Human thrombin-derived host defense peptides inhibit neutrophil recruitment and tissue injury in severe acute pancreatitis

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Human thrombin-derived host defense peptides inhibit neutrophil recruitment and tissue injury in severe acute pancreatitis. Am J Physiol Gastrointest Liver Physiol 307: G914–G921, 2014. First published September 11, 2014; doi:10.1152/ajpgi.00237.2014.—Severe acute pancreatitis (AP) is characterized by leukocyte infiltration and tissue injury. Herein, we wanted to examine the potential effects of thrombin-derived host defense peptides (TDPs) in severe AP. Pancreatitis was provoked by infusion of taurocholate into the pancreatic duct or by intraperitoneal administration of L-arginine in C57BL/6 mice. Animals were treated with the TDPs GKY20 and GKY25 or a control peptide WFF25 30 min before induction of AP. TDPs reduced blood amylase levels, neutrophil infiltration, hemorrhage, necrosis, and edema formation in the inflamed pancreases. Treatment with TDPs markedly attenuated the taurocholate-induced increase in plasma levels of CXCL2 and interleukin-6. Moreover, administration of TDPs decreased histone 3, histone 4, and myeloperoxidase levels in the pancreas in response to taurocholate challenge. Interestingly, administration of TDPs abolished neutrophil expression of Mac-1 in mice with pancreatitis. In addition, TDPs inhibited CXCL2-induced chemotaxis of isolated neutrophils in vitro. Fluorescent-labeled TDP was found to directly bind to isolated neutrophils. Finally, a beneficial effect of TDPs was confirmed in L-arginine-induced pancreatitis. Our novel results demonstrate that TDPs exert protective effects against pathological inflammation and tissue damage in AP. These findings suggest that TDPs might be useful in the management of patients with severe AP.

MATERIALS AND METHODS


Address for reprint requests and other correspondence: Henrik Thorlacius, Dept. of Clinical Sciences, Section of Surgery, Malmö, Lund Univ., S-205 02 Malmö, Sweden (e-mail: henrik.thorlacius@med.lu.se).
Switzerland) and 25 mg of xylazine (Janssen Pharmaceutica, Beerse, Belgium) per kg body wt. The TDPs, GKY25 (GKYGFTYHVFRLKKWIQKVVIDQFE), GKY20 (GKYGYHTYHVFRLKWIQKVKY1), and the control peptide WFF25 (WFFYYLY1IGGVTHQQRKKKDKDE) were synthesized by Biopeptide, (San Diego, CA). The purity (>95%) of these peptides was confirmed by mass spectral analysis (MALDI-ToF Voyager; Applied Biosystems, Foster City, CA).

**Taurocholate-induced pancreatitis.** Anaesthetized mice underwent midline laparotomy, and the second part of duodenum and papilla of Vater were identified. Traction sutures were placed 1 cm from the papilla, and a small puncture was made through the duodenal wall with a 23-G needle in parallel to the papilla of Vater. A nonradioopaque polyethylene catheter connected to a microinfusion pump (CMA/100; Carnegie Medicine, Stockholm, Sweden) was inserted through the punctured hole in the duodenum and 1 mm into the common bile duct. The common hepatic duct was identified at the liver hilum and clamped with a neobulldog clamp. A sample (10 μl) of 5% sodium taurocholate (Sigma Chemical, St. Louis, MO) was retrogradely infused for 10 min into the pancreatic duct. After completion, the catheter was withdrawn, and the common hepatic duct clamp was removed. The duodenal puncture was closed with a purse-string suture. The traction sutures were removed, and the abdomen was closed in two layers. Using this protocol, we observed a total mortality rate of 5% within 24 h of pancreatitis induction. Animals were allowed to wake up and were given free access to food and water. Animals were treated i.p. with 0.5 mg of WFF25 (n = 6), GKY20 (n = 6), GKY25 (n = 6), or vehicle (n = 6) 30 min before bile duct cannulation. Peptides were dissolved in sterile Tris buffer (10 mM Tris, 0.15 M NaCl, pH 7.4) and diluted just before injection. Sham mice undergoing laparotomy and sodium chloride infusion into the pancreatic duct were pretreated with vehicle (Sham, n = 6). All animals were killed 24 h after pancreatitis induction and assessed for all parameters included in this study.

