Radical Scavengers of Indapamide in Prostacyclin Synthesis in Rat Smooth Muscle Cell

Yoshio Uehara, Hiroaki Shirahase, Taiji Nagata, Toshihiko Ishimitsu, Shigeyoshi Morishita, Seimei Osumi, Hiroaki Matsuoka, and Tsuneaki Sugimoto

Indapamide, a nonthiazide diuretic, exhibits direct vasodilator action as well as natriuretic and diuretic effects. Although calcium antagonist-like activity has been addressed so far, the mechanisms for vasodilator effect are still uncertain. To understand the wide range of indapamide actions, we examined the effects of indapamide on the vascular eicosanoid generation and investigated its mechanisms by using rat vascular smooth muscle cells in culture. Indapamide uniquely increased the prostacyclin generation in the vascular smooth muscle cells in a dose-dependent manner, whereas it did not affect the vasoconstrictor thromboxane A2. Thiazide diuretics lowered the prostacyclin generation, while nonthiazide derivatives did not affect the biosynthesis. Enzymatic analysis revealed that indapamide affected neither \[^{14}C\]arachidonate liberation nor prostacyclin synthase of the smooth muscle cells. Indapamide eliminated a stable free radical in a cell-free system, lowered the formation of malondialdehyde from lipid peroxides in rat brain homogenate, and reduced lipid peroxidation by the free radical generating system of xanthine-xanthine oxidase. Indeed, the scavenging action of indapamide significantly attenuated the inhibitory activity of 15-hydroperoxy-arachidonate to prostacyclin synthase activity. These results indicate that indapamide diuretic increases prostacyclin generation in the vascular smooth muscle cells possibly through antioxidant effects and that the enhanced prostacyclin generation is partly responsible for its direct vasodilator action. (Hypertension 1990;15:216–224)

A n-o-chlorobenzenesulfonamide molecule is a prototype of various diuretics including thiazide and nonthiazide diuretics (Figure 1). Although natriuretic and diuretic effects are mainly due to the common structure of o-chlorobenzenesulfonamide, varied side chains give the diuretics characteristic properties. In this context, indapamide diuretic comprises an indoline molecule and uniquely exhibits a direct vasodilator action as well as a natriuretic effect on the cortical collecting duct in the kidney.1–4 The vasodilator effect is assumed to be caused by its calcium channel blocker–like activity4 or endothelium-derived relaxing factor–mediated mechanism.6 However, the exact mechanism is not fully understood.

On the other hand, the biosynthetic system for eicosanoid (prostaglandins and thromboxanes) is well demonstrated in the vascular smooth muscle cells (VSMC) of rats.7,8 Prostacyclin (PGI2), a major product of arachidonate metabolism in VSMC, has a powerful vasodilator action and attenuates the vasoconstrictor response to vasoactive substances.9 The eicosanoid metabolism is a major component participating in the regulation of the constriction-relaxation mechanism of VSMC.

To understand the wide range of indapamide effects, it seems important to disclose the relevance of the eicosanoid system in the vascular wall to the extrarenal indapamide effects. Thus in this study, we investigated the influences of indapamide on the eicosanoid generation in VSMC by using cultured VSMC of rats and attempted to define the mechanisms for its alteration.

