Tolerance to nitroglycerin-induced preconditioning of the endothelium: a human in vivo study

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Submitted 23 December 2008; accepted in final form 28 October 2009

Gori T, Dragoni S, Di Stolfo G, Sicuro S, Liuni A, Luca MC, Thomas G, Oelze M, Daiber A, Parker JD. Tolerance to nitroglycerin-induced preconditioning of the endothelium: a human in vivo study. Am J Physiol Heart Circ Physiol 298: H340–H345, 2010. First published November 20, 2009; doi:10.1152/ajpheart.01324.2008.—Damage and dysfunction of the vascular endothelium critically influence clinical outcomes after ischemia and reperfusion (I/R). Brief exposure to organic nitrates can protect the vascular endothelium from I/R injury via a mechanism that is similar to ischemic preconditioning and is independent of hemodynamic changes. The clinical relevance of these protective effects clearly depends on whether they can be sustained over time. Twenty-four healthy (age 25–32) male volunteers were randomized to receive 1) transdermal nitroglycerin (GTN; 0.6 mg/h) administered for 2 h on 1 day only, 2) transdermal GTN for 2 h/day for 7 days, or 3) continuous therapy with transdermal GTN for 7 days. Eight volunteers underwent continuous GTN therapy followed by intra-arterial infusion of the antioxidant vitamin C. Finally, five additional subjects underwent no therapy and served as controls. Endothelial function measurements were performed before and after induction of I/R of the arm. I/R caused a significant blunting of the flow responses to acetylcholine in the control group (P < 0.01 vs. before I/R). A single 2-h GTN dosage, given 24 h before I/R, prevented I/R-induced endothelial dysfunction [P = not significant (NS) vs. before I/R], but this protective effect was completely lost after 1 wk of GTN administration 2 h/day (P < 0.05 vs. before I/R; P = NS vs. control). In subjects who received continuous GTN, endothelial responses were blunted before I/R, and I/R did not cause further endothelial dysfunction. Finally, vitamin C normalized acetylcholine responses and prevented the loss of preconditioning associated with prolonged GTN. In a separate experimental model using isolated human endothelial cells, short-term incubation with GTN caused upregulation of heme oxygenase, an effect that was lost after prolonged GTN administration. Although a single administration of GTN is able to protect the endothelium from I/R-induced endothelial dysfunction, this protection is lost upon prolonged exposure, likely via an oxidative mechanism.

ischemia and reperfusion; ischemic preconditioning; endothelial function

ORGANIC NITRATES HAVE LONG been used for their vasodilator effects. Of interest, recent lines of research have shown that exposure to these drugs has protective properties that do not depend on their hemodynamic changes and are mediated by the induction of a mechanism similar to ischemic preconditioning. In human studies, short-term exposure to nitroglycerin (GTN) has been associated with reduced evidence of ischemia during both exercise stress testing and angioplasty (3, 20) as well as with a reduced endothelial dysfunction after ischemia and reperfusion (I/R) injury (8, 11). Although the mechanisms of these effects remain incompletely understood, it has been proposed that the incubation with organic nitrates (and other nitric oxide donors) may trigger a cascade of phenomena that ultimately lead to the induction of protective genes and pathways (2). In line with this, we previously reported that incubation of cultured endothelial cells with nitrates induces the expression of heme oxygenase-1 (8), a protective enzyme that appears to play an important role in both ischemic and pharmacological preconditioning (19). Therefore, GTN may also be able to protect both the myocardium and the vascular endothelium via nonhemodynamic effects.

The clinical applicability of these effects of nitrates is clearly dependent on whether they can be maintained upon prolonged therapy. Previous animal studies addressing this question have produced conflicting results: one study demonstrated that exposure to tolerance-inducing doses of GTN was associated with a loss in the capacity to develop ischemic preconditioning (31), but other studies have described preconditioning-mimetic effects of GTN despite the induction of nitrate tolerance (6, 18). To date, this question has not been studied in humans.

