The Role of Oxygen-Derived Free Radicals in Augmented Relaxations to Levchromakalim in the Aorta From Hypertensive Rats

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ABSTRACT—Hydrogen peroxide and peroxynitrite induce relaxations via ATP-sensitive K⁺ channels, indicating that oxygen-derived free radicals may activate these channels. Levels of free radicals are increased throughout the arterial wall in animal models of atherosclerosis, and therefore, vasorelaxation via ATP-sensitive K⁺ channels may be augmented in chronic hypertension. The present study was designed to determine whether relaxations to an ATP-sensitive K⁺ channel opener, levchromakalim, are increased in the aorta from spontaneously hypertensive rats (SHR) and whether free radical scavengers reduce these relaxations. Rings of aortas without endothelium taken from age-matched Wistar-Kyoto rats (WKY) and SHR were suspended for isometric force recording. Relaxations to levchromakalim (10⁻⁸ to 10⁻⁵ M), which are abolished by glibenclamide (10⁻⁵ M), were augmented in the aorta from SHR, compared to those in the aorta from WKY. In the aorta from SHR, catalase (1200 U/ml), but neither superoxide dismutase (150 U/ml) nor deferoxamine (10⁻⁴ M), reduced relaxations to levchromakalim, whereas in the aorta from WKY, the free radical scavengers did not affect these relaxations. These results suggest that in chronic hypertension, vasorelaxation to an ATP-sensitive K⁺ channel opener is augmented and that hydrogen peroxide produced in smooth muscle cells may partly contribute to these relaxations.

Keywords: ATP-sensitive K⁺ channel, Catalase, Glibenclamide, Hydrogen peroxide

Hydrogen peroxide and peroxynitrite can induce relaxations via ATP-sensitive K⁺ channels in cerebral arterioles (1). A recent study in cerebral blood vessels has also demonstrated that non-selective free radical scavengers, such as salicylate and L-cysteine, inhibit vasodilation induced by ATP-sensitive K⁺ channel openers, cromakalim and pinacidil (2). In cardiac myocytes, ATP-sensitive K⁺ channel currents produced by the reaction between xanthine and xanthine oxidase, are abolished by superoxide dismutase and catalase (3). These results suggest that in these preparations, oxygen-derived free radicals may play a role in activation of ATP-sensitive K⁺ channels. Recent studies have demonstrated increased levels of oxygen-derived free radicals throughout the wall of arteries taken from animal models of atherosclerosis (4). Therefore, in atherosclerotic blood vessels, vasorelaxation via ATP-sensitive K⁺ channels may be augmented by the increased production of these radicals. Since ATP-sensitive K⁺ channel openers can be used as vasodilators for the treatment of cardiovascular

disorders, it is crucial to determine whether vasodilator effects induced by these openers are modified in chronic hypertension, which can progressively aggravate atherosclerotic lesions in the arterial walls (5). Indeed, a recent study demonstrated that relaxations to ATP-sensitive K⁺ channel openers are augmented in arteries from hypertensive rats, compared to those from normotensive rats (6). However, the role of oxygen-derived free radicals in augmented relaxations to ATP-sensitive K⁺ channel openers in chronic hypertension has not been studied.

Levchromakalim is one of the currently available ATP-sensitive K⁺ channel openers, and it produces potent and selective vasodilator effects via these channels (7, 8). The clinically relevant concentrations of this ATP-sensitive K⁺ channel opener may induce the augmented vasodilation in hypertensive animals. Therefore, the present study was designed to examine the mechanisms of augmented relaxations to an ATP-sensitive K⁺ channel opener in chronic hypertension by determining in the aorta from hypertensive rats, whether relaxations to levchromakalim are increased and whether oxygen-derived free radical scavengers reduce these relaxations.

