Concomitant Phosphodiesterase 5 Inhibition Enhances Myocardial Protection by Inhaled Nitric Oxide in Ischemia-Reperfusion Injury

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ABSTRACT

Enhanced cyclic guanosine monophosphate (cGMP) signaling may attenuate myocardial ischemia-reperfusion injury (I/R) and improve left ventricular (LV) functional recovery after myocardial infarction (MI). We investigated the cardioprotection afforded by inhaled NO (iNO), the phosphodiesterase 5 (PDE5)-specific inhibitor tadalafil (TAD), or their combination (iNO + TAD) in C57Bl/6J mice subjected to 6-minute left anterior descending artery ligation followed by reperfusion. We measured plasma and cardiac concentrations of cGMP during early reperfusion, quantified myocardial necrosis and inflammation by serial troponin-I (TnI) and myeloperoxidase-positive cell infiltration at day 3, and evaluated LV function and remodeling after 4 weeks using echocardiography and pressure-conductance catheterization. Administration of iNO, TAD, or both during I/R was safe and hemodynamically well tolerated. Compared with untreated mice (CON), iNO + TAD increased plasma and cardiac-cGMP levels during early reperfusion (80 ± 12 versus 36 ± 6 pmol/ml and 0.15 ± 0.02 versus 0.05 ± 0.01 pmol/mg protein, P < 0.05 for both). Moreover, iNO + TAD reduced TnI at 4 hours to a greater extent (P < 0.001 versus CON) than either alone (P < 0.05 versus CON) and was associated with significantly less myocardial inflammatory cell infiltration at day 3. After 4 weeks and compared with CON, iNO + TAD was associated with increased fractional shortening (43 ± 1 versus 33 ± 2%, P < 0.01), larger stroke volumes (14.9 ± 1.2 versus 10.2 ± 0.9 µl, P < 0.05), enhanced septal and posterior wall thickening (P < 0.05 and P < 0.001, respectively), and attenuated LV dilatation (P < 0.001), whereas iNO or TAD alone conferred less benefit. Thus, iNO + TAD has superior efficacy to limit early reperfusion injury and attenuate adverse LV remodeling. Combination of inhaled NO with a long-acting PDE5 inhibitor may represent a promising strategy to reduce ischemic damage following reperfusion and better preserve LV function.

Introduction

Acute myocardial infarction (MI) and subsequent loss of myocardial contractile function remains a major health challenge despite timely state-of-the-art recanalization strategies and guideline-recommended medical treatment. This is in part accounted for by a paradoxical phenomenon termed reperfusion injury (Yellon and Hausenloy, 2007). Multiple humoral and cellular pathways contribute to this paradoxical injury, including: activation and infiltration of neutrophils and platelets, generation of excess free radicals, and release of proinflammatory and proapoptotic cytokines (Carden and Granger, 2000; Vinten-Johansen, 2004; Heusch et al., 2008; McAlindon et al., 2015). In concert, these pathways result in impaired intracellular calcium homeostasis, mitochondrial damage, and cardiac myocyte necrosis with further loss of contractile function (Yellon and Hausenloy, 2007; Heusch et al., 2008; Chen and Zweier, 2014). Therefore, targeted strategies to reduce supplemental cardiac myocyte necrosis may enhance post-reperfusion functional recovery (Carden and Granger, 2000; Yellon and Hausenloy, 2007; Garcia-Dorado et al., 2009). Administration of inhaled nitric oxide (iNO) reduces ischemia-reperfusion injury (I/R) and improves collateral blood flow in rodent and porcine models of myocardial infarction without systemic hypotension (Liu et al., 2007; Nagasaka et al., 2008; Neye et al., 2012). The cardioprotective
effects of NO are mediated in part via activation of soluble guanylate cyclase, its major downstream intracellular receptor, with generation of the second messenger cGMP (Nagasaka et al., 2011). Elevated levels of cGMP and subsequent protein kinase G (PKG)-dependent phosphorylations regulate contractile function, Ca$^{2+}$- influx, Na$^+$/Ca$^{2+}$ exchange of cardiac myocytes, and prevent opening of the mitochondrial transition pore, thereby limiting apoptosis and necrosis. Enhanced cGMP signaling also stimulates endothelial proliferation, induces smooth muscle relaxation, and inhibits neutrophil and platelet activation (Bloch et al., 2007; Garcia-Dorado et al., 2009; Tsai and Kass, 2009).

