentration-response curves for PEA in the absence (■, $n = 19$) and, in separate tissues, in the presence of prazosin (1 μM, ♦) ($n = 5$). Responses are plotted as the increase in tension expressed as a percentage of the maximum contraction to KCl (60 mM).

In the presence of the selective α_1-adrenoceptor antagonist, prazosin (1 μM), added 30 min before the CRC for PEA, the CRC was not significantly different from that in tissues in the absence of prazosin (Fig. 2). The –logEC40 values of 3.55 ± 0.09 in the absence ($n = 19$) and 3.65 ± 0.15 ($n = 5$) in the presence of prazosin were not significantly different. We have previously shown that this concentration of prazosin caused a 30.5-fold shift of the CRC for phenylephrine in guinea-pig aorta [4].

3.2. Effects of phenylephrine and PEA in endothelium-intact and denuded aorta

The CRCs for phenylephrine were virtually superimposed for endothelium-intact and endothelium-denuded aortae. There were no significant differences in any responses between the CRCs, the maxima in intact and denuded tissues being 80.4 ± 3.0 and 77.1 ± 8.1%, respectively (Fig. 3A). The –logEC$_{40}$ value of denuded tissues (5.54 ± 0.17) ($n = 6$) was not significantly different from the value in intact tissues (5.87 ± 0.18) ($n = 11$). Similarly, the CRCs for PEA in endothelium-intact and denuded aortae were virtually superimposed with no significant differences in responses between the CRCs, the maxima in intact and denuded tissues being 71.7 ± 3.3 and 60.2 ± 3.4%, respectively (Fig. 3B). The –logEC$_{40}$ values for PEA in endothelium-intact (3.56 ± 0.09) ($n = 22$) and denuded aortae (3.36 ± 0.12) ($n = 16$) were not significantly different.

3.3. Effect of L-NAME in intact and endothelium-denuded aorta

In endothelium-intact aortae, L-NAME (100 μM) potentiated the responses to PEA. The maximum response was significantly raised from 59.1 ± 3.6 to 87.5 ± 2.8% ($n = 7$) in the presence of L-NAME (Fig. 4A) and the –logEC$_{40}$ value was significantly increased from 3.40 ± 0.16 to 3.91 ± 0.15. In control experiments receiving no L-NAME between CRCs, there was a small non-significant potentiation of the second curve maximum response to PEA from 65.7 ± 6.0 to 74.8 ± 1.4%. The potentiation by L-NAME was significantly greater than in the controls. The phenylephrine CRC was also significantly elevated by L-NAME (Fig. 4B), the maximum response increasing from 75.8 ± 2.2 to 94.0 ± 0.8% ($n = 5$). However, the –logEC$_{40}$ values before (6.01 ± 0.26) and in the presence of L-NAME (6.16 ± 0.14)

