Effect of thrombin fragment (TP508) on myocardial ischemia-reperfusion injury in hypercholesterolemic pigs

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Osipov RM, Robich MP, Feng J, Clements RT, Liu Y, Glazer HP, Wagstaff J, Bianchi C, Sellke FW. Effect of thrombin fragment (TP508) on myocardial ischemia-reperfusion injury in hypercholesterolemic pigs. J Appl Physiol 106: 1993–2001, 2009. First published April 16, 2009; doi:10.1152/japplphysiol.00071.2009.—Myocardial ischemia-reperfusion (IR) injury occurs frequently in the setting of hypercholesterolemia. We investigated the potential efficacy of a novel thrombin fragment (TP508) on IR injury in a hypercholesterolemic porcine model. Twenty-one hypercholesterolemic male Yucatan pigs underwent 60 min of mid-left anterior descending coronary artery occlusion followed by 120 min of reperfusion. Pigs received either placebo (control, \( n = 7 \)) or TP508 in two doses (TP508 low dose, \( n = 7 \), as bolus of 0.5 mg/kg 50 min into ischemia and an infusion of 1.25 mg·kg\(^{-1}\)·h\(^{-1}\) during reperfusion period or TP508 high dose, \( n = 7 \), a double dose of TP508 low-dose group). Myocardial function was monitored throughout the experiment. The area at risk and myocardial necrosis were determined by Monastryl blue/tetrazolium chloride staining. Apoptosis in the ischemic territory was assessed. Coronary microvascular reactivity to endothelium-dependent and -independent factors was measured. Myocardial necrosis was lower in both TP508-treated groups vs. control (\( P < 0.05 \)). Regional left ventricular function was improved only in the TP508 high-dose group (\( P < 0.05 \)). Endothelium-dependent coronary microvascular reactivity was greater in both TP508-treated groups (\( P < 0.05 \)) vs. control. The expression of proteins favoring cell survival, 90-kDa heat shock protein and phospho-Bad (Ser112) was higher in the TP508 high-dose group (\( P < 0.05 \)). This study demonstrates that myocardial IR injury in the setting of hypercholesterolemia may result in more myocardial necrosis and apoptosis compared with what is observed in normcholesterolemic animals (5, 38). In addition, our laboratory’s recent study in a porcine model of hypercholesterolemia showed a greater than 40% increase in necrosis after myocardial IR injury (24).

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. It is known that TP508 interacts with high-affinity binding sites found on fibroblasts, neutrophils, monocytes, endothelium, and epithelial cells (9). Studies conducted in human umbilical vein endothelial cell cultures have shown that TP508 antagonizes thrombin-induced deleterious cellular effects via specific \( \alpha_\text{v}\beta_\text{3} \)-integrin binding in a RGD (Arg-Gly-Asp) sequence-dependent manner (36). However, the possibility exists for alternative TP508 receptors. In addition, TP508 alone has been shown to induce protective responses during surgically induced dural ischemia (21, 37). Our laboratory has demonstrated that TP508 protects the heart after myocardial IR injury in normocholesterolemic pigs (25).

In light of these studies, we hypothesized that TP508 may offer potential benefits in ameliorating myocardial IR injury in a more rigorous and novel hypercholesterolemic model, which more closely mimics what may occur in the clinical setting.

MATERIALS AND METHODS

Experimental design. Twenty-one intact male Yucatan mini-swine (20 wk old) were fed with a high-fat/high-cholesterol diet for 1 mo (Sinclair Research Center, Columbia, MO) (34). All animals were subjected to 1 h of ischemia, followed by 2 h of reperfusion. Animals received intravenously either placebo (control, \( n = 7 \)) or TP508 as a bolus (TP508 low dose 0.5 mg/kg and TP508 high dose 1 mg/kg) 50 min into ischemia (over 2 min), followed by a continuous TP508 infusion during the entire period of reperfusion (TP508 low dose 1.25 mg·kg\(^{-1}\)·h\(^{-1}\) and TP508 high dose 2.5 mg·kg\(^{-1}\)·h\(^{-1}\)) (Harvard Apparatus, Holliston, MA). There was no significant difference in animal body weight among groups (control 21.4 ± 2.2 kg vs. TP508 low dose 21.6 ± 2.1 kg vs. TP508 high dose 20 ± 1.9 kg, \( P = 0.3 \)).

