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

Susheel Gundewar, John W. Calvert, John W. Elrod, and David J. Lefer

Department of Medicine, Division of Cardiology, and Department of Pathology, Albert Einstein College of Medicine, Bronx, New York

Submitted 29 March 2007; accepted in final form 13 July 2007


First published July 13, 2007; doi:10.1152/ajpheart.00392.2007.

---

**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 \textit{Guide for the Care and Use of Laboratory 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 \textit{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+/+ Lepr<sup><s>ab</s></sup> /J (db/db), and B6.V-Lep<sup><s>y</s></sup>/<s>y</s> (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 regions 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 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 (80 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% 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 1.0% 2,3,5-triphenyltetrazolium chloride for 5 min at 37°C to demarcate the viable and nonviable myocardium within the risk zone of the heart. 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 mouse (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 transthoracic 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 30 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 (Bio-Rad 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-ε (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/db</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/db.
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 ± 53.51 vs. 672.8 ± 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 ± 4.41 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.
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/db 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.

### 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.

**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 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.

### 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>Baseline</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>
</tr>
<tr>
<td></td>
<td>Post-MI/R</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>
</tr>
<tr>
<td></td>
<td>TMS</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>
</tr>
</tbody>
</table>

Values are means ± SE at baseline and 72 h postmyocardial ischemia-reperfusion (post-MI/R); n = 11 in vehicle group and n = 10 in N,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.
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
been known to play an important role in endothelial dysfunction and reperfusion injury (14, 60) by disrupting endothelial cell integrity and inactivation of endothelium-derived nitric oxide synthase (eNOS) (52). Studies have previously shown that TMS attenuates the surface expression of P-selectin on coronary endothelial cells during reperfusion of the ischemic heart (55) and attenuates leukocyte-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 aberrant regulation of cellular signaling pathways (57). These conditions are characterized by a high concentration of inflammatory 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 demonstrated 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 lipidic peroxidation pathways. In addition, glycated proteins activate membrane receptors 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 responses (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 protection against acute inflammatory injury and may be partially responsible for the beneficial effects of TMS therapy following 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, myocardial 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
In our study, we have demonstrated that there is increased cell death following I/R injury in diabetic animals. This may therefore be a likely signaling mechanism for TMS-mediated cardioprotection. In addition, Inagaki and colleagues (29, 30) have shown that administration of a selective PKC-δ inhibitor protects against I/R injury in isolated perfused rat hearts. In our study we have demonstrated that there is increased translocation of PKC-δ from the cytosol to the mitochondria during I/R. We have also demonstrated that administration of TMS at the onset of ischemia inhibits the translocation of PKC-δ in healthy mice but not in db/db diabetic mice. This may therefore be a signaling mechanism for TMS-mediated cardioprotection.

Hyperglycemia activates the glycolytic pathway and increases the production of diacylglycerols (DAG) (31). PKC-δ belongs to a novel family of PKCs that are maximally activated by DAG in the absence of calcium and increased DAG levels and PKC activity (53). Increased activity of PKC-δ isoforms in the retina, aorta, heart, renal glomeruli, and macrophages has been reported in diabetes (13). Activation of the DAG/PKC-δ pathway may therefore lead to upregulation of endothelial cell adhesion molecules, accumulation of leukocytes and platelets, increased production of ROS, increased endothelial permeability, and endothelial dysfunction, all of which may contribute toward an increase in cell death following I/R injury in diabetic animals. In our study we have demonstrated that there is increased translocation PKC-δ to the mitochondria in db/db mice. In 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 hyperinsulinemia 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 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-δ 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, 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 antioxidant 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.

ACKNOWLEDGMENTS

We thank Mark R. Duranski for expert technical assistance during the course of these studies.
GRANTS

This work was supported by National Heart, Lung, and Blood Institute Grant 2R01 HL-6049, American Diabetes Association Grant 7-04-RA-59, and a grant from the Japan Science and Technology Agency (to D. J. Lefer).

REFERENCES


2. Bastard JP, Maachais M, Lagothu C, Kim MJ, Caron M, Vidal H, Capeau J, Febe V. Recent advances in the relationship between obesity, inflammation, and insulin resistance. *Eur Cytokine Netw* 17: 4–12, 2006.