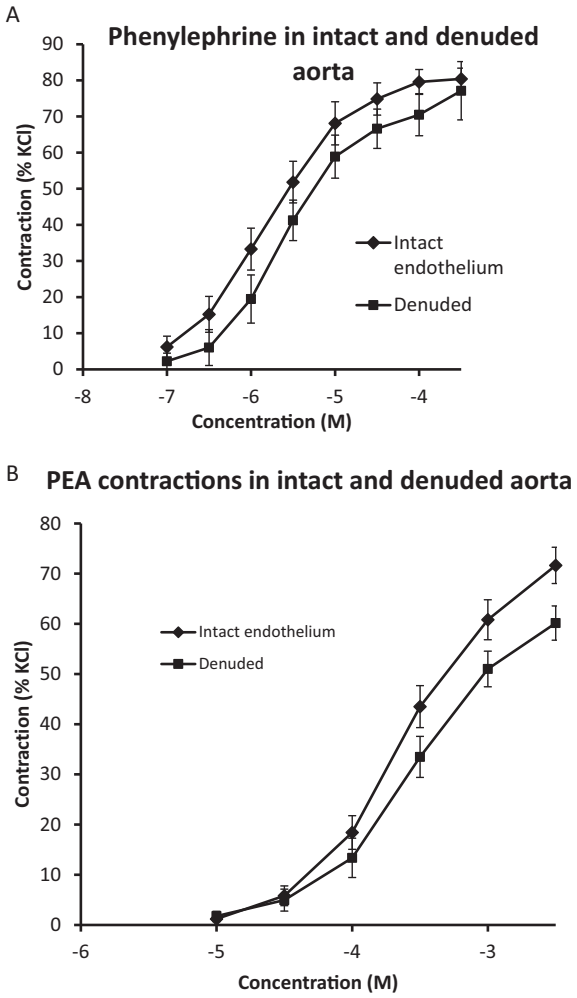

Fig. 3. Effect of denuding guinea-pig aorta on contractions to phenylephrine and β-phenylethylamine (PEA). A. Mean concentration-response curves for phenylephrine in aortae with intact endothelium (◆, $n = 11$) and, in separate tissues, with denuded endothelium (■, $n = 6$). B. Mean concentration-response curves for PEA in aortae with intact endothelium (◆, $n = 22$) and, in separate tissues, with denuded endothelium (■, $n = 16$). Responses are plotted as the increase in tension expressed as a percentage of the maximum contraction to KCl (60 mM).

were not significantly different. In endothelium-denuded aortae, L-NAME (100 μM) also potentiated both PEA and phenylephrine. In the presence of L-NAME, the CRC for PEA was increased, the maximum significantly increasing from 60.8 ± 6.9 to 86.9 ± 2.3% ($n = 6$) (Fig. 4C) and the $-\log EC_{40}$ value in the presence of L-NAME (3.80 ± 0.13) was significantly higher than in its absence (3.33 ± 0.26). In control endothelium-denuded aortae that received no L-NAME between CRCs, there was a small significant potentiation of the second curve to PEA, the maximum increasing from 68.4 ± 2.9 to 80.4 ± 2.9%. However, the potentiation by L-NAME was significantly greater than in the controls. The CRC for contractions to phenylephrine was also potentiated by L-NAME, the maximum significantly increasing from 70.3 ± 6.8 to 88.9 ± 3.7% ($n = 5$) (Fig. 4D). The $-\log EC_{40}$ value in the presence of L-NAME (5.97 ± 0.15) was significantly higher than in its absence (5.46 ± 0.22).

3.4. Effect of baseline and plotting method on the action of L-NAME

The baseline tension after washout of the first exposure to PEA was less than the starting baseline tension before the first CRC. In endothelium-intact aortae, the baseline tension before commencing the first CRC for PEA was 1.52 ± 0.06 g and this fell significantly to 1.38 ± 0.06 g after washout and before adding the L-NAME. In endothelium-denuded aortae, the baseline tension before commencing the PEA CRC was 1.42 ± 0.07 g and this also significantly fell to 1.20 ± 0.09 g after washout and before addition of the L-NAME. It was therefore possible that the potentiation of the contractions to PEA was simply a reflection of the lower baseline from which the contractions commenced in the presence of L-NAME. To test this possibility, experiments were performed to examine PEA in denuded aortae before and in the presence of L-NAME but with the baseline readjusted to 1.5 g after washout of the first CRC to PEA. In these experiments, the baseline tensions before commencing the first CRC for PEA was 1.39 ± 0.06 g and this fell to 1.18 ± 0.03 g after washout ($n = 5$). The mean baseline after readjustment was 1.51 ± 0.04 g. L-NAME again potentiated the contractile responses to PEA, the maximum response was significantly increased from 49.4 ± 6.0% to 83.6 ± 3.9% in the presence of L-NAME and the –logEC40 was significantly increased from 3.08 ± 0.25 to 3.72 ± 0.13 in the presence of L-NAME (Fig. 5A).

It was also possible that plotting the responses as a percentage of the maximum response to KCl added at the end of the experiment may have influenced the interpretation of the data, especially if the KCl response was affected by the presence of L-NAME. Therefore we plotted the responses as change in tension (g) for the examination of L-NAME on PEA responses in aortae with intact endothelium. The CRC was significantly enhanced and at the maximum, the response significantly increased from 1.26 ± 0.23 g to 2.16 ± 0.40 g ($n = 7$) in the presence of L-NAME (Fig. 5B).