**L-arginine-induced pancreatitis.** In separate experiments, AP was induced by administration of L-arginine (4 g/kg per dose) i.p. twice at an interval of 1 h as described in detail previously (10). Sample (0.5 mg) of WFF25 (n = 6), GKY20 (n = 6), GKY25 (n = 6), and vehicle (n = 6) were given i.p. to each mouse before the first dose of L-arginine. Saline-treated animals served as negative controls. All animals were killed 72 h after pancreatitis induction.

**Amylase measurements.** Blood amylase levels were determined in blood collected from the tail vein by use of a commercially available assay (Reflotron; Roche Diagnostics, Mannheim, Germany).

**Myeloperoxidase activity.** A piece of the pancreatic head and lung tissue were harvested for myeloperoxidase (MPO) measurements. All frozen pancreatic and lung tissues were prewashed and homogenized in 1 ml of mixture (4:1) of PBS and aprotonin 10,000 kallikrein inactivator units per milliliter (Trasylo; Bayer HealthCare, Leverkusen, Germany) for 1 min. The homogenate samples were centrifuged (15,339 g, 10 min), and the supernatant was stored at −20°C; the pellet was used for MPO assay as previously described (19). All pellets were mixed with 1 ml of 0.5% hexadecyltrimethylammonium bromide. Next, the samples were frozen for 24 h and then thawed, sonicated for 90 s, and put in a water bath 60°C for 2 h, after which the MPO activity of the supernatant was measured. The enzyme activity was determined spectrophotometrically as the MPO-catalyzed change in absorbance in the redox reaction of H₂O₂ (450 nm, with a reference filter of 540 nm, 25°C). Values are expressed as MPO units per gram of tissue.

**Tissue histology.** Samples of pancreatic head were fixed in 4% formaldehyde phosphate buffer overnight and then dehydrated and paraffin embedded. Six-micron sections were stained (hematoxylin and eosin) and examined by light microscopy. The severity of pancreatitis was evaluated in a blinded manner by use of a preexisting scoring system including edema, acinar cell necrosis, hemorrhage, and neutrophil infiltrate on a zero (absent) to four (extensive) scale as previously described in detail (26).

**ELISA.** Histone 3 and histone 4 levels in the pancreas as well as CXC2L2 and IL-6 levels in the plasma were analyzed by use of double-antibody Quantikine enzyme-linked immunosorbent assay kits (R & D Systems Europe, Abingdon, UK) using recombinant murine histone 3, histone 4, CXC2L2, and IL-6 as standards.

**Flow cytometry.** For analysis of surface expression of Mac-1 on circulating neutrophils, blood was collected from the inferior vena cava (1:10 acid citrate dextrose) 24 h after taurocholate induction and incubated (10 min at room temperature) with an anti-CD16/CD32 antibody blocking Fcγ III/II receptors to reduce nonspecific labeling and then incubated with phycoerythrin (PE)-conjugated anti-Gr-1 (clone RB6-8C5, rat IgG2b; eBioscience, Frankfurt, Germany) and FITC-conjugated anti-Mac-1 (clone M1/70, integrin αM China, rat IgG2b; BD Biosciences Pharmingen, San Jose, CA) antibodies. Cells were fixed, erythrocytes were lysed, and neutrophils were recovered following centrifugation. Flow-cytometric analysis was performed by first gating the neutrophil population of cells based on forward and side scatter characteristics, and then Mac-1 expression was determined on Gr-1⁺ in these gated cells on a FACSComp flow cytometer (Becton Dickinson, Mountain View, CA) and analyzed with CellQuest Pro software (BD Bioscience). A viable gate was used to exclude dead and fragmented cells.

**Chemotaxis assay.** Neutrophils isolated form bone marrow of healthy mice by use of Ficoll-Paque TM were incubated with 100 μM of WFF25, GKY20, or GKY25 for 30 min. 1.5 × 10⁶ neutrophils were placed in the upper chamber of the Transwell inserts (5-μm pore size; Corning Costar, Corning, NY). Inserts were placed in wells containing medium alone (control) or medium plus CXCL2 (100 ng/ml, R & D Systems). After 120 min, MPO levels were determined in the bottom wells as described above.

