The tripeptide analog feG ameliorates severity of acute pancreatitis in a caerulein mouse model

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Departments of 1Surgery, Centre for Neuroscience, 2Anatomy and Histology, and 3Anatomical Pathology, Flinders University, Flinders Medical Centre, Bedford Park, South Australia, Australia; and 4Department of Physiology and Biophysics, University of Calgary, Calgary, Alberta, Canada

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Rifai Y, Elder AS, Carati CJ, Hussey DJ, Li X, Woods CM, Schloithe AC, Thomas AC, Mathison RD, Davison JS, Toouli J, Saccone GT. The tripeptide analog feG ameliorates severity of acute pancreatitis in a caerulein mouse model. Am J Physiol Gastrointest Liver Physiol 294: G1094–G1099, 2008. First published February 28, 2008; doi:10.1152/ajpgi.00534.2007.—Acute pancreatitis (AP) is a common disease with significant morbidity and mortality. The incidence of AP is 100,000 individuals and is increasing (5, 11). In severe cases mortality is increased at sites of inflammation. Blocking ICAM-1 expression has been shown to have a protective effect against local and systemic organ damage in various experimental models of AP (2). Furthermore, ICAM-1 knockout mice are protected against AP and AP-associated lung injury (8, 16). Taken together, the inhibition of ICAM-1 expression is likely to be a potential therapeutic target in AP.

The minimally active sequence for the peptide SGPT (Thr-Ile-Phe-Glu-Gly-Gly), derived from submandibular gland of Sprague-Dawley rats, which protects against endotoxic and anaphylactic shock (19), is the tripeptide FEG (20). The D-enantiomeric and metabolically stable form of FEG, feG [D(Phe)-(D)-Glu]-Gly] modulates immune and inflammatory responses in several animal models (6, 20–24). No adverse or inherent toxicities of feG were identified in these studies. feG has also been shown to restrict neutrophil movement and chemotaxis of human and rat neutrophils (24) and to reduce the production of superoxide production by activated neutrophils (25). The ability of feG to prevent PAF-induced upregulation of CD 11B and CD 16B (low-affinity IgG receptor), which are known to be involved in the activation of leukocytes and chemotaxis, has also been demonstrated (21). These properties of feG led us to hypothesize that this peptide may be beneficial in the treatment of AP.

Our overall aim of this study was to determine whether feG is able to modulate the severity of AP in a mouse caerulein model. The specific aims of this study were to determine whether the administration of a single dose of feG at 0 h (prophylactic) or 3 h after AP onset (therapeutic) would decrease plasma amylase and lipase, pancreatic MPO, and pancreatic damage and 2) downregulate ICAM-1 mRNA expression in the pancreas during AP.

METHODS AND MATERIALS

For these studies we used the caerulein mouse model initially described by Niderau and colleagues (27). Male Swiss mice 6–8 wk...
old and weighing 25–30 g were used. Mice were fasted for 12 h before the AP induction and had free access to water. Mice were allocated into eight groups (Table 1).

On the day prior to the AP induction, a blood sample was collected from each mouse by retroorbital sinus bleeding under anesthesia. This anesthesia was induced with an injection of ketamine (75 mg/kg ip; Parnell Laboratory, Australasia, Auckland, New Zealand) and medetomidine (1 mg/kg; Novartis Animal Health, North Ryde, Australia). Anesthesia was reversed by an injection of atipamezole (1 mg/kg ip; Novartis). Blood was collected in microhematocrit tubes and centrifuged at 4,000 g for 5 min at room temperature (Cellokrit 2, Linson, UK), and the plasma was collected.

Mice received caerulein (50 μg/kg ip) (American Peptide, Sunnyvale, CA) in 150 μl of 0.9 NaCl at hourly intervals 0–12 h (total 13 injections). Each mouse received a subcutaneous injection of 0.1 mg/kg of buprenorphine HCl (Temgesic Inject, Reckitt Benckiser Health Care UK, Hull, UK) at the time of the first caerulein injection. This dose provided analgesia for the duration of the experimental period.

