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# Altered Responsiveness of Guanylyl Cyclase to Nitric Oxide Following Treatment of Cardiomyocytes with *S*-Nitroso-D,L-acetylpenicillamine and Sodium Nitroprusside

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**The stimulation of cardiomyocyte guanylyl cyclase by nitric oxide (NO)-donor drugs was examined before and after exposure of these cells to the NO-donor drugs: S-nitroso-d,l-acetylpenicillamine (SNAP) and sodium nitroprusside (SNP). Short- (2-hr) and long-term (24-hr) exposure attenuated the maximal stimulation of GC by either SNAP or SNP by up to 80% ("desensitization"). However this "desensitization" of the myocardial GC was atypical in nature in that the reduction in maximal NO-stimulated GC activity was associated with an increase in the affinity of the GC towards either NO-donor, a finding not as yet reported. There was also evidence of "cross-desensitization" of GC (e.g., SNAP exposure decreasing the stimulatory effect of SNP). Further, this is the first time that SNAP-induced desensitization of GC has been observed.** 

Cytosolic guanylyl cyclase is readily and potently stimulated by nitric oxide (NO) in all tissues including the myocardium (1). The myocardium is chronically exposed to NO during organic nitrate therapy commonly used in the treatment of angina and congestive heart failure (2). In septic shock endogenous NO synthesis is dramatically increased, including in the myocardium, as a result of cytokine mediated induction of NO synthase type II (3). Whether the regulation of guanylyl cyclase by NO is modulated by chronic exposure of the tissues to NO or to NO-donor drugs, particularly the myocardium has not been delineated. This will be of interest in view of the documented role of the NO/GC/cGMP system in myocardial inotropic and chronotropic responses (4-6). The present study examined responsiveness of GC in cardiomyocytes exposed for short and long periods to the NO-donors, SNAP and SNP. The results, which clearly document unique alterations in the GC characteristics consequent to such exposures, are presented along with their potential significance.

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## MATERIALS AND METHODS

*Materials.* SNAP was obtained from Research Biochemicals (Natick, Mass.). Collagenase Type II was from Worthington Enzymes (Freehold, NJ) and fetal bovine serum was from Gibco (Burlington, ON). AG50W-X4 resin was purchased from Bio-Rad (Mississauga, ON). [$^{32}$P-$\alpha$] GTP and [$^{3}$H] cGMP were obtained from Dupont-NEN (Mississauga, ON). Ready Gel scintillation fluid was from Beckman (Mississauga, ON). All other reagents were obtained from Sigma Chemical Co. (St. Louis, MO).

*Isolation and culture of adult rat ventricular cardiomyocytes.* All animals were cared for under the guidelines provided by the Canadian Council on Animal Care. Ventricular cardiomyocytes were isolated from male Wistar rats (250-350g) utilizing a previously described methodology (7). The myocytes were then suspended in culture medium [Dulbecco's modified Eagle's medium supplemented with 2 % fetal bovine serum, 5 mM creatine, 2 mM L-carnitine, 5 mM taurine, 0.1 $\mu$M insulin, 250 U/ml penicillin G and 250 $\mu$g/ml streptomycin sulfate] and placed in 100 mm polycarbonate tissue culture dishes. Cardiomyocytes were maintained at 37°C in a humidified tissue culture incubator under an atmosphere of 5% $CO_2$/95% air. For the NO-donor treatments SNAP or SNP was dissolved in culture medium and then added to the dishes containing cardiomyocytes. The final concentration of cardiomyocytes for all treatments was 250,000/ml.

*Preparation of cardiomyocyte cytosolic fraction.* The cardiomyocytes (control and treated) were harvested by centrifugation for 30 sec at 25 × g, and then the cell pellet was then washed three times by resuspension in 5 ml of oxygenated Earle's balanced salt solution [117 mM NaCl, 5.3 mM KCl, 0.8 mM $MgCl_2$, 1 mM $NaH_2PO_4$, 1.26 mM $CaCl_2$, 26 mM sodium bicarbonate, 5.6 mM D-glucose, 25 mM HEPES, pH 7.45], followed by centrifugation as described above. The cells were then suspended in homogenization buffer [50 mM HEPES, 1 mM dithiothreitol (DTE) and 0.1 mM phenylmethylsulfonylfluoride, pH 7.4] at a concentration of $2.5 \times 10^6$ cells/ml and homogenized using a Polytron homogenizer. A cytosolic fraction was obtained, as a supernatant, by centrifuging the homogenate for 1 hr at 4°C and 100,000 × g. The protein concentration of the cytosolic fractions ranged from 1.5 - 2 mg/ml.



