Nitric oxide: a trigger for classic preconditioning?

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Received 16 March 2000; accepted in final form 6 July 2000

Although the exact mechanism of classic ischemic preconditioning remains to be established, it is now generally accepted that multiple triggers and signal transduction pathways are involved. For example, activation of protein kinase (PKC) (16, 47) as well as of the β-adrenergic pathway (18, 29) may contribute to the protection elicited by short episodes of ischemia-reperfusion. Lochner et al. (19) previously hypothesized that characterization of events during the preconditioning protocol will yield more insight into the triggers and signaling pathways and subsequently showed that a 3 × 5-min preconditioning protocol caused cyclic elevations in the cyclic nucleotides cAMP and cGMP. The latter observation as well as the fact that nitric oxide (NO) synthase (NOS) activity is increased after 5 min of global ischemia (6) suggested the possibility that NO may also be a trigger in preconditioning.

Involvement of NO in the late phase of preconditioning is well established. A recent study (4) suggested a dual role for NO in this scenario: initially as trigger and subsequently as mediator of protection. Triggering the development of late preconditioning involves generation of NO on day 1 (3) and subsequent activation of PKC (27) and protein tyrosine kinase (14). There is also increasing evidence that the cardioprotective effects of late preconditioning observed on day 2 are due to upregulation of inducible NOS (38). A recent study (45) showed that NO promotes nuclear factor-κB activation in the heart, which, in turn, was demonstrated as playing an important role in the development of late preconditioning. Further convincing evidence of a role for NO in late preconditioning is that NO donors mimic and NOS inhibitors (39) abolish late protection.

The role of NO as a trigger or mediator in the phenomenon of classic preconditioning is, however, much less well established, and the few studies done thus far focused mainly on outcomes such as reperfusion dysrhythmias. The results obtained were controversial: some studies reported that the antiarrhythmic effects of preconditioning were independent of NO (20, 35) and that NOS inhibition by Nω-nitro-L-arginine methyl ester (L-NAME) had no effect on the preconditioning-induced reduction in infarct size (44) or the improvement in functional recovery (43); others showed that NO donors or NOS inhibitors could mimic (2) or abolish (40) the protective effects of preconditioning on dysrhythmias respectively. Further evidence for involvement of NO in preconditioning was the recent observation that cardiac NO biosynthesis was essential to trigger but not to mediate protection (5). Thus far, the effect of NO donors on outcomes such as infarct size or functional recovery during reperfusion has not been studied.

Although there is ample evidence for the generation of NO and peroxynitrite during ischemia-reperfusion (5, 42, 43, 46), no increase in tissue NO could be demonstrated during the preconditioning protocol itself (5). However, in view of the observation that NOS activation (6) and increases in cGMP (19) occur within the cardiac tissue during ischemia-reperfusion (21) and the fact that increased NO protects the organism (23), it is likely that NO plays a role in preconditioning.

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5 min of global ischemia, we hypothesized that NO acts as a trigger in classic preconditioning. The aim of this study was, therefore, to evaluate the roles of NO and cGMP, in particular, in classic preconditioning by 1) investigating the changes in the cyclic nucleotides cGMP and cAMP during a three-episode preconditioning protocol and during sustained ischemia and 2) mimicking or abolishing the above changes in cGMP by either exogenous administration of NO donors or inhibition of the L-arginine-NO pathway either before and during or after the preconditioning protocol, which will allow evaluation of the role of NO and thus cGMP as a trigger or mediator. Functional recovery during reperfusion of the globally ischemic heart and tissue cyclic nucleotides at the end of sustained ischemia were used as indicators of protection. In addition, the functional reserve of non preconditioned and preconditioned hearts and hearts pretreated with NO donors was evaluated by administration of adrenaline during reperfusion.

MATERIALS AND METHODS

Animals

Male Wistar rats (220−250 g) were used in all experiments. Before anesthesia (30 mg of pentobarbital sodium ip), the rats were allowed free access to food and water. This investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH Publication No. 85-23, Revised 1985).

Compounds

S-nitroso-N-penicillamine (SNAP), sodium nitroprusside (SNP), L-arginine, L-NAME, and N-nitro-L-arginine (L-NNA) were obtained from Sigma (St. Louis, MO). 1H-[1,2,4]oxadiazo- zol-[4,3-α]quinoxaline-1-one (ODQ) was purchased from Tocris (Bristol, UK). All other chemicals were of Analar grade and obtained from Merck (Cape Town, South Africa). SNAP and ODQ were dissolved in dimethyl sulfoxide (DMSO).

Perfusion Technique

The perfusion technique and measurement of mechanical activity were performed as previously described (23). Briefly, after removal, the hearts were perfused retrogradely for 15 min via the aorta in a nonrecirculating manner at a pressure of 100 cmH2O. During this time, the left atrium was canulated to allow subsequent atrial perfusion according to the working heart model of Neely as modified by Opie et al. (26). Coronary and aortic flow rates were measured manually by collecting fluid at timed intervals. Aortic pressure (P\textsubscript{a}) was obtained through a side-arm of the aortic cannula, which was connected to a Statham pressure transducer (P23 D6). The peak systolic pressure, mean pressure, and heart rate were obtained from the recordings made. The mean external power (W\textsubscript{e}; in milliwatts) produced by the left ventricle was calculated according to the formula of Kannengieser et al. (15): \[ W\textsubscript{e} = 0.002222(P\textsubscript{a0} - 11.25)(CO), \] where CO is cardiac output.