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MATERIALS AND METHODS

The study was approved by the institutional animal care and use committee. Male age-matched Wistar-Kyoto rats (WKY) and spontaneously hypertensive rats (SHR) (16 to 18 weeks of age) were obtained from Charles River Japan, Inc. (Yokohama). Rats were anesthetized with inhalation of 1% halothane, and systemic blood pressure at the abdominal aorta was measured by cannulating from the femoral artery with the 24 G Teflon catheter, connecting it to the pressure transducer and this in turn to a recorder (RMC-1100; Nihon Kohden Inc., Tokyo). Mean arterial pressure was greater in SHR compared to WKY (141 ± 10 vs 89 ± 5 mmHg, $P < 0.05$), whereas heart rate did not significantly differ (280 ± 19 vs 273 ± 22 bpm, NS). After these measurements, the rats were sacrificed by exsanguination and thoracic aortas were harvested. Thoracic aortic rings of 3 mm in length were studied in modified Krebs-Ringer bicarbonate solution (control solution) of the following composition: 119 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl_2 , 1.17 mM MgSO_4 , 1.18 mM KH_2PO_4 , 25 mM NaHCO_3 and 11 mM glucose. In all rings, the endothelium was removed mechanically. Several rings cut from the same artery were studied in parallel. Each ring was connected to an isometric force transducer and suspended in an organ chamber filled with 10 ml control solution (37°C , pH 7.4) bubbled with 95% O_2 –5% CO_2 gas mixture. The artery was gradually stretched to the optimal point of its length-tension curve as determined by the contraction to phenylephrine (3×10^{-7} M). In most of the studied arteries, optimal tension was achieved approximately at 1.5 g. The endothelial removal was evaluated by the absence of relaxation induced by acetylcholine (10^{-5} M). Preparations were equilibrated for 90 min. During submaximal contractions to phenylephrine (3×10^{-7} M), concentration-response curves to levcromakalim (10^{-8} to 10^{-5} M) were obtained in the absence or in the presence of indomethacin (10^{-5} M), superoxide dismutase (SOD, 150 U/ml), catalase (1200 U/ml), deferoxamine (10^{-4} M), glibenclamide (10^{-5} M). Concentration-response curves were obtained in a cumulative fashion. Only one concentration-response curve was made from each ring. SOD and catalase, deferoxamine and glibenclamide, and indomethacin were given 5, 15 or 30 min before addition of phenylephrine (3×10^{-7} M), respectively. The relaxations were expressed as a percentage of the maximal relaxations to papaverine (3×10^{-4} M), which is added at the end of experiments to produce maximal relaxations (= 100%) of the arteries.

Drugs

The following pharmacological agents were used: catalase, deferoxamine, glibenclamide, indomethacin, papaverine hydrochloride, phenylephrine, superoxide dismutase

(from bovine erythrocytes) (Sigma, St. Louis, MO, USA). Levcromakalim was a generous gift from SmithKline Beecham Pharmaceutical Co. (Betchworth, Surrey, Great Britain). Drugs were dissolved in distilled water such that volumes of $<60 \mu\text{l}$ are added to the organ chambers. Stock solutions of levcromakalim (10^{-5} M) was prepared in DMSO (3×10^{-4} M). A stock solution of indomethacin (10^{-5} M) was prepared in equal molar concentrations of Na_2CO_3 . The concentrations of drugs are expressed as final molar (M) concentration.

Statistical analyses

The data were expressed as means $\pm$ S.D.; n refers to the number of rats from which the aorta was taken. Statistical analysis was performed by repeated measures ANOVA, followed by Scheffe's F test for multiple comparison. Differences were considered to be statistically significant when P is <0.05 .

Fig. 1. Concentration-response curves to levcromakalim (10^{-8} to 10^{-5} M) in the absence and in the presence of glibenclamide (10^{-5} M) in the aorta from WKY or SHR, respectively. Data are shown as means $\pm$ S.D. and expressed as a percent of the maximal relaxation induced by papaverine (3×10^{-4} M; 100% = 1277 ± 163 mg [$n = 6$] and 1180 ± 483 mg [$n = 6$], 1197 ± 201 mg [$n = 6$] and 928 ± 115 mg [$n = 5$] for control rings from WKY and rings from WKY treated with glibenclamide, and control rings from SHR and rings from SHR treated with glibenclamide, respectively). *Difference between control rings and rings treated with glibenclamide and #difference between control rings from WKY and control rings from SHR are statistically significant ($P < 0.05$).