Measurements of cGMP bioavailability in vivo following iNO administration are not readily available. However, these data are critically important, because the bioactive pool of cGMP is tightly regulated and inactivated by compartmentalized phosphodiesterases (PDEs) (Tsai and Kass, 2009; Mika et al., 2012). Five different PDE isoforms have been identified in the cardiovascular system, of which PDE5 and PDE9 share cGMP selectivity (Kass et al., 2007b). Moreover, pharmacological NO-based therapy has been associated with increased PDE5 activity, further attenuating cGMP signaling (Kakkar et al., 2002; Vandeput et al., 2009). Recently, PDE5 inhibitors, initially marketed for erectile dysfunction, have been shown to protect the heart against I/R and improve outcome in small animal models (Elrod et al., 2007; Das et al., 2008, 2015a; Salloum et al., 2008; Kukreja et al., 2011).

We hypothesized that concomitant stimulation of cGMP generation using iNO and prevention of degradation through PDE5-inhibition may contribute to a more powerful and persistent increase in cGMP bioavailability during I/R and may represent a novel, synergistic strategy to protect against cardiac dysfunction at longer term follow up.

**Materials and Methods**

**Induction of I/R and Experimental Design.** Animal experiments were approved by the Ethics Committee for Animal Experimentation (KU Leuven) and were performed in accordance with the Guide for Care and Use of Laboratory Animals (NIH). C57Bl6J mice (Charles River Laboratories, Chatillon-sur-Chalaronne, France) were housed in temperature- and light-cycle–controlled facilities and had access to rodent chow and water ad libitum. Age-matched (8–10 weeks old) adult male mice (20–35g) were anesthetized using sodium pentobarbital (40–60 mg/kg, i.p., Nembutal; Ceva Santé Animale, Brussels, Belgium) and ventilated with room air using 250 μl tidal volume at 150 strokes/min (MiniVent; Hugo Sachs Elektronik, Germany). Depth of anesthesia was controlled by pedal withdrawal reflex during the entire procedure. Following a left-sided thoracotomy, ischemia was induced by 60-minute transient ligation of the left anterior descending artery (LAD) using 7-0 silk suture (Ethicon/Johnson & Johnson, Brussels, Belgium). After 1 hour, the ligation was released and blood flow restored. Wounds were closed using 6-0 TiCron suture (Sherwood, VA, USA) supplemented with morphine hydrochloride (0.5–1 mg/kg, s.c., Stellosphine; Sterop Group, Brussels, Belgium) and ventilated with room air using 250 μl tidal volume at 150 strokes/min (MiniVent; Hugo Sachs Elektronik, Germany). Depth of anesthesia was controlled by pedal withdrawal reflex during the entire procedure. Following a left-sided thoracotomy, ischemia was induced by 60-minute transient ligation of the left anterior descending artery (LAD) using 7-0 silk suture (Ethicon/Johnson & Johnson, Brussels, Belgium). After 1 hour, the ligation was released and blood flow restored. Wounds were closed using 6-0 TiCron suture (Sherwood—Davis & Geck, Quebec, Canada) and animals were allowed to recover in temperature-controlled cages. Postoperative pain suppression (buprenorphine, 100 g/kg, i.p., Temgesic; Schering-Plough, Hull, UK) was administered during the first 2 postoperative days.

Mice were randomized after I/R into four treatment groups and followed for 4 weeks (4w): untreated control (CON, n=9), inhaled nitric oxide (iNO, n=9), tadalafil (TAD, n=9), and combination treatment with iNO and TAD (iNO+TAD, n=9). An additional subset of mice in these four groups was studied after 3 days to evaluate infarct size and determine inflammatory cell infiltration. Nitric oxide (80 ppm in room air; Ino Therapeutics LLC, Hampton, NJ) was administered through an intratracheal tube during mechanical ventilation and was started 30 minutes prior to and continued for 20 minutes after reperfusion. Dose and timing of NO inhalation was selected on the basis of previously reported results (Hataishi et al., 2006). Tadalafil (Kempprotec Ltd., Cumbria, UK) dissolved in 30% solution of 2-hydroxypropyl-β-cyclodextrin (Sigma-Aldrich, St. Louis, MO) was administered via gastric gavage (4 mg/kg) 1 hour prior to I/R, which was determined on the basis of previously reported interspecies dose extrapolations (Ahmad et al., 2009; Salloum et al., 2009; Koka et al., 2010). To determine acute cardiac cGMP responses to myocardial ischemia during different treatment regimens, an additional subset of animals (n=6–8 per treatment arm) was euthanized 20 minutes after reperfusion (Fig. 1A).

**Echocardiography.** Transthoracic echocardiography was performed using a MS400 transducer (18–38 MHz) connected to a Vevo 2100 scanner (VisualSonics Inc., Toronto, Canada) in anesthetized (2% isoflurane in oxygen; Ecuphar, Oostkamp, Belgium), temperature-controlled mice. Recordings were evaluated using the Vevo dedicated cardiac software, and LV dimensions at end-diastole (LVIDd) and end-systole (LVIDs), interventricular septum and LV posterior wall thickness increase in cGMP bioavailability during I/R and may be facilitated by these mechanisms. Pharmaceutical PDE5-inhibition may contribute to a more powerful and persistent increase in cGMP bioavailability during I/R and may represent a novel, synergistic strategy to protect against cardiac dysfunction at longer term follow up.
thickness at end-diastole and end-systole (IVSd, IVSd, LVPWd, LVPWs) were measured. Wall-thickening and fractional shortening (FS) were calculated.

Invasive Hemodynamic Measurements. Invasive blood pressure measurement was performed in all animals at day 3 prior to vital staining of the myocardium. In mice followed for 4 weeks, invasive pressure-conductance hemodynamic recordings were performed using urethane, etomidate, and morphine hydrochloride (1000, 1 and 0.5–1 mg/kg body weight i.p.) anesthesia supplemented with pancuronium bromide (Pavulon, 2 mg/kg i.p.) neuromuscular blockade. Mechanical ventilation was performed using room air at tidal volume 7 μl/kg body weight (MiniVent). Fluid homeostasis was supported by 80–100 μl/100 g infusion of 15% albumin in physiologic saline, and body temperature was maintained at 37°C using an infrared lamp connected to a T-thermocouple rectal probe (Hugh Sachs Elektronik). A 1.0-F pressure-conductance catheter (Millar, Houston, TX) was inserted in the LV via the right carotid artery, and steady-state LV pressure-volumes were recorded after a 10-minute stabilization. To obtain occlusion loops with progressively lowered preload, the inferior vena cava was compressed between liver and diaphragm with a cotton swab without opening the abdomen. Parallel conductance, attributable to cardiac muscle and connective tissues, was recorded after infinitesimal volume (5–6 μl 15% NaCl solution) i.v. injection and deducted from the total volume. After all measurements were completed, blood was withdrawn from the inferior vena cava using a 24G heparinized needle and used for cuvette calibration. Recorded measurements were evaluated using the P-V module of the LabChart software version 7.5 (ADInstruments, Oxford, UK). All data were inferred from the average of measurements with breath-holding during the expiratory phase. Each measurement represents at least 10 successive baseline loops. Indices of systolic and diastolic function were calculated, including stroke volume, cardiac output (CO), ejection fraction, stroke work, preload-recruitable stroke work, arterial elastance (Ea), ventricular-arterial coupling (Ea/Ees ratio), maximum and minimum peak rates of systolic pressure rise (dP/dtmax) or decline (dP/dtmin), and the time constant of isovolumic relaxation (τ) according to Weiss’ method.

Measurement of Nitric Oxide–Derived Oxidative End Products. In blood samples collected 20 minutes after I/R, plasma was separated and supplemented with N-ethylmaleimide (8 mM final concentration) to protect thiol-groups and stored frozen at −80°C until analysis. Nitrites (NO2−) were reduced using the tri-iodide reagent, and nitrites, nitrates, and S-nitroso compounds expressed as NOx were converted using vanadate (H3)Cl to NO, followed by ozone-based chemiluminescence measurement in line with the Sievers Model 280 Analyzer (GE Analytical Instruments, Boulder, CO) as described previously (Yang et al., 2003; MacArthur et al., 2007). Cardiac tissue collected 20 minutes after reperfusion was homogenized under liquid nitrogen and extracted in T-PER reagent (Thermo Fisher Scientific, Sunnyvale, CA). Protein concentration was measured by bicinchoninic acid assay (Pierce™ BCA Protein Assay Kit, Thermo Fisher Scientific) and adjusted to 5 mg/ml. 3-Nitrotyrosine content was determined in cardiac extracts and plasma using OxiSelect Nitrotyrosine ELISA Kit (Cell Biolabs Inc., San Diego, CA).

Histologic and Immunohistochemical Determination of Collagen Deposition and Myeloperoxidase-Positive Cell Infiltration. To assess mononuclear cell infiltration 3 days after I/R, myeloperoxidase (MPO) staining was performed using rabbit anti-human MPO antibody (Dako, Belgium). Mosaic scans of MPO-stained LV sections were used to count the number of cells infiltrating the LV septal and LV free walls at three different planes distal to the site of LAD ligation. Collagen deposition was measured in a semiquantitative manner on Sirius red-stained myocardial sections at three different planes and related to LV tissue area. Analysis was performed using color thresholding; Red color attributable to interstitial collagen was quantified, related to LV area, and expressed as the average relative collagen percentage for each animal. Mosaic images were scanned using AxioVer 200 microscope with AxioVer 4.6 software, (Carl Zeiss, N.V., Zaventem, Belgium) and different myocardial planes were evaluated using the ImageJ software (ver. 1.47s; NIH, Bethesda, MD).

Statistical Analysis. All data are expressed as mean ± S.E.M. Differences between groups were determined using one-way analysis of variance with Bonferroni’s posthoc test. For time-dependent follow up of cTnI, two-way analysis of variance with Dunnnett’s test for multiple comparisons versus untreated CON was applied. Non-Gaussian distributed MPO-cell infiltration data were compared using Kruskal-Wallis method with Dunn’s posthoc test. Probability value of P < 0.05 for all tests was considered statistically significant. Statistical analysis was performed using GraphPad Prism 6 software (ver. 6.04; GraphPad Inc., La Jolla, CA).

Results

Combined Therapy with iNO and TAD Confers Synergistic Myocardial Protection after I/R. To investigate the respective treatment effect on cardiac myocyte necrosis markers, we measured peak troponin I release at 4 hours and at 24 hours after reperfusion. After 4 hours, peak

<table>
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<tr>
<th>Table 1</th>
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<tr>
<td>Treatment</td>
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<tr>
<td>CON</td>
</tr>
<tr>
<td>TAD</td>
</tr>
<tr>
<td>INO</td>
</tr>
<tr>
<td>INO+TAD</td>
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BPM, Beats per minute; dP/dtmax and dP/dtmin, peak rate of systolic pressure rise and decline; HR, heart rate; LVDPmax, left ventricular maximal pressure.
plasma TnI levels were significantly reduced by iNO (n = 9, 17.6 ± 5.1 ng/l) and TAD (n = 7, 17.3 ± 5.0 ng/l) compared with untreated CON animals (n = 9, 24.6 ± 5.3 ng/l). This effect was further amplified in the combined iNO-TAD treatment arm (n = 9, 11.4 ± 2.4 ng/l), resulting in greater than 50% reduction in peak TnI levels 4 hours after reperfusion (Fig. 1B).

To evaluate the effect of pharmacologic intervention on myocardial inflammatory cell infiltration and tissue damage, we determined the number of myeloperoxidase-positive cells and the extent of infarcted area relative to risk area 3 days after I/R. The AAR encompassed 57% of the LV area in all groups and did not compromise hemodynamic status or early survival (Table 1). The TTC-stained nonviable area within the AAR was significantly smaller in TAD (n = 8), iNO (n = 5), and iNO-TAD (n = 5) animals (27 ± 4%, 22 ± 3%, and 24 ± 4%, respectively, versus 43 ± 2% in nontreated CON mice (n = 7); P < 0.05 for all). Limited discriminatory power of TTC stains and small sample size did not allow further differentiation between treatment groups (Fig. 2).

To evaluate the effect of iNO and TAD during I/R on subsequent long-term LV structural and functional remodeling, we measured LV dimensions and fractional shortening after 4 weeks using transthoracic echocardiography, and we performed pressure-conductance catheter analysis. The iNO+TAD intervention significantly reduced adverse remodeling and improved fractional shortening, whereas either therapy alone failed to do so (Fig. 3). Measurements of interventricular septal and posterior wall thickness in the infarct border zone
revealed less hypertrophy and better preserved wall thickening in all treatment groups (Table 2), with the most significant improvement in regional systolic function in the iNO and iNO+TAD groups. Pressure-volume analysis showed a significantly greater stroke volume in mice treated with iNO+TAD, resulting in a proportionately higher cardiac output at heart rates comparable between groups (Table 3). Mice who inhaled NO had an intermediate response with a lesser increase in stroke volume and CO, but higher LV end-systolic pressure and stroke work. Increased preload-recruitable stroke work, a load-independent parameter of contractility, failed to reach statistical significance in iNO+TAD, suggesting that increased stroke volumes may also be accounted for by altered loading conditions (e.g., lower arterial elastance, an index of ventricular afterload) independent of better preserved inotropy. Ventricular elastance, Ees, which defines the end-systolic pressure-volume relation and LV end-systolic stiffness and is considered a useful marker of acute changes in contractile function (Pacher et al., 2008), did not differ appreciably between groups in the chronic postinfarction phase 4 weeks after reperfusion and remained in what is considered the normal range in mice. This is consistent with the absence of overt systolic heart failure in the present I/R model. Consequently, ventricular-vascular coupling indexed by the Ea/Ees ratio did not show major differences between groups. Diastolic function parameters, including left ventricular end-diastolic pressure (LVEDP), dP/dt\text{min}, and isovolumic relaxation-time index (\( \tau \)) were comparable between CON and treated groups (Table 3) and consistent with comparable interstitial collagen deposition pattern (Supplemental Fig. 1).

### Table 2

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No.</th>
<th>IVSd (mm)</th>
<th>IVSs (mm)</th>
<th>WTiVs (%)</th>
<th>LVPd (mmHg)</th>
<th>LVPWs (mmHg)</th>
<th>WTPw (%)</th>
<th>HR (BPM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CON</td>
<td>11</td>
<td>1.07 ± 0.05</td>
<td>1.21 ± 0.004</td>
<td>24 ± 6</td>
<td>1.05 ± 0.03</td>
<td>1.23 ± 0.004</td>
<td>18 ± 4</td>
<td>385 ± 16</td>
</tr>
<tr>
<td>TAD</td>
<td>12</td>
<td>0.93 ± 0.02**</td>
<td>1.30 ± 0.01</td>
<td>40 ± 3</td>
<td>0.99 ± 0.01</td>
<td>1.23 ± 0.005</td>
<td>25 ± 1</td>
<td>452 ± 17</td>
</tr>
<tr>
<td>iNO</td>
<td>10</td>
<td>0.90 ± 0.03**</td>
<td>1.32 ± 0.002</td>
<td>48 ± 5**</td>
<td>0.94 ± 0.03*</td>
<td>1.27 ± 0.015**</td>
<td>35 ± 5**</td>
<td>442 ± 14</td>
</tr>
<tr>
<td>iNO+TAD</td>
<td>13</td>
<td>0.87 ± 0.02**</td>
<td>1.32 ± 0.007</td>
<td>52 ± 3***</td>
<td>0.93 ± 0.02**</td>
<td>1.23 ± 0.003</td>
<td>32 ± 2*</td>
<td>450 ± 19</td>
</tr>
</tbody>
</table>

BPM beats per minute; HR, heart rate in; IVSd and IVSs, diastolic and systolic interventricular septal thickness; LVPd and LVPWs, diastolic and systolic left ventricular posterior wall thickness; WTiVs and WTPw, percentage of interventricular septal and posterior wall thickening.

### Table 3

Pressure-volume analysis of cardiac function at 4 weeks

All data are presented as mean ± S.E.M. Significance is presented as: *\( P < 0.05 \); **\( P < 0.01 \); ***\( P < 0.001 \) versus CON.