3.5. Testing for the presence of intact endothelium and endothelium removal

The comparisons of PEA and phenylephrine in endothelial intact and denuded preparations had shown no significant differences and this raised the question whether the endothelium played a role in the contractile responses to these agonists or whether endothelial removal had been effective. Endothelium removal was therefore tested by adding acetylcholine (Ach, 100 μM) to a sample of tissues contracted with U46619 (1 or 0.5 μM). In endothelium-intact aortae, 5 out of 14 tissues responded with small relaxations to Ach (Fig. 6A). The mean relaxation was 0.08 ± 0.02 g, which represented only 7.6 ± 3.3% of the U46619 contraction (1.44 ± 0.29 g). No relaxations occurred in denuded aortae ($n = 6$) (Fig. 6B). The lack of substantial relaxations to Ach in U46619-contracted aortae made us question whether the tissues were capable of demonstrating relaxation responses to any drug. We therefore examined the nitric oxide donating vasodilator, sodium nitroprusside in U46619-pre-contracted tissues. Aortae were contracted with U46619 (1 μM) and at the plateau, a CRC for sodium nitroprusside was commenced. Concentration-related relaxations occurred, the tissue relaxing to 97.3 ± 5.1% of the U46619-induced contraction (1.58 ± 0.21 g) ($n = 4$) at 3×10^{-5}M (Fig. 6C). We compared the magnitude of the contractions to U46619 (1 μM) in endothelium intact and denuded aortae. Intact tissues contracted by 1.35 ± 0.11 g ($n = 17$) whereas denuded tissues contracted by 1.09 ± 0.26 g ($n = 6$), which was not significantly different.

In uncontracted aortae, Ach caused small concentration-related contractions rather than relaxations. We therefore determined the effects of removal of the endothelium on these contractile responses by performing a CRC in aortae with intact endothelium and then repeating a CRC in the same tissue after removing the endothelium. The CRC for the contractile response to Ach was potentiated, with the response to 10^{-4}M significantly increased from 18.4 ± 5.4 to 34.7 ± 11.2% (Fig. 7A). Since the maximum response before rubbing was only 24.3 ± 7.9%, it was not possible to measure $-\log EC_{40}$ values. We therefore measured $-\log EC_{15}$ to accommodate all CRCs. The $-\log EC_{15}$ values before and after rubbing to remove endothelium were 4.3 ± 0.37 and 5.3 ± 0.54 ($n = 5$), respectively which were not

