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*J Immunol* 2012; 189:5047-5056; Prepublished online 3 October 2012; doi: 10.4049/jimmunol.1200290
http://www.jimmunol.org/content/189/10/5047

**Supplementary Material**

http://www.jimmunol.org/content/suppl/2012/10/04/jimmunol.1200290.DC1

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Small β2-Glycoprotein I Peptides Protect from Intestinal Ischemia Reperfusion Injury

Michael R. Pope,* Urska Bukovnik, † John M. Tomich, † and Sherry D. Fleming* 

Intestinal ischemic events, which are followed by reperfusion, induce significant tissue damage and frequently result in multiple organ failure, with >70% mortality. Upon reperfusion, excessive inflammation leads to exacerbated tissue damage. Previous studies indicated that binding of the serum protein, β2-glycoprotein I, to the endothelium initiates a cascade of inflammatory molecules that is required for damage. We hypothesized that peptides derived from the binding domain (domain V) of β2-glycoprotein I would attenuate ischemia/reperfusion-induced damage and inflammation in a therapeutic manner. Using a mouse model of intestinal ischemia/reperfusion, we administered peptides either prior to ischemia or at clinically relevant time points during reperfusion and evaluated intestinal tissue damage and inflammation after 2 h of reperfusion. We demonstrate that multiple peptides attenuate injury and inflammation in a dose-dependent manner and, perhaps more significantly, are efficacious when administered up to 30 min after the onset of reperfusion. In addition, an all D-amino acid retro-inverso peptide was biologically active. Thus, the β2-glycoprotein I-derived peptides attenuate injury and inflammation when administered in a therapeutic manner in intestinal ischemia/reperfusion injury. The Journal of Immunology, 2012, 189: 5047–5056.

Although a lack of blood flow (ischemia) and, therefore, lack of oxygen (hypoxia) result in cellular death and tissue damage, the return of blood flow (reperfusion) to the cell or organ significantly magnifies the tissue damage and may lead to damage in other organs, resulting in multiple organ failure. Several clinical conditions result in ischemia/reperfusion (I/R)-induced injury, ranging from myocardial infarction, intestinal I/R, cardiac bypass surgery, stroke, hemorrhage, and transplantation. Because myocardial infarction and stroke are two of the leading causes of death in the United States, and ~15,000 transplants and 30,000 cases of intestinal I/R (mortality rate of 60–80%) occur each year in the United States (1), I/R is a significant clinical condition.

Multiple components of the innate immune response play a role in reperfusion-induced injury, including activation of the complement cascade, cellular infiltrations, and secretion of cytokines and eicosanoids (2–8). The current literature suggests that during the ischemic period, reactive oxygen species, NO, Ca2+, lipases, and mitochondrial changes expose neoantigens on the cellular surface (9–11). During reperfusion, naturally occurring Ab (NAb) recognizes newly expressed neoantigens, including multiple phospholipid-binding proteins and the serum protein, β2-glycoprotein I (β2-GPI) (6, 7, 12, 13). The NAb are critical to initiating an excessive complement response and subsequent inflammation and tissue damage (6, 7). Additional data indicate that both neutrophil infiltration and complement activation are required for tissue damage, because the absence of either attenuates tissue injury (reviewed in Ref. 14).

As a component of the innate immune system, β2-GPI, also known as apolipoprotein H, is a 43-kDa serum protein that is abundant in the plasma (4–5 μM) (15–17). The protein contains five short consensus repeats with homology to the complement regulatory domains. Each domain consists of ~60 aa, with domain V containing additional residues on the C terminus (18). Domain V binds to anionic phospholipids, DNA, or other negatively charged molecules (18). Multiple carbohydrate structures stabilize the different conformations of β2-GPI (19). Each conformation is proposed to have distinct biological activity (19, 20). The primary conformation circulating in the blood is a closed or circular form. Within the plasma, β2-GPI exists in multiple oxidation states, which appear to regulate platelet adhesion and protect endothelial cells from oxidative stress (21, 22). Recent studies also identified β2-GPI as an inhibitor of angiogenesis (23, 24). Activation of β2-GPI induces a “fishhook” conformation when domain V binds to anionic phospholipids and exposes a neoantigen in domains I and II, which is recognized by NAb (19). Subsequently, the NAb–β2-GPI complex acts as an opsonin for the clearance of apoptotic cells by phagocytes (18, 25).

