A Diffusible Substance(s) Mediates Endothelium-Dependent Contractions in the Aorta of SHR

Di Yang, Michel Féletou, Nigel Levens, Ji Nan Zhang, Paul M. Vanhoutte

Abstract—A modified bioassay system was designed to demonstrate the diffusible nature of endothelium-derived contracting factor(s) released by acetylcholine in the aorta of spontaneously hypertensive rat. In “sandwich”-like layered preparation, isometric tension was recorded from a bioassay strip (without endothelium) in the presence of \( \text{N}^\circ \)-nitro-L-arginine and tetrahydrobiopterin to selectively potentiate endothelium-dependent contractions. A donor strip (with or without endothelium) was stitched on the bioassay tissue so that it did not directly contribute to the recorded contractions. Acetylcholine induced contractions that occurred only when the donor strip was with endothelium. Superoxide dismutase did not affect but catalase and the combination of superoxide dismutase plus catalase significantly decreased the endothelium-dependent contraction. The contractions in the layered preparations were abolished when the donor strip with endothelium was treated previously with valeryl salicylate, an irreversible cyclooxygenase-1 inhibitor, but remained unaffected when the bioassay strip was treated with the compound. Previous treatment of the bioassay strip alone with \( S \text{ 18886} \) abolished the contractile response, whereas treatment of the donor strip with endothelium by the selective TP receptor antagonist only produced a moderate inhibition. These results indicate that in the aorta of spontaneously hypertensive rats, endothelium-dependent contractions to acetylcholine involve a diffusible substance(s) released by the endothelium. The production of this contracting factor(s) requires the activation of endothelial cyclooxygenase-1, and its action the activation of TP receptors on the vascular smooth muscle cells. (Hypertension. 2003;41:143-148.)

Key Words: cyclooxygenase ■ endothelium-dependent contraction ■ tetrahydrobiopterin ■ free radicals ■ rats, spontaneously hypertensive ■ receptors, thromboxane

The endothelium-derived contracting factors (EDCF) identified so far include superoxide anions, endoperoxides, thromboxane \( A_2 \), and endothelin-1. In the aorta of the spontaneously hypertensive rat (SHR), endothelium-dependent contractions to acetylcholine involve reactive oxygen species that activate the cyclooxygenase-1 pathway with the production of endoperoxide(s), which stimulate TP receptors on the aortic vascular smooth muscle. Of the products of the cyclooxygenase pathway, endoperoxide(s) (in particular prostaglandin \( H_2 \)) are likely candidates as EDCF in the SHR aorta. Both endothelial cells and smooth muscle cells express cyclooxygenase and TP receptors. The precise location of cyclooxygenase-1 and TP receptors activated during endothelium-dependent contractions is unknown. There is no direct evidence at hand showing that EDCF is a diffusible factor. Inhibitors of nitric oxide (NO) synthase augment endothelium-dependent contractions to acetylcholine in the SHR. This potentiation is augmented further by exogenous tetrahydrobiopterin, an essential cofactor of endothelial NO synthase. The mechanism underlying this effect of tetrahydrobiopterin involves superoxide generation by the autoxidation of tetrahydrobiopterin. This potentiation is selective for the endothelium-dependent contractions observed in SHR in the presence of \( \text{L} \)-arginine analogues, inhibitors of the NO synthase. This effect of tetrahydrobiopterin in the presence of an inhibitor of NO synthase makes it potentially a useful tool to bioassay EDCF. The purpose of this study was to design a bioassay system permitting the detection of the EDCF in SHR aorta and thus to determine the location (endothelium or smooth muscle) of cyclooxygenase-1 and TP receptors,
which contribute to the endothelium-dependent response to acetylcholine in the hypertensive vascular wall.

Methods

Experiments were performed on thoracic aortas from 35-week-old male SHR (358 ± 8 g, n = 37). The rats were anesthetized with sodium pentobarbital (50 mg/kg IP). Arterial blood pressure was measured from the carotid artery, and rats with a systolic blood pressure <170 mm Hg were excluded from the experiments (systolic blood pressure, 204 ± 5 mm Hg, n = 37). The aorta was dissected free, excised, and placed in cold modified Krebs-Ringer bicarbonate solution of the following composition (mmol/L): NaCl 118, KCl 4.7, CaCl₂ 2.5, MgSO₄ 1.2, KH₂SO₄ 1.2, NaHCO₃ 25.0, and edetate calcium di-sodium 0.026; glucose 11.1 (control solution).

