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The bimodal regulation of vascular function by superoxide anion: role of endothelium

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Reactive oxygen species (ROS) are implicated in vascular homeostasis. This study investigated whether $O_2^{\cdot-}$, the foundation molecule of all ROS, regulates vasomotor function. Hence, vascular reactivity was measured using rat thoracic aortas exposed to an $O_2^{\cdot-}$ generator (pyrogallol) which dose-dependently regulated both α-adrenergic agonist-mediated contractility to phenylephrine and endothelium-dependent relaxations to acetylcholine. Pyrogallol improved and attenuated responses to acetylcholine at its lower (10 nM - 1 μM) and higher (10 - 100 μM) concentrations, respectively while producing the inverse effects with phenylephrine. The endothelial inactivation by L-NAME abolished acetylcholine-induced vasodilatations but increased phenylephrine and KCl-induced vasoconstrictions regardless of the pyrogallol dose used. Relaxant responses to sodium nitroprusside, a nitric oxide donor, were not affected by pyrogallol. Other ROS i.e. peroxynitrite and H_2O_2 that may be produced during experiments did not alter vascular functions. These findings suggest that the nature of $O_2^{\cdot-}$-evoked vascular function is determined by its local concentration and the presence of a functional endothelium. [BMB reports 2008; 41(3): 223-229]

INTRODUCTION

The endothelium plays a pivotal role in the maintenance of normal vascular tone. However, its properties in pathological conditions change leading to a phenomenon termed "endothelial dysfunction" which is characterized by impaired endothelium-dependent relaxation (1). Endothelial dysfunction has been reported in conduit and resistance arteries of patients with vascular diseases including hypertension and in various animal models of human diseases (2-4). Despite current data on the etiology of endothelial dysfunction being rather diverse, much of the recent data have implicated oxidative stress in its pathogenesis (5, 6). Oxidative stress is associated with enhanced production of reactive oxygen species (ROS) in particular superoxide anion ($O_2^{\cdot-}$) that is normally converted to hydrogen peroxide (H_2O_2) by superoxide dismutases (SODs). H_2O_2 is in turn metabolized to H_2O by antioxidant enzymes; catalase and glutathione peroxidase (7). However, in pathological states the balance between the synthesis and metabolism of ROS may be impaired thus leading to generation of oxidative stress. $O_2^{\cdot-}$ can elicit direct vasoconstriction and scavenge nitric oxide (NO), the most potent endogenous vasodilator, to produce peroxynitrite ($OONO^-$) which oxidizes proteins, breaks DNA strands and reduces intracellular antioxidants such as glutathione and cysteine (1, 8). Contrary to these findings, other studies have revealed that low levels of $O_2^{\cdot-}$ are required for normal vascular cell proliferation and migration and contribute to endothelium-dependent vasodilatation in some vascular beds particularly after dismutation to H_2O_2 (9, 10).

In the light of these contradictory findings, the aim of the current study was to investigate whether local concentrations of $O_2^{\cdot-}$ determine the nature of vascular functions using aortic rings from Sprague Dawley (SD) rats. To this end, relaxant responses to endothelium-(in)dependent agents and contractile responses to an α-adrenergic agonist phenylephrine (PE) and receptor-independent constrictor KCl were investigated in vessels exposed to incremental concentrations of an $O_2^{\cdot-}$-generating agent, pyrogallol. The requirement for an intact endothelium and the involvement of other ROS including H_2O_2 and $OONO^-$ in putative functional changes were also examined in the study.

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RESULTS

Effects of pyrogallol on levels of $O_2^{\cdot-}$ in aortic rings

Pyrogallol produced dose-dependent increases in the levels of aortic $O_2^{\cdot-}$ as detected by lucigenin enhanced-chemiluminescence, cytochrome C reduction and DHE oxidation assays without altering the level of LDH release, an indicator of cellular damage (supplemental Table 1).

