Nitroglycerin Induces Late Preconditioning Against Myocardial Infarction in Conscious Rabbits Despite Development of Nitrate Tolerance

Michael Hill, PhD; Hitoshi Takano, MD; Xian-Liang Tang, MD; Eitaro Kodani, MD; Gregg Shirk, BA; Roberto Bolli, MD

Background—Recent studies suggest that the late phase of ischemic preconditioning (PC) can be mimicked by pretreatment with NO donors. The ability of clinically relevant NO donors to induce PC against infarction, however, has not been evaluated. Furthermore, it is unknown whether tolerance to the hemodynamic actions of nitrates also extends to their PC effects.

Methods and Results—Conscious rabbits underwent a 30-minute coronary occlusion and 3 days of reperfusion. A 60-minute intravenous (IV) infusion of nitroglycerin (NTG) ending 1 hour before occlusion reduced infarct size, indicating an early PC effect. When the time interval between NTG infusion and occlusion was extended to 24 or 72 hours, the infarct-sparring action of NTG became even more pronounced, indicating a robust late PC effect. Transdermal NTG patches elicited a late PC effect that was (1) equivalent to that induced by IV NTG, demonstrating the efficacy of transdermal NTG as an alternative form of NTG delivery for inducing late PC, and (2) similar in nitrate-tolerant and -nontolerant rabbits, demonstrating that tolerance does not extend to the PC effects of NTG.

Conclusions—In conscious rabbits, administration of NTG via either the IV or the transdermal route elicits a robust protective effect against infarction that lasts for 72 hours. The magnitude of NTG-induced cardioprotection is equivalent to that observed during the late phase of ischemic PC and is not affected by the development of tolerance. These findings reveal a new action of nitrates and support novel applications of these drugs for protecting the ischemic myocardium in patients. (Circulation. 2001;104:694-699.)

Key Words: nitroglycerin ■ ischemia ■ reperfusion ■ myocardial infarction ■ nitrates
coronary artery disease and because it can be administered both by the intravenous (IV) and the transdermal (TD) routes. To fully characterize the PC-mimetic potential of NTG, we examined the following specific questions: (1) Does a brief IV infusion of NTG induce delayed protection against infarction? (2) If so, how long does this protective effect persist? (3) Is NTG-induced late PC abrogated by the development of nitrate tolerance? (4) Can a late PC effect be induced by TD, rather than IV, administration of NTG? (5) Can NTG induce an early phase of PC as well? In a well-established rabbit model of ischemic PC, a comprehensive investigation was performed that included 3 consecutive phases (1, 2, and 3) and 12 experimental groups. All studies were conducted in conscious animals to eliminate the potentially confounding influence of factors associated with open-chest preparations, such as anesthesia, surgical trauma, abnormal hemodynamics, elevated catecholamine levels, fluctuations in body temperature, exaggerated formation of reactive oxygen species, and release of cytokines, which could, in themselves, interfere with the development of myocardial infarction and with the phenomenon of PC. The results demonstrate, for the first time, that NTG can faithfully recapitulate the infarct-sparing effects of the late phase of ischemic PC despite the development of nitrate tolerance, suggesting novel potential applications of nitrates in patients.

Methods

The conscious rabbit model of myocardial ischemia has been described in detail previously. Phase 1: Effect of IV NTG

Group 1 (control group) underwent the 30-minute occlusion without any treatment or PC protocol (Figure 1). Groups 2, 3, and 4 were preconditioned with a sequence of six 4-minute coronary occlusion/4-minute reperfusion cycles ending 10 minutes (early PC-10 minutes group), 1 hour (early PC-1 hour group), or 24 hours (late PC group) before the 30-minute occlusion (Figure 1). Groups 5 to 8 were studied to examine the ability of NTG to induce PC. In group 5 (NTG-1 hour group), rabbits were pretreated with a 60-minute IV infusion of NTG (2 μg·kg⁻¹·min⁻¹) ending 1 hour before the 30-minute occlusion. In groups 6 (NTG-24 hours group), 7 (NTG-72 hours group), and 8 (NTG-96 hours group), the animals were pretreated with the same dose of NTG as in group 5, but the time interval between NTG infusion and onset of the 30-minute occlusion was extended to 24, 72, and 96 hours, respectively (Figure 1). The dose of NTG used in this investigation was chosen because pilot studies demonstrated that infusion rates >2 μg·kg⁻¹·min⁻¹ caused significant hypotension and tachycardia.