The salivary tripeptide analog fG and the control peptide CP were kindly provided by Salpep Biotechnology, Calgary, Alberta, Canada. A single dose of either tripeptide (100 μg/kg ip) was administered at 0 h or at 3 h post-AP onset. This dose of fG was commonly used in previous studies (6, 21, 23).

Mice were euthanized 1 h after the final caerulein administration under anesthesia as follows. Mice were anesthetized for retroorbital sinus bleeding as described above, and, following blood collection for plasma, the mice were euthanized by cervical dislocation. The pancreas was then harvested and divided into two pieces; one piece was used for amylase assays and the other for histology. The pancreas was processed and embedded in paraffin wax. Three randomly selected sections (5 μm) were mounted on glass slides and stained with hematoxylin-eosin.

Histology analysis of pancreatic damage. Fixed pancreatic tissue was processed and embedded in paraffin wax. Three randomly selected sections (5 μm) were mounted on glass slides and stained with hematoxylin-eosin.

The histological analysis was performed by using a point-counting morphometry method (13). From each slice, five randomly chosen microscopic fields (magnification ×40) were imaged with a digital camera (DP70 Olympus, Japan). Grids of 100 points (10 × 10) were superimposed on each image by using the Microsoft Flash software. Each grid represented 100 μm². In a pilot study we assessed the degree of necrosis, acinar cell vacuolization, and intra-acinar and interstitial edema. On the basis of these data, we determined that the most evident changes were in the proportion of abnormal cells and the degree of overall edema. Intercellular space represented a small fraction compared with other interstitial space and was combined with the latter to give total interstitial space. Consequently, normal and abnormal acinar cells and interstitial space were identified under each grid point and counted. Abnormal cells were defined as having small, dense, darker colored, or abnormally pigmented nuclei, which were surrounded by a “halo”; they had a ruptured membrane boundary or were swollen and misshapen and often contained excessive vacuoles or disrupted cytoplasm. Abnormal acinar cells are expressed as the percentage of total acinar cells counted. Interstitial space is expressed as a proportion of the number of points counted. Data for all mice per group (means ± SE) were then combined.

RNA extraction. Mice were euthanized by cervical dislocation. Following a midline abdominal incision to expose the viscera, the pancreas was harvested and snap frozen in liquid N2. To extract total RNA from mouse pancreata the frozen tissue was ground to a powder with a mortar and pestle prechilled with liquid N2. From each sample, RNA extraction was a modification of that described by Bhatia and coworkers (3). Frozen pancreatic tissue (100–300 mg) were thawed on ice and homogenized in 1.5 ml of 50 mM phosphate buffer pH 6 with a handheld tissue grinder (Wheaton, NJ). For each suspension, a 5-min freeze-and-thaw cycle was carried out three times. The suspension was then ultrasonicated (Branson Sonic Power, Danbury, CT) for two 10-s periods with at least 5 min between each ultrasonication step. The suspension was then recentrifuged then the pellet was resuspended with 500 μl of 50 mM phosphate buffer pH 6. The suspension was recentrifuged then the pellet was removed and the pellet was suspended in 1 ml of 50 mM phosphate buffer pH 6 with a handheld tissue grinder (Wheaton, NJ) for two 10-s periods with at least 5 min between each ultrasonication step. The suspension was then ultrasonicated (Branson Sonic Power, Danbury, CT) for two 10-s periods with at least 5 min between each ultrasonication step. The suspension was then recentrifuged then the pellet was resuspended with 500 μl of 50 mM phosphate buffer pH 6. The suspension was then ultrasonicated (Branson Sonic Power, Danbury, CT) for two 10-s periods with at least 5 min between each ultrasonication step. The suspension was then recentrifuged then the pellet was resuspended with 500 μl of 50 mM phosphate buffer pH 6. The suspension was then ultrasonicated (Branson Sonic Power, Danbury, CT) for two 10-s periods with at least 5 min between each ultrasonication step. The suspension was then recentrifuged then the pellet was resuspended with 500 μl of 50 mM phosphate buffer pH 6. The suspension was then ultrasonicated (Branson Sonic Power, Danbury, CT) for two 10-s periods with at least 5 min between each ultrasonication step. The suspension was then recentrifuged then the pellet was resuspended with 500 μl of 50 mM phosphate buffer pH 6. The suspension was then ultrasonicated (Branson Sonic Power, Danbury, CT) for two 10-s periods with at least 5 min between each ultrasonication step. The suspension was then recentrifuged then the pellet was resuspended with 500 μl of 50 mM phosphate buffer pH 6.