Effects of $O_2^{\cdot-}$ on vasorelaxations

In PE-precontracted aortic rings, both acetylcholine (ACh) and SNP produced concentration-dependent relaxations where endothelial inactivation by mechanical (by rubbing) or pharmacological (via incubation with 50 μM of L-NAME) means selectively inhibited endothelium-dependent but not endothelium-independent vascular relaxations (Supplemental Fig. 1).

Pyrogallol regulated endothelium-dependent relaxant responses in aortic rings in a dose-dependent manner in that it improved and attenuated ACh-mediated relaxations at its lower (10 nM - 1 μM) and higher (10 and 100 μM) concentrations, respectively (Fig. 1A-B). However, pyrogallol did not affect relaxant responses to SNP. It is of note that the inactivation of endothelium selectively abrogated ACh- but not SNP-mediated relaxant responses in the presence of pyrogallol (data not shown).

To investigate whether pyrogallol-induced effects are attributable to its $O_2^{\cdot-}$-releasing function, the aortic rings were pretreated with one of the two structurally different $O_2^{\cdot-}$ scavengers, namely Tiron (4, 5-dihydroxy-1, 3-benzenedisulfonic acid, 1 μM) or MPG (mercaptopropionylglycine, 1 μM) for 20 min. Both agents prevented pyrogallol-mediated (10 μM) endothelium-dependent relaxations without affecting the basal relaxations determined in the absence of pyrogallol (Fig. 2A-B). To support these findings, experiments with exogenous SOD (150 U / ml) or an endogenous inhibitor of SOD, namely diethyldithiocarbamate (DETCA, 1 - 10 mM) were conducted. Pretreatments with either agent alone for 20 min failed to alter the ACh-mediated relaxant responses while the combination of DETCA and 10 μM pyrogallol worsened these responses and the SOD and pyrogallol combination was found to be ineffective (Fig. 2C-D). To establish whether the effects of low concentrations of pyrogallol are also mediated by $O_2^{\cdot-}$, similar experiments were conducted in the presence of 10 nM of pyrogallol where Tiron and MPG suppressed low dose pyrogallol-induced increases in ACh-mediated relaxant responses by almost 22% while DETCA and exogenous SOD failed to alter these responses in a significant manner.

Experiments performed under identical conditions with Tiron, MPG, SOD or DETCA revealed that none of these treatments had an impact on endothelium-independent relaxant responses.

Putative involvement of other ROS in $O_2^{\cdot-}$-mediated vasorelaxations

To examine the relative contributions of hydroxyl radical ($^{\cdot}OH$), H_2O_2 or $OONO^-$ that may be generated during incubation with pyrogallol to relaxant changes, the aortic rings were treat-

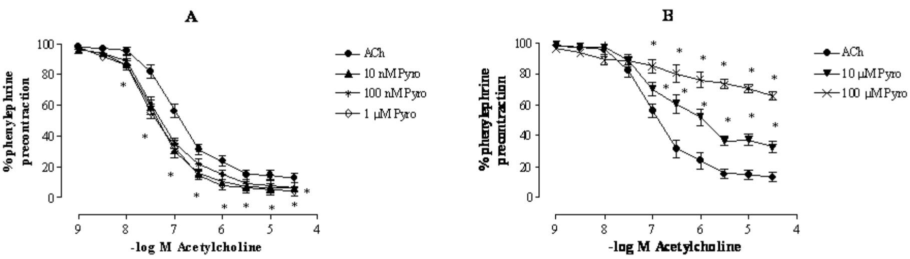

Fig. 1. (A) Effects of low dose pyrogallol (Pyro) on ACh-induced aortic relaxations. (B) Effects of high dose pyrogallol on ACh-induced aortic relaxations. Results are expressed as mean ± SEM from 8 separate experiments. $^*P < 0.05$ compared to ACh-induced relaxations.

