Vitamin D$_2$-induced Arteriosclerosis in Spontaneously Hypertensive Rats and Protection by Diltiazem, a Calcium Antagonist

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In male spontaneously hypertensive rats fed an atherogenic diet and treated with vitamin D$_2$ (320,000 I.U./kg/day) for 4 days, arteriosclerosis of Mönckeberg type was produced in the aorta with multiple massive hemorrhage in the periaortic adipose and subdural tissues. The production of vascular changes and hemorrhages was inhibited by oral administration of a calcium antagonist, diltiazem (60 mg/kg-twice/day) for 7 days starting at the first day of vitamin D$_2$ treatment.—Key words: Arteriosclerosis, Diltiazem, Vitamin D$_2$, SHR.


Disturbance of calcium metabolism has been already suggested to be a risk factor of arteriosclerosis by Ham and Lewis [11]. Recently Fleckenstein et al. [7] indicated that calcium overload in the arterial walls is as important as cholesterol accumulation for production of arteriosclerotic damages.

On the other hand, vitamin D (VD) plays an important role in calcium metabolism inducing pathological changes of the cardiovascular system in rats [3], dogs [14], rabbits [24] and horses [12]. Excess intake of VD seems to play a major role in producing certain types of human arteriosclerosis [21, 23].

Our previous studies [18, 19, 25] revealed that arteriosclerosis of so-called Mönckeberg type characterized by necrosis of smooth muscles and calcification in the media was produced in the aorta of rats after administration of a mixture of massive dose of vitamin D$_2$ (VD$_2$) and cholesterol for 4 days, a method being similar to Altman’s [1]. Electron microscopy revealed that edema was produced during the early stage in the aortic intima by plasma infiltration due to hypercalcemia with calcium deposit in the media [25]. By means of the short term loading with VD$_2$ and cholesterol, however, the production of severe arteriosclerosis during the chronic phase could not be expected without involvement of some other additional factors.

In the present study, spontaneously hypertensive rats (SHRs) were fed an atherogenic diet (AD) supplemented with massive dose of VD$_2$, and effects of a calcium antagonist diltiazem, $d$-3-acetoxy-cis-2, 3-dihydro-1, 5-[2-((dimethylamino)-ethyl]-2-(p-methoxyphenyl)-1, 5-benzothiazepine-4 (5H)-one hydrochloride, on arteriosclerosis of Mönckeberg type were investigated as compared to the findings of its effects on hypervitaminosis D in normotensive rats [7].

MATERIALS AND METHODS

Fifty-four male SHRs (Charles River Japan Inc.), 7 to 8 months of age, weighing about 380 g and with a mean systolic blood pressure of 212 mmHg, were used. Two or three rats were housed in a metal cage. The animals were randomly divided into 6 groups as shown in Fig. 1. Groups I and II were fed
normal diet (ND) and AD, respectively, as controls. ND was a commercial diet (CE-2, CLEA Japan Inc., Tokyo), and AD consisted of 89.2% ND, 7% hydrogenated oil, 3% cholesterol, 0.5% cholic acid and 0.3% thio-uracil. The diets and water were given *ad libitum*.

Group III of AD-fed animals were given orally 8 mg (320,000 I.U.) of VD2 (E.Merck, Darmstadt) dissolved in 2 ml of olive oil per kg of body weight for four consecutive days starting at the 1st day of experiment. To groups IV and VI of VD2-loaded and AD-fed animals diltiazem (Tanabe Seiyaku Co., Ltd., Osaka) in an aqueous solution was orally administered at daily doses of 60 (group IV) and 120 mg/kg (group VI), respectively, 1 hr after VD2 dosing, while animals of group V were treated with 60 mg/kg of the drug twice 1 and 7 hr after VD2 dosing, daily for 7 days.

On day 8 of the experiment, blood samples were collected from the abdominal aorta under ether anesthesia and animals were sacrificed by exsanguination. Prothrombin time (PT) was determined by Quick’s one step method using tissue thromboplastin (Ortho Diagnostic Systems, Inc., Tokyo). Serum calcium and cholesterol levels were assayed by OCPC method [2] using a Ca-set (IATRON Laboratories Inc., Tokyo) and enzyme assay using Determiner TC-5 (Kyowa Medex Co., Ltd., Tokyo), respectively. The aorta and heart were removed and fixed in 10% neutral buffered formalin solution, and paraffin sections were stained with hematoxylin-eosin and von Kossa’s method.

