Thromboxane and Vascular Smooth Muscle Cell Growth in Genetically Hypertensive Rats

TOSHIHIKO ISHIMITSU, YOSHIO UEHARA, MASAO ISHII, TOSHIO IKEDA, HIROAKI MATSUOKA, AND TSUNEAKI SUGIMOTO

SUMMARY The vascular wall has the capacity to produce thromboxane A₂. However, the role of vascular thromboxane A₂ generation and vascular smooth muscle cell growth in spontaneously hypertensive rats (SHR). Vascular thromboxane A₂ generation was significantly enhanced by 49% in 5-week-old and by 117% in 15-week-old SHR as compared with age-matched Wistar-Kyoto rats (WKY). Thromboxane A₂ generation was also significantly enhanced by 59% in the cultured vascular smooth muscle cells of SHR when compared with production in WKY. Vascular smooth muscle cells of SHR exhibited a significantly shortened doubling time (by 32%) and greater [³H]thymidine uptake (by 56%), as compared with those of WKY. OKY 046 (10⁻¹° M), a thromboxane synthase inhibitor, significantly tempered the rapid vascular smooth muscle cell growth in SHR by 9% for doubling time and by 10% for [³H]thymidine uptake. OKY 046 did not influence the doubling time of WKY. Conversely, a stable analogue of thromboxane A₂ dose-dependently stimulated the [³H]thymidine uptake by vascular smooth muscle cells of WKY, and, at a concentration of 10⁻⁵ M, shortened the doubling time of vascular smooth muscle cells of WKY by 11%, whereas it showed slight effects on SHR. These data indicate that vascular thromboxane A₂ is involved in the regulatory mechanism of vascular smooth muscle cell growth and that enhanced vascular thromboxane A₂ generation is partly responsible for the rapid proliferation of vascular smooth muscle cells of SHR. The alterations of vascular thromboxane production may be a key trait for genetic hypertension.

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KEY WORDS • thromboxane • vascular smooth muscle cell growth • thromboxane inhibitor • hypertension • spontaneously hypertensive rats

S PONTANEOUSLY hypertensive rats (SHR) in the prehypertensive stage show hyperplasia or hypertrophy in the cardiovascular system.¹⁻³ Thickening of the vascular wall increases the wall’s stiffness and narrows the vascular lumens, thereby producing an elevation of peripheral vascular resistance. Because some humoral substances, such as catecholamines, steroids, platelet-derived growth factor, and insulin, are reported to influence cell proliferation in various cell lines, these factors may be responsible to some extent for the vascular thickness observed in SHR. However, since vascular smooth muscle cells (VSMCs) of SHR sustain an enhanced proliferative activity even if they are cultured in an artificial medium, alterations of the biological properties of VSMCs are assumed to elicit the thickening of the vascular wall.

The vascular wall produces vasodepressor prostacyclin (PGI₂). Moreover, some evidence has been provided that vasoconstrictor thromboxane A₃ (TXA₃) is produced in the vascular wall.⁸ In addition, experimental models of genetic hypertension have been shown to possess an enhanced vascular TXA₂ generation.⁹ ¹⁰ Further, prostaglandins exert an influence on the proliferation of various cells,¹¹ and thromboxane B₂ (TXB₂) stimulates the proliferations of cell lines such as 3T3 cells¹² and melanoma cells.¹³ Thus, we propose that enhanced vascular TXA₂ generation to some extent participates in the increase in vascular wall thickness in SHR, which may be partly manifested by hyperplasia of VSMCs. In the present study, to test our hypothesis, we examined vascular TXA₂ generation in SHR and investigated the effects of TXA₂ synthase inhibitor, OKY 046 (sodium [E]-3-[4-(1-imidazolylmethyl)phenyl]-2-
Thromboxane and Smooth Muscle Cell Growth in SHR

Jishimitsu et al.

Materials and Methods

Experiment 1: Vascular Capacity to Produce Thromboxane A2

Twenty-four 5-week-old and twenty 15-week-old SHR and WKY (from the University of Tokyo breeding colony) were used for the experiment. After systolic blood pressures were measured by the tail-cuff method, the rats were killed by decapitation and the descending thoracic aortas were isolated. The aortas were placed in an ice-chilled Dulbecco's phosphate-buffered saline solution (D-PBS), and the surrounding connective tissue was gently removed. The prepared strips were incubated in 3 ml of oxygenized D-PBS (pH 7.4) at 37 °C for 30 minutes. TXA2 released in the medium was measured in the form of TXB2, using a direct radioimmunoassay method previously described. Antiserum was raised in rabbits in our laboratory. Measurements were normalized to the dry tissue weights of aorta.