In certain experiments, the blood vessels were cut into rings (4 mm in length), suspended in organ chambers containing control solution (37°C), and aerated with 95% O₂ and 5% CO₂. The rings were connected to a fixed hook and the other to a vertical transducer (Gould, model 7100). The rings were stretched progressively to reach the optimal point of their length-active tension relation.

For bioassay experiments, a modified "sandwich"-like bioassay system was designed. The aortas were cut into rings (7 to 8 mm in length). The endothelium of some aortic rings was removed by inserting the tip of a small forceps into the lumen and rolling the ring back and forth on a wetted paper towel. The rings were then cut into strips along the longitudinal axis. In the bioassay strip from which the aorta was recorded, the endothelium was removed and two small metal stirrups were sewed to the two rims of the preparation. The donor strips could be with or without endothelium and were rotated 90° in reference to the bioassay strip. To obtain a layered preparation, a donor strip was stitched onto the bioassay strip to minimize the gap between the two intimal sides. Then, the assembly of the two stitched strips was placed in organ chambers filled with oxygenated modified Krebs-Ringer solution. One strip was connected to a fixed hook and the other to a vertical transducer (Gould, UC2) to record changes in isometric tension. The axis of the lumen of the bioassay strip was horizontal, that is, perpendicular in reference to the axis of the transducer. The strips were stretched progressively to a passive tension of 2 g and contracted twice with KCl (40 mmol/L). A reference contraction to KCl (60 mmol/L) was then obtained. The duration of the whole experiment (equilibration period and the experimental protocol) was shortened as much as possible and lasted between 120 to 150 minutes. All the experiments were performed in the presence of N°-nitro-l-arginine (10⁻⁴ mol/L) and tetrodotoxin (10⁻⁴ mol/L).

In preliminary experiments, to confirm whether or not this layered preparation could bioassay a diffusible substance, strips from the aorta of normotensive Wistar-Kyoto rats were prepared in the same manner. They were contracted with phenylephrine, and endothelium-dependent relaxation to acetylcholine (10⁻⁹ mol/L) was recorded (relaxation, 46 ± 9% of phenylephrine-induced contraction, n = 3), indicating detection of the release of NO.

Cyclooxygenase and TP Receptors

Preliminary experiments were performed to identify an inhibitor of cyclooxygenase and a TP receptor antagonist, with an activity sustained for at least 3 hours after washing out. These experiments were performed in intact rings with endothelium incubated for 60 to 120 minutes with various inhibitors of cyclooxygenase (valeryl salicylate: 5 × 10⁻⁷ mol/L; indomethacin: 5 × 10⁻⁷ mol/L; aspirin: 10⁻⁷ mol/L) and different TP receptor antagonists (S 1886: 10⁻⁷ to 10⁻⁴ mol/L; SQ 29548: 3 × 10⁻⁸ mol/L). After repeated rinses, the rings were sustained in organ chamber and stretched progressively and contracted with KCl (60 mmol/L) to mimic the experimental protocol used with the layered preparations. Then, the rings were contracted with acetylcholine (10⁻⁹ to 10⁻⁴ mol/L) or the α₁-adrenoceptor agonist phenylephrine (10⁻⁹ to 10⁻⁴ mol/L). In the presence of each of these inhibitors or antagonists tested, the contractions to acetylcholine were abolished. However, valeryl salicylate was the only inhibitor of cyclooxygenase to provide a complete inhibition of the contractions to acetylcholine up to 3 hours after washing out. This inhibition was specific, as the contraction to phenylephrine was not affected by the preferential cyclooxygenase-1 inhibitor. Therefore, valeryl salicylate (3 × 10⁻⁷ mol/L) was chosen to inhibit cyclooxygenase-1 in the bioassay experiments.