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

**Surgical protocol.** Pigs were sedated with Telazol (4 mg/kg im) and weighed before the induction of anesthesia, followed by endotracheal intubation and ventilation with a volume-cycled ventilator (North American Dragger, Telford, PA). General endotracheal anesthesia was established and maintained with 2.0% isoflurane (Ultane; Abbott Laboratories). The right common femoral artery was accessed and used for arterial blood sampling and continuous monitoring of intra-arterial blood pressure (Millar Instruments, Houston, TX). Arterial blood-gas analysis, hematocrit, and core temperature were assessed every 30 min. Before left anterior descending (LAD) coronary artery occlusion, each animal received a 1-liter bolus of lactated Ringer solution, followed by an infusion at a rate of 15 ml·kg⁻¹·h⁻¹. A phenylephrine drip (0.25 μg·kg⁻¹·min⁻¹) to prevent hypotension induced by isoflurane. Heparin (80 U/kg bolus), and lidocaine (1.5 mg/kg) to control ventricular dysrhythmia, were administered. A median sternotomy was performed, and a pericardial cradle was created. A catheter-tipped manometer (Millar Instruments) was introduced through the apex of the left ventricle (LV) to record LV pressure. The LAD was occluded 3 mm distal to the origin of the second diagonal branch by a Rommel tourniquet. After 60 min of ischemia, the tourniquet was released, and the myocardium was reperfused for 120 min. Blood flow was monitored by a transonics Doppler probe on the LAD. At the end of reperfusion, the LAD was relegated, and monastryl blue pigment was diluted in PBS (1:150) (Engelhard, Louisville, KY) and injected into the aortic root to demarcate the area at risk (AAR). The heart was rapidly excised and sliced into 1-cm-thick slices perpendicular to the axis of the LAD up to the point of ligation. Tissue from the second slice was used in molecular and coronary microvascular reactivity studies. The remaining tissue was incubated in 1% triphenyl tetrazolium chloride (Sigma Chemical, St. Louis, MO) solution in PBS for 30 min, and infarct size was assessed as previously described (35). Ventricular dysrhythmia [ventricular fibrillation (VF) or pulseless ventricular tachycardia (VT)] events were recorded and treated with immediate electrical cardioversion with 20–50 J.

**Measurement of global and regional myocardial function.** Indexes of global and regional myocardial function were monitored during the entire experiment. Mean arterial pressure, developed LV pressure, and coronary blood flow were recorded continuously. LV pressure and arterial pressure were monitored using a Statham 23C transducer (Statham, Calif). LV pressure pulse width ratio was calculated as the ratio of the LV pressure pulse duration at 50% of the peak systolic pressure to the total LV pressure pulse duration. LV pressure pulse duration was measured as the time from the onset of the peak systolic pressure to the point of the return to 50% of peak systolic pressure. Correlation analysis was performed to determine relationships between serum levels of TP508 and infarct size among groups.

### Table 1. Plasma lipid profile

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>TP508 Low Dose</th>
<th>TP508 High Dose</th>
<th>P Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholesterol</td>
<td>13.8±2</td>
<td>13.2±2</td>
<td>12.4±1</td>
<td>0.9</td>
</tr>
<tr>
<td>d-LDL</td>
<td>5.5±0.7</td>
<td>5.5±0.1</td>
<td>4.2±0.6</td>
<td>0.5</td>
</tr>
<tr>
<td>d-HDL</td>
<td>1.8±0.1</td>
<td>1.7±0.06</td>
<td>1.5±0.2</td>
<td>0.5</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>0.3±0.03</td>
<td>0.35±0.03</td>
<td>0.2±0.02</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

Values are mean ± SE in mmol/l. P values are with one-way ANOVA.
positive (+dP/dt) and negative first derivatives of LV pressure (−dP/dt), and longitudinal and horizontal segmental shortening in the AAR were recorded for 10 sequential beats at baseline and then every 30 min (during LAD occlusion: 30 and 60 min, and reperfusion: 30, 60, 90, and 120 min) using a Sonometrics system (Sonometrics London, ON, Canada), as previously described (33).