Experiment 2: Vascular Smooth Muscle Cell Culture

VSMCs were isolated from the thoracic aortas of sixteen 7-week-old SHR and WKY according to the method of Ross. Briefly, thoracic descending aortas were obtained, and the adventitia and intima were carefully stripped off with forceps and gauze. Then, explants of about 5 mm2 were placed on a 94x21-mm polystyrene dish with the intimal side attached to the dish, and 10 ml of Dulbecco's modified Eagle's medium (DMEM; Gibco Laborato ries, Grand Island, New York, USA), supplemented with 20% (vol/vol) fetal calf serum (FCS; Gibco), penicillin (100 U/ml), and streptomycin (100 µg/ml), was gently added. The dishes were incubated at 37 °C in a humidified atmosphere of 95% air and 5% CO2. The cells reached confluency 10 days after the inoculation. They were harvested by brief exposure to Hanks' solution supplemented with 0.05% (wt/vol) trypsin and 0.02% (wt/vol) EDTA and transferred into a new dish. At this stage, the cells were designated as the first passage. The percentage of FCS was switched to 10% (vol/vol) in the subsequent media. Harvesting was repeated when the cell growth reached confluency. VSMCs from Passages 4 to 10 were used in the studies. The VSMCs in culture showed the characteristic "hills and valleys" growth pattern, and the occurrence of cytoplasmic myofilaments was confirmed by electron microscopy.

Measurement of Thromboxane A2 Generation by Vascular Smooth Muscle Cells

VSMCs in the sixth passage were washed three times with FCS-free D-PBS. Thereafter, VSMCs were cultured in D-PBS or D-PBS supplemented with 10-6 M arachidonic acid for 1 hour. TXA2 released in the medium was directly radioimmunoassayed as TXB2. The cross-reaction of anti-TXB2 serum was less than 0.00001% with arachidonic acid. The antiserum was highly specific to TXB2, so that other arachidonate metabolites did not interfere with the radioimmunoassay in this experimental condition.

Measurement of Vascular Smooth Muscle Cell Doubling Time

To assess the doubling time of VSMCs, approximately 5 x 103 cells were seeded onto 9-cm2 dishes with 2 ml of DMEM containing 10% (vol/vol) FCS. After 24 hours, the medium was replaced with DMEM containing 10% (vol/vol) FCS and a given concentration of OKY 046 or STA2. The medium was renewed every day. The number of cells was counted using a light microscope before and 48 hours after the addition of the compounds. The doubling time of cultured VSMCs was calculated according to the following equation:

\[ t = \frac{(t_1 - t_0) \times \log 2}{\log N_1 - \log N_2} \]

In this expression, \( t \) and \( t_2 \) are the times when cell numbers are counted and \( N \), and \( N_2 \) are the cell numbers at \( t_0 \) and \( t_2 \), respectively.

Incorporation of [3H]Thymidine into DNA

DNA turnover rate was determined by measuring the incorporation of [3H]thymidine into VSMCs. Approximately 103 cells were inoculated in a 16 x 17-mm 24-well polystyrene dish (Corning-Iwaki Glass, Tokyo, Japan) and cultured with 0.5 ml of DMEM containing 10% (vol/vol) FCS for 24 hours. The incubation medium was switched to DMEM with 10% (vol/vol) FCS and a given concentration of the compounds. Thereafter, 0.25 µCi of [3H]thymidine (20-30 Ci/mmol) was added to the medium. Twenty-four hours after the incubation, the cells were repeatedly washed with D-PBS without Ca2+ and Mg2+ at pH 7.35 and treated with 5% perchloric acid. The radioactivity in the acid-insoluble fractions was measured by an automatic liquid scintillation counter.

Reagents

The authentic eicosanoids, STA2 and OKY 046 were courteously offered by Ono Pharmaceutical Co., Osaka, Japan. [3H]TXB2 and [3H]thymidine were purchased from Amersham International, Buckinghamshire, England.

Statistical Analysis

Values are expressed as means ±SEM. The difference was assessed by the unpaired or paired t test or one-way analysis of variance.
### Table 1. Averaged Systolic Blood Pressure and Weight of the Aorta of SHR and WKY

<table>
<thead>
<tr>
<th>Strain</th>
<th>Systolic blood pressure (mm Hg)</th>
<th>Weight of Aorta (mg dry tissue/cm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-week-old</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SHR (n=12)</td>
<td>119 ± 2*</td>
<td>5.4 ± 0.2†</td>
</tr>
<tr>
<td>WKY (n=12)</td>
<td>109 ± 3</td>
<td>4.8 ± 0.1</td>
</tr>
<tr>
<td>15-week-old</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SHR (n=10)</td>
<td>189 ± 4†</td>
<td>6.3 ± 0.2†</td>
</tr>
<tr>
<td>WKY (n=10)</td>
<td>134 ± 3</td>
<td>5.2 ± 0.3</td>
</tr>
</tbody>
</table>

Values are means ± SEM. n = number of studies. *p < 0.01, †p < 0.003, ‡p < 0.001, compared with value for respective control WKY.

