Repeated daily dosing with sildenafil provides sustained protection from endothelial dysfunction caused by ischemia and reperfusion: a human in vivo study

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McLaughlin K, Lytvyn Y, Luca MC, Liuni A, Gori T, Parker JD. Repeated daily dosing with sildenafil provides sustained protection from endothelial dysfunction caused by ischemia and reperfusion: a human in vivo study. Am J Physiol Heart Circ Physiol 307: H888–H894, 2014. First published July 25, 2014; doi:10.1152/ajpheart.00215.2014.—Sildenafil and nitroglycerin (GTN) are effective pharmacological preconditioning agents, protecting from the adverse effects of ischemia and reperfusion (I/R). The objective of the present study was to determine whether repeated, daily administration of sildenafil or GTN provides sustained preconditioning from I/R in the human forearm vasculature. Thirty-six healthy volunteers participated in this investigator-blind, randomized, placebo-controlled trial. Subjects received transdermal GTN (0.6 mg/h, 2 h/day), sildenafil (50 mg once daily), or placebo. Twenty-four hours after the first dose of medication, subjects underwent an assessment of flow-mediated dilation (FMD) before and after I/R (15 min of upper arm ischemia followed by 15 min of reperfusion). Subjects continued their study medication for 7 days, at which point FMD measurements were repeated before and after I/R. Venous blood samples were obtained for the determination of myeloperoxidase, P-selectin, or myoglobin before and after each I/R episode. Twenty-four hours after the first dose, both sildenafil and GTN (but not placebo) provided protection from the adverse effects of I/R. After 7 days of repeated daily doses and 24 h after the last dose, FMD was significantly blunted after I/R in placebo- and GTN-treated groups. In contrast, repeated daily administration of sildenafil provided continued protection from the adverse effects of I/R on endothelial function. There was no significant change in plasma levels of myeloperoxidase, P-selectin, or myoglobin at any time point. In conclusion, the present study establishes, for the first time in humans, that sildenafil, but not GTN, provides sustained pharmacological preconditioning of the endothelium and protection from adverse I/R effects on vascular function.

Although reperfusion is vital for the survival of ischemic tissue, restoration of blood flow after a period of ischemia is associated with further tissue damage (26). This phenomenon, termed ischemia-reperfusion (I/R) injury, has been the focus of intense research for decades as investigators have attempted to reduce infarct size in a variety of organs. It is now understood that exposure of the heart (and a number of other tissues) to repeated, short episodes of ischemia before a period of lethal ischemia provides potent protection, limiting consequent infarct size. This approach to protection has been termed ischemic preconditioning (3). Ischemic preconditioning is a biphasic phenomenon, with an early phase lasting 2–3 h (early window) and a late phase apparent 12–24 h after application and lasting 2–3 days (late window) (22). A number of pharmacological agents have been found to stimulate a similar protective phenotype in human models of I/R injury, including, but not limited to, organic nitrates (7), opioid agonists (37), angiotensin-converting enzyme inhibitors (27), volatile anesthetics (15), 3-hydroxy-3-methyl-glutaryl-CoA reductase inhibitors (21), and adenosine (18), although the mechanisms of these effects remain incompletely understood. Of note, late window pharmacological preconditioning is classically induced by short-term drug exposure 24–48 h before I/R (8, 21). As such, it does not depend on continued, therapeutic plasma concentrations of the preconditioning drug to manifest its protective effect.

Nitroglycerin (GTN) has clear late window preconditioning effects in humans undergoing percutaneous coronary intervention and in the setting of exercise testing (5, 19). We have recently demonstrated that a single 2-h exposure to GTN protected the endothelium 24 h after administration from I/R-induced endothelial dysfunction in both forearm resistance and conduit vessels (7, 8). Subsequently, we observed that daily, 2-h exposure to GTN therapy for a period of 5–7 days led to loss of this protective effect in forearm resistance vessels (8). Sildenafil has also been explored as a preconditioning agent. Animal models have demonstrated the ability of sildenafil to precondition tissue against I/R injury and reduce infarct size (28). Our laboratory confirmed these results in humans, providing the first evidence that oral sildenafil induces acute early window preconditioning against I/R-induced endothelial dysfunction (10).