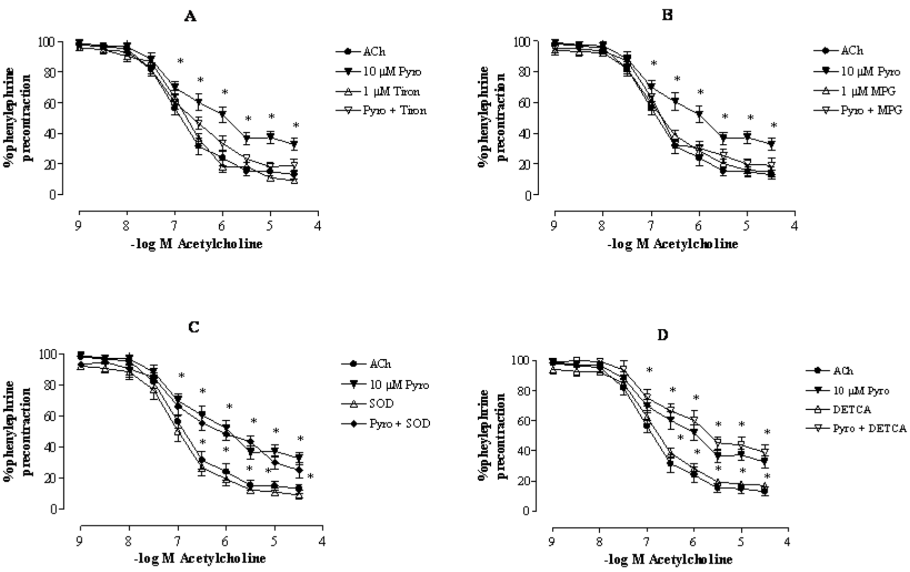

Fig. 2. Effects of $O_2^{\cdot-}$-scavengers, Tiron (A) and MPG (B) and exogenous superoxide dismutase (SOD, C) and the inhibitor of SOD (DETCA, D) on ACh-induced aortic relaxations in the absence and presence of pyrogallol (Pyro). Results are expressed as mean ± SEM from 8 separate experiments. $^*P < 0.05$ compared to ACh-induced relaxations.

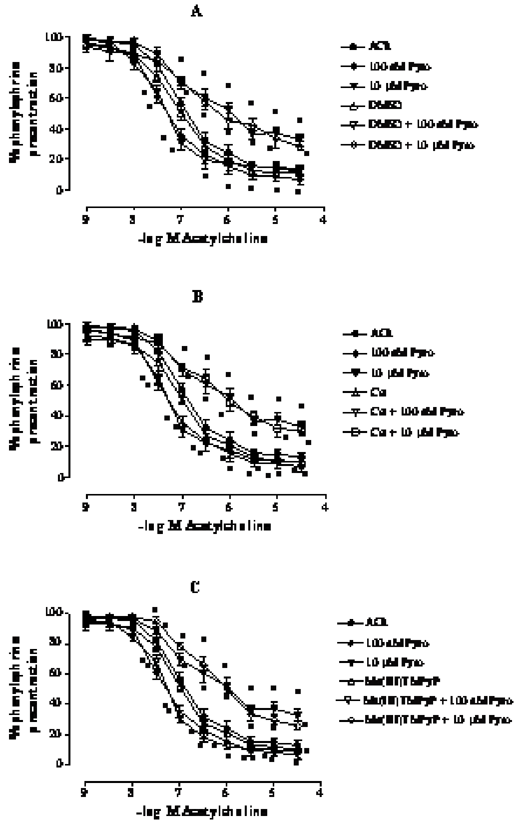

Fig. 3. Effects of DMSO (A), catalase (Cat, B) and Mn(III)TMPyP (C) on ACh-induced aortic relaxations in the absence and presence of pyrogallol (Pyro). Results are expressed as mean ± SEM from 8 separate experiments. *$P < 0.05$ compared to ACh-induced relaxations.