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*Guanylyl cyclase assay using cardiomyocyte cytosolic fraction.* Guanylyl cyclase activity was measured in the cytosolic fraction essentially as described previously (8,9). A 30 $\mu$l aliquot of protein (50 $\pm$ 10 $\mu$g) was added to the assay mixture containing the following: 50 mM HEPES pH 7.5, 1 mM EGTA, 1 mM 3-isomethyl-1-butylxanthine, 2 mM $MgCl_2$, 1 mM DTE, 1 mM cGMP, 5 mM phosphocreatine, 50 $\mu$g creatine phosphokinase and 2000-3000 dpm of [$^3$H]-cGMP in a final assay volume of 0.15 ml. The tubes were preincubated 5 min at 30°C, and then either $H_2O$ (for basal activity) or NO-donor (SNAP or SNP) was added and the tubes were incubated for another 5 min at 30°C. The reaction was started by the addition of [$^{32}$P-$\alpha$]-GTP (0.2 mM final, specific activity 20-25 dpm/pmol), was carried out for 15 min at 30°C and then was terminated by the addition of 0.15 ml stop solution (2% SDS containing 1mM GTP). [$^{32}$P]-cGMP was then isolated by ion exchange chromatography using Dowex (AG 50W-X4, 200-400 mesh, $H^+$ form) and neutral alumina columns (8). [$^{32}$P]-cGMP formation was determined by liquid scintillation counting using Ready Gel scintillation cocktail. Using this chromatographic protocol the recovery of cGMP, as measured using [$^3$H]-cGMP as a marker, was in the 55-65% range. The formation of [$^{32}$P]-cGMP was found to be linear with respect to time of incubation and protein concentrations used. Protein concentrations were determined using the Lowry method.

*Lactate dehydrogenase [LDH] assay.* LDH activity of the culture medium and cardiomyocytes was determined according to the instructions provided with a commercially available kit (Sigma).

*Statistical analysis.* Student's t-tests were performed on the data using the computer program Statview 4.01 for Macintosh. A $p < 0.05$ was considered to be statistically significant.

## RESULTS

### *Short-term (2 hr) NO-Donor Treatment*

Treatment of cardiomyocytes for 2 hr with SNAP or SNP had no significant effect on basal GC activity as shown in Table 1, but the treatment did result in some very interesting effects upon the ability of the NO-donors to produce a subsequent stimulation of GC. Concentration-response curves for SNAP and SNP from a representative experiment are shown in Figure 1, Panels A and B. From these graphs and the cumulative data from 4-7 similar experiments (see Table 1) it is apparent that the maximal SNAP and SNP stimulated GC activity was lower in the cytosols from NO-donor treated cardiomyocytes. Treatment of the cardiomyocytes with SNAP decreased both SNAP and SNP stimulated GC activity by 20-25%, while SNP treatment resulted in a reduction of maximal GC activity for both NO-donors of approximately 33% (Table 1). To determine $EC_{50}$ values for SNAP and SNP the data in Figure 1, Panels A and B was normalized and replotted in Figure 1, Panels C and D. From these graphs it is evident that 2 hr treatment of cardiomyocytes with SNAP or SNP produced a leftward shift of the concentration-response curves for both SNAP and SNP. Cumulative $EC_{50}$ values from 4-7 experiments are presented in Table 2. While 2 hr treatment of the cardiomyocytes with either NO-donor produced a significant ($p < 0.05$) decrease in the $EC_{50}$ values for SNAP, there was a much more pronounced reduction of the $EC_{50}$ values for SNP. The described changes in the responsiveness of GC to NO resulting from the 2 hr treatment with SNAP or SNP were not associated with change in the LDH activity of either the culture medium or the cells (data not shown). Thus, the decreased maximal NO-stimulated GC activity is not a result of a loss of cardiomyocyte viability during this treatment protocol.