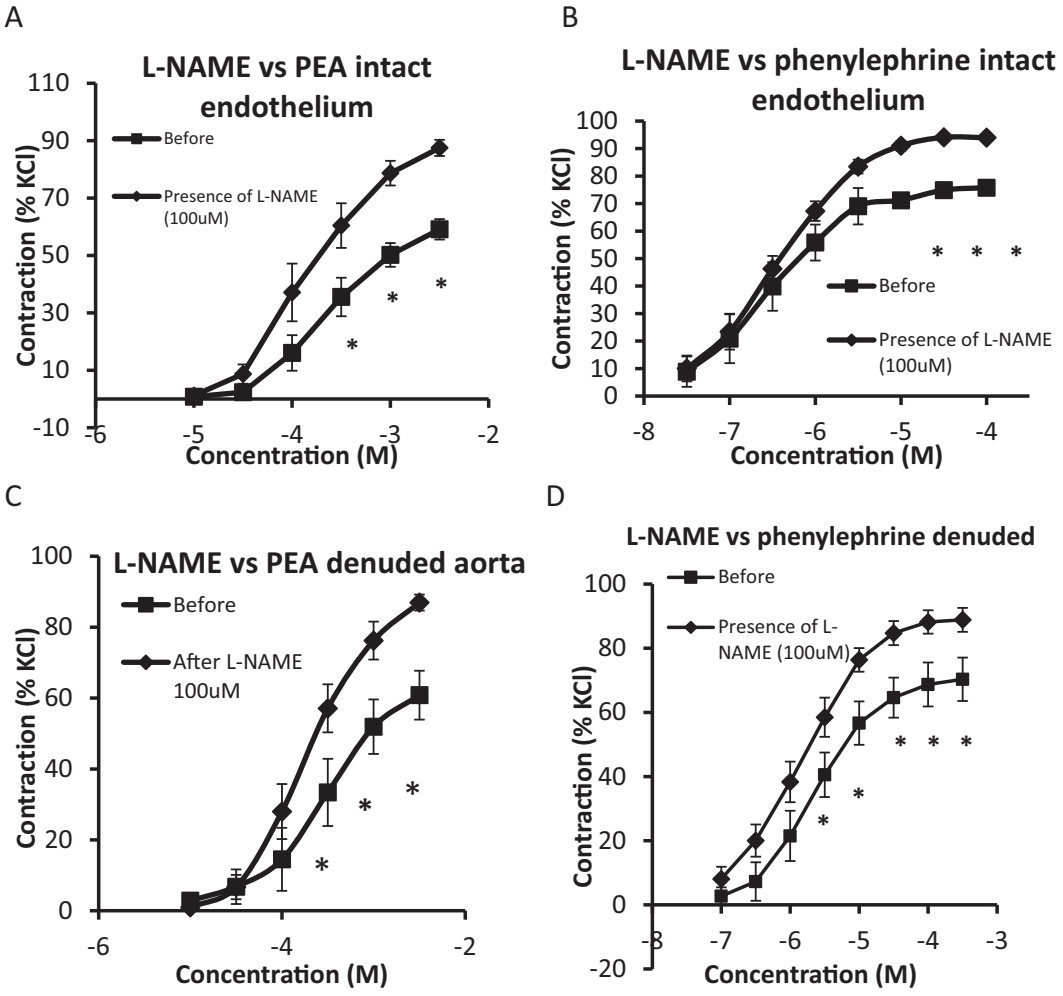

Fig. 4. Effects of L-NAME (100 μM) on contractions of guinea-pig aorta to β-phenylethylamine (PEA) and phenylephrine. A. Mean concentration-response curves for PEA in endothelium-intact aortae before (■) and in the presence of L-NAME (♦) (n = 7). B. Mean concentration-response curves for phenylephrine in endothelium-intact aortae before (■) and in the presence of L-NAME (♦) (n = 5). C. Mean concentration-response curves for PEA in endothelium-denuded aortae before (■) and in the presence of L-NAME (♦) (n = 6). D. Mean concentration-response curves for phenylephrine in endothelium-denuded aortae before (■) and in the presence of L-NAME (♦) (n = 5). Responses are plotted as the increase in tension expressed as a percentage of the maximum contraction to KCl (60 mM). * Significant difference between with and without L-NAME $P < .05$.

significantly different.

This design of experiment was next applied to the contractile responses of the aorta to phenylephrine. A CRC to phenylephrine was constructed in endothelium-intact aortae and after washout and rubbing off the endothelium, a second CRC was obtained. The CRC for phenylephrine was potentiated after rubbing the endothelium. The maximum response was significantly increased from 66.9 ± 6.2 to 83.6 ± 1.5% (n = 6) (Fig. 7B) but the increase in the -logEC_{40} from 5.22 ± 0.29 to 5.64 ± 0.06 was not significant.

4. Discussion

We previously showed that the contractile responses of guinea-pig aorta to the α_1-adrenoceptor agonist, phenylephrine, and the trace amine, β-phenylethylamine (PEA), were potentiated by the NOS inhibitor L-NAME [4]. This indicated that they release nitric oxide (NO) causing a vasodilatation to oppose the predominant vasoconstriction. Since the aortae had intact endothelium, the source of the NO was presumed to be the endothelium, as explained for the potentiation by L-NAME of responses to phenylephrine in rat aorta (Taberno et al., 1996). In the present study we tested this hypothesis in the guinea-pig aorta by comparing the effects of L-NAME in aortae with intact or denuded endothelium. It was therefore necessary to test for the presence or absence of endothelium functionally by recording responses to Ach. Ach is known to relax the aorta of rat [2,7] and rabbit (Furchgott and Zawadski, 1980; [11]) through an endothelium-dependent mechanism which involves primarily the release of the endothelium-derived relaxant factor (EDRF), NO [19]. Ach was added to a sample of tissues preconstricted with the thromboxane TxA_2 mimetic, U46619, which has been shown to yield substantial relaxation responses to Ach in rat aorta with intact endothelium [10]. To our surprise, Ach did not consistently relax the guinea-pig aorta and when a response was recorded, it was small and amounted to only 7.6% of the U46619 contraction. It was considered that the contraction to 1 μM of U46619 might be too large for Ach to overcome – we used Ach at 100 μM compared with 1 μM used by Folger et al. [10]. However, when lower concentrations of U46619 (0.1 μM) were used, there were still no relaxations. We found one previous study that used guinea-pig aorta and they also observed only small relaxation responses to Ach which were precontracted with noradrenaline or high potassium solution rather than U46619 [13]. Thus, it is unlikely that the use of U46619 was responsible for the poor relaxation by Ach. The poor relaxation by Ach was not because the tissue fails to relax to any vasorelaxant since they showed marked relaxations to substance P [13]. We also showed complete relaxation of

