Inhibition of N-Ethylmaleimide–Sensitive Factor Protects Against Myocardial Ischemia/Reperfusion Injury

John W. Calvert, Susheel Gundewar, Munekazu Yamakuchi, Pierce C. Park, William M. Baldwin III, David J. Lefer, Charles J. Lowenstein

Abstract—Exocytosis of endothelial granules promotes thrombosis and inflammation and may contribute to the pathophysiology of early reperfusion injury following myocardial ischemia. TAT-NSF700 is a novel peptide that reduces endothelial exocytosis by inhibiting the ATPase activity and disassembly activity of N-ethylmaleimide–sensitive factor (NSF), a critical component of the exocytic machinery. We hypothesized that TAT-NSF700 would limit myocardial injury in an in vivo murine model of myocardial ischemia/reperfusion injury. Mice were subjected to 30 minutes of ischemia followed by 24 hours of reperfusion. TAT-NSF700 or the scrambled control peptide TAT-NSF700scr was administered intravenously 20 minutes before the onset of ischemia. Myocardial ischemia/reperfusion caused endothelial exocytosis, myocardial infarction, and left ventricular dysfunction. However, TAT-NSF700 decreased von Willebrand factor levels after myocardial ischemia/reperfusion, attenuated myocardial infarct size by 47%, and preserved left ventricular structure and function. These data suggest that drugs targeting endothelial exocytosis may be useful in the treatment of myocardial injury following ischemia/reperfusion. (Circ Res. 2007;101:1247-1254.)

Key Words: exocytosis | vesicle trafficking | microvascular obstruction | endothelial | myocardial infarction

Reperfusion of an obstructed coronary artery limits the extent of myocardial necrosis.1 The effects of reperfusion are complex and paradoxically include deleterious effects such as vascular inflammation.2 The inflammatory response that follows reperfusion involves interactions of leukocytes with the coronary vascular endothelium, arterial wall, and cardiomyocytes.3 A key component of reperfusion related injury is the trafficking of polymorphonuclear leukocytes.4 Neutrophil-mediated microvascular injury results from the release of inflammatory mediators, as well as a progressive decrease in blood flow caused by microvascular obstruction (“no-reflow” phenomenon).5,5

The pathogenesis of microvascular obstruction is not well understood but may be caused by endothelial exocytosis. Ischemia/reperfusion (I/R) injury to endothelial cells trigger endothelial exocytosis, which activates the initial stage of leukocyte trafficking. Endothelial granules, termed Weibel–Palade bodies, contain both proinflammatory and prothrombotic mediators, such as P-selectin and von Willebrand factor (vWF).5–11 The release of vWF into the lumen promotes the adhesion and aggregation of platelets,12 whereas the translocation of P-selectin to the luminal surface promotes leukocyte rolling along the vessel wall through interactions with its ligand P-selectin glycoprotein-I on the surface of leukocytes.13 Following activation of endothelial exocytosis, further inflammatory stimuli activate leukocytes and endothelial cells, which lead to the expression of intracellular adhesion molecules and the activation of integrin ligands, mediating tight adherence to the vessel wall, followed by diapedesis of leukocytes across the endothelium and migration to the site of injury.

Endothelial exocytosis is regulated by the cellular machinery that mediates vesicle trafficking.14 This process involves the targeting of a vesicle or granule to a specific membrane, priming of the vesicle, and membrane fusion followed by recycling of the trafficking components.15 Several sets of proteins have been identified in this process, including N-ethylmaleimide–sensitive factor (NSF), SNAREs (soluble NSF attachment receptors), Sec/Munc proteins, and members of the Rab superfamily.16 NSF in particular plays a critical role in this complex process by hydrolyzing ATP to prepare the vesicle for fusion and by disassembling SNARE complexes to promote the recycle of the vesicle component.17–20

Therapies that inhibit NSF may limit endothelial exocytosis, thus decreasing leukocyte trafficking and inflammation. We developed a set of novel polypeptides that can cross cell membranes and inhibit NSF.21,22 These peptides consist of an amino-terminal TAT domain (that permits the peptide to
cross endothelial cell exocytosis in vitro and inhibit leukocyte rolling and trafficking in a murine model of experimental peritonitis.\(^{23}\) Here we tested the potential cardioprotective effects of TAT-NSF700 in an in vivo murine model of myocardial I/R.