Quantification of myocardial infarct size. Heart slices (1-cm-thick) were incubated in 1% triphenyl tetrazolium chloride solution, as previously described (33). Briefly, necrotic (pale), AAR (bright red), and the nonischemic portion of the heart specimens (purple) were photographed and measured. Percentage of the AAR and necrosis within the AAR were calculated in each individual slice by planimetry (Image J 1.4, NIH, Bethesda, MD) using the following equations: [(LV necrosis surface area + nonischemic AAR surface area)/LV total surface] × 100 and [(LV necrosis surface area/LV AAR surface area) × 100], respectively.

Coronary microvascular reactivity studies. Coronary microvascular reactivity was examined in the ischemic territory, as previously described (33). Briefly, coronary arterioles (140–160 μm) were dissected with a ×40 microscope. Microvessels were mounted on glass micropipettes and examined in a pressurized, isolated microvessel chamber. ADP (1 μM–100 μM), substance P (0.1–10 nM), A23187 (1 μM–10 μM), and sodium nitroprusside (1 μM–100 μM) were applied extraluminally after precontraction to 25–50% of the baseline diameter with the thromboxane A2 analog U-46619 (0.1–100 nM–100 μM), respective ly. Levels of B-cell lymphoma 2 (Bcl-2), caspase-3, cleaved caspase-3, phospho-caspase 9, poly-ADP ribose polymerase (PARP), cleaved PARP, Akt, phospho-Akt (Ser473 and Thr308), Bcl-2 adenovirus E1B 19-kDa interacting protein (BNip-3), apoptosis inducing factor (AIF), Bad, phospho-Bad (Ser112), endothelial nitric oxide synthase (eNOS), phospho-eNOS (Ser1177) (Cell Signaling Technology, Beverly, MA), 90-kDa heat shock protein (HSP90), HSP70 (or HSP72), HSC70 (heat shock cognate protein or HSP73), HSP27, and αB-crystallin (Stressgen, Ann Arbor, MI) were assessed. Ponceau staining was used to confirm equal protein loading. Band intensities were normalized to Ponceau. Data are presented as means ± SE in arbitrary density units. TP508 plasma levels were measured by ELISA kit (Capstone Therapeutics, Tempe, AZ).

TUNEL staining. Apoptotic cells were identified using the ApoTag detection kit according to manufacturer’s specifications (Chemicon, Temecula, CA). At least 1 cm² of tissue from the AAR was analyzed from each animal (4 per group). The number of terminal deoxynucleotidyl transferase dUTP-mediated nick-end labeling (TUNEL)-positive cardiomyocytes is expressed as positive cells per centimeter squared.

Fig. 2. Global and regional myocardial function. A: mean arterial pressure (MAP; P = 0.4). B: developed left ventricular pressure (DLVP; P = 0.4). C: positive first-order derivative of ventricular pressure (+dP/dt; P = 0.2). D: negative first-order derivative of ventricular pressure (−dP/dt; P = 0.2). Regional myocardial function is shown as percentage of segmental shortening (SS) in the horizontal axis (E) and in the longitudinal axis (F) (P = 0.3). Pre, baseline; O1 and O2, 30 and 60 min in ischemia/left anterior descending (LAD) occlusion, respectively; R1, R2, R3, and R4: 30, 60, 90 and 120 min into reperfusion, respectively. Data are presented as means ± SE and compare control (n = 7), low-dose (n = 7), and high-dose (n = 7) groups. *P < 0.05 (two-way repeated-measures ANOVA).
Statistical analysis. Functional and microvascular reactivity data were analyzed using two-way repeated-measures ANOVA. Infarct size and densitometry were analyzed using one-way ANOVA. Data are reported as means ± SE, and P < 0.05 was considered significant (Systat, San Jose, CA).