Phase 2: Effect of TD NTG

TD NTG patches that released 0.3 mg/h NTG continuously for a period of 24 hours were applied on the rabbits’ skin. After removal of the patch, a 3-day interval was allowed to elapse before rabbits were subjected to a 30-minute occlusion followed by 72 hours of reperfusion (Figure 2). The reason for selecting a 3-day interval was to determine whether a single TD NTG treatment is sufficient to provide long-lasting protection against subsequent infarction. The dose of NTG chosen in phase 2 (132 μg·kg⁻¹·h⁻¹) is similar to that previously used in several studies of NTG in rabbits.

Phase 3: Nitrate Tolerance

To induce nitrate tolerance, rabbits were given 1 TD NTG patch/d (0.3 mg/h) continuously over a period of 28 days (Figure 2). Seventy-two hours after removal of the last patch (day 31), nitrate-tolerant rabbits were subjected to a 30-minute coronary occlusion followed by 72 hours of reperfusion. Previous studies have shown that use of TD NTG patches at this dose (120 μg·kg⁻¹·h⁻¹) is associated with the development of tolerance to the vasodilator effects of NTG in rabbits.

Measurement of Regional Myocardial Function and Infarct Size

The total deficit of systolic wall thickening over the 3-day reperfusion period (an integrative assessment of the overall severity of contractile dysfunction during this time interval) was calculated as described. The total deficit of wall thickening provides a tetrazolium-independent index of protection. The size of the occluded-reperfused coronary vascular bed (region at risk) and of the infarction was determined by a previously described postmortem perfusion technique followed by staining with triphenyltetrazolium chloride and by computerized planimetry.

Statistical Analysis

Data are reported as mean±SEM. Heart rate and thickening fraction were analyzed by a 2-way repeated-measures ANOVA (time and group). Infarct sizes and risk region sizes were analyzed by a 1-way ANOVA followed by Student’s t tests for unpaired data with the Bonferroni correction.

Results

Of the 99 conscious rabbits instrumented for the study, 13 (13%) were excluded for the reasons specified in Table 1. As shown in Table 2, there were no significant differences among the various groups with respect to the size of the region at risk. In all 3 phases of this study, the effects of NTG-induced PC were compared with those of ischemia-induced PC.
Phase 1: Effect of IV NTG
No significant changes in heart rate or arterial pressure were observed during or after the 60-minute infusion of 2 μg·kg⁻¹·min⁻¹ of NTG, which is consistent with previous studies in rabbits. As expected, when rabbits underwent a 30-minute coronary occlusion either 10 minutes or 1 hour after the 6 occlusion-reperfusion cycles, infarct size was markedly reduced, indicating an early PC effect of ischemia (Figure 3). In rabbits undergoing the 30-minute occlusion 24 hours after ischemic PC, infarct size was also reduced (late PC) (Figure 3). When rabbits were pretreated with a 60-minute IV infusion of NTG ending 1 hour before the 30-minute occlusion, infarct size was significantly decreased compared with controls (Figure 3), indicating that NTG induced an early PC effect. This is in agreement with previous observations with SNAP in isolated rabbit hearts. When the time interval between the NTG infusion and the 30-minute occlusion was extended to 24 or 72 hours, the reduction in infarct size became even more pronounced, demonstrating that NTG elicited a robust late PC effect. When the interval between NTG infusion and the 30-minute occlusion was further extended to 96 hours, however, infarct size was indistinguishable from that measured in the control group, indicating that the NTG-induced late PC effect against infarction had disappeared.