RESULTS

During submaximal contractions to phenylephrine (3×10^{-7} M), relaxations to levcromakalim (10^{-8} to 10^{-5} M) were augmented in aortas from SHR, compared to those in the artery from WKY (Fig. 1). The relaxations in both arteries were abolished by a selective ATP-sensitive K^+ channel inhibitor, glibenclamide (10^{-5} M) (Fig. 1). In aortas from SHR, a hydrogen peroxide scavenger (catalase, 1200 U/ml; Fig. 2a), but neither a superoxide anion scavenger (SOD, 150 U/ml; Fig. 2b) nor a hydroxyl radical scavenger (deferoxamine, 10^{-4} M; Fig. 2c), reduced relaxations to levcromakalim. In contrast, in the aorta from WKY, the free radical scavengers did not affect these relaxations (Table 1). In addition, these free radical scavengers did not have any effects on baseline tension in the aorta from hypertensive rats as well as normotensive rats (data not shown). The cyclooxygenase inhibitor indomethacin (10^{-5} M) did not alter relaxations to levcromakalim in arteries from SHR (data not shown).

DISCUSSION

In the present study, relaxations to clinically relevant

Table 1. Effects of catalase (1200 U/ml), superoxide dismutase (SOD, 150 U/ml), and deferoxamine (10^{-4} M) on relaxations to levcromakalim in the aorta from Wistar-Kyoto rats

	n	$-\log EC_{50}$	% of maximal relaxation
Control	6	6.61 ± 0.09	-72.2 ± 5.3
Catalase (1200 U/ml)	6	6.63 ± 0.06	-74.4 ± 6.9
SOD (150 U/ml)	6	6.71 ± 0.13	-76.6 ± 6.3
Deferoxamine (10^{-4} M)	6	6.68 ± 0.04	-78.7 ± 5.1

Data are shown as means $\pm$ S.D.

concentrations of levcromakalim (from 3×10^{-8} M) were augmented in the aorta from SHR, compared to those in the artery from WKY. Since relaxations to levcromakalim in both arteries were abolished by a selective ATP-sensitive K^+ channel inhibitor glibenclamide (9–11), our results suggest that in chronic hypertension, vasorelaxation in response to clinically relevant concentrations of an ATP-sensitive K^+ channel opener is augmented. This conclusion is supported by a recent study demonstrating that relaxations in response to ATP-sensitive K^+ channel openers other than levcromakalim are augmented in arteries from hypertensive rats, compared to those from normotensive rats (6).

Fig. 2. Concentration-response curves to levcromakalim (10^{-8} to 10^{-5} M) in the absence and in the presence of catalase (1200 U/ml, a), superoxide dismutase (SOD, 150 U/ml, b) or deferoxamine (10^{-4} M, c), obtained in the aorta from SHR. Data are shown as means $\pm$ S.D. and expressed as a percent of the maximal relaxation induced by papaverine (3×10^{-4} M; 100% = 1100 ± 96 mg [n = 5] and 1108 ± 172 mg [n = 5], 1130 ± 113 mg [n = 6] and 1017 ± 204 mg [n = 6], 944 ± 84 mg [n = 5] and 992 ± 166 mg [n = 5] for control rings and rings treated with catalase, control rings and rings treated with SOD, and control rings and rings treated with deferoxamine, respectively). *Difference between control rings and rings treated with catalase is statistically significant ($P < 0.05$).

In the aorta from SHR, catalase reduced relaxations to levcromakalim, indicating that hydrogen peroxide may contribute to relaxations in response to levcromakalim in hypertensive rats (12, 13). This conclusion is concurrent with recent studies on blood vessels and renal epithelial cells demonstrating that hydrogen peroxide can induce vasorelaxation or currents via ATP-sensitive K^+ channels, respectively (1, 14). Therefore, it is likely that hydrogen peroxide can activate ATP-sensitive K^+ channels in these preparations. In the present study, catalase did not have any effects on baseline tension in the aorta from SHR, suggesting that in chronic hypertension, basally released hydrogen peroxide may not play a role in augmented vasorelaxation in response to levcromakalim. Instead, it may be possible that in the aorta from hypertensive rats, levcromakalim itself is capable of activating production of hydrogen peroxide in vascular smooth muscle cells. In addition, we can not completely rule out the possibility that the sensitivity of ATP-sensitive K^+ channels to hydrogen peroxide is augmented in smooth muscle cells in chronic hypertension. However, molecular mechanisms of the activation of ATP-sensitive K^+ channels induced by this oxygen-derived free radical, remain to be determined. In contrast to the results with catalase, SOD did not reduce relaxations to levcromakalim in the aorta from SHR, suggesting that superoxide anion does not mediate augmented relaxations via ATP-sensitive K^+ channels in chronic hypertension (12, 13). Inability of an iron chelator deferoxamine to affect relaxations to levcromakalim indicates that hydroxyl radical may not be a mediator of augmentation of the vasodilator effect via ATP-sensitive K^+ channels in these arteries (12, 13). In our experimental condition, in the aorta from a normotensive animal, these free radical scavengers did not affect relaxations to levcromakalim. This indicates that in the normotensive rat aorta, oxygen-derived free radicals are not responsible for relaxations via ATP-sensitive K^+ channels in smooth muscle cells and supports the selectivity of free radical scavengers used in the present study.

