Augmentation of Adenosine-Induced Relaxation Response with Hydralazine in Aortic Strips

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Abstract—Adenosine produced a slight but concentration-dependent relaxation in rabbit aortic strips preconstricted with norepinephrine. The effect of adenosine was markedly augmented in the presence of hydralazine. On the other hand, the adenosine-induced relaxation was attenuated by 8-phenyltheophylline, but was unaffected by indomethacin, nordihydroguaiaretic acid and quinacrine, indicating that adenosine acts via purinergic receptors and that vasodilating metabolites of arachidonic acid are not involved in the relaxation. The adenosine-induced relaxation remained unaffected by S-(p-nitrobenzyl)-6-thioguanosine (NBTG) or 2'-deoxycoformycin (2'DCF), alone or combined. NBTG significantly inhibited the incorporation of [3H] adenosine, while the content of [3H] compound was increased by 2'DCF, but was unchanged by hydralazine. Hydralazine also augmented the 2-chloroadenosine-induced relaxation. These results suggest that the augmentation of adenosine-induced relaxation with hydralazine does not result from an inhibition of adenosine transport and/or adenosine deaminase. When adenosine was added, relaxation was elicited with concomitant increase in cAMP, but with no significant change in cGMP. In the presence of hydralazine, the cAMP increasing effect of adenosine was augmented, and the level of cGMP increased with adenosine. These changes in cyclic nucleotide levels might at least in part explain the augmentation of adenosine-induced relaxation with hydralazine.

Hydralazine is assumed to exert its blood pressure lowering effect by a direct action on arteriolar smooth muscle cells (1). However, indirect mechanisms have also been discussed: for example, the induction of a release of the vasodilator prostaglandin E2 and I2 (2) or an interference with sympathetic transmitter release at the level of the vascular smooth muscle (3). Recently, Spokas et al. (4) have presented convincing evidence that the endothelial component of the hydralazine response represents a major contribution to the net relaxant effect on vascular smooth muscle, particularly at low concentrations that are of clinical relevance. On the other hand, several different mechanisms for the intracellular action of hydralazine have been suggested by many workers (5–8). However, the mechanism responsible for the antihypertensive action of hydralazine has been the subject of considerable controversy and remains to be clarified.

We have recently found that the adenosine-induced relaxation in rabbit aortic strips preconstricted with norepinephrine is markedly augmented in the presence of hydralazine at concentrations which do not exert any relaxation activity. Adenosine is considered to be an important regulator of blood flow in a variety of organs. This function of adenosine is based on its relaxant effect on arteries. The present experiments were, therefore, undertaken to investigate the mechanism of the augmentation in connection to the mechanism responsible for the antihypertensive action of hydralazine.

Materials and Methods

Chemicals: [2-3H] Adenosine (specific activity: 16.4 Ci/mmol) and [14C(U)]
sucrose (specific activity: 671.0 mCi/mmol) were purchased from New England Nuclear (Boston, MA, U.S.A.). Hydralazine hydrochloride, adenosine, 2-chloroadenosine, S-(p-nitrobenzyl)-6-thioguanosine (NBTG), nordihydroguaiaretic acid (NDGA), I-nor-epinephrine bitartrate (NE), 8-phenylthio-phylline (8PT) and quinacrine were all from Sigma. Cocaine hydrochloride was purchased from Takeda Pharmaceutical Co. 2'-Deoxycoformycin (2'DCE) was a generous gift from Dr. K. Kodama, Yamasa Shoyu Co. (Choshi, Japan). Indomethacin (Merck) and NBTG were dissolved in dimethylsulfoxide (DMSO, Art. 2950), which was present in a final concentration of 0.5% v/v or less in all experiments with these agents, and this concentration had no effect on any parameters studied.