Phase 2: Effect of TD NTG
Application of the TD NTG patch resulted in a transient decrease in mean arterial pressure at 3, 6, 9, and 12 hours; however, arterial pressure returned to baseline values by 24 hours (Table 3). In rabbits that were subjected to the 30-minute coronary occlusion 72 hours after six 4-minute occlusion/reperfusion cycles, infarct size was significantly reduced (Figure 4), indicating that ischemic PC produces a sustained infarct-sparing effect that lasts ≥72 hours in this conscious rabbit model. An even greater infarct-sparing effect was observed in the rabbits that received a single NTG patch ending 72 hours before the 30-minute occlusion; the reduction in infarct size in this group (−41%) compared favorably with that observed at 72 hours after ischemic PC (−41%), indicating that pretreatment with TD NTG resulted in a delayed protective action at least equivalent to, if not greater than, that induced by ischemia.

Phase 3: Nitrate Tolerance
No significant changes in heart rate or arterial pressure were observed on days 14 and 28 of the 28-day continuous TD NTG treatment (Table 3). Thus, the early hypotensive effects of TD NTG seen in phase 2 did not persist during daily application of TD NTG patches, indicating tolerance. The development of nitrate tolerance was confirmed in a separate

### TABLE 1. Reasons for Exclusion

<table>
<thead>
<tr>
<th>Group</th>
<th>Phase 1</th>
<th>Phases 2 and 3</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 2 3 4 5 6 7 8</td>
<td>9 10 11 12</td>
<td></td>
</tr>
<tr>
<td>Malfunction of the balloon occluder</td>
<td>... ... ... ...</td>
<td>... ... ...</td>
<td>1</td>
</tr>
<tr>
<td>Ventricular fibrillation during occlusion</td>
<td>2 1 ... 2 ... ...</td>
<td>2 ... 1 ...</td>
<td>8</td>
</tr>
<tr>
<td>Small region at risk (≤10% of left ventricle)</td>
<td>... ... ... ... ... ...</td>
<td>... ... 1 ...</td>
<td>1</td>
</tr>
<tr>
<td>Technical problems during postmortem perfusion</td>
<td>... ... ... ... ... ...</td>
<td>... ... ... 2</td>
<td>2</td>
</tr>
<tr>
<td>Paraplegia</td>
<td>... ... ... ... ... ...</td>
<td>1 ... ... ...</td>
<td>1</td>
</tr>
<tr>
<td>Total number of rabbits excluded</td>
<td>2 1 ... 2 1 2 2</td>
<td>2 2 2</td>
<td>13 (13%)</td>
</tr>
<tr>
<td>Total number of rabbits assigned to each group</td>
<td>10 6 6 8 10 8 7 8</td>
<td>9 9 9 9</td>
<td>99</td>
</tr>
<tr>
<td>Rabbits that completed the protocol</td>
<td>8 6 5 8 8 6 7</td>
<td>7 9 7 7</td>
<td>86 (87%)</td>
</tr>
</tbody>
</table>
impressive advances in our understanding of the pathophysiology and mechanism of PC in experimental models, no widely applicable PC mimic has yet been developed for clinical use. Most agents that elicit a late PC-like protection have significant side effects that limit their clinical applicability.25,26 In contrast, NO donors, such as nitrates, are widely used clinically and relatively well tolerated.

The present study provides significant new information that is relevant to the development of PC-mimetic therapies. The salient results can be summarized as follows: (1) In conscious rabbits, a brief IV infusion of a clinically relevant NO-releasing agent (NTG) elicits a powerful infarct-sparing effect 24 hours later; (2) this infarct-sparing effect is sustained for 72 hours and is at least equivalent to that of ischemia-induced late PC; (3) the same degree of cardioprotection as afforded by IV NTG can be elicited by TD administration of NTG (NTG patch); (4) NTG-induced late PC is not affected by the development of nitrate tolerance; and (5) in addition to a late PC effect, NTG also induces an early PC effect against infarction. Taken together, these results support a potential role of nitrates as PC mimetics in humans and provide a framework for testing these agents in the clinical arena. To the best of our knowledge, this is the first group of 5 rabbits by the finding that on days 14 and 28 of continuous TD NTG treatment, the hypotensive response to IV NTG was markedly attenuated compared with that observed immediately before the application of the first TD NTG patch (Figure 5). In the rabbits that underwent the 30-minute coronary occlusion 72 hours after the end of a continuous 28-day TD NTG treatment (NTG-tolerant group), infarct size was significantly smaller than in controls and was indistinguishable from that measured in the late ischemic PC groups (Figure 4), indicating that tolerance to the hemodynamic actions of NTG does not extend to the PC effects of this drug.

In all 3 phases of this study, the results of the measurements of infarct size were corroborated by the analysis of the relationship between infarct size and risk region size (ANCOVA) and by the measurements of the total deficit of wall thickening over the 3-day reperfusion period (data not shown).

**Discussion**

The ultimate goal of studying PC is to exploit this phenomenon for the protection of the ischemic myocardium in patients with coronary artery disease. Unfortunately, despite impressive advances in our understanding of the pathophysiology and mechanism of PC in experimental models, no widely applicable PC mimic has yet been developed for clinical use. Most agents that elicit a late PC-like protection have significant side effects that limit their clinical applicability. In contrast, NO donors, such as nitrates, are widely used clinically and relatively well tolerated.

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study to determine that a form of therapy widely used in patients elicits both an early and a late PC effect against infarction. In addition, this is the first study to indicate that NO donors induce cardioprotection at a distance of >24 hours and that this salubrious effect is preserved despite nitrate tolerance.

Having found in phase 1 that IV infusion of NTG induces late PC, in phase 2 we tested the efficacy of TD NTG patch treatment and compared the degree of cardioprotection afforded by this route of administration with that achieved by the IV route. TD NTG patches provide a vehicle for NTG delivery that is simple, noninvasive, does not require blood pressure monitoring, and is potentially widely applicable among patients with coronary artery disease. The results of phase 2 demonstrate that a single NTG patch is sufficient to induce a cardioprotective effect 72 hours later that is similar in magnitude to that achieved with IV NTG infusion. If these results are found to be applicable to the clinical arena, the PC-mimetic actions of NTG could be exploited in ambulatory patients without the need for IV treatment or hospital admission.

The development of nitrate tolerance is an important factor limiting the therapeutic efficacy of NTG in the treatment of patients with ischemic heart disease.\(^\text{15,16,27}\) Accordingly, in phase 3 of this study, we investigated whether tolerance to the hemodynamic actions of NTG extends to its PC-mimetic actions. To this end, rabbits were subjected to daily application of TD NTG patches for 4 consecutive weeks, and infarction was produced 72 hours after removal of the last patch. We reasoned that if nitrate tolerance prevents NTG from inducing late PC against infarction is not hampered by the development of tolerance, even after 28 days of continuous NTG administration resulting in marked attenuation of the vasodilator response to this drug. The dissociation between the PC-mimetic and hemodynamic actions of NTG after long-term treatment is important not only for its therapeutic implications but also from a conceptual standpoint, because it implies that different cellular mechanisms account for the 2 effects.

