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Beneficial effects of *N*,*N*,*N*-trimethylsphingosine following ischemia and reperfusion in the isolated perfused rat heart[1]

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Abstract

Objective: Ischemia followed by reperfusion in the presence of polymorphonuclear leukocytes (PMNs) results in cardiac contractile dysfunction as well as myocardial injury. These deleterious effects are due in large part to endothelial dysfunction leading to an upregulation of cell adhesion molecules and subsequent neutrophil-induced cardiac injury. At physiologically relevant concentrations, *N*,*N*,*N*-trimethylsphingosine (TMS), a synthetic *N*-methylated sphingosine derivative, has been shown to attenuate leukocyte–endothelial cell interactions. We wanted to test the effects of TMS on neutrophil-mediated cardiac dysfunction in ischemia/reperfusion. **Methods:** This study examines the effects of TMS in a neutrophil-dependent isolated perfused rat heart model of ischemia (I) (20 min) and reperfusion (R) (45 min) injury. **Results:** Administration of TMS (20 μg/kg) to I/R hearts perfused with PMNs improved coronary flow and preserved left ventricular developed pressure as an index of cardiac contractile function (95±5%) in comparison to those I/R hearts receiving only vehicle (60±7%) ($P<0.001$). In addition, TMS significantly reduced PMN accumulation in the ischemic myocardium, as evidenced by an attenuation in cardiac myeloperoxidase activity from 1.12±0.04 in untreated hearts to 0.01±0.02 in treated hearts ($P<0.001$). However, TMS did not directly stimulate nitric oxide (NO) release from rat vascular endothelium. **Conclusion:** These results provide evidence that TMS is a potent and effective cardioprotective agent that inhibits leukocyte–endothelial cell interactions and preserves cardiac contractile function and coronary perfusion following myocardial ischemia and reperfusion.

Keywords: Neutrophils; Myocardium; Cell adhesion molecules; Sphingosine; Rat; NO electrode

1. Introduction

Early reperfusion of the ischemic myocardium plays an important role in minimizing myocardial tissue injury associated with acute myocardial infarction. However, reperfusion of the myocardium itself results in enhanced myocardial injury [1,2]. The process of reperfusion injury is characterized by an inflammatory response in which polymorphonuclear leukocytes (PMNs) are believed to play an important role [2,3]. Upon reperfusion, many activated PMNs accumulate in the microvasculature, resulting in microvascular plugging and an impairment in coronary perfusion [4,5]. The activated PMNs induce tissue injury by the release of a variety of cytotoxic substances, including oxygen-derived free radicals, inflammatory cytokines and proteolytic enzymes [6]. Many of these substances may mediate vascular endothelial dysfunction as well as contribute to myocardial injury [7]. This is also consistent with evidence that either decreasing the number of circulating PMNs or the administration of monoclonal antibodies directed against cell adhesion molecules can lead to significant cardioprotection against reperfusion injury [8–11].

Sphingoglycolipids are known to play a physiological role in the function of membrane ion transporters and exert key effects on cell signal transduction pathways [12]. Recently, it has become evident that the biological activity of these compounds also applies to some of their derivatives and metabolites [13]. Several studies have shown that

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not only sphingosine but a synthetic *N*-methylated analogue, *N,N,N*-trimethylsphingosine (TMS), demonstrates an inhibitory effect on protein kinase C (PKC) activity [13,14]. TMS also reduces transendothelial migration of neutrophils and attenuates platelet aggregation at physiologic concentrations [15,16]. It has previously been shown that TMS attenuates myocardial necrosis and endothelial dysfunction in feline myocardial ischemia/reperfusion (MI/R) [17]. Therefore, TMS is considered to be a potentially effective agent in attenuating myocardial reperfusion injury. The major purpose of this study was to investigate if the administration of TMS is able to protect against cardiac contractile dysfunction and PMN accumulation associated with ischemia reperfusion injury in a carefully controlled model of rat MI/R, which is dependent upon neutrophils to mediate the cardiac contractile dysfunction. A second purpose of this study was to investigate the interaction between TMS and endothelial nitric oxide (NO).

2. Methods

All experiments reported in this study conform to Thomas Jefferson University IACUC guidelines regarding the use of animals in the laboratory as well as the standard in the Guide for the Care and Use of Laboratory Animals (NIH Publication Number 85-23, revised 1985).