Measurements of the mechanical responses of aortic strips: Measurements of the mechanical responses of aortic strips were performed as described in the previous report (9). Briefly, male albino rabbits weighing about 2.5 kg were sacrificed by exsanguination and the thoracic aorta was rapidly excised. After removal of the adventitial connective tissue, helical strips 3 mm wide and 25 mm long were prepared. These preparations were denuded of a functional endothelium (10) to avoid any complicating effects of endothelium and mounted vertically in a 20 ml jacketed organ bath filled with modified Krebs' solution at 37°C and gassed with 95% O2 and 5% CO2. The composition of the modified Krebs' solution was as follows: 115.0 mM NaCl, 4.7 mM KCl, 1.2 mM MgSO4·7H2O, 2.5 mM CaCl2·2H2O, 1.2 mM KH2PO4, 25.0 mM NaHCO3 and 10.0 mM glucose. One end of each strip was attached to the lower end of a stainless rod which was mounted independently from the organ bath. The other end was attached to a force-displacement transducer (SB-1T, Nihon Kohden Kogyo Co., Tokyo). Isometric changes in tension were recorded on a pen-writing oscillograph (Wi-681G, Nihon Kohden Kogyo Co.). Length of the strips was adjusted several times until a stable tension of 2 g was attained. Before beginning the experiments, strips were allowed to equilibrate for at least 60 min in the bathing solution and during this period, the bathing solution was replaced every 20 min with fresh solution.

To compare the relaxation activity, strips were exposed to 10−6 M NE for 10 min in the presence of 3×10−8 M cocaine and 5.7×10−4 M ascorbic acid. During this period, the strips had attained a steady state level of tone. Then, adenosine or 2-chloroadenosine in a single concentration was added to the bathing solution. Cumulative dose-response curves for adenosine were obtained by a stepwise increase in the concentration as soon as a steady response was attained with the preceding concentration. The dose-response curves for adenosine were obtained prior to and following hydralazine at different concentrations in the same tissue. After studying the effects of the first concentration of hydralazine, the tissue was washed out until the second control response was recorded.

Relative relaxation value was calculated by taking the percentage relaxation with relaxation agonists against 10−6 M NE-induced contraction in the absence of test agents as a value of 1.0. Unless otherwise stated, agents to be tested were added 10 min prior to NE (i.e., 20 min prior to relaxation agonists). It has been determined by the preliminary experiments that this incubation time is sufficient to augment the adenosine-induced relaxation; that is, the magnitude of augmentation was not significantly different when hydralazine was added 10, 20 or 50 min prior to NE.

Uptake studies: Uptake studies were carried out by the following method: Aortic spiral strips prepared as described above were weighed and preincubated at 37°C for 5 min in 5 ml modified Krebs' solution gassed with 95% O2 and 5% CO2. The composition of the modified Krebs' solution was as follows: 115.0 mM NaCl, 4.7 mM KCl, 1.2 mM MgSO4·7H2O, 2.5 mM CaCl2·2H2O, 1.2 mM KH2PO4, 25.0 mM NaHCO3 and 10.0 mM glucose. One end of each strip was attached to the lower end of a stainless rod which was mounted independently from the organ bath. The other end was attached to a force-displacement transducer (SB-1T, Nihon Kohden Kogyo Co., Tokyo). Isometric changes in tension were recorded on a pen-writing oscillograph (Wi-681G, Nihon Kohden Kogyo Co.). Length of the strips was adjusted several times until a stable tension of 2 g was attained. Before beginning the experiments, strips were allowed to equilibrate for at least 60 min in the bathing solution and during this period, the bathing solution was replaced every 20 min with fresh solution.
solution, rinsed twice and blotted with filter paper. Each strip was dissolved in 1 ml of Soluene-350 (United Technologies Packard) by heating for 60 min at 60°C. After cooling, 4 ml of Aquazol-2 (New England Nuclear) was added, and the radioactivity of the sample counted in a liquid scintillation counter (Packard 460-C). The radioactivity of 1 ml of medium was determined in a liquid scintillation counter. Four milliliters of Aquazol-2 were used as a scintillant. The blank values were obtained by incubation of the aortic strips at 0°C and were consistently between 8 and 11% of the total uptake. The uptake of $[^3$H] adenosine was calculated as follows: $[^3$H] uptake = $([^3$H] in tissue at 37°C $-[^3$H] in media $/[^1$C] in media $) \times [^1$C] in tissue at 37°C $-[^3$H] in tissue at 0°C $-[^3$H] in media $/[^1$C] in media $) \times [^1$C] in tissue at 0°C $).