Methods

Vascular Smooth Muscle Cell Culture

VSMC were isolated from the thoracic aortas of 7-week-old Wistar rats according to the method of...
Ross et al. Briefly, rats were decapitated without anesthesia. The descending aortas were immediately obtained, and the surrounding connective tissue was carefully removed with forceps and gauze. Then, explants of ~5 mm² were placed on a 94x21 mm polystyrene dish (Corning-Iwaki Glass Co., Tokyo, Japan) with the intimal side attached to the dish. Ten milliliters Dulbecco's modified Eagle medium (DMEM) (GIBCO Labs., Grand Island, New York), supplemented with 20% (vol/vol) fetal bovine serum (FBS) (GIBCO Labs.), penicillin (100 units/ml), and streptomycin (100 μg/ml), was gently added. The dishes were kept at 37°C in a humidified atmosphere of 95% air and 5% CO₂. The cells reached confluency 10 days after inoculation. They were harvested by brief exposure to Hanks' medium, supplemented with 0.05% (wt/vol) trypsin and 0.02% (wt/vol) ethylenediaminetetraacetic acid disodium, and transferred into a new dish. At this stage, the cells were designated as the first passage. FBS was reduced to 10% (vol/vol) in the subsequent media. Harvesting was repeated when the cell growth became confluent. VSMC from the fourth to the sixth passage were used in the following studies.

Figure 2A shows the appearance of the cells in primary culture and in the sixth generation (Figure 2B) on a phase contrast microscope (model IMT Olympus microscope, Olympus Optical Co., Tokyo, Japan). The cultured VSMC were characterized by the "hills and valleys" growth pattern on a light microscope, and the occurrence of cytoplasmic myofilaments was confirmed by electron microscopy. Actin molecules were demonstrated by antiantin monoclonal antibody (Amersham International plc, Buckinghamshire, England), antimuscle actin immunoglobulin G (IgG) (Biomed. Technol. Inc., Stoughton, Massachusetts), and peroxidase-labeled anti-immunoglobulin M (IgM) or anti-IgG antibody (Kirkegaard & Perry Labs., Inc., Gaithersburg, Maryland).

Effects of Diuretics on Eicosanoid Generation in Vascular Smooth Muscle Cells

The confluent VSMC in the 35x18 mm six-well polystyrene dish were washed three times with FBS-free Dulbecco's balanced phosphate-buffered saline solution (D-PBS) (GIBCO Labs.). Then, the VSMC were continuously cultured for 1 hour in D-PBS with 10⁻⁶ M arachidonic acid and a given concentration of various diuretics. Eicosanoid released in a D-PBS medium was measured by direct radioimmunoassay. The cells were detached with brief exposure to 0.05% trypsin solution and washed repeatedly with D-PBS. The cells were homogenized with a Polytron blender (Kinematica GmbH, Littau, Switzerland), and the protein content was measured by the method of Lowry et al.

Direct radioimmunoassay was performed according to the previous method. Briefly, the assay mixture consisted of 0.1 ml [³H]eicosanoid (10⁻⁴ disintegrations per minute), 0.1 ml diluted sample or standard solution, and 0.1 ml diluted antibody solution. The assay mixture was incubated at 4°C for 24 hours. To separate the bound from the free [³H]eicosanoid, 0.1 ml dextran-coated charcoal solution (2.5% charcoal and 0.25% dextran) was added, and the mixture was immediately centrifuged at 1,000g at 4°C for 5 minutes. Radioactivity of the bound [³H]eicosanoid was counted with an automatic liquid scintillation counter.
Anti-6-keto-prostaglandin F$_{1\alpha}$ (6-k-PGF$_{1\alpha}$) serum cross-reacted 1% with prostaglandin E$_2$ (PGE$_2$), 2% with prostaglandin F$_{2\alpha}$ (PGF$_{2\alpha}$), 0.08% with prostaglandin D$_2$ (PGD$_2$), 0.01% with thromboxane B$_2$, and less than 0.001% with arachidonate. Anti-thromboxane B$_2$ serum cross-reacted 0.005% with 6-k-PGF$_{1\alpha}$, 0.007% with PGE$_2$, 0.018% with PGF$_{2\alpha}$, 0.06% with PGD$_2$, and less than 0.0001% with arachidonate.