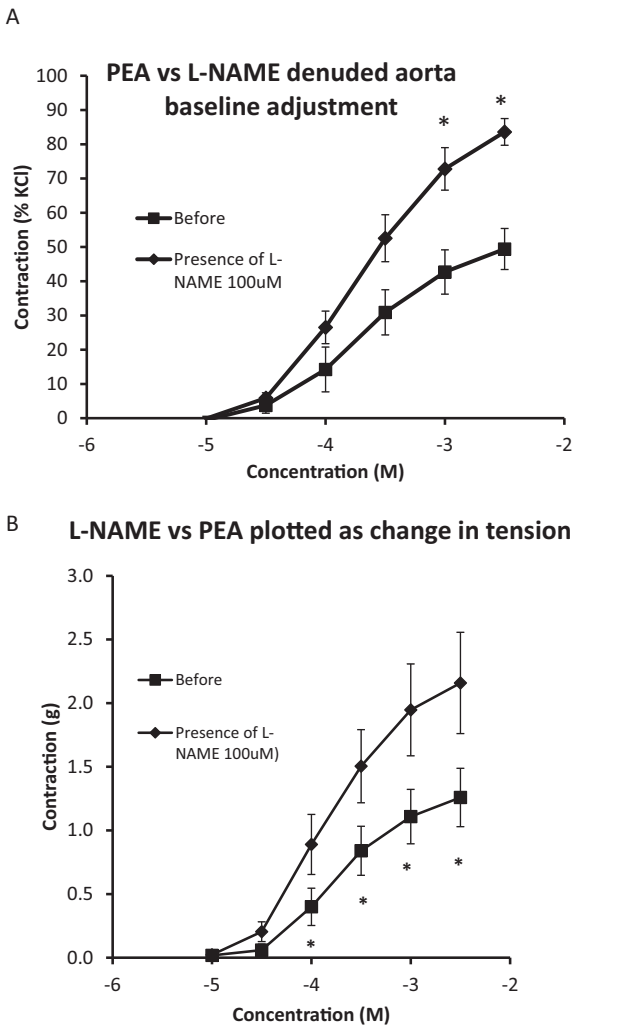

Fig. 5. Effects of baseline and plotting method on the action of L-NAME (100 μM) on contractions of guinea-pig aorta to β-phenylethylamine (PEA). A. Mean concentration-response curves for PEA in endothelium-denuded aortae before (■) and in presence of L-NAME (♦) (n = 5) with the baseline readjusted to 1.5 g before construction of the second curve. Responses are plotted as the increase in tension expressed as a percentage of the maximum contraction to KCl (60 mM). B. Mean concentration-response curves for PEA in endothelium-intact aortae before (■) and in the presence of L-NAME (♦) (n = 7). Responses are plotted as the change in tension from the pre-curve baseline. * Significant difference between with and without L-NAME $P < .05$.