Table 1. Experimental groups: protocols and animal numbers

<table>
<thead>
<tr>
<th>Group</th>
<th>Protocol</th>
<th>Mice/Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>AP alone</td>
<td>21</td>
</tr>
<tr>
<td>2</td>
<td>AP + CP at 0 h</td>
<td>18</td>
</tr>
<tr>
<td>3</td>
<td>AP + CP at 3 h</td>
<td>11</td>
</tr>
<tr>
<td>4</td>
<td>fG alone at 0 h</td>
<td>11</td>
</tr>
<tr>
<td>5</td>
<td>fG alone at 3 h</td>
<td>11</td>
</tr>
<tr>
<td>6</td>
<td>AP + fG at 0 h</td>
<td>20</td>
</tr>
<tr>
<td>7</td>
<td>AP + fG at 3 h</td>
<td>11</td>
</tr>
<tr>
<td>8</td>
<td>Saline control</td>
<td>13</td>
</tr>
</tbody>
</table>

Acute pancreatitis (AP) was induced by 13 hourly injections of caerulein (50 μg/kg ip). Control peptide (CP) or fG was administered as a single dose (100 μg/kg ip) at 0 or 3 h after AP onset. The saline control group received 13 hourly injections of saline (ip).
in the RT and real-time PCR reactions, each group of samples included a water control (no RNA sample), and each sample had a negative control (the same RT treatment without SuperScript III).

ICAM-1 mRNA and 18S RNA were detected by using 6 μl of cDNA in real-time PCR with an annealing temperature of 55°C (ICAM-1) or 57°C (18S). All reaction volumes were 20 μl, constituting 10 μl Quantitect 2× SYBRgreen master mix (Qiagen, Doncaster, Australia) and 2 μl each forward primer (5'-TAAGAG-GACTCGGTGGATGG) and reverse primer (5'-GCAGGG-GCAATAGGAATGA) of ICAM-1. The stock concentration of each primer was 5 μM. The RT-PCR was performed on the Rotor-gene 3000 (Corbett Life Science, Eight Mile Plains, Australia). PCR products were verified by nucleotide sequencing at the Southpath and Flinders Sequencing Facility.

The amplification efficiency of each primer pair was calculated from a real-time PCR dilution curve generated by using serial twofold dilutions of genomic DNA. Quantitative real-time RT-PCR analysis was then performed using Q-Sense software (1). ICAM-1 expression data was normalized by dividing the target quantity of ICAM-1 by the target quantity of 18S. 18S was selected as an appropriate housekeeping gene because its expression was unaffected during experimentally induced AP (31).

Statistical analysis. The data are reported as means ± SE (n = 3–11 mice). Statistical analysis employed nonparametric tests, Mann-Whitney rank or Kruskal-Wallis where appropriate. A P value of <0.05 was regarded as significant.

This study was approved by the Animal Welfare Committee of Flinders University.