<table>
<thead>
<tr>
<th></th>
<th>CON</th>
<th>TAD</th>
<th>iNO</th>
<th>iNO+TAD</th>
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<tbody>
<tr>
<td>n</td>
<td>11</td>
<td>13</td>
<td>12</td>
<td>13</td>
</tr>
<tr>
<td>HR (BPM)</td>
<td>601 ± 12</td>
<td>604 ± 14</td>
<td>603 ± 15</td>
<td>609 ± 13</td>
</tr>
<tr>
<td>LVESP (mmHg)</td>
<td>78 ± 3</td>
<td>85 ± 4</td>
<td>89 ± 2*</td>
<td>82 ± 3</td>
</tr>
<tr>
<td>LVEDP (mmHg)</td>
<td>2.4 ± 0.6</td>
<td>2.1 ± 0.3</td>
<td>3.5 ± 0.7</td>
<td>2.3 ± 0.5</td>
</tr>
<tr>
<td>SV (( \mu l ))</td>
<td>10.2 ± 0.9</td>
<td>11.0 ± 1.1</td>
<td>13.6 ± 1.1</td>
<td>14.9 ± 1.2*</td>
</tr>
<tr>
<td>CO (( \mu l/\text{min} ))</td>
<td>6129 ± 566</td>
<td>6637 ± 713</td>
<td>8277 ± 707</td>
<td>9156 ± 773*</td>
</tr>
<tr>
<td>EF (%)</td>
<td>50.6 ± 4.6</td>
<td>54 ± 4</td>
<td>54 ± 4</td>
<td>52 ± 3</td>
</tr>
<tr>
<td>SW (mmHg x ( \mu l ))</td>
<td>771 ± 85</td>
<td>932 ± 91</td>
<td>1205 ± 97*</td>
<td>1134 ± 119</td>
</tr>
<tr>
<td>PRSW</td>
<td>69 ± 8</td>
<td>71 ± 6</td>
<td>75 ± 10</td>
<td>94 ± 8</td>
</tr>
<tr>
<td>Ea (mmHg/( \mu l ))</td>
<td>7.8 ± 1.1</td>
<td>7.0 ± 0.5</td>
<td>6.3 ± 0.7</td>
<td>5.7 ± 0.3</td>
</tr>
<tr>
<td>Ees (mmHg/( \mu l ))</td>
<td>10.1 ± 2.5</td>
<td>10.3 ± 1.5</td>
<td>6.7 ± 1.1</td>
<td>9.1 ± 2.2</td>
</tr>
<tr>
<td>Ea/Ees</td>
<td>1.01 ± 0.16</td>
<td>0.79 ± 0.09</td>
<td>1.10 ± 0.14</td>
<td>0.83 ± 0.13</td>
</tr>
<tr>
<td>dP/dtmax (mmHg/( s ))</td>
<td>8795 ± 946</td>
<td>9956 ± 646</td>
<td>11899 ± 879</td>
<td>10617 ± 969</td>
</tr>
<tr>
<td>dP/dtmin (mmHg/( s ))</td>
<td>-7498 ± 538</td>
<td>-8248 ± 586</td>
<td>-8930 ± 822</td>
<td>-7837 ± 396</td>
</tr>
<tr>
<td>( \tau ) (ms)</td>
<td>5.2 ± 0.2</td>
<td>5.2 ± 0.2</td>
<td>5.0 ± 0.3</td>
<td>5.1 ± 0.2</td>
</tr>
</tbody>
</table>

BPM, Beats per minute; CO, cardiac output; dP/dt\text{max} and dP/dt\text{min}, peak rate of systolic pressure rise and decline; Ea, arterial elastance; Ea/Ees ratio, ventricular-arterial coupling; Ees, left ventricular end-systolic elastance; EF, ejection fraction; HR, heart rate in; LVESP and LVEDP, left ventricular end systolic and end diastolic pressures; PRSW, preload-recruitable stroke work; SV, stroke volume; SW, stroke work; \( \tau \), tau time constant of isovolumic relaxation according to Weiss' method.
increase after iNO and TAD treatments [48 ± 3 pmol/ml (n = 8) and 51 ± 7 pmol/ml (n = 6), respectively] versus 38 ± 6 pmol/ml (n = 7) in CON, but nearly doubled after iNO-TAD [80 ± 12 pmol/ml (n = 7), P < 0.01, versus CON and iNO, P < 0.05 versus TAD]. In parallel, cardiac tissue cGMP level increased significantly only after iNO-TAD treatment [iNO-TAD 0.15 ± 0.02 pmol/mg (n = 7) versus 0.05 ± 0.01 pmol/mg in CON (n = 7), P < 0.01], whereas the increase with either iNO 0.07 ± 0.01 pmol/mg (n = 6) or TAD 0.10 ± 0.02 pmol/mg (n = 8) alone did not reach statistical significance (Fig. 4, E and F).