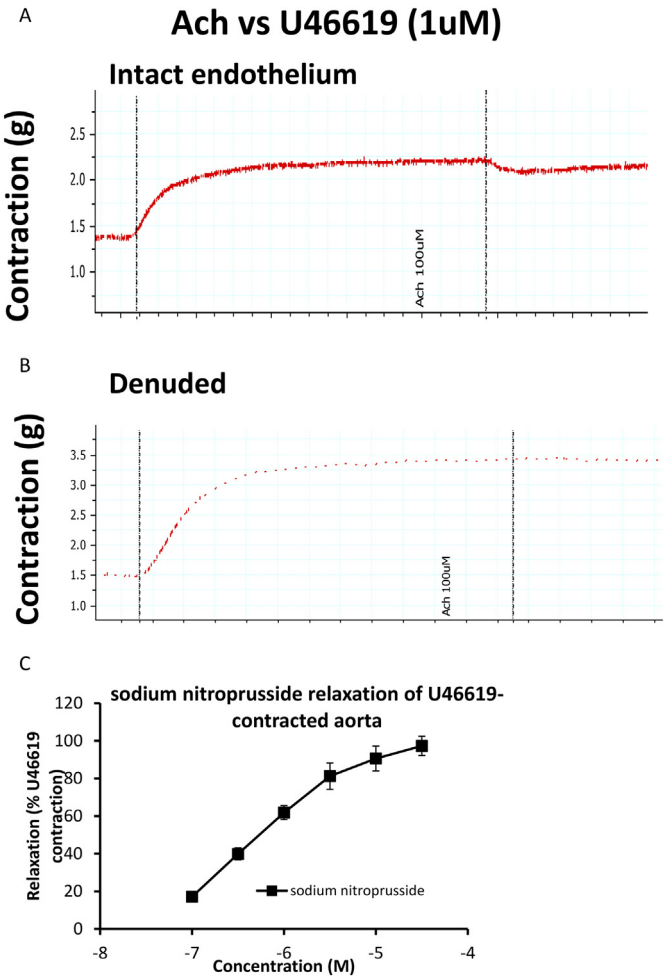

Fig. 6. Effects of acetylcholine and sodium nitroprusside on guinea-pig aorta. A. Typical response to acetylcholine (100 μM) of aorta with intact endothelium preconstricted with U46619 (1 μM). B. Typical lack of response to acetylcholine (100 μM) of endothelium-denuded aorta preconstricted with U46619 (1 μM). C. Mean concentration-response curves for the relaxation responses to sodium nitroprusside of endothelium-intact aortae pre-constricted with U46619 (1 μM) (n = 4). Responses are plotted as the reduction in tension expressed as a percentage of the contraction to U46619.

the U46619-induced contraction by the nitric oxide donor, sodium nitroprusside, thus demonstrating adequate reactivity to NO. The reason for the minimal response to Ach in guinea-pig aorta was not further examined. Although Hozumi et al. [13] attribute this to low production of cGMP, this does not explain the low sensitivity. It is possible that there are low levels of endothelial M_3 muscarinic receptors in guinea-pig aorta [2,15].

When Ach was added to uncontracted aortae, a small contractile response was observed. In rabbit aorta this contraction is endothelium-independent and is mediated via M_2 muscarinic receptors on smooth muscle cells [15]. In rat aorta there is also an endothelium-dependent contraction mediated via M_3 muscarinic receptors that is observed when the vasodilator response is abolished by L-NAME [2]. We examined this contractile response in the same tissue before and after rubbing to remove the endothelium. This approach does not appear to have been used before. Rubbing the endothelial surface potentiated the contractions to Ach confirming that the endothelium had been removed and that a small underlying vasodilator response to Ach had also been abolished. When the same experimental approach was applied to phenylephrine, the vasoconstrictor response was also potentiated when the CRC was repeated after rubbing to remove the endothelium. This establishes that phenylephrine releases an endothelial vasodilator, probably NO, which when removed leads to an enhanced vasoconstriction, as observed for noradrenaline in rat aorta [7]. The potentiation of Ach and phenylephrine contractions by endothelial rubbing therefore confirms the effectiveness of the rubbing process in removing endothelium even though the underlying vasodilator responses to these agonists are weak.