The normal physiological function of β2-GPI remains unclear (26). Anti–β2-GPI Ab are associated with autoimmune diseases, and β2-GPI is the major antigenic target for anti-phospholipid Ab found in the serum of patients with anti-phospholipid Ab syndrome or systemic lupus erythematosus (27). Furthermore, increased anti–β2-GPI Ab titer correlated with an increased risk for ischemic stroke or heart disease in patients with anti-phospholipid Ab syndrome or systemic lupus erythematosus, respectively (28, 29).

Previously, we demonstrated that, during reperfusion, β2-GPI binds to ischemic cell membranes, allowing Ab recognition that is necessary for subsequent complement activation and inflammation (13). Importantly, long peptides (25 aa) derived from the β2-GPI sequence attenuated β2-GPI binding to hypoxic endothelial cells.
and attenuated intestinal damage and inflammation when administered prior to I/R (13). Other β2-GPI–derived peptides also decrease fetal loss in a murine model of anti-phospholipid Ab syndrome (30, 31). However, the previous studies administered peptide prior to an ischemic event (13) or simultaneously with the Ab that induce fetal loss (30, 31). These treatment regimens are not feasible therapeutic interventions. Therefore, studies administering peptide at time points after the ischemic event or Ab induction are needed. In addition, reducing the size of the peptide would provide a more cost effective therapeutic, because the cost of production is not a linear function relative to length of the peptide. Finally, the peptides provide a useful tool for examining additional mechanisms of β2-GPI–induced damage in response to I/R.

We hypothesized that smaller peptides derived from β2-GPI would therapeutically attenuate the injury and inflammation in a mouse model of intestinal I/R. We demonstrate that the peptides (and smaller derivatives) are biologically active not only when administered prior to ischemia but also when administered during the reperfusion event at 1–10-fold excess of the native protein concentrations. Additionally, biologically active, reversed sequences of D-amino acids (retro inverse) appear to be more effective, possibly as the result of an increased half-life. These data indicate that the β2-GPI–derived peptides are clinically relevant and may provide a therapy for intestinal I/R injury.

Materials and Methods

Mice

Originally obtained from The Jackson Laboratory, C57BL/6 mice were bred and maintained in the Division of Biology at Kansas State University. Housed in a 12-h light-to-dark, temperature-controlled room, mice were allowed food and water ad libitum. All mice were kept in specific pathogen-free conditions (*Helicobacter* spp., mouse hepatitis virus, minute virus of mice, mouse parvovirus, Sendai virus, murine norovirus, *Mycoplasma pulmonis*, Theiler’s murine encephalomyelitis virus, and endo- and ecto-parasites). All research was approved by the Institutional Animal Care and Use Committee and conducted in compliance with the Animal Welfare Act and other federal statutes and regulations concerning animals.

Peptides

Previously reported studies using peptides 296 and 305 attenuated tissue injury (13). Smaller peptides contained within these regions of the β2-GPI sequence were designed and synthesized (Table I). Peptide p7 contained the consensus sequence for lipid binding found in peptide 296, with a Cys-to-Ser substitution (SKNKEKK) (32). Peptides p9 (SSTYVEAHS) and p11 (KSSTYVEAHS) from peptide 305 also contained the Cys-to-Ser substitution from original peptide 305. Additional forms of p9 were created using D-amino acids (D-p9) or the retro-inverso of D-p9 (Retro D-p9: SHAEVYSS). All peptides were generated at the Kansas State University Biochemistry Core Lab by solid-phase synthesis with 9-fluorenylmethoxycarbonyl chemistry, as described previously (33). The peptides were purified by reversed-phase HPLC and characterized by MALDI-TOF mass spectroscopy. All lyophilized peptides were stored at −20°C until use.