The postulated irreversible antagonist of TP receptors, benextra-mine, could not be studied because of its poor potency and selectivity (inhibition of TP receptor: pKB ~4, and inhibition of contraction to KCl, 60 mmol/L, at 10⁻⁹ mol/L: 84%). SQ 29548 (3 × 10⁻⁶ mol/L), a potent inhibitor of TP receptor and endothelium-dependent contraction, could not be considered because its inhibitory effect had completely disappeared after 1 hour of washout. The duration of action of the specific TP receptor antagonist S 1886 was longer than that of SQ 29548, but nevertheless it did not behave as an irreversible antagonist. After repeated rinses, a slow, time-dependent fading away of the antagonism was observed. This was attributed to the slow dissociation of the antagonist from its binding site and was evidenced by the release of the antagonist in the organ bath with two negative effects. The inhibitory effect on the receptor targeted slowly vanished with time, and in the bioassay configuration the released compound could bind to the TP receptors on the strip that had not been treated, in essence the contamination of the nontreated strip by the antagonist slowly dissociated from the treated strip. To minimize these two experimental pitfalls, the concentration of S 1886 chosen was the lowest effective concentration that produced a significant inhibition of acetylcholine-induced contractions in intact rings treated with S 1886 and washed repeatedly for 1 hour. The concentration of S 1886 chosen was 5 × 10⁻⁷ mol/L. Under the experimental conditions used, S 1886 up to 10⁻⁷ mol/L did not affect phenylephrine or KCl-induced contractions.

Drugs

Acetylcholine hydrochloride, benextramine, deferoxamine, mannitol, N°-nitro-l-arginine, papaverine, phenylephrine superoxide dismutase, and catalase were purchased from Sigma Chemical Company. S 1886 (3-[6-(amino-(4-chlorobenzensulfanyl))-2-methyl-5,6,7,8-tetrahydronaphth]-1-yl)propionic acid) was synthesized at the Institut de Recherches Servier. Valeryl salicylate was purchased from Cayman Chemical Company. SQ 29548, tetrodotoxin, and U46619 (9,11-dideoxy-9α,11α-epoxyethano prostaglandin F₂α) were purchased from Alexis Biochemicals. Drug concentrations were expressed as final molar concentrations in the bath solution.

Data Analysis and Statistics

Contractions to acetylcholine were obtained in quiescent preparations. Changes in tension are expressed as the percentage of the reference response to KCl (60 mmol/L) or as the absolute changes in tension (g). The data are given as mean ± SEM; n refers to the number of rats from which the aortas were taken. Statistical analysis was performed by 2-tailed Student t test for control and treatment comparisons and by ANOVA 1 and 2 analyses for multiple comparisons where appropriate. Differences were considered to be statistically significant at a level of P < 0.05.

Results

Bioassay of EDCF

To rule out any contribution of the donor strip in the contractile response recorded from the sandwich preparation, some preliminary experiments were performed. The tension was recorded directly from the donor strip with endothelium incubated under those two experimental conditions, there was no measurable contraction in response to KCl (60 mmol/L) or as the absolute changes in tension (g). The data are given as mean ± SEM; n refers to the number of rats from which the aortas were taken. Statistical analysis was performed by 2-tailed Student t test for control and treatment comparisons and by ANOVA 1 and 2 analyses for multiple comparisons where appropriate. Differences were considered to be statistically significant at a level of P < 0.05.
Involvement and Localization of Cyclooxygenase-1 and TP Receptors

The assay strip (without endothelium) or the donor strip (with endothelium) were treated (or not) individually with either valeryl salicylate (3×10⁻³ mol/L) for 2 hours or S 18886 (5×10⁻³ mol/L) for 1 hour. After repeated rinses, the layered preparation was assembled and contractions to acetylcholine were recorded.

The contractions to acetylcholine (10⁻⁴ mol/L) were abolished when the donor tissue with endothelium had been treated with valeryl salicylate but remained unaffected when the bioassay strip had been exposed previously to the cyclooxygenase-1 inhibitor (Figure 3). Conversely, the contractile response to acetylcholine (10⁻⁴ mol/L) was nearly abolished when the bioassay strip was treated with S 18886, whereas the exposure of the donor strip with endothelium to the TP receptor antagonist produced only a moderate inhibition of the contractions (Figure 4). In both experimental protocols, acetylcholine produce no or minor contractions in the presence of a donor strip without endothelium.