**Materials and Methods**

**Animals**  
Male C57BL6/J mice, 8 to 10 weeks of age, were purchased from a commercial breeder (The Jackson Laboratory, Bar Harbor, Me). The animals were housed in the vivarium at Albert Einstein College of Medicine before any experimentation. All experimental mouse procedures were approved by the Institute for Animal Care and Use committee at Albert Einstein College of Medicine.

**Materials**  
TAT-NSF700 is a fusion polypeptide consisting of a human immunodeficiency virus transactivator of transcription (TAT) protein transduction domain (YGRKKRRQRRR), a poly-glycine linker (GGG), and an NSF homohexamerization domain starting at amino acid residue 700 (LLDYVPGRFSNLVLQALLVL).\(^{24}\) The entire sequence of TAT-NSF700 is: YGRKKRRQRRR-GGG-LLDYVPGRFSNLVLQALLVL. We also designed a control peptide TAT-NSF700scr, which consists of the intact TAT domain and glycine linker, followed by the NSF amino acids in a random order. TAT-NSF700 or TAT-NSF700scr were dissolved in saline and injected directly into the left ventricle lumen 20 minutes before myocardial ischemia at a final concentration of 0.5 mg/kg in a final volume of 100 \(\mu\)L.

**Myocardial I/R Protocol**  
Surgical ligation of the left coronary artery (LCA) was performed similar to methods described previously.\(^{24}\) Briefly, mice (n=12/group) were anesthetized with intraperitoneal injections of ketamine (50 mg/kg) and pentobarbital sodium (50 mg/kg), orally intubated, and ventilated. Core body temperature was maintained constantly at 37°C. A median sternotomy was then performed using an electric cautery. TAT-NSF700 or TAT-NSF700scr was injected into the left ventricle either 20 minutes before I/R or directly after ischemia but before reperfusion, as described in the text. Twenty minutes later, the proximal LCA was visualized and ligated. The coronary artery remained occluded for 30 minutes, after which the suture was cut and the vessel was allowed to reperfuse. The sternum and skin was closed separately, and the animals were allowed to recover.

**Myocardial Area-at-Risk and Infarct Size Determination**  
Measurement of area at risk and infarct size was performed as reported previously.\(^{25,26}\) In brief, at 24 hours of reperfusion, the mice were anesthetized, ventilated, and catherized through the common carotid artery. A median sternotomy was performed, and the LCA was religated in the same location as before. Evans blue dye (1.2 mL of a 4.0% solution; Sigma) was injected into the carotid artery catheter into the heart to delineate the ischemic zone from the nonischemic zone. The heart was rapidly excised and serially sectioned and incubated in 1.0% 2,3,5-triphenyltetrazolium chloride (Sigma). Each of the 5, 1-mm thick myocardial slices were weighed and the areas of infarction, risk, and nonischemic left ventricle were assessed by a blinded observer using computer-assisted planimetry (NIH Image J 1.37).

**Echocardiographic Assessment of Left Ventricular Structure and Function**  
Baseline echocardiography images were obtained in a separate group of mice (n=8) 1 week before and after LCA ischemia, as described previously.\(^{25,26}\)

**Histological Analysis of Infarct Size**  
After echocardiographic assessment, the mice (n=8/group) 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 mol/L phosphate buffer). After 12 hours in 4% paraformaldehyde, the heart was cut into 1-mm thick sections, as detailed above. The slices were dehydrated and embedded in paraffin, then cut into 4-\(\mu\)m slices, which were heated overnight in 60°C incubator. The sections were dewaxed and stained with hematoxylin and eosin. Digital images of the slides were then captured and analyzed using computer-assisted planimetry with NIH Image J 1.37 software to measure the area of infarct or scar relative to the left ventricle.

