ISSN 0253-9756 Acta Pharmacol Sin 中国药理学报 1999 Dec; 20 (12): 1087 - 1092
http://www.simm.ac.cn; www.chinainfo.gov.cn/periodical

Increased response to prostaglandin H_2 precedes changes in PGH synthase-1 expression in the SHR aorta

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KEY WORDS acetylcholine; prostaglandin-endoperoxide synthase; inbred SHR; inbred WKY; endothelium-dependent contractions; polymerase chain reaction; Western blotting; nitroarginine

ABSTRACT

AIM: To determine the expression of PGH synthase-1 and the sensitivity of vascular smooth muscle to PGH_2 in the aorta from the SHR at an age when no endothelium-dependent contractions to acetylcholine are observed under control conditions. **METHODS**: All experiments were performed in parallel on aortas from 20-wk-old SHR and Wistar-Kyoto normotensive rats (WKY). Rings, with or without endothelium, were suspended in conventional organ chambers for the recording of changes in isometric force. The expression of PGH synthase-1 was evaluated by reverse transcription-polymerase chain reaction (RT-PCR) and Western blot analysis. **RESULTS**: Under control conditions acetylcholine did not cause contractions of rings with or without endothelium. However, in the presence of nitro-*L*-arginine (*NLA*, an inhibitor of nitric-oxide synthase), it evoked endothelium-dependent contraction in the SHR but not in the WKY aortas. The expression of PGH synthase-1 was comparable in the aortas of both strains (with and without endothelium). PGH_2 caused greater contractions in rings without endothelium from the SHR than those from WKY, while U46,619 evoked a comparable response, in aortas from both strains. **CONCLUSION**: In the aorta of 20-wk-old SHR, endothelium-dependent contractions to acetylcholine are observed only when the production of nitric oxide is prevented. They are associated with an augmented sensitivity of the smooth muscle to PGH_2, but not with an increased expression of PGH synthase-1.

INTRODUCTION

In the aorta of the spontaneously hypertensive rat (SHR), acetylcholine causes endothelium-dependent contractions, which are mediated by activation of thromboxane-endoperoxides receptors[1-5]. In the SHR aorta, the phenomenon is age-dependent: it can also be observed in arteries from old normotensive rats[6-11]. The occurrence of endothelium-dependent contractions to acetylcholine in the aorta of the 35-wk-old SHR is explained both by an increased expression of prostaglandin H synthase-1, resulting in a larger production of prostaglandin H_2 (PGH_2) and a hypersensitivity of the vascular smooth muscle to the constrictor effect of the endoperoxide[5]. The present experiments were designed to examine the expression of PGH synthase-1 and the responsiveness of vascular smooth muscle to PGH_2 in SHR and WKY aortas at age of 20-wk old, at which endothelium-dependent contractions to acetylcholine do not occur under control conditions in the aorta of the hypertensive strain.

MATERIALS AND METHODS

Animals The experiments were performed on thoracic aortas from male inbred SHR and WKY (20 wk old; Harlan Sprague Dawley, Indianapolis, IN, USA). All procedures using animals were in accordance with the guidelines of the Animal Protocol

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Received 1999-03-17 Accepted 1999-09-12

ISSN 0253-9756 Acta Pharmacol Sin 中国药理学报 1999 Dec; 20 (12)
E-mail aps@server shcnc.ac.cn Phn/Fax 86-21-6474-2629

Review Committee of Baylor College of Medicine. Systolic arterial blood pressure was measured by the tail cuff method, and averaged (137.1 ± 4.7) and (229.3 ± 2.3) mmHg in WKY and SHR, respectively (n = 7, $P < 0.05$). The rats were anesthetized with pentobarbital sodium (50 mg/kg, ip). The thoracic aorta was dissected free, excised, and cleaned of adherent connective tissue in cold modified Krebs-Ringer bicarbonate solution (control solution; composition in mmol/L: NaCl 118.3; KCl 4.7; $MgSO_4$ 1.2; KH_2PO_4 1.2; $CaCl_2$ 2.5; $NaHCO_3$ 25.0; calcium disodium edetate (EDTA) 0.026; and glucose 11.1; pH 7.4). For organ chambers experiments, the preparations were immersed in control solution and used immediately. For RNA and protein extraction, the vessels were frozen in liquid nitrogen and kept in a freezer (−70 ℃) until the assays were performed.