GKY25 binding to neutrophils. To determine whether GKY25 can bind to neutrophils, blood was collected into 1-ml syringes containing acid citrate dextrose solution (1:10). Blood samples were incubated with an anti-CD16/CD32 blocking antibody for 10 min at room temperature to reduce nonspecific labeling. Blood cells were then incubated with tetramethylrhodamine (TAMRA)-labeled GKY25 peptide, FITC-conjugated anti-Gr-1, and APC-conjugated anti-CD14 antibodies for 30 min at 37°C followed by red blood cell lysis and fixation by BD lysis buffer. Neutrophils were defined as Gr-1⁺/CD14⁺ and gated for TAMRA-GKY25 binding. Flow cytometry was performed as described above.

**Statistics.** Data are presented as means ± SE. Statistical evaluations were performed by using nonparametrical tests and Kruskal-Wallis one-way analysis of variance on ranks followed by multiple comparisons. P < 0.05 was considered significant, and n represents the number of animals.

**RESULTS**

**TDPs inhibit tissue damage in AP.** Examination of tissue morphology revealed normal pancreatic microarchitecture in sham animals (Fig. 1), whereas taurocholate induction caused significant destruction of the pancreatic tissue structure characterized by acinar cell necrosis, edema formation, and neutrophil accumulation (Fig. 1). Treatment with GKY20 and GKY25 attenuated taurocholate-induced tissue damage (Fig. 1). For example, GKY20 decreased taurocholate-provoked acinar cell necrosis by 67% and edema by 80% in the pancreas (Fig. 1, B and C). Additionally, GKY20 reduced the number of extravascular neutrophils by 78% in the inflamed pancreas (Fig. 1E). Moreover, it was found that retrograde infusion of taurocholate in the pancreatic duct increased blood amylase levels by sevenfold (Fig. 2A). Treatment with GKY20 and GKY25 reduced taurocholate-induced blood amylase levels by 41% and 44%, respectively (Fig. 2A). Histones have been
Fig. 1. Thrombin-derived host defense peptides (TDPs) regulate tissue damage in acute pancreatitis (AP). A: representative hematoxylin & eosin sections of the pancreas. Shown are data for acinar cell necrosis (B), edema formation (C), hemorrhage (D), and extravascular neutrophils (E). Animals were treated with 0.5 mg of WFF25, GKY20, and GKY25 or saline before taurocholate challenge. Mice treated with saline alone served as sham animals. Samples were harvested 24 h after pancreatitis induction. Asterisks indicate necrosis, and arrows indicate neutrophils. Data represent means ± SE, and n = 6. \#P < 0.05 vs. sham and \$P < 0.05 vs. WFF25 + taurocholate.
shown to exert potent cell cytotoxic effects (2, 25). Herein, we observed that taurocholate challenge markedly increased histone 3 and histone 4 levels in the pancreas (Fig. 2, B and C). Administration of GKY20 and GKY25 significantly decreased histone levels in the inflamed pancreas (Fig. 2, B and C). For example, GKY25 reduced pancreatic levels of histone 3 by 78% and histone 4 by 56% in mice exposed to taurocholate (Fig. 2, B and C). The specifically designed peptide WFF25 (WFFFYYLIIGGGVVTHQQRKKKKDE) did not show significant inhibitory effects on these parameters. This peptide has the same amino acid composition as the endogenous sequence GKY25, but with the amino acids sorted after hydrophobicity to eliminate sequence dependence and create an amphipathic linear structure (16).

**TDPs inhibit formation of proinflammatory mediators in AP.** Taurocholate infusion into the pancreatic duct increased plasma levels of CXCL2 and IL-6 by 20-fold and 9-fold, respectively (Fig. 2, D and E). Treatment with GKY20 decreased taurocholate-induced plasma levels of CXCL2 by 57% and IL-6 by 36% (Fig. 2, D and E). Moreover, administration of GKY25 reduced CXCL2 and IL-6 levels in plasma by 60% and 30%, respectively, in animals exposed to taurocholate (Fig. 2, D and E).