**Immunohistochemistry of Murine Myocardial Infarction**  
Following the induction of myocardial ischemia, an additional set of mice (n=6/group) was euthanized at 20 minutes of reperfusion. The hearts were fixed in acid methanol (10% glacial acetic acid, 60% methanol, and 30% water) and embedded in paraffin. Sections were then stained with antibody to vWF. The extent of vWF expression was scored in a blinded fashion by 2 observers using a scale from 0 to 3. The scale was based on the intensity and the area of staining. In particular, a score of 0 represented vWF confined to granules within endothelial cells in the infarct region; a score of 1 corresponded to vWF released into the subendothelial area in less than 50% of the infarct region; a score of 2 corresponded to vWF release into subendothelial areas in more than 50% of the infarct region; a score of 3 indicated vWF release into subendothelial areas in more than 50% of the infarct region and also extending into the myocardium, with aggregates of platelets and leukocytes clearly present in vessels.

**Statistical Analysis**  
All of the data in this study are expressed as means±SEM. Differences in data between the groups were compared using Prism 4 (GraphPad Software Inc), with Student’s paired 2-tailed \(t\) test or 1-way ANOVA where appropriate. For the ANOVA, if a significant variance was found, the Tukey test was used as the post hoc analysis. A probability value <5% was considered significant.

**Results**  
**TAT-NSF700 Decreased Endothelial Exocytosis After Myocardial I/R**  
We first explored the effects of TAT-NSF700 on endothelial exocytosis following myocardial I/R. Mice were treated with TAT-NSF700 or the scrambled control peptide 20 minutes before ischemia, subjected to 30 minutes of myocardial ischemia, and then reperfused for 24 hours. Myocardial I/R increased the release of vWF into the injured myocardium, whereas TAT-NSF700 significantly attenuated this release (\(P=0.043\)) (Figure 1). The effects of the single dose of TAT-NSF700 on endothelial exocytosis appear to be brief, because no difference between the experimental groups was observed at 4 hours of reperfusion (data not shown).

Higher magnification of the ischemic myocardium in control treated mice shows vWF deposition in the subendothelial space and vWF extending into the underlying myocardium; no granules containing vWF were visible in endothelial cells. Platelets were adjacent to the endothelium and were coated with vWF (Figure 1C, arrowheads). Large numbers of margined mononuclear cells and neutrophils were present in the vein (Figure 1C, arrows). In contrast, higher magnification of the TAT-NSF700–treated myocardium showed vWF largely confined to granules in endothelial cells (Figure
A decrease in endothelial exocytosis would be expected to diminish neutrophil recruitment into the myocardium. Accordingly, we used immunohistochemistry to measure the neutrophil marker myeloperoxidase in myocardium from treated and control mice. TAT-NSF700 decreased infiltration of myeloperoxidase-positive cells by a nonsignificant 10% after 20 minutes ($P=0.38$) and by a nonsignificant 27% after 240 minutes ($P=0.28$) (Figure I in the online data supplement at http://circres.ahajournals.org).

We had previously shown that peptides targeting NSF decrease endothelial exocytosis. For example, TAT-NSF700 decreases vWF release from endothelial cells treated with thrombin. To test the ability of TAT-NSF700 to suppress exocytosis during hypoxia, we exposed human umbilical vein endothelial cells to hypoxia (1% oxygen) or normoxia (21% oxygen) for 6 hours and then placed in normoxia for 1 hour, and the amount of vWF in the media measured by ELISA ($n=3$; $P<0.001$ vs TAT-NSFscr). Some cells were pretreated for 15 minutes with 1 $\mu$mol/L TAT-NSF700 or the scrambled control peptide TAT-NSFscr.
Mice were subjected to 30 minutes of myocardial ischemia, treated with TAT-NSF700 or the scrambled control peptide 0.5 mg/kg, and then reperfused for 24 hours. AAR with respect to the left ventricle was similar between both groups. TAT-NSF700 treatment significantly attenuated myocardial infarct size by 38% ($P=0.025$ versus TAT-NSF700scr) with respect to the left ventricle (infarct size/left ventricle) and by 35% ($P=0.023$ versus TAT-NSF700scr) relative to the AAR (infarct size/AAR) (Figure 2C). Thus treatment with TAT-NSF700 after ischemia (Figure 2C) was slightly less effective than treatment with TAT-NSF700 before ischemia (Figure 2B).