### Results

**Experiment 1**

The averaged systolic blood pressures and the averaged dry tissue weights of aortas are given in Table 1. Five-week-old SHR were still in the prehypertensive stage, although they showed 9% higher systolic blood pressure than the age-matched WKY. Fifteen-week-old SHR, which were in the established stage of hypertension, possessed a 41% higher blood pressure than the controls. The difference in averaged systolic blood pressure became greater with age. The dry tissue weight of aorta was significantly increased by 12% for 5-week-old SHR and by 21% for 15-week-old SHR when compared with the respective WKY.

**Experiment 2**

There was no difference in TXA2 generation between VSMCs of SHR and those of WKY when they were cultured in arachidonate-free medium (8.44 ± 0.28, n = 7, vs 7.83 ± 0.19 pg/10⁶ cells/hr, n = 7). As shown in Figure 2, however, when prostaglandin production was stimulated by 10⁻⁶ M arachidonic acid, vascular TXA2 generation was significantly enhanced by 59% in SHR, as compared with that in WKY (24.98 ± 1.21 vs 15.63 ± 0.64 pg/10⁶ cells/hr).

Doubling times of VSMCs of SHR and WKY are presented on the left side of Figure 3. The doubling times were significantly shortened in VSMCs of SHR (by 24% at the 5th passage and by 32% at the 10th passage) when compared with those of the control WKY (23.6 ± 1.0 vs 31.2 ± 1.6 hours for the 5th passage and 23.4 ± 0.8 vs 34.3 ± 3.4 hours for the 10th passage). The enhanced proliferative activity in VSMCs of SHR was also confirmed by...
THROMBOXANE AND SMOOTH MUSCLE CELL GROWTH IN SHR/Ishimitsu et al.

FIGURE 4. Effects of thromboxane synthase inhibitor (OKY 046) on proliferation and DNA synthesis of vascular smooth muscle cells in WKY (○) and SHR (●). n = number of studies.

The incorporation of [3H]thymidine into DNA in VSMCs is shown on the right side of Figure 3. VSMCs of SHR at the 5th and 10th passages incorporated a significantly larger amount of [3H]thymidine than did VSMCs of WKY (18.9 ± 2.1 vs 12.1 ± 1.6 x 10^3 dpm/10^4 cells/24 hr for the 5th passage and 17.6 ± 0.8 vs 14.2 ± 1.1 x 10^3 dpm/10^4 cells/24 hr for the 10th passage).

Next, to assess whether the increased vascular TXA2 generation is connected to the rapid proliferation of VSMCs of SHR, we explored the effects of a thromboxane synthase inhibitor, OKY 046, and the effects of a stable TXA2 analogue, STA2, on the proliferation of VSMCs. When OKY 046 (10^-3 M) was added to the culture medium, the doubling time was significantly prolonged in VSMCs of SHR (from 24.4 ± 0.8 to 26.4 ± 1.1 hours), whereas the doubling time of VSMCs of WKY was unaltered (Figure 4). Similarly, OKY 046 significantly reduced [3H]thymidine uptake by VSMCs of SHR (from 15.7 ± 1.2 to 14.7 ± 1.2 x 10^3 dpm/10^4 cells/24 hr), although it did not affect the uptake in WKY.

On the other hand, the effects of STA2, a stable analogue of TXA2, on VSMC growth are given in Figure 5. STA2 promoted the incorporation of [3H]thymidine into DNA in VSMCs of WKY in a dose-dependent manner. At an STA2 concentration of 10^-5 M, the [3H]thymidine uptake was almost maximally stimulated by 39%. Thus, we employed this concentration of STA2 to examine the effects on the proliferation of VSMCs of SHR. At a concentration of 10^-3 M, STA2 significantly shortened the doubling time of VSMCs of WKY (from 33.7 ± 1.0 to 30.0 ± 0.7 hours) and increased incorporation of [3H]thymidine (from 13.0 ± 0.9 to 15.2 ± 1.6 x 10^3 dpm/10^4 cells/24 hr; Figure 6). In contrast, STA2 produced a slight decrease in the doubling time of VSMCs of SHR, but [3H]thymidine uptake was unaltered.