In conclusion, this study reveals a new, heretofore unappreciated action of nitrates that has considerable potential usefulness for the treatment of coronary artery disease. Data obtained in experimental animals must obviously be extrapolated to the clinical situation with caution. Nevertheless, the notion that in addition to its immediate anti-ischemic effects, NTG can also trigger a sustained phenotypic change that renders the heart resistant to infarction at a distance of 24 to 72 hours suggests novel applications of nitrates for protecting

TABLE 3. Heart Rate and Blood Pressure Before and During Administration of TD NTG

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>1 h</th>
<th>3 h</th>
<th>6 h</th>
<th>9 h</th>
<th>12 h</th>
<th>24 h</th>
<th>Day 14</th>
<th>Day 28</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Heart rate, bpm</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>12-h NTG patch group (n=5)*</td>
<td>244.6±8.3</td>
<td>276.0±5.9†</td>
<td>271.2±5.1†</td>
<td>252.0±7.7</td>
<td>249.6±7.8</td>
<td>243.2±11.4</td>
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<td>...</td>
<td>...</td>
</tr>
<tr>
<td>1-day NTG patch group (n=7)</td>
<td>255.0±5.3</td>
<td>...</td>
<td>...</td>
<td>...</td>
<td>...</td>
<td>...</td>
<td>...</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>28-day NTG patch (n=7) (NTG tolerant)</td>
<td>240.0±11.3</td>
<td>...</td>
<td>...</td>
<td>...</td>
<td>...</td>
<td>...</td>
<td>...</td>
<td>232.0±9.3</td>
<td>246.7±4.0</td>
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<tr>
<td><strong>Mean arterial pressure, mm Hg</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12-h NTG patch group (n=5)*</td>
<td>100.9±3.0</td>
<td>94.3±2.2</td>
<td>85.5±2.1†</td>
<td>78.7±1.5†</td>
<td>83.3±1.5†</td>
<td>85.1±3.4†</td>
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<tr>
<td>1-day NTG patch group (n=7)</td>
<td>82.7±2.8</td>
<td>...</td>
<td>...</td>
<td>...</td>
<td>...</td>
<td>...</td>
<td>...</td>
<td>84.6±1.8</td>
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<tr>
<td>28-day NTG patch (n=7) (NTG tolerant)</td>
<td>88.5±3.8</td>
<td>...</td>
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<td>...</td>
<td>87.7±2.1</td>
<td>85.6±3.0</td>
</tr>
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</table>

In the 1-day NTG patch group, measurements were taken before application of the NTG patch (baseline) and 24 h after application of the patch. In the NTG-tolerant group, measurements were taken before application of the first NTG patch (baseline) and on days 14 and 28 of the 28-day continuous TD NTG treatment. Data are mean±SEM.

*In a separate group of 5 rabbits, measurements were taken before application of the first NTG patch (baseline) and at 1, 3, 6, 9, and 12 h after application of the patch.
†P<0.05.
the ischemic myocardium in patients. Thus far, these drugs have been used mainly for their antianginal and preload-reducing properties. The present findings support the novel idea that nitrates might be equally useful, or perhaps even more useful, for the prophylaxis of ischemic myocardial death. Such an effect could be as important as, or even more important than, their short-term effects.

If nitrates exert PC-mimetic actions in humans, the IV route of NTG administration examined in phase 1 would be particularly feasible in patients who are hospitalized for acute coronary syndromes or for mechanical revascularization (CABG, PTCA). However, the results of phases 2 and 3 demonstrating that the same degree of infarct size reduction can be achieved with TD NTG delivery suggest that at least in principle, this therapy might be clinically feasible in a much larger spectrum of patients. The prolonged duration of NTG-induced cardioprotection observed in this study supports the hypothesis (which remains to be tested) that a protracted or even chronic PC state could be implemented by administering appropriate doses of IV or TD NTG on a regular basis every 2 to 3 days. Thus, the present results provide a rationale for studies aimed at investigating the usefulness of NTG and other nitrates as prophylactic treatments against infarction in patients with coronary artery disease. In all clinical studies that have examined the effect of nitrates in acute coronary syndromes, treatment was started either during or immediately after the index ischemic insult.28,29 We suggest that it might be fruitful to reexplore the role of nitrate therapy given before the onset of ischemia.

Acknowledgments

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References

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