Organ chambers experiments Thoracic aorta from WKY and SHR were cut into rings (4 − 5 mm wide). In some preparations, the endothelium was removed by gently rubbing the intimal surface with a small forceps. In the remaining rings, care was taken not to touch the inner surface of the blood vessel. The presence or absence of endothelial cells was confirmed by the presence or absence of relaxation in response to thrombin (1 kU/L), respectively. The rings were suspended horizontally between two stainless wires in organ chambers filled with control solution (37 ℃) aerated with 95 % O_2 + 5 % CO_2. The preparations were connected to force transducers (Statham Universal UC2 or Grass FT03C, Quincy, MA, USA) for recording of changes in isometric force. Prior to experimentation, the preparations were stretched progressively and exposed to KCl (45 mmol/L) at each level of force until the optimal point of the length-active force relationship was reached. After this procedure the rings were allowed to equilibrate for 40 min. All rings were then exposed to KCl 60 mmol/L to determine their maximal responsiveness. There were no significant differences in response to KCl 60 mmol/L between aortas of WKY and SHR (data not shown).

The contractions to acetylcholine were examined in parallel rings with or without endothelium[11]. In some preparations, rings were incubated with N^G-nitro-*L*-arginine (0.1 mmol/L; Aldrich, Milwaukee, WI) for 20 min before addition of acetylcholine[12]. Contractions to increasing concentrations of PGH_2 (Biomol, Plymouth Meeting, PA) and the thromboxane analog U46,619 (Cayman Chemical Co, Ann Arbor, MI) were obtained in parallel in rings without endothelium from WKY and SHR aortas[5].

All drugs were prepared daily (stock solution 10 mmol/L) and further diluted in distilled water, except prostaglandin H_2, which was obtained as a solution in acetone (100 mg/L; −70 ℃) and was diluted further in acetone. The highest concentration of acetone was 1.2 % of the final chamber volume (to reach PGH_2 0.1 μmol/L in the bath solution). At this concentration, acetone decreased vascular tone to a comparable extent in both strains (data not shown).

RT-PCR RNA preparation Total RNA was extracted with RNA STAT-30 kit (Tel-Test "B", Inc, Friendswood, Texas) and the concentration was measured two times for each sample in a spectrophotometer (UV160U, Shimadzu). Aliquots of RNA were loaded on a denatured formamide agarose gel to check the RNA quality[5].

RT-PCR The message RNA expression of PGH synthase-1 in WKY and SHR was evaluated by reverse transcription-polymerase chain reaction (RT-PCR; 5). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was amplified parallelly as an internal control. Total RNA of 2 μg from each rat aorta was reverse-transcribed into cDNA by using the SuperScript Preamplification System (Gibco BRL, Grand Island, NY). Ten microliters of cDNA aliquots were amplified by PCR with either PGH synthase-1 or GAPDH specific primers in a DNA thermal cycler (Perkin Elmer, Norwalk, CT). The primers for PGH synthase-1 were obtained from Baylor College of Medicine, Molecular Core Facility[5]. The primers for GAPDH were purchased from Clontech, Palo Alto, CA. The PCR mixture included 200 μmol/L of each dNTP plus 0.37 MBq of α-^{32}P-dCTP (110 TBq/mol), 1 μmol/L of each primer, 2 units of *Taq* polymerase (Perkin Elmer, Norwalk, CT), in PCR buffer (Tris-HCl 20 mmol/L, $MgCl_2$ 2.5 mmol/L, KCl 50 mmol/L, and BSA 100 μg/L), with a final volume of 50 μL. For quantitative purpose, the cDNA were amplified by 28 cycles (each cycle consisting of DNA denaturation at 94 ℃ for 40 s, primer annealing at 54 ℃ for 40 s and primer extension at 72 ℃ for 90 s). Under these conditions, the yield of the amplified product was linear with respect to the amount of input

RNA for the specific pair of primers[5]. The amplified cDNA (212 and 452 bp for PGH synthase-1 and GAPDH, respectively) were resolved on 8 % polyacrylamide gels and quantitated by a Beta-Scope 603, blot analyzer (Betaken, Mountain View, CA).