ed with two different inhibitors of each agent in the absence or presence of a lower (100 nM) or a higher (10 μM) concentration of pyrogallol. To this end, desferrioxamine (100 μM) or DMSO (50 nM), catalase or glutathione peroxidase (100 U / ml for each agent) and Mn(III)TMPyP (10 μM) or uric acid (100 μM) were used as inhibitors of $^{\cdot}OH$, H_2O_2 and $OONO^-$. It was revealed that none of these agents displayed significant effects on vasorelaxations. Fig. 3A-C selectively show the effects of DMSO, catalase and Mn(III)TMPyP on ACh-mediated relaxations in the presence of 10 μM of pyrogallol. To ensure whether pyrogallol treatments led to increases in intracellular levels of $OONO^-$ or H_2O_2, the levels of these ROS were measured in aortic vessels which showed steady increases to incremental concentrations of pyrogallol (supplemental Table 2). Given that $OONO^-$ or H_2O_2 (10 nM - 100 μM for each agent) *per se* produced dose-dependent increases in vascular relaxation in vessels with or without endothelium (Supplemental Fig. 2), it was safe to suggest that the pyrogallol-mediated increases in $OONO^-$ or H_2O_2 did not reach the lower levels that were used in the latter experiments. Similar experiments with the listed inhibitors revealed their inefficacy in altering endothelium-independent relaxant responses to SNP.

Effects of $O_2^{\cdot-}$ on vasocontractility

PE (0.003 - 10 μM) induced contractile responses in a dose-dependent manner in isolated aortic rings with / out endothelium (Fig. 4A). In endothelium-intact vascular rings, the lower concentrations of pyrogallol (10 nM - 1 μM) diminished PE-mediated contractile responses - significantly between 1 nM to 1 μM logarithmic concentrations of PE - without changing the E_{max}. However, the higher concentrations of pyrogallol (10 and 100 μM) significantly enhanced PE-mediated contractility (Fig. 4B). Pretreatment of vascular rings with L-NAME (50 μM) for 20 min significantly enhanced PE-mediated contractile responses regardless of the concentration of pyrogallol used (Fig. 4C). Similar to endothelium-intact rings, pyrogallol produced dose-dependent changes in endothelium-denuded aortic rings (data not shown).

The receptor-independent contractile responses of pyrogallol were determined through use of KCl (120 mM) that produced similar contractions in isolated aortic rings with or without endothelium in the absence or presence of pyrogallol (Fig. 4D-E). However, endothelial inactivation by L-NAME dramatically enhanced KCl-induced contractile responses in the presence of pyrogallol (Fig. 4F).

Putative involvement of other ROS in $O_2^{\cdot-}$-mediated vascular contractility

Tiron and MPG did not affect basal contractility to PE. However, they diminished pyrogallol-induced (10 μM) contractile responses to PE by almost 50 % ($P < 0.05$). Inactivation of endothelium with L-NAME (50 μM) did not influence inhibitory effects of these components on pyrogallol-evoked contractions (Supplemental Fig. 3). In contrast to $O_2^{\cdot-}$ scavengers, treatments with exogenous SOD, DETCA, catalase, DMSO and Mn(III) TMPyP in the presence of pyrogallol (100 nM or 10 μM) did not significantly affect PE-mediated contractile responses and generated between 3 to 5 % difference in these responses.

DISCUSSION

Several factors including diminished production of NO due to inefficient utilization of substrate L-arginine or impaired activity of endothelial NO synthase have been implicated in the pathogenesis of endothelial dysfunction (11, 12). However, oxidative stress characterized by the excessive vascular presence of $O_2^{\cdot-}$, has recently been associated with the pathogenesis of this phenomenon in a variety of diseases with vascular complications such as hypertension and hypercholesterolemia (2, 6). $O_2^{\cdot-}$ under pathological conditions may trigger the formation of other ROS such as $OONO^-$, $^{\cdot}OH$ and H_2O_2 that exert opposing i.e. relaxant or contractile effects on endothelium through activating different pathways and / or modifying mem-