2.1. Isolated rat heart experiments

Male Sprague-Dawley rats (250–300 g) were anesthetized with 40 mg/kg sodium pentobarbital and 1000 U of sodium heparin were administered i.p. (Elkins Sinn, Cherry Hill, NJ, USA). Following a midline thoracotomy, the hearts were rapidly excised, the ascending aorta was cannulated, and retrograde perfusion of the non-working heart was initiated with a modified Krebs buffer maintained at 37°C and at a constant pressure of 80 mmHg. The Krebs buffer had the following composition (in mmol/l): glucose, 17; NaCl, 120; $NaHCO_3$, 25; $CaCl_2$, 2.5; EDTA, 0.5; KCl, 5.9 and $MgCl_2$, 1.2. The perfusate was aerated with 95% O_2+5% CO_2, which was equilibrated at a pH of 7.3 to 7.4. Two sidearms of the apparatus in the perfusion line just proximal to the heart inflow cannula allowed the infusion of PMNs and plasma, with or without TMS, directly into the coronary inflow line. To assess cardiac contractile function, a 2.5 Fr microtip catheter transducer (Millar Instruments, Houston, TX, USA) was inserted directly into the left ventricular cavity, as previously reported [18,19]. The left ventricular pressure, the maximal rate of development of left ventricular developed pressure (+dP/dt max) and coronary flow were all recorded using a MacLab data acquisition system (ADI Diagnostics, Castle Hill, NSW, Australia) in conjunction with a Power Macintosh 7600 computer (Apple Computers, Cupertino, CA, USA). All of the data were stored and analyzed at the end of each experiment.

2.2. Rat neutrophil isolation

Sprague-Dawley rats (300–350 g), which were used as neutrophil donors, received a 10-ml injection of 0.5% oyster glycogen i.p. (Sigma, St. Louis, MO, USA). Eighteen hours later, the rats were anesthetized using ethyl ether and the neutrophils were harvested by peritoneal lavage in 20 ml of phosphate-buffered saline (PBS). The peritoneal lavage was centrifuged at 2000×*g* at 4°C for 10 min and washed twice, as described previously [19]. Finally, the PMNs were resuspended in Krebs buffer and counted using a hemocytometer and microscope. These neutrophil preparations were >95% pure, and >95% viable using 0.3% trypan blue exclusion as the criterion for viability. Furthermore, PMNs obtained by this method have been found to respond normally in cell adhesion tests [19]. Additional PMNs were isolated from rat blood by the method of Williams et al. [20] using the hetastarch exchange transfusion method in pentobarbital-anesthetized (40 mg/kg, i.p.) rats. This method yielded 110–130×10^6 PMNs per rat, which were >95% pure and >95% viable. These PMNs were washed five to six times to remove the hetastarch prior to perfusion into rat hearts.

2.3. Rat plasma preparation

An intracardiac puncture was performed in anesthetized rats with a 20-ml plastic syringe and a 20 gauge needle (Becton Dickinson, Franklin Lakes, NJ, USA) containing 2.0 ml of sodium citrate–phosphate–dextrose solution, to obtain whole blood. The blood was centrifuged immediately in a refrigerated centrifuge (GSGR; Beckman Instruments, Palo Alto, CA, USA) at 2000×*g* for 10 min, and the plasma was decanted. The plasma was infused along with the PMNs in order to more closely simulate the conditions present in vivo.

2.4. Perfused heart experimental protocol

After the first 15 min of perfusion, baseline left ventricular developed pressure (LVDP), +dP/dt max and coronary flow were measured every 5 min for 15 min, to ensure complete equilibration of the hearts. LVDP was defined as left ventricular systolic minus left ventricular end-diastolic pressure. In all of the hearts, at both the initial and final reading, end-diastolic pressure was 4 to 8 mmHg. The first derivative of left ventricular pressure (+dP/dt max) was recorded from instantaneous left ventricular pressure. Flow of the Krebs buffer was then reduced to zero, creating a state of total global ischemia. This ischemia was maintained for 20 min. The flow was then allowed to return to values near control levels by re-establishing coronary perfusion at 80 mmHg. At reperfusion, 100×10^6 PMNs

and 5 ml of plasma were infused directly into the coronary circulation over a period of 5 min via a set of side ports situated just proximally to the heart in the perfusion line. The PMNs were suspended in 5.0 ml of Krebs buffer in a 5.0-ml syringe. The plasma was also placed in a 5.0 ml syringe located just proximal to the inflow port to the coronary circulation. The hearts were allowed to reperfuse for a total of 45 min, during which time the data were collected every 5 min for the first 30 min and at the 45 min time point. TMS was diluted in Krebs buffer according to previously described methods [21]. We determined that 20.0 μg/kg body weight of this agent was an effective cardioprotective dose, and this dose was infused with the plasma over the first 5 min of reperfusion. Preliminary studies using 0.2 μg/kg of TMS were without any cardioprotective effect, and 2 μg/kg were only moderately protective.