To date, human investigations have almost always examined the preconditioning effect of a single dose or short-term exposure to the agent of interest. Clinical studies of preconditioning interventions have involved clinical situations where an acute event has already occurred (such as in the setting of an acute myocardial infarction) or a situation in which myocardial damage is predictable (as in the setting of cardiac surgery or elective percutaneous coronary intervention) (19, 23). An important and incompletely explored facet of pharmacological preconditioning is the potential for a repeated pharmacological stimulus to induce and maintain the preconditioned phenotype over time. As such, daily pharmacological preconditioning

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could be used as a “treatment,” potentially in patients at high risk of cardiac events. The purpose of the present investigation was to examine whether the acute preconditioning effects of GTN and sildenafil are maintained after a sustained period of daily exposure to these drugs.

METHODS

Population

Thirty-six healthy male volunteers (age: 18–29 yr, average: 22 yr) were recruited for this investigator-blind, randomized, placebo-controlled trial. Exclusion criteria included any active or history of medical illness, the use of medications (including supplemental vitamins and caffeine during the duration of the study), prior hypersensitivity to GTN or sildenafil, and risk factors for cardiovascular disease, such as hypertension or smoking. There was no significant difference in blood pressure between groups at recruitment, with an average blood pressure of 126/74 mmHg.

Study Protocol

All study visits were conducted in a quiet temperature- and humidity-controlled environment. Subjects were invited to participate in the study and provided written informed consent in all cases. This study was reviewed and approved by the Human Subjects Review Committee of Mount Sinai Hospital (MSH REB no. 11-0020-A). Before each study visit, subjects were required to fast for 12 h and abstain from alcohol. After subjects had been screened and admitted into the study, an informed consent was reviewed and approved by the Human Subjects Review Committee. Before each study visit, subjects were required to fast for 12 h and abstain from alcohol. After subjects had been screened and admitted into the study, standing blood pressure and heart rate were recorded using an automatic, calibrated sphygmomanometer (GE Healthcare). A designated study nurse randomized subjects to one of the following three treatments: transdermal GTN (Trinipatch, Paladin, 0.6 mg/h, 2 h/day), sildenafil (Viagra tablet, Pfizer, 50 mg once daily), or matching placebo (1 tablet daily). All study medications were taken daily at 9 AM. Subjects remained in the same treatment group for both the acute and chronic treatment periods. The design of the protocol is shown in Fig. 1.

Acute treatment period. Participants came to the laboratory at ~9 AM. After 30 min of rest in a temperature- and humidity-controlled environment, baseline standing blood pressure and heart rate were recorded. Subjects received one dose of their respective agent and remained in the laboratory for 2 h for monitoring. Standing blood pressure and heart rate were recorded at 2 h, and the subject was discharged from the laboratory. Subjects returned to the laboratory the following day, 24 h after drug administration. A venous blood sample was obtained from the antecubital vein of the study arm, and flow-mediated dilation (FMD) of the radial artery was measured as previously described (7, 10). To induce forearm ischemia, a pneumatic cuff was inflated to 250 mmHg for 15 min at the level of the brachial artery. Subsequently, reperfusion was allowed for 15 min after cuff deflation and was followed by a repeat FMD measurement. A second venous blood sample from the study arm was obtained at the completion of the FMD measurement. Subjects then continued into the chronic treatment period. This series of I/R does not cause impairment of endothelium-independent vasodilation; rather, it specifically causes blunting of endothelium-dependent vasodilation (16).

Chronic treatment period. Subjects administered one dose of their respective agents daily at 9 AM for 7 days. Participants returned 24 h after their final dose for pre- and post-I/R FMD measurements as well as venous blood collection.

