Cytoprotective effects of N,N,N-trimethylsphingosine during ischemia-reperfusion injury are lost in the setting of obesity and diabetes

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First published July 13, 2007; doi:10.1152/ajpheart.00392.2007.—N,N,N-Trimethylsphingosine chloride (TMS), a stable N-methylated synthetic sphingolipid analog, has been shown to modulate protein kinase C (PKC) activity and exert a number of important biological effects, including inhibition of tumor cell growth and metastasis, inhibition of leukocyte migration and respiratory burst, and inhibition of platelet aggregation. We hypothesized that TMS would be cytoprotective in clinically relevant in vivo murine models of metastasis, inhibition of leukocyte migration and respiratory burst, biological effects, including inhibition of tumor cell growth and protein kinase C (PKC) activity and exert a number of important biological effects, including inhibition of tumor cell growth and metastasis (15), inhibition of neutrophil respiratory burst (37), inhibition of leukocyte migration (37), and inhibition of platelet aggregation (49), suggesting potent anti-inflammatory properties. Previous studies (6, 44, 55) performed in feline and rat models have reported that TMS protects against myocardial I/R injury by reducing myocardial necrosis. In addition, TMS therapy promotes coronary vascular function and attenuates the inflammatory response following myocardial reperfusion injury by attenuating cardiac accumulation of neutrophils and neutrophil superoxide production, as well as coronary vascular P-selectin expression (6, 44, 55). Therefore, TMS is considered to be a potentially effective agent in attenuating myocardial reperfusion injury in healthy animal models.

Cardiovascular (CV) disease is the leading cause of diabetes-related death (20). Persons with diabetes mellitus tend to suffer unduly from premature and severe coronary atherosclerosis. Postmortem studies of diabetic hearts have also independently confirmed that coronary heart disease is increased three- to fourfold compared with nondiabetic specimens (18, 51). The decade-spanning study of the Framingham population has shown an increased mortality rate, as well as reinfarction rates, following myocardial infarction (MI) in diabetics compared with nondiabetic patients (50). Similarly, obesity is reaching epidemic proportions worldwide (32) and is associated with an increased risk of premature death (1). Individuals with a central deposition of adipose tissue experience elevated cardiovascular morbidity and mortality, including stroke, congestive heart failure, myocardial infarction, and cardiovascular death. The increase in CV disease incidence is independent of the association between obesity and other cardiovascular risk factors (36, 38). The purpose of the present study was to investigate the potential cytoprotective effects of TMS in clinically relevant models of hepatic and myocardial I/R injury involving obesity and type 2 diabetes mellitus.

MATERIALS AND METHODS

Animals. All animals received humane care in compliance with the “Principles of Laboratory Animal Care” formulated by the National Society of Medical Research and the Guide for the Care and Use of Laboratory Animals. The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
Laboratory Animals published by the National Institutes of Health (NIH Publication No. 85-23, Revised 1996). The experimental protocol was reviewed and approved by the Institutional Animal Care and Use Committee of Albert Einstein College of Medicine. The mice utilized in the present studies were C57BL/6J, B6.Cg-m-/+ Lep<sup>ob</sup>/J (db/db), and B6.V-Lep<sup>ob</sup>/J (ob/ob) mice at 8–10 wk of age, obtained from Jackson Laboratories (Bar Harbor, ME).

Drugs. TMS (stock no. T6067) was purchased from Sigma-Aldrich (St. Louis, MO) and prepared as previously described (15, 27), dissolved at 1 mg/ml in ethanol-water (50:50 vol/vol) solution. Solutions at a concentration varying from 0.001 to 1 mg/kg were prepared from the stock solution, and a volume of 100 μl was injected into the left ventricle (LV) or femoral vein using a 30-gauge needle. An equal volume of the vehicle solution was injected into control animals. All surgeons were blinded to treatment until all analyses were performed.

Myocardial ischemia and reperfusion. The myocardial I/R protocol is depicted in Fig. 1A and has been described previously (12, 14, 25). Briefly, mice were weighed and blood obtained via tail snip was screened using a SureStep glucose-monitoring system (LifeScan). Mice were then anesthetized via an intraperitoneal injection of pentobarbital sodium (50 mg/kg) and ketamine (60 mg/kg). In addition, mice were given heparin sodium (200 U/kg) before surgery to prevent clot formation and allow for consistent and complete reperfusion postligation. The mice were then placed in a supine position with paws taped to the operating table. Animals were intubated with an endotracheal tube (PE-60) and ventilated with 100% oxygen (0.5 l/min) by a rodent ventilator (MiniVent, model 845; Hugo Sachs) at a rate of 110 strokes/min with a tidal volume of 230 μl. Effective ventilation was visually confirmed by vapor condensation in the endotracheal tube and rhythmic rising of the chest. Mice were maintained at a constant temperature of 37°C, monitored via a rectal probe connected to a Digi-Sense K-type digital thermometer. The mice were then exfoliated using Nair hair removal lotion, and the exposed areas were wiped with alcohol and betadine solution. A midline incision was made along the sternum, exposing the ribcage. Next, a thoracotomy was performed just to the left of the midline utilizing a thermal cautery unit (Geiger). The second and third ribs were cauterized, creating a small vertical opening ~1 cm in size. The left coronary artery (LCA) was then visually identified with the aid of an Olympus SZ61 stereomicroscope with a Schott ACE1 fiber optic light source. The LCA was ligated with a 7-0 silk suture passed with a tapered BV-1 needle in close approximation just under the coronary artery. A small piece of PE-10 tubing was then placed inline with the LCA, and the 7-0 suture was tightly tied, compressing the LCA and rendering the LV ischemic. Ischemia was visually confirmed by cyanosis of the affected LV. During the ischemic period the incision was covered with parafilm, creating an effective barrier against desiccation and dehydration. Just before ischemia, vehicle (saline) or TMS was injected into the lumen of the LV at a final volume of 100 μl. After 30 min of ischemia, the LCA was allowed to reperfuse by the removal of the 7-0 suture, and the sternum and skin were closed separately with 5-0 BIOSYN glycomer monofilament suture. In a separate group of mice, 100 μl of vehicle (saline) or TMS were injected into the lumen of the LV at the time of reperfusion. Animal recovery was supplemented by 100% oxygen and butorphanol (0.3 mg/kg) analgesia, as well as a single dose of the antibiotic cefazolin (50 mg/kg) to prevent infection. In the surgical recovery area, a heat lamp was utilized to maintain the appropriate body temperature of the mice. In addition, food and water were made available immediately and for the remainder of the first 24 h of recovery. In all the experiments of myocardial infarct size, coronary occlusion was maintained for 30 min, followed by removal of the suture and reperfusion for 24 h. In the experiments of cardiac function, the period of reperfusion was 72 h after coronary occlusion for 30 min.

Myocardial infarct size determination. All of the procedures for the LV area at risk (AAR) and infarct size determination have been described previously (34). At 24 h of reperfusion, the mice were reanesthetized, intubated, and connected to a rodent ventilator. A catheter (PE-10 tubing) was placed in the common carotid artery to allow for Evans blue dye injection. A median sternotomy was performed, and the LCA was religated in the same location as before. Evans blue dye (1 ml of a 2% solution) was injected via the carotid artery catheter into the heart to delineate the ischemic zone from the nonischemic zone. The heart was then rapidly excised and serially sectioned into five 1-mm-thick sections that were then incubated in 1% 2,3,5-triphenyltetrazolium chloride for 5 min at 37°C to demarcate the viable and nonviable myocardium within the risk zone of the mice. Each of the five 1-mm-thick myocardial slices was imaged and weighed. Images were captured using a Q-Capture digital camera connected to an Apple computer. Images were then analyzed in a blinded fashion by using computer-assisted planimetry with ImageJ-1.37 software to measure the areas of infarction, risk, and nonischemic LV.

Echocardiographic assessment of LV structure and function. Baseline echocardiography images were obtained in a separate group of mice (n = 11 in vehicle group, n = 10 in TMS group) 1 wk before LCA ischemia in a blinded fashion. The mice were lightly anesthetized with isoflurane in 100% O<sub>2</sub> and in vivo transatlhoracic echocardiography of the LV using a 30-MHz RMV scanhead interfaced with a Vevo 770 (Visualsonics, Toronto, Canada) was used to obtain high-resolution two-dimensional ECG-based kilohertz visualization (EKV) B-mode images acquired at the rate of 1,000 frames/s over 7 min. LV end-diastolic dimensions (LVEDDs), LV end-systolic dimensions (LVESDs), and heart rate were measured from analysis of the EKV images. High-resolution B-mode images were used to calculate the ejection fraction and fractional shortening. One week after the baseline images were acquired, the mice were subjected to 30 min of LCA occlusion followed by 72 h of reperfusion. After 72 h of reperfusion, post-MI echocardiographic images were obtained and analyzed in a blinded fashion.

Histological analysis of myocardial infarct size. After echocardiographic assessment, the mice were reanesthetized, intubated, and connected to a rodent ventilator. A median sternotomy was performed, and the heart was rapidly excised and fixed in conventional fixing solutions (4% paraformaldehyde and 1% glutaraldehyde in 0.1 M phosphate buffer). After 12 h in 4% paraformaldehyde, the heart...
was cut into 1-mm-thick slices as detailed above. The slices were dehydrated and embedded in paraffin, then cut into 4-μm slices that were heated overnight in a 60°C incubator. The sections were dewaxed and stained with hematoxylin and eosin. For each heart, we analyzed four sections taken from the midventricle and then averaged these numbers to obtain a single infarct per LV (%) measurement for each animal. Digital images of the slides were then captured and analyzed in a blinded fashion using computer-assisted planimetry with Image J-1.37 software to measure the area of infarct or scar relative to the LV.

**Hepatic I/R protocol.** The hepatic I/R protocol is depicted in Fig. 1B and has been described previously (25, 26). Mice were anesthetized with a combination of ketamine (100 mg/kg) and xylazine (8 mg/kg) through an intraperitoneal injection. Mice were then injected with heparin (100 μg/kg ip) to prevent blood clotting. A midline laparotomy incision was performed to expose the liver. TMS was injected intravenously through the femoral vein, and the left lateral and median lobes of the liver were rendered ischemic by complete clamping of the hepatic artery and the portal vein using microaneurysm clamps. This experimental model results in a segmental (70%) hepatic ischemia. This method of partial ischemia prevents mesenteric venous congestion by allowing portal decompression throughout the right and caudate lobes of the liver. The liver was then repositioned in the peritoneal cavity in its original location for 45 min. The liver was kept moist with gauze soaked in 0.9% normal saline. In addition, body temperature was maintained at 37°C using a heat lamp and monitoring of body temperature with a rectal temperature probe. The duration of hepatic ischemia was 45 min in all experiments, after which the microaneurysm clamps were removed and the abdominal wall was sutured. The total duration of hepatic reperfusion was 5 h.

**Liver enzyme determinations.** To evaluate the extent of hepatic injury, mice were reanesthetized with ketamine (100 mg/kg) and xylazine (8 mg/kg) at 5 h following reperfusion. At this time a blood sample was obtained by insertion of a 20-gauge needle into the inferior vena cava. Serum was obtained from each blood sample to analyze for hepatic alanine aminotransferase (ALT) levels. This enzyme is liver specific and is released from the liver during injury (25, 26). Serum samples were analyzed for ALT using a spectrophotometric method (Sigma-Aldrich) (23).

**Western blot analysis.** Hearts of sham, I/R plus vehicle, and I/R plus TMS mice from both the nondiabetic and db/db groups were excised 1 h following myocardial I/R. Samples of the LV (75 mg) were homogenized in 1 ml of ice-cold RIPA lysis buffer. The homogenate was then centrifuged at 1,330 g for 2 min to remove cellular debris. The supernatant was then transferred into a new tube and centrifuged at 16,000 g for 20 min at 4°C. The resultant supernatant was collected (cytosolic fraction). The resulting pellet (mitochondrial fraction) was resuspended in 300 μl of RIPA lysis buffer. Protein concentrations of both fractions were measured using the DC protein assay (BioRad Laboratories, Hercules, CA). Equal amounts of protein (50 μg) were loaded in each lane of polyacrylamide-SDS gels. The gels were electrophoresed, followed by a transfer of the protein to a nitrocellulose membrane. The membrane was then blocked with a blocking solution and then probed with primary antibodies specific for PKC-α, PKC-β, PKC-δ, PKC-ε, PKC-θ, and PKC-λ (Santa Cruz Biotechnology) overnight at 4°C. Immunoblots were next processed with secondary antibodies (Amersham) for 1 h at room temperature. Immunoblots were then probed with an ECL Plus chemiluminescence reagent kit (Amersham) to visualize signal, followed by exposure to X-ray film.

**Statistical analysis.** All data in this study are means ± SE. Differences in data between the groups were compared using Prism 4 (GraphPad Software) with Student’s paired two-tailed t-test or one-way analysis of variance (ANOVA) where appropriate. For the ANOVA, if a significant variance was found, the Tukey test was used as the post hoc analysis. A P value <0.05 was considered significant.

### Table 1. Body weight and blood glucose measurements of wild-type, ob/ob, and db/db mice

<table>
<thead>
<tr>
<th>Strain</th>
<th>Sample Size</th>
<th>Body Weight, g</th>
<th>Blood Glucose, mg/dl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>19</td>
<td>26±1</td>
<td>125±5</td>
</tr>
<tr>
<td>ob/ob</td>
<td>52</td>
<td>48±1*</td>
<td>222±8*</td>
</tr>
<tr>
<td>db/db</td>
<td>44</td>
<td>44±1*†</td>
<td>353±10*†</td>
</tr>
</tbody>
</table>

Values are means ± SE. *P < 0.001 vs. wild type. †P < 0.001 vs. ob/ob.

## RESULTS

**Body weight and blood glucose in wild-type, ob/ob, and db/db mice.** Data for body weight and blood glucose are presented in Table 1. Measurements demonstrated that the body weights in ob/ob obese mice (n = 52) and db/db diabetic mouse (n = 44) were significantly increased (P < 0.001) compared with those in nondiabetic control mice (n = 19). Fasted blood glucose levels were measured in wild-type, ob/ob, and db/db mice. Blood glucose levels were significantly (P < 0.001) elevated in the ob/ob (222 ± 8 mg/dl) and db/db mice (353 ± 10 mg/dl) compared with those in nondiabetic control animals (125 ± 5 mg/dl). Furthermore, there was a significant difference (P < 0.001) in the blood glucose measurements between the ob/ob and db/db mice.

**TMS and myocardial infarct size in wild-type mice.** We initially investigated the effect of varying dosages of TMS on myocardial infarct size following 30 min of ischemia and 24 h of reperfusion (Fig. 2). TMS or vehicle was administered just before the onset of ischemia. The AAR per LV area was similar in the vehicle- and TMS-treated mice (P = nonsignificant (NS)). TMS decreased myocardial necrosis relative to the AAR at doses ranging from 0.001 to 1.0 mg/kg. A peak effect was observed at a dose of 0.025 mg/kg with a myocardial infarct size reduction of 66% (50.83 ± 1.89 vs. 17.32 ± 2.11%, P < 0.001 vs. vehicle) at this dose.

**TMS and myocardial infarct size in wild-type mice at 72 h.** We also measured the infarct area relative to the left ventricle following myocardial I/R at 72 h of reperfusion by histological analysis (Fig. 3). Compared with the vehicle group, TMS-treated mice displayed a smaller area of infarct. Infarct size per AAR at doses ranging from 0.001 to 1.0 mg/kg. A peak effect was observed at a dose of 0.025 mg/kg with a myocardial infarct size reduction of 66% (50.83 ± 1.89 vs. 17.32 ± 2.11%, P < 0.001 vs. vehicle) at this dose.

**TMS and LV chamber dimensions and function.** We next investigated the effect of TMS on LV dimensions. Mice were treated with 0.025 mg/kg TMS or vehicle, subjected to 30 min of myocardial ischemia, and then reperfused for 72 h. M-mode echocardiography was used to measure LVEDD and LVESD before I/R and after 72 h of reperfusion. The area and volume of the LV were measured from two-dimensional high-resolution B-mode images. Myocardial I/R increased LVESD, LV systolic area, and LV systolic volume (Table 2). However, TMS limited this effect and attenuated LV dilation during systole by 67% (limiting the LVESD increase to 0.34 instead of 1.04 mm, P < 0.05). There were no significant differences (P = NS) in LVESD, LV diastolic area, and LV diastolic volume between the study groups.

We subsequently determined whether the reduction of infarct size and preservation of LV dimensions would lead to improvements in LV function. LV ejection fraction and fractional shortening were measured at baseline and 72 h post-
myocardial I/R. Ejection fraction and fractional shortening post-MI decreased in both groups following myocardial I/R (Fig. 4, A and B). Nevertheless, TMS significantly ($P < 0.01$) improved the ejection fraction by 51% and fractional shortening by 52% compared with the vehicle.

Myocardial infarct size following TMS therapy at reperfusion. In a separate group of mice (Fig. 5), TMS was administered at the time of reperfusion. In those mice, myocardial infarct size was reduced by 37% (39.87 ± 4.61 vs. 25.19 ± 3.12%, $P = 0.04$ vs. vehicle), which represents a 44% loss of efficacy compared with administration before ischemia.

TMS and hepatic I/R injury. We next investigated the effects of administration of TMS on hepatic cell injury following I/R (Fig. 6). We examined a range of TMS dosages from 0.001 to 1 mg/kg. TMS at a dose of 0.025 mg/kg limited serum elevations of the liver-specific enzyme ALT by 43% (385.4 ± 51.37 U/l, $P = 0.05$) compared with vehicle, demonstrating a marked decrease in hepatic cell injury with TMS at that dose. TMS, however, had a narrow therapeutic range in the liver and at dosages higher than 0.2 mg/kg increased serum ALT levels compared with the vehicle (not shown).

TMS and myocardial infarct size in ob/ob mice. We investigated the cardioprotective effects of TMS following 30 min of ischemia and 24 h of reperfusion (Fig. 7, A and B) in ob/ob mice. The AAR per LV area was similar ($P = NS$) in the vehicle- and the TMS-treated mice. TMS significantly decreased the infarct size relative to the AAR at all the administered doses. A peak effect was observed at the dose of 0.025 mg/kg with a myocardial infarct size reduction of 36% (53.41 ± 4.61 vs. 34.24 ± 3.54%, $P < 0.05$ vs. vehicle).

TMS and hepatic I/R injury in ob/ob mice. We investigated the effects of TMS on hepatic cell injury following I/R in ob/ob mice following 30 min of ischemia and 24 h of reperfusion. Representative midventricular photomicrographs of wild-type hearts are shown after 30 min of myocardial ischemia and 24 h of reperfusion. Areas of the myocardium that appear blue represent the areas of myocardium that are at risk for infarction. In contrast, the areas of myocardium that stain red (i.e., TTC positive) represent viable myocardium that was at risk for infarction. Myocardium that appears pale (i.e., TTC negative) indicates areas of myocardium at risk that are necrotic (i.e., infarcted). TMS treatment significantly reduced myocardial infarction. B: myocardial infarct size per area at risk for hearts receiving doses of TMS ranging from 0.001 to 1 mg/kg. TMS significantly reduced myocardial infarct size compared with vehicle. Values are means ± SE. Numbers inside bars indicate the number of animals investigated in each group. **$P < 0.01$; ***$P < 0.001$ vs. vehicle.
mice. TMS was administered at a dose of 0.025 mg/kg (Fig. 7C). This dose did not result in a significant reduction in serum ALT levels following I/R. These data suggest that TMS does not ameliorate hepatic I/R injury in ob/ob mice.

**TMS and myocardial infarct size in db/db mice.** Next, we investigated the effects of TMS on myocardial infarct size following 30 min of ischemia and 24 h of reperfusion in db/db mice (Fig. 8, A and B). The AAR per LV area was similar (P = NS) in the vehicle- and the TMS-treated mice. TMS did not provide beneficial effects in diabetic mice subjected to myocardial I/R injury.

**TMS and hepatic I/R injury in db/db mice.** Last, we investigated the effects on TMS on hepatic cell injury following I/R in the db/db mouse (Fig. 8C). TMS (0.025 mg/kg) did not attenuate serum ALT levels following I/R. These data suggest that at the doses investigated, TMS does not ameliorate cell injury in the liver of db/db mice.

**TMS and PKC-δ translocation to the mitochondria following myocardial I/R in wild-type and diabetic hearts.** Western blot analysis (Fig. 9) of heart tissue taken from wild-type mice revealed that following MI/R, the expression of PKC-δ decreased in the cytosolic fraction and increased in the mitochondrial fraction, indicating that PKC-δ translocates from the cytosol to the mitochondria following MI/R (P < 0.05 vs. sham). The administration of TMS was found to significantly decrease this translocation (P < 0.05 vs. I/R plus vehicle), as evidenced by a greater expression of PKC-δ in the cytosolic fraction accompanied by a lesser expression in the mitochondrial fraction compared with vehicle-treated samples. The analysis of heart samples taken from sham-operated db/db mice revealed that the expression of PKC-δ was significantly higher at the level of mitochondria compared with sham-operated wild-type mice, suggesting that the more PKC-δ resides at the mitochondria of a diabetic heart at baseline (P < 0.05 vs. wild type). As observed in the hearts of wild-type mice, PKC-δ was also found to translocate from the cytosolic fraction to the mitochondrial fraction following MI/R in the diabetic heart (P < 0.05 vs. sham). However, TMS failed to attenuate translocation in the diabetic heart.

**DISCUSSION**

The present study clearly demonstrates a significant cytoprotective effect of TMS in models of myocardial and hepatic I/R injury in healthy mice. The conferred cardioprotection was characterized by a reduction in myocardial infarct size, preservation of LV ejection fraction, and fractional shortening,

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**Table 2. Two-dimensional echocardiographic measurements at baseline and 72 h post-MI/R**

<table>
<thead>
<tr>
<th>Group</th>
<th>Heart Rate, beats/min</th>
<th>LVEDD, mm</th>
<th>LVESD, mm</th>
<th>LV Area-D, mm²</th>
<th>LV Area-S, mm²</th>
<th>LV Volume-D, μl</th>
<th>LV Volume-S, μl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>508 ± 32</td>
<td>3.89 ± 0.13</td>
<td>2.60 ± 0.18</td>
<td>17.12 ± 0.95</td>
<td>9.00 ± 1.17</td>
<td>43.75 ± 3.40</td>
<td>16.15 ± 3.92</td>
</tr>
<tr>
<td>Baseline</td>
<td>471 ± 33</td>
<td>4.43 ± 0.18*</td>
<td>3.63 ± 0.20†</td>
<td>24.75 ± 1.91†</td>
<td>21.15 ± 1.75‡</td>
<td>80.47 ± 9.83†</td>
<td>62.94 ± 8.62‡</td>
</tr>
<tr>
<td>Post-MI/R</td>
<td>477 ± 26</td>
<td>4.09 ± 0.06</td>
<td>2.74 ± 0.14</td>
<td>17.99 ± 1.25</td>
<td>10.26 ± 1.06</td>
<td>46.28 ± 4.24</td>
<td>18.99 ± 2.75</td>
</tr>
<tr>
<td>Post-MI/R</td>
<td>498 ± 17</td>
<td>4.25 ± 0.08</td>
<td>3.08 ± 0.10‡</td>
<td>20.87 ± 1.13</td>
<td>14.79 ± 1.21§</td>
<td>59.47 ± 4.91</td>
<td>33.87 ± 4.19§</td>
</tr>
</tbody>
</table>

Values are means ± SE at baseline and 72 h post-MI/R. n = 11 in vehicle group and n = 10 in N,N-trimethylsphingosine (TMS) group. LVEDD, left ventricular (LV) end-diastolic dimension; LVESD, LV end-systolic dimension; D, diastole; S, systole. *P < 0.05; †P < 0.01; ‡P < 0.001 vs. baseline. §P < 0.05 vs. vehicle.

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**Fig. 4.** TMS and LV function in wild-type mice. Ejection fraction (A) and fractional shortening (B) were calculated using 2-dimensional echocardiography images at baseline and 72 h following 30 min of LCA ischemia. Both groups of mice displayed a significant reduction in ejection fraction and fractional shortening at 72 h following myocardial ischemia. However, mice treated with 0.025 mg/kg TMS displayed significantly less reduction than control peptide-treated mice. Values are means ± SE. Numbers inside bars indicate the number of animals investigated in each group. ***P < 0.01 vs. vehicle. **P < 0.05 vs. vehicle.

**Fig. 5.** TMS and myocardial infarct size in wild-type mice. TMS was administered at the onset of reperfusion. Myocardial infarct size was determined following 30 min of LCA ischemia and 24 h of reperfusion. Comparison of infarct area (INF), area at risk (AAR), and LV area showed that TMS treatment significantly attenuated myocardial infarct size by 37% (P = 0.04 vs. vehicle) relative to the area at risk (INF/AAR). Values are means ± SE. Numbers inside bars indicate the number of animals investigated in each group.
resulting in a significant attenuation of cardiac dysfunction. TMS also ameliorated the extent of hepatocellular injury following I/R in healthy mice. However, these cytoprotective effects did not fully extend to hepatic or myocardial tissue in obese and diabetic mice following I/R injury. Although TMS did reduce myocardial infarct size in ob/ob obese mice, the extent of infarct size reduction was less significant than that observed in the healthy wild-type mice.

Murohara et al. (44) have previously demonstrated that TMS therapy reduces myocardial infarct size in an in vivo feline model of myocardial I/R injury. They investigated the beneficial effects of a single dose (60 μg/kg) of TMS therapy in adult healthy cats and demonstrated a significant reduction in infarct size following a short (270 min) period of reperfusion. Campbell et al. (6) also demonstrated in a Langendorff-perfused heart model of myocardial I/R that 20 μg/kg TMS significantly maintained postreperfusion coronary flow and LV developed pressure. In our study we have investigated the effect of TMS therapy on myocardial infarct size and cardiac function after a much longer period of myocardial reperfusion (72 h) in vivo. We also investigated its therapeutic effect across varying dosages (0.001 to 1 mg/kg), demonstrating a complete dose-response relationship. In addition, we have also investigated the effects of varying dosages of TMS in hepatic tissue following I/R. Our data are consistent with previous data confirming the beneficial effects of TMS in healthy animals. Importantly, however, our study elucidates the effects of TMS therapy in clinically relevant models of diabetes and obesity with contradictory results and describes the likely mechanism of its cytoprotective action.

It has long been known that an inflammatory response involving leukocyte infiltration plays an important role in the pathogenesis of myocardial infarction following I/R (16, 35, 42, 48). In addition, oxygen-derived free radicals have

Fig. 6. TMS and serum ALT levels in wild-type mice. Serum ALT levels were measured in wild-type mice receiving doses of TMS ranging from 0.001 to 0.2 mg/kg following 45 min of hepatic ischemia and 5 h of reperfusion. TMS (0.025 mg/kg) reduced serum ALT levels by 43%. Values are means ± SE. Numbers inside bars indicate the number of animals investigated in each group. *P < 0.05 vs. vehicle.

Fig. 7. TMS and I/R injury in ob/ob obese mice. A: representative photomicrographs of ob/ob hearts after 30 min of myocardial ischemia and 24 h of reperfusion. TMS treatment significantly reduced myocardial infarction after 30 min of myocardial ischemia and 24 h of reperfusion. B: myocardial infarct size per AAR for mouse hearts receiving TMS ranging from 0.025 to 1 mg/kg. TMS therapy significantly reduced myocardial infarct size compared with vehicle. *P < 0.05 vs. vehicle. C: serum ALT levels in ob/ob mice receiving 0.025 mg/kg TMS after 45 min of hepatic ischemia and 5 h of reperfusion. TMS did not reduce serum ALT levels in ob/ob mice. Values are means ± SE. Numbers inside bars indicate the number of animals investigated in each group.
been known to play an important role in endothelial dys-
function and reperfusion injury (14, 60) by disrupting en-
dothelial cell integrity and inactivation of endothelium-
derived nitric oxide synthase (eNOS) (52). Studies have
previously shown that TMS attenuates the surface expres-
sion of P-selectin on coronary endothelial cells during
reperfusion of the ischemic heart (55) and attenuates leuko-
cyte-endothelium interactions (37), leukocyte migration,
and a subsequent inflammatory response (37), inhibiting the
generation of oxygen-derived free radicals (37).

Diabetes and obesity are metabolic disease states with ab-
errant regulation of cellular signaling pathways (57). These
conditions are characterized by a high concentration of inflam-
matory mediators such as IL-6 and TNF-α (2). In addition,
studies have demonstrated that eNOS-derived NO release and
eNOS-dependent vasoreactivity is significantly impaired in
diabetes (5, 10). Our laboratory has previously reported that the
extent of myocardial infarction is significantly increased in the
db/db diabetic mouse following I/R (33). We have also dem-
onstrated that therapies aimed at reducing the extent of I/R
injury may not be beneficial and may even prove to be harmful
in this animal model of type 2 diabetes (14). Hyperglycemia
during diabetes mellitus increases nonenzymatic glycation,
characterized by the binding of glucose or its by-products to
amino groups of proteins. This reaction leads to the formation
of complex compounds, advanced glycation end products
(AGEs), which alter structure and functions of proteins (56).
Glycation and oxidative stress are closely linked, and both
phenomena are referred to as “glycoxidation” (3). All steps of
glycoxidation result in the generation of oxygen free radicals,
some of them being common with lipid peroxidation path-
ways. In addition, glycated proteins activate membrane recep-
tors such as RAGE through AGEs, and induce an intracellular
oxidative stress and a proinflammatory status (40). Glycated
proteins, therefore, may modulate functions of cells involved in
oxidative metabolism and induce inappropriate signaling re-
sponses (64).