Western blot Frozen thoracic aortas were ground as described for RNA extraction, then homogenized in phosphate buffered saline (PBS) (NaCl 135 mmol/L; KCl 2.7 mmol/L; Na_2HPO_4 10 mmol/L; KH_2PO_4 1.8 mmol/L) with Triton-X 100 0.1 %, phenylmethylsulfonyl fluoride 1 mmol/L, EDTA 0.01 %, and leupeptin 0.03 % (pH = 7.4); the protein-concentration was measured in the supernatant using the Micro BCA protein assay (Pierce, Rockford, IL). Samples (50 μg protein) were electophoresed on a 8 % polyacrylamide gel under reducing and denaturating conditions, and transferred onto a nitrocellulose-ECL membrane (Amersham, Arlington Heights, IL). Non-specific binding sites were blocked overnight (4 ℃) in a 0.05 % Tween-20 PBS buffer (T-PBS; pH 7.4) containing 5 % non-fat milk (Biorad, Hercules, CA). The blots were then incubated with either the monoclonal anti-prostaglandin H synthase-1 antibody (dilution 1:200; Oxford Biomedical Research, Oxford, MI) or the monoclonal anti-α-smooth muscle actin (as an internal standard, dilution 1:2000, Sigma, St Louis, MO). Both PGH synthase-1 and α-smooth muscle actin were revealed with ECL Western blotting detection reagents following exposure to the secondary antibody according to the instructions given by the manufacturer (Amersham, Arlington Heights, IL). Films were analyzed by a densitometor (Biomed Instruments, Inc). Data are expressed as the ratio of the peak absorbance for PGH synthase-1 and α-smooth muscle actin.

Statistical analysis All experiments were performed in parallel on preparations from 20-wk-old WKY and SH rats; n represents the number of preparations from different animals. Results are given as mean ± SEM for organ chambers experiments and measures of PGH synthase expression, statistical analysis was performed using Student's t-test for paired and unpaired observations or using a two-way analysis of variance (ANOVA, followed by a Bonferroni test) when more than two means were compared. All data were analyzed using Instat software (GraphPad, San Diego, CA). Differences were considered to be statistically significant when P was less than 0.05.

RESULTS

Organ chamber studies Under control conditions, acetylcholine (10 nmol/L – 10 μmol/L) did not cause significant changes in tension in aortic rings, with and without endothelium, of either WKY and SHR. However, after incubation with nitro-*L*-arginine (inhibitor of nitric-oxide synthase; 30 μmol/L), acetylcholine evoked significant endothelium-dependent increases in tension in the SHR, but not in the WKY aorta (Fig 1).

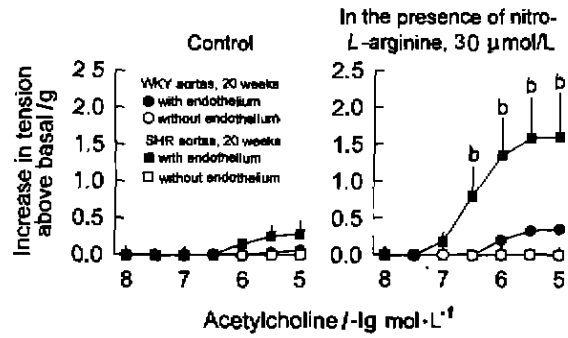

Fig 1. Effect of increasing concentrations of acetylcholine in aortic rings (with and without endothelium) from spontaneously hypertensive (SHR) and normotensive Wistar-Kyoto (WKY) rats, in control solution (left) and after incubation with nitro-*L*-arginine (30 μmol/L, right). The changes in tension above basal are shown as means ± SEM. There is a significant difference ($^bP < 0.05$) in response between rings with endothelium of SHR and WKY.

