Studies of Hypertension-Induced Vascular Hypertrophy in Cultured Smooth Muscle Cells from Spontaneously Hypertensive Rats

Toshimi Kanbe, M.S., Yasuo Nara, Ph.D., Motoki Tagami, M.D., and Yukio Yamori, M.D.

SUMMARY Mechanisms of vascular hypertrophy induced by hypertension were studied in cultured aortic smooth muscle cells from spontaneously hypertensive rats (SHR) and stroke-prone SHR (SHRSP) and compared with those from normotensive Wistar-Kyoto (WKY) rats. Fetal calf serum-stimulated ornithine decarboxylase (ODC) activity of cultured smooth muscle cells was greater in SHR and SHRSP than in WKY. Beta- but not alpha-adrenergic agonist stimulated ODC activity acutely in cultured smooth muscle cells from WKY, and isoprenaline-induced activation was blocked by the beta-blocker, propranolol, and enhanced by the phosphodiesterase inhibitor, l-methyl-3-isobutylxanthine. These results indicate that cultured vascular smooth muscle cells from SHR and SHRSP are more prone to increase the protein synthesis than those from WKY through the trophic induction of ODC activity and that the regulation of ODC activity by catecholamines is mediated through beta-agonistic effect in cultured smooth muscle cells. (Hypertension 5: 887-892, 1983)

Key Words • catecholamine • cultured vascular smooth muscle cells • ornithine decarboxylase • stroke-prone spontaneously hypertensive rats • spontaneous hypertension

It is now established that functional as well as structural vascular changes are important in the pathogenesis of genetic hypertension. Medial hypertrophy of arterial walls is generally regarded as being secondary to blood pressure rise but involved in the further development and maintenance of hypertension. However, in spontaneously hypertensive rats (SHR), the thickening of the arterial walls is induced at the early stage or prehypertensive stage of hypertension. Furthermore, we found that vascular noncollagen and collagen protein synthesis was enhanced not only by the blood pressure rise itself but also by genetic predisposition and neural activation, and that vascular hypertrophy in SHR was prevented by the prolonged treatment of a low dose of beta-blocker which did not obviously affect blood pressure.

Under tissue culture condition free from the influence of blood pressure, smooth muscle cells from SHR and stroke-prone SHR (SHRSP) grew faster than those from normotensive Wistar-Kyoto (WKY) rats, and labelled thymidine or leucine incorporation was also significantly increased. In the present study, to determine whether vascular smooth muscle cells from SHR and SHRSP are genetically predisposed to hypertrophy, we measured the activity of ornithine decarboxylase (ODC), which is the rate-limiting enzyme for polyamine biosynthesis and thus regarded as an indicator of cellular hypertrophy or hyperplasia. Furthermore, because we had found that sympathetic innervation was important in the increased synthesis of vascular protein during the early hypertensive phase in SHR, acute effects of alpha- and beta-adrenergic agonists or antagonists on the ODC activity were studied in cultured smooth muscle cells.

Methods

Isolation and Culture of Rat Aortic Smooth Muscle Cells

Cultured smooth muscle cells were obtained by an explant method from the thoracic aortas of age-matched, 3- to 6 month-old SHR, SHRSP, and WKY. The media was newly removed, and the explants, which were cut off approximately into 1 mm² sections, were transferred into tissue culture flasks (Falcon) to which 5 ml of modified medium 199 supplemented with 10% fetal calf serum (GIBCO) was added carefully to avoid the detachment of explants from the flasks.
These flasks were incubated at 37°C in a humidified incubator (95% air/5% CO2 atmosphere). Cells migrating from these explants after 30 days of incubation were trypsinized and subcultured by repeated passage. The medium was changed every 4 days, and confluent cultured smooth muscle cells (passage 3–9) were used for the following experiments.