Discussion

The vascular wall has a large capacity to generate vasodepressor prostaglandin, which may participate in blood pressure regulation through direct actions on VSMCs. The vascular wall has been demonstrated to produce small amounts of vasconstrictor TXA2 as well.8 In addition, there is some evidence that vascular TXA2 production is enhanced in genetically hypertensive rat models (e.g., SHR and Dahl salt-sensitive strains).9, 10 In this study, we clearly showed that vascular TXA2 generation was increased not only in adult hypertensive SHR, but also in 5-week-old prehypertensive SHR, which also showed a marked increase in aortic weight per square centimeter, as compared with the age-matched WKY. Moreover, we also demonstrated that the enhanced TXA2 generation in VSMCs of SHR could be unveiled when prostaglandin production was stimulated by arachidonic acid.
The elevation of blood pressure brings about growth of the vascular wall. In this context, the difference in averaged blood pressure value could explain the difference in weight of the aorta between SHR and WKY. Five-week-old SHR had markedly heavier aortic walls than the age-matched WKY, although the difference in blood pressure was relatively small. Accordingly, the blood pressure difference alone seems unable to account for the entire difference in the vascular weight between the young SHR and WKY; other factors (e.g., an age factor, hormones, various growth factors derived from platelets or endothelial cells, or sympathetic innervation) may participate in the vascular hypertrophy in SHR.

In conjunction with these results, we tested our hypothesis that the enhanced vascular TXA2 generation might be responsible to some extent for the increased aortic wall thickness in SHR by using VSMCs of the aortic walls of SHR and WKY. In this study, we clearly demonstrated that VSMCs of SHR possessed increased proliferative activity, as compared with WKY. These results are consistent with the previous work of Yamori et al. Next, we demonstrated that the rapid VSMC growth of SHR was significantly tempered by OKY 046, a specific inhibitor of TXA2 synthase, although it was not brought back to the normal level of WKY. These results indicate that endogenous vascular TXA2 is partly responsible for the rapid VSMC growth in SHR and that, besides TXA2, other intrinsic factors such as alterations of membranous function are additionally involved in the rapid cell proliferation in SHR. Conversely, STA2, a stable analogue of TXA2, showed a stimulatory effect on VSMC proliferation in WKY, whereas it was less effective in SHR, the cell proliferation of which was assumed to be maximized through endogenous TXA2 generation by VSMCs. These results indicate that TXA2 potentiates the cell growth of vascular smooth muscle and that the enhanced endogenous TXA2 generation observed in VSMCs of SHR becomes a cause of the rapid VSMC growth in SHR.

The increase of vascular wall thickness contributes to the elevation of high blood pressure. Thus, enhanced TXA2 generation may participate in the development of hypertension in SHR through its effects on VSMC growth. Indeed, there is some evidence in vivo that TXA2 synthase inhibitors (e.g., OKY 046 or UK 38485) ameliorate the development of hypertension in SHR. Moreover, Purkerson et al. have reported protective effects of OKY 046 against sclerotic changes in renal arterioles of SHR. However, since there are some conflicting studies concerning the hypotensive action of UK 38485, any conclusions should be withheld until more evidence is accumulated.

The mechanisms for the stimulatory effect of TXA2 on VSMC growth remain to be elucidated. However, there are at least two possibilities. One is that TXA2 potentiates various humoral substances that participate in the regulatory mechanism for cell proliferation, such as catecholamines, steroids, platelet-derived growth factor, or insulin. The other possibility is a direct action of TXA2 on VSMC growth. TXA2 reportedly raises the intracellular Ca2+ concentration and triggers the calcium messenger system. Among the members of the calcium messenger system, protein kinase C has been assumed to promote cell proliferation. TXA2 may influence the activity of protein kinase C through the elevation of cytosolic Ca2+ concentration or by the activation of phosphoinositol-specific phosphodiesterase, which produces diacylglycerol, an activator of protein kinase C.

In this study, we employed VSMCs of the aortic wall and focused on hyperplasia of aortic smooth muscle cells. The aorta is a kind of conduit for the cardiovascular system, and there may be some distinctions in the biological properties of VSMCs from the aortic wall and those from the peripheral resistant vessels. Moreover, there is much evidence that hypertrophy of VSMCs is a main factor in the increase of vascular wall thickness in SHR. Hence, we may not be able to extrapolate our results to the levels of smaller arteries, which play a key role in the development of hypertension. Further studies are required to address these questions more directly.

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References

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