![Figure 1](https://www.jimmunol.org/)

**FIGURE 1.** Peptides 296c-s and 305 attenuate injury and eicosanoid production when administered after ischemia. C57BL/6 mice were subjected to sham treatment or I/R, with or without injection of 40 μM β2-GPI peptide, either prior to ischemia or 5, 15, or 30 min postischemia. Mid-jejunal sections were scored (75–150 villi/animal) (A, D) and V/C were measured (B, E) from C57BL/6 mice, with or without injection of 40 μM β2-GPI peptides 296c-s (A, B) or 305 (D, E), at the appropriate time points. PGE2 or LTB4 production was measured in intestinal sections from C57BL/6 mice, with or without injection of β2-GPI peptides 296c-s (C) or 305 (F), prior to sham or I/R treatment. Data are pg/mg of intestinal protein. Each symbol in (A) and (D) represents an individual animal. Each bar in (B), (C), (E), and (F) is representative of 5–10 animals, and each treatment was performed on at least two separate days. *p ≤ 0.05 versus sham + peptide, †p ≤ 0.05 versus I/R treatment animals not receiving peptides.
**I/R procedure**

As described previously, I/R was performed on ketamine (16 mg/kg)/xylazine (80 mg/kg)-anesthetized male mice (8–16 wk old), with buprenorphine administered for pain (34). Following a midline laparotomy, the body temperature of the mice was equilibrated for 30 min on a 37°C water-circulating heat pad, and moistened gauze prevented desiccation. The superior mesenteric artery was identified and isolated; a small vascular clamp was applied for 30 min and removed prior to 2 h of reperfusion. Ischemia was confirmed by observing the intestine changing from a pink to a gray color. Sham-treated animals, with or without peptide, underwent the same procedure as did the ischemic mice, without occlusion of the superior mesenteric artery. Sham-treated survival rate was 100%, with or without peptide treatment. After 2 h of reperfusion, the mice were euthanized, and multiple sections of the small intestine, beginning ∼10 cm distal to the gastroduodenal junction, were collected for histological and other analyses. The sequence of sections used for each analysis was maintained for each animal. All mice received 80–100 μl normal saline or peptide. Mice treated with the various β2-GPI peptides underwent the same procedure with i.v. administration of the peptides. Most studies administered 40 μM (80 nmol/mouse) peptide at 5 min prior to ischemia. Dose-response studies administered 1–40 μM peptide at 5 min prior to ischemia. These concentrations are 1–10-fold excess of the native protein concentration (15–17). Time course studies administered 40 μM peptide at 5 min prior to ischemia or at 15, 30, or 60 min posts ischemia.

**Histology and injury scoring**

After euthanization, a 2-cm mid-jejunum tissue section ∼10 cm distal to the gastroduodenal junction was immediately fixed in 10% buffered formalin and embedded in paraffin; 8-μm sections were cut transversely and stained with H&E. Mucosal injury was graded on a six-tiered scale adapted from Chiu et al. (35), as described previously (34). Briefly, the average damage score of each intestinal section (75–150 villi) was determined after grading each villus from 0 to 6. Normal villi were assigned a score of 0; villi with tip distortion were assigned a score of 1; a score of 2 was assigned when Guggenheims’ spaces were present; villi with patchy disruption of the epithelial cells were assigned a score of 3; a score of 4 was assigned to villi with exposed, but intact, lamina propria with epithelial sloughing; a score of 5 was assigned when the lamina propria was exuding; and villi that displayed hemorrhage or were denuded were assigned a score of 6. Using the same slides, the villus height/crypt depth ratio (V/C) was measured at ×200 from 20–25 villi/animal using a Nikon DS camera with a DS-L2 Controller and software (Nikon, Melville, NY). The software was calibrated with a slide micrometer. Cryosections were stained with rat anti-mouse C3, IgM, or myeloperoxidase mAb, followed by Texas Red conjugated donkey anti-rat Ab, and mounted with Prolong Gold (Invitrogen, Grand Island, NY). Photomicrographs were obtained at room temperature using a Plan Fluor 20×/0.5 objective on a Nikon 80i microscope equipped with a Photometrics CoolSNAP cf camera using MetaVue software. All microphotographs were analyzed by Image software (National Institutes of Health, Bethesda, MD) using the fluorescent area fraction after setting threshold for each experiment. The average of the isotype control was subtracted from each photo. The average of five photos/tissue from three or four animals/treatment group is reported.