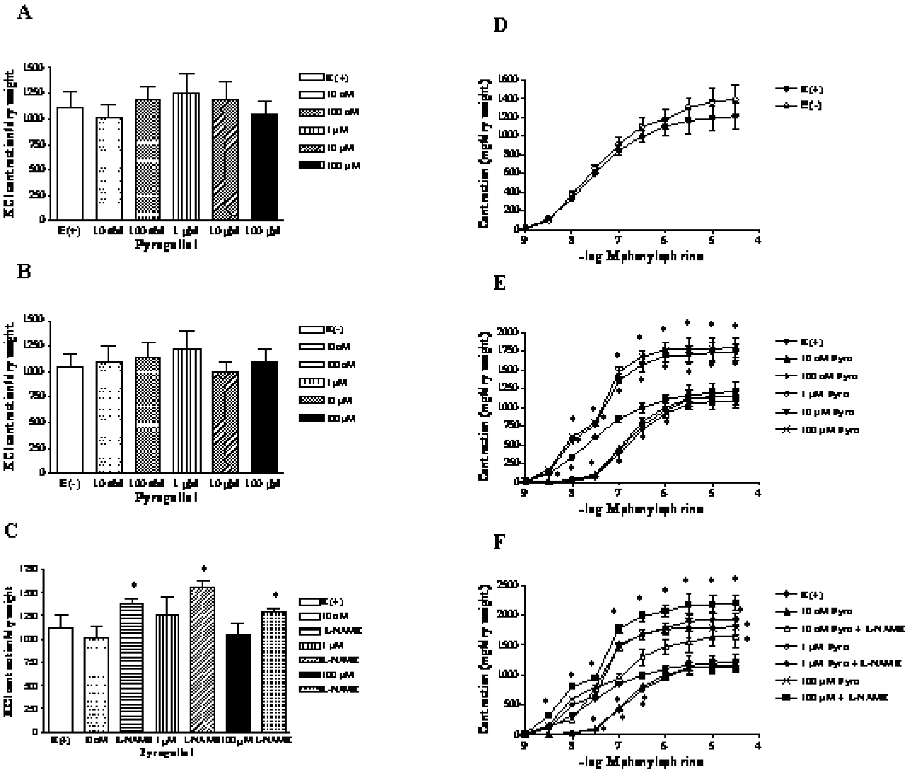

Fig. 4. (A) Phenylpehrine (PE)-induced contractile responses in endothelium-intact [E (+)] and endothelium-denuded [E (-)] aortic rings. (B) Effects of different doses of pyrogallol (Pyro) on PE-induced contractile responses in E (+) aortic rings. (C) Effect of pharmacological inactivation of endothelium by L-NAME (50 μM) on PE-induced contractility in the presence of different concentrations of pyrogallol in E (+) aortic rings. (D) KCl-induced aortic contractile responses in endothelium- intact [E (+)] aortic rings in the absence and presence of pyrogallol. (E) KCl-induced aortic contractile responses in endothelium-denuded [E(-)] aortic rings in the absence and presence of pyrogallol. (F) KCl-induced aortic contractile responses in L-NAME-treated E (+) in the absence and presence of pyrogallol. Results are expressed as mean ± SEM from 8 separate experiments. *$P < 0.05$ compared to PE-mediated contractile responses in E (+) or compared to sole KCl-induced contractile response.

brane permeability to macromolecules (1, 10, 13-15).

