The effect of substituted sydnonimines on coronary smooth muscle relaxation and cyclic guanosine monophosphate levels

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ABSTRACT In vitro experiments on precontracted canine coronary arteries were performed to study the direct relaxant effects of mosidomine (MOLS) and its active metabolite, SIN-1, and to determine if there is a relationship between effect and cGMP level elevations. The effects of MOLS and SIN-1 were compared with those of a classic vasodilator, nitroglycerin (NTG). At equimolar doses (10^{-6}M) SIN-1 exerted greater relaxant effect than NTG (80 \pm 2\% and 60 \pm 5\%, respectively) in spite of the fact that it produced less of an increase in cyclic guanosine monophosphate (cGMP) levels. cGMP levels fell rapidly after they peaked, but relaxation was maintained. cGMP elevation preceded the induction of relaxation by NTG but not that induced by SIN-1. Relaxation occurred faster after NTG than after SIN-1. Since SIN-1 has a greater relaxant effect than NTG in spite of the fact that SIN-1 induces less of an increase in cGMP levels and the fact that the peak elevation does not precede the onset of relaxation, the causal nexus between GMP level elevation and relaxation effect after sydnonimines should be challenged.

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SUBSTITUTED SYDNONIMINES were recently introduced for the treatment of angina pectoris. Molsidomine (MOLS), an inactive compound, must be converted in the liver to SIN-1, which in turn is nonenzymatically transformed into SIN-1A in the blood stream. SIN-1A is finally transformed into SIN-1C by the release of nitric oxide, which is apparently responsible for the smooth muscle relaxation elicited by these compounds and by nitrates and nitrates. The chemical structures of these compounds are shown in figure 1. The mechanisms leading to coronary smooth muscle relaxation after exposure to these drugs are also a matter of controversy. Increased levels of 3′5′-guanosine monophosphate (cGMP) were found in arteries relaxed by nitrates, nitrates, and substituted sydnonimines. Some authors have therefore suggested that cGMP is the intracellular mediator of the relaxant action of these drugs, but such a role was denied or considerably deemphasized in other studies.

Clinical and experimental studies suggest that MOLS acts like nitroglycerin (NTG), but that the effects of MOLS last longer. However, whereas the direct relaxant effect of NTG on coronary muscle has been demonstrated in in vitro and in vivo studies, less information is available concerning substituted sydnonimines. Considering that an opposite effect of NTG and MOLS on myocardial blood flow has been described, the in vitro action of substituted sydnonimines on coronary smooth muscle deserves further study.

We carried out in vitro experiments to determine the effects of substituted sydnonimines on isolated coronary smooth muscle and to study the possible role of cGMP as intracellular mediator of the pharmacologic action of these drugs. MOLS and its derivative, SIN-1, were used, and the results were compared with those obtained with a classic vasodilator, NTG. Our initial results in these experiments have been presented in preliminary form.

Methods
Mongrel dogs of both sexes were anesthetized with sodium pentobarbital (35 mg/kg) and killed by rapid excision of the heart. Sections of either the circumflex or the anterior descending branches of the left coronary artery were obtained and
stripped of loose fat and connective tissue. Helical strips (2 to 3 mm wide, 0.2 to 0.3 mm thick, and 15 to 20 mm long) were cut and vertically mounted in a water-jacketed muscle bath filled with Krebs-Henseleit (K-H) solution of the following composition (in mM): NaCl 118, KCl 5.32, NaH₂PO₄ 1.54, MgSO₄ 1.19, NaHCO₃ 24.9, CaCl₂ 2.53, EDTA 0.01, glucose 5.6. This solution was aerated with 5% CO₂ in O₂ (pH 7.42 ± 0.01) and maintained at 37°C. The inferior end of the strip was attached to a stationary post and the other end was rigidly suspended from a Grass FT.03C force displacement transducer, the output of which was driven to an oscillographic recorder. The strips were stretched 40% beyond their initial or unstretched length to obtain an average resting tension of 1.78 ± 0.14 g after 1 hr of stabilization. This stretch was found to be the optimal for tension development and was used in all experiments. At the end of the stabilization period a-contracture was elicited by replacing the standard K-H solution with another containing 35 mM KCl; the solution was kept isosmotic by equimolar reduction of the NaCl concentration. Active tension induced by KCl depolarization was similar in the different experimental groups. After 15 min the contracture stabilized and the following interventions were carried out.