**Ex vivo eicosanoid and cytokine generation**

Immediately after collection, a 2-cm intestinal section ∼14 cm distal to the gastroduodenal junction was minced, washed, resuspended in 37°C oxygenated Tyrode’s buffer (Sigma-Aldrich, St. Louis, MO) and incubated at 37°C for 20 min; the supernatants were collected. Leukotriene B4 (LTB4) and PGE2 concentrations were determined using enzyme immunoassay kits (Cayman Chemical, Ann Arbor, MI). Cytokines present in the supernatants were determined using a Milliplex MAP immunoassay kit and read on a Milliplex Analyzer (both from Millipore, Bedford, MA). All LTB4, PGE2, and cytokine concentrations were standardized to the total tissue protein content determined by BCA assay (Pierce, Rockford, IL) adapted to microtiter plates.

**Statistical analysis**

Data are presented as mean ± SEM, and significance (p < 0.05) was determined by one-way ANOVA with Newman–Keuls post hoc analysis (GraphPad/InStat Software).

**FIGURE 2.** Peptides 296c-s and 305 attenuate I/R-induced injury and inflammation in a dose-dependent manner. C57BL/6 mice were subjected to sham treatment or I/R, with or without injection of β2-GPI peptides (1–40 μM final concentration). Mid-jejunal sections were scored (75–150 villi/animal) (A, D) and V/C were measured (B, E) from C57BL/6 mice, with or without injection of β2-GPI peptide 296c-s (A, B) or 305 (D, E) prior to sham or I/R treatment. PGE2 or LTB4 production was measured in intestinal sections from C57BL/6 mice, with or without injection of β2-GPI peptide 296c-s (C) or 305 (F) prior to sham or I/R treatment. Values are represented as pg/mg of intestinal protein. Each symbol in (A) and (D) represents an individual animal. Each bar in (B), (C), (E), and (F) is representative of 3–10 animals, and each treatment was performed on at least two separate days. *p ≤ 0.05 versus sham + peptide, #p ≤ 0.05 versus I/R treatment animals not receiving peptides.
Results

Therapeutically administered peptides 296c-s and 305 protect from I/R-induced damage

Previous studies indicated that both peptide 296c-s and peptide 305 prevented intestinal I/R-induced tissue injury and PGE2 production (13). However, the peptides were administered prior to I/R-induced injury, which is not possible in most clinical situations. Therefore, we determined the optimal time of peptide administration. Similar to previous studies, I/R induced significant injury, with an average injury score of 2.5 ± 0.12 compared with sham treatment injury (0.7 ± 0.06) (Fig. 1A). The V/C decreased with I/R as another measure of intestinal injury (Fig. 1B). As indicated in Fig. 1 and Supplemental Fig. 1, both peptides appear to be efficacious in preventing intestinal injury when administered at either 5 min prior to reperfusion or 15 min into the reperfusion period in this 2-h evaluation period. In addition, treatment at both 30 and 60 min postischemia significantly decreased intestinal injury (Fig. 1A, 1D). Specifically, peptide 296c-s decreased injury scores to 1.64 ± 0.19 and 1.40 ± 0.11 when administered at 30 and 60 min after initiation of reperfusion, respectively (Fig. 1A). In addition, the V/C remained high at 30 min postischemia, indicating tall villi (Fig. 1B). When administered at similar time points, peptide 305 decreased injury scores to 1.58 ± 0.08 (30 min) and 1.24 ± 0.06 (60 min) (Fig. 1D). The V/C also remained high when peptide 305 was administered at 30 min into the reperfusion period, but it had decreased by 60 min postischemia (Fig. 1E).