Since the majority of the previous studies investigating the effects of ROS on vascular functions have focused mainly on exogenous H_2O_2 (10, 13-16), the current study aimed to investigate the direct effects of O_2^-, the foundation molecule of all ROS, on vasomotor functions using aortas from SD rats. This study primarily demonstrated that pyrogallol, a potent O_2^--generator, produced significant dose-dependent increases in intracellular levels of O_2^- without affecting the tissue viability as determined by the measurements of LDH release. These findings were particularly important to ascertain that the putative differences that might be observed in the study would not be due to cellular damage. It also demonstrated that both ACh and SNP produced dose-dependent vasodilatations in aortic rings and that the inactivation of endothelium by mechanical or pharmacological means selectively abolished relaxations to ACh thereby indicating the existence of a functional vascular smooth muscle layer following endothelial damage by either means. The investigation of the impact of O_2^- on vasomotor function revealed that the endothelium-derived vasodilatations to ACh were improved and attenuated by lower (10 nM - 1 μM) and higher (10-100 μM) concentrations of pyrogallol, respectively and also that the inactivation of endothelium abrogated solely ACh-mediated responses regardless of the concentration of pyrogallol. Taken together these data implied a prerequisite for endothelial presence in modulation of beneficial activities of pyrogallol. This hypothesis was supported by the findings illustrating that the endothelial inactivation dramatically increased PE-induced contractile responses irrespective of the pyrogallol dose used despite initially recording significant decreases with lower concentrations (10 nM - 1μM) of pyrogallol in PE-evoked contractility.

It is noteworthy here that the combination of PE and pyrogallol generated similar effects in endothelium-intact and -denuded rings, findings were in agreement with previous ones suggesting that the smooth muscle cells or α1-mediated responses may be the sites of action of O_2^- (17, 18). Moreover, the additional contractility studies with KCl (120 mM) alone or with different doses of pyrogallol (10 nM - 100 μM) produced similar contractions in vascular rings with or without endothelium. However, pretreatment of aortic rings with L-NAME significantly enhanced KCl-mediated contractile responses to pyrogallol selectively in endothelium-intact segments whereby indicated that the accentuation of pyrogallol-mediated contractility was due to endothelial inactivation but not to L-NAME itself. These data were rather surprising given that KCl induces smooth muscle contractions by depolarizing the cell membrane via a mechanism depending on calcium influx through the voltage-dependent channels (19).

It has been well-documented that NO is involved in much of the endothelium-derived vasodilatation and is readily neutralized by O_2^- (1, 8, 20). The direct correlation between O_2^-

and vascular reactivity was further assessed in this study using $O_2^{\cdot-}$ scavengers i.e. Tiron and MPG which prevented pyrogallol-induced impairments in ACh-mediated relaxant responses without altering the basal endothelium-dependent relaxations. Nonetheless, while the beneficial effects of $O_2^{\cdot-}$ scavengers were not mimicked by exogenous SOD, an irreversible inhibitor of endogenous SOD i.e. DETCA worsened relaxant responses when used in combination with pyrogallol. These findings suggest that an increase in endogenous $O_2^{\cdot-}$ stemmed from the inhibition of SOD alone is not sufficient to neutralize intracellular NO to deteriorate vascular relaxations.

It is inevitable that the incubation of aortic rings with pyrogallol would lead to generation of other ROS including $^{\cdot}OH$, H_2O_2 and $OONO^-$. Indeed, exposure of aortas to incremental concentrations of pyrogallol produced steady increases in intracellular levels of both H_2O_2 and $OONO^-$ levels. To investigate the contributions of these free radicals to vascular relaxant and contractile responses, the relevant experiments were repeated in the presence of the specific inhibitors of $^{\cdot}OH$ (DMSO; 50 nM or desferrioxamine; 100 μM), H_2O_2 (catalase or glutathione peroxidise; 100 U / ml for each agent) or $OONO^-$ (Mn(III)TMPyP; 10 μM or uric acid; 100 μM) that failed to alter the vascular responses when used alone or in the presence of pyrogallol (100 nM or 10 μM). In contrast, studies with $OONO^-$ and H_2O_2 on vasculature showed that both agents could produce relaxations in a dose-dependent manner in vessels with or without endothelium thereby indicating direct vasodilatory effects of these compounds on smooth muscle cells. In previous studies, $OONO^-$-mediated relaxations have been assigned to elevation of cGMP levels, interference with Ca^{2+} movement and membrane hyperpolarization via K^+-channel activation while those of H_2O_2 have been attributed to the NO synthesis and activation of smooth muscle K^+ channels (21, 22). Although, the elucidation of the molecular mechanisms involved in $O_2^{\cdot-}$-mediated vascular responses was out of the scope of the current study, it is possible that some of these mechanisms may be associated with $O_2^{\cdot-}$-induced relaxant responses. In the current study, the inability of the ROS inhibitors to alter vascular responses may be explained by the existence of a delicate balance between their relaxant and contractile effects e.g. $OONO^-$, in addition to its vasodilator effects, also generates vascular contractions by reducing intracellular antioxidants including glutathione and oxidizing tetrahydrobiopterin, a critical cofactor for NO synthesis (1).