2.5. Determination of cardiac tissue myeloperoxidase

At the end of the reperfusion period of each experiment, left ventricular tissue samples were obtained, frozen at −70°C and subsequently analyzed for PMN accumulation using myeloperoxidase (MPO) activity as a marker for PMNs. Myocardial tissue MPO is an enzyme occurring exclusively in neutrophils [22] and, therefore, increased cardiac MPO activity indicates a significant accumulation of PMNs in the myocardium. One unit of MPO is defined as the quantity of enzyme that hydrolyzes 1 mmol of peroxide per minute at 25°C. MPO was determined spectrophotometrically by the method of Bradley et al. [23], as modified by Mullane et al. [22]. All of the assays were performed without prior knowledge of the group from which each sample originated. In six additional hearts, three ischemic–reperfused with vehicle and three ischemic reperfused with 20 μg/kg TMS at reperfusion, histological sections were made according to previously described techniques for the counting of infiltrating PMNs [19].

2.6. Isolated rat aortic rings

Rat aortae were isolated from pentobarbital-anesthetized (pentobarbital sodium, 40 mg/kg, i.p.) rats and carefully dissected into rings that were 2 to 3 mm in length, preserving the integrity of the endothelium. The rings were suspended in oxygenated 10 ml organ baths and subjected to a 1.0-g resting force, as described previously [24]. TMS (0.6 μg/ml) was added to the bath alone and prior to the addition of 100 nM acetylcholine or 100 μM acidified $NaNO_2$ (pH 2.0). $NaNO_2$ at pH 7.4 exerted no vasoactive effects. Aortic rings were precontracted with 10 nM U46619 (Biomol Laboratories, Plymouth Meeting, PA, USA; 9,11-methano-epoxy PGH_2). Additional aortic rings, flattened out in cell culture dishes, were placed in contact with a calibrated self-grounded selective nitric oxide electrode (World Precision Instruments, Orlando, FL, USA) according to the method of Weyrich et al. [24]. The fluid surrounding these aortic segments with intact endothelium were tested for NO release in the presence and absence of TMS.

2.7. Statistical analysis

All data in the text and figures are presented as the mean±S.E.M. The data on left ventricular function and coronary flow were analyzed by ANOVA, incorporating repeated measures. The MPO data were compared with an ANOVA using post-hoc analysis with Fisher's corrected *t*-test. Probability values of 0.05 or less were considered to be statistically significant.

3. Results

3.1. Experimental protocol

To determine if physiologically relevant concentrations of TMS, in a well characterized model of myocardial ischemia reperfusion, can attenuate leukocyte–endothelial interactions and improve cardiac contractile function, we perfused rat hearts at control flow for 80 min, or for 15 min of control flow followed by 20 min of total global ischemia and 45 min of reperfusion at control flows either with or without PMNs and plasma. Perfusion of rat hearts with TMS was performed at a perfusion pressure of 80 mmHg for 80 min. Perfusion with TMS at 80 mmHg during sham ischemia or during ischemia/reperfusion without PMNs resulted in no change in coronary flow (CF), LVDP or the first derivative of LVDP (+dP/dt max) at the end of the observation period, indicating that TMS did not exert any direct effects on cardiodynamics (Figs. 2–5). Also, perfusion of sham ischemic hearts with PMNs did not alter any of the indices of cardiac function measured, indicating that PMNs do not induce cardiac dysfunction in normal non-ischemic hearts. Only in ischemic reperfused rat hearts perfused with PMNs was there a marked reduction in cardiac contractile function and coronary flow. Only a slight degree of protection was observed when TMS was infused 20 min after reperfusion with PMNs. Final LVDP decreased to 53±3% of the initial value in untreated ischemic–reperfused hearts and to 64±10% of control in ischemic–reperfused rat hearts treated with 20 μg/kg TMS starting 20 min following reperfusion. These values are not significantly different.

Ischemic–reperfused hearts perfused with PMNs and plasma exhibited significant cardiac dysfunction in these hearts ($P<0.01$). In a direct comparison with PMNs isolated from the peritoneal fluid and PMNs isolated from blood, the degree of cardiac dysfunction was very similar. Fig. 1 illustrates the initial and final LVDPs of rat hearts subjected to ischemia–reperfusion and perfused with either