Unlike the above results, when we compared the vasoconstrictor responses to phenylephrine in aortae that were either intact or with denuded endothelia from the outset (i.e. in different tissues from different animals), the CRCs were not significantly different. Similarly, when we compared contractions to PEA and U46619 in separate aortae with or without endothelium, the responses were not significantly different. When different tissues are compared, clearly the between-tissue variations are sufficient to obscure the small potentiating effect of endothelium removal – only by comparing within the same tissue can the potentiation be observed. In rat aorta, the effect of the endothelium is more marked and the potentiation by endothelial removal can be seen

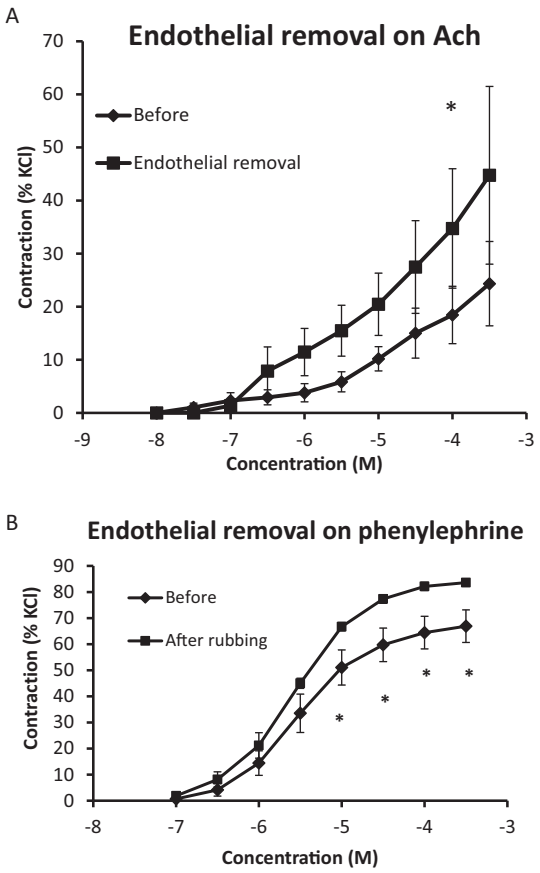

Fig. 7. Effect of endothelium removal on contractile responses of guinea-pig aorta to acetylcholine and phenylephrine. A. Mean concentration-response curves for the contractions to acetylcholine obtained before (♦) and, in the same preparation, after removal of the endothelium (■) (n = 5). B. Mean concentration-response curves for the contractions to phenylephrine obtained before (♦) and, in the same preparation, after removal of the endothelium (■) ($n = 6$). Responses are plotted as the increase in tension expressed as a percentage of the maximum contraction to KCl (60 mM). * Significant difference between with and without endothelium $P < .05$.

in a between-tissue comparison [24]. No previous studies appear to have compared blood vessels with and without endothelium in the same tissue.

In both endothelium-intact and denuded guinea-pig aortae, L-NAME potentiated the contractile responses to both phenylephrine and PEA. The potentiation of PEA in endothelium-denuded aortae was even more evident when the baseline was restored to the initial level prior to commencing the second CRC in the presence of L-NAME. Correction of the resting tension does not appear to have been undertaken in previous studies. Thus, both agonists must release NO from non-endothelial sites to cause an opposing vasodilatation which when removed by L-NAME led to potentiation of the contractions. This confirms our previous observation in endothelium-intact guinea-pig aortae [4]. Previous studies in endothelium-intact rat aorta also showed potentiation by L-NAME [23,24], but not in denuded tissues [24]. The most likely source of this non-endothelial NO is the vascular smooth muscle. Vascular smooth muscle in various pig blood vessels has been shown to express all three isoforms of NOS [6] and thus be capable of drug-induced generation of NO. Neuronal NOS has also been shown to be expressed in vascular smooth muscle cells of rat carotid artery rings [1]. The question arises whether the non-endothelial release of NO in guinea-pig aorta by phenylephrine and PEA, is a common phenomenon to all vasoconstrictors. Is it related to the change in tone in the vessel wall or to common intracellular events occurring during the contractions? For example, a rise in intracellular Ca^{2+} is initiated by the phosphatidylinositol cascade which is a common pathway for a number of receptors mediating smooth muscle contraction, including α-adenoceptors [22]. A rise in endothelial Ca^{2+} has also been linked with NO release [25]. Although the potentiation of PEA and phenylephrine by L-NAME is most likely due to its inhibition of NOS, an off-target action of L-NAME should be considered. For example, inhibition of a vasodilator substance released by these vasoconstrictors, such as substance P or bradykinin or β-adrenoceptor agonism, could explain the potentiation. However, this possibility seems most unlikely since inhibition of NO is the primary action of L-NAME.