Previous studies have also shown that PKC-mediated
signal transduction pathways are involved in P-selectin
upregulation on platelet and endothelial cell surfaces (45)
and that inhibitors of PKC activation cause attenuation of
leukocyte migration (7), platelet aggregation (24, 58, 61–
63), and oxygen-derived free radical production (26). Since
TMS is a potent inhibitor of PKC activity (27), this may be
an important underlying signaling mechanism in the protec-
tion against acute inflammatory injury and may be partially
responsible for the beneficial effects of TMS therapy fol-
lowing I/R. In our study we have demonstrated a mechanism
of TMS-mediated cytoprotection involving the inhibition of
PKC-δ.

PKC-δ is among the predominant forms of PKC in cardiac
ventricles and has been implicated in heart failure, myocar-
dial hypertrophy, and ischemic preconditioning (21, 30, 41).
Previous studies have shown that translocation of PKC from
the cytosol to different subcellular sites is a hallmark of
PKC activation by various stimuli, and ischemia induces the
addition, the administration of TMS does not significantly inhibit translocation of PKC-δ to the mitochondria in db/db mice. The failure to inhibit the chronic activation of DAG/PKC transduction pathway in hyperglycemic states may be responsible for the apparent lack of benefit with TMS in these animals.

In the ob/ob obese mouse, we observed a reduction in myocardial infarct size following I/R; however, this reduction was not as significant as that observed in the wild-type mice. Although both diabetes and obese models are characterized by hyperphagia, obesity, hyperglycemia, and hyper-insulinemia associated with characteristic pancreatic lesions, diabetes causes the more severe condition. In the db/db mouse, marked hyperglycemia appears as early as 6 wk and is sustained until death at 5–7 mo. In contrast, ob/ob mice have less marked and usually transient hyperglycemia and continue to gain weight during a nearly normal life span (11). The significantly lower fasting blood glucose in the ob/ob compared with the db/db mice (19) may explain the sustained benefit of TMS following myocardial I/R in the ob/ob model. The liver of these ob/ob mice, however, is characterized by higher levels of triglycerides and an increased severity of steatosis (54). Hyperlipidemia can lead to an increase in the intracellular accumulation of DAG and subsequent activation of the DAG/PKC pathway (12). Therefore, activation of the DAG/PKC pathway in the liver in the ob/ob mice may have accounted for the lack of any benefit with TMS therapy following hepatic I/R in these mice.

In conclusion, we have demonstrated that acute administration of TMS attenuates cell necrosis related to hepatic and myocardial I/R injury in healthy mice. TMS attenuated the extent of myocardial cell necrosis, preserved cardiac function, and limited hepatic cell injury following I/R, thereby providing conclusive proof of its cytoprotective effects in healthy mice. We have also shown that inhibition of PKC activation and translocation may be a likely mechanism for TMS-mediated cardioprotection. The beneficial effects of TMS did not fully extend to myocardial and hepatic tissue in the ob/ob obese and db/db diabetic mouse models following I/R injury. It is likely that the loss of cytoprotection may be secondary to derangements in sphingolipid-PKC signaling mechanisms in insulin-resistant and steatotic states. Our laboratory has previously shown that pretreatment with simvastatin initiated 5 days before myocardial I/R was protective in db/db mice. Further studies are needed to investigate whether chronic therapy with TMS, days ahead of I/R, is beneficial in these obese and diabetic mice. In addition, since increased oxidative stress in diabetes and obesity may be partially responsible for the loss of beneficial effects with TMS, another approach would be to test the efficacy of TMS therapy in models of I/R injury when administered in combination with antioxidants agents. Future studies are also required to determine the precise mechanisms of MI and hepatic I/R in ob/ob and db/db mice. In the meantime, caution must be exercised in the translation of preclinical results to patient populations.

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REFERENCES

2. Bastard JP, Maachi M, Lagathu C, Kim MJ, Caron M, Vidal H, Capeau J, Feve B. Recent advances in the relationship between obesity, inflammation, and insulin resistance. Eur Cytokine Neu 17: 4–12, 2006.