RESULTS

Plasma lipid profile. Levels of plasma cholesterol, α-LDL, and α-HDL were not significantly different among groups. Triglyceride level was lower in the TP508 high-dose group compared with the other groups (Table 1).

Arterial blood-gas, hematocrit, and core temperature. There were no significant differences among groups at any time point recorded with respect to arterial blood gas, hematocrit, and core temperature.

Incidence of VF or pulseless VT. There was no difference in incidence of VF/VT during ischemia (control 8/7 vs. low dose 10/7 vs. high dose 7/7; χ² P = 0.38) and reperfusion (control 4/7 vs. low dose 1/7 vs. high dose 0/7; χ² P = 0.39). Generally, VF/VT appeared 20 min into ischemia. All dysrhythmias were successfully terminated with intravenous lidocaine and electrical cardioversion. There was no mortality in any of the groups.

Myocardial infarct size. The size of the AAR was not significantly different among groups (Fig. 1A), whereas the area of necrosis decreased in a dose-dependent manner in both TP508 groups compared with the control group (Fig. 1B). The area of necrosis inversely correlated with serum levels of the TP508 (Fig. 1F).

Hemodynamic parameters and LV function. The mean arterial blood pressure, developed LV pressure, and +dP/dt and −dP/dt were not significantly different among groups (Fig. 2, A–D). Regional myocardial function in the AAR was improved in the TP508 high-dose group compared with the other groups in the horizontal axis, starting 1 h into reperfusion (Fig. 2E), whereas no difference was observed in the longitudinal axis among groups (Fig. 2F).

Coronary microvascular reactivity in the ischemic territory and LAD blood flow. The baseline diameters were 144 ± 5, 147 ± 4, and 157 ± 5 μm in the control, TP508 low-dose, and TP508 high-dose groups, respectively (P = 0.12). The percent precontractions were −34.5 ± 1, −35.7 ± 1, and −32.8 ± 1.2 in the control, TP508 low-dose, and TP508 high-dose groups, respectively (P = 0.16). Receptor-mediated, endothelium-dependent relaxations to ADP and substance P were improved in both TP508-treated groups compared with the control group (Fig. 3, A and B), whereas nonreceptor-mediated, endothelium-dependent relaxation to the calcium ionophore A23187 was higher in the TP508 high-dose group (Fig. 3C). Endothelium-independent relaxation to sodium nitroprusside was marginally higher in the
TP508 high-dose group vs. the TP508 low-dose and control groups (Fig. 3D). LAD blood flow was higher in the TP508 low-dose group compared with the other groups (Fig. 3E).

Profile of proteins favoring cell survival. The expression of HSP90 was higher in the TP508 high-dose group compared with the other groups (Fig. 4A). The expression of HSP70 was lower in the TP508 low-dose group vs. TP508 high-dose and control groups. The expression of HSC70 was not different among groups (Fig. 4, B and C). The expression of HSP27 and αB-crystallin trended higher in the TP508 high-dose group compared with the other groups, but were not statistically significant (Fig. 4, D and E).

While the expression of total Akt and phospho-Akt (Ser473) was not significantly different among groups, the expression of phospho-Akt (Thr308) was lower in the TP508 high-dose group vs. the TP508 low-dose and control groups (Fig. 5, A and B). The expression of Bcl-2, eNOS, and phospho-eNOS (Ser1177) was not significantly different among groups (Fig. 5, C and D). The expression of phospho-Bad (Ser112) was higher in the TP508 high-dose group vs. the TP508 low-dose and control groups (Fig. 5E).