The thromboxane analog U-46,619 evoked comparable responses in rings without endothelium of WKY and SHR aortas whereas PGH_2 (30 nmol/L – 10 μmol/L) induced significantly greater contractions in the SHR than in the WKY aorta (Fig 2).

Expression of PGH synthase-1 in WKY and SHR aorta PGH synthase-1 and GAPDH mRNA were reverse transcribed and amplified by polymerase chain reaction; there was no significant difference in the ratio of PGH synthase-1/GAPDH cDNA between WKY and SHR preparations (Fig 3).

The expression of PGH synthase-1 in the WKY and SHR aorta were compared at the protein level by Western blotting (Fig 4). The densitometric analysis

ISSN 0253-9756 Acta Pharmacol Sin 中国药理学报 1999 Dec; 20 (12)
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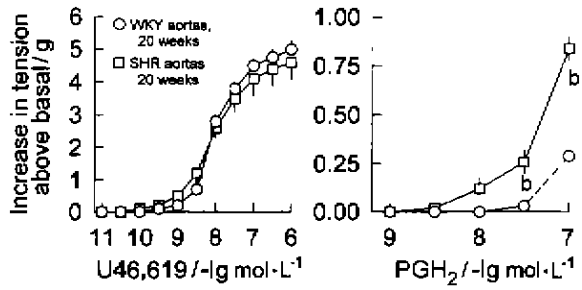

Fig 2. Contractions of aortic rings without endothelium from Wistar-Kyoto (WKY) and spontaneously hypertensive (SHR) rats to increasing concentrations of U46,619 (left) and prostaglandin H_2 (right). The responses to both agonists were obtained in parallel on rings of the same animals. Data were shown as means ± SEM. There was a significant difference ($^bP < 0.05$) between preparations from SHR and WKY rats.

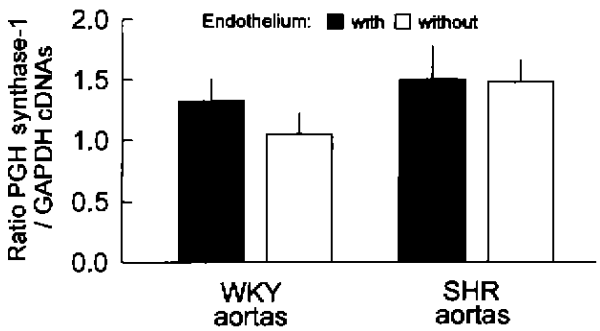

Fig 3. Comparison of reverse transcriptase-polymerase chain reaction (PCR) analysis of prostaglandin H synthase-1 (PGH-1) and GAPDH in RNA expression in aortas, with (open bars) and without (hatched bars) endothelium, of Wistar-Kyoto (WKY) and spontaneously hypertensive (SHR) rats. The data are expressed as ratio between the prostaglandin H synthase-1 and GAPDH cDNA, and shown as means ± SEM. There were no statistically significant differences between the experimental groups.

showed that the ratio of PGH synthase-1 *vs* α-smooth muscle actin was 0.79 ± 0.04 and 0.81 ± 0.06 in WKY and SHR, respectively ($n = 3$). There was no significant difference in the amount of PGH synthase-1 between the two rat strains.