**Observation of Smooth Muscle Cells in Culture**

Smooth muscle cells were examined with phase contrast microscopy (Nikon DIAPHOT-TMD, Tokyo, Japan). For electron microscopy, cells grown on films were fixed by 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) at room temperature. After osmication by 2% osmium tetroxide for 2 hours, the cells were stained with 2% uranyl acetate in 50% ethanol for 1 hour at 4°C, dehydrated in graded ethanol, and embedded in Epon 812. Thin sections were cut on a Sorvall Porter-Blum, MT-2B ultratome (Sorvall Porter-Blum, Inc., Newtown, Connecticut), and stained with lead citrate. These sections were examined by a transmission electron microscope (Hitachi HS-9, Tokyo, Japan).

**Measurement of Ornithine Decarboxylase Activity in Cultured Smooth Muscle Cells**

The assay of ODC was carried out as described by Bachrach.16 Fetal calf serum (10%) or catecholamines (2 μg/ml) with or without beta-adrenergic blocker (5 μg/ml) or l-methyl-3-isobutylxanthine (0.3 mM) were added to the cultured smooth muscle cells which had been placed in fetal calf serum-free medium for 16–18 hours. At selected time intervals the cells were washed with phosphate-buffered saline and suspended in a solution containing 5 mM dithiothreitol, 0.2 mM pyridoxal phosphate, 4 mM EDTA, and 50 mM Tris-HCl (pH 7.4). After sonication and centrifugation, the supernatant fraction was assayed for ODC activity by the method of Lowry et al.17 using bovine serum albumin as the standard. ODC activity was calculated as pmol of 14CO2 produced/mg protein hr−1 incubation at 37°C. Student’s t test was applied for statistical analysis.

**Results**

**Observation of Smooth Muscle Cells in Culture**

Smooth muscle cells that migrated from explants were of ribbon or spindle shape with an oval- or sausage-shaped nucleus. Ultrastructurally, cells in primary culture had numerous myofilaments (fig. 1), and there was no apparent difference between the ultrastructure of cultured smooth muscle cells from SHR and SHRSP and those from WKY.

**Fetal Calf Serum-Stimulated ODC Activity in Cultured Aortic Smooth Muscle Cells from SHR, SHRSP, and WKY**

The time course of ODC activation by fetal calf serum is shown in figure 2. Maximum enzyme activity occurred approximately 5 hours after the addition of 10% fetal calf serum with a 30- to 40-fold augmentation of activity above control values. ODC activity assayed under this condition was 1.5 and 1.7 times greater in smooth muscle cells from SHR and SHRSP, respectively, than those from WKY (fig. 3).

**Effects of Catecholamines on the ODC Activities of Cultured Aortic Smooth Muscle Cells from WKY**

The time course of ODC activation by the addition of catecholamines to smooth muscle cells is shown in figure 4. After 1 hour, the ODC activity was slightly higher in cultured smooth muscle cells given isoprenaline. The peak rise in ODC activity occurred approximately 2 to 3 hours after the application of isoprenaline and was about 3 times greater than the control values. The enzyme activity then decreased but was still higher than the control 5 hours after the application. The control enzyme activity did not change over this period. On the other hand, the ODC activity of cultured smooth muscle cells was only slightly increased after the application of norepinephrine and not affected by phenylephrine.

**Effects of Beta-Adrenergic Antagonist on ODC Activity of Cultured Smooth Muscle Cells**

The effect of propranolol and the combined effect of propranolol and catecholamines are demonstrated in table 1. The Beta-adrenergic antagonist, propranolol, (5μg/ml), given 30 minutes before catecholamine, completely blocked isoprenaline activation 3 hours after the exposure, but did not affect basal enzyme activity.