**RESULTS**

No animals died during the experimental period, although most of the VD2-loaded animals (groups III, IV, V, VI) were anorectic and wasted with time. At autopsy, marked hemorrhages were observed at various sites of the VD2-loaded rats including the adipose tissue around the aorta as well as in subdural, labyrinthis, thymic, subepicardial, and subcutaneous (cervical, axillary, thoracic, femoral, etc.) areas (Table 1,Figs. 2–4). When VD2-loaded and AD-fed animals were treated with diltiazem (groups IV-VI), the incidence and severity of hemorrhages were markedly decreased. Especially in group V, periaortic hemorrhage appeared only in 1 out of 11 animals (Table 1, Fig. 5).
Table 1. Incidence of hemorrhages

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Group&lt;sup&gt;a&lt;/sup&gt;</th>
<th>I</th>
<th>II</th>
<th>III</th>
<th>IV</th>
<th>V</th>
<th>VI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subdural</td>
<td>0/5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0/5</td>
<td>8/12</td>
<td>8/12</td>
<td>0/11</td>
<td>4/9</td>
<td></td>
</tr>
<tr>
<td>Periaortic</td>
<td>0/5</td>
<td>0/5</td>
<td>7/12</td>
<td>5/12</td>
<td>1/11</td>
<td>0/9</td>
<td></td>
</tr>
<tr>
<td>Subcutaneous</td>
<td>0/5</td>
<td>0/5</td>
<td>6/12</td>
<td>4/12</td>
<td>0/11</td>
<td>1/9</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> See the text.
<sup>b</sup> Number of rats with hemorrhages/number of rats examined.

Fig. 2. Subdural hemorrhage in cerebellum (arrow) of a rat in group III.

Fig. 3. Subcutaneous hemorrhages in cervical and axillary regions (arrows) of a rat in group III.

Fig. 4. Hemorrhages around the aorta of a rat in group III.

As shown in Table 2, the AD-fed rats (group II) showed a marked increase in serum cholesterol level as compared with the ND-fed ones (group I), without differences in other items. On the other hand, the food consumption, body weight and serum cholesterol of groups III-VI rats were lower as compared with group II rats. PT was markedly prolonged in group III. In groups V and VI, having received diltiazem there was no significant prolongation of PT as compared with groups I and II. Serum calcium levels in groups III to VI were significantly higher than in groups I and II, but there were no significant differences among these groups.
Table 2.

<table>
<thead>
<tr>
<th></th>
<th>I</th>
<th>II</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>392.0 ±15.7b)</td>
<td>371.6 ±10.0</td>
</tr>
<tr>
<td></td>
<td>(385.2 ±15.9)</td>
<td>(372.8 ± 9.7)</td>
</tr>
<tr>
<td>Food consumption (g/head/day)</td>
<td>20.7</td>
<td>17.5</td>
</tr>
<tr>
<td>Prothrombin time (sec)</td>
<td>14.7 ± 0.1</td>
<td>14.5 ± 0.2</td>
</tr>
<tr>
<td>Serum Ca (mEq/l)</td>
<td>5.18± 0.05</td>
<td>5.19±0.06</td>
</tr>
<tr>
<td>Serum cholesterol (mg/dl)</td>
<td>63.6 ± 4.2</td>
<td>198.2 ±9.0**</td>
</tr>
</tbody>
</table>

a) See the text.

b) Mean±S.E. at the end of the experiment with that at the onset of
*: p<0.05, **: p<0.01 compared to control (Student's t test)

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Fig. 5. No hemorrhage is seen around the aorta and in trachea, heart, lung and kidneys of a rat in group V.

The aorta of most animals in group III showed remarkable arteriosclerosis of so-called Mönckeberg type with calcification at the elastica and disorganization and necrosis of the smooth muscle cells in the inner and middle layers of the media. These changes were more intense at the sites of ramification. The lumen of the aorta was conspicuously dilated and the wall was thinned. Considerable hemorrhages were occasionally observed around the blood vessels with infiltration of leukocytes and proliferation of fibroblasts (Fig. 6). In animals of groups IV to VI VD₂-loaded and treated with diltiazem, the incidence and severity of the aortic changes were significantly decreased, particularly in animals of group V (Fig. 7). No changes

Fig. 6. Thoracic aorta of a rat in group III. Thinning of vascular wall, calcification (darker stained area) in the media and hemorrhage in and around the adventitia are apparent. von Kossa stain. ×150. L: Lumen.
Clinicopathological data