DISCUSSION

The present experiments demonstrate that in SHR aorta, the hypersensitivity of vascular smooth muscle to

Fig 4. Densimetric analysis showing detection prostaglandin H synthase-1 (PGH-1, top) and α-smooth muscle actin (actin, bottom) in protein homogenous form aortas (without endothelium) of Wistar-Kyoto (WKY) and spontaneously hypertensive (SHR) rats. The estimated molecular mass values were 70 and 45 kDa a for the protein detected with the PGH synthase-1 antibody and that detected with the α-smooth muscle actin monoclonal antibody, respectively. Similar results were obtained with two other sets of WKY and SHR aortas.

endoperoxides (PGH_2) precedes the occurrence of endothelium-dependent contractions to acetylcholine. Indeed, in 20-wk-old SHR rats, no endothelium-dependent contractions are observed unless the formation of nitric oxide is prevented. In older SHR, such inhibition is not required[1,5] although inhibitors of the *L*-arginine-NO pathway potentiate the response[12]. The potentiation is explained best whether cyclooxygenase-dependent contracting factor and nitric oxide interact chemically and inactivate each other[12-14].

The cyclooxygenase-dependent contracting factor is likely to be PGH_2, the production of which is regulated by PGH synthase-1 in SHR aorta[2,3,5]. In the 35-wk-old SHR, differences in PGH synthase-1 expression are associated with the occurrence of endothelium-dependent contractions in the aorta of SHR rats. Indeed, at that age the SHR aorta has a two-fold greater expression of PGH synthase-1 and a resulting significantly larger release of PGH_2 and $PGF_{2\alpha}$ upon acetylcholine stimulation than that of age-matched WKY[5]. By contrast in the present study, such difference was not observed in the aorta of 20-wk-old hypertensive rats, since a comparable measurement of PGH synthase-1 expression was obtained at both the mRNA and the protein level. The fact that hypertension was already well established at 20 wk of age argues againts a direct regulation of PGH synthase-1

expression by the increase in arterial blood pressure. The likely explanation for this difference between 20- and 35-wk-old SHR is that increase in PGH synthase expression is a gradual process during aging.

The endothelium-dependent contractions to acetylcholine in 35-wk-old SHR were caused by an augmented production of PGH_2 resulting from the increased expression of PGH synthase-1 but also by an enhanced responsiveness of vascular smooth muscle to the constrictor effect of PGH_2[5]. In 20-wk old SHR, the contractions to PGH_2 are also augmented when compared with age-matched WKY while the response to U46,619, a stable analog of thromboxane A_2, is comparable between the two strains as observed in 35-wk-old rats. The different responsiveness to PGH_2 and U46,619 could be explained by an alteration of either PGH_2 metabolism into vasoactive prostaglandins or the prostanoid receptors signal transductions in the SHR vascular wall[1,15]. Alternatively, PGH_2 and the thromboxane analog may activate different subtypes of thromboxane-endoperoxide receptors on the smooth muscle[16]. Despite the hyperresponsiveness of the hypertensive vascular smooth muscle to PGH_2, the physiological antagonism exerted by nitric oxide released together with endoperoxides during stimulation with acetylcholine is sufficient to prevent the occurrence of endothelium-dependent contractions in 20-wk old SHR aorta.

In conclusion, the augmented responsiveness of hypertensive vascular smooth muscle precedes the occurrence of endothelium-dependent contractions in SHR aorta. However, increase in both prostaglandin synthase-1 expression and PGH_2 contractions appears to be required to elicit endothelium-dependent contractions in response to acetylcholine[5]. Although the role of this endothelial dysfunction remains uncertain in the regulation of blood pressure in animal models of hypertension, the improvement of the blood flow response to acetylcholine-infusion following inhibition of cyclooxygenase with indometacin in essential hypertensive patients suggests an important role for PGH synthase-1 metabolites, or their receptors on vascular smooth muscle, in the curtailed endothelium-dependent vasodilatation characteristic of the disease[17].

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ISSN 0253-9756 Acta Pharmacol Sin 中国药理学报 1999 Dec; 20 (12)
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自发性高血压大鼠主动脉对前列腺素 H_2 反应的增加先于前列腺素 H 合酶 1 表达的变化

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关键词 乙酰胆碱; 前列腺素内过氧化物合酶; 近交 SHR 大鼠; 近交 WKY 大鼠; 内皮依赖性收缩; 聚合酶链反应; 蛋白质印迹; 硝基精氨酸

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