Measurements of cyclic nucleotide levels:
For measurements of cyclic nucleotide levels, aortic strips were suspended as described previously in an organ bath under 2 g load and allowed to equilibrate for at least 60 min. At any time in experiments, the organ bath could be instantly dropped down, leaving the supporting preparation accessible for immediate freezing in liquid N2. After 10 min-exposure to 10$^{-6}$ M NE, each strip was treated with 10$^{-4}$ M adenosine for 2 min and then frozen in liquid N2. Frozen tissues were homogenized in ice-cold 6% trichloroacetic acid, and extracts were assayed for cyclic AMP and cyclic GMP by a sensitive radioimmunoassay procedure (11) using commercially available kits (Yamasa Shoyu Co., Japan). All assays were performed in duplicate. To test the effect of hydralazine, it was added 10 min prior to NE (i.e., 20 min prior to adenosine).

Statistical analyses: Results in the text, Table and Figures are expressed as the mean±S.E. For statistical evaluation, data were analyzed by Student's t-test.

Results
Augmentation of the adenosine-induced relaxation with hydralazine: Results are shown in Fig. 1. Adenosine (10$^{-6}$ to 10$^{-4}$ M) produced a slight but concentration-dependent relaxation in rabbit aortic strips preconstricted with 10$^{-6}$ M norepinephrine in a concentration-dependent manner (●, n=17). The relaxation induced by 10$^{-6}$ M adenosine in the control was taken as 1.0 (dotted horizontal line). The adenosine-induced relaxation was markedly augmented in the presence of hydralazine at concentrations of 10$^{-6}$ M (○, n=5), 3×10$^{-6}$ M (♦, n=5) and 10$^{-5}$ M (△, n=7). Vertical bars show the S.E.

Fig. 1. Augmentation of the adenosine-induced relaxation with hydralazine. Adenosine produced relaxation of aortic strips preconstricted with 10$^{-6}$ M norepinephrine in a concentration-dependent manner (●, n=17). The relaxation induced by 10$^{-6}$ M adenosine in the control was taken as 1.0 (dotted horizontal line). The adenosine-induced relaxation was markedly augmented in the presence of hydralazine at concentrations of 10$^{-6}$ M (○, n=5), 3×10$^{-6}$ M (♦, n=5) and 10$^{-5}$ M (△, n=7). Vertical bars show the S.E.

with 10$^{-6}$ M NE. The maximum concentration of adenosine (10$^{-4}$ M) produced 18.9±1.4% (n=17) relaxation against 10$^{-6}$ M NE-induced contraction (3.98±0.26 g, n=17). The adenosine-induced relaxation was markedly augmented by pretreatment with hydralazine even at a concentration of 10$^{-6}$ M. The magnitude of augmentation increased with increasing concentrations of hydralazine (10$^{-6}$, 3×10$^{-6}$ and 10$^{-5}$ M).

Hydralazine at a concentration of 10$^{-6}$ M or less, per se, did not produce relaxation in the preconstricted aorta. However, relaxation of the strips to 3×10$^{-5}$, 10$^{-4}$ and 3×10$^{-4}$ M of the antihypertensive agent was determined as 5.6±1.6, 21.9±3.4 and 40.9±2.0% (n=9), respectively, against 10$^{-6}$ M NE-induced contraction (4.19±0.21 g, n=9).
Effects of some agents on the adenosine-induced relaxation: Adenosine (10^{-4} M)-induced relaxation in the preconstricted aortic strips was markedly augmented by 3 \times 10^{-6} M hydralazine, while it was attenuated (68.0 \pm 5.1\%, n=6) in the presence of 10^{-5} M 8PT, a potent adenosine receptor antagonist (12). In addition, the augmented relaxation in the presence of 3 \times 10^{-6} M hydralazine was clearly attenuated (48.6 \pm 5.4\%, n=6) by 10^{-5} M 8PT. However, neither indomethacin (10^{-5} M), NDGA (10^{-4} M) nor quinacrine (10^{-5} M) had effects on the relaxation response to adenosine (data not shown).

Figure 2 shows the effects of hydralazine, 2'-DCF, an irreversible and potent inhibitor of adenosine deaminase (13), and NBTG, an inhibitor of adenosine uptake (14), on the adenosine-induced relaxation. Hydralazine (3 \times 10^{-7} to 10^{-5} M) produced a concentration-dependent augmentation of the relaxation. In contrast, 2'-DCF and NBTG at the concentrations tested did not produce significant augmentation. In addition, when 2'-DCF (10^{-5} M) was given in combination with NBTG (10^{-5} M), the adenosine-induced relaxation was not remarkably affected (data not shown).

Effects of hydralazine, 2'-DCF and NBTG on [3H] adenosine uptake by the aortic strips: Hydralazine even at concentrations sufficient to augment the adenosine-induced relaxation did not produce any effect on the tissue level of [3H] compound, whereas the level was significantly (P<0.05 and P<0.005) decreased by pretreatment with NBTG at concentrations of 3 \times 10^{-6} to 3 \times 10^{-5} M. In contrast, the tissue level of [3H] compound was increased by 2'-DCF (Fig. 3).

Effects of hydralazine on the cyclic nucleotide levels: Results are shown in Table 1. Hydralazine (10^{-6}, 3 \times 10^{-6} and 10^{-5} M), per se, did not produce any changes in cyclic AMP and cyclic GMP levels and tension in the aortic strips preconstricted with 10^{-6} M NE. When 10^{-4} M adenosine was added, relaxation was elicited with concomitant increase in cyclic AMP (P<0.05), but with no significant change in cyclic GMP. The cyclic AMP increasing effect of adenosine was slightly but significantly (P<0.005) augmented in the presence of hydralazine at concentrations of 3 \times 10^{-6} and 10^{-5} M, which were sufficient to augment the adenosine-induced relaxation. Although the cyclic GMP level was not significantly
changed by adenosine alone, the level was slightly but significantly (P<0.05) increased by adenosine in combination with hydralazine.

**Discussion**

The adenosine-induced relaxation response in rabbit aortic strips in the presence of adenosine was markedly attenuated by 8-phenyltheophylline, a potent purinergic receptor antagonist (12). Thus, adenosine seems to act primarily via purinergic receptors. A previous report (15) had confirmed the existence of vascular smooth muscle purinergic receptors. In addition, the facts that indomethacin, an inhibitor of cyclooxygenase; nordihydroguaiaretic acid, a lipooxygenase inhibitor (16); and quinacrine, an inhibitor of phospholipase A2 (17), failed to modify the adenosine-induced relaxation rule out the possible involvement of local generation of the endogenous metabolites of arachidonic acid in the relaxation to adenosine.

Huang and Daly (14) reported that S-(P-nitrobenzyl)-6-thioguanosine (NBTG) and other inhibitors of adenosine transport into brain slices increased the cyclic AMP response to adenosine. These authors suggested that these agents increased the effective concentration of adenosine at extracellular sites. On the other hand, the adenosine-induced relaxation remained unaffected in the presence of NBTG which significantly inhibited the transport of \(^{3}\)H adenosine into aortic smooth muscle. The difference between effects of NBTG in aortic preparations and brain slices may be explained by the absence of a diffusion barrier limiting access of adenosine to the cell membrane in the former (18).

The use of an inhibitor of adenosine deaminase often appears to cause a significant accumulation of adenosine to trigger biological responses (19). In the present

**Table 1. Effects of hydralazine on cyclic nucleotide levels in aortic strips**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Cyclic AMP (pmol/g wet wt.)</th>
<th>n</th>
<th>Cyclic GMP (pmol/g wet wt.)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>10^{-6} M Norepinephrine (NE)</td>
<td>129.8±4.4</td>
<td>14</td>
<td>6.4±0.9</td>
<td>9</td>
</tr>
<tr>
<td>NE+10^{-4} M Adenosine (Ad)</td>
<td>151.5±10.9(^a)</td>
<td>14</td>
<td>5.1±0.6</td>
<td>9</td>
</tr>
<tr>
<td>10^{-6} M Hydralazine+NE+Ad</td>
<td>168.3±8.6</td>
<td>14</td>
<td>7.8±1.0(^c)</td>
<td>9</td>
</tr>
<tr>
<td>3×10^{-6} M Hydralazine+NE+Ad</td>
<td>191.1±8.4(^b)</td>
<td>14</td>
<td>7.4±1.3</td>
<td>9</td>
</tr>
<tr>
<td>10^{-6} M Hydralazine+NE+Ad</td>
<td>213.3±9.0(^b)</td>
<td>14</td>
<td>8.1±1.0(^c)</td>
<td>9</td>
</tr>
</tbody>
</table>

\(^a\): P<0.05 vs. NE; \(^b\): P<0.005 vs. NE+Ad; \(^c\): P<0.05 vs. NE+Ad. Values are given as the means±S.E. For measurements of cyclic nucleotide levels, aortic strips were suspended in an organ bath under 2 g load. After 10 min-exposure to cyclic nucleotide levels, aortic strips were suspended in an organ bath under 2 g load. After 10 min-exposure to 10^{-6} M norepinephrine, each strip was treated with 10^{-4} M adenosine for 2 min and then frozen in liquid N\(_2\). To test the effect of hydralazine, it was added 10 min prior to norepinephrine (see text).
experiments, however, 2′-deoxycoformycin (2′DCF), an irreversible and potent inhibitor of adenosine deaminase (13) at concentrations sufficient to increase the tissue level of [3H] compound ([3H] adenosine and/or its metabolite) had no effect on the adenosine-induced relaxation. In addition, when 2′DCF was given in combination with NBTG, the adenosine-induced relaxation was not remarkably affected. Thus, augmentation of the adenosine-induced relaxation with hydralazine probably did not result from an inhibition of adenosine transport and/or adenosine deaminase with the agent. This speculation seems to be supported in part by the findings that hydralazine also augments the relaxation response to 2-chloroadenosine, which is not a substrate for uptake (20) and adenosine deaminase (21).

With respect to the cyclic nucleotide metabolism, no effects of hydralazine alone were observed on cyclic AMP and cyclic GMP levels at a concentration of 10⁻⁵ M or less. On the other hand, many studies have shown adenosine-elicited increases in intracellular cyclic AMP levels in vascular smooth muscle strips and cultured vascular smooth muscle cells (22). It is also well established that agents which stimulate the guanylate cyclase and consequently elevate the level of cyclic GMP are capable of relaxing the vascular smooth muscle (23, 24). In the present experiments, when adenosine was added to the preconstricted aortic strips, relaxation was elicited with concomitant increase in cyclic AMP, but with no significant change in cyclic GMP level. The cyclic AMP increasing effect of adenosine was slightly but significantly augmented in the presence of hydralazine. The cyclic GMP level was slightly but significantly increased by adenosine in combination with hydralazine. These changes in cyclic nucleotide levels may at least partly relate to the augmentation of adenosine-induced relaxation with hydralazine.

After concluding the present experiments, a report by Kurtz (25) was published. He demonstrated that low concentrations of adenosine alone increased cyclic GMP levels in isolated vascular smooth muscle cells from rat aorta and suggested that adenosine exerted relaxing effects by stimulating guanylate cyclase. These findings are consistent in part with the present results, although experimental conditions are different in detail.

It has been proposed that adenosine decreases vascular smooth muscle membrane permeability to calcium, thus lowering the intracellular calcium availability for the initiation and maintenance of smooth muscle contraction. Electrophysiological and ⁴⁶Ca²⁺ flux studies in vascular smooth muscle have provided corroborative evidence for the hypothesis (26, 27). On the other hand, since chemically skinned renal arteries, contracted by the addition of ATP and calmodulin, did not respond to 10⁻³ M hydralazine, it has been suggested that the agent has probably no direct effect on the level of the regulatory and contractile proteins (28). However, Khayyal et al. (7) have suggested that an action of hydralazine on excitation-contraction coupling, perhaps on the movement of intracellular calcium, was the most important. In these respects, the mechanism of synergism between adenosine and hydralazine in vascular smooth muscle remains to be elucidated. In addition, whether or not hydralazine selectively augments the adenosine-induced relaxation also remains to be clarified.

References
5 Diamond, J. and Shaikh, M.J.: Effect of hy-