**TDPs inhibit neutrophil recruitment in AP.** Tissue levels of MPO were used as an indicator of neutrophil infiltration. Taurocholate challenge enhanced pancreatic MPO levels by ninefold (Fig. 3 A). Administration of GKY20 and GKY25 decreased taurocholate-induced MPO activity in the pancreas by 56% and 66%, respectively (Fig. 3 A). As part of a systemic inflammatory response in severe AP, activated neutrophils accumulate in the pulmonary microvasculature. Indeed, challenge with taurocholate markedly increased the MPO levels in the lung (Fig. 3 B). Treatment with GKY20 and GKY25 reduced the increased MPO activity in the lung by 55% and 65%, respectively (Fig. 3 B).
TDPs inhibit neutrophil activation and chemotaxis. Mac-1 is a useful marker of neutrophil activation. Taurocholate caused a significant upregulation of Mac-1 on neutrophils in the blood (Fig. 4A). Treatment with GKY20 and GKY25 reduced Mac-1 expression on neutrophils in pancreatitis mice (Fig. 4A). Thus mean fluorescence intensity values of Mac-1 on neutrophils decreased from 234 ± 14 to 177 ± 9 and 184 ± 12 in pancreatitis mice treated with GKY20 and GKY25, respectively (Fig. 4A). We next asked whether TDPs might directly regulate neutrophil chemotaxis in vitro. Neutrophils were isolated from the bone marrow and stimulated with CXCL2. It was observed that 100 ng/ml CXCL2 caused a clear-cut increase in neutrophil migration over a time period of 120 min (Fig. 4B). Coincubation of neutrophils with GKY20 and GKY25 decreased CXCL2-mediated migration of neutrophils by more than 85% (Fig. 4B). Next, we found that TAMRA-labeled GKY25 binds directly to isolated neutrophils (Fig. 4, C and D).

TDPs inhibit tissue damage in l-arginine-induced AP. To examine whether TDPs might also regulate tissue injury and neutrophil infiltration in an alternative experimental model of pancreatitis, l-arginine was used to trigger AP. Administration of 4 g/kg per dose l-arginine caused wide-spread tissue injury and infiltration of neutrophils in the pancreas (Table 1 and Fig. 5). Treatment with GKY20 and GKY25 significantly reduced l-arginine-induced acinar cell necrosis, edema, and hemorrhage in the pancreas (Table 1) and protected against l-arginine-provoked damage of the tissue microarchitecture in the pancreas (Fig. 5). In addition, administration of GKY20 and GKY25 attenuated l-arginine-induced MPO activity and the number of extravascular neutrophils in the pancreas (Table 1), as well as MPO levels in the lung (Table 1).

DISCUSSION

Clinical management of patients with severe AP is largely limited to supportive care and poses a major challenge to clinicians. Novel therapeutic options are needed to improve the outcome of patients with AP. The present study demonstrates that HDPs derived from thrombin protect against tissue injury in AP via inhibition of neutrophil infiltration. Thus our results elucidate novel and important protective mechanisms exerted by TDPs in the inflamed pancreas and suggest a therapeutic potential of GKY20 and GKY25 in AP.