In layered preparations, the addition of U 46619 (10⁻⁴ mol/L) produced a slowly developing contraction that was equivalent to the amplitude of the endothelium-dependent contraction to acetylcholine (10⁻⁴ mol/L) under the same conditions. The donor strip with endothelium or the bioassay strip were exposed, or not, to S 18886 (5×10⁻⁹ mol/L) before layering the preparation. The contractions to U 46619 (10⁻⁴ mol/L) were significantly decreased both in preparations in which the bioassay tissue or the donor strip were previously treated with S 18886 (ANOVA2, followed by Bonferroni post hoc test). However, the inhibition was significantly smaller when the donor strips were treated than when the bioassay strips were exposed to the TP receptor antagonist (Figure 5).
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Figure 3. Inhibition of cyclooxygenase-1 by previous treatment with valeryl salicylate (VAS: 3×10\(^{-5}\) mol/L) and endothelium-dependent contractions to acetylcholine (10\(^{-4}\) mol/L) in layered preparations from SHR aorta in the presence of N\(^{-}\)nitro-L-arginine (10\(^{-4}\) mol/L) and tetrahydrobiopterin (10\(^{-4}\) mol/L). Valeryl salicylate produced significant inhibition of acetylcholine-induced contraction only when donor tissue had been treated, indicating that cyclooxygenase-1, involved in endothelium-dependent contractions, is located on endothelial cells. Data are shown as mean±SEM (n=7). *Statistically significant difference (P<0.05) when compared with untreated control.

**Discussion**

This study demonstrates the existence of a bioassayable EDCF in the SHR aorta. Furthermore, it demonstrates that acetylcholine-induced endothelium-dependent contractions involve stimulation of endothelial cyclooxygenase-1 that in turn produces a diffusible EDCF(s), capable of activation TP receptors on the vascular smooth muscle cells.

Figure 4. TP receptor antagonism by previous exposure to S 18886 (5×10\(^{-5}\) mol/L) and endothelium-dependent contraction to acetylcholine (10\(^{-4}\) mol/L) in layered preparations from SHR aorta in the presence of N\(^{-}\)nitro-L-arginine (10\(^{-4}\) mol/L) and tetrahydrobiopterin (10\(^{-4}\) mol/L). S 18886 produced significant inhibition of acetylcholine-induced contraction only when donor tissue had been treated, indicating that cyclooxygenase-1, involved in endothelium-dependent contractions, is located on endothelial cells. Data are shown as mean±SEM (n=8). *Statistically significant difference (P<0.05) compared with untreated control rats. †Statistically significant difference (P<0.05) between treatment of donor strips and that of bioassay strips.

The current experiments were performed in the presence of N\(^{-}\)nitro-L-arginine, an analogue of L-arginine, and tetrahydrobiopterin, an essential cofactor for the activity of NO synthase, to amplify the endothelium-dependent contractions. Inhibitors of NO synthase augment endothelium-dependent contractions. In preliminary experiments, it has been shown that tetrahydrobiopterin selectivity augments the endothelium-dependent contractions to acetylcholine, only in SHR and only in the presence of N\(^{-}\)nitro-L-arginine. This potentiation, associated with the generation of superoxide anion by the autoxidation of tetrahydrobiopterin, does not alter the main characteristics of the endothelium-dependent contractions, for example, involvement of oxygen-derived free radicals, stimulation of cyclooxygenase-1, and the resulting activation of TP receptors.