### *Long-Term (24 hr) NO-Donor Treatment*

24 hr treatment of the cardiomyocytes with SNAP or SNP produced a similar pattern of changes to those seen following 2 hr treatment. The results of a representative experiment are shown in Figure 2. As was the case in the 2 hr treatment, the curves were left-shifted when compared to control (Figure 2, Panels C and D). The $EC_{50}$ values for both SNAP and SNP were significantly reduced compared to control but there was no difference between the 24 hr and 2 hr treatment $EC_{50}$s (Table 2). The long-term exposure to either NO-donor also produced a reduction in the maximal SNAP and SNP stimulated GC activity which was significantly greater in magnitude than that resulting from the 2 hr exposure (Table 1). Once again, the degree of this reduction was dependent upon the NO-donor utilized in the treatment. SNAP treatment produced a 60% reduction for both SNAP and SNP stimulated GC activity while in the case of SNP treatment, the reduction was approximately 80%. 24 hr treatment of the cardiomyocytes with either SNAP or SNP resulted in a modest, but statistically significant (12%), increase in the LDH activity of culture medium (data not shown), which further indicated that the vast majority of the cardiomyocytes were viable at the time of the above noted unique alterations in the cardiomyocyte GC.

## DISCUSSION

The results of this study demonstrate that NO-stimulated cGMP synthesis in adult rat ventricular cardiomyocytes is significantly decreased following short or long term exposure to NO-donors. Desensitization of GC to NO was seen as a reduction in the maximal NO-stimulated GC activity in the cytosols from the treated cells (Table 1). The magnitude of the desensitization was dependent upon the duration of exposure to the NO-donors with the 24 hr treatment resulting in a 2 to 3-fold greater degree of GC desensitization than the 2 hr treatment. The time course of GC desensitization observed in this study is similar to that seen in a recent report where SNP-induced GC desensitization in rat kidney cells began after 3 hrs and was maximal between 15-27 hrs of SNP treatment (10). In our study the degree of desensitization was also dependent upon the NO-donor used during the treatment. SNP produced a larger desensitization than SNAP for both treatment durations. It is unclear why SNP is more