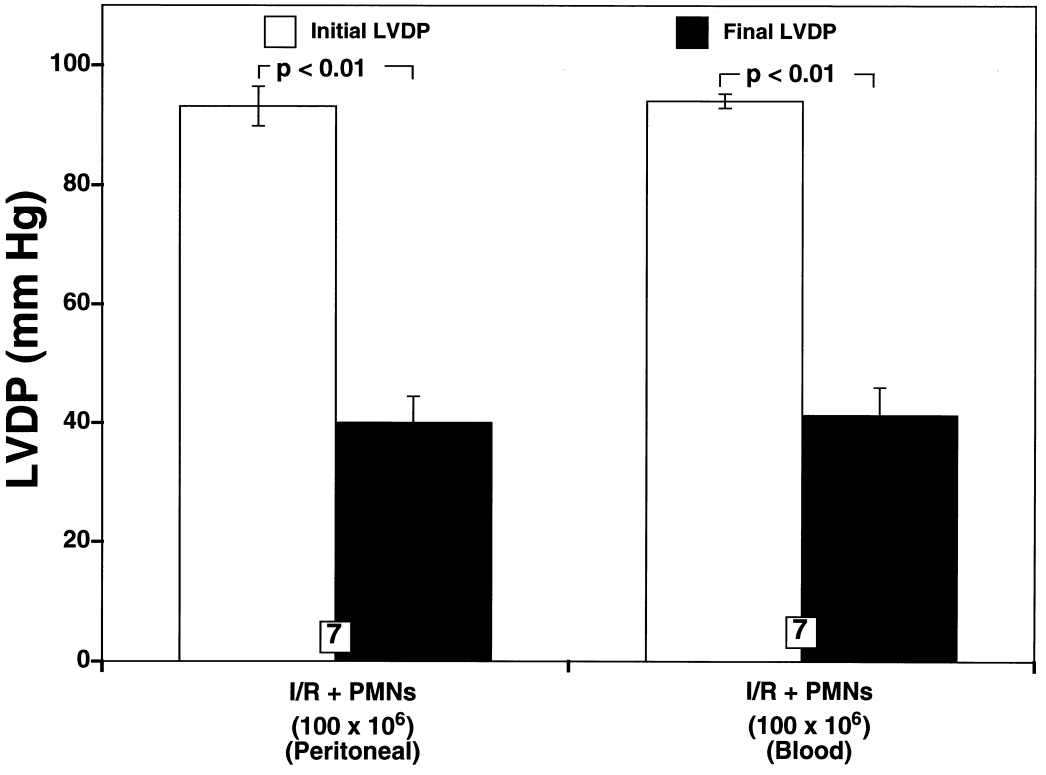

Fig. 1. Initial and final left ventricular developed pressure (LVDP), expressed in mmHg, in isolated perfused rat hearts that were subjected to ischemia/reperfusion with 100×10^6 PMNs isolated from peritoneal fluid or blood. All values are expressed as the mean±S.E.M. for seven hearts in each group. $^{**}P<0.01$ from initial value.

peritoneal PMNs or blood PMNs. These results indicate that the source of PMNs is not a significant factor in this model of myocardial ischemia–reperfusion. Coronary flow, maintained at zero during ischemia, showed a recovery of 80±2% of control in the presence of PMNs (Fig. 2). However, with the same number of PMNs under the same conditions in the presence of TMS, coronary flow recovered almost completely (Fig. 2).

Changes in left ventricular function (i.e., LVDP) were similar to those of coronary flow. In ischemic reperfused rat hearts perfused with PMNs, LVDP recovered only partially following reperfusion stabilizing at a deficit in LVDP of 50±5% ($P<0.001$) (Fig. 3). However, in hearts perfused with the same number of PMNs under the same I/R conditions, TMS showed a significant protective effect in LVDP, to 96±3% of initial values.

These same relationships were obtained with the first derivative of LVDP (+dP/dt max). There was a 55±4% reduction in the final +dP/dt max in untreated PMN perfused hearts subjected to ischemia/reperfusion ($P<$ 0.001; Fig. 4). However, ischemic/reperfused hearts given TMS showed a significantly marked recovery of cardiac contractility, comparable to that of control values (i.e., 98±2% of control).

In all of the control hearts and non-PMN-perfused ischemia/reperfusion hearts, no cardiac MPO activity could be detected, indicating that, in non-ischemic hearts or in I/R hearts without the addition of PMNs, there are very few resident PMNs. However, I/R hearts perfused in the presence of PMNs showed a highly significant MPO activity, signifying PMN accumulation ($P<0.001$). Furthermore, when these ischemic/reperfused hearts were perfused with PMNs and given TMS after reperfusion, there was a significant attenuation of the MPO activity ($P<0.001$; Fig. 5), indicating an anti-neutrophil effect of TMS. These effects on MPO activity are reflective of PMN infiltration, since the direct addition of 0.6 μg/ml TMS to PMN pellets did not significantly alter MPO activity. In four such pairs of experiments, TMS-treated PMN pellets exhibited an MPO activity of 0.17±0.03 U/g vs. 0.11±0.03 for the Krebs buffer-treated PMN pellets. These values are not significantly different. Moreover, in three ischemic–reperfused hearts given vehicle, there were 13.0±1.2 PMNs/field compared with 1.1±0.3 PMNs/field in ischemic reperfused rat hearts given 20 μg/kg TMS ($P<0.001$), thus confirming the MPO data.

In eight isolated rat aortic rings, TMS at 0.6 μg/ml exerted no direct vasoactive effects; 1±1% relaxation in eight aortic rings. Moreover, incubation of eight rat aortic rings with 0.6 μg/ml TMS for 45 min did not significantly alter the vasorelaxation response to either acetylcholine (94±2 vs. 90±2%, TMS vs. its vehicle, i.e., Krebs buffer) or $NaNO_2$. For $NaNO_2$, the comparable percentage relaxation responses were 99±1 vs. 100±1. Neither of these

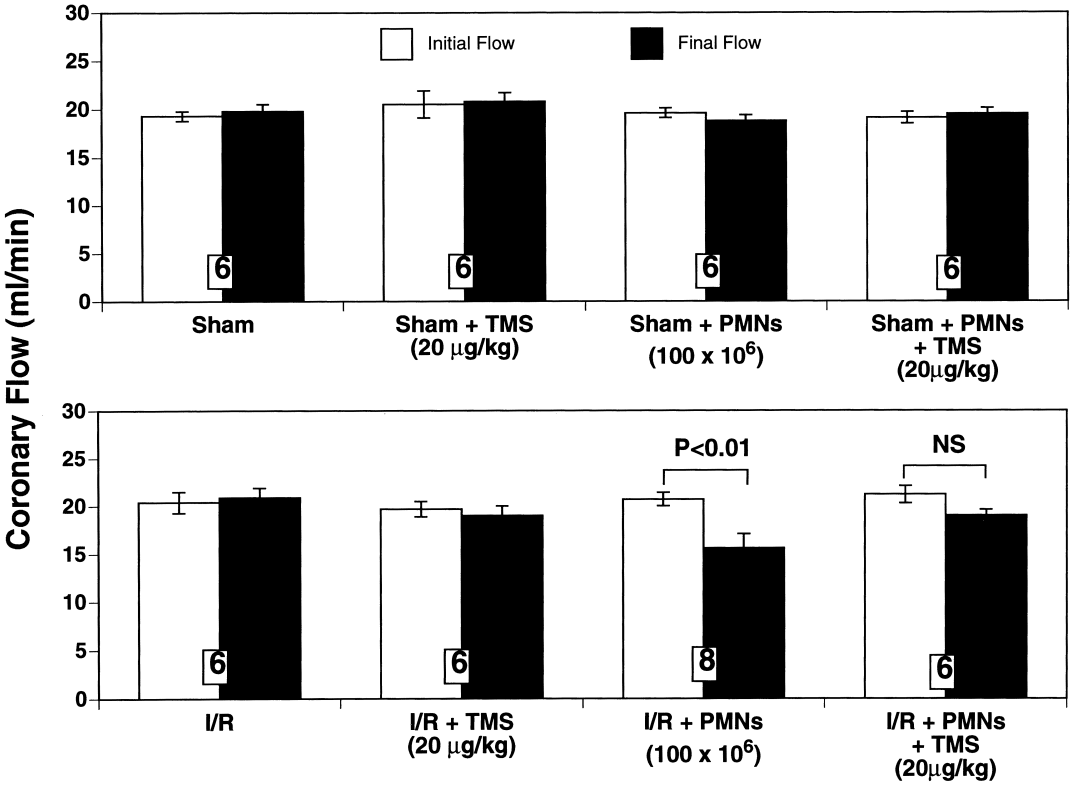

Fig. 2. Initial and final coronary flow (CF), expressed in ml/min, in isolated perfused rat hearts that were subjected to global total ischemia for 20 min and to 45 min of reperfusion. Ischemic hearts were perfused in the presence or absence of PMNs (100×10^6). All values are expressed as the mean±S.E.M. The number at the bottom of the bars represents the number of hearts.

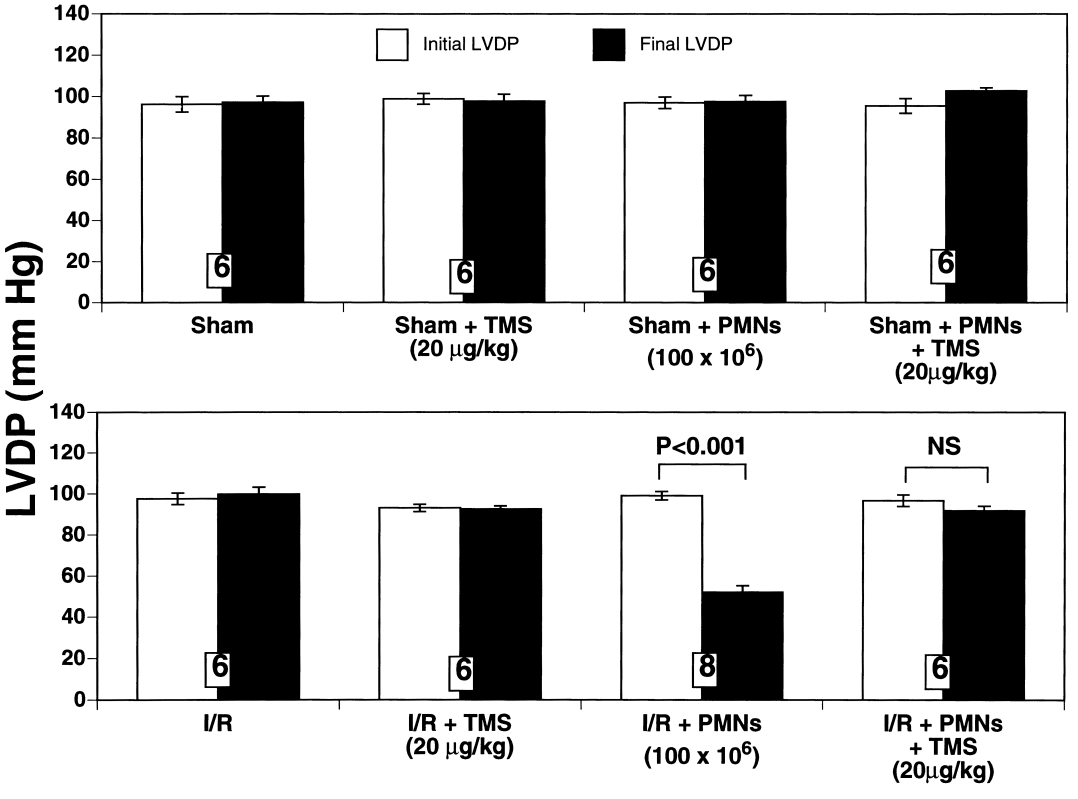

Fig. 3. Initial and final left ventricular developed pressure (LVDP), expressed in mmHg, in isolated perfused rat hearts prior to ischemia and following reperfusion. Ischemic hearts were perfused in the presence or absence of PMNs. PMNs caused a marked contractile dysfunction, which was attenuated by the TMS. All values are expressed as the mean±S.E.M. The number at the bottom of the bars represents the number of hearts.

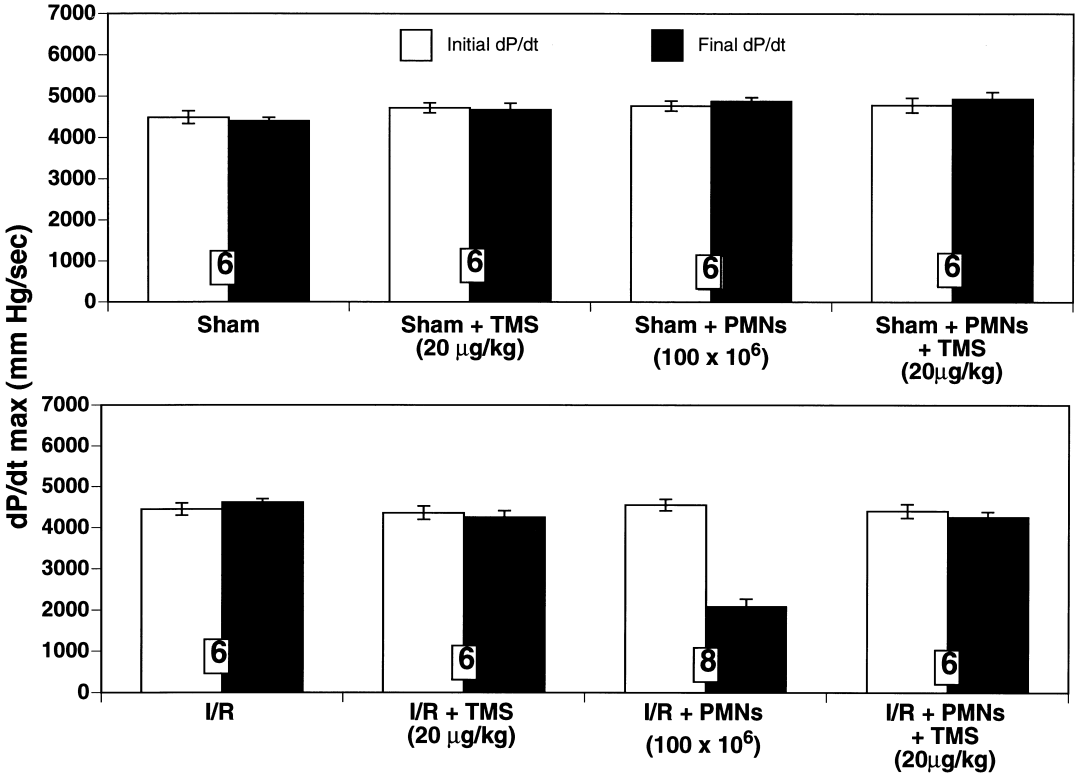

Fig. 4. Initial and final first derivative of LVDP (+dP/dt max), expressed in mmHg/s in rat hearts that were subjected to ischemia and reperfusion. Ischemic hearts were perfused in the presence or absence of PMNs. PMNs caused a significant impairment, which was eliminated by the TMS. All values are expressed as the mean±S.E.M. The number at the bottom of the bars represents the number of hearts. The final dP/dt max was significantly lower ($P<0.01$) only in the I/R+PMNs group.

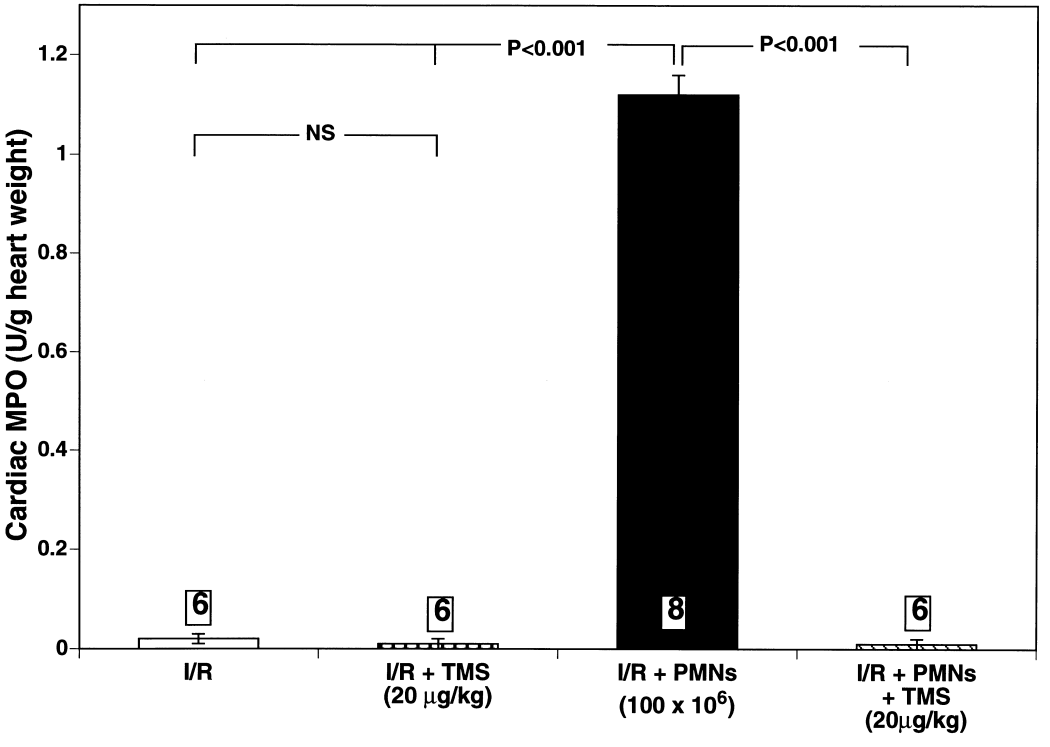

Fig. 5. Cardiac myeloperoxidase (MPO) activity in cardiac samples obtained from ischemic–reperfused rat hearts either in the presence or absence of PMNs and TMS. TMS significantly attenuated the increase of MPO in ischemic reperfused hearts with PMNs. MPO activity is expressed in units per gram of wet tissue weight. All values are expressed as the mean±S.E.M. The number at the bottom of the bars represents the number of hearts.

comparisons are significantly different from each other, and suggest that TMS does not release physiologically meaningful amounts of NO from the vascular endothelium. To ascertain if TMS could directly release NO, we tested TMS in aortic segments with a NO electrode. Basal NO release was 18±2 vs. 20±2 pmol NO/mg tissue in the presence of 0.6 μg/ml TMS in five isolated rat aortic segments. In the presence of 100 μM L-arginine, to stimulate NO production, these values were 41±5 vs. 45±4 pmol NO released/mg tissue. Neither of these comparisons were statistically significant.

4. Discussion

This study clearly demonstrates a significant cardioprotective action of low concentrations of TMS in a Langendorff perfused heart model of myocardial ischemia/reperfusion. This cardioprotection was characterized by significant maintenance of post-reperfusion coronary flow, left ventricular developed pressure and the first derivative of left ventricular developed pressure (i.e., dP/dt max) indicating a significant attenuation of cardiac dysfunction by this phospholipid. It is unlikely that TMS exerted its cardioprotective effects by directly influencing hemodynamics (i.e., inducing coronary vasodilation or increased cardiac contractility) since TMS does not affect hemodynamic parameters when administered in vivo [17]. This concept is also consistent with the data obtained in the present study, showing that there were no increases in coronary flow or left ventricular developed pressure in the non-ischemic hearts infused with TMS.

Since myocardial reperfusion injury has been shown to be related to PMNs infiltrating into ischemic cardiac tissue [2,10,25], one very important component of the protection afforded by TMS is due to its inhibition of PMN accumulation in the ischemic myocardium. Approximately 90% attenuation of cardiac MPO activity was observed in TMS-treated ischemic–reperfused hearts compared to those I/R hearts given only the vehicle. This attenuation of leukocyte–endothelium interaction appears to be the key cardioprotective effect of TMS. Consistent with this concept, Murohara et al. [17] demonstrated that TMS exhibited a 50% protection in PMN adherence to thrombin-stimulated coronary endothelium. Without TMS, neutrophils adhere to the endothelium of the vasculature and release cytotoxic substances, such as proteases, eicosanoids, cytokines and oxygen-derived free radicals [6], each of which can mediate tissue injury and exacerbate endothelial dysfunction. These humoral mediators have been found to lead to coronary endothelial injury, disruption of the endothelial basement membranes, PMN extravasation and myocardial necrosis [5,6,26,27]. Our finding of reduced MPO accumulation supports the concept that TMS attenuates PMN–endothelial cell interactions.

Recruitment of PMNs to inflammatory tissue consists of a series of sequential processes that are mediated by different cell adhesion molecules located either on the PMNs or the endothelial cells [28]. Upon reperfusion, an increased number of PMNs start to roll along the endothelial surface, a process mainly mediated by P-selectin upregulation on the endothelium and to a lesser extent by constitutively expressed L-selectin on the PMNs [28,29]. Rolling PMNs are then able to engage in firm adhesion to the endothelium, mainly by β_2 integrin (i.e., CD11/CD18) interaction with intercellular adhesion molecule-1 (ICAM-1), which is located on the endothelium [28]. After firm adhesion, many of the PMNs then become activated, flatten out and undergo transendothelial migration to the injured or inflamed tissue [30]. It is known that the respiratory burst of PMNs (i.e., the production of oxygen-derived free radicals) at the onset of reperfusion coincides with a marked reduction in nitric oxide release from the endothelium [31], which may in turn lead to an enhanced expression of P-selectin on the endothelial surface [32–34]. In recent studies, in vivo administration of monoclonal antibodies against various cell adhesion molecules was shown to significantly attenuate PMN adherence to the endothelium and to curtail myocardial necrosis after MI/R [8,10]. In this regard, TMS has been shown to attenuate surface expression of P-selectin on coronary endothelial cells during reperfusion of the ischemic heart [21].

In the present study, TMS protected cardiac function after ischemia and reperfusion. It is known that endothelial dysfunction occurs within 2.5 to 5 min of the onset of reperfusion and is characterized by a reduction in the release of biologically active NO [2]. However, TMS did not directly promote the release of NO by the vascular endothelium, suggesting that TMS protects by preserving endothelial function rather than by directly stimulating the release of additional NO from the endothelium. Studies have shown that the loss of endothelium-derived NO further facilitates PMN adherence to the vascular endothelium [35,36]. These adherent PMNs are activated and release mediators that aggravate endothelial dysfunction, resulting in an increased PMN adherence to the vascular endothelium and myocardial necrosis [36]. Because NO inhibits PMN adherence to the endothelium, the diminished PMN adherence to the coronary endothelium after reperfusion may be explained, at least in part, by preservation of the endothelial NO production by TMS, eventually preventing myocardial necrosis and cardiac dysfunction [35].

In conclusion, we have demonstrated that low doses of a sphingolipid membrane derivative, TMS, can attenuate cardiac contractile dysfunction related to myocardial ischemia–reperfusion injury in vitro, by interfering with neutrophil–endothelial cell interactions. This study provides strong evidence that TMS, an *N*-methylated derivative of a sphingolipid, has important beneficial effects on acute inflammatory tissue injury, such as that occurring in the reperfusion of the ischemic myocardium. These studies

show that, in addition to previously shown attenuation of myocardial cell necrosis by TMS in ischemia/reperfusion, TMS preserves cardiac mechanical function by an anti-neutrophil mechanism.

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