RESULTS

The induction of AP produced about a sevenfold increase in plasma amylase activity (Fig. 1). Prophylactic treatment with feG reduced plasma amylase activity by ~45% compared with the activity in the AP-alone group (P < 0.05), whereas feG administered at 3 h post-AP induction was without effect (Fig. 1). Administration of a control peptide at or 3 h after AP induction did not significantly change the plasma amylase compared with AP alone (Table 2). Injection of saline or feG (either prophylactically or therapeutically) alone did not change the plasma amylase activity compared with preinduction activity (Fig. 1). These data suggest that feG treatment at the onset of AP reduces hyperamylasemia.

As expected, the AP group exhibited about a 10-fold increase in MPO activity in the pancreas (P < 0.05; Fig. 2). Prophylactic feG treatment significantly decreased the MPO activity by ~80% of that in the AP-alone group, whereas therapeutic treatment with feG was ineffective (Fig. 2). Pancreatic MPO activity in the feG alone (0 or 3 h) groups was low and comparable to that observed in the saline group (Fig. 2). The administration of the control peptide at AP induction or 3 h after did not alter the AP-induced pancreatic MPO activity (Table 2). These data suggested that prophylactic but not therapeutic feG treatment reduced pancreatic MPO activity.

Histological assessment of pancreatic damage revealed the presence of a greater percentage of abnormal acinar cells and interstitial edema (Figs. 3–5). The AP group displayed ~90% of abnormal acinar cells compared with ~1% in the saline group (P < 0.05; Fig. 4). The prophylactic and therapeutic treatment with feG significantly reduced the percentage of abnormal acinar cells by ~30 and 10%, respectively, compared with the percentage in the AP-alone group (both P < 0.05; Fig. 4). In contrast, the administration of control peptide at 0 or 3 h after AP onset did not alter the percentage of abnormal acinar cells compared with the percentage in the AP-alone group (Table 2).

Table 2. Effects of prophylactic and therapeutic administration of control peptide

<table>
<thead>
<tr>
<th>Parameter</th>
<th>AP Alone</th>
<th>AP + CP (0 h)</th>
<th>AP + CP (3 h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma amylase activity, fold change</td>
<td>7.43 ± 0.59 (21)</td>
<td>6.17 ± 0.52 (18)</td>
<td>10.75 ± 1.65 (11)</td>
</tr>
<tr>
<td>Pancreatic MPO activity, IU/mg protein</td>
<td>0.062 ± 0.007 (16)</td>
<td>0.050 ± 0.007 (16)</td>
<td>0.074 ± 0.005 (8)</td>
</tr>
<tr>
<td>Abnormal acinar cells, % of total</td>
<td>88.68 ± 1.16 (16)</td>
<td>87.40 ± 1.28 (15)</td>
<td>91.66 ± 0.63 (8)</td>
</tr>
<tr>
<td>Interstitial space, per 100 points</td>
<td>21.05 ± 0.72 (16)</td>
<td>21.55 ± 0.76 (15)</td>
<td>21.33 ± 0.76 (8)</td>
</tr>
<tr>
<td>ICAM-1 expression, normalized to 18S</td>
<td>0.96 ± 0.04 (6)</td>
<td>0.57 ± 0.32 (3)</td>
<td>0.96 ± 0.08 (3)</td>
</tr>
</tbody>
</table>

Data are presented as means ± SE (n animals). All parameters in the AP + CP groups were not statistically different from those in the AP-alone group.
tion or 3 h later produced a very small but statistically significant increase in the percentage of abnormal acinar cells to 3.2 and 3.4%, respectively, compared with 1.3% in the saline group (both \( P < 0.05 \); Fig. 4). On balance, these data suggest that prophylactic and therapeutic feG treatment reduced the pancreatic acinar cell damage induced by AP.