**TAT-NSF700 Preserved LV Chamber Dimensions**

We then explored the effect of TAT-NSF700 on LV dimensions. Mice were treated with TAT-NSF700 or control peptide, subjected to 45 minutes of myocardial ischemia, and then reperfused for 7 days. M-mode echocardiography was used to measure LV end-diastolic dimensions and LV end-systolic dimensions (LVESDs) before I/R and after 7 days of reperfusion (Table). The area and volume of the left ventricle were measured from 2D high-resolution B mode images. Myocardial I/R increased LVESD, LV systolic area, and LV systolic volume. However, TAT-NSF700 limited the effect of I/R (Table). TAT-NSF700 improved the LVESD by 41% (limiting the LVESD increase to 0.31 instead of 0.54 mm, $P<0.05$) and improved LV systolic volume by 41% (reducing the LV systolic volume increase to 6.4 instead of 15.4 $\mu$L, $P<0.05$).

**TAT-NSF700 Improved LV Function**

We subsequently determined whether the reduction of infarct size and preservation of LV dimensions would lead to an improved LV function. LV function was assessed through the analysis of 2D high-resolution B-mode images acquired at baseline and 1 week after myocardial I/R. Ejection fraction and fractional shortening at baseline and 1 week post–myocardial I/R were calculated. The ejection fraction and fractional shortening post–myocardial infarction decreased in mice after myocardial I/R in mice treated with TAT-NSF700 or TAT-NSF700scr control (Figure 3). Nevertheless, TAT-NSF700 significantly improved the ejection fraction by 50% ($P<0.001$) and improved the fractional shortening by 46% ($P<0.01$), compared with the control TAT-NSF700scr treatment (Figure 3).

Treatment with the TAT-NSF700 peptide also improved stroke volume and cardiac output 1 week after myocardial I/R (Figure 4). Both groups of mice displayed a significant decrease in stroke volume ($P<0.001$) and cardiac output ($P<0.01$) post–myocardial infarction. However, TAT-NSF700 significantly improved both the stroke volume by 36% ($P=0.014$) and cardiac output by 52% ($P=0.02$), compared with control peptide treatment. The heart rate of the 2 groups of mice was not different at baseline (Table). Both groups of mice did exhibit an elevated heart rate 1 week after myocardial I/R, but the TAT-NSF700scr control mice was the only group that had a significant increase from baseline ($P<0.05$).

**TAT-NSF700 Decreased Infarct Scar Formation**

We also measured the infarct area relative to the entire left ventricle. For each heart, we analyzed 4 sections taken from the midventricle and then averaged these numbers to obtain a single infarct size/left ventricle measurement for each animal.
TAT-NSF700scr–treated mice displayed a large area of scar formation extending from the midmyocardium to the epicardium (arrows in Figure 5A). Conversely, TAT-NSF700–treated mice displayed a smaller area of scar formation, mostly localized to the epicardium (arrowhead in Figure 5B). Analysis from the multiple midventricular sections per animal revealed that TAT-NSF700 decreased infarct size/left ventricle by 27% ($P/0.038$ versus TAT-NSF700scr).

Table. Two-Dimensional Echocardiography Measurements at Baseline and Seven Days After Myocardial I/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, mm$^2$</th>
<th>LV Area-S, mm$^2$</th>
<th>LV Volume-D, $\mu$L</th>
<th>LV Volume-S, $\mu$L</th>
</tr>
</thead>
<tbody>
<tr>
<td>TAT-NSF700scr</td>
<td>Baseline</td>
<td>406±16</td>
<td>3.81±0.03</td>
<td>2.77±0.05</td>
<td>19.25±0.39</td>
<td>8.72±0.66</td>
<td>49.28±1.54</td>
</tr>
<tr>
<td></td>
<td>Post</td>
<td>522±35*</td>
<td>4.15±0.14</td>
<td>3.31±0.21†</td>
<td>17.15±0.62</td>
<td>13.63±0.47†</td>
<td>44.14±2.85</td>
</tr>
<tr>
<td>TAT-NSF700</td>
<td>Baseline</td>
<td>422±14</td>
<td>3.80±0.06</td>
<td>2.65±0.08</td>
<td>18.22±1.13</td>
<td>8.26±0.91</td>
<td>46.70±4.25</td>
</tr>
<tr>
<td></td>
<td>Post</td>
<td>480±28</td>
<td>3.87±0.10</td>
<td>2.97±0.19¶</td>
<td>16.11±1.09</td>
<td>10.45±1.09¶</td>
<td>40.65±3.32</td>
</tr>
</tbody>
</table>

Means±SEM; n=8 per group. LVEDD indicates LV end diastolic dimension; D, diastole; S, systole. *$P<0.05$ vs baseline, †$P<0.01$ vs baseline, ‡$P<0.001$ vs baseline, ¶$P<0.05$ vs TAT-NSF700scr.

Discussion

The major finding of this study is that a single administration of TAT-NSF700, a compound that blocks endothelial exocytosis, transiently attenuated myocardial vWF levels in response to myocardial I/R, decreased myocardial infarct size, and preserved LV structure and LV function. This is the first...
fully elucidated, the accumulation of neutrophils in the underlying mechanisms of this phenomenon have not been characterized by the absence of tissue perfusion despite both microvasculature. This “no-reflow” phenomenon is caused by the release of inflammatory mediators, as well as through the obstruction of smaller vessels.

Neutrophils cause injury through the release of specific adhesion molecules on the surface of both neutrophils in the microcirculation.28,29 Once neutrophil trapping in the microvascular via the interaction of critical to the progression of vascular inflammation.28,29 Once trapped, neutrophils cause injury through the release of inflammatory mediators, as well as through the obstruction of the microvasculature. This “no-reflow” phenomenon is characterized by the absence of tissue perfusion despite both epicardial coronary artery patency and flow.30 Although the underlying mechanisms of this phenomenon have not been fully elucidated, the accumulation of neutrophils in the microvascular resulting in microvascular vasoconstriction and obstruction has been suggested to be a key factor.31 Therefore, strategies aimed at reducing this entrapment during the early stages of reperfusion might attenuate early inflammatory responses, as well as prevent the plugging of small vessels.

Our data show that I/R trigger endothelial exocytosis, extending prior studies showing that hypoxia induces exocytosis in vitro.32 Endothelial exocytosis plays a role in vascular inflammation by releasing P-selectin and vWF from the endothelial cell to promote leukocyte and platelet tethering to the endothelium. TAT-NSF700 treatment appears to decrease infarct size by 47%, with more sparing of the epicardial and endocardial regions. This treatment, which decreases endothelial exocytosis, might be expected to decrease infarction most in regions supplied by distal arterial branches, because smaller vessels might be more susceptible to vWF-driven platelet adherence and P-selectin–driven neutrophil adherence. These results suggest that ischemic injury to endothelial cells may play a role in the development of microvascular obstruction.

Our data also suggest that the pathway of endothelial exocytosis may be a therapeutic target. The process of endothelial exocytosis involves the targeting of Weibel–Palade bodies to the luminal membrane of the endothelial cell, priming of the vesicle, and membrane fusion followed by recycling of the trafficking components.33 NSF plays a critical role in vesicle trafficking and therefore is a potential therapeutic target.34 A temperature-sensitive NSF mutation decreases neurovesicle release in Drosophila.35 Antibodies against NSF inhibit exocytosis in human endothelial cells and platelets.21,36 NO inhibits exocytosis in part by chemically modifying NSF.18–21,23 The antiinflammatory effects of NO are mediated in part by its ability to decrease endothelial exocytosis.21,37 Taken together, these prior studies suggested that NSF is a potential therapeutic target for diseases that involve vascular inflammation. Our current study validates this concept, suggesting that NSF can be therapeutically targeted following myocardial I/R.

TAT-NSF700 might decrease vWF staining in vivo for a number of reasons including: less endothelial exocytosis, less platelet exocytosis, less platelet adherence to the endothelium, less platelet aggregation with other platelets, or a combination of these factors. Prior in vitro data show that TAT-NSF700 inhibits endothelial exocytosis but not platelet exocytosis.23 In theory, TAT-NSF700 might also decrease platelet adherence to endothelial cells by decreasing the release of endothelial vWF (which interacts with the platelet receptor GPIb/IX/V) and diminishing the translocation of P-selectin (which binds to the platelet receptors PSGL-1 and GPIba). Decreased vWF release would also diminish platelet aggregation mediated by the GPIIb/IIIa receptor. TAT-NSF700 decreases hypoxic triggered release of vWF from cultured endothelial cells (Figure 1E). Our current in vivo study supports several of these mechanisms but does not distinguish between them. TAT-NSF700 maintains vWF inside endothelial cells (Figure 1C, arrowheads), whereas control peptide permits vWF release into the subendothelial space (Figure 1D). TAT-NSF700 also decreases platelet adherence that would otherwise occur in ischemic tissue (Figure 1D, arrowheads). Because the total amount of tissue vWF is less in treated mice than in control mice, it is likely that TAT-NSF700 limits the synthesis and release of additional vWF from platelets and endothelial cells. Thus TAT-NSF700 probably decreases infarct size through several related mechanisms.