Numerous reports have shown that endogenous antimicrobial peptides or proteins may exert pleiotropic immunomodulatory actions besides killing bacteria (9, 11, 12). In this context, it is notable that, among the experimental animal
models published at present, the majority of these has so far dealt with either isolated endotoxin models of septic shock or included infection models with a single microbe, as exemplified by previous work on invasive infections with *Pseudomonas aeruginosa* (16). Therefore, the present results represent, to our best knowledge, the first study demonstrating a significant protective effect of TDPs in a clinically relevant noninfectious model. Severe AP is characterized by massive activation of the host immune system, causing local and systemic complications (20, 27, 29). Herein, we show that treatment with two peptides derived from thrombin attenuate neutrophil activation and tissue damage in the inflamed pancreas. For example, administration of GKY20 and GKY25 decreased taurocholate-provoked increase in blood amylase by more than 41% and edema formation by more than 80%, suggesting that these TDPs effectively protect against tissue damage in severe AP. Previous studies have reported that GKY20 and GKY25 can inhibit vascular leakage in an endotoxin model of murine lung injury (16, 17). Considering that Toll-like receptor 4 (TLR4) activation seems to be important during development of AP (4) and that TDPs can directly bind endotoxin, a well-known TLR4 agonist (17), it is possible that TLR4-dependent interactions might be involved in the beneficial effects of GKY20 and GKY25, also in AP. A potential contribution of ionic interactions would likely be of minor importance because our control peptide, WFF25, of identical net charge and amino acid composition (16), had no effect on tissue injury in the inflamed pancreas. Thus this indicates that a specific mode of action mediates the protective effects of TDPs in AP. Convincing data have established neutrophil accumulation as a key component in the pathophysiology of AP (1, 5). In the present study, we could demonstrate that GKY20 and GKY25 reduced pancreatic activity of MPO, a marker of neutrophil infiltration, by more than 56% in response to taurocholate challenge. This inhibitory effect was also observed for acinar cell necrosis, extravascular neutrophils, and hemorrhage (Fig. 5).

### Table 1. *l*-arginine-induced pancreatitis

<table>
<thead>
<tr>
<th></th>
<th>Saline</th>
<th>Vehicle + <em>l</em>-arginine</th>
<th>WFF25 + <em>l</em>-arginine</th>
<th>GKY20 + <em>l</em>-arginine</th>
<th>GKY25 + <em>l</em>-arginine</th>
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<tr>
<td>Blood amylase, µkat/l</td>
<td>86 ± 1</td>
<td>310 ± 8*</td>
<td>356 ± 2</td>
<td>157 ± 0.8†</td>
<td>207 ± 0.8†</td>
</tr>
<tr>
<td>MPO in the pancreas, U/g</td>
<td>0.49 ± 3</td>
<td>5.0 ± 0.1*</td>
<td>7.9 ± 2</td>
<td>1.5 ± 0.4†</td>
<td>1.1 ± 0.6†</td>
</tr>
<tr>
<td>MPO in the lung, U/g</td>
<td>1.2 ± 0.5</td>
<td>12.0 ± 0.3*</td>
<td>9.4 ± 0.4</td>
<td>1.5 ± 0.1†</td>
<td>1.9 ± 0.3†</td>
</tr>
<tr>
<td>Acinar cell necrosis (0–4)</td>
<td>0.5 ± 0</td>
<td>3.3 ± 0.1*</td>
<td>3.1 ± 0.18</td>
<td>1.6 ± 0.1†</td>
<td>1.4 ± 0.2†</td>
</tr>
<tr>
<td>Extravascular neutrophils (0–4)</td>
<td>0.8 ± 0.2</td>
<td>3.1 ± 0.1*</td>
<td>2.9 ± 0.18</td>
<td>1.4 ± 0.1†</td>
<td>1.4 ± 0.1†</td>
</tr>
<tr>
<td>Edema (0–4)</td>
<td>0.7 ± 0.2</td>
<td>3.0 ± 0.5*</td>
<td>2.5 ± 0.5</td>
<td>1.2 ± 0.2†</td>
<td>1.2 ± 0.1†</td>
</tr>
<tr>
<td>Hemorrhage (0–4)</td>
<td>0.8 ± 0.5</td>
<td>2.7 ± 0.3*</td>
<td>2.9 ± 0.5</td>
<td>1.0 ± 0.7†</td>
<td>1.1 ± 0.5†</td>
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Data represent means ± SE. Blood amylase and myeloperoxidase (MPO) levels in the pancreas and lung as well as histology scoring in saline and *l*-arginine-exposed mice pretreated with vehicle or 0.5 mg of WFF25, GKY20, and GKY25 or PBS 30 min before *l*-arginine challenge. *P* < 0.05 vs. sham and †P < 0.05 vs. WFF25 + pancreatitis; n = 5.