In phenylephrine-contracted, layered preparations prepared with aortic strips of normotensive WKY rats, acetylcholine evoked relaxation when the donor strips contained endothelium, indicating that the present “sandwich”-like preparations can detect diffusible NO. Likewise, in the presence of N\(^{-}\)nitro-L-arginine and tetrahydrobiopterin, acetylcholine produced a contraction only when the donor strip of SHR aorta contained endothelium. Preliminary experiments, in which isometric tension was measured directly from the donor strip with endothelium alone, show that there was no contractile response recorded in response not only to acetylcholine but also to more potent stimuli such as KCl or phenylephrine. This was to be expected because of the 90° rotation of the donor strip in relation to the length axis of the transducer, which does not allow proper recording of contractile force from the mainly circularly running smooth muscle bundles in the aortic wall. Furthermore,
experiments performed with the bioassay preparation, destroyed by a prolonged incubation in distilled water, layered with an intact donor strip, did not respond either to acetylcholine, KCl or phenylephrine, demonstrating that even when stitched to the bioassay strip, the donor strip does not contribute to the development of tension as recorded by the transducer. Therefore, the contractions to acetylcholine, recorded in the used layered preparation was indeed due to a diffusible endothelium-derived contracting factor(s) and not to artifacts linked to the experimental procedure.

The involvement of oxygen-derived free radicals in endothelium-dependent contraction has been demonstrated in canine basilar arteries and in the SHR aorta. These earlier findings were confirmed in the current study since in the layered preparations, the contractions were reduced by the acute treatment with catalase or the combination of superoxide dismutase plus catalase. In earlier experiments involving the contraction of isolated intact aortic rings from SHR, superoxide dismutase plus catalase, at the same concentration as used in the current study, did not prevent endothelium-dependent contractions unless the animals were treated chronically in vivo with dimethylthiourea, a scavenger of hydroxyl radicals. These observations were interpreted by the poor ability of these enzymes to access the site where the oxygen-derived free radicals are generated and therefore to be able to scavenge enough of the reactive oxygen species. This interpretation is confirmed by the current study, showing nearly full blockade of the endothelium-dependent contractions to acetylcholine by catalase or by the combination of the superoxide dismutase plus catalase. This observation strongly suggests the presence of reactive oxygen species in the extracellular space. The lack of activity of superoxide dismutase alone indicates that superoxide anions per se are not sufficient enough to evoke the contractions, although they must be the source of the secondary reactive oxygen species, such as hydrogen peroxide and hydroxyl radicals. Since superoxide dismutase did not affect the contractions, the effect of catalase alone or the combination of superoxide dismutase plus catalase would suggest that hydrogen peroxide or its product, hydroxyl radicals, is mainly responsible for the endothelium-dependent response. Iron chelation by deferoxamine prevents the formation of the hydroxyl radical from H2O2 (Fenton reaction). The inhibition produced by deferoxamine is in favor of a role for the hydroxyl radical. However, the absence of effect of mannitol, a scavenger of the hydroxyl radical, is somehow puzzling. Mannitol is a poorly cell-permeable agent, and the very short half-life of the hydroxyl radical may have prevented mannitol from scavenging the hydroxyl radical under the current experimental conditions. Earlier studies have reported an improved endothelial function by deferoxamine and no significant effect of mannitol. Obviously, the respective role of H2O2 or/and the generation of hydroxyl radical in the production of endothelium-dependent contractions deserve further investigation.

The reactive oxygen species are likely to activate cyclooxygenase(s). Endothelium-dependent contractions to acetylcholine are inhibited by either nonspecific inhibitors of cyclooxygenase or a preferential inhibitor of cyclooxygenase-1 but not by a preferential inhibitor of cyclooxygenase-2. The current study confirms these observations as valeryl salicylate, an irreversible inhibitor of cyclooxygenase-1, abolished the contractions. This inhibitor was chosen for its long duration of action and its specificity. These results demonstrated that under the present experimental conditions, endothelial cyclooxygenase plays a pivotal role in mediating endothelium-dependent contractions, as a previous study suggested.