The clinical use of nitrates is limited by the development of tolerance, i.e., the loss of hemodynamic and symptomatic effects that invariably occurs upon prolonged (>12 h) treatment. Tolerance appears to be a complex phenomenon involving vascular, biochemical, and autonomic changes in which oxidative stress appears to play a central role (for review see Refs. 7 and 15), as demonstrated by the fact that administration of the antioxidant vitamin C prevents or reverses these modifications (1, 32). To avoid tolerance, organic nitrates are administered intermittently, i.e., allowing a 10-h daily drug-free interval. However, studies have consistently demonstrated that even this treatment schedule is not devoid of implications, since intermittent administration of GTN (or isosorbide mononitrate), in doses that do not cause the development of hemodynamic tolerance, has been associated with endothelial dysfunction, increased sensitivity to vasoconstrictors, and, most importantly, with a paradoxical decrease in the ischemic threshold in many patients (26). Therefore, any preconditioning effect of nitrates is offset, in patients receiving GTN 12 h daily, by the appearance of these counter-regulatory mechanisms.

Given this scenario, the present study was designed to test, in humans, whether chronic continuous therapy with GTN is associated with the persistence of the preconditioning-mimetic effects of GTN on the vascular endothelium or, alternatively,
whether these effects could be maintained in the setting of repeated daily short-term (2 h/day) GTN therapy.

METHODS

The Ethics Committee of the Mount Sinai Hospital (Toronto, Canada) approved the protocol. Written informed consent was obtained in all cases. As detailed in Fig. 1, 24 healthy (age range, 25–32) male volunteers were randomized in an investigator-blinded parallel trial to one of three groups: 1) transdermal GTN (0.6 mg/h) administered for 2 h on one single occasion, with the dosage administered 24 h before the second visit; 2) transdermal GTN for 2 h/day for 7 days, with the last administration given 24 h before I/R; and 3) continuous therapy (24 h/day) with transdermal GTN for 7 days with the transdermal preparation changed each day at 9 h. Five additional subjects underwent no therapy to serve as controls. Seven days after the randomization, all subjects underwent measurement of endothelium-dependent forearm blood flow responses to intra-arterial infusions of acetylcholine chloride (7.5, 15, and 30 μg/min, infused at 0.4 ml/min) using venous occlusion strain gauge plethysmography. Endothelial function measurements were performed before and after induction of local I/R (15 min of ischemia followed by 15 min of reperfusion). Finally, another eight subjects underwent chronic continuous GTN therapy (0.6 mg/h) for 7 days. On the last day, before the study of endothelial function and I/R, they received an intra-arterial infusion of vitamin C at a rate of 24 mg/min for 15 min. This infusion rate, which is expected to achieve a local plasma concentration of 5 mM (i.e., ~50 times the normal concentration), has been used in previous studies to demonstrate a role of reactive oxygen species in the pathogenesis of endothelial dysfunction in patients with cardiovascular disease (17). Data were analyzed in a randomized, investigator-blinded fashion, and forearm blood flow was measured as milliliter per minute per 100 ml forearm volume as previously described (14).

Cell culture and treatment. Human umbilical vein endothelial cells (HUVECs) were obtained from the Academic Teaching Hospital of Frankfurt am Main/Höchst and the St. Vincenz Hospital in Mainz. HUVECs were isolated by collagenase digestion as previously described (25) and cultured in endothelial basal medium containing 20% fetal calf serum with penicillin (100 IU/ml) and streptomycin (100 μg/ml), 10 ng/ml epidermal growth factor, and L-glutamine at 37°C and 5% CO2. The cells were seeded in six-well plates at a density of 2 × 10^5 cells/well and grown to 60–70% confluence. Cells were kept in the medium and exposed to GTN (nitronal infusion; Pohl-Boskamp, Hohenlockstedt, Germany; 45 or 90 μM for 2 h) or vehicle (normal saline) either in one single occasion or for 4 consecutive days. Twenty-four hours after the last exposure to GTN, the medium was removed; cells were scraped and placed into 0.4 ml Laemmli buffer and frozen at −80°C until analysis.