**Combined iNO and TAD Therapy Attenuates Myocardial Leukocyte Infiltration, But Not Myocardial Fibrosis.** To investigate additional mechanisms of cardioprotection, we analyzed myocardial infiltration of myeloperoxidase-positive cells using immunohistochemical stains, an established marker of the inflammatory reaction after I/R. After 3 days, the number of MPO-positive cells relative to midventricular transversal tissue areas was significantly lower in iNO+TAD-treated mice (P = 0.02 versus CON; Fig. 5) but not in iNO- or TAD-treated mice.

Four weeks after I/R, we measured collagen fiber deposition in ~7% of the ischemic LV area, which was mostly restricted to the midventricular wall and did not vary with treatment assignment. In the absence of transmural infarction, the interstitial fibrosis did not result in extensive adverse LV remodeling causing significant impairment in contractile function, as is usually observed after permanent LAD ligation.

**Discussion**

In this study we report that combining inhalation of NO with oral administration of tadalafil is safe and confers incremental myocardial protection during I/R in mice. For a similar risk area, combination therapy was associated with a greater reduction in troponin release during the acute phase, and less inflammatory cell infiltration compared with either treatment alone. The early benefit at the time of reperfusion translated into markedly improved functional and structural remodeling after 4 weeks. LV end-systolic dimensions following combination treatment were reduced and were associated
with a better-preserved regional LV function on transthoracic echocardiography. In addition, invasive pressure volume analysis using conductance catheter technology confirmed improved contractile performance in mice treated with iNO and TAD with significantly higher stroke volumes. Finally, the superiority of the combination therapy was associated with significantly greater nitrite plasma concentration, a trend for lower cardiac nitrosative stress levels, and significantly higher cGMP bioavailability in the heart and in the circulation, emphasizing the importance of this second messenger system in cardioprotection.

Several laboratories have previously reported that NO inhalation during ischemia and reperfusion effectively reduces myocardial infarct size and improves cardiac function in rodent and porcine models of I/R (Liu et al., 2007; Nagasaka et al., 2008; Neye et al., 2012). Moreover, when administered at 80 ppm for 24 hours, iNO prevented early increase in left ventricular dimensions and helped to preserve ejection fraction (Hataishi et al., 2006). In the bloodstream, iNO is rapidly converted to nitrite, nitrate, or to various S- and N-nitrosated proteins (Bloch et al., 2007; Bhatraju et al., 2015). Red blood cells serve as a major reservoir of nitrite with a bidirectional reversible flux between red blood cell and plasma compartments. When exposed to lower hemoglobin oxygen saturations and acidic pH in ischemic myocardium, NO can be released from nitrites, hem-nitrosyls, and S-nitrosothiols. Such a selective release of bound NO increases its bioavailability in areas of impaired perfusion and could confer cardioprotection (McMahon and Doctor, 2006; Neye et al., 2012; Terpolilli et al., 2012; Schumacker, 2013). In this study, we have observed 2.5- to 3-fold increase in plasma nitrite levels and 6- to 7-fold increase in total NOx levels, which may serve as source of NO. Conversely, NO may interact with superoxide radicals to generate peroxynitrites and partially offset cardioprotection or may scavenge free radicals and transiently reduce nitrosative stress, as suggested by reduced cardiac 3-nitrotyrosine levels in the early post-reperfusion phase in mice treated with inhaled NO (Fig. 4D) (Ferdinandy et al., 2000; Szabó et al., 2007; Giels et al., 2011).

At the same time, inhibition of PDEs, the class of enzyme responsible for cGMP degradation, significantly reduced MI size and cardiac dilatation and preserved global LV function in mice (Kass et al., 2007a,b; Das et al., 2015a). Under physiologic conditions, PDE5 expression levels in the cardiovascular system are very low and mainly confined to smooth muscle cells. Uregulation and activation of PDE5 was reported in ischemic and failing myocardium, in part via cGMP-dependent and PKG-I mediated phosphorylation (Kass et al., 2007a,b; Das et al., 2015a), setting the stage for successful administration of selective PDE5 inhibitors, such as sildenafil, in heart failure with reduced ejection fraction (Guazzi et al., 2011; Redfield et al., 2013; Lukowski et al., 2014; Das et al., 2015a, p. 5).