Methods of FMD Measurement

Details of our approach to FMD measurement can be found in previous reports (7, 9, 10). Our laboratory has previously reported the repeatability (range of variation) and intraclass correlation coefficient of the FMD technique as 1.7% and 0.68, respectively (9). Files of FMD imaging sequences were coded and subsequently analyzed in a blinded fashion, where the operator was not aware of either the treatment group or period. During this analysis, four subjects were found to have poor-quality images in one or both treatment periods and, because of this, their images were not included in the analysis.

Biochemical Analysis

Venous blood from the antecubital vein of the study arm was collected into tubes containing EDTA at baseline and after I/R in both treatment periods. Venous levels of myeloperoxidase (MPO), P-selectin, and myoglobin were quantified using xMAP bead-based, multiplex technology (Luminex) with MILLIPLEX MAP Human Cardiovascular Disease Magnetic Bead Panel 2 (EMD Millipore). Briefly, diluted samples (25 µL, 1:100 dilution) and antibody-immobilized beads (50 µL) specific for MPO, P-selectin, and myoglobin were combined in wells on the capture plate. Fluorescently tagged detection antibodies to MPO, P-selectin, and myoglobin were then added to the wells followed by streptavidin-phycocerythrin (50 µL). Sheath fluid (Luminex) was added to all wells, and the plate was run on the compact analyzer MAGPIX instrument (EMD Millipore), which detected median fluorescent intensity. This assay provides detection limits for MPO, P-selectin, and myoglobin of 0.036, 0.051, and 0.004 ng/ml, respectively. Intraclass coefficients of variation of

![Image of experimental design](http://ajpheart.physiology.org/)
MPO, P-selectin, and myoglobin measurements were 6.8%, 5.6%, and 3.4%, respectively.

Statistical Analysis

Normally distributed data are presented as means ± SE. Sample size estimates assumed 1 − β = 0.2 and a two-sided α of 0.05. For our model of I/R injury, sample size estimates were made based on data from a previous report from our laboratory (10), in which I/R decreased FMD from 7.9 ± 3.3% to 1.2 ± 2.3%. Prevention of 50% of this impairment via GTN or sildenafil preconditioning at 80% power required a sample size of eight subjects in each treatment group. However, to take a more conservative approach and to account for potential dropouts, 12 subjects were recruited in each treatment group.

Analysis was performed in SAS (version 9.4, SAS Institute, Cary, NC). A two-period repeated-measures design was used (proc GLM with a Manova statement) to test for both the effect of time and treatment within and between groups. For the majority of time points, the biomarker data were not normally distributed. Therefore, differences over time and between treatment groups for plasma concentrations of MPO, P-selectin, and myoglobin levels were analyzed using a Kruskal-Wallis test. P values of <0.05 were set as the threshold for significance. A small number of biomarker values appeared to be measurement errors, with values greater than four times the median value of the remainder of the group at certain time points. These values were excluded from the analysis. Missing data points were replaced by imputation. SAS (version 9.1.3, SAS Institute) was used for all statistical analyses.

Table 1. Blood pressure and heart rate data

<table>
<thead>
<tr>
<th></th>
<th>Systolic/Diastolic Blood Pressure, mmHg</th>
<th>Heart Rate, beats/min</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Acute treatment period</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Placebo</td>
<td>124/70 ± 2</td>
<td>86 ± 4</td>
<td>NS</td>
</tr>
<tr>
<td>GTN</td>
<td>125/75 ± 2</td>
<td>78 ± 3</td>
<td>NS</td>
</tr>
<tr>
<td>Sildenafil</td>
<td>120/69 ± 3</td>
<td>82 ± 4</td>
<td>NS</td>
</tr>
<tr>
<td><strong>Chronic Treatment Period</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Placebo</td>
<td>125/70 ± 3</td>
<td>89 ± 4</td>
<td>NS</td>
</tr>
<tr>
<td>GTN</td>
<td>124/74 ± 2</td>
<td>80 ± 5</td>
<td></td>
</tr>
<tr>
<td>Sildenafil</td>
<td>119/70 ± 3</td>
<td>84 ± 4</td>
<td></td>
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</table>

Data are means ± SE. Systolic and diastolic blood pressure and heart rate before ischemia-reperfusion (I/R) are shown. GTN, nitroglycerin; NS, not significant.