All drugs were administered via a sidearm into the aortic cannula while the heart was perfused retrogradely at a constant pressure of 100 cmH2O. NO donors were prepared fresh before each experiment.

Biochemical Analyses

Hearts were freeze-clamped at various times during the perfusion protocol with precooled Wollenberger tongs and immediately plunged into and stored in liquid nitrogen. For tissue cAMP analyses, tissue (100–200 mg) was extracted with 1.2 ml of 6% perchloric acid. The extracts were neutralized, and cAMP content was determined with the Amersham \[^{3}H\]cAMP assay system. For cGMP, ~100 mg of tissue were extracted with 1.2 ml of 5% trichloroacetic acid, and the extracts were washed four times with ether before analysis with the Amersham \[^{125}I\]labeled cGMP assay system. All samples were analyzed in duplicate.

Experimental Protocol

General description. All hearts were stabilized by perfusing retrogradely for 15 min followed by 15 min of perfusion in the working mode (Fig. 1A). The hearts were then perfused in the retrograde manner for 30 min during which time they either received no treatment, were preconditioned, or received inhibitors or agonists to manipulate tissue cyclic nucleotides. This was followed by 25 min of sustained global ischemia and reperfusion for 30 min (10 min for retrograde perfusion and 20 min for the working heart).

Characterization of changes in tissue cyclic nucleotides during preconditioning protocol. After stabilization, non preconditioned hearts were subsequently perfused retrogradely for a further 30 min, whereas preconditioned hearts were subjected to three 5-min episodes of global ischemia (designated PC1−, PC2−, and PC3−) alternating with three 5-min episodes of retrograde perfusion (designated PC1+ , PC2+ , and PC3+ ; Fig. 1B). Non preconditioned hearts were freeze-clamped at 30 and 60 min of total perfusion time, whereas the preconditioned hearts were freeze-clamped at the beginning and end of each period of 5-min ischemia. At least six hearts were freeze-clamped at each time point.

Manipulation of tissue cyclic nucleotides before sustained ischemia. After stabilization (as described in General description), the NO donors SNAP, SNP, or L-arginine or the solvent DMSO was then administered for 3 × 5 min alternating with 5 min of reperfusion with normal buffer (Fig. 1C). Hearts were freeze-clamped at the end of the first and third episodes of administration (1 × 5 min and 3 × 5 min) and in the case of SNAP and SNP also after 5 min of reperfusion (1 × 5 min plus reperfusion and 3 × 5 min plus reperfusion).

An inhibitor of guanylyl cyclase, ODQ, was administered 5 min before the onset of the first 5-min episode of ischemic preconditioning (PC1−) and during each 5 min of reperfusion after PC1−, PC2−, and PC3−. Hearts were freeze-clamped at PC1−, PC1+, PC3−, and PC3+ as indicated in Fig. 1C. L-NAME and L-NNA were administered as described for ODQ. L-NAME-treated hearts were freeze-clamped at the end of PC3− only.
Pilot studies were done to ensure that the concentration of donors used yielded cGMP levels equal to or exceeding those observed during the preconditioning protocol, whereas the concentration of the inhibitors was such that it prevented the rise in cGMP during preconditioning.

Appropriate control hearts were perfused similar to the nonpreconditioned hearts as described in Characterization of changes in tissue cyclic nucleotides during preconditioning protocol and freeze-clamped at 30 and 60 min of total perfusion time.

At least six hearts were freeze-clamped at each time point. The tissues were analyzed for cyclic nucleotide contents.

Evaluation of cyclic nucleotides at the end of 25 min of sustained ischemia. Five series of hearts (nonpreconditioned, preconditioned, or pretreated with SNAP (50 μM), SNP (100 μM), or ODQ (20 μM) as described in Manipulation of tissue
D

Non-preconditioned hearts:

Preconditioned hearts:

NO donors (SNAP or SNP):

E

Non-preconditioned hearts + Inhibitors (In):

Preconditioned hearts + Inhibitors:

Fig. 1. Experimental protocols. A: general perfusion protocol. Reperf, reperfusion. B: characterization of changes in cyclic nucleotides during preconditioning protocol. PC1−, PC2−, and PC3−, three 5-min episodes of global ischemia; PC1+, PC2+, and PC3+, three 5-min episodes of reperfusion. Arrows, times of freeze-clamping of hearts. C: manipulation of tissue cyclic nucleotides before onset of sustained ischemia. Nitric oxide (NO) donors S-nitroso-N-penicillamine (SNAP) and sodium nitroprusside (SNP) were administered for 3 × 5 min alternating with perfusion with normal buffer. Inhibitors 1H-[1,2,4]oxadiazol-[4,3-a]quinoxaline-1-one (ODQ), N-nitro-L-arginine methyl ester (l-NAME), and N-nitro-L-arginine (l-NNA) were administered 5 min before the onset of PC1− and during each reperfusion period. In nonpreconditioned hearts, the inhibitors were administered for 4 × 5 min before sustained ischemia. Arrows, times of freeze-clamping of hearts. D: effect of preconditioning and NO donors on functional recovery during reperfusion after 25 min of global ischemia. E: effect of inhibitors (ODQ, l-NAME, or l-NNA) on functional recovery during reperfusion after 25 min of global ischemia.
cyclic nucleotides before sustained ischemia were subsequently subjected to 25 min of global sustained ischemia and freeze-clamped (>6 hearts/series; Fig. 1C).

Roles of NO and cGMP in eliciting protection against ischemic damage: functional recovery during reperfusion. NO donors. After a stabilization period of 30 min, the hearts were subjected to either 30 min of retrograde perfusion (non preconditioned), 3 × 5 min of global ischemia (preconditioned), 3 × 5 min of SNAP (10 or 50 μM), 3 × 5 min of SNAP (100 μM), or 3 × 5 min of L-arginine (10 mM) before 25 min of sustained global ischemia followed by 30 min of reperfusion as described above (Fig. 1D).

In all series, functional performance was evaluated before the onset of sustained ischemia (at 25 and 30 min of total perfusion time) and during reperfusion (at 105 and 115 min of total perfusion time).

Inhibition of guanylyl cyclase or NOS. To establish whether NO acted as a trigger and/or mediator in preconditioning (Fig. 1E), the following protocols were used. As a mediator/trigger, in the preconditioned hearts, either L-NAME (50 μM), L-NNA (50 μM), or ODQ (20 μM) was administered 5 min before the onset of PC1− and during reperfusion after PC1−. In non preconditioned hearts, each of the drugs was administered for 4 × 5 min alternating with 5 min of reperfusion with normal buffer before the onset of sustained ischemia. All hearts were subsequently subjected to 25 min of global ischemia followed by 30 min of reperfusion (10 min for retrograde perfusion and 20 min for a working heart).

As a mediator, the hearts were preconditioned as described in Characterization of changes in cyclic nucleotides during preconditioning protocol, and L-NAME (50 μM) or ODQ (20 μM) was administered for 5 min during reperfusion after the third preconditioning episode (PC3) before the onset of sustained ischemia. In non preconditioned hearts, the drugs were given for 5 min before the onset of sustained ischemia. The hearts were preconditioned as described in Perfusion Technique, and L-NAME (50 μM) or ODQ (20 μM) was administered for 15 min during reperfusion after PC3 and before the onset of sustained ischemia. In non preconditioned hearts, the drugs were administered for 15 min before the onset of sustained ischemia.

As a trigger, in the preconditioned hearts, either L-NAME (50 μM) or ODQ (20 μM) was administered 5 min before the onset of PC1− and during reperfusion after PC1− and PC2−. PC3− was followed by 10 min of perfusion with buffer only (to wash out the inhibitors) before the onset of sustained ischemia. In non preconditioned hearts, the inhibitors were administered for 3 × 5 min alternating with perfusion with buffer.

Effect of epinephrine on mechanical recovery. Non preconditioned, preconditioned, and SNAP- or SNP-treated hearts were perfused as described in Roles of NO and cGMP in eliciting protection against ischemic damage: functional recovery during reperfusion until 20 min of reperfusion (at 105 min of total perfusion time). After registration of mechanical performance at this time point, epinephrine (10−6 M) was added, and mechanical activity was monitored after 5 and 10 min (at 110 and 115 min of total perfusion time).

Statistics

All data are means ± SE. Multiple comparisons were analyzed by one way analysis of variance, and the Bonferroni correction was applied. A P value of <0.05 was regarded as significant.

RESULTS

Tissue Cyclic Nucleotides During the Preconditioning Protocol

Hearts exposed to three 5-min episodes of global ischemia showed significant increases in cAMP at PC1−, PC2−, and PC3− compared with the control values (Table 1). During reperfusion (PC1+, PC2+, and PC3+), cAMP levels returned to those of control hearts. Furthermore, in each of the three episodes of preconditioning, tissue cAMP levels were significantly higher at the end of ischemia (PC1−, PC2−, and PC3−) than after reperfusion (PC1+, PC2+, and PC3+).

Tissue cGMP levels at the end of each ischemic episode showed the same tendency, namely a significant increase. With the exception of PC1+, reperfusion caused a significant reduction in tissue cGMP values, although not to baseline values. Of particular interest is the observation that the percent increases in cGMP at PC2− and PC3− (140 and 99%, respectively) greatly exceeded those in cAMP (16.3 and 16.8%, respectively).

Manipulation of Tissue Cyclic Nucleotides Before Sustained Ischemia

The cyclic increases in tissue cGMP during a three-episode ischemic preconditioning protocol could be mimicked by 3 × 5-min administration of the NO donors SNAP and SNP in the absence of ischemia (Table 2). In these studies, cGMP and cAMP were measured at the beginning and the end of the first and third episodes of administration (corresponding to PC1−, PC1+, PC3−, and PC3+ of the ischemic preconditioning protocol; Fig. 1).

The effect of SNAP on tissue cGMP levels is dose dependent; a 5-min administration of 10 μM SNAP increased cGMP levels to 38.77 ± 7.56 pmol/g wet wt compared with 52.44 ± 7.13 pmol/g wet wt with 50 μM SNAP (P < 0.001). Cyclic increases in cGMP occurred

Table 1. Effects of preconditioning on tissue cyclic nucleotides before onset of sustained ischemia

<table>
<thead>
<tr>
<th></th>
<th>cGMP</th>
<th>cAMP</th>
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<tbody>
<tr>
<td>Control</td>
<td>8.42 ± 0.35</td>
<td>317.0 ± 9.27</td>
</tr>
<tr>
<td>Preconditioning</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PC1−</td>
<td>12.18 ± 0.71</td>
<td>402.8 ± 13.23</td>
</tr>
<tr>
<td>PC1+</td>
<td>12.71 ± 1.37</td>
<td>307.0 ± 13.51</td>
</tr>
<tr>
<td>PC2−</td>
<td>20.22 ± 0.91</td>
<td>368.7 ± 13.84</td>
</tr>
<tr>
<td>PC2+</td>
<td>13.12 ± 1.65</td>
<td>283.0 ± 10.34</td>
</tr>
<tr>
<td>PC3−</td>
<td>16.79 ± 1.12</td>
<td>370.2 ± 9.74</td>
</tr>
<tr>
<td>PC3+</td>
<td>12.54 ± 0.69</td>
<td>277.1 ± 9.16</td>
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</tbody>
</table>

Values are means ± SE in pmol/g wet wt; n = 11 control hearts and ≥9 preconditioned hearts/series. Control hearts were perfused for 60 min (15-min retrograde, 15-min working heart, and 30-min retrograde). Similar values were obtained at 30 min of control perfusion conditions. Preconditioned (PC) hearts were subjected to three 5-min episodes of global ischemia (PC1−, PC2−, and PC3−) alternating with three 5-min periods of retrograde perfusion (PC1+, PC2+, and PC3+). *P < 0.05 vs. control (untreated). †P < 0.05 vs. respective PC−.
The effect of inhibition of cGMP generation during the ischemic preconditioning protocol was studied with ODQ, which was administered 5 min before the onset of PC1− as well as during each reperfusion phase (Table 3). Because ODQ was dissolved in DMSO, a control series was included where DMSO (0.04%) plus ODQ (20 μM) or DMSO alone was administered retrogradely for 3 × 5 min after the usual equilibration period of 30 min. Although DMSO alone had no significant effect on tissue cGMP and cAMP levels, the combination DMSO and ODQ significantly lowered tissue cAMP while increasing cGMP levels. These controls were therefore used for comparing the effects of preconditioning on tissue cyclic nucleotides in the presence of ODQ. ODQ prevented the significant rise in cGMP occurring during the preconditioning protocol at PC1− and PC3−, whereas tissue cAMP increased in a similar manner as observed before.

L-NAME, an inhibitor of NOS, also caused a reduction in cGMP when measured at PC3− (11.32 ± 0.85 pmol/g wet wt (n = 6 hearts); PC3− (untreated) 16.79 ± 1.12 pmol/g wet wt (n = 9 hearts); P < 0.05).

Tissue Cyclic Nucleotides at the End of 25 min of Sustained Ischemia

Comparison of cGMP and cAMP values at the end of 25 min of sustained ischemia showed that an ischemic preconditioning protocol caused a concomitant significant increase in tissue cGMP and reduction in cAMP compared with those in nonpreconditioned hearts (Table 4). The NO donors SNAP (50 μM) and SNP (100 μM) also caused significant increases in cGMP at the end of sustained ischemia compared with those in nonpreconditioned hearts. However, although SNP significantly lowered tissue cAMP at this time point, the reduction induced by SNAP administration was not significant. ODQ (20 μM), compared with its own control series, was characterized by no change in tissue cGMP and a significant increase in tissue cAMP. Of interest is the similarity between the percent increase in cAMP content of nonpreconditioned (93%) and ODQ-treated (97%) hearts compared with their respective controls.
controls. Cyclic nucleotides at this stage of the protocol were not assessed in L-NAME- or L-NNA-treated hearts.

**Effect of Ischemic Preconditioning, Inhibitors of Guanylyl Cyclase and NOS, and NO Donors on Functional Recovery After 25 Min of Sustained Ischemia**

Because DMSO was used as solvent for both SNAP and ODQ (SNP and L-NAME being water soluble), a series of nonpreconditioned and preconditioned hearts was studied in which DMSO alone was administered either for 4 × 5 min or for 15 min before the onset of sustained ischemia (Tables 5 and 6; Figs. 2–4; see also Fig. 1E). DMSO alone had no effect on any parameter of functional recovery in both series (data not shown).

In all series studied, exposure of the heart to 25 min of sustained global ischemia caused a significant decline in all parameters of function measured during reperfusion compared with values obtained before arrest. However, comparison of the functional performance during reperfusion revealed significant differences among the groups. Preconditioning by 3 × 5 min

| Table 5. Effect of ischemic preconditioning and NO donors on functional recovery after 25-min normothermic ischemic cardiac arrest |
|-----------------|-----------------|-----------------|-----------------|-----------------|
|                 | n               | Coronary Flow, ml/min | Cardiac Output, ml/min | Peak Systolic Pressure, mmHg |
| Control         | 9               | 12.4 ± 0.6           | 54.6 ± 2.0           | 101 ± 2          | 278 ± 9          |
| Non-PC          | 6               | 7.9 ± 3.0*           | 9.7 ± 3.5*           | 64 ± 21*         | 173 ± 63*        |
| PC              | 9               | 11.7 ± 0.6           | 32.2 ± 1.9*†         | 91 ± 27†         | 259 ± 10†        |
| SNP (100 µM)    | 10              | 9.4 ± 0.8            | 30.5 ± 1.3*†         | 94 ± 2†          | 227 ± 5          |
| SNAP (50 µM)    | 9               | 8.5 ± 0.4            | 29.0 ± 2.6*†         | 89 ± 1†          | 245 ± 15         |
| l-Arg (10 mM)   | 5               | 9.4 ± 0.5            | 14.8 ± 0.8*‡         | 82 ± 1*          | 255 ± 13         |