The importance of an inflammatory response in the development of myocardial infarction has been recognized and studied for more than 30 years.38–41 Leukocytes release a
variety of inflammatory mediators that contribute to the development of myocardial injury. Additionally, many experimental studies have shown that anti-inflammatory strategies are cardioprotective. Unfortunately, the promising results of these experimental studies have not translated into clinical success, and these disappointing results have led to criticism regarding the usefulness of strategies targeting a single mediator within the inflammatory cascade in myocardial infarction. Inhibition of endothelial exocytosis limits the externalization or release of the contents of endothelial granules, including vWF, which would otherwise promote platelet adhesion, and P-selectin, which mediates leukocyte trafficking, as well as other proinflammatory and prothrombotic compounds.

In summary, our findings demonstrate that a novel peptide that inhibits NSF decreases myocardial vWF levels during the early period of reperfusion, leading to a decrease in myocardial necrosis and a preservation of LV structure and function. Drugs directed at the exocytic machinery of endothelial cells may prove useful in the treatment of myocardial ischemia and other diseases involving vascular inflammation.

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Disclosures

The Johns Hopkins University has filed a provisional patent for the use of TAT-NSF peptides for the treatment of inflammatory diseases, with C.J.L. listed as a coinventor.

References

1. Buja LM. Myocardial ischemia and reperfusion injury. Circulatio

2. Vakeva AP, Agah A, Rollins SA, Matis LA, Li L, Stahl GL. Myocardial

3. Vinten-Johansen J. Involvement of neutrophils in the pathogenesis of


5. Palazzo AJ, Jones SP, Girod WG, Anderson DC, Granger DN, Lefer DJ.

6. Jones SP, Greer JJ, Kakkar AK, Ware PD, Turnage RH, Hicks M, van

7. Lowenstein CJ, Tsuda H. N-ethylmaleimide-sensitive factor: a redox


9. Bonfanti R, Furie BC, Furie B, Wagner DD. PADGEM (GMP140) is a


11. Bonfanti R, Furie BC, Furie B, Wagner DD. PADGEM (GMP140) is a

12. Ruggeri ZM. Von Willebrand factor, platelets and endothelial cell inter-

13. Frenette PS, Wagner DD. Insights into selectin function from knockout


15. Mellman I, Warren G. The road taken: past and future foundations of

16. ruggeri ZM. Von Willebrand factor, platelets and endothelial cell inter-

17. Lowenstein CJ, Tsuda H. N-ethylmaleimide-sensitive factor: a redox


20. Burgoyne RD, Morgan A. Analysis of regulated exocytosis in arterial

21. Matsushita K, Morrell CN, Cambien B, Yang SX, Yamakuchi M, Bao C, Hara MR, Quick RA, Cao W, O’Rourke B, Lowenstein JM, Pevsner J, Wagner DD, Lowenstein CJ. Nitric oxide regulates exocytosis by S-nitrosylation of N-ethylmaleimide-sensitive factor. Cell. 2003;115:139–150.


31. Alfayourim F, Srinivasan V, Geller M, Gradman A. The no-reflow phe-


34. Iwakiri Y, Satoh A, Chatterjee S, Toomre DK, Chalouni CM, Fulton D, Hara MR, Quick RA, Lowenstein JM, Pevsner J, Wagner DD, Lowenstein CJ. Nitric oxide regulates exocytosis by S-nitrosylation of N-ethylmaleimide-sensitive factor. Cell. 2003;115:139–150.