Table I. β2-GPI peptide sequences

<table>
<thead>
<tr>
<th>Peptide Name</th>
<th>Sequence (5′ → 3′ Direction)</th>
<th>Residue Numbers</th>
<th>Molecular Mass (Da)</th>
</tr>
</thead>
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<tr>
<td>296 Cys-Ser</td>
<td>H-IHFYSKKEKKSSYTVEAHSRDGTI-OH</td>
<td>296–320</td>
<td>2925</td>
</tr>
<tr>
<td>p7</td>
<td>H-SKNKEKK-OH</td>
<td>300–306</td>
<td>861</td>
</tr>
<tr>
<td>305</td>
<td>H-KKCSYTVEAHRCEIPSFKEHS-OH</td>
<td>305–330</td>
<td>2969</td>
</tr>
<tr>
<td>322</td>
<td>H-IPSCFKEHSSLAPWDASELPC-NH2</td>
<td>322–345</td>
<td>2698</td>
</tr>
<tr>
<td>p9</td>
<td>H-SYSTVEAHS-OH</td>
<td>307–315</td>
<td>980</td>
</tr>
<tr>
<td>p11</td>
<td>H-KKSSYTVEAHS-OH</td>
<td>305–315</td>
<td>1236</td>
</tr>
<tr>
<td>D-p9</td>
<td>H-SYSTVEAHS-OH with D-amino acids</td>
<td>307–315</td>
<td>980</td>
</tr>
<tr>
<td>Scrambled</td>
<td>H-AQCPIDVRIQTA-NH2</td>
<td></td>
<td>1331</td>
</tr>
</tbody>
</table>

* Amino acid sequence is a modification of National Center for Biotechnology Information sequence AAB30789, as described in Materials and Methods.
Because eicosanoids LTB₄ and PGE₂ play significant roles in I/R-induced intestinal inflammation, we evaluated these molecules in peptide-treated mice. Treatment with peptides 296c-s and 305 attenuated PGE₂ production when administered at 15 min postischemia (Fig. 1C, 1F). However, PGE₂ increased when peptide was administered at later time points (Fig. 1C, 1F). I/R-induced LTB₄ was significantly inhibited by peptide 296c-s at 15 and 30 min postischemia (Fig. 1C). Peptide 305 only attenuated LTB₄, a chemotactic factor for inflammatory cells, when administered prior to ischemia (Fig. 1F). These results suggest that both peptides may be appropriate therapeutics for I/R-induced injury.

Peptides 296c-s and 305 attenuate I/R-induced injury in a dose-dependent manner

To determine the optimal dose of each peptide when administered prior to I/R, we treated at least three mice with 1, 4, 10, or 40 μM of either peptide optimally attenuated injury and PGE₂ production. Peptide 296c-s also attenuated intestinal damage at 4 μM, as determined by low injury scores and high V/C (Fig. 2A, 2B). In contrast, peptide 305 at 4 μM did not provide protection, as determined by the injury score, despite maintaining high V/C (Fig. 2D, 2E). In addition, similar concentrations of peptide 296c-s attenuated PGE₂ production (Fig. 2C, 2F).

Interestingly, peptide 305 reduced eicosanoid production at all concentrations tested, despite being unable to prevent injury at 1–4 μM (Fig. 2D, 2F). Peptide 296c-s treatment only attenuated LTB₄ production at 40 μM peptide, whereas peptide 305 attenuated LTB₄ production at all concentrations tested (Fig. 2C, 2F).

These studies indicate that both peptides, 296c-s and 305, are effective when administered at lower concentrations than previously reported and suggest that the peptides differentially alter the inflammatory response.

Multiple short peptides attenuate I/R-induced tissue damage and inflammation

Because smaller peptides are therapeutically more cost effective, we examined peptides 296c-s and 305, which share 16 of 25 aa, for smaller sequences that contain similar inhibitory activity. As such, we tested three peptides, 7–11 aa in length, for the ability to provide intestinal protection from I/R-induced injury and inflammation (Fig. 3, Table I). Multiple short peptides (40 μM; 80 nmol/animal) were administered i.v. to wild-type, C57BL/6 mice 5 min prior to ischemia. As indicated in Fig. 3A, 3B, and 3E, peptide treatment resulted in significantly decreased intestinal epithelial injury compared with similar mice that were subjected to I/R in the presence of saline or a scrambled peptide. Importantly, none of the mice treated with short peptides p7, p9, or p11 sustained injury scores or V/C that were significantly different from mice treated with 40 μM peptide 296c-s prior to ischemia (Figs. 1A, 1B, 3A, 3E). In addition, the V/C were similar to sham-treated animals, indicating that the small peptides attenuated intestinal I/R-induced tissue damage (Fig. 3B).

Similar to the large peptides, we evaluated eicosanoid production in shorter peptide-treated mice. Similar to the intestinal damage, the intestines of p7-, p9-, or p11-treated mice secreted similar concentrations of LTB₄ (Fig. 3C). In addition, the LTB₄ secreted differed significantly from I/R-treated or scrambled peptide-
treated mice and was similar to that found in mice subjected to sham treatment in the presence or absence of peptide treatment (Fig. 3C). Interestingly, all peptides attenuated I/R-induced PGE₂ production; however, only peptide p7 returned PGE₂ levels to those found in sham-treated animals (Fig. 3D). These data suggest that the shorter peptides may provide therapeutic efficacy that is similar to or better than that of the larger peptides.

Similar to the larger peptides, time course and concentration studies were performed to determine the clinical relevance of peptide p9 (Fig. 4). Peptide p9 appeared efficacious in preventing intestinal injury when administered at either 5 min prior to reperfusion (1.50 ± 0.09) or 15 min postischemia (1.72 ± 0.25) in this 2-h evaluation period (Fig. 4A, Supplemental Fig. 2B). In addition, treatment at 30 min postischemia also significantly decreased intestinal injury, with an injury score of 1.60 ± 0.08 (Fig. 4A). Finally, the V/C remained elevated even after 30 min postischemia (Fig. 4B). When the dose response was evaluated, only 40 μM attenuated intestinal damage, as determined by decreased injury score and increased V/C (Fig. 4C, 4D, Supplemental Fig. 2B).

Similar to the larger peptides, peptide p9 attenuated injury and PGE₂ production, even when administered at 30 min postischemia (Fig. 5A). In addition, LTB₄ production was significantly decreased after administration of peptide p9 at 15 and 30 min postischemia (Fig. 5A). Surprisingly, attenuated injury required 80 ng (40 μM) of p9/mouse, whereas as little as 8 ng (4 μM) reduced eicosanoid production (Fig. 5D). During the inflammatory process, the ischemic tissue also releases cytokines in response to I/R-induced NF-κB mobilization (36). Analysis of IL-6 and MCP-1 indicated that I/R induced intestinal secretion of both cytokines. Importantly, peptide p9 attenuated cytokine release when administered at any of the time points or at lower concentrations (Fig. 5B, 5C, 5E, 5F). Together, these data indicate that peptide p9 inhibits the I/R-induced inflammatory response.

**Peptide retro-inverso D-p9 attenuates intestinal I/R-induced damage and inflammation**

Mammalian proteins consist almost exclusively of L-amino acids. As a result of changes in the location of the amino acid side groups, proteins consisting of D-amino acid are generally not biologically active and are not degraded as rapidly by hydrolases. In contrast, reversal of the D-amino acid sequence (retro-inverso) orients the majority of the side groups of a peptide to a conformation that is similar to the L-amino acid sequence. Thus, the retro-inverso sequences frequently have a longer half-life as a result of the D-amino acids but are functionally similar to the L-amino acid sequence. We hypothesized that D-sequences substituted with all D-amino acids would not protect against I/R-induced damage but that the retro-inverso peptide may protect from I/R-induced tissue damage, similar to p9. Thus, prior to I/R, we treated mice with peptide D-p9 (p9 substituted with D-amino acids) and Retro D-p9 (D-p9 in the reverse sequence; Table I). Surprisingly, treatment with either peptide D-p9 or peptide Retro D-p9 attenuated I/R-induced intestinal injury and maintained V/C similar to peptide p9 treatment (Fig. 6A, 6B, 6G, 6H).