Interstitial edema was increased about threefold in the AP group compared with the saline group (both \( P < 0.05 \); Fig. 5). The prophylactic and therapeutic treatment with feG significantly reduced the percentage of interstitial edema by \( \sim 40 \) and 20%, respectively, compared with that measured in the AP-alone group (both \( P < 0.05 \); Fig. 5). As with the other indexes of AP, the administration of control peptide fdG with caerulein did not change the acinar architecture compared with that in AP alone (top right). In the AP plus feG at 0 h and 3 h groups (bottom middle and bottom right, respectively), the morphology of nucleus in acinar cells varied; some appeared normal and were surrounded with an intact membrane but others were severely disrupted. Interstitial space and inflammatory cells were still present, although less in degree than in the AP + fdG groups (original magnification \( \times 40 \)).

Fig. 3. Representative histology images of pancreas harvested from the various experimental groups. The architecture of acinar cells in saline (top left) and feG alone groups (bottom left) was normal. Induction of AP (top middle) displayed abnormal acinar cells with a shrunken nucleus, increased interstitial space, and marked neutrophil and other white blood cell infiltration. Coadministration of the control peptide fdG with caerulein did not change the acinar architecture compared with that in AP alone (top right). In the AP plus feG at 0 h and 3 h groups (bottom middle and bottom right, respectively), the morphology of nucleus in acinar cells varied; some appeared normal and were surrounded with an intact membrane but others were severely disrupted. Interstitial space and inflammatory cells were still present, although less in degree than in the AP + fdG groups (original magnification \( \times 40 \)).

Fig. 4. Percentage of abnormal pancreatic acinar cells in different experimental groups. Induction of AP resulted in a significant increase in the percentage of abnormal acinar cells. feG treatment at 0 h and at 3 h reduced the percentage of abnormal acinar cells compared with that in the AP-alone group. The saline group displayed few abnormal pancreatic acinar cells. Administration of feG alone did result in a small but statistically significant increase in the percentage of abnormal acinar cells compared with the saline group. Data are presented as means ± SE (\( n = 4–8 \)). *\( P < 0.05 \) compared with the saline group; †\( P < 0.05 \) compared with the AP-alone group.

Fig. 5. Pancreatic interstitial edema, as indicated by interstitial space, in different experimental groups. There was a significant difference in the interstitial space in the AP-alone group compared with the saline group. Treatment with feG at 0 and 3 h reduced the interstitial space. Data are presented as means ± SE (\( n = 4–8 \)). *\( P < 0.05 \) compared with the saline group; †\( P < 0.05 \) compared with the AP-alone group.
Pancreatic ICAM-1 mRNA expression, as determined by real-time PCR, showed an 7.5-fold increase in the AP group compared with the saline group ($P < 0.05$; Fig. 6). Prophylactic and therapeutic feG treatment significantly reduced the expression to ~50% of that observed in the AP-alone group. As with the other parameters listed in Table 2, the administration of the control peptide did not significantly alter the expression of ICAM-1 mRNA. Administration of feG alone did not significantly alter the basal expression of ICAM-1 mRNA (Fig. 6).

Taken together these data show that feG treatment ameliorates AP and this effect is associated with reduced ICAM-1 expression in the pancreas.

**DISCUSSION**

This study has demonstrated that prophylactic treatment with feG reduced the elevated markers of AP including pancreatic damage. Therapeutic treatment with feG, although less effective, also reduced pancreatic damage (abnormal acinar cells and edema). Pancreatic ICAM-1 mRNA expression was upregulated in AP, and this was reduced by prophylactic and therapeutic treatment with feG, suggesting that feG may ameliorate the inflammation cascade involved in the onset and progression of AP.

Modulation of the inflammatory response as a treatment strategy for AP is the basis of this study as this response leads to systemic complications with a mortality rate of 20–30% (38). The beneficial effects of feG in reducing inflammation in other systems have been previously demonstrated for allergic inflammation (6) and endotoxin-provoked perturbation of intestinal motility and inflammation (23). These effects are probably mediated by feG’s ability to regulate calcium and phospholipid-dependent protein kinase C (known to regulate the release of superoxide) in circulating neutrophils (6) and neutrophil adhesion (21), two neutrophil functions that are increased in AP patients (1, 37).