The involvement of endogenous agonists of TP receptors, other than thromboxane A2, in endothelium-dependent contractions to acetylcholine in the SHR aorta is also very likely as TP receptor antagonists but not inhibitors of thromboxane synthase abolish these responses. The current study also confirms these earlier conclusions. However, to determine the exact localization of the TP receptor was more difficult than to demonstrate the endothelial nature of the cyclooxygenase involved. Indeed, the available specific TP receptor antagonists caused reversible inhibitions. The antagonist with the longer remnant duration of action was S 18886. However, if previous exposure of the bioassay tissue to S 18886 fully inhibited the contraction to acetylcholine that of the endothelium also produced a moderate but significant inhibition of the response. This could be interpreted by either a role for TP receptors on both endothelium and vascular smooth muscle or by the artifactual contamination of the untreated strip by the TP receptor antagonist slowly dissociating from the previously treated strip. This latter interpretation is likely to be correct since the contractions to exogenous U 46619, a potent TP receptor agonist, were

Figure 6. Endothelium-dependent contractions to acetylcholine in SHR aorta. Unbroken lines: Current study demonstrates that in response to acetylcholine, reactive oxygen species (scavenged by superoxide dismutase, SOD, and catalase) generated by endothelial cells from an unknown source (X, which is not necessarily different from cyclooxygenase-1) activates the endothelial cyclooxygenase-1 pathway (COX-1, inhibited by valeryl salicylate, VAS). The later produces endoperoxide(s) (EP) such as prostaglandin H2 (PGH2) or isoprostane(s) to activate TP receptors on smooth muscle cells (inhibited by S 18886). Broken lines, Hypothesis to reconcile present data and earlier studies. Contraction obtained under bioassay conditions is only 50% of contraction observed in intact rings. Therefore, an additional pathway can be suggested. In intact rings, cyclooxygenase-1 situated on smooth muscle could, because of the closer proximity between endothelial and smooth muscle cells, also be activated by diffusing reactive oxygen species. Activation of smooth muscle cyclooxygenase-1 would contribute to acetylcholine-induced contraction However, at the present time, there is no experimental evidence for this hypothesis.
inhibited when the donor tissue was previously treated with S 18886. As the donor tissue does not contribute to the contraction, the only explanation for this inhibition of the response to U 46619 is the dissociation of the antagonist from the binding sites on the donor strip which results to the diffusion and blockade of the receptors present on the untreated assay strip.

Altogether, these results indicate that acetylcholine stimulates the endothelial production of reactive oxygen species, most likely hydrogen peroxide and possibly the hydroxyl radical, from a yet unknown source. These oxygen species activate endothelial cyclooxygenase-1 that releases a diffusible substance(s), possibly prostaglandin H₂ or an isoprostane,²³ which in turn stimulates TP receptors on the vascular smooth muscle cells (Figure 6).

It seems unlikely that under the experimental conditions of the current study, the totality of the released EDCF was bioassayed. In intact aortic rings of SHR, in the presence of N⁶-o-nitro-arginine and tetrahydrobiopterin, the amplitude of the maximal endothelium-dependent contraction to acetylcholine represents 90% of the reference response to KCl,¹⁰ whereas in the current study in the layered preparations, the maximal responses to acetylcholine averaged only 45% of the KCl contraction. This difference might be due to the experimental paradigm per se (endothelial degradation linked to the manipulation needed to prepare the sandwich-like preparations, for instance) or due to the increased inactivation of EDCFs (reactive oxygen species and endoperoxides) as a consequence of the increased distance between the endothelium and the vascular smooth muscle cells. A third hypothesis can also be proposed. In intact rings from SHR aorta, H₂O₂ can theoretically diffuse more easily from the endothelial cells to the vascular smooth muscle cells than in the sandwich preparation. Exogenous H₂O₂ produces (like the co-administration of xanthine plus xanthine oxidase) greater contractions in SHR than in WKY in both control and endothelium-denuded aortic segments. These contractions share the same characteristics as the endothelium-dependent contractions produced by acetylcholine because they are sensitive to catalase, cyclooxygenase inhibitors, and TP receptor antagonists.²⁴,²⁵ Therefore, in intact rings, considering that the expression of cyclooxygenase-1 is larger in aortic smooth muscle of SHR when compared with WKY,³ the additional activation of cyclooxygenase-1 in the vascular smooth muscle by endothelium-derived reactive oxygen species cannot be excluded (Figure 6).

Perspectives

The existence of a diffusible EDCF and the possibility to bioassay this endothelial factor should provide new means for its characterization and identification. Only if this is achieved will the relevance of this, a factor in the pathophysiology of hypertension, be properly assessed.

References

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