We hypothesized that during acute ischemic disease insufficient NO bioavailability to stimulate cGMP generation argues against the use of PDE5 inhibition as a viable strategy to sufficiently boost cGMP levels. Therefore, in this study we investigated whether combined iNO-induced stimulation of soluble guanylate cyclase with selective inhibition of PDE5-catalyzed hydrolysis can safely confer cardioprotection. Ligation of the proximal LAD resulted in a territory at risk that encompasses more than 50% of the left ventricle. This degree of myocardial injury did not compromise hemodynamic stability or survival. Stable or very modest reductions in blood pressure were previously reported after application of iNO in acute MI (Bloch et al., 2007; Nagasaka et al., 2008; Ichinose, 2013). Conversely, single-dose administration of the long-acting tadalafil was reported to have variable effects on blood pressure. The applied dose of tadalafil, 4 mg/kg via gastric delivery, was approximated from human studies and was reported to reach protective plasma concentrations in rodents (Sesti et al., 2007; Ahmad et al., 2009; Salloum et al., 2009, 2014; Koka et al., 2014). In this study, monotherapy with iNO and TAD or combined application did not compromise blood pressure, one of the most critical parameters during I/R treatment protocols. However, rigorous dose escalation
studies would be required to determine the most effective dose and the safety margins.

We measured a significant rise in plasma and cardiac cGMP levels only in animals that received combination therapy. Plasmatic cGMP does not necessarily reflect the cGMP bioavailability in organs, but increased cGMP signaling in the ischemic heart confers protection by attenuating β-adrenergic-stimulated contractility, preventing progression of Ca2+ waves and limiting gap-junction communication during cell-to-cell propagation of necrosis (Francis et al., 2010). In addition to direct effects on cardiac myocytes, cGMP also affects multiple other pathways involved in I/R, including platelet activation, vasorelaxation, expression of adhesion proteins, endothelial permeability, and neutrophil activation (Garcia-Dorado et al., 2009). The latter effect together with monocyte-to-macrophage differentiation is reflected by increased MPO-levels (Hataishi et al., 2006; Liu et al., 2007). We observed reduction in MPO-positive cell infiltration exclusively after combined treatment. At the molecular level, cGMP-dependent activation of PKG-I enhances extracellular signal–regulated kinase signaling and results in reduced opening probability of mitochondrial permeability transition pore (Francis et al., 2010). The latter represents a final common switch in the reperfusion injury salvage kinase (RISK) pathway for cellular protection against ischemic damage and may be mediated partially through hydrogen sulfide (Salloum et al., 2009; Das et al., 2015b). In our study, combination treatment improved LV fractional shortening and prevented cardiac remodeling during the 4-week follow-up period, and either treatment alone had only partial effects.

Limitations. First, the relatively short follow up period and limited transmurality of the infarction in our mice precludes extension of findings to survival and development of heart failure. Second, TTC-based planimetry of infarct size was not sensitive enough to discriminate between the three treatment groups. To delineate infarct size with greater precision, detailed morphometric analysis or high flux density magnetic resonance imaging would be required. Third, administration of TAD 60 minutes prior to I/R does not represent a therapeutic situation in mice, but the concept may be useful during clinical translation using the time window between MI detection and reperfusion.

Conclusion

Combined NO inhalation and selective PDE5 inhibition using tadalafil during myocardial ischemia-reperfusion confers superior protection against I/R in mice. The associated increase in cGMP-signaling after the combined treatment suggests the importance of this pathway for beneficial long-term structural and functional remodeling. Combined therapy may represent a promising strategy for translational research to improve the outcome of ischemia-reperfusion injury in patients.

Authorship Contributions

Participated in research design: Lux, Pokreisz, Janssens.
Conducted experiments: Lux, Pokreisz, Swinnen, Caluwe, Gillijns.
Performed data analysis: Lux, Pokreisz, Swinnen.
Wrote or contributed to the writing of the manuscript: Lux, Pokreisz, Janssens, Szelid, Merkely.

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