There were no significant differences in baseline resting blood pressure or heart rate between groups in either treatment period (Table 1). There were no significant differences in baseline levels of creatine kinase, cholesterol, triglycerides, HDL, LDL, or cholesterol ratios (total cholesterol/HDL) between the three treatment groups in either treatment period, and all were within normal range (data not shown). There were no significant differences in baseline radial artery diameter, baseline blood flow, reactive hyperemia, or FMD before I/R between groups in either treatment period (Table 2).

Overall Effect of Treatment with Placebo, GTN, and Sildenafil

Two-period ANOVA revealed a significant effect of time (P = 0.033), a highly significant effect of treatment (P < 0.001), which varied as a function of treatment. This analysis revealed a significant difference between treatment groups and that this effect varied as a function of treatment period. Differences between individual treatment groups within each treatment period are described below.

Acute Treatment Period: Effect of Acute Administration of Placebo, GTN, and Sildenafil on Endothelial Dysfunction Induced by I/R

Baseline radial artery diameter and radial artery blood flow were not significantly different before versus after I/R during both treatment periods in any of the treatment groups [P = not significant (NS); Table 2]. Furthermore, there were no significant differences in reactive hyperemia after I/R in any of the groups (Table 2). In the acute treatment period, specific group comparisons revealed that FMD was significantly blunted after I/R in the placebo treatment group (pre-I/R: 7.9 ± 1.1% and post-I/R: 2.5 ± 0.9%, P = 0.0006; Fig. 2 and Table 3). Comparatively, impairment of FMD by I/R was prevented by treatment with GTN (pre-I/R: 9.0 ± 0.7% and post-I/R: 7.0 ± 0.9%, P = NS; Fig. 2 and Table 3) and sildenafil (pre-I/R: 8.0 ± 1.2% and post-I/R: 7.5 ± 1.8%, P = NS; Fig. 2 and Table 3).

Table 2. Radial artery diameter and blood flow data

<table>
<thead>
<tr>
<th></th>
<th>Radial Artery Diameter Before I/R</th>
<th>Radial Artery Diameter After I/R</th>
<th>Blood Flow Before I/R</th>
<th>Blood Flow After I/R</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline diameter, mm</td>
<td>Change in diameter after wrist cuff release, mm</td>
<td>Baseline diameter, mm</td>
<td>Change in diameter after wrist cuff release, mm</td>
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<tr>
<td><strong>Acute treatment period</strong></td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>Placebo</td>
<td>2.37 ± 0.09</td>
<td>0.19 ± 0.03</td>
<td>2.36 ± 0.09</td>
<td>0.06 ± 0.02</td>
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<td>GTN</td>
<td>2.25 ± 0.06</td>
<td>0.20 ± 0.02</td>
<td>2.29 ± 0.07</td>
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<td>Sildenafil</td>
<td>2.27 ± 0.06</td>
<td>0.18 ± 0.03</td>
<td>2.38 ± 0.07</td>
<td>0.17 ± 0.04</td>
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<tr>
<td><strong>Chronic treatment period</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Placebo</td>
<td>2.33 ± 0.10</td>
<td>0.18 ± 0.03</td>
<td>2.43 ± 0.12</td>
<td>0.06 ± 0.01†</td>
</tr>
<tr>
<td>GTN</td>
<td>2.40 ± 0.09</td>
<td>0.17 ± 0.02</td>
<td>2.57 ± 0.09</td>
<td>0.04 ± 0.03†</td>
</tr>
<tr>
<td>Sildenafil</td>
<td>2.34 ± 0.09</td>
<td>0.19 ± 0.03</td>
<td>2.39 ± 0.13</td>
<td>0.13 ± 0.03</td>
</tr>
</tbody>
</table>

Data are means ± SE. Radial artery diameter and blood flow before and after I/R are shown. *P < 0.0005 and †P < 0.005 vs. the corresponding value before I/R.