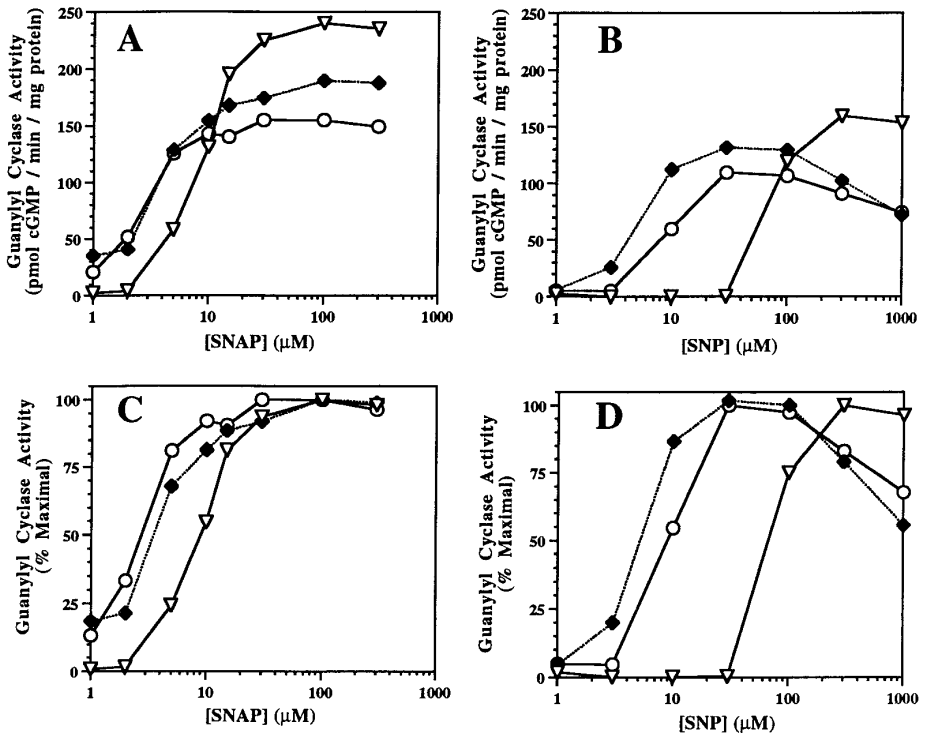


**FIG. 1.** NO-donor stimulated GC in cytosols from 2 hr treated cardiomyocytes. Panels A and B show the results of a representative experiment in which the cytosols from cardiomyocytes treated for 2 hrs in the absence (open triangle) or presence of 1 mM SNAP (closed diamond) or 1 mM SNP (open circle) were incubated with varying concentrations of SNAP or SNP. The data from Panels A and B respectively were normalized with the maximal SNAP (Panel A) and SNP (Panel B) stimulated GC activity for each cardiomyocyte treatment set at 100% and then plotted in Panels C and D.

effective at producing the desensitization. The maximal SNAP stimulated GC activity was greater than that elicited by SNP and this might suggest that perhaps more NO was released from SNAP than SNP under the experimental conditions used. If this were true, then the results raise the possibility that NO-independent mechanisms may have also contributed to the observed SNP-induced GC desensitization.

"Cross-desensitization" of GC was produced by treatment of the cardiomyocytes with either NO-donor. That is, SNP or SNAP treatment produced desensitization of GC to both SNAP and SNP. Interestingly, the degree of desensitization to SNAP and SNP was similar for a given treatment. For example, 2 hr SNAP treatment decreased maximal SNAP and SNP stimulated GC activity by 22 and 25% respectively. Desensitization and

**TABLE 1**

Effect of SNAP or SNP Treatment of Cardiomyocytes upon Basal GC Activity and Maximal SNAP- or SNP-Stimulated GC Activity

| | Guanylyl Cyclase Activity (pmol cGMP/min/mg protein) | | |
|---|---|---|---|
| Treatment | Basal | Maximal SNAP | Maximal SNP |
| 2 hr | | | |
| Control | 0.52 ± 0.36 | 261.00 ± 25.78 | 172.65 ± 16.97 |
| 1 mM SNAP | 8.08 ± 3.67 | 202.59 ± 27.00† | 128.73 ± 19.60* |
| 1 mM SNP | 4.20 ± 1.40 | 173.64 ± 9.76* | 115.65 ± 5.72† |
| 24 hr | | | |
| Control | 1.28 ± 0.50 | 260.57 ± 27.78 | 166.52 ± 13.61 |
| 1 mM SNAP | 8.84 ± 2.30* | 102.89 ± 22.78*§ | 64.87 ± 10.74*$\pi$ |
| 1 mM SNP | 3.71 ± 1.09† | 46.50 ± 6.89*$\Delta$ | 32.38 ± 4.31†$\Delta$ |

*Note.* The values in the table represent the mean ± SEM of 4–7 experiments. (*$p < 0.05$, †$p < 0.01$ vs control), (§$p < 0.05$, $\pi p < 0.01$, $\Delta p < 0.001$ vs 2 hr treatment).

**TABLE 2**

$EC_{50}$ Values for SNAP and SNP in Cytosols from Untreated (Control) and NO-Donor Treated Cardiomyocytes

| | $EC_{50}$ ($\mu$M) | |
|---|---|---|
| Treatment | SNAP | SNP |
| 2 hr | | |
| Control | 9.06 ± 0.07 | 69.43 ± 7.62 |
| 1 mM SNAP | 4.13 ± 0.99* | 6.58 ± 3.15* |
| 1 mM SNP | 3.05 ± 0.93* | 6.20 ± 2.05* |
| 24 hr | | |
| Control | 9.43 ± 1.83 | 57.1 ± 7.68 |
| 1 mM SNAP | 1.84 ± 0.31† | 0.16 ± 0.06† |
| 1 mM SNP | 2.75 ± 0.83* | 2.80 ± 0.55† |