Table 1. Blood pressure changes in response to transdermal GTN

<table>
<thead>
<tr>
<th></th>
<th>No Therapy</th>
<th>2 h For 1 Day</th>
<th>2 h For 7 Days</th>
<th>Continuous For 7 Days</th>
<th>Continuous For 7 Days + Vitamin C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Visit 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Systolic blood pressure</td>
<td>118±8</td>
<td>117±4</td>
<td>125±5</td>
<td>114±2</td>
<td>117±4</td>
</tr>
<tr>
<td>Diastolic blood pressure</td>
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<td>71±2</td>
<td>72±2</td>
<td>66±2</td>
<td>69±2</td>
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<tr>
<td>Heart rate</td>
<td>80±3</td>
<td>70±2</td>
<td>81±6</td>
<td>78±6</td>
<td>81±4</td>
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<tr>
<td>2 h after randomization</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Systolic blood pressure</td>
<td>117±9</td>
<td>105±3*</td>
<td>113±5*</td>
<td>103±4*</td>
<td>108±4*</td>
</tr>
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<td>Diastolic blood pressure</td>
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<td>57±3*</td>
<td>66±1*</td>
<td>55±2*</td>
<td>60±1*</td>
</tr>
<tr>
<td>Heart rate</td>
<td>87±17</td>
<td>77±3*</td>
<td>88±5*</td>
<td>87±8*</td>
<td>100±6*</td>
</tr>
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<td>Visit 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Systolic blood pressure</td>
<td>111±3</td>
<td>112±3*</td>
<td>119±6*</td>
<td>115±3*</td>
<td>115±4*</td>
</tr>
<tr>
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<td>67±4*</td>
<td>69±4*</td>
<td>67±3*</td>
<td>67±3*</td>
</tr>
<tr>
<td>Heart rate</td>
<td>72±3</td>
<td>72±3</td>
<td>81±4</td>
<td>79±7</td>
<td>88±5</td>
</tr>
<tr>
<td>Blood flow, infused arm</td>
<td>2.6±0.3</td>
<td>4.0±0.6</td>
<td>4.0±0.3</td>
<td>3.7±0.4</td>
<td>2.1±0.2</td>
</tr>
<tr>
<td>Blood flow, n.i. arm</td>
<td>2.9±0.4</td>
<td>3.8±0.5</td>
<td>3.7±0.3</td>
<td>2.9±0.3</td>
<td>2.4±0.4</td>
</tr>
</tbody>
</table>

Values are means ± SE. Blood pressure is measured in mmHg; forearm blood flow is measured in milliliters per minute per 100 ml of forearm volume. Blood pressure decreased in response to transdermal nitroglycerin (GTN). Immediately before endothelial function measurements (visit 2), blood pressure was not different across groups, confirming the presence of nitrate tolerance in the group undergoing continuous treatment. n.i., Noninfused. *P < 0.05, compared with visit 1; †P = not significant, compared with visit 1 and across groups.
Western blot analysis. Laemmli cell samples were subjected to Western blot analysis. Proteins were separated by SDS-PAGE and blotted onto nitrocellulose membranes. Immunoblotting was performed with antibodies against α-actinin (100 kDa, 1:2,500; Sigma-Aldrich) as controls for loading and transfer and against the enzyme heme oxygenase-1 (1:5,000, monoclonal; Stressgen, San Diego, CA) (33). Detection was performed by enhanced chemiluminescence with peroxidase conjugated anti-mouse (1:10,000; Vector, Burlingame, CA) secondary antibody. The antibody-specific bands were quantified by densitometry (33).

Results are expressed as means ± SE. Within- and between-group differences for all variables were evaluated with ANOVA for repeated measures, as appropriate. P < 0.05 was set as the threshold for significance. Post hoc comparisons were done with the Bonferroni correction.

RESULTS

Plethysmography data. Data are presented in Table 1 and in Fig. 2. Two hours after the first administration of GTN, blood pressure was significantly lower in all groups. Demonstrating the presence of nitrate tolerance, this effect was lost at the end of the 1-wk therapy with continuous GTN. As such, all subjects had similar hemodynamic parameters on visit 2.

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Fig. 2. Responses to intra-arterial infusions of acetylcholine in the 4 groups. Forearm blood flow (FBF) is expressed as the ratio of infused to noninfused arm. Each column presents the percent change in FBF from baseline (reported in Table 1) in response to each infused concentration of acetylcholine (7.5, 15, and 30 μg/min). A: in the control group, I/R caused a significant blunting in the responses to acetylcholine. B: GTN, administered in a single occasion for 2 h 24 h before I/R, protected endothelial function from I/R. C: this effect was lost after a 7-day treatment with GTN (2 h/day). D: acetylcholine responses were blunted already before I/R in nitrate tolerant subjects. E: coinfusion of vitamin C normalized acetylcholine responses and reinstated the preconditioning effect of GTN. NS, not significant.
Before I/R, the responses to acetylcholine in the subjects who received transdermal GTN for 2 h (either on 1 day only or daily for 7 days) were not different from those measured in the control group (P = not significant (NS)). In contrast, administration of GTN in tolerance-inducing doses was associated with a significant reduction in these responses (P < 0.01 vs. control) (14). Finally, confirming the role of oxygen free radicals in the development of nitrate-induced endothelial dysfunction, acetylcholine responses in tolerant subjects were normalized by coinfusion of the antioxidant vitamin C (P = NS compared with control).

I/R caused a significant blunting of the endothelial responses in the control group (Fig. 2A; P < 0.01 vs. before I/R). A single 2-h dosage of GTN prevented this I/R-induced endothelial dysfunction (Fig. 2B; P = NS vs. before I/R), which confirmed the preconditioning-mimetic properties of GTN (11). Importantly, this protective property of GTN was lost when subjects were exposed to transdermal GTN for 2 h/day for 7 days (Fig. 2C; P < 0.05 vs. before I/R and P = NS vs. control group after I/R). Finally, in those randomized to receive continuous treatment with GTN for 7 days (Fig. 2D), acetylcholine responses were blunted already before I/R, and GTN did not cause further evidence of endothelial dysfunction. In contrast, in those who received 7 days continuous GTN followed by intra-arterial vitamin C (Fig. 2E), I/R did not significantly blunt acetylcholine responses (P = NS vs. before I/R and vs. 2 h GTN after I/R; and P < 0.05 compared with no therapy after I/R).

Gene expression data. Data are presented in Fig. 3. Incubation with both dosages of GTN in one single occasion was associated with a significant increase in the expression of the protective enzyme heme oxygenase-1. In contrast, repeated GTN incubations were associated with a loss of this effect and the expression of the protein for the heme oxygenase-1 was not different from control.

**DISCUSSION**

Ischemic and pharmacological preconditioning are protective phenomena that occur in virtually every tissue and experimental model. While being particularly sensitive to I/R injury, endothelial integrity is of critical importance to obtain effective reperfusion and survival of tissues exposed to I/R (23); for instance, I/R-induced endothelial dysfunction is felt to be responsible for the no-reflow phenomenon, a condition where, despite effective recanalization, reperfusion (and tissue survival) are limited by (endothelium-dependent) phenomena such as platelet and leukocyte activation, endothelial swelling, and (micro)vascular thrombosis. Studies of I/R injury are very complex in humans. Of importance, the human in vivo forearm model described here has been previously used by our group and others (10, 11, 22) to specifically study I/R-induced endothelial dysfunction while leaving smooth muscle responses unaltered, and the I/R-induced endothelial dysfunction observed in this model can be prevented by ischemic and pharmacological (including GTN-mediated) preconditioning (11, 22).

Although studies in the last 20 years have investigated the mechanism(s) of preconditioning in response to both physical and pharmacological stimuli (including a single 2–4 h exposure to organic nitrates (2, 5, 11)), it remains to be seen whether prolonged exposure to these stimuli continues to exert protective effects. This question is central to the practical clinical application of preconditioning, and it is particularly complex in the setting of nitrate-induced preconditioning. Indeed, it is well accepted that intermittent (i.e., 10 h daily) GTN administration may cause sympathetic activation, increased reactivity to vasoconstrictors (12), and endothelial dysfunction (32), leading to a paradoxical decrease in the ischemic threshold in many patients, a phenomenon termed rebound ischemia (28). These complex changes offset any beneficial preconditioning-mimetic effect when GTN is administered intermittently. Similarly, the exposure to continuous therapy with organic nitrates causes a number of vascular abnormalities that include uncoupling of the nitric oxide synthase, production of reactive oxygen species, mitochondrial dysfunction, and inactivation of prostaglandin synthesis (all reviewed in Refs. 7 and 15). Thus there is evidence that suggests that tolerance-inducing dosages of GTN should be avoided in clinical practice. Given this background, the present study was designed to determine whether a daily short (2 h) exposure to GTN could allow maintaining the previously demonstrated benefit of GTN-preconditioning on the vascular endothelium while not causing the side effects of prolonged therapy and whether tolerance is associated with the loss of the preconditioning properties of GTN.