41. Alfayourim F, Srinivasan V, Geller M, Gradman A. The no-reflow phe-


44. Iwakiri Y, Satoh A, Chatterjee S, Toomre DK, Chalouni CM, Fulton D, Hara MR, Quick RA, Lowenstein JM, Pevsner J, Wagner DD, Lowenstein CJ. Nitric oxide regulates exocytosis by S-nitrosylation of N-ethylmaleimide-sensitive factor. Cell. 2003;115:139–150.


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On-Line Supplement
Materials and Methods

Myocardial Ischemia-Reperfusion (I/R) Protocol. Briefly, mice (n=12/group) were anesthetized with intraperitoneal injections of ketamine (50 mg/kg) and pentobarbital sodium (50 mg/kg). The animals were then attached to a surgical board with their ventral side up, orally intubated with PE-60 polyethylene tubing, and connected to a rodent ventilator (Minivent type 845, Hugo Sachs Elektronik). A medial sternotomy was then performed using an electric cautery. TAT-NSF700 or TAT-NSF700scr was injected into the left ventricle either 20 min before ischemia/reperfusion, or directly after ischemia but before reperfusion, as described in the text. Twenty minutes later the proximal left coronary artery was visualized and completely ligated at the atrial appendage with a 7-0 silk suture mounted on a tapered needle (BV-1, Ethicon). Core body temperature was maintained constantly at 37°C throughout the entire surgical procedure and myocardial I/R protocol. The coronary artery remained occluded for 30 minutes after which the suture was cut and the vessel was allowed to reperfuse. Mice were administered heparin (200 U/kg) to aid in the reperfusion of the coronary artery. The sternum and skin was closed separately with 4-0 BIOSYN monofilament suture. Animal recovery was supplemented by 100% oxygen and
buprenorphine (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 immediately made available.

**Myocardial Area-at-Risk and Infarct Size Determination.** At 24 hours of reperfusion, the mice were anesthetized with ketamine (50 mg/kg) and pentobarbital sodium (50 mg/kg), intubated, and connected to a rodent ventilator (Minivent type 845, Hugo Sachs Elektronik). 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 left coronary artery was re-ligated in the same location as before. Evans Blue dye (1.2 mL of a 4.0% solution, Sigma) was injected into the carotid artery catheter into the heart to delineate the ischemic zone from the non-ischemic zone. The heart was rapidly excised and serially sectioned along the long axis in five, 1 mm thick sections that were then incubated in 1.0% 2,3,5-triphenyltetrazolium chloride (TTC, Sigma) for 5 minutes at 37°C to demarcate the viable and nonviable myocardium within the ischemic zone. Each of the five, 1 mm thick myocardial slices were weighed and the areas of infarction, risk, and non-ischemic left ventricle were assessed by a blinded observer using computer-assisted planimetry (NIH ImageJ 1.37).

**Echocardiographic Assessment of Left Ventricular Structure and Function.**
Baseline echocardiography images were obtained in a separate group of mice one week prior to LCA ischemia. The mice (n=8/group) were lightly anesthetized with isoflurane in 100% O\textsubscript{2} and \textit{in vivo} transthoracic echocardiography of the left ventricle (LV) using a 30-MHz RMV scanhead interfaced with a Vevo 770 (Visualsonics) was used to obtain M mode images, as well as, high-resolution two-dimensional ECG based kilohertz visualization (EKV) B mode images acquired at the rate of 1000 frames/sec over 7 minutes. LV end-diastolic dimensions (LVEDDs), LV end-systolic dimensions (LVESDs) and heart rate were measured from the M-mode images. High-resolution B-mode images were used to measure the area and volume of the LV and to calculate the ejection fraction (EF), fractional shortening (FS), stroke volume (SV), and cardiac output (CO). One week after the baseline images were acquired, the mice were subjected to 45 minutes of LCA occlusion followed by reperfusion as described above. After 1 week of reperfusion, post I/R echocardiographic images were obtained and analyzed.
Supplemental Fig. S1. TAT-NSF and myeloperoxidase staining of myocardium after ischemia and reperfusion. Mice were pre-treated with TAT-NSF peptides and then subjected to ischemia and reperfusion as above. The presence of neutrophils was assessed by staining myocardial sections with antibody to myeloperoxidase (MPO) (n=6). The TAT-NSF700 peptide decreases MPO staining by a non-significant amount.