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Confirmed that the preconditioning effect of transdermal GTN is an effective preconditioning agent in humans. Finally, we consistent with our previous report (10) showing that sildenafil nafil is preserved during sustained, daily dosing. This finding is documentation that the vascular preconditioning effect of sildena-fil. We also provide the first continued pharmacodynamic effect. This study addresses a true preconditioning response and not a therapeutic. As such, for both early and late window periods, when the plasma concentrations of both drugs are no longer single exposure to both sildenafil and GTN during the late window of preconditioning. The half-life of GTN is very short (60–90 s), whereas sildenafil, with first-order clearance kinet-ics, has a reported half-life of 3– 4 h. Therefore, the observed (pre-I/R: 7.7 ± 1.2% and post-I/R: 2.7 ± 0.5%, \( P = 0.0012 \); Fig. 2 and Table 3). Sustained treatment with GTN did not prevent the impairment of FMD after I/R (pre-I/R: 7.4 ± 0.7% and post-I/R: 1.6 ± 1.2%, \( P = 0.0004 \); Fig. 2 and Table 3). However, sustained treatment with sildenafil prevented the impairment of FMD after I/R (pre-I/R: 8.1 ± 1.1% and post-I/R: 5.8 ± 1.1%, \( P = NS \); Fig. 2 and Table 3).

Effects of I/R and Treatment Group on Biomarker Levels

There were no differences in baseline values (before I/R) of MPO, P-selectin, or myoglobin during both acute and chronic treatment periods of the protocol in any of the three treatment groups. Furthermore, I/R had no significant effect on these biomarkers in any of the treatment groups during either acute or chronic treatment periods (Fig. 3).

DISCUSSION

In the present study, we confirmed the beneficial effect of a single exposure to both sildenafil and GTN during the late window of preconditioning. The half-life of GTN is very short (60–90 s), whereas sildenafil, with first-order clearance kinet-ics, has a reported half-life of 3–4 h. Therefore, the observed late window preconditioning effects were found at a time point when the plasma concentrations of both drugs are no longer therapeutic. As such, for both early and late window periods, this study addresses a true preconditioning response and not a continued pharmacodynamic effect.

This is the first human study to report a late window preconditioning effect of sildenafil. We also provide the first documentation that the vascular preconditioning effect of sildena-fil is preserved during sustained, daily dosing. This finding is consistent with our previous report (10) showing that sildenafil is an effective preconditioning agent in humans. Finally, we confirmed that the preconditioning effect of transdermal GTN in the conduit radial artery is attenuated after repeated exposure for 7 days, consistent with our previous reports (8, 20).