Reproducing previous observations from our group (8), we show that a single short-term exposure to GTN protects the endothelium from I/R-induced endothelial dysfunction. Insight into the mechanism of this observation is also provided, since evidence from human endothelial cell culture studies demonstrates that a single short-term (2 h) exposure to GTN causes upregulation of the gene for heme oxygenase-1, a gene involved in ischemic preconditioning. Of note, caution should always be applied when interpreting data from in vitro models, since a number of potential sources of artifacts (higher concentrations of the drug studied, experimental conditions, etc.) may influence the applicability of the results to humans in vivo. Despite this caveat, the present cell culture data are consistent with the existence of GTN-induced preconditioning and with the concept that this protective phenotype is not maintained over prolonged administration. In our forearm model, GTN-
induced endothelial preconditioning was lost when GTN was administered 2 h daily for a week, and in line with this, the expression of heme oxygenase-1 after repeated short-term exposure to GTN was not different from that seen in controls. Importantly, previous studies showed that heme oxygenase is actually inactivated after in vivo models of tolerance (35). Thus the preconditioning properties of GTN on the vascular endothelium appear to be lost when the exposure to the drug is protracted (or repeated) beyond a single 2-h administration. This evidence is compatible with previous data from our laboratory reporting that even after an 8-h exposure (8), this gene induction effect of GTN is absent. Thus, although the present data provide mechanistic evidence for the preconditioning effect of GTN, they also emphasize the importance of the timing of exposure to the nitrate: while brief (2 h) GTN causes endothelial preconditioning, incubation times of 8 h, or repeated daily short-term incubation, do not. In sum, our data suggest that this important nonhemodynamic effect of GTN may be lost upon repeated GTN administration (19).

Our plethysmography data in subjects during chronic continuous GTN administration confirm that GTN tolerance is associated with an endothelial dysfunction that is quantitatively similar to that induced by I/R injury. Caution should thus be used in avoiding prolonged continuous exposure to these drugs. Of importance, the intra-arterial infusion of vitamin C in doses known to have potent antioxidant effects normalized endothelial responses, which confirms the role of oxidative stress in nitrate-induced endothelial dysfunction (reviewed in Ref. 15). Furthermore, vitamin C, administered before I/R in a group of subjects who underwent GTN therapy in tolerant-inducing dosages, was associated with only a mild, statistically nonsignificant, blunting in acetylcholine responses after I/R.

Collectively, the above data emphasize the complex relationship between endothelial preconditioning and reactive oxygen species. Release of small quantities of oxygen free radicals appear to play a crucial role in the development of ischemic and pharmacological preconditioning (16, 24, 30), and we have previously demonstrated that the preconditioning-mimetic properties of GTN can be abolished when this drug is administered along with an antioxidant (8). However, an excess of reactive oxygen species might have the opposite effect, since preconditioning appears to be lost, or blunted, in conditions such as diabetes, aging, and hyperhomocysteinemia (and now, possibly, nitrate tolerance) that are known to be associated with chronic oxidative stress (9, 21). Thus excessive exposure to reactive oxygen species might play a role in the loss of this protective effect. Although a number of mechanisms remain to be elucidated, the present data provide the first human demonstration that preconditioning can be recaptured by removing, immediately before I/R, this oxidative damage.

The present findings have a number of implications. First, coupled with the evidence from previous studies that intermittent GTN therapy (i.e., 10 h/day exposure to transdermal GTN) might cause a paradoxical decrease in the ischemic threshold (29), they complicate the possibility of using GTN to induce a preconditioning-mimetic protection in patients at risk of, or with, overt coronary artery disease. Future studies will have to test whether this can be obtained with other drugs of the same or other classes. Second, the fact that I/R did not cause further endothelial dysfunction in patients who had received continuous GTN emphasizes the potential negative impact of long-term nitrate treatment on vascular function. Finally, we propose a mechanistic explanation for the observed loss of endothelial preconditioning properties during repeated GTN administration and suggest that, although the important role of reactive oxygen species in preconditioning is acknowledged, an excess of these toxic mediators might cause this loss. A number of issues remain open as to the molecular mechanisms involved in the present observations, their clinical relevance, and whether these findings can be transferred to myocardial tissue (4), to the cardiac circulation and to conduit arteries [which have been reported to be more resistant against nitrate tolerance (13, 27)]. Although vascular endothelial dysfunction is a potent determinant of outcome after an infarction, we acknowledge the fact that our study does not provide insight directly into I/R-induced myocardial dysfunction, arrhythmias, infarct size, and, ultimately, mortality. These end points will need to be tested in the future. For the moment, our data suggest that any future study investigating the preconditioning effect of a physical or pharmacological intervention should also test whether this effect can be maintained over a prolonged period.

ACKNOWLEDGMENTS

We thank Angelika Karpi, Richard Schell, and Sebastian Steven for expert technical assistance.

GRANTS

This study was supported by a grant from the Canadian Institute for Health Research. John Parker holds a Career Investigator award from the Heart and Stroke Foundation of Ontario.

DISCLOSURES

No conflicts of interest are declared by the author(s).

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