Dose-effect curves. SIN-1 or NTG was added to the bath to increase the drug concentration in 10-fold steps from 10⁻⁹ to 10⁻³ M. The relaxation induced by each dose was determined and plotted as a percentage of the previous contracture. From the curve created by data from each experiment the concentration that produced definite percentages of the maximal relaxation (from 10% to 90% in increments of 10) were interpolated. These concentrations (i.e. ED₁₀, ED₂₀, ED₃₀, and so on) were then averaged and plotted as a function of the amount of relaxation obtained (figure 2). This method permitted the statistical evaluation of the difference between the effective doses determined for both compounds. The contractures (NTG = 1.86 ± 0.6 g, SIN-1 = 1.93 ± 0.7 g) and amounts of maximal relaxation obtained (NTG = 76 ± 2% of previous contracture, SIN-1 = 83 ± 5%) did not differ significantly between the groups, making valid the comparison between effective doses. Each strip of artery was exposed to only one drug.

Single-dose experiments. SIN-1 or NTG were added to the bath to obtain a final concentration of 10⁻⁶ M, and the mechanical effects were followed for up to 7 hr. In this type of experiment the amount of relaxation obtained was calculated as a percentage of the previous contracture and plotted as a function of time (figures 3, 4, 6, and 7). Contractions that occurred without the addition of drug during the 7 hr were used as controls. The time to reach half-maximal relaxation (t½) was calculated in each experiment. Each strip of artery was exposed to only one drug.

Experiments to detect changes in cyclic adenosine monophosphate (cAMP) and cGMP levels. These studies were carried out with the use of the same mechanical method, but at specific times after the addition of drug the strips were frozen, pulverized, and stored at −85°C. Control experiments were conducted simultaneously in a second organ bath and the strips were frozen at the same time, but the drug addition step was omitted. The organ baths could be quickly dropped and the strips were immediately frozen with a precooled clamp and then pulverized while immersed in liquid nitrogen. The entire procedure took about 2 sec. To measure cGMP levels about 20 mg of the frozen tissue samples was homogenized at 4°C in 500 µl of 6% trichloroacetic acid with ³H-cGMP (approximately 1500 counts/min) as a tracer. The homogenate was centrifuged at 12,000 g at 4°C for 20 min and the supernatant was extracted four times with 5 volumes of water-saturated diethyl ether. The ether phase was discarded and the aqueous phase was lyophilized and resuspended in 50 mM acetate buffer, pH 6.2. The same procedure was followed to measure cAMP levels, but about 5 mg of the frozen tissue samples was homogenized in 400 µl of 6% trichloroacetic acid with approximately 6000 counts/min of ³H-cAMP. cGMP and cAMP levels were determined in duplicate by radioimmunoassay31, 32 with acetylation of the samples33 as supplied by New England Nuclear. For cAMP antiserum the number of picomoles necessary to produce 50% displacement of the labeled cAMP were, with acetylation of the samples, 0.08 for cAMP and 200 for cGMP. For cGMP antiserum the same values were 0.1 for cGMP and 60 for cAMP. The protein contents of the samples were calculated34 and cyclic nucleotide content was calculated as picomoles per gram of wet tissue (pmol/gt) and as picomoles per milligram of protein (pmol/mg). The same results were obtained when either form of expression was used, and for this reason we used only the more usual notation (pmol/gt).

Drugs. NTG solution was freshly prepared every day by diluting standard commercial tablets (NTG tablets USP, Eli Lilly & Co., Indianapolis) crushed to powder in K-H solution. MOLS and SIN-1 were used as supplied by the manufacturer (Cassella AG, Frankfurt).

Comparison between groups was carried out by the analysis of variance35 and a modified t statistic for comparison between two specific means. All the data were expressed as mean ± SEM. Significance was accepted at the p < .05 level.

Results

Figure 2 shows dose-effect curves for NTG and SIN-1. A concentration of 10⁻⁶ M elicited similar amounts of relaxation with both drugs. However, when administered as a single dose of 10⁻⁶ M, NTG produced relaxation coincident with that shown in the dose-effect curve (60 ± 5% and 60 ± 4%, respectively), whereas SIN-1 produced a greater effect than would be predicted from the illustrated dose-effect relationship (86 ± 2% and 65 ± 4%, respectively). The interval of almost 2 hr between the first and the last doses of SIN-1 in the cumulative dose-response curve could be the reason for this discrepancy, the delay causing either some degree of inactivation of the compound or the

**FIGURE 1.** Chemical structure of substituted sydnomines. Note that only SIN-1 and SIN-1A activate guanylate cyclase and increase intracellular GMP. GTP = guanosine triphosphate.
overshoot phenomenon illustrated in figure 3. Whatever the reason for this difference, it does not seem to change the conclusions of our study.