Profile of proapoptotic proteins. The expression of PARP and cleaved PARP tended to be lower in the TP508 low-dose animals (Fig. 6, A and B), but did not reach statistical significance. While BNip-3 expression was not different among groups, expression of AIF was lower in the TP508 low-dose group vs. the TP508 high-dose and control groups (Fig. 6, C and D). The expression of total caspase-3 was not significantly different among groups ($P=0.2$), whereas the expression of cleaved caspase-3 was lower in both TP508-treated groups vs. the control group (Fig. 6E).

TUNEL staining. The apoptotic cell count in the AAR was 26-fold higher in the control group compared with both TP508-treated groups (Fig. 6F). Most apoptotic cells were cardiomyocytes and mainly located near the necrotic area. The nonischemic area was devoid of TUNEL-positive cells.

DISCUSSION

In the past decades, several studies have examined the effects of various drugs on myocardial IR injury in animal models, and many have reported positive results (32, 33).
However, there is currently no drug used in the clinic that has been demonstrated to provide a significant improvement in heart function or a reduction in myocardial necrosis for the treatment of acute myocardial infarction or during cardiac surgery (28). In addition, it is not known how results in these animal models mimic the clinical situation.

The key finding of this study is that the novel drug TP508 provides significant myocardial protection in the setting of hypercholesterolemia during IR injury in a dose-dependent manner. TP508 administration led to a greater than 50% reduction of infarct size, improved regional LV function, favored expression of cell survival proteins, and attenuated apoptosis. Our laboratory’s previous study has demonstrated that hypercholesterolemia alone increases myocardial necrosis by 45% in pigs subjected to acute IR (24). In addition, another study conducted in normocholesterolemic pigs demonstrated that TP508 at low dose decreases myocardial necrosis by 45% (25). The combined data from those studies suggest that high dose of TP508 may offset the negative effects of hypercholesterolemia on myocardial survival after IR injury.

While TP508 significantly improves regional LV function during IR injury in the TP508 high-dose group, we did not observe any differences in the global LV function among groups. One possible explanation for this discrepancy is that thrombin has been shown to depress cardiomyocyte contractility and interfere with β-adrenergic responsiveness. This effect may be mimicked by thrombin fragment administration (13). Thus protection by TP508 seen in this study may fail to be reflected in indexes of global LV function due to a transient, negative inotropic effect of the peptide. However, improved regional LV function suggests that a higher dose of TP508 may attenuate ischemia-induced myocardial stunning in the setting of hypercholesterolemia (2, 16, 42). In contrast, in a previous study conducted in normocholesterolemic pigs, TP508 administration did not produce any difference in regional myocardial function in animals treated with the low dose of TP508 used in the present study (25). In addition, hypercholesterolemic pigs have shown higher baseline indexes of LV function (+dP/dt), but greater deterioration of regional function during reperfusion compared with normocholesterolemic pigs (24).

Several previous studies have reported impaired endothelium-dependent and -independent coronary microvascular relaxation in the setting of hypercholesterolemia (15, 26, 27). In the
present study, the high dose of TP508 resulted in a significant improvement in endothelium-dependent and -independent coronary microvascular relaxations, in the absence of increased phospho-eNOS expression. Previous studies conducted on normocholesterolemic pigs in both acute and chronic ischemia models have demonstrated that low-dose TP508 improves microvascular relaxation and increases the expression of phospho-eNOS (7, 25). However, in previous studies, hypercholesterolemia was shown to lead to increases in myocardial nitrotyrosine staining in the ischemic territory (4, 24). These changes may be indicative of increased oxidative stress, as well as potential NOS uncoupling (31). Overall, these data suggest that, in the setting of hypercholesterolemia, higher doses of TP508 are needed to improve vasodilatation and enhance subsequent reperfusion to limit the extent of myocardial infarction following myocardial IR injury. Furthermore, TP508 may enhance coronary microvascular relaxation via pathways, in addition to the modulation of NO signaling.