**FIGURE 5.** Peptide p9 attenuates I/R-induced intestinal eicosanoid and cytokine release in a therapeutic and dose-dependent manner. (A–C) C57BL/6 mice were subjected to sham or I/R treatment, with or without injection of 40 μM p9 peptides, either 5 min prior to ischemia or 15 or 30 min postischemia. (D–F) Additional mice were subjected to sham or I/R treatment, with or without injection of 4–40 μM p9 peptide. (A and D) PGE₂ and LTB₄ production was measured in intestinal sections from C57BL/6 mice, with or without injection of peptides prior to sham or I/R treatment. IL-6 (B, E) and MCP-1 (C, F) release was determined by multiplex analysis of intestinal supernatants. Values are represented as pg/mg of intestinal protein in 20 min. Each bar is representative of 4–10 animals, and each treatment was performed on at least two separate days. *p ≤ 0.05 versus sham + peptide, #p ≤ 0.05 versus I/R treatment animals not receiving peptides.
In response to I/R, IgM recognizes and binds multiple neo-antigens prior to complement activation. Complement component C3 is rapidly deposited and required for I/R-induced intestinal damage. Therefore, we examined the ability of the D-stereoisomer of p9 peptides to attenuate IgM binding and C3 deposition after I/R.

Intestinal I/R induced both IgM (Fig. 7A) and C3 (Fig. 7B) deposition in untreated mice. Importantly, significantly less IgM and C3 was deposited when mice were treated with p9, and few to no deposits were found in mice treated with Retro D-p9 (Fig. 7A, 7B). These data suggest that peptide Retro D-p9 has similar function as the L-amino acid peptide p9.

In contrast to injury, peptide D-p9 did not attenuate IgM deposition (Fig. 7A) or C3 deposition (Fig. 7B). To further investigate the mechanism of D-p9 peptide protection from I/R-induced injury, we examined the eicosanoid and cytokine response. As indicated in Fig. 6C–F, both p9 and Retro D-p9 attenuated PGE2 and LTB4, as well as cytokine (IL-6 and MCP-1) production, compared with untreated mice with I/R or control peptide-treated mice. Similar to peptides p9 and Retro D-p9, PGE2 and LTB4 did not induce IL-6 or MCP-1 production (Fig. 6E, 6F). However, only L-amino acid containing p9 and Retro D-p9 attenuated LTB4 and IL-6 production in response to I/R (Fig. 6C, 6D). Peptide D-p9 induced significant LTB4 and IL-6 production (Fig. 6C, 6D).

Because LTB4 is chemotactic for neutrophils, and I/R-induced injury requires both complement activation and a neutrophilic inflammatory response, we examined neutrophil infiltration by examining the myeloperoxidase-positive cells. As indicated in Fig. 7C, I/R induced significant neutrophil infiltration compared with sham treatment. However, none of the small p9 peptides induced neutrophil infiltration, as determined by myeloperoxidase staining. Thus, despite complement and IgM deposition, D-p9 attenuated injury and decreased neutrophil infiltration.

**Discussion**

I/R-induced injury requires activation of a cascade of innate immune components, ranging from NAb recognition of a neoantigen and complement activation to inflammatory cell infiltration (reviewed in Ref. 14). Although normally a protective response, during reperfusion the innate response is excessive and damaging. Thus, inhibition of any of these components (complement activation or neutrophil infiltration) may decrease the immune response to nondamaging levels. We hypothesized that inhibiting an initiator of the cascade would allow each component of the response to remain intact for further insults while preventing the excessive response. The current studies demonstrate that peptide inhibition of β2-GPI attenuates I/R-induced intestinal damage and inflammation when administered up to 30 min postischemia. To our knowledge, this is the first study to therapeutically administer β2-GPI–derived peptides that are small enough to be cost effective for routine use. Finally, the optimized p9 peptide retains activity when synthesized in the retro-inverso form that may extend the therapeutic half-life. Together, these studies extend the possibilities of peptide therapy to intestinal I/R injury and provide evidence that the peptides are clinically relevant.