In the setting of I/R, sildenafil has consistently exhibited protective effects across different animal models and tissue types. This protective phenotype appears to be mediated through augmentation of the nitric oxide (NO)-mediated sig-naling cascade (4, 6, 28). Other phosphodiesterase (PDE) inhibitors, including tadalafil and cilostazol, have also been demonstrated to have a preconditioning response in animal models, indicating a class effect (1, 2). After sildenafil treat-ment, mRNA levels of both endothelial and inducible NO synthase (NOS) are increased, with protein levels elevated after 24 h (33). The resulting increase in NO is thought to be one of the primary mediators of the subsequent protective signaling cascade in delayed preconditioning. These effects, in concert with decreased catabolism of cGMP, are felt to augment the opening of ATP-sensitive K\(^+\) (K\(_{ATP}\)) channels through the activation of PKG (12, 34, 36). Our laboratory has previously demonstrated the acute (early window) precondi-tioning effect of sildenafil in humans at the level of the radial artery and that this effect was dependent on the opening of K\(_{ATP}\) channels, as inhibition of these channels with a sulfonyl-urea prevented the endothelial protection by sildenafil (10). Animal models examining the second window of preconditioning with sildenafil therapy have reported that these later pro-ective effects also appear to be mediated through the opening of mitochondrial K\(_{ATP}\) channels (28). In the present report, we have not addressed augmentation of NO bioavailability as a potential mechanism of the observed sustained preconditioning response. Although this might be explored with the use of a NOS inhibitor, such as N\(^2\)-monomethyl-L-arginine (L-NMMA), the FMD model does not lend itself to this approach in experiments examining I/R injury. The administration of L-NMMA markedly inhibits FMD responses, which, in turn, makes the demonstration of I/R-induced abnormalities of endo-thelial dysfunction difficult to demonstrate (11, 14). Therefore, although augmented NO bioavailability may be involved in the sustained preconditioning actions of repeated exposure to sildenafil, the experimental approach we used does not lend itself to a mechanistic exploration of that process.

To date, no animal experiment has examined the effect of repeated exposure to sildenafil on responses to I/R. However,
the sustained cardioprotective effects of the Bristol Meyers Squibb (BMS) compound BMS-191095, a selective opener of mitochondrial K\textsubscript{ATP} channels (35), has been explored. After 4 days of repeated administration, BMS-191095 significantly improved left ventricular function and decreased infarct size after I/R injury compared with controls. Coadministration of BMS-191095 with inhibitors of either phosphatidylinositol 3-kinase (wortmannin), K\textsubscript{ATP} channels (5-hydroxydecanoic acid), or NOS (L-arginine methyl ester) abolished this protection. This study provides evidence showing that protection against I/R injury, mediated by the opening of K\textsubscript{ATP} channels, can be sustained with chronic therapy in an animal model.

Although both pharmacological interventions studied here modify the NO/cGMP pathway, they do so in different ways. Sildenafil augments the effects of NO via inhibition of cGMP degradation and, potentially, by upregulation of NOS (33). In contrast, GTN acts as a direct NO donor or by generation of some NO-based moiety. In the setting of I/R injury, the action of sildenafil as a preconditioning agent appears to be mediated primarily by opening of mitochondrial K\textsubscript{ATP} channels, whereas the protective effects of GTN appear to be initiated by an increase in the bioavailability of free radical species (7, 10). The fact that the preconditioning effect of GTN is lost over time may be secondary to an adaptation to repeated increases in free radical production. In a previous report (8), we found that a single, short-term exposure of cultured endothelial cells to GTN was associated with upregulation of heme oxygenase-1. However, the upregulation of this protective enzyme was attenuated during repeated, daily exposure to GTN, leading to the hypothesis that repeated, daily increases in ROS were responsible for loss of the protective phenotype. The loss of the preconditioning effects of GTN in conduit arteries during repeated daily therapy with GTN is confirmation of this phenomenon in our previous reports (8, 20). Since our previous study examined the preconditioning effects of GTN in the resistance vasculature, we felt it important to explore this question in conduit arteries, since this vascular distribution is more relevant to the clinical effects of GTN. Of note, in both this report and our previous publication with GTN (8), we used a transdermal GTN preparation with the same delivery rate (0.6 mg/h). It is possible that the loss of preconditioning effects of GTN during repeated exposure might be dose dependent and that smaller or larger delivery rates might have different effects.