**Effects of Diuretics on Liberation of [14C]Arachidonate From Vascular Smooth Muscle Cells**

Cells (10$^5$) were seeded onto a 35×18 mm six-well polystyrene dish with 2 ml DMEM containing 10% (vol/vol) FBS. When the VSMC became confluent, the medium was reduced to 2 ml DMEM with 5% (vol/vol) FBS and 0.5 μCi [14C]arachidonate (50-60 mCi/mmol) and maintained for 1 hour to incorporate [14C]arachidonate into the cellular membrane of the VSMC. The VSMC labeled with [14C]arachidonate were repeatedly washed with D-PBS supplemented with 10$^{-5}$ M indomethacin. The cells were cultured in D-PBS with 10$^{-5}$ M indomethacin and a given concentration of indapamide (10$^{-8}$ to 10$^{-4}$ M), bradykinin (10$^{-8}$ to 10$^{-3}$ M) (Peptide Institute, Inc., Osaka, Japan), or calcium ionophore A23187 (10$^{-9}$ to 10$^{-5}$ M) (Boehringer Mannheim GmbH, Mannheim, FRG). [14C]Arachidonate released in the medium for 1 hour was extracted with Folch's solvent (chloroform : methanol, 2:1, vol:vol) and purified with silicic acid thin layer chromatography. The radioactivity in the zone corresponding to arachidonate was counted by an automatic scintillation counter. The recovery rate of this extraction method averaged 96±1% (n=6).

Protein content of the cells was measured with the same procedure as described previously.

**Effects of Diuretics on PGI$_2$ Synthase Activity**

PGI$_2$ synthase activity was determined with use of a modified method of Salmon et al. Briefly, 50 μl of the microsomal fraction (10$^5$ g pellet) of VSMC, enriched with PGI$_2$ synthase, was preincubated in 50 μl 25 mM Tris (hydroxymethyl)aminomethane HCl (Tris-Cl) (Sigma Chemical Co., St. Louis, Missouri) containing 100 mM NaCl and a given concentration of diuretic at 4°C for 15 minutes (pH 7.5). The reaction was initiated at 37°C by addition of 10 μl prostaglandin H$_2$ (PGH$_2$) (0.5 μg in acetone) (Ono Pharmaceutical Co., Ltd, Osaka, Japan) and terminated with 50 μl of 50 mM FeCl$_3$ solution after 3 minutes. The generated PGI$_2$ was measured by the radioimmunoassay as 6-k-PGF$_{1\alpha}$. To measure the nonspecific conversion of PGH$_2$ to PGI$_2$, we measured the PGI$_2$ generation by boiled enzymatic fraction. The nonspecific conversion was less than 1%. Moreover, the amount of PGI$_2$ generated by the microsomal fraction was negligible when the substrate PGH$_2$ was not added to the assay mixture.

**Measurements of Scavenging Effects**

The antioxidant activity of indapamide and some diuretics, or α-tocopherol, was assessed by using a stable free radical, α,α-diphenyl-β-picrylhydrazyl (DPPH) (Wako Pure Chemical Industries, Osaka, Japan), according to the method of Blois. The reduction of 10$^{-4}$ M DPPH was initiated by addition of a given concentration of the test compound. The conversion of DPPH to its reduced form was recorded at 25°C for 30 minutes by a Hitachi Model U-3200 spectrophotometer (Hitachi, Ltd, Tokyo, Japan) at 517 nm wavelength.
The antioxidant activity of indapamide was assessed by an inhibitory activity to the formation of malondialdehyde (MDA) in rat brain homogenate, which according to Kubo et al., is an end product of lipid peroxides. In brief, the brains from 7-week-old male Wistar rats (Kitayama LABES Co., Kyoto, Japan) were isolated after the rats were decapitated. The cortical tissues were homogenized in ice-chilled 50 mM phosphate-buffered saline solution at pH 7.4 using an Ultra-Disperser (Yamato Scientific Co., Ltd, Tokyo, Japan). The homogenate was centrifuged at 1,300g for 10 minutes at 4°C. The supernatant was diluted with the buffer to 2 mg/ml for the protein concentration. This prepared supernatant was incubated at 37°C for 30 minutes with a given concentration of diuretics or α-tocopherol. The reaction was terminated by addition of 20% trichloroacetic acid (1:1, vol:vol), which was spun again at 1,200g for 5 minutes at 4°C. The supernatant was added to the ice-chilled mixture was treated at 100°C for 10 minutes. Absolut eα-butanol was added to the ice-chilled mixture (1:1, vol:vol), which was spun again at 1,200g for 5 minutes at 4°C. The MDA in the organic phase was measured at 532 nm by the spectrophotometer. MDA in a medium before the assay was determined so that the net MDA production during the reaction could be measured. Protein concentration was measured according to the method of Lowry et al.

We also explored the antioxidant effects of indapamide using the xanthine-xanthine oxidase free radical generating system. A given concentration of the test compound in 15 μl was mixed with 1.5 ml 50 mM linolenic acid (Sigma Chemical Co.) and 0.15 ml 0.5 mM xanthine (Sigma Chemical Co.) in 10 mM phosphate-buffered solution including 1% sodium dodecyl sulfate at pH 7.4. The reaction mixture was spun at 37°C for 30 minutes with 0.15 ml (5 units/ml) xanthine oxidase (Sigma Chemical Co.) and terminated with 0.4 ml 0.12 M thiobarbituric acid solution. The resulting mixture was mixed with 0.2% butylated hydroxytoluene and 0.02 M thiobarbituric acid solution. The mixture was heated at 100°C for 15 minutes in a mixture of 50 μl of 25 mM Tris-Cl buffered solution with 100 mM NaCl and 15-HPETE of 25 mM Tris-Cl buffered solution with 100 mM NaCl and 15-HPETE (0-2,000 ng/ml) in the absence or presence of 5x10^{-5} M diuretics. PGI2 synthase activity was determined according to the procedure described in this section.

Reagents

The reagents were all of analytical grade. Authentic eicosanoids and related compounds were a gift from Ono Pharmaceutical Co., Ltd (Osaka, Japan). Mefuroside was supplied by Yoshitomi Pharmaceutical Co., Ltd (Osaka, Japan); furosemide, trichloromethiazide, and hydrochlorothiazide were obtained from Sigma Chemical Co. (St. Louis, Missouri), and radioactive tritiated water was purchased from Amersham International PLC, Buckinghamshire, UK.

The cortical tissues were homogenized in ice-chilled 50 mM phosphate-buffered saline solution at pH 7.4 using an Ultra-Disperser (Yamato Scientific Co., Ltd, Tokyo, Japan). The homogenate was centrifuged at 1,300g for 10 minutes at 4°C. The supernatant was added to the ice-chilled mixture (1:1, vol:vol), which was spun again at 1,200g for 5 minutes at 4°C. The MDA in the organic phase was measured with a spectrophotometer at 532 nm. The nonspecific oxidation in this assay system was checked under the experimental condition without xanthine-xanthine oxidase. Moreover, we confirmed that indapamide did not interfere with the xanthine-xanthine oxidase reaction by measuring the formation of uric acid in the assay system by a modified colorimetric method with the uricase-catalase system of Kageyama (Uric Acid C-Test, Wako Pure Chemical Industries, Ltd., Osaka, Japan).

Finally, we attempted to examine the effects of diuretics on the inhibitory activity of 15-hydroperoxyeicosatetraenoic acid (15-HPETE) to PGI2 synthase. Enzymatic fraction (50 μl) was preincubated at 4°C for 15 minutes in a mixture of 50 μl of 25 mM Tris-Cl buffered solution with 100 mM NaCl and 15-HPETE (0-2,000 ng/ml) in the absence or presence of 5x10^{-5} M diuretics. PGI2 synthase activity was determined according to the procedure described in this section.

### Table 1. Effects of Indapamide on Vascular Eicosanoid Generation

<table>
<thead>
<tr>
<th>Concentration (M) (n=5)</th>
<th>TXA2 (n=5)</th>
<th>PGE2 (n=5)</th>
<th>PGF2α (n=5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.129±0.008</td>
<td>0.173±0.079</td>
<td>0.320±0.088</td>
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<tr>
<td>10^{-9}</td>
<td>0.129±0.010</td>
<td>0.446±0.203</td>
<td>0.331±0.087</td>
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<tr>
<td>10^{-7}</td>
<td>0.128±0.014</td>
<td>0.787±0.341</td>
<td>0.127±0.035</td>
</tr>
<tr>
<td>10^{-5}</td>
<td>0.128±0.021</td>
<td>0.873±0.298*</td>
<td>0.332±0.083</td>
</tr>
</tbody>
</table>

Values are expressed as nanograms per milligrams protein per hour. n, number of rats; TXA2, thromboxane A2; PGE2, prostaglandin E2; PGF2α, prostaglandin F2α.

*p<0.1 versus control (0 M) value.
TABLE 2. Effects of Thiazide Diuretics on Vascular Eicosanoid Generation

<table>
<thead>
<tr>
<th>Concentration (M)</th>
<th>Hydrochlorothiazide</th>
<th>Trichloromethiazide</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PG12 (n=5)</td>
<td>TXA2 (n=5)</td>
</tr>
<tr>
<td>0</td>
<td>1.20±0.04</td>
<td>0.14±0.02</td>
</tr>
<tr>
<td>10⁻⁹</td>
<td>1.19±0.08</td>
<td>0.16±0.04</td>
</tr>
<tr>
<td>10⁻⁷</td>
<td>1.11±0.12</td>
<td>0.15±0.02</td>
</tr>
<tr>
<td>10⁻⁵</td>
<td>0.98±0.08*</td>
<td>0.15±0.03</td>
</tr>
</tbody>
</table>

*p<0.05 versus respective control (0 M) values.

Values are expressed as nanograms per milligram protein per hour. Dose-dependency is assessed by one-way analysis of variance. NS, statistically not significant; n, number of rats; PG12, prostaglandin I2; TXA2, thromboxane A2.

Statistical Analysis

The values were expressed as mean±SEM. The difference was assessed by the Student's t test or one-way analysis of variance.

Results

The direct effect of indapamide, a nonthiazide diuretic, on the vasodilator PG12 generation in VSMC is shown in Figure 3. The PG12 production was significantly stimulated with the indapamide diuretic in a dose-dependent manner, increasing by 54% at a concentration of 10⁻⁸ M when compared with the control value. In contrast, the diuretic did not influence thromboxane biosynthesis in the VSMC (Table 1). The VSMC had a small capacity in the generation of PGE2 and PGF2α. Indapamide tended to increase vasodilator PGE2 generation in VSMC, although we could not find a significant dose-dependency. PGF2α generation was not altered by the diuretic (Table 1).

The PG12 stimulatory effect was uniquely observed in indapamide alone. As shown in Table 2, the thiazide diuretics, that is, trichloromethiazide and hydrochlorothiazide, produced a decrease in PG12 generation, whereas the thromboxane generation was not altered. The effects on PG12 generation varied among the nonthiazide diuretics. Mefruisde, a sulfonyl group diuretic (Figure 1), did not influence the PG12 biosynthesis in the VSMC (Table 3). Although furosemide is classified as an aminocarbonyl group, which is structurally related to indapamide (Figure 1), it did not affect the PG12 generation (Table 3). Furosemide exhibited a significant increase in thromboxane A2 production when tested at a higher concentration.

To explore the mechanisms for the unique PG12 stimulatory effect of indapamide, we analyzed enzymatically the eicosanoid biosynthesis. First, we investigated the liberation of [¹⁴C]arachidonate from the cellular membrane of VSMC, into which the radioactive arachidonate had been incorporated. In comparison, bradykinin and the calcium ionophore A23187, both of which are the stimulators of phospholipase A2, were also examined. As shown in Figure 4, the control compounds were found to stimulate, in a dose-dependent manner, the [¹⁴C]arachidonate liberation in this assay system. However, the indapamide diuretic did not produce a significant change in the arachidonate liberation.

Second, we examined the direct effect of indapamide on PG12 synthase in the microsomal fraction of the VSMC (Figure 5). The PG12 synthase activity was not influenced by indapamide at concentrations ranging from 10⁻⁹ to 10⁻⁴ M. Furosemide, the control compound, also did not affect the PG12 synthase activity.

Next, we explored the antioxidant effects of indapamide on the eicosanoid generation. Indapamide diuretic significantly promoted the reduction of a stable free radical, a,a-diphenyl-β-picrylhydrazyl, in a dose-dependent manner (Figure 6). However, neither trichloromethiazide nor furosemide facilitated the reduction.

TABLE 3. Effects on Nonthiazide Diuretics on Vascular Eicosanoid Generation

<table>
<thead>
<tr>
<th>Concentration (M)</th>
<th>Mefruisde (n=8)</th>
<th>Furosemide (n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PG12</td>
<td>TXA2</td>
</tr>
<tr>
<td>0</td>
<td>1.20±0.07</td>
<td>0.16±0.02</td>
</tr>
<tr>
<td>10⁻⁹</td>
<td>1.12±0.06</td>
<td>0.14±0.01</td>
</tr>
<tr>
<td>10⁻⁷</td>
<td>1.21±0.10</td>
<td>0.15±0.01</td>
</tr>
<tr>
<td>10⁻⁵</td>
<td>1.33±0.07</td>
<td>0.20±0.03</td>
</tr>
</tbody>
</table>

*p<0.001 versus control value (0 M).

Values are expressed as nanograms per milligram protein per hour. Dose-dependency is assessed by one-way analysis of variance. NS, statistically not significant; n, number of rats; PG12, prostaglandin I2; TXA2, thromboxane A2.
We examined the indapamide effects on MDA formation in the rat brain homogenate (Figure 7). Indapamide was found to markedly reduce the MDA formation in the rat brain homogenate. Moreover, the dose for equipotency of the scavenging effect was significantly smaller in indapamide than a conventional scavenger such as α-tocopherol. The thiazide diuretic and furosemide did not exhibit the antioxidant effects on the MDA formation in the homogenate.

To ascertain the possible scavenging property of indapamide, we examined its effects on the peroxidation of linolenic acid by the free radical generating system of xanthine-xanthine oxidase. We found that this system caused the peroxidation of linolenic acid, which was inhibited 37% with superoxide dismutase coupled with catalase (4.505±0.155 vs. 2.820±0.232 nmol MDA/ml/10 min, p<0.005). By using this assay system, we assessed the scavenging effects of indapamide. As depicted in Figure 8, indapamide reduced the formation of MDA in a dose-dependent manner, which became 29% of the control value at 10⁻⁴ M. In contrast, neither trichloromethiazide nor furosemide affected the formation.

In this context, we examined whether the compound could attenuate the inhibitory activity of free radicals to PGI₂ synthase (Figure 9). As shown in the open circles, 15-HPETE, a free radical provider and potent inhibitor to PGI₂ synthase, suppressed the PGI₂ synthase activity in a dose-dependent manner. However, when 5 x 10⁻⁵ M indapamide occurred in the assay mixture, the inhibitory activity of 15-HPETE was significantly lowered, thereby shifting the dose-response curve toward the right. Furosemide did not produce any alteration in the dose-response curve.

FIGURE 6. Plot showing antioxidant effects of indapamide on α,α-diphenyl-β-picrylhydrazyl. Circles, squares, and triangles represent indapamide (IDP), trichloromethiazide (TCMT), and furosemide (FRS), respectively. Value is an average of four studies. Dose-dependency is analyzed by one-way analysis of variance. F values are 5.524 for indapamide (p<0.001), 1.00 for trichloromethiazide (p>0.1) and 2.49 for furosemide (p>0.1).

FIGURE 7. Plot showing effects of indapamide on malondialdehyde formation in rat brain homogenate. •, □, △ and × represent indapamide (IDP), trichloromethiazide (TCMT), furosemide (FRS), and α-tocopherol (α-TOCO), respectively. Value is an average of four studies. Dose-dependency is analyzed by one-way analysis of variance. F values are 240 for indapamide (p<0.001), 4.63 for α-tocopherol (p<0.01), 0.02 for trichloromethiazide (p>0.1), and 0.96 for furosemide (p>0.1).

FIGURE 5. Plot showing effects of indapamide on PG I₂ synthase activity. Circles and triangles represent indapamide (IDP) and furosemide (FRS), respectively. Value is an average of six studies. Dose-dependency is analyzed by one-way analysis of variance. F values are 0.46 for indapamide (p>0.1) and 0.35 for furosemide (p>0.1).

FIGURE 4. Plot showing effects of indapamide on arachidonate liberation from vascular smooth muscle cells (VSMC). •, □, ○, △, and × represent indapamide (IDP) diuretic; ○, △ represent bradykinin (BK) and calcium ionophore (A23187), respectively. Value is an average of six rats. [¹⁴C]Arachidonate incorporated into membrane of VSMC for 1 hour averaged 15,449±423 (n=6) dpm/10⁴ cells/hr. Dose-dependency is analyzed by one-way analysis of variance. F values are 0.887 for indapamide (p>0.1), 4.22 for bradykinin (p<0.02), and 5.29 for calcium ionophore (p<0.005). *p<0.05; *+p<0.025 versus respective control values (0 M).

FIGURE 8. Plot showing antioxidant effects of indapamide on malondialdehyde formation in rat brain homogenate. Circles, squares, and triangles represent indapamide (IDP), trichloromethiazide (TCMT), and furosemide (FRS), respectively. Value is an average of six studies. Dose-dependency is analyzed by one-way analysis of variance. F values are 5.524 for indapamide (p<0.001), 1.00 for trichloromethiazide (p>0.1) and 2.49 for furosemide (p>0.1).
Discussion

The VSMC were primarily cultured and maintained according to the standard method of Ross et al. The cells were characterized by various properties of growth pattern, which are characteristic of vascular smooth muscle cells. Moreover, the origin of vascular smooth muscle was confirmed as well by light and electron microscopy and immunohistochemical findings.

In this study, we demonstrated that indapamide was a potent stimulator of vasodilator PGI2 generation in VSMC. Indapamide did not affect the vasoconstrictor thromboxane A2, thereby shifting the PGI2-to-thromboxane A2 ratio toward a vasodilator state. The enhanced PGE2 generation, although a small amount, is likely to produce vasodilation as well. The direct vasodilator action of indapamide is reportedly observed at the relatively higher concentrations, that is, 10^-3 to 10^-4 M. The concentrations that elicit vasodilation are completely correspondent with those that stimulate PGI2 generation in VSMC. PGI2 and PGE2 potentiate adenylate cyclase activity, which finally leads to a decrease in the cytosolic calcium concentration. Conversely, vasoconstrictor thromboxane A2 enhances the cytosolic calcium through phosphoinositide metabolism and a receptor-mediated calcium channel mechanism. Although the involvement of the eicosanoid system in the direct vasodilation by indapamide has not been proven so far, the increase in the PGI2-to-thromboxane A2 ratio observed in the VSMC could be one of the factors that cause vasodilation. To address these questions more directly, however, we might be required to test the prostaglandin inhibitors in the direct vasodilator action of the indapamide diuretic.

We showed that indapamide enhanced the elimination of the stable free radical DPPH and reduced the formation of MDA in the rat brain homogenate. More directly, we demonstrated that indapamide lowered the oxidation of linoleic acid in the xanthine-xanthine oxidase system. It has been reported that xanthine-xanthine oxidase is a free radical-generating system and that the oxidation of fatty acids is a free radical-mediated reaction. We also showed that 0.5 unit superoxide dismutase inhibited the oxidation 37% in this assay system. These data clearly indicate that indapamide has marked antioxidant effects particularly at the relatively higher concentrations. In addition, since such antioxidant properties were found in neither trichloromethiazide nor furosemide, the scavenging effect is unique to the indapamide diuretic.

Free radicals are more destructive to PGI2 synthase and the cyclooxygenase-hydroperoxidase complex becomes the target as well. Indeed, ONO-3144 (Ono Pharmaceutical Co., Ltd, Osaka, Japan), a potent antioxidant and anti-inflammatory drug, as well as MK-44727, reportedly enhances PGI2 and PGE2 formation while thromboxane A2 generation is somehow lowered in guinea pig macrophages. The enhanced PGI2 generation could be partly explained by a diversion of endoperoxide from MDA formation to PGI2 synthesis. However, it seemed probable that the antioxidant effect of indapamide protects PGI2 synthase from the attacks of endogenous free radicals, thereby increasing PGI2 formation in VSMC. In fact, the enzyme analyses revealed that arachidonate liberation was not responsible for the enhanced PGI2 generation in indapamide-stimulated VSMC. Moreover, we found that indapamide failed to directly activate PGI2 synthase in a cell-free assay system. In contrast, this diuretic attenuated PGI2 synthase inhibitory activity of 15-HPETE, a provider of free radicals and a powerful inhibitor of PGI2 synthase. These data are supportive of the involvement of scavenging effects in the enhanced PGI2 generation by indapamide diuretic.
In this context, it is reported that PGH synthase generates superoxide in the presence of reduced nicotinamide-adenine dinucleotide or nicotinamide-adenine dinucleotide 3'-phosphate. The elimination of superoxide generated in the formation of PGH₂ is expected to enhance PGH₂ production, which would lead to increased PGE₂ generation. Hence, the fact that indapamide tended to increase the PGE₂ generation in VSMC could be accounted for by such scavenging effects on the PGH₂ formation.

From a viewpoint of a structure-activity relation, the common structure of o-chlorobenzenesulfonamide, the basal component for natriuretic effects, is irrelevant to PG₁ stimulation observed in indapamide (Figure 1). Because PG₁ stimulation and scavenging effects were found in neither mefruside nor furosemide, the side chains appeared to be largely responsible for these various properties. Moreover, there are naturally a few types of free radicals, that is, superoxide, hydroxy radical, singlet oxygen, or peroxide. It is still uncertain what type of free radicals is scavenged by the indapamide diuretic. Electrochemical analyses are needed to reveal the mechanisms for the antioxidant effects of this diuretic.

It has been reported that the oral dosage of indapamide for obtaining sufficient antihypertensive effects in rats is more than 1 mg/kg body wt, which is about 10 times as much as the dosage for related compounds, and Yoshitomi Pharmaceutical Co., Ltd, Tokyo, Japan, for the supply of mefruside.

Finally, indapamide exhibited the enhanced PG₁ generation and scavenging effects in vitro. These properties are assumed to be protective from the arteriosclerotic changes or tissue damages caused by blood pressure elevation. We do not have in vivo data available for the indapamide effects. In humans, however, it has been reported that indapamide does not deteriorate renal function even when given to patients with renal impairment. Thus, our next goal is to test whether the indapamide diuretic exhibits in vivo protective effects against organ damage in hypertensive models. Experiments are in progress to address these questions.

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Key Words • prostacyclin • vascular smooth muscle • thromboxane • diuretic therapy
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