Values are means ± SE; n, no. of hearts. *P < 0.05 vs. before arrest (control values). †P < 0.05 vs. non-PC. ‡P < 0.05 vs. PC.

| Table 6. Effect of ischemic preconditioning, L-NAME, L-NNA, and ODQ on functional recovery after 25-min normothermic ischemic cardiac arrest |
|-----------------|-----------------|-----------------|-----------------|-----------------|
|                 | n               | Coronary Flow, ml/min | Cardiac Output, ml/min | Peak Systolic Pressure, mmHg |
| Control         | 7               | 14.1 ± 0.4           | 56.1 ± 1.5           | 104 ± 3          | 271 ± 15         |
| Non-PC during reperfusion |
| Untreated       | 6               | 7.9 ± 3.0*           | 9.7 ± 3.5*           | 64 ± 21*         | 173 ± 63*        |
| Trigger + mediator |
| 4 × 5 min l-NAME | 6               | 8.8 ± 2.4*           | 10.45 ± 2.6*         | 69 ± 13*         | 183 ± 37*        |
| 4 × 5 min ODQ   | 7               | 5.4 ± 1.7*           | 5.4 ± 1.7*           | 32 ± 9†          | 120 ± 31*        |
| 4 × 5 min l-NNA | 4               | 8.6 ± 1.2*           | 9.7 ± 1.8*           | 68 ± 9*          | 271 ± 13†        |
| Mediator |
| 5 min l-NAME    | 7               | 5.8 ± 0.9*‡          | 10.59 ± 3.2*         | 59 ± 12*         | 175 ± 8*         |
| 5 min ODQ       | 9               | 10.5 ± 1.6*          | 18.1 ± 3.7*          | 80 ± 7*          | 207 ± 10*        |
| Trigger |
| 3 × 5 min l-NAME | 6               | 9.9 ± 0.8*           | 9.4 ± 3.4*           | 85 ± 4           | 243 ± 15         |
| 3 × 5 min ODQ   | 7               | 8.6 ± 0.5*           | 9.2 ± 1.0*           | 70 ± 5*          | 197 ± 17*        |
| PC during reperfusion |
| Untreated       | 9               | 11.7 ± 0.6           | 33.9 ± 2.2*          | 91 ± 2           | 259 ± 10         |
| Trigger + mediator |
| 4 × 5 min l-NAME | 8               | 9.3 ± 1.0*           | 16.6 ± 3.6*‡         | 77 ± 6*          | 229 ± 21         |
| 4 × 5 min ODQ   | 6               | 10.6 ± 0.7*          | 21.6 ± 1.7*‡         | 86 ± 3*          | 210 ± 10*        |
| 4 × 5 min l-NNA | 6               | 7.1 ± 0.5*           | 14.85 ± 2.1*‡        | 89 ± 3*          | 202 ± 23         |
| Mediator |
| 5 min l-NAME    | 6               | 10.8 ± 0.8*          | 28.1 ± 1.6*          | 90 ± 4           | 207 ± 19*        |
| 5 min ODQ       | 6               | 11.8 ± 0.7*          | 29.0 ± 3*            | 92 ± 3           | 209 ± 17*        |
| Trigger |
| 3 × 5 min l-NAME | 7               | 9.0 ± 0.7*           | 16.8 ± 2.8*‡         | 83 ± 3*          | 240 ± 19         |
| 3 × 5 min ODQ   | 9               | 10.0 ± 1.0*          | 18.6 ± 2.5*‡         | 86 ± 2*          | 219 ± 15*        |

Values are means ± SE; n, no. of hearts. l-NAME, N-nitro-l-arginine methyl ester; l-NNA, N-nitro-l-arginine. *P < 0.05 vs. before arrest (control values). †P < 0.05 vs. non-PC. ‡P < 0.05 vs. PC.
of global ischemia caused a significant improvement in all parameters studied compared with those in nonpreconditioned hearts.

**NO Donors**

Interruption administration (3 × 5 min) of the NO donors SNAP (10–100 μM) or SNP (100 μM) before 25 min of sustained ischemia significantly improved functional recovery during reperfusion (values obtained with 50 μM SNAP are shown in Table 5) compared with that in nonpreconditioned hearts. The protection afforded by these interventions was similar to that elicited by prior preconditioning; aortic output and total work performed (Fig. 2) and peak systolic pressure, heart rate, and CO (Table 5) were similar in these groups. On the other hand, L-arginine (10 mM, 3 × 5 min) did not improve functional recovery during reperfusion, and values similar to those for nonpreconditioned hearts were obtained.

**Inhibitors**

Preconditioned hearts. Inhibitors administered before and during the preconditioning protocol up to the onset of sustained ischemia (Fig. 1E) allow evaluation of the roles of NO and cGMP as a trigger as well as a mediator of protection. Table 6 and Figs. 3 and 4 summarize the effects of the inhibitors L-NAME, L-NNA, and ODQ on mechanical recovery during reperfusion. Inhibition of NOS activity in this manner by either L-NAME or L-NNA caused partial inhibition of the protection conferred by preconditioning (Fig. 4). Administration of L-NAME or ODQ after the preconditioning protocol for either 5 (Figs. 3 and 4) or 15 (data not shown) min before the onset of sustained ischemia was similar to that in preconditioned hearts, suggesting that NO probably does not act as a mediator.

Both L-NAME and ODQ when given during the preconditioning protocol but washed out for 10 min before the onset of sustained ischemia (Fig. 1E) caused a significant reduction in aortic output and work performance compared with those in untreated preconditioned hearts. These results suggest a role as a trigger.

Nonpreconditioned hearts. Administration of L-NAME had no significant effect on aortic output and work performance during reperfusion of nonpreconditioned hearts regardless of the protocol used (Fig. 3). Similarly, with ODQ, none of the protocols caused a significant difference in aortic output and work performance compared with those for untreated nonpreconditioned hearts (Fig. 4). However, administration of ODQ for 5 min before the onset of sustained ischemia (Fig. 1D) yielded values significantly higher than when administered for 3 × 5 min or 4 × 5 min.

**Effect of Epinephrine on Recovery Potential**

To assess and compare the contractile reserve of hearts subjected to preconditioning and treatment with NO donors, epinephrine (10–6 M) was administered during the reperfusion phase and the changes in function were monitored. The data obtained during reperfusion of nonpreconditioned, preconditioned, SNP-treated, and SNAP-treated hearts are shown in Table 5 and Fig. 2, and the results expressed as the percent increase induced by
epinephrine compared with the values obtained during reperfusion before stimulation are shown in Table 7. The percent increases induced by epinephrine in all parameters were similar in preconditioned and nonpreconditioned hearts. However, SNAP-treated hearts showed significantly higher increases in coronary flow rate, CO, peak systolic pressure, and Wt compared with those in nonpreconditioned and preconditioned hearts. Similarly, SNP treatment also caused significantly higher increases in CO and Wt.

DISCUSSION

The observation that a multicycle preconditioning protocol elicits cyclic increases in both cGMP and cAMP (Table 1) suggests the possibility of a role for both as triggers in the phenomenon of ischemic preconditioning (see Fig. 5). To establish the relevance of the significant increases in cGMP per se, appropriate donors that allowed evaluation of such changes in the absence of a concomitant elevation in cAMP were employed in the present study (Table 2, Fig. 5). The results obtained suggest that NO and subsequent cGMP generation also act as a trigger in the phenomenon of classic preconditioning; the non-endothelium-dependent NO donors SNAP and SNP both elicited protection against ischemic damage (Fig. 2, Table 5), whereas prevention of ischemic preconditioning-induced cyclic elevations in cGMP (by inhibition of either guanylyl cyclase or NOS activation) attenuated protection (Table 6, Figs. 3 and 4).

Experimental Model

The isolated perfused working rat heart was chosen as the experimental model for this study. Although
recognition that mechanistic information on preconditioning in rats may not always be transferable to other species, the rat heart model has been well characterized (23) and is widely used. Although infarct size reduction remains the gold standard as the end point in preconditioning (12a), impaired recovery of contractile function during reperfusion is frequently used as an alternative (23, 30). Myocardial function may perhaps be the more physiological indicator of the beneficial effects of preconditioning. Furthermore, the use of triphenyltetrazolium chloride staining alone for measurement of infarct size has recently been questioned (10, 37).

After 25 min of global ischemia in the rat heart, varying degrees of stunning, apoptosis, and necrosis

Table 7. Effect of epinephrine on mechanical performance during reperfusion

<table>
<thead>
<tr>
<th></th>
<th>Coronary Flow</th>
<th>Cardiac Output</th>
<th>Peak Systolic Pressure</th>
<th>Heart Rate</th>
<th>Work Performance</th>
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<tr>
<td>Non-PC</td>
<td>22.7 ± 7.3</td>
<td>53.9 ± 25.5</td>
<td>14.2 ± 3.7</td>
<td>52.8 ± 19.1</td>
<td>96 ± 29</td>
</tr>
<tr>
<td>PC</td>
<td>41.3 ± 4.50</td>
<td>40.7 ± 4.0</td>
<td>7.2 ± 2.0</td>
<td>46.7 ± 2.5</td>
<td>54.4 ± 7.6</td>
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<tr>
<td>SNAP</td>
<td>62.9 ± 7.5†</td>
<td>122.1 ± 18.0‖</td>
<td>17.0 ± 0.5*</td>
<td>50.2 ± 2.9</td>
<td>167.6 ± 22.7*</td>
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<tr>
<td>SNP</td>
<td>44.0 ± 4.6</td>
<td>114.8 ± 13.3‖</td>
<td>16.6 ± 1.3</td>
<td>53.1 ± 6.5</td>
<td>154.8 ± 16.8*</td>
</tr>
</tbody>
</table>

Values are means ± SE in percent increase of values obtained after 20 min of reperfusion. Epinephrine (10⁻⁶ M) after 20 min of reperfusion, and mechanical performance was monitored after 5 and 10 min. Hearts were preconditioned or treated with NO donors as shown in Fig. 1. *P < 0.05 vs. PC. †P < 0.05 vs. non-PC.
may contribute to the reduced mechanical performance during reperfusion. Moolman et al. (23) have previously shown that the ischemia-induced structural damage was reduced by prior preconditioning in this model.

**Mimicking of Preconditioning by Use of NO Donors**

As far as we know, this is the first direct demonstration that repeated transient administration and washout of NO donors before the onset of sustained ischemia improves functional recovery during reperfusion (Table 5, Fig. 2). These results are consistent with the finding that NO donors mimic the effect of preconditioning on reperfusion arrhythmias in rat hearts (2).

To distinguish between the contribution of cAMP and cGMP as triggers, it was a prerequisite that the NO donor used should not increase tissue cAMP levels before the onset of sustained ischemia (cAMP elevation per se also acts as trigger (18]). Although SNAP caused dose-dependent increases in cGMP (see Table 2), it had no significant effect on tissue cAMP at 50 μM. However, at 10 μM, SNAP caused slight but not significant increases in cAMP except when administered for 1 × 5 min followed by reperfusion. Low concentrations of SNAP (1 μM) have also been shown to elevate the cAMP content of isolated adult myocytes and to increase contractility, whereas at 100 μM, the drug had no effect on cAMP (41). Vila-Petroff et al. (41) attributed the increase in cAMP induced by low levels of NO to a novel cGMP-independent activation of adenylyl cyclase. In view of the above, it is possible but unlikely that generation of NO (as suggested by the cyclic increases in cGMP) during a multiphase preconditioning protocol in isolated rat hearts contributes to the characteristic ischemia-induced increases in cAMP (Table 1).

The improvement in functional recovery induced by NO donors was not dose dependent in the range of tissue cGMP levels observed; the elevation in cGMP to 52.44 ± 7.13 (1 × 5 min with 50 μM SNAP) or 18.96 ± 3.67 (1 × 5 min with 100 μM SNP) pmol/g wet wt elicited the same degree of protection (Fig. 2, Table 5). Because 20.22 ± 0.91 pmol/g wet wt was the highest level to which cGMP increased during the preconditioning protocol (Table 1), it seems as if maximal protection is achieved with cGMP levels in that range. Failure of L-arginine (10 mM) to elicit protection (Fig. 2) was surprising in view of the previous findings that this substrate protects against ischemia-reperfusion injury (9, 32). A possible explanation may be the failure of intermittent administration of L-arginine to increase cGMP (Table 2). On the other hand, it has also been shown that intracoronary administration of L-arginine aggravates myocardial stunning through the production of peroxynitrite in dogs (24).

Interestingly, the contractile reserve of hearts preconditioned with SNP or SNAP was significantly higher than that of ischemic preconditioned hearts; for example, adrenaline (10⁻⁶ M) caused an approximately threefold higher percent stimulation of CO and Wt compared with that in preconditioned hearts. This may indicate a greater degree of protection in the NO donor preconditioned hearts that was not apparent when evaluating CO and Wt alone at the end of reperfusion. Although the possibility of NO donors contributing to improved function by reduction of stunning.

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**Fig. 5.** Preconditioning via cyclic ischemia leads to β-adrenergic stimulation and activation of NO synthase (NOS), causing cyclic elevations in tissue cAMP and cGMP levels, respectively. The role of NO in classic PC was studied with the inhibitors L-NAME, L-NNA, and ODQ, whereas SNAP, SNP, and L-Arg were used as NO donors.
has been shown by others in large animals (9, 11, 12), this remains to be elucidated in our model.

Involvement of NO and cGMP as a trigger in ventricular overdrive pacing preconditioning has been demonstrated in the isolated working rat heart (36). Ferdinandy et al. and Csonka et al. also showed that l-NAME inhibited the cardioprotective effect of ventricular overdrive pacing (11) and that intact basal NO synthesis was required to trigger preconditioning (5).

**Abolishment of Protection by Prevention of Cyclic Increases in cGMP During Preconditioning**

The significance of elevation in tissue cGMP during the preconditioning protocol was further investigated with l-NAME or l-NNA, inhibitors of NOS, as well as with ODQ, an inhibitor of soluble guanylyl cyclase. The latter has no effect on NOS activity and does not inactivate NO (13). ODQ dissolved in DMSO (as used in the present study) caused a significant increase in basal cGMP levels while lowering cAMP (Table 3). An explanation for these unexpected findings is not readily available. However, ODQ-DMSO effectively prevented the cyclic increases in cGMP during PC1− and PC3−, whereas the increases in cAMP occurred as before.

With the use of appropriate protocols (Fig. 1E), it was shown convincingly that NO acts only as a trigger in the phenomenon of preconditioning; both l-NAME and ODQ, if present before and throughout the preconditioning procedure but washed out before sustained ischemia, significantly attenuated functional protection during reperfusion of preconditioned hearts while having no inhibitory effects when added after the preconditioning protocol and before the onset of sustained ischemia (Figs. 3 and 4). Similar conclusions were made by Csonka et al. (5).

In contrast to our results, Weselcouch et al. (43) failed to demonstrate involvement of endothelium-derived NO as a trigger of preconditioning using functional recovery and enzyme release as indicators. This discrepancy may be due to several minor but possibly important differences in 1) protocol (3 × 5 min vs. 4 × 5 min), 2) perfusion model (working hearts vs. retrogradely perfused hearts fitted with an intraventricular balloon), and 3) concentration of l-NAME administered (50 vs. 30 μM).

Inhibition of NOS activation by l-NAME had no effect on the functional recovery of nonpreconditioned hearts regardless of the protocol used (Fig. 3), suggesting that inhibition of NO generation during sustained ischemia did not protect against cell damage in our model. Similarly, ODQ did not improve functional recovery in nonpreconditioned hearts when administered for 3 × 5 min or 4 × 5 min. In fact, when administered in this manner, ODQ significantly impaired functional recovery in nonpreconditioned hearts compared with functional recovery observed with the protocol with a shorter (5 min) total exposure to ODQ. It would seem that repeated administration of the drug could have a detrimental effect.

These results are in contrast to other studies (7, 25, 44) where NOS inhibition protected against ischemia-reperfusion. This discrepancy could be due to differences in species and experimental protocol: coronary ligation (44), low-flow ischemia (7) of isolated rabbit hearts, and retrogradely perfused rat hearts (25) versus the isolated working rat heart in the present study. The time of administration may also be important because in the retrogradely perfused heart, l-NAME was infused for 30 min before the onset of sustained ischemia (25), whereas in the rabbit heart, l-NAME was administered continuously during 45 min of coronary artery ligation (44). In our study, l-NAME was administered for 5 min only or for three to four periods alternating with reperfusion.

**How Does cGMP Trigger Protection?**

Establishing the relative importance of a trigger is difficult because pharmacological mimicking of a trigger often elicits maximal protection similar to that observed with ischemic preconditioning. Also, in this study, cyclic elevations in cGMP and cAMP occur simultaneously during the preconditioning protocol (Table 1), although elevation in both nucleotides is not a prerequisite for subsequent protection against ischemic damage; the elevation in cAMP per se by α-adrenergic stimulation (18) or in cGMP per se by NO donors (Table 2) could induce protection to the same extent as that induced by ischemic preconditioning (Table 5). On the other hand, prevention of these cyclic increases in cAMP (18) or cGMP (Table 3, Figs. 3 and 4) only partially suppressed preconditioning-induced protection, confirming involvement of more than one trigger.

Considerable “cross talk” may occur between the signal transduction pathways of the released triggers. For example, NO (and thus cGMP) attenuate β-receptor-mediated responses (8), whereas inhibition of NOS activity could enhance the β-response to isoproterenol in myocytes (1). However, the very significant increases in cGMP during the preconditioning protocol suggests an important role for NO and could possibly account for the significantly smaller increases in cAMP occurring simultaneously.

Possible mechanisms of action of NO in the preconditioning process have recently been reviewed by Rakhit et al. (28). NO acts as a free radical, combining with superoxide to generate peroxynitrite, which, in turn, could activate PKC. It has recently been shown (27) that activation of PKC during a 6 × 4-min preconditioning protocol is NO dependent and that exogenous NO donors can translocate PKC-ε and -η in the absence of ischemia.

Further downstream events remain to be elucidated. The mitochondrial ATP-sensitive K+ channel may be involved because activation of these channels by NO has recently been demonstrated (31), whereas it also potentiates the ATP-sensitive K+ current induced by K+ channel openers in ventricular cells (34).
Cyclic Nucleotides and Protection During Sustained Ischemia

As observed before (19, 22), preconditioning significantly attenuates the increase in tissue cAMP and stimulates an increase in cGMP in response to sustained ischemia (Table 4). It is not yet known whether these changes are merely the consequence of or whether they contribute to the protection induced by ischemic preconditioning. Elevated cGMP may act by reducing the influx of Ca\(^{2+}\) through L-type channels (33) and by stimulation of cGMP-sensitive phosphodiesterase, with resultant lowering of cAMP. It is possible that these two nucleotides act synergistically during sustained ischemia; in view of their opposing effects on the Ca\(^{2+}\) slow channel, the simultaneous lowering of tissue cAMP and elevation in cGMP may reduce Ca\(^{2+}\) influx (33) during ischemia as well as during reperfusion. In addition to the above, there is substantial pharmacological evidence that the guanylyl cyclase-cGMP-phosphodiesterase system contributes to cardioprotection. For example, Ljusegren and Axelsson (17) described that an increase in myocardial cGMP with SNP, atrial natriuretic peptide, or zaprinast, an inhibitor of cGMP-specific phosphodiesterase-γ, caused a reduction in lactate accumulation in isolated hypoxic rat ventricular myocardium.

In summary, the results obtained suggest that NO and thus the generation of cGMP act as a trigger in classic preconditioning. Cyclic elevation in cGMP by NO donors effectively protects against ischemic damage, whereas inhibition of NOS or guanylyl cyclase partially abolishes the beneficial effects of preconditioning.

We thank the Harry Crossley Foundation, the South African Medical Research Council, and the National Foundation for Research for financial support.

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