*Note.* $EC_{50}$ values were derived graphically as described in Methods. Data are expressed as mean ± SEM from 4-7 experiments. (*$p < 0.05$, †$p < 0.01$ vs control.

cross-desensitization of GC resulting from *in vitro* and *in vivo* SNP treatment has previously been reported in vascular tissue (11,12,13), rat lung fibroblasts (14) and rat kidney cells (10). To our knowledge, ours is the *first* study to document cross-desensitization of GC to SNAP resulting from treatment with SNP. In fact, the studies to date have shown that SNAP-stimulated GC activity is not affected by prior exposure to any NO-donor including SNAP itself. *In vitro* (<1hr) and *in vivo* (5-24 hr) SNAP treatments have been shown to have very little (15) or no effect (16, 17,18) upon the vasodilatory/ hypotensive action of SNAP. Since NO is believed to elicit vasodilatation through GC stimulation these studies indirectly suggest that SNAP treatment does not desensitize GC. A recent *in vivo* study also demonstrated that SNAP-mediated increases in vascular tissue cGMP levels were not altered following administration of SNAP intravenously for 16 hr (13). It is possible that adult rat ventricular cardiomyocytes possess a unique NO-stimulatable GC which is readily desensitized to SNAP.

While our study has not investigated the mechanisms by which GC desensitization is produced other reports have found that it can result from a decrease in the amount of GC or a stable modification of GC. Desensitization of GC in rat kidney cells by an 18 hr SNP treatment was associated with reduced levels of mRNA for both the $\alpha_1$ and $\beta_1$ subunits of GC. Furthermore, in the same study the GC desensitization was shown to be cGMP dependent (10). In another study, GC desensitization as a result of decreased GC abundance was postulated because the reversal of the desensitization required *de novo* synthesis of a protein, per-