Previous studies with β2-GPI–derived peptides 296c-s and 305 demonstrated efficacy when administered prior to ischemic events, such as in planned surgical procedures, including coronary artery bypass surgery (13). In a mouse model of antiphospholipid syn-
drome, fetal loss was decreased when other β2-GPI-derived peptides were administered either simultaneously with pathogenic Ab or up to 3 h prior to injection of Ab (30, 31). Finally, in vitro studies also demonstrated an effect when cells were treated with peptide prior to stimulation with anti-β2-GPI Ab (30, 31). In the current studies, peptide treatment was efficacious when administered at 15 and 30 min into the 2-h reperfusion period. Additionally, peptide treatment was efficacious at equal molar concentrations up to a 10-fold excess of the circulating native protein concentrations. Hence, administration of the peptides during the reperfusion period extends the clinical relevance to therapeutics, in addition to pretreatments. To our knowledge, this is the first demonstration of β2-GPI-derived peptide efficacy in preventing ischemic injury when administered in a clinically relevant mode. Thus, these peptides may be useful in acute scenarios in addition to planned and routine surgical procedures and organ transplants.

Previous studies demonstrated that complement is required for I/R-induced injury (7), whereas other studies indicated that neutrophil infiltration is required (3, 4), suggesting that multiple innate components are required for tissue damage, and the absence of any one component attenuates injury. The peptides appear to have distinct properties, suggesting that treatment with multiple small peptides may be more effective. For example, peptides 305 and p9 (encompassed within 305) appear to be more critical for inhibiting the inflammatory response (eicosanoids and neutrophil infiltration), and peptides 296c-s and p7 (within 296c-s) attenuate actual epithelial injury more effectively. Hence, a combination of p7 and p9 may be optimal therapeutically.

Previous studies indicated that intestinal PGE2 production is necessary for I/R-induced intestinal injury but that PGE2 alone was not sufficient for I/R-induced intestinal injury (8). The current data support the correlation at high peptide doses and when administered early in the reperfusion period. In addition, PGE2 production increases prior to intestinal injury in the time course of peptide administration, supporting the concept that PGE2 is necessary, but not sufficient, for I/R-induced injury (8, 37). Previous studies indicated that 5-lipoxygenase is required for I/R-induced tissue damage (38). As one of the 5-lipoxygenase products, we examined LTB4 production, a potent chemoattractant factor and

**FIGURE 7.** Peptide Retro D-p9 attenuates IgM and C3 deposition in response to I/R. Prior to sham or I/R treatment, C57BL/6 mice were treated or not with injection of 40 μM β2-GPI peptides p9, D-p9, or Retro D-p9. Representative intestinal sections were stained for IgM (A) or C3 (B) deposition. (C) Additional sections were stained for myeloperoxidase. Fluorescent intensity was quantitated using ImageJ software, with the threshold set on isotype-control tissues. Each bar is the average ± SEM (n = 5–8 animals/treatment and n = 4–6 photographs/animal). Microphotographs are representative of three or four animals stained in at least three independent experiments (original magnification ×200). *p ≤ 0.05 versus sham + peptide, #p ≤ 0.05 versus I/R treatment animals not receiving peptides.
activator of neutrophils (39). The dose response data suggest that 
LTβ 1 is not required for intestinal damage. Because LTβ 1 pri-
mari ly recruits neutrophils, these data correlate with previous
studies indicating that the neutrophil infiltration is not sufficient for intestinal damage (5).

Interestingly, the D-amino acid form of p9 (D-p9) attenuated intestinal injury and PGE 2 production similar to the L-amino acid form. Although many D-amino acid peptides are not biologically active, other examples of biologically active D-peptides include the D-PIE12-trimer and an antagonist of p53. As a leading anti-

References


7. Williams, J. P., T. T. Pechet, M. R. Weiser, R. Reid, L. Kobzik, F. D. Moore, J., M. C. Carroll, and H. B. Hechtman. 1999. Intestinal reperfusion injury is me-