The delicate balance between cell death and cell survival proteins plays a crucial role in cell fate after myocardial IR.

Fig. 7. Effect of TP508 on the ischemic myocardium during ischemia-reperfusion (IR) injury in the setting of hypercholesterolemia.
injury (41). This study demonstrates that TP508 treatment induces the expression of specific cell survival proteins in the ischemic myocardium, such as HSPs. Members of the HSP family are known to play an important role in cell survival by stabilizing protein structure during stress and binding and sequestering activated caspases and AIF, leading to improved cell survival (1, 18, 22, 29). HSP90, specifically, has been shown to complex with and stabilize Akt. This kinase is known to serve many prosurvival functions in cardiomyocytes (8).

In contrast, the expression of HSP90 was not different between TP508-treated (low-dose) and control groups in the normocholesterolemic pigs (data not shown), while the TP508-treated group had a higher expression of another protective HSP, HSC70 (HSP73) in the same study (25). While we found lower expression of phospho-Akt(Thr308) after myocardial IR injury in the TP508-treated groups, phosphorylation and inactivation of proapoptotic protein Bad (Ser112) was higher in the TP508 high-dose group (43). Bcl-2 is known to play a crucial role in preventing apoptosis, by blocking the activation of executioner caspases-3, -6, and -7 (23), and the release of mitochondrial cytochrome-c (11). Although hypercholesterolemia, by lowering Bcl-2 expression, may increase myocardial apoptosis after myocardial IR injury (24, 38), we did not find any significant changes in the expression of Bcl-2 in the TP508-treated groups. In a previous study conducted in normocholesterolemic pigs, our laboratory demonstrated that Bcl-2 expression was higher in the ischemic territory of TP508-treated pigs (25).

Another important finding in this study is that TP508-treated animals have lower expression of proapoptotic proteins, such as PARP, cleaved caspase-3, and AIF. PARP is a nuclear enzyme activated by DNA damage, which catalyzes the synthesis of poly (ADP) ribose from nicotine adenine dinucleotide. Overactivation of PARP results in the depletion of nicotine adenine dinucleotide and a subsequent decrease in ATP formation, leading to cell death (3). PARP inhibition has been shown to ameliorate myocardial damage in response to IR injury (40). Caspase-3 plays a central role in activation of a wide array of proteins involved in apoptosis (20). The lower expression of proapoptotic proteins supports the observed decreased TUNEL-positive cell counts in the TP508-treated animals. A previous study has demonstrated that hypercholesterolemia alone increases the expression of PARP and was associated with increased TUNEL-positive cell counts in the ischemic territory (24). In addition, nonischemic ventricular tissue from normocholesterolemic and hypercholesterolemic pigs demonstrated that the expression of PARP, BNip-3, and phospho-Akt (Ser473) was higher in the hypercholesterolemic pigs, whereas the expression of cleaved PARP and Bcl-2 was lower compared with normocholesterolemic animals. The expression of total Akt, caspase-3, and cleaved caspase-3 was not different among groups (24).

While providing important functional and molecular data about the role of hypercholesterolemia in acute IR, this study has several limitations. Our time course for tissue harvest (3 h after the onset of ischemia) could not account for long-term effects of IR on myocardial function and apoptosis. Furthermore, a short-term diet induction of hypercholesterolemia was performed, which does not account for the chronic effects of hypercholesterolemia on the myocardium. Whether this model is similar to long-standing endogenous or exogenous hypercholesterolemia in patients needs to be further evaluated.

In conclusion, this study demonstrates that parenteral administration of TP508 provides significant myocardial protection in hypercholesterolemic pigs following acute myocardial IR injury. 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 cell survival proteins, and the attenuation of apoptotic signaling (Fig. 7). Thus the novel thrombin fragment TP508 may have a therapeutic role in limiting myocardial injury after acute myocardial infarction, followed by interventions to restore blood flow in hypercholesterolemic patients. However, this will need to be verified in clinical trials.

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DISCLOSURES

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