A recent study on cultured smooth muscle cells from the rat aorta demonstrated that angiotensin II, which is one of the important vasoconstrictors involved in the pathophysiology of chronic hypertension, mediates production of superoxide anion generated by the NADH/NADPH oxidase, followed by an increase in intracellular levels of hydrogen peroxide (15). It has been also documented that monocytes, which are well known to be capable of producing large amounts of hydrogen peroxide, are present in the aortic wall of hypertensive animals (16). These previous results and ours suggest that in chronic hypertension, hydrogen peroxide contributes to modification of the intracellular redox state of vascular smooth muscle cells. In contrast to these studies, cumulative findings documented the crucial role of superoxide anion in impaired endothelium-de-

pendent vasorelaxation in chronic hypertension (17, 18). Indeed, in arteries from hypertensive animals, a significant amount of superoxide anion is produced in the presence of endothelium (19). We do not have a clear explanation for why superoxide anions have no role in augmented relaxations to levcromakalim in the present study. However, the present results were obtained in the aorta without endothelium, indicating that at least in our experimental condition, vascular smooth muscle cells may not be responsible for the production of superoxide anions. Previous studies demonstrated that ATP-sensitive K^+ channels are expressed in endothelial cells as well as smooth muscle cells, suggesting that levcromakalim may also produce activation of these channels on endothelial cells (20). Therefore, precaution may be needed to interpret the role of oxygen-derived free radicals in vasorelaxation mediated by ATP-sensitive K^+ channels in hypertensive animals because our results were obtained in preparations without endothelium.

In the aorta from SHR, relaxations in response to levcromakalim were still augmented compared to those in arteries from normotensive animals, even in the presence of catalase. These results suggest that in chronic hypertension, levcromakalim is capable of inducing relaxations via ATP-sensitive K^+ channels in a hydrogen peroxide-independent fashion. It appears that distribution and/or sensitivity of these channels may be increased in this diseased state, resulting in augmented relaxations to levcromakalim. Indeed, increased expression and enhanced sensitivity of Ca^{2+} -dependent K^+ channels have been demonstrated in arterial smooth muscle cells from SHR (21, 22). However, those of ATP-sensitive K^+ channels have not been determined in chronic hypertension.

Prostanoids play a major role in reduced endothelium-dependent relaxations in animal models of chronic hypertension (23). Therefore, we suspected that prostanoids may contribute to increased relaxations to levcromakalim in chronic hypertension. However, indomethacin did not alter relaxations to levcromakalim in the aorta from SHR, suggesting no role of prostanoids in relaxations to an ATP-sensitive K^+ channel opener in our preparation.

The therapeutic ranges of plasma concentrations of levcromakalim used as an antihypertensive drug, have been reported as 1×10^{-8} to 5×10^{-8} M, indicating that some concentrations of levcromakalim used in the present study are within plasma concentrations in the clinical situation (unpublished observation by SmithKline Beecham Pharmaceutical Co.). ATP-sensitive K^+ channel openers are clinically available vasodilators for the treatment of cardiovascular disorders, including hypertension (5). In addition, it has been well-known that in patients with hypertension, endothelium-dependent vasodilation is reduced (24). Therefore, our results indicate that in hypertensive patients, vasodilator effects of ATP-sensitive K^+ channel openers

may be augmented and this may be in turn one of the compensatory mechanisms to maintain vasorelaxation in the diseased state where endothelial function is impaired.

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