Fig. 5. Representative hematoxylin & eosin sections of the pancreas. A: saline animals served as negative controls. AP was induced by administration of 4 g/kg per dose of *l*-arginine i.p. twice at an interval of 1 h. Animals were treated with vehicle (B), 0.5 mg of WFF25 (C), or GKY20 (D) and GKY25 (E) before the first dose of *l*-arginine. Asterisks indicate necrosis, and arrows indicate neutrophils. Tissue samples were obtained 72 h after pancreatitis induction.
TDPs directly control neutrophil chemotaxis. In addition, we markedly decreased by GKY20 and GKY25, suggesting that direct effect of TDPs on neutrophil chemotaxis. Interestingly, potent regulators of CXC chemokine formation in the plasma, regulation on neutrophils and our observation that TDPs are neutrophils, which may help to explain the inhibitory effect in the present study, it was found that GKY20 and GKY25 decreased pulmonary MPO activity, suggesting that these TDPs also attenuate systemic activation and infiltration of neutrophils in the lung in severe AP. Accumulating data indicate that histones released during inflammation can cause tissue damage (2, 25). In fact, a recent study showed that histones evoe significant damage to epithelial and endothelial cells (25). It was therefore of interest to study levels of histones in the inflamed pancreas. Indeed, we found that taurocholate challenge markedly increased histone levels in the pancreas. In addition, administration of GKY20 and GKY25 significantly decreased levels of histones in the inflamed pancreas. These novel findings suggested that it could be important to define the functional role of histones in the pathophysiology of AP in future studies. In this context, it is interesting to note that a recent study reported that IL-6 seems to be an important link between local inflammation in the pancreas on one hand and systemic inflammation and lung damage on the other hand (28). We therefore examined plasma levels of IL-6 in pancreatitis animals and found that taurocholate challenge markedly increased the circulating levels of IL-6. Notably, taurocholate-induced plasma levels of IL-6 were greatly reduced in TDP-treated animals. Whether this TDP-regulated formation of IL-6 plays a mechanistic role in pancreatitis-associated lung damage and mortality remains to be studied in the future.

Numerous studies have shown that specific adhesion molecules control the extravasation process of leukocytes in the pancreas (3, 5). Although the detailed role of adhesion molecules in facilitating leukocyte accumulation in the pancreas is relatively unclear, several reports have documented that Mac-1 is a dominating molecule in mediating tissue infiltration of neutrophils (3, 15). In the present study, it was found that GKY20 and GKY25 reduced taurocholate-induced expression of Mac-1 on circulating neutrophils, which may help to explain the inhibitory effect of TDPs on taurocholate-induced pancreatic neutrophilia. However, knowing that CXC chemokines trigger Mac-1 up-regulation on neutrophils and our observation that TDPs are potent regulators of CXC chemokine formation in the plasma, it is possible that the reduced Mac-1 expression observed in pancreatitis mice treated with GKY20 and GKY25 might be an indirect effect. It was therefore of great interest to examine the direct effect of TDPs on neutrophil chemotaxis. Interestingly, we found that CXCL2-evoked migration of neutrophils was markedly decreased by GKY20 and GKY25, suggesting that TDPs directly control neutrophil chemotaxis. In addition, we found that GKY25 binds to the surface of neutrophils, which further support a direct action of TDPs on neutrophils. Considered together with the evidence above showing that GKY20 and GKY25 regulate CXC chemokine production, these results suggest that GKY20 and GKY25 regulate neutrophil accumulation in the inflamed pancreas lung at two distinct levels, i.e., indirectly via formation of CXC chemokines and directly via inhibition of neutrophil chemotaxis. Importantly, we found that GKY20 and GKY25 also decreased neutrophil infiltration and tissue damage in an alternative model of AP, i.e., i-arginine-induced pancreatitis.

In conclusion, this study provides new evidence that that GKY20 and GKY25, not only exert potent anti-inflammatory effects, but also ameliorate tissue damage in AP. One key effect of TDPs was inhibition of neutrophil recruitment in the inflamed pancreas. Taken together, our novel findings indicate that TDPs might be useful in the treatment of patients with severe AP.

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DISCLOSURES
Dr. Schmidtchen owns shares in in2cure, a company that develops thrombin-derived peptides for therapeutic use. The other authors state no conflict of interest.

AUTHOR CONTRIBUTIONS

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