<table>
<thead>
<tr>
<th>Group</th>
<th>II</th>
<th>IV</th>
<th>V</th>
<th>VI</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>300.2 ± 5.8**</td>
<td>292.7 ± 5.1**</td>
<td>312.5 ± 6.1**</td>
<td>306.7 ± 6.9**</td>
</tr>
<tr>
<td></td>
<td>(374.3 ± 6.1)</td>
<td>(367.3 ± 6.1)</td>
<td>(374.9 ± 5.9)</td>
<td>(380.2 ± 5.3)</td>
</tr>
<tr>
<td></td>
<td>3.6</td>
<td>3.4</td>
<td>6.5</td>
<td>4.2</td>
</tr>
<tr>
<td></td>
<td>60.0 ± 9.8**</td>
<td>49.8 ± 4.1**</td>
<td>26.5 ± 4.5</td>
<td>29.9 ± 4.6</td>
</tr>
<tr>
<td></td>
<td>5.77 ± 0.07**</td>
<td>5.74 ± 0.10**</td>
<td>5.87 ± 0.07**</td>
<td>5.80 ± 0.13**</td>
</tr>
<tr>
<td></td>
<td>94.3 ± 3.6*</td>
<td>95.9 ± 3.5**</td>
<td>130.6 ± 11.2**</td>
<td>114.0 ± 7.6**</td>
</tr>
</tbody>
</table>

the experiment in parenthesis.

![Fig. 7. Thoracic aorta of a rat in group V, showing nearly normal appearance. von Kossa stain. ×150.](image1)

![Fig. 8. Heart of a rat in group III. Marked myocardial necrosis, hemorrhage and round cell infiltration are seen around a branch of coronary artery with distorted configuration. H-E stain. ×150.](image2)

were noticeable in rats of groups I and II.

In animals of group III, remarkable changes were seen in the coronary arteries and myocardium. The wall of the coronary arteries was edematous and medial smooth muscle cells were disorganized. The vascular lumen was dilated and with proliferation of fibroblasts and myocytes around the blood vessels. Some large branches of the coronary arteries showed obvious calcification of the internal elastic membrane. Focal necrosis (Fig. 8) and granulomatous lesions were present in the myocardium. In these necrotic or granulomatous lesions and the subepicardium, hemorrhages were frequently encountered. In animals of groups IV to VI, which were treated with diltiazem, the incidence and
minated hemorrhages, marked prolongation of PT was recorded, implying exhaustion of blood coagulating factors due to the abrupt and massive hemorrhages.

In VD2-loaded and diltiazem-treated groups IV to VI the production of clinical and pathological changes was inhibited, and the protective effect of the drug was most evident in animals which had received 60 mg/kg of diltiazem twice a day (group V).

Calcium antagonists such as diltiazem, verapamil and MgCl2 have potent blocking actions against the increased uptake of calcium into the arterial wall of animals given a high dose of VD [7–9] or norepinephrine [6]. Diltiazem might stabilize or protect the surface of endothelial cells as calcium antagonist and protect arteries from sclerotic changes.

Striking hypercalcemia induced by high doses of VD [4, 5, 10, 13] also causes damage of the endothelial cells as reported previously [25]. While diltiazem does not affect the occurrence of VD2-induced hypercalcemia, the resulting injuries might be inhibited by an ion channel blocking by the drug [15, 17]. In fact, in a separated experiment under the same conditions, the serum calcium levels of VD2-loaded animals on day 5 were 7.21±0.33 mEq/l without diltiazem and 7.14±0.25 mEq/l with diltiazem (unpublished data).

Moreover, such actions of diltiazem on physicochemical properties of cell membrane as shown by inhibition of platelet aggregation [22] as well as improvement of deformability and inhibition of hypotonic hemolysis [20] of red blood cells, or the hypotensive activity of diltiazem [16] may also play an important role in the prevention of arteriosclerosis and multiple hemorrhages.

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REFERENCES


要約

SHRにおけるVitamin D₃誘発動脈硬化症および抗Ca剤diltiazemの効果: 岩崎仁・北村和之・土井邦雄1・岡庭様（田辺製薬薬理研究所，1)東京大学農学部実験動物学教室）——320,000単位のビタミンD₃を高脂肪食飼育の雄SHRに4日間投与することにより,大動脈壁に特徴的なMönckeberg型の動脈硬化症が惹起された。大動脈周囲脂肪組織や脳硬膜下組織をはじめ全身性に著明な出血がみられた。ビタミンD₃と同時に抗Ca剤diltiazem（60, 2×60,および120mg/kg/日）を7日間与えた結果,ビタミンD₃誘発による血管病変ならびに出血の発現は阻止された。とくにdiltiazem（60mg/kg）を1日2回投与した動物では防御効果はほぼ完全であった。