The mechanisms of I/R-induced endothelial dysfunction remain uncertain; however, previous human studies have implicated ROS, coagulation factors, and inflammatory markers (13, 24, 31). We examined the effect of forearm I/R on plasma concentrations of MPO, an enzyme with potent oxidant activity that is released from neutrophils upon their activation, as well as the cellular adhesion molecule P-selectin, which is rapidly released from granule storage upon endothelial cell activation (17, 25). Our purpose was to determine if pharmacological preconditioning with sildenafil or GTN (compared with placebo) would modify the response of these biomarkers to I/R. However, there were no significant changes in the venous concentration of either MPO or P-selectin after I/R injury in any treatment group. This neutral response may indicate that these biomarkers are not responsive to the I/R stimulus used; however, it could also be the result of either a washout effect secondary to local hyperemia after the episode of ischemia and/or a sequestration of inflammatory markers in the reperfused vascular bed (16). It is possible that the concentration of these biomarkers in systemic venous blood samples, rather than the local venous effluent from the postischemic forearm, might have identified evidence of an inflammatory response. We also measured myoglobin levels in an effort to demonstrate the skeletal muscle response to ischemic conditions. The observation that our model of I/R injury has no effect on myoglobin levels suggests that the ischemic stimulus we used did not affect smooth muscle cell integrity, a finding that is consistent with the observation that forearm resistance vessel responses to endothelium-independent vasodilators are not impaired after I/R (16).

The present findings have potential clinical implications, as sustained pharmacological preconditioning through intermittent sildenafil therapy could be exploited as a prophylactic treatment providing protection from unexpected episodes of cardiac ischemia. In contrast to opioids, which have demonstrated the ability to sustain cardioprotection against I/R injury in mouse models (30), sildenafil exhibits a good safety profile.
with few adverse effects. It is recognized that the daily use of a PDE5 inhibitor would complicate the management of acute cardiac events, limiting the free use of GTN and other organic nitrates. However, it has been demonstrated that intravenous GTN can be given safely after the administration of sildenafil, although there is a clear increase in the dose-blood pressure response relationship (29). The approval of daily administration tadalafil, in the management of erectile dysfunction and for symptoms of benign prostatic hypertrophy, suggests that daily administration of a PDE5 inhibitor is both feasible and safe for the majority of stable patients at risk of cardiovascular events (32).

We acknowledge that our study has a number of limitations. The forearm model of I/R injury in normal volunteers may not be representative of the effects of I/R injury, nor the impact of pharmacological preconditioning interventions, in the coronary circulation of patients with significant abnormalities of endothelial function, such as those with atherosclerosis, diabetes, hypertension, and/or hyperlipidemia. Nevertheless, the present findings provide a strong rationale for further investigations of the role of PDE5 inhibitors as preconditioning agents in patients with coronary artery disease. Furthermore, the model used here has provided consistent and reliable data regarding endothelial protection against I/R injury (7, 10, 16). Although our previous human study (10) confirmed an important role for mitochondrial KATP channels as mediators of the preconditioning effects of PDE5 inhibitors, we did not address this or other potential mechanisms in the present study. Although concurrent therapy with a sulfonylura could be used to explore the role of KATP channels in the sustained preconditioning effect of PDE5 inhibition, the hypoglycemic effect of these agents makes such a study difficult, if not impossible, in the human model. Finally, although our study had sufficient statistical power to detect protection from both GTN and sildenafil, the response after repeated dosing with sildenafil was variable (Table 3). Therefore, it is possible that the observed lack of I/R-induced endothelial dysfunction may represent a type II error.

In summary, the present study demonstrates, for the first time in humans, that repeated, daily exposure to sildenafil is associated with sustained preconditioning effects after chronic treatment, whereas daily exposure to intermittent transdermal GTN in conduit vessels is associated with the loss of the protection seen with acute exposure. These findings are supported by previous animal and human models, which have demonstrated that sildenafil is capable of providing protection to a multitude of tissue types. Any potential mechanistic differences that may exist between classic and sustained preconditioning have yet to be elucidated and should be the focus of future research.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS
Author contributions: K.M., M.C.L., A.L., T.G., and J.D.P. conception and design of research; K.M. and Y.L. performed experiments; K.M. analyzed data; K.M., Y.L., A.L., and J.D.P. interpreted results of experiments; K.M. prepared figures; K.M. drafted manuscript; K.M., Y.L., M.C.L., A.L., T.G.,
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