<table>
<thead>
<tr>
<th>Agent</th>
<th>ODC activity</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3.82 ± 0.42</td>
<td></td>
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<tr>
<td>Isoprenaline</td>
<td>10.94 ± 2.15</td>
<td>p &lt; 0.01 vs control</td>
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<tr>
<td>Norepinephrine</td>
<td>5.83 ± 1.21</td>
<td>ns vs control</td>
</tr>
<tr>
<td>Phenylephrine</td>
<td>3.86 ± 0.94</td>
<td>ns vs control</td>
</tr>
<tr>
<td>Propranolol</td>
<td>3.73 ± 0.57</td>
<td>ns vs control</td>
</tr>
<tr>
<td>Isoprenaline +</td>
<td>5.36 ± 0.53</td>
<td>p &lt; 0.05 vs isoprenaline</td>
</tr>
<tr>
<td>propranolol</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Norepinephrine +</td>
<td>4.33 ± 0.85</td>
<td>ns vs norepinephrine</td>
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</table>

ODC activity (14CO2 pmol per mg protein/hr) was assayed 3 hours after cultured smooth muscle cells were incubated with isoprenaline (2 μg/ml), norepinephrine (2 μg/ml), or phenylephrine (2 μg/ml). Propranolol (5 μg/ml) was given 30 minutes before the addition of sympathetic agonists. Results are means ± se of six experiments.
FIGURE 1. Transmission electron micrographs of smooth muscle cells from SHRSP in primary culture. A. Ultrastructure reveals parallel layering of myofilaments (arrows). Cytoplasm contains lysosome (Lys), mitochondria (mit), and rough endoplasmic reticulum. B. Ultrastructure of cells with myofilaments (arrows) and nucleus (N). × 48,000
FIGURE 2. Time course of ODC activity in cultured smooth muscle cells from SHR, SHRSP, and WKY after a treatment with 10% fetal calf serum (FCS). Values are means of duplicate determination.

TABLE 2. Effect of 1-Methyl-3-isobutylxanthine (MIX) and Dibutyryl Cyclic AMP (DB-cAMP) on Basal and Isoprenaline-Stimulated ODC Activity of Cultured Smooth Muscle Cells from WKY

<table>
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<th>Agent</th>
<th>ODC activity</th>
<th>Comments</th>
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<td>Control</td>
<td>3.08 ± 0.44</td>
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<tr>
<td>Isoprenaline</td>
<td>17.82 ± 0.65</td>
<td>p &lt; 0.001 vs control</td>
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<tr>
<td>MIX</td>
<td>2.85 ± 0.68</td>
<td>ns vs control</td>
</tr>
<tr>
<td>DB-cAMP</td>
<td>5.68 ± 1.30</td>
<td>ns vs control</td>
</tr>
<tr>
<td>Isoprenaline + MIX</td>
<td>31.71 ± 2.39</td>
<td>p &lt; 0.001 vs isoprenaline</td>
</tr>
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</table>

ODC activity ((14)CO2 pmol per mg protein/hr) was assayed 3 hours after cultured smooth muscle cells were incubated with isoprenaline (2 μg/ml), MIX (0.3 mM), and dibutyryl cyclic AMP (0.5 mM). Results are means ± SE of four experiments.

Effect of Phosphodiesterase Inhibitor and a Cyclic AMP Analog

The phosphodiesterase inhibitor, 1-methyl-3-isobutylxanthine (0.3 mM), significantly augmented isoprenaline activation of ODC activity, although it did not affect basal ODC activity (table 2). The cyclic AMP analog, dibutyryl cyclic AMP (0.5 mM), slightly increased ODC activity.

Discussion

Medial hypertrophy of blood vessels is the main structural change induced by hypertension and is generally regarded as being secondary to hypertension. However, the early development of structural changes in spontaneous hypertension suggests that such changes are enhanced by the blood pressure rise itself and also by other factors such as genetic predisposition and the trophic effect of neural innervation. Under tissue culture condition free from the influence of blood pressure, smooth muscle cells from SHR and SHRSP grew faster than those from normotensive WKY, and labelled thymidine or leucine incorporation was also significantly increased in cultured smooth muscle cells from SHR and SHRSP.

Cultured vascular smooth muscle cells grown from arterial explants usually lost their ability to contract or relax and were therefore not used for functional studies. In spite of such a limitation, they have been used as a model for cell proliferation following arterial injury and biochemical properties associated with such. Since increased growth rate, DNA, or protein synthesis of cultured smooth muscle cells in vitro from hypertensive rats compared with those from normotensive rats corresponded well to early development of vascular hypertrophy observed in vivo, it is suggested that cultured smooth muscle cells are proper materials for studying the biochemical properties related to cell proliferation and the influence of catecholamines on such properties.

Since ODC activity is regarded as a sensitive indicator of protein synthesis, this enzyme activation induced by fetal calf serum application was measured in cultured smooth muscle cells. Fetal calf serum-stimu-
vascular hypertrophy in spontaneously hypertensive rats/Kanbe et al.

Isoxsuprine, phenylephrine, and norepinephrine were used to study the effect of catecholamines with or without beta-blocker on ODC activity in cultured smooth muscle cells. Isoprenaline but not phenylephrine stimulated ODC activity in cultured smooth muscle cells similarly to its effect in rat myocardial cells in vivo. Furthermore, since accumulating studies using SHR in vivo indicate the important role of neural activation of protein synthesis in vascular walls, the effect of catecholamines with or without beta-blocker on the ODC activity of cultured smooth muscle cells was investigated. Isoprenaline but not phenylephrine stimulated ODC activity significantly in smooth muscle cells from WKY. These results demonstrated that beta-adrenoceptor stimulation activated protein synthesis of cultured vascular smooth muscle cells similarly to its activation of ODC activity in rat myocardial cells in vivo. In contrast to our findings under tissue culture conditions free from the effect of blood pressure, Thadan et al. reported that the sympathetogenic regulation of ODC activity levels is mediated through alpha-receptor in the rat aorta in vivo. ODC activation by alpha-stimulation in vivo may be due to blood pressure elevation. Norepinephrine slightly stimulated ODC activity, suggesting that norepinephrine-induced ODC activation may be partially mediated by beta-adrenoceptor stimulation. Furthermore, beta-blockers may completely blocked the isoprenaline-induced ODC activation, suggesting that beta-adrenoergic activity may modulate vascular structural changes in hypertension. The augmentation of isoprenaline-induced ODC activation by a phosphodiesterase inhibitor further indicates that the cyclic AMP system is involved in the activation of protein synthesis by beta-adrenoceptor stimulation. Increased circulating epinephrine levels parallel cardiac hypertrophy, and the denervation of the adrenal medulla totally prevents cardiac hypertrophy after aortic coarctation in the dog. Moreover, Baker et al. reported that circulating levels of epinephrine, but not norepinephrine, achieve Kd values sufficient for cardiac beta-receptor stimulation. This is further supportive evidence that epinephrine but not norepinephrine released from the adrenal medulla as a circulating hormone may be responsible for the medial cell hypertrophy of vasculatures in hypertension in addition to raised blood pressure itself. Copeland et al. reported that beta-adrenoceptors were selectively coupled to the regulation of murine cardiac ODC activity following catecholamine simulation. It is therefore interesting to know whether beta- or beta-adrenoceptors are coupled to the induction of ODC activity in cultured smooth muscle cells. Further studies on the effects of beta- and beta-agonists and antagonists on ODC activity in cultured vascular smooth muscle cells are in progress.

We may conclude that the acceleration of vascular protein synthesis in genetic hypertension is induced not only by the blood pressure level itself but also by other factors such as genetic predisposition and increased sympathetic tone through beta mechanisms of catecholamines from the adrenal medulla and/or nerve endings, and results in an increase in peripheral vascular resistance. Thus, beta blockers may be important for reducing and preventing cardiovascular structural changes in hypertension.

References


FIGURE 4. Time course of ODC activity in cultured smooth muscle cells from WKY after a treatment with catecholamines. Cultured smooth muscle cells were incubated with isoprenaline (2 µg/ml), norepinephrine (noradrenaline) (2 µg/ml) or phenylephrine (2 µg/ml) for varying intervals and then harvested for subsequent determination of ODC activity. Values are means of duplicate determination.
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