In AP, the normal secretory pathway of the acinar cell appears to fail. Zymogen granules are discharged into the intracellular spaces from the basolateral cell surface rather than into the duct lumen, and trypsinogen is prematurely converted into trypsin. The release of activatedzymogens and the presence of trypsin and other activated proteases as well as amylase and lipase contribute to acinar cell injury (9). The rise in the activity of plasma amylase in AP thus reflects vascular absorption of proteases leaking from the pancreatic acinar cells and the duct system. feG treatment in this study was associated with reduced hyperenzymemia. The mechanism(s) underlying feG’s beneficial effect on AP is incompletely understood, but the present findings provide some insight. Considering the complexity of AP, it is likely that more than one mechanism is involved, but one identified was reduced influx of neutrophils into the pancreas (i.e., lower MPO activity) consequent to reduced ICAM-1 expression with prophylactic administration of feG. First of all, in AP, neutrophils adhere to the venules, which can lead to capillary plugging with resultant accumulation of neutrophils in a time-dependent manner between 0 and 7 h after AP induction (15). feG may be able to prevent the adhesion and plugging components if administered prophylactically, but to a lesser degree when administered therapeutically since significant neutrophil activation will have already occurred (hence elevated MPO activity).

Secondly, the expression level of ICAM-1, which is upregulated in the pancreas during experimental AP, perhaps induced by intrapancreatic trypsin activity (12), usually correlates with the severity of the disease (8, 37), and ICAM-1 gene knockout mice are protected against AP and AP-associated lung injury (8, 30, 33). Unfortunately, the mechanisms involved are incompletely understood, although NK1 receptors are involved (18, 29). Furthermore, several adhesion molecules are upregulated during AP (18). In addition to feG’s possible action on ICAM-1 expression, other mechanisms involving altered neutrophil function are possible. Previous studies have shown that feG restricts the movement of neutrophils into inflamed endothelium in the rat lung by reducing tumor necrosis factor-α (TNF-α) levels in bronchoalveolar lavage fluid derived from macrophages (6). It is known that during the onset of AP, high levels of cytokines, e.g., TNF-α and its receptor TNF-α receptor 1 are produced (2). TNF-α is known to upregulate ICAM-1 on several cell types (14, 18, 36) due to its direct cellular toxic effect (35). The overproduction of cytokines will exacerbate the subsequent inflammatory cell infiltration during AP.

Wertheimer and colleagues (34) reported that the rapid accumulation of ICAM-1 on the endothelial cell surface by TNF-α was detected as early as 30 min and peaked by 2 h whereas others report that TNF-α was elevated at very early time (~2 h) of AP induction (10). These studies may be relevant to the possible mechanism by which feG downregulates ICAM-1 mRNA expression. One possible explanation is that prophylactic and therapeutic feG administration might reduce elevated TNF-α levels in pancreatic acinar cells and then prevent ICAM-1 from accumulating on the endothelial surface.

The acinar cell abnormality, demonstrated in the present study, was likely characterized by the progressive loss of cell membrane integrity, rupture of cells, and rapid influx of water, resulting in cytoplasmic swelling and pyknotic nuclei. These data are consistent with a previous study that reported interstitial edema developed early in the pancreas after caerulein infusion and was marked at 12 h (32).

![Fig. 6. ICAM-1 mRNA expression in the pancreas from the various experimental groups. The induction of AP resulted in pancreatic ICAM-1 expression. Administration of feG alone at 0 and at 3 h did not significantly change ICAM-1 expression compared with the saline group. Treatment of AP with feG at 0 and 3 h decreased ICAM-1 expression. The ICAM-1 expression is normalized to the expression of 18S. Data are presented as means ± SE (n = 3). *$P < 0.05$ compared with the saline group; †$P < 0.05$ compared with the AP-alone group.](image-url)
Therapeutic administration of feG at the dose of 100 μg/kