Effect of Thrombin Fragment (TP508) on Myocardial Ischemia Reperfusion Injury in a Model of Type 1 Diabetes Mellitus

Louis M. Chu, MD; Robert M. Osipov, MD; Michael P. Robich, MD; Jun Feng, MD, PhD; Michael R. Sheller, PhD; Frank W. Sellke, MD, FAHA, FACS

Background—We investigated the efficacy of novel thrombin fragment TP508 on ischemia-reperfusion injury using a porcine model of type 1 diabetes mellitus.

Methods and Results—Alloxan-induced diabetic male Yucatan swine underwent 60 minutes of mid-left anterior descending coronary artery occlusion, followed by 120 minutes of reperfusion. Fifty minutes into ischemia, animals received either placebo (DM; n=8) or TP508 as a bolus of 1 mg/kg followed by infusion at 2.5 mg/kg per hour (DMT; n=8). Hemodynamic parameters and myocardial function were monitored. Monastryl blue/triphenyl tetrazolium chloride staining was used to assess sizes of the areas at risk and infarction. Coronary microvascular reactivity was measured and expression of cell survival and proapoptotic proteins quantified. Preoperative serum glucose values were similar between groups (309±57 mg/dL in DM versus 318±67 mg/dL in DMT; P=0.92). Infarct size was smaller in the TP508-treated group (5.3±1.9% in DMT versus 19.4±5.6% in DM; P=0.03). There was no statistically significant difference in global or regional left ventricular function between groups. Endothelium-dependent microvessel relaxation was moderately improved in the DMT group (P=0.09), whereas endothelium-independent relaxation was similar between groups. The expression of cell survival proteins Akt, phospho-p38, and mammalian target of rapamycin was higher in the areas at risk of DMT animals compared with DM animals (P<0.05), and expressions of proapoptotic glycogen synthase kinase 3β and caspase 3 were lower in the DMT group (P<0.05).

Conclusions—This study demonstrates that, in type 1 diabetic swine, TP508 reduces infarct size after ischemia-reperfusion. Thus, TP508 may offer a novel approach in cardioprotection from ischemia-reperfusion injury in diabetic patients. (Circulation. 2010;122[suppl 1]:S162–S169.)

Key Words: ischemia ■ reperfusion ■ diabetes mellitus ■ pharmacology

Previous studies have examined the effects of various interventions on myocardial ischemia-reperfusion (IR) in an attempt to decrease myocardial infarction (MI). Although many drugs have demonstrated a significant benefit in reducing myocardial necrosis in preclinical studies, there is no drug on the market that has been found to lessen myocardial IR injury in clinical trials.1–4 A reason for the disparate effects of various drugs in animal models and clinical trials may be that preclinical studies are performed on homogeneous, relatively young, and otherwise healthy animals, whereas clinical trials are performed on elderly patients with multiple comorbidities, including diabetes mellitus.

TP508 (also known as rusalatide acetate or Chrysalin) is a 23-amino acid peptide from a portion of highly conserved, noncatalytic, receptor-binding domain in the native thrombin molecule, of which the half-life in serum is 10 to 14 minutes. It is known that TP508 interacts with high-affinity binding sites found on fibroblasts, neutrophils, monocytes, endothelial cells, and epithelial cells.5 There are data to suggest that TP508 may antagonize thrombin-induced deleterious cellular effects via specific α,β3 integrin binding in an arginine-glycine-aspartate sequence-dependent manner.6 In addition, TP508 alone has been shown to induce protective responses during surgically induced dermal ischemia7–8 and to provide benefits in diabetic foot ulcer and fracture healing.9 Recently, our laboratory has reported that TP508 may reduce IR injury and MI size in both normal10 and hypercholesterolemic11 pigs, but no studies to date have examined its effects in diabetic animals. We hypothesized that TP508 may also offer potential benefits in ameliorating myocardial IR injury in a type 1 diabetic porcine model of IR that more closely mimics the clinical setting than a normal pig model.

Methods

Experimental Design

Sixteen intact male Yucatan mini-swine were divided into 2 groups: diabetic control (DM; n=8) and diabetic plus TP508 (DMT; n=8).
All of the animals were fed normal chow (S11 Purina). Diabetes mellitus was induced in all of the animals by a single intravenous injection of alloxan (200 mg/kg) at age 15 weeks (Sinclair Research Center, Inc). Alloxan-treated animals that maintained serum glucose levels >250 mg/dL were considered diabetic and used in the study. At 20 weeks of age (5 weeks after induction of diabetes mellitus), animals were subjected to acute ischemia by occluding the left anterior descending coronary artery (LAD) for 60 minutes, followed by release of the LAD and reperfusion for 120 minutes. After 50 minutes of ischemia, DM animals received an intravenous infusion of placebo (1X PBS, BioRad Laboratories), whereas animals in the DMT group received TP508 (Harvard Apparatus) in a dose established previously to be efficacious in swine.11 Arterial blood gas, arterial blood pressure, hematocrit, left ventricular (LV) pressure, heart rate, ECG, oxygen saturation, and core body temperature were measured and recorded. Myocardial segmental shortening in the longitudinal axis (parallel to the LAD) and horizontal axis (perpendicular to the LAD) were recorded at baseline and every 30 minutes thereafter. At the completion of the protocol, the heart was excised, and tissue samples were collected for microvessel studies and molecular analyses as described below.

**Animals**

Swine were housed individually and provided with normal chow and water ad libitum. All of the experiments were approved by the Beth Israel Deaconness Medical Center Institutional Animal Care and Use Committee and conformed to the National Institutes of Health guidelines regulating the care and use of laboratory animals (National Institutes of Health publication 5377-3, 1996).

**Surgical Protocol**

Swine at age 20 weeks were sedated with Telazol (1.5 mg/kg, IM) and weighed before endotracheal intubation and ventilation with a volume-cycled ventilator (North American Drager). Anesthesia was established and maintained with 2.0% isoflurane (Abbott Laboratories). A 5-F arterial sheath was passed into the right common femoral artery via direct cutdown and used for arterial blood sampling and continuous monitoring of arterial blood pressure. Arterial blood gas, hematocrit, and core temperature were measured at the start of surgery and every 30 minutes thereafter. Each animal received a 1-L bolus of Lactated Ringer solution, followed by continuous infusion (15 mL/kg per hour). A phenylephrine drip (0.25 µg/kg per minute) to prevent isoflurane-induced hypotension, heparin (80 U/kg of bolus), and lidocaine (1.5 mg/kg of bolus) to prevent ventricular dysrhythmias were administered. A median sternotomy was performed. A catheter-tipped manometer (Millar Instruments) was introduced through the apex of the heart to record LV pressure. Segmental shortening in the ischemic area at risk (AAR) was assessed with a digital ultrasonic crystal measurement system (Sonometrics Corp) using four 2-mm digital ultrasonic probes assessed with a digital ultrasonic crystal measurement system (Sonometrics Corp) was used for data recording (Cardiosoft software). Myocardial segmental shortening in the ischemic area at risk (AAR) was assessed with a digital ultrasonic crystal measurement system (Sonometrics Corp) using four 2-mm digital ultrasonic probes assessed with a digital ultrasonic crystal measurement system (Sonometrics Corp) was used for data recording (Cardiosoft software). Myocardial segmental shortening in the ischemic area at risk (AAR) was assessed with a digital ultrasonic crystal measurement system (Sonometrics Corp) using four 2-mm digital ultrasonic probes assessed with a digital ultrasonic crystal measurement system (Sonometrics Corp) was used for data recording (Cardiosoft software). Myocardial segmental shortening in the ischemic area at risk (AAR) was assessed with a digital ultrasonic crystal measurement system (Sonometrics Corp) using four 2-mm digital ultrasonic probes assessed with a digital ultrasonic crystal measurement system (Sonometrics Corp) was used for data recording (Cardiosoft software). Myocardial segmental shortening in the ischemic area at risk (AAR) was assessed with a digital ultrasonic crystal measurement system (Sonometrics Corp) using four 2-mm digital ultrasonic probes assessed with a digital ultrasonic crystal measurement system (Sonometrics Corp) was used for data recording (Cardiosoft software). Myocardial segmental shortening in the ischemic area at risk (AAR) was assessed with a digital ultrasonic crystal measurement system (Sonometrics Corp) using four 2-mm digital ultrasonic probes assessed with a digital ultrasonic crystal measurement system (Sonometrics Corp) was used for data recording (Cardiosoft software). Myocardial segmental shortening in the ischemic area at risk (AAR) was assessed with a digital ultrasonic crystal measurement system (Sonometrics Corp) using four 2-mm digital ultrasonic probes assessed with a digital ultrasonic crystal measurement system (Sonometrics Corp) was used for data recording (Cardiosoft software).

**Quantification of Myocardial Infarct Size**

The LV (including septum) was isolated, cut into 1-cm-thick slices, and immediately immersed in 1% triphenyl tetrazolium chloride in PBS (Boston Bioproducts) at 38°C for 30 minutes. The infarct area (characterized by absence of staining), noninfarcted AAR (characterized by bright red tissue staining), and the nonischemic ventricle (characterized by purple tissue staining) were photographed and measured. AAR as a percentage of total LV surface area and percentage of infarction in the AAR were calculated in each individual slice by planimetry (Image J 1.4) using the following equations: AAR size = (AAR area surface/LV total surface area)×100 and infarct size = (LV infarct surface area/LV AAR surface area)×100.

**Coronary Microvascular Reactivity Studies**

Coronary microvascular reactivity was examined in the ischemic territory as described previously.13 Briefly, coronary arterioles (diameter 140 to 160 µm in diameter) were isolated from ischemic myocardium using a ×40 microscope. Microvessels were mounted on dual-glass micropipettes and examined in a pressurized, isolated microvessel chamber. Adenosine diphosphate (ADP; 1 nmol/L to 100 µmol/L) and sodium nitroprusside (1 mmol/L to 100 µmol/L) were applied extraluminally after preconstriction to 25% to 50% of the baseline diameter with the thromboxane A2, analog U-46619 (0.1 to 1.0 µmol/L), and the vasodilatory response to each drug was measured.

**Western Blotting**

Samples of ischemic myocardium from both groups were homogenized in radioimmunoprecipitation assay buffer (Boston Bioproducts) and total protein concentration determined by bicinchoninic acid assay (Pierce). Equal amounts of protein (40 µg) were subjected to SDS-PAGE and immunoblotting as described previously.14 Primary antibodies were used according to the manufacturer’s recommendation. Levels of Akt, phospho-Akt (Ser473), phosphorlated endothelial NO synthase (eNOS), p38 and phospho-p38 (Thr180/Tyr182), glycogen synthase kinase-3β (GSK-3β) and phospho-GSK-3β (Ser9), apotosis inducing factor (AIF), mammalian target of rapamycin (mTOR) and phospho-mTOR (Ser2448). B cell lymphoma 2, extracellular signal–regulated kinase 1/2 and phospho-extracellular signal–regulated kinase 1/2 (Thr202/Tyr204), 4E–binding protein 1 and phospho–4E–binding protein 1 (Thr37/46), p70S6K1 and phospho-p70S6K1 (Thr389), p70S6K1 and phospho-p70S6K1 (Thr389), p70S6K1 and phospho-p70S6K1 (Thr389), p70S6K1 and phospho-p70S6K1 (Thr389), p70S6K1 and phospho-p70S6K1 (Thr389), p70S6K1 and phospho-p70S6K1 (Thr389), p70S6K1 and phospho-p70S6K1 (Thr389), p70S6K1 and phospho-p70S6K1 (Thr389), p70S6K1 and phospho-p70S6K1 (Thr389), p70S6K1 and phospho-p70S6K1 (Thr389), and glycogen synthase kinase/c-Jun NH2-terminal kinase, nuclear factor-κB and phospho–nuclear factor-κB (Ser536), phospho-Bad, (Cell Signaling Technology), heat shock protein 1 (HSP) 27, HSP70, HSP90, and heat shock cognate 70 (Stressgen) were assessed. Band intensities were normalized to Ponceau staining.

**Statistical Analysis**

Hemodynamic, microvessel, and global and regional LV functional data were analyzed using 2-way repeated-measures ANOVA (Systat), with group and time as the factors in the hemodynamic and functional analyses and group and drug dose the factors in the microvessel analysis. Multiple-comparison Bonferroni posttest was...
Results

Diabetic Swine
DM and DMT were not significantly different in comparing body weight, arterial blood gas measurements, hematocrit, or body temperature. Preoperative serum glucose values were similar between the 2 groups as well (309 ± 57 mg/dL in DM versus 318 ± 67 mg/dL in DMT; P = 0.92), and serum glucose values remained relatively stable during the experiment (Figure 1).

Ventricular Dysrhythmias
There was no significant difference in the incidence of ventricular fibrillation/ventricular tachycardia events during ischemia between groups (median of 4.0 events per animal in DM versus 3.5 events in DMT; P = 0.8). The DM group did require more electric cardioversion attempts on average to restore sinus rhythm (median 3 shocks per animal in DM versus 1 shock in DMT; P = 0.03). All of the dysrhythmias were successfully terminated with intravenous lidocaine with or without electric cardioversion. There was no premature death in either group.

Hemodynamic Parameters
There was no statistically significant difference in heart rate between the 2 groups. MAP was similar between the 2 groups at all of the time points except at 90 minutes and 120 minutes of reperfusion, when the MAP was higher in the DMT group (P < 0.05; Figure 2A). LAD blood flow was significantly greater immediately after reperfusion (hyperemic response) in DMT animals versus controls (P < 0.05; Figure 2B). There were statistically significant interactions between group and time on both MAP and LAD flow (P = 0.09 and 0.01, respectively).

Global and Regional Myocardial Function
There was no statistically significant difference between groups when looking at DLVP, +dP/dt, longitudinal axis segmental shortening, or horizontal axis segmental shortening (Figure 2C through 2F).

Myocardial Infarct Size
The size of the AAR was not significantly different between groups (34.3 ± 1.7% in DM versus 33.8 ± 1.1% in DMT; P = 0.8; Figure 3A). The infarct area was smaller in the DMT group compared with the DM group (5.3 ± 1.9% in DMT versus 19.4 ± 5.6% in DM; P = 0.03; Figure 3B).

Coronary Microvessel Reactivity Studies
The baseline microvessel diameters ranged between 120 and 200 μm. The percentages of preconstrictions were −32 ± 2% in the DM group and −30 ± 4% in the DMT group (P = 0.6). Endothelium-dependent relaxation to ADP treatment was slightly improved in the DMT group compared with the DM group, but the difference was only of borderline significance (P = 0.09; Figure 4A). Endothelium-independent relaxation to sodium nitroprusside treatment was similar between groups (Figure 4B).

Western Blotting
The expression of Akt was higher in the DMT group (P = 0.04; Figure 5A), but expressions of its phosphorylated forms phospho-Akt (Ser473) and phospho-Akt (Thr308) were similar between groups (Figure 5B and 5C). Phospho-eNOS expression was similar between groups as well (Figure 5D). Caspase 3 expression was lower in DMT animals (P = 0.04; Figure 5E). Although total p38 was similar between groups (Table), phospho-p38 was more highly expressed in the DMT group (P = 0.04; Figure 5F). The expression of both GSK-3β (P < 0.001) and phospho—GSK-3β (P = 0.04) was lower in the DMT group (Figure 6A and 6B), whereas the ratio of phospho—GSK-3β to total GSK-3β was similar between groups (Table). AIF was more highly expressed in the DMT group (P = 0.02; Figure 6C). mTOR expression was higher in the DMT group as well (P = 0.03; Figure 6D), but phospho-mTOR expression was similar between groups (Table). There was no statistically significant difference between groups in the expression of B cell lymphoma 2, ERK 1/2, phospho-ERK 1/2, 4E-BP1, phospho—4E-BP1, p70S6K, phospho-p70S6K, SAP/c-Jun NH2-terminal kinase, nuclear factor-κB, phospho—nuclear factor-κB, phospho-Bad, HSP90, heat shock cognate 70, HSP70, or HSP27 (Table).

Discussion
There have been numerous studies examining the effects of drugs on myocardial IR injury in animal models, and nearly all have reported positive results.13–15 However, none of these drugs have been shown to clearly provide a clinically significant improvement in heart function or a reduction in myocardial necrosis for the treatment of acute MI or during cardiac surgery.16 It is likely that normal animal models of IR do not mimic clinical reality. Thus, we used a swine model of type 1 diabetes mellitus to investigate the efficacy of the novel drug TP508.

Figure 1. Serum glucose values. Shown are glucose values for DM (n = 8) and DMT (n = 8) groups at different time points in the experiment. Pre indicates baseline; O1, 30 minutes of ischemia; O2, 60 minutes of ischemia; R1, 30 minutes of reperfusion; R2, 60 minutes of reperfusion; R3, 90 minutes of reperfusion; R4, 120 minutes of reperfusion. Data are presented as mean ± SEM. P for group = 0.9 (2-way repeated-measures ANOVA).
The key finding of this study is that TP508 provides significant myocardial protection in a type 1 diabetic swine during IR injury. Recently, we examined the effects of type 1 diabetes mellitus alone in a porcine model of IR and found that, surprisingly, diabetes mellitus alone was associated with decreased myocardial necrosis. This is likely attributed to increased energy substrate availability and an increase in expression of survival proteins compared with that observed in nondiabetic animals. The current study demonstrates that treatment with TP508 in the setting of type 1 diabetes mellitus offers additional cardioprotection against IR injury, reducing infarct size by >70% compared with diabetic control animals. TP508 also moderately improved endothelium-dependent relaxation of coronary arterioles, but it did not improve global or regional LV function.

Hemodynamic parameters were also largely similar between the 2 groups, with the exception of a slightly higher MAP at the end of reperfusion in the DMT group. The hyperemic response after release of the LAD ligation and endothelium-dependent relaxation to ADP was greater in the DMT group versus the control group, suggesting improved vascular protection with TP508. Interestingly, even with the reduction in infarct size in the DMT group, no improvement in global or regional function or hemodynamics was witnessed. This could be because of the global effects of diabetes mellitus on both groups. In our previous study comparing infarct size in diabetic and nondiabetic animals, the diabetic animals displayed worsened global function and minimally improved regional function, although infarct size was diminished. Both groups in the current study likely similarly experience some measure of diabetic cardiomyopathy.

IR injury has been shown to increase the incidence of cardiac arrhythmias. Interestingly, although the incidence of ventricular dysrhythmias was similar between groups, the DMT group did require significantly fewer electric cardioversions to maintain sinus rhythm, likely as a consequence of the cardioprotective properties of TP508 against reperfusion injury.

The balance between cell death and cell survival proteins plays a crucial role in cell fate after myocardial IR injury.
This study demonstrates that TP508 treatment induces the expression of several cell survival proteins in the ischemic myocardium, including Akt, phospho-p38, and mTOR, each of which have been shown to have cardioprotective properties. Akt has been shown to protect myocardium from IR by phosphorylating and inactivating a number of proapoptotic proteins, including caspase and GSK-3β, both of which demonstrated decreased expression in the DMT group in this experiment. GSK-3β, in particular, is a target for a large number of kinases and, when active, phosphorylates many downstream effectors of apoptosis. GSK-3β is inactivated by phosphorylation, and inactivation of GSK-3β has been shown to be cardioprotective. We found that both total and phosphorylated GSK-3β were decreased in the DMT group but that the ratio of phosphorylated to total GSK-3β was similar between groups. Thus, TP508 may provide cardioprotection not by increasing phosphorylation of GSK-3β but rather by decreasing expression of the protein. Paradoxically, AIF was increased in the DMT group compared with control. However, recent studies have shown that AIF also serves to...
scavenge free radicals in mitochondria and that downregulation of AIF sensitizes myocardium to oxidative stress-related cell death. It is evident that a complex interaction of many different proteins is likely responsible for the cardioprotection afforded by TP508.

TP508 has also been found to stimulate NO production in human endothelial cells in vitro, similar to vascular endothelial growth factors,\textsuperscript{25} and to increase the expression of phospho-eNOS in normal animals.\textsuperscript{10,26} Treatment of endothelial cells with TP508 in the presence of a \textsuperscript{3H}H-specific inhibitory antibody has also been shown to reduce NO production compared with TP508 treatment alone.\textsuperscript{27} However, in the present study there was no statistically significant difference in the expression of phospho-eNOS between the DM and DMT groups, a finding that is echoed in our previous study examining TP508 in hypercholesterolemic animals.\textsuperscript{11} Furthermore, although endothelium-dependent microvessel relaxation was somewhat improved by treatment with TP508, the difference was only of borderline significance. It is likely that TP508 exerts its cardioprotective effect via multiple mechanisms, of which the NO-vasorelaxation pathway is only one.

While providing important functional and molecular data about the role of diabetes mellitus in acute IR, this study has several limitations. Our time course for tissue harvest (3 hours after the onset of ischemia) is not able to account for long-term effects of diabetes mellitus on myocardial function and apoptosis. Furthermore, a short-term induction of hyperglycemia was performed using alloxan. Although this model is adept at examining changes in myocardial necrosis attributed to hyperglycemia alone, the chronic secondary effects of diabetes mellitus may not yet have developed. Diabetic patients experiencing coronary insufficiency and MI clearly have poorer outcomes in the clinical setting. Whether this model is applicable to long-standing endogenous or exogenous hyperglycemia in human patients needs to be further evaluated.

Recently, the National Institutes of Health, the Food and Drug Administration, and the pharmaceutical industry have given increased attention to the use of clinically relevant large animal models for preclinical trials, because it has been observed on many occasions that preclinical efficacy and safety studies do not reflect what is observed in clinical trials. This is true of many trials examining the effects of drugs on

### Table.

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<th>Selected Protein Expression</th>
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*Data presented in arbitrary density units as mean±SEM.
IR injury, regenerative therapies, and other cardiovascular mediations. We feel that studies using such clinically relevant models will in the long run save on the use of laboratory animals for preclinical studies and better predict the results of planned clinical studies.

In conclusion, this study demonstrates that parenteral administration of TP508 provides significant myocardial protection in hyperglycemic pigs after acute myocardial IR. Although the exact mechanisms of TP508-induced responses are unclear, it appears that these beneficial effects may be related to improved coronary microvascular responses, higher expression of certain cell survival proteins, and attenuation of apoptotic signaling. Thus, the novel thrombin fragment TP508 may have a therapeutic role in limiting myocardial injury after acute MI followed by interventions to restore blood flow in patients. However, this will need to be verified in clinical trials.

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Disclosures
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