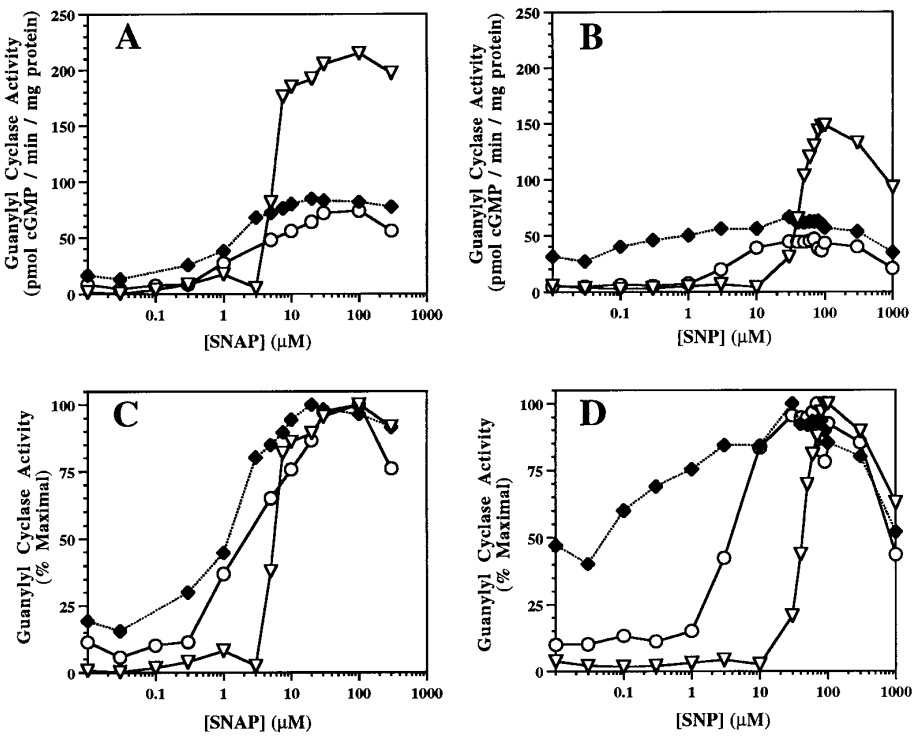


**FIG. 2.** NO-donor stimulated GC in cytosols from 24 hr treated cardiomyocytes. Panels A and B show the results of a representative experiment in which the cytosols from cardiomyocytes treated for 24 hrs is the absence (open triangle) or presence of 1 mM SNAP (closed diamond) or 1 mM SNP (open circle) were incubated with varying concentrations of SNAP or SNP. The data from Panels A and B respectively were normalized with the maximal SNAP (Panel A) and SNP (Panel B) stimulated GC activity for each cardiomyocyte treatment set at 100% and then plotted in Panels C and D.

haps GC (14). Desensitization of GC resulting from a stable modification of GC was first hypothesized by Waldman *et al* (19). Subsequently it has been postulated that oxidation of critical free thiol groups of GC is the mechanism by which some NO-donors produce desensitization of GC (13). In our experiments however, the reducing agent DTE (1 mM) was present during homogenization and in the GC assay.

For the *first time* we report leftward shifts in the concentration-response curves of GC for both SNAP and SNP following NO-donor treatment of cardiomyocytes for 2 or 24 hrs. One possible explanation for this finding is that the NO-donor was taken up by the cardiomyocytes during the treatment period, which following washing, homogenization and centrifugation of the cells, may have been carried over into the subsequent GC assay. Experiments in our laboratory using N-acetyl-d,l-penicillamine (AP), a NO-deficient SNAP analog, have provided observations which do not support the "NO-donor uptake hypothesis" (Davis, Vo and Sulakhe, unpublished). Furthermore, the stimulation of GC by NO-donors is extremely powerful and thus, if the NO-donors were being taken up by the treated myocytes in any significant amounts, a much larger increase in basal GC activity relative to that observed would have been anticipated.

An alternate postulate to explain the left shift of the concentration response curves is that NO itself and not the NO-donors is being carried over into the GC assay. When NO comes into contact with a cell it will interact with several different "targets" including thiols, oxygen free radicals, heme and probably other targets unidentified as yet (20). Within a cell there is a fixed number of "NO-binding sites/targets", each target possessing its own affinity for NO. In our experimental conditions, the two targets of NO that will be considered to be of importance are protein and non-protein thiols and the heme-containing enzyme GC. We propose here that the heme moiety of GC is the low affinity binding site, which constitutes a very small proportion of the total NO-binding sites in the cell, while the thiols account for the majority of NO-binding sites and also are the high affinity sites. During the course of the 2 or 24 hr NO-donor treatment of the cardiomyocytes, the millimolar concentrations of SNAP or SNP used would be releasing levels of NO which would, in all probability, saturate both the high and low affinity NO-binding sites. Following NO-donor treatment, the processing of the cells to isolate the cytosolic fraction took approximately 3 hr (see Methods). In that time period NO would dissociate first from the GC (low affinity) and be lost. The lack of NO binding to GC would be reflected by the absence of an increase in the basal GC activity. During the 3 hr interval NO would also be lost from a portion but not all of the thiol-NO complexes (S-nitrosothiols). The remaining cytosolic S-nitrosothiols would be carried over into the GC assays and therefore less SNAP or SNP would be required in the GC assay to saturate the unbound high affinity sites and to stimulate GC. Our results, which document leftward shifts of the concentration-response curves of GC for SNAP and SNP, are compatible with this postulate.

The present results raise an important question of the altered GC responsiveness in the tissues following their exposure to NO-donor drugs during organic nitrate therapy, and also in the case of septic shock. In a recent report, the modulation of cardiac contractility by NO has been shown to be biphasic in nature and cGMP dependent (5). Low levels of NO and therefore cGMP correlated with a positive inotropic effect while negative inotropy was seen with higher levels of NO/cGMP. Elevated endogenous NO production has been shown to contribute to the myocardial depression associated with septic shock (3). The desensitization of GC which we report in this study may be important from the perspective of adaptation of the myocardium to the pathophysiological levels of NO.

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