Figure 3 shows that the KCl-elicited contracture was stable for the first hour and then on decayed progressively, stabilizing at 83 ± 6% of the control values after the fourth hour. cGMP levels also varied during this period (figures 4 and 5); content was 31.4 ± 8.5 pmol/gt before the contracture and a progressive fall was observed thereafter. The fall became significant at 10 and 20 min (7.2 ± 2.9 pmol/gt and 7.5 ± 3.1 pmol/gt, respectively). The first hour value was not significantly lower than that before contracture (17.6 ± 2.9 pmol/gt), but the seventh hour value showed a decrease (8.6 ± 1.7 pmol/gt; p < .05). In view of these results the data obtained after pharmacologic intervention were compared with control data obtained at the corresponding time points in KCl-contracted arteries not exposed to a drug.

Figure 3 illustrates the effects over 7 hr of 10^{-6}M doses of the drugs used. MOLS elicited slight but significant relaxation after 1 hr (16 ± 7%). At this time SIN-1 and NTG induced 85 ± 2% and 60 ± 5% relaxation, respectively (p < .01). A spontaneous recovery of tension occurred after 7 hr of NTG, whereas an overshoot with respect to control values was observed after 7 hr of SIN-1.

Figure 5 shows the cAMP and cGMP levels after 1 and 7 hr of pharmacologic intervention. No significant variations in cAMP levels were detected at the first hour, while a significant decrease in cAMP was present in the MOLS-treated strips after 7 hr (from 363 ± 76 pmol/gt to 113 ± 24 pmol/gt). cGMP in the MOLS-treated strips increased significantly at the first hour, from 17.6 ± 2.9 to 29.4 ± 5.0 pmol/gt, while we were unable to detect any significant variation after SIN-1 and NTG at this particular time. At the seventh hour cGMP levels were not significantly altered in MOLS- or SIN-1-treated strips, but they did increase from 8.6 ± 1.7 to 32.0 ± 9.7 pmol/gt (p < .05) in NTG-exposed strips.

During the first 20 min of exposure to a single dose of 10^{-6}M MOLS, tension remained unchanged and

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**FIGURE 2.** Dose-effect curves for NTG (n = 5) and SIN-1 (n = 6) in coronary artery strips contracted with 35 mM KCl. ED_{50} for the two drugs did not differ significantly, but at lower concentrations NTG induced greater relaxation than SIN-1. | M | = molar concentration, *p < .05. Bars represent 1 SEM.

**FIGURE 3.** Time course of tension in high KCl-contracted coronary arterial strips not exposed to pharmacologic intervention (control, n = 33) and after the addition of a single dose of 10^{-6}M MOLS (n = 11), SIN-1 (n = 13), and NTG (n = 14). DT = developed tension. Vertical bars represent 1 SEM.

**FIGURE 4.** Tension developed in coronary arterial strips exposed to high KCl and cGMP contents at specific time points after stabilization of contracture. Number of experiments for control, and 1, 3, 10, and 20 min were 15, 12, 9, 8, and 8, respectively. Abbreviations are as in figures 2 and 3. Note that the cGMP axis has been expanded four times with respect to those on figures 6 and 7.
cGMP levels showed a tendency to increase, although not significantly so at these particular times.

Figure 6 depicts the time course of tension and cGMP levels during the first 20 min of exposure to a single dose of 10^{-6}M SIN-1. Significant relaxation was present, and the $t_{1/2}$ was 249 ± 27 sec. cGMP levels were lower than in control arteries in the first minute, not significantly augmented in the third, reached a peak value after 5 min (214 ± 28 pmol/gt; p < .05), and decreased after 20 min of the intervention.

Figure 7 depicts the time course of tension and cGMP levels during the first 20 min of exposure to a single dose of 10^{-6}M NTG. There was significant relaxation with a $t_{1/2}$ of 54 ± 7 sec; cGMP level values peaked at 15 sec (203 ± 34 pmol/gt; p < .05) and decreased thereafter.

The relationship between milligrams of tissue and milligrams of protein did not change significantly (9.20 ± 1.24 to 11.07 ± 1.30; NS) after exposure to high-KCl solution and remained within the same range after contracture was established.

**Discussion**

After 1 hr of pharmacologic intervention a slight but significant decay compared with control contracture was present in arteries treated with MOLS, while a frank relaxation of 85 ± 2% was elicited by SIN-1. This greater effect of SIN-1 could be expected in view of the metabolic transformations that MOLS must undergo in order to be active. The small direct relaxant action of MOLS, however, is difficult to explain, and could have been due to some degree of breakdown of the compound used. This fact could also explain the increase in cGMP levels detected at this time in MOLS-treated strips.

When the mechanical effect was analyzed over 7 hr, no significant relaxation was present with MOLS after the first hour. The relaxant effect of SIN-1 and NTG progressively faded out, and the former produced an overshoot with respect to control levels of contracture. An explanation for this phenomenon did not emerge from this study, but might have some similarity to the "rebound" described in man after NTG^{17} or nitroprusside^{38} withdrawal.
Cyclic nucleotide content was analyzed to correlate it with the mechanical effects observed. The values reported in our study are similar to those found by some investigators,4,7, 11, 12 but differ from those reported by others.8, 9 Reasons for these discrepancies might be the different species, tissues, methods, or control values used.

After 1 hr cAMP and cGMP levels were not significantly altered by SIN-1 and NTG, despite the fact the drugs exerted a pronounced relaxant effect. On the contrary, cGMP levels were increased with MOLS at this time, and this was accompanied by a slight relaxant action. Seven hours after pharmacologic intervention cAMP levels significantly decreased in the MOLS-treated strips. At this time cGMP levels showed no variations after MOLS or SIN-1, in spite of the lack of relaxant action of the former and the overshoot in tension produced by the latter. NTG, on the other hand, produced an increase in cGMP levels compared with control, and there was no significant change in developed tension after 7 hr of exposure to the drug. Based on these results we could not demonstrate an unequivocal relationship between increase in cyclic nucleotide levels and relaxation after drug intervention since the phenomena were often dissociated.

Additional cGMP determinations were carried out to explore the possibility of earlier changes in cGMP levels, as shown by others.4-6, 8, 11-13, 15, 24 MOLS produced a slow and progressive elevation in cGMP levels up to the first hour, which was the only statistically significant value compared with control. NTG and SIN-1 produced transient elevations in cGMP, with peaks at 30 sec and 5 min, respectively. After these peaks cGMP levels began to decrease in both cases, while relaxation was still present and progressing. Relaxation reached the maximum and then diminished up to the seventh hour. Loss of relaxation was accompanied by an elevation in cGMP levels in the case of NTG-exposed arteries, but these levels did not change significantly in the case of the SIN-1-exposed segments. Time courses for cGMP concentrations similar to those described here after NTG were recently reported.15

When, in in vivo experiments, the effects of MOLS on myocardial blood flow were studied, a decrease in myocardial blood flow was observed.20 The coronary blood flow is the result of the interplay of several variables, some acting directly and others acting through changes in myocardial oxygen consumption. These factors are perhaps the most important in determining the arteriolar tone, which is modulated through local concentration of adenosine. The decrease in myocardial oxygen consumption brought about by MOLS has been reported26 and probably reflects the decrease in wall tension produced by the decrease in both cardiac size and aortic pressure. With this consideration in mind, a decrease in total coronary blood flow with a dilatation of conduit arteries would be an expected finding after exposure of arteries to this compound. This in fact was mentioned by Dr. W. Schaper as an unpublished finding in a recent article.22

The cellular mechanism by which these compounds are eliciting smooth muscle relaxation is not apparent to us at this time. Whenever relaxation was induced, cGMP levels were transiently elevated. cGMP levels fall rapidly after a peak, whereas relaxation is maintained. This fact suggests either that there is no cause-effect relationship between the phenomena or that cGMP elevation is triggering intermediate events leading to smooth muscle relaxation. cGMP elevation precedes the relaxation elicited by NTG, but not the one following SIN-1. Furthermore, the magnitude of elevations in cGMP is greater after NTG than after SIN-1 whereas the relaxation effect is smaller with NTG than with SIN-1. Relaxation took place faster after NTG than after SIN-1.

The results of the experiments presented here show a direct effect of the metabolite SIN-1 on coronary relaxation of precontracted coronary arteries and suggest that, in the presence of coronary obstruction localized to a single territory, substituted sydnonimines would augment collateral blood flow through vasodilation of collateral vessels or of the conduit arteries from which they arise. Also, in presence of severe coronary narrowing or spasm the relief of ischemia by these drugs may be due to relaxation of smooth muscle at the site of the stenosis. Whether this is a principal or contributory reason for their benefits will depend on the clinical circumstances.59

In summary, we demonstrate a strong direct relaxant effect of sydnonimines on precontracted canine coronary arteries. In equimolar doses, the relaxant effect of these compounds is greater than that of NTG, although the increase in cGMP levels is smaller. The transitory increase in cGMP levels does not precede the onset of relaxation. Based on our findings, the previously proposed theory of cGMP level elevation as the mechanism producing coronary relaxation following sydnonimines should be challenged.

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