Abstract—The vasorelaxing effects of nicorandil (NCR), isosorbide dinitrate (ISDN) and nitroglycerin (NTG) were studied in isolated canine coronary arteries. In rings of coronary arteries precontracted with prostaglandin F₂α (3×10⁻⁶ M) or KCl (30 mM), removal of the endothelium significantly augmented the relaxing effects of NCR, while it did not affect those of ISDN and NTG. In un rubbed rings precontracted with KCl (30 mM), methylene blue (5×10⁻⁶ M) significantly inhibited vasorelaxing responses to the three drugs. The order of the inhibition was as follows: NTG > ISDN > NCR. When the un rubbed tissue was incubated with NTG (10⁻⁵ M) or ISDN (10⁻⁴ M) for 10 min, it developed acute tolerance in relaxing response to NTG or ISDN. Unlike NTG and ISDN, NCR did not develop any tolerance. The treatment with N-acetylcysteine (5×10⁻⁵ M) tended to potentiate relaxant effects of NTG and to reduce the degree of acute tolerance to NTG. The results suggest that cGMP plays a role in the relaxation of the coronary artery induced by the drugs and furthermore that the mode of the vasorelaxing action of NCR may be somewhat different from that of NTG or ISDN.

Although nitrates are the oldest and most frequently used medications for the urgent treatment of angina pectoris (1), the mechanism of its vasodilator action still remains uncertain. Needleman and Johnson (2) first hypothesized that nitrates induce vasodilatation by interacting with sulfhydryl groups on a nitrate “receptor” on the cell membrane. Even though possible modulation of this process by sulfhydryl availability may occur at several points, available data have suggested that S-nitrosothiol compounds, formed by interaction of nitrates with tissue sulfhydryls, activate guanylate cyclase, leading to production of cyclic guanosine monophosphate (cGMP) (3-5). Nicorandil, N-(2-hydroxyethyl)nicotinamide nitrate (ester), is a newly developed, orally efficacious antianginal drug (6) with strong coronary vasodilating (7-10) and vasospasmolytic effects (9, 11). On the basis of the view that nicorandil includes a nitrate moiety in its chemical structure, which plays an essential role in its pharmacological activity (7, 12), it chemically belongs to the class of nitrate compounds. Holzmann (13) reported in isolated circular strips of bovine coronary arteries that there is a close correlation between increases in cGMP and muscle relaxation obtained with nicorandil, but its correlation is steeper than that obtained with other nitrates, and suggested that nicorandil has an additional relaxant effect which does not depend on cGMP production.

Our present purpose, therefore, was to further evaluate the vasorelaxing effects of nicorandil, compared with those of isosorbide dinitrate and nitroglycerin, in isolated
coronary arteries of the dog.

Materials and Methods

General

Beagle dogs of both sexes, weighing 9 to 10 kg, were anesthetized with sodium pentobarbital (35 mg/kg, i.v.). Immediately after the animals were sacrificed by bleeding from the cannulated femoral arteries, the heart was quickly removed. The left circumflex coronary artery was dissected out, immersed in cold Krebs-Henseleit (K-H) solution, and cleaned of surrounding connective tissue. After storage overnight at 4°C, the artery was cut into rings of 2.5 mm in length. Great care was taken to avoid injuring the endothelium. The ring was mounted under a resting tension of 1.5 g in an organ bath (10 ml capacity) containing a modified K-H solution (standard) of the following composition (mM): NaCl, 120; KCl, 5.6; CaCl2, 2.5; KH2PO4, 1.5; MgSO4, 1.2; NaHCO3, 25.0; dextrose, 11.0. The solution was equilibrated with a gas mixture containing 95% O2 and 5% CO2. The oxygen tension of the solution was about 600 mmHg, and the pH was approximately 7.4 at 37°C (measured with a blood gas analyzer, ABL2 Acid-Base Laboratory, Radiometer, Copenhagen). One side of the ring preparation was fixed to the bottom of the bath and the other end was connected by a hook at the level of a force-displacement transducer (Nihon Kohden, SB-1T). Before the initiation of the experiments, all preparations were allowed to equilibrate for at least 2 hr at 37°C, with washes every 20 min. The drugs were added to the bath in a volume less than 0.1 ml. A series of sequential doses was administered, each subsequent one being introduced when the effect of the preceding one had reached a steady value. Cumulative concentration-percentage maximal response curves for muscle relaxation of drugs were generated after contraction with 30 mM KCl or 3 x 10^{-6} M prostaglandin F2α. The 'high K' K-H solution was made by hyperosmotically adding 23 mM KCl to standard K-H solution. Submaximal (50–60% of maximum) contractions induced reproducibly by these agonists were maintained. When the experiments had been completed, tissues were exposed to 10^{-4} M papaverine, and the point to which they relaxed was defined as 100% relaxation. Then, preceding relaxation induced by drugs was expressed in percent with reference to relaxation induced by 10^{-4} M papaverine. The value of ED50 was defined as the concentration of the drugs which induced a relaxation of 50% of the KCl- or PGF2α-induced muscle contraction. Isometric tension was recorded on a self-balancing potentiometric recorder (Yokogawa, 3066).

Experimental protocols

Experiments were carried out in the following series:

Series 1: In the preparation with or without endothelium relaxant effects of nicorandil, isosorbide dinitrate and nitroglycerin were examined. One of two coronary arterial rings obtained from the same dog was used as a control, and the intimal surface of the other ring was gently rubbed with a wooden stick. Functional integrity of the endothelium was confirmed in each ring by the presence or the absence of relaxation induced by acetylcholine (3 x 10^{-6} M) during contraction with 30 mM KCI or 3 x 10^{-6} M PGF2α.

Series 2: In unrubbed vessels precontracted with 30 mM KCl, effects of methylene blue on nitrate-induced relaxation were evaluated. According to the report of Griffith et al. (14) using aortic strip preparations of the rabbit, 5 x 10^{-6} M of methylene blue was used in the present study. Before the addition of nicorandil, isosorbide dinitrate or nitroglycerin, the rings precontracted with 30 mM KCl were treated with methylene blue for 15 min. Then, cumulative concentration-response curves for the nitrates were constructed.

Series 3: To induce acute tolerance, the unrubbed coronary ring preparations were incubated with nicorandil (10^{-4} M), isosorbide dinitrate (10^{-4} M) or nitroglycerin (10^{-5} M) at pH 7.4 for 10 min. After incubation, the drug was washed out five times; and 20 min later, the tissues were contracted with 30 mM KCl. Cumulative concentration-response curves for the nitrates were constructed by comparing the relaxing effects in preparations with or
without incubation with the nitrates. The effect of N-acetylcysteine (NAC) on tolerance to nitroglycerin was examined as follows: according to the report of Torresi et al. (15) using isolated bovine coronary artery preparations, $5 \times 10^{-5}$ M of NAC was added to the bath, just before incubation with nitroglycerin. Immediately after nitroglycerin was washed out five times, $5 \times 10^{-5}$ M NAC was added to the bath again, and the tissues were contracted with $30$ mM KCl. Then, cumulative concentration-response curves for nitroglycerin were constructed.

**Drugs used**

Nicorandil, N-(2-hydroxyethyl)nicotinamide nitrate (ester) (M.W. 211.18, $C_8H_{10}N_3O_4$), was synthesized in our Research Laboratories. Isosorbide dinitrate was extracted from commercially available tablets (Eisai). Nitroglycerin in commercially available ampoules was used (Nippon Kayaku). The other drugs were prostaglandin (PG) $F_{2\alpha}$ and N-acetylcysteine (both Sigma), methylene blue (Wako Junyaku), papaverine hydrochloride (Tokyo Kasei) and acetylcholine chloride (Daiichi Seiyaku). Isosorbide dinitrate and $PGF_{2\alpha}$ were dissolved in $99.5\%$ ethyl alcohol at concentrations of $10$ mg/ml and $1$ mg/ml, respectively. Methylene blue and papaverine hydrochloride were dissolved in distilled water. The other drugs were dissolved in and diluted to the desired concentrations with standard K-H solution. Drug solutions were added to the bath in a volume of $30-100$ $\mu$l using individual syringes (Terumo Co.), and the final bath concentrations are given.

**Statistical analysis**

Data are presented as values of the mean±S.E. Intergroup differences were analyzed by Student's $t$-test. When a $P$ value was less than 0.05 in the $F$-test, the differences between mean values were analyzed by Welch's $t$-test. A $P$ value less than 0.05 was considered statistically significant.

**Results**

Effects of nicorandil (NCR), isosorbide dinitrate (ISDN) and nitroglycerin (NTG) in the presence or the absence of the endothelium. In coronary arterial rings precontracted with $3 \times 10^{-6}$ M $PGF_{2\alpha}$, the addition of NCR ($10^{-7}-3 \times 10^{-4}$ M), ISDN ($10^{-7}-10^{-4}$ M) or NTG ($10^{-9}-3 \times 10^{-8}$ M) caused a concentration-dependent relaxation. Removal of the endothelium did not affect the relaxant response to either ISDN or NTG, while it significantly augmented the response to NCR. No significant difference was observed between increments in tone caused by $3 \times 10^{-6}$ M $PGF_{2\alpha}$ in the preparations with and without the endothelium. The concentration-response curves for the three drugs are depicted in Fig. 1A, and the ED50 values for relaxation are demonstrated in the upper part of Table 1.

Similarly, in the arterial rings with and without the endothelium precontracted with $30$ mM KCl, cumulative concentration-

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**Fig. 1.** Concentration-response curves for nicorandil (NCR, circle), isosorbide dinitrate (ISDN, triangle) or nitroglycerin (NTG, square) in coronary arteries with (open symbols) and without (closed symbols) the endothelium precontracted with A) $3 \times 10^{-6}$ M prostaglandin (PG)$F_{2\alpha}$ or B) $30$ mM KCl. Each point represents the mean±S.E. ($n=8$). *$P<0.05$, **$P<0.01$, ***$P<0.001$ vs. unrubbed preparations. There was no significant difference between increments in tone induced by $PGF_{2\alpha}$ or KCl in the presence (open columns) and the absence of endothelium (solid columns) (see the right side).
Table 1. Effects of removal of the endothelium on the relaxant responses to nicorandil (NCR), isosorbide dinitrate (ISDN) and nitroglycerin (NTG) in coronary arteries precontracted with prostaglandin (PG)F₂α or KCI

<table>
<thead>
<tr>
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<th>Endothelium-intact</th>
<th>Removal of endothelium</th>
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<tr>
<td></td>
<td>-log ED50</td>
<td></td>
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<tr>
<td>with 3x10⁻⁶ M PGF₂α</td>
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<tr>
<td>NCR</td>
<td>5.40±0.14</td>
<td>5.92±0.09**</td>
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<tr>
<td>ISDN</td>
<td>6.17±0.01</td>
<td>6.24±0.09</td>
</tr>
<tr>
<td>NTG</td>
<td>7.87±0.07</td>
<td>7.92±0.09</td>
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<tr>
<td>with 30 mM KCl</td>
<td></td>
<td></td>
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<tr>
<td>NCR</td>
<td>4.81±0.03</td>
<td>5.26±0.15*</td>
</tr>
<tr>
<td>ISDN</td>
<td>5.74±0.10</td>
<td>5.61±0.13</td>
</tr>
<tr>
<td>NTG</td>
<td>7.19±0.09</td>
<td>7.29±0.08</td>
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Values represent the mean±S.E. (n=8). *P<0.05, **P<0.01 vs. unrubbed preparations.

response curves for NCR (3x10⁻⁷–3x10⁻⁴ M), ISDN (10⁻⁷–10⁻⁴ M) or NTG (10⁻⁹–3x10⁻⁵ M) were obtained. As shown in Fig. 1B, there were no significant differences between the vasorelaxant effects of ISDN or NTG in the presence and the absence of the endothelium. On the other hand, NCR caused significantly larger relaxation in the absence than in the presence of the endothelium. The ED50 values for relaxation are shown in the lower part of Table 1. Between increments in tone induced by 30 mM KCl in the preparations with and without the endothelium, there were no significant differences.

Effects of methylene blue on relaxant responses to nicorandil (NCR), isosorbide dinitrate (ISDN) and nitroglycerin (NTG): In resting preparations, methylene blue at a concentration of 5x10⁻⁶ M increased the tone by approximately 1 g. The concentration of methylene blue further increased the force in preparations contracted with 30 mM KCl. The rise in tension did not affect the relaxant response (% relaxation) to papaverine, since the concentration-response curves for papaverine (10⁻⁷–10⁻⁴ M) were almost identical in the presence and the absence of methylene blue (not shown). Figure 2 shows that methylene blue (5x10⁻⁶ M) caused significant rightward shift of concentration-response curves for NCR, ISDN and NTG, indicating that in the precontracted preparations, concentration-dependent relaxation in response to the three drugs was significantly inhibited by acute addition of methylene blue. When evaluated in ED50 values (Table 2), the preparations treated with methylene blue were approximately 6.6, 3.5 and 2.6 times less sensitive to the relaxant effects of NTG, ISDN and NCR, respectively, than those not treated with methylene blue.

Effect of N-acetylcysteine (NAC) on nitrate tolerance: After ring preparations were incubated for 10 min with either nicorandil (NCR, 10⁻⁴ M), isosorbide dinitrate (ISDN, 10⁻⁴ M), or nitroglycerin (NTG, 10⁻⁵ M) and contracted with 30 mM KCl, concentration-response curves for the three drugs were constructed. As demonstrated in Fig. 3, the incubation with ISDN and NTG caused comparable 9- and 30-fold shifts, respectively, to the right of the corresponding
Table 2. Effects of methylene blue (MB) on the relaxant responses to nicorandil (NCR), isosorbide dinitrate (ISDN) and nitroglycerin (NTG), and development of tolerance to the three drugs in coronary arteries precontracted with 30 mM KCl.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>MB-treated</th>
<th>Tolerant</th>
</tr>
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<tbody>
<tr>
<td>NCR</td>
<td>4.81±0.03</td>
<td>4.43±0.06**</td>
<td>4.86±0.02</td>
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<tr>
<td>ISDN</td>
<td>5.74±0.10</td>
<td>5.26±0.13**</td>
<td>4.76±0.07***</td>
</tr>
<tr>
<td>NTG</td>
<td>7.19±0.09</td>
<td>6.32±0.05***</td>
<td>5.72±0.10***</td>
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Values represent the mean±S.E. (n=8). **P<0.01, ***P<0.001 vs. controls.

Fig. 3. Concentration-response curves for nicorandil (NCR, circle), isosorbide dinitrate (ISDN, triangle) or nitroglycerin (NTG, square) after incubation with the three nitrates, respectively, in coronary arteries precontracted with 30 mM KCl. Open symbols, not incubated (control vessels); solid symbols, incubated. Each point represents the mean±S.E. (n=8). *P<0.05, **P<0.01, ***P<0.001 vs. controls. There was no significant difference between the extent of KCl-induced contraction of incubated rings (solid columns) and non-incubated ones (open columns).

Concentration-response curve, indicating development of acute tolerance. Tolerance to ISDN and NTG was also defined as a significant increase in ED50 values relative to controls. The incubation with NCR did not develop any tolerance (Table 2). Among the three drugs, NTG was chosen to determine whether NAC might reverse acute tolerance to NTG, and the effect of addition of 5×10⁻⁶ M NAC together with 10⁻⁵ M NTG was assessed. In preparations incubated and not incubated with 10⁻⁵ M NTG, the addition of 5×10⁻⁶ M NAC tended to reduce the degree of acute tolerance to NTG and to potentiate relaxant effects of NTG, although the differences were not significant as seen from changes in ED50 values of NTG which were as follows: without preincubation with 10⁻⁵ M NTG: 7.68±1.66 (×10⁻⁸) M in NTG-treated vessels and 4.23±1.08 (×10⁻⁸) M in NTG/NAC-treated ones, P>0.05, with preincubation with 10⁻⁵ M NTG: 2.26±0.41 μM in NTG-treated vessels, 1.79±0.41 μM in NTG/NAC-treated ones, P>0.05; each n=8.

Discussion

In the isolated coronary arteries precontracted with PGF₂α or KCl, nicorandil as well as isosorbide dinitrate and nitroglycerin caused a concentration-dependent relaxation of the muscle rings. Removal of the endothelium significantly enhanced the relaxant response to nicorandil, while it had no influence on response to isosorbide dinitrate and nitroglycerin. According to the recent report of Shirasaki and Su (16), in the aortic ring segments of the rat precontracted with norepinephrine, the relaxant responses to sodium nitroprusside and sodium nitrite are also significantly augmented by removal of the endothelium. These findings are of interest, but further investigation is needed to elucidate its possible mechanism.

Needleman et al. (17) observed that in rabbit aortic strips incubated with the sulfhydryl alkylating agent, ethacrynic acid, sensitivity to nitroglycerin was reduced. Furthermore, it was suggested that the tolerance to nitroglycerin could be induced by oxidation of sulfhydryl groups (2). These observations had led to the suggestion that the vasorelaxant action of nitrates is closely linked to the availability of critical sulfhydryl groups in vascular smooth muscle. Indeed, it seems that nitrates combine with sulfhydryls within smooth muscle, where they are converted to short-lived compounds, S-nitrosothiols. Consequently, these induce an increase in cGMP production,
which trigger vasodilation (3). Actually, a number of experiments indicated that in bovine coronary arterial smooth muscle, nitrates can relax the muscle and activate the arterial guanylate cyclase under appropriate conditions, and that both muscle relaxation and guanylate cyclase activation are inhibited by methylene blue, an inhibitor of soluble guanylate cyclase (3–5, 18).

In the present experiments, the relaxant response to nicorandil, like nitroglycerin and isosorbide dinitrate, was significantly inhibited by methylene blue, although the degree of inhibition for nicorandil was the weakest among the three drugs. Holzmann (13) investigated some correlation between increases in cGMP and muscle relaxation induced by six nitrocompounds in isolated circular strips of bovine coronary arteries and found a steeper correlation for nicorandil, compared with the other nitrocompounds, suggesting that nicorandil has an additional relaxant component of action which does not depend on cGMP production. Furthermore, it has been reported in both in vitro (19) and in vivo preparations (20–22) that unlike conventional nitrates, nicorandil does not develop either tolerance or cross-tolerance to the other nitrates in terms of vasodilating response. Similar findings were obtained in the present study. Although the experiments by Needleman and Johnson (2) provided the pharmacological basis of nitrate tolerance, it is unlikely that their hypothesis can simply explain the phenomenon (the lack of tolerance or cross-tolerance to the other nitrates) obtained with nicorandil which is uncharacteristic of conventional nitrates. Thus, additional mechanisms for vasorelaxation of nicorandil should be taken into consideration.

According to the first documentation of Yanagisawa and Taira (23) in canine atrial muscle, nicorandil increases potassium conductance of the sarcolemma without mediation through muscarinic receptors there. Successively, Furukawa et al. (24), using the microelectrode and double sucrose gap methods, investigated the effects of nicorandil on smooth muscle cells of porcine and guinea-pig coronary arteries, and they suggested that the vasorelaxant action of nicorandil, unlike that of nitroglycerin or Ca<sup>2+</sup>-antagonists, is mainly due to an increase in the potassium conductance in the membrane of coronary smooth muscle. More recently, the pharmacological characteristics of nicorandil have also been observed in the isolated guinea-pig trachealis (25) and in a variety of responses of the aorta and portal vein of the rat (26). Taken the unique properties of nicorandil together into consideration, it might be possible to partly explain the additional vasorelaxant mechanism of nicorandil.

In the present experiments, it was noted that tolerance to nitroglycerin was not reversed significantly by N-acetylcysteine, although the concentration of N-acetylcysteine (5 x 10<sup>-5</sup> M) used here was similar to that associated with therapeutic use of the drug (27). Our present results are inconsistent with those obtained in humans (27) and in rings of bovine coronary artery (15). N-acetylcysteine is of interest as a sulfhydryl, and in particular, a cysteine source. However, it is likely that even though hydrolysis to cysteine was extensive in vivo (28), it presumably occurs only to small extent in isolated vessel preparations, leading to little appreciable reversion of nitrate tolerance. Actually, Lieberman et al. (29) could not reverse tolerance to nitroglycerin by pretreatment with N-acetylcysteine in the isolated bovine intrapulmonary artery.

In summary, nicorandil is chemically an organic nitrate, and its vasodilator effect depends on a nitrate moiety in the chemical structure (7, 12). However, it appears that the mode of vasodilator action of nicorandil is somewhat different from that of nitroglycerin or isosorbide dinitrate. In the present experiments, it was particularly interesting that the relaxant response to nicorandil, unlike that to nitroglycerin or isosorbide dinitrate, was significantly augmented by removal of the endothelium. The integrity of the vascular endothelium is clearly important for the maintenance of normal vascular function, and the damage of the endothelium may possibly become the site of coronary spasm (30). Therefore, it is expected that the augmented relaxant effect of nicorandil observed in the rubbed preparations may play some role in the antianginal therapy, even though the
precise mechanism remains to be elucidated.

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