In summary, the major conclusions to be drawn from this study are firstly that the vascular relaxant and contractile functions are regulated by $O_2^{\cdot-}$ in a dose-dependent fashion. The similar bimodal regulation of vascular function has previously been reported with H_2O_2 in endothelium-intact and endothelium-denuded rat aortas (21). Secondly, a functional endothelium is required for the regulation of $O_2^{\cdot-}$-mediated dilator and contractile responses to ACh and PE, respectively. Finally, $O_2^{\cdot-}$ may exert a direct effect on smooth muscle cells via stimulation of α1-adrenergic receptors.

MATERIALS AND METHODS

All the experiments described in this article were performed in accordance with the UK "Home Office Guidance on the Operation of the Animals (Scientific Procedures) Act 1986".

Drugs and chemicals

All chemicals, other than Mn(III)TMPyP (IDS Ltd., UK), used in this study were from Sigma Chemical Company (UK).

Animals

The studies were conducted using the thoracic aortas obtained from 12-14 week old male SD rats. The aortas were removed from anticoagulated rats (100 U heparin, IV) under deep pentobarbitone sodium anesthesia (100 mg / kg body, IP) and carefully cleaned of adhering tissue.

Vascular reactivity studies

Thoracic aortas were cut into six 2-3 mm rings and mounted horizontally on stainless hooks in 25 ml organ baths filled with carbonated (95 % O_2 / 5 % CO_2) Krebs buffer [in (mM): NaCl 118.3, KCl 4.7, $MgSO_4$ 1.2, KH_2PO_4 1.22, $CaCl_2$ 2.5, $NaHCO_3$ 25, glucose 11.1, pH 7.4]. Rings were equilibrated for 90 min under 2 g resting tension and only one vasodilator was used for each ring experiment. Changes in isometric tension were detected and recorded by a force transducer and an 8 channel transducer data acquisition system (LE-TR201 and PowerLab / 8S, ADInstruments), respectively. After initial equilibration period, ordinary Krebs solution was replaced with fresh solution containing 1 μM indomethacin to dismiss the relaxant effects of prostanoids. All experiments were performed in the latter solution.

At the beginning of each experiment dose-response curves to PE (0.003-10 μM) were obtained. Rings were then washed and equilibrated prior to contraction with submaximal concentration of PE before detecting endothelium-dependent or endothelium-independent relaxant responses to acetylcholine (ACh, 0.03-1 μM) and sodium nitroprusside (SNP, 0.01-10 μM), respectively. For contractility studies, the rings were incubated with cumulative concentrations of PE (0.003-10 μM) or a single dose of KCl (120 mM) until the respective contractions reached a plateau. For pyrogallol experiments, rings that were used in the abovementioned experiments were washed and equilibrated prior to 20 min incubation with the required concentration of pyrogallol [$C_6H_6O_3$; 1,2,3-trihydroxybenzene] and repetition of the same experiments. This experimental pattern allowed each vascular ring to serve as its own control for a given chemical.

At the end of each experiment, the rings were blotted dry, weighed and the lengths were measured to calculate tension as normalized for cross-sectional area as previously reported (23).

Detection of $O_2^{\cdot-}$

$O_2^{\cdot-}$ generation in aortic rings was measured using three different approaches. Firstly, thoracic aortic rings (~10 mm) were placed in Krebs solution for 30 min at 37°C prior to transfer into Krebs solution containing 5 μM of lucigenin. The chemiluminescence was recorded every 60 sec for 10 min using a luminometer and the levels of $O_2^{\cdot-}$ were calculated by taking into account the differences in the readings before and after addition of the aortic segments to the buffer (24).

Secondly, level of $O_2^{\cdot-}$ was measured by SOD-inhibitable cytochrome *C* reduction assay. Briefly, aortic homogenates were incubated with 50 μM cytochrome *C* for 60 min at 37°C in Krebs buffer with / out SOD (125 U / ml). Absorbances were recorded using a GENios plate reader (TECAN) at 550 nm with 60 sec intervals for 8 min. Production of $O_2^{\cdot-}$ was calculated as previously described and results were expressed as pmole $O_2^{\cdot-}$ per mg tissue (25).

Finally, the level of $O_2^{\cdot-}$ was measured using dihydroethidium (DHE) assay. DHE is oxidized by $O_2^{\cdot-}$ thereby yielding a fluorescent compound whose emission relates to the amount of $O_2^{\cdot-}$ present in the system. This assay was performed in 96-well microplates. Each well contained 100 μl of homogenate obtained from aortas (~10 mm) exposed to different concentrations of pyrogallol (10 nM - 100 μM) in phosphate buffer (pH 7.8) plus 10 μM of DHE in a volume of 100 μl of phosphate buffer. The reaction was performed at 25°C and the extent of DHE oxidation was followed by measuring the increase of the fluorescence using a microplate reader for 60 min (excitation = 485 nm and emission = 595 nm). Three independent experiments were performed for each concentration of pyrogallol. The percentage generation of fluorescence with aortic homogenates was calculated using the pyrogallol-untreated aortic homogenates as positive controls.

Detection of H_2O_2

H_2O_2 synthesis was measured using 2',7'-dichlorodihydrofluorescein (DCHF) diacetate (DA) as a fluorescent probe for intracellular H_2O_2 measurement (26). DCHF-DA diffuses readily to the intracellular compartment, where it is desacetylated to the non-membrane-permeable DCHF. Then, during the cellular production of H_2O_2, DCHF is oxidized and emits a fluorescent signal. To this end, the normal and pyrogallol-incubated aortic segments were homogenized and loaded with 20 μM DCHF-DA for 30 min at 37°C and fluorescence generation at 488 nm (excitation) and 525 nm (emission) was detected. The fluorescent signal was registered as a function of the time.

Detection of $OONO^{\cdot-}$

Thoracic aortas were incubated with different concentrations of pyrogallol (10 nM - 100 μM) in the presence of 20 μM 123-dihydrorhodamine (123-DHR) for 2 h at 37°C in Krebs solution. Following incubation, 123-DHR conversion to 123-rhodamine was measured by fluorometric analysis at excitation / emission wavelengths of 485 and 530 nm, respectively. Fluorescence due to autooxidation of 123-DHR was deducted from the original measurements (27).

LDH measurement

The vascular rings were exposed to different concentrations of pyrogallol in a 3 mL of phenol-free cell culture medium (DMEM) for 3 h at 37°C in a humidified tissue culture incubator (5 % CO_2 / 95 % air) prior to removal of an 100 μl of aliquot. The LDH release was measured spectrophotometrically from the changes in absorbance at 560 nm using 0.25 mM NADH and 0.75 mM pyruvate as substrates following subtraction of the background values obtained from the tubes without rings (28).

Statistical analysis

Data are expressed as mean ± SEM. Each of the vascular reactivity was performed using 8 rats. Dose-response curves were fitted by non-linear regression with simplex algorithm and E_{max} values, the maximum contractile response of the tissue that indicates the potency, were calculated. Relaxant responses were given as the percentages of PE precontraction. Comparisons of dose-response curves were evaluated by two-way analysis of variance (ANOVA) for repeated measures. Molecular biological experiments were performed using aortic samples from 3 different rats. Statistical significances for LDH release and ROS production were assessed by two-way ANOVA followed by Bonferroni-Dunn's *post hoc* analysis. P values less than 0.05 were considered to be statistically significant.

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