Phenylephrine exerts its vasoconstrictor effect in rat aorta via α_1-adrenoceptors of the α_{1D}-subtype [14,16] while in the guinea-pig aorta they are of the α_{1L}-adrenoceptor subtype [4]. In contrast, the vasoconstriction by the trace amine, PEA, was not antagonised by the α_1-adrenoceptor antagonist, prazosin, in guinea-pig aorta with intact endothelium. This confirms our previous observations in guinea-pig aorta [4], in rat aorta [5,9] and in pig coronary arteries [12]. Narang et al. [21] showed that PEA enhances nerve-evoked vasoconstriction of rat perfused mesenteric beds most likely due to blockade of α_2-adrenoceptors since it also binds to both α_1- and α_2-adrenoceptors in rat brain homogenates. The contraction of guinea-pig aorta to PEA would be an agonist rather than antagonist action at α_2-adrenoceptors. This is an unlikely mechanism for the contractile response to PEA. Furthermore, we have eliminated α_2-adrenoceptors in rat aorta, since the contractions were not inhibited by yohimbine, which antagonised contractions to clonidine [5]. We conclude that the response to PEA is mediated not via α-adrenoceptors but through trace amine-associated receptors (TAARs) which we have shown to be present in rat aorta [9].

A further indication of the different receptors mediating the vasoconstrictor responses to PEA and phenylephrine was revealed by the character of the vasoconstriction. Phenylephrine produced rapid onset responses that peaked and then waned while the agonist was still present, whereas PEA contractions were slow in onset and sustained. A similar phenomenon was observed in rat aorta with the sympathomimetic amine, octopamine. Normally, octopamine constricts the aorta via α_1-adrenoceptors, however, in the presence of prazosin to block α_1-adrenoceptors, a non-α-adrenoceptor-mediated contraction occurs, probably via TAARs. The α_1-adrenoceptor-mediated response is rapid in onset, whereas the prazosin-resistant response is slow in onset and sustained [5]. Thus, sympathomimetic amines may have dual mechanisms of action depending upon the particular amine (e.g. PEA, octopamine, tyramine) and the vascular bed on which it acts – indirect/direct sympathomimetic actions via α-adrenoceptors and directly through interaction with TAARs. Because the TAAR-mediated actions are slower in onset, they may have been underestimated in previous studies. Here we allowed the contractions to fully develop. Why should Mother Nature provide for two independent mechanisms? Our hypothesis is that there is a need for an immediate circulatory response to these amines as they are released endogenously, which is the sympathetic 'Fight or Flight' response. However, this response is not usually sustained and there is need for a more gradual and prolonged response to continued exposure, for example, after a meal. This is the TAAR-mediated response. A slow onset response may reflect different kinetics of the drug-receptor interaction or post-receptor cascade.

4.1. Conclusions

The guinea-pig thoracic aorta exhibits only weak endothelium-dependent vasodilator responses to acetylcholine. Removal of the endothelium can be demonstrated by potentiation of the vasoconstrictor responses to acetylcholine and to the α_1-adrenoceptor agonist, phenylephrine. This occurs through the removal of an opposing vasodilator response which is also weak in the aorta of this species. These are novel findings for guinea-pig aorta which suggest that it is a poor tissue for study of endothelial responses. In endothelium-denuded aortae, the

NOS inhibitor, L-NAME, potentiates the vasoconstrictor responses to phenylephrine and the trace amine, PEA. This indicates that the vasoconstriction by both mechanisms (α_1-adrenoceptors and TAARs, respectively) is accompanied by the release of NO which exerts an opposing vasodilator response to suppress the vasoconstriction. The novel finding from this study is that the vasoconstrictors, phenylephrine and PEA, release NO from non-endothelial sites presumably the vascular smooth muscle. It does raise the important question whether in human blood vessels NO is also released from non-endothelial sites and studies into this are therefore indicated. The weak endothelial responses of this species make it suitable for further evaluation of non-endothelial NO release.

Author contributions

Both authors contributed to the drafting the work and revising it critically for intellectual content. KJB made substantial contributions to the concept of the work and KJB and HDB both contributed to the design of the work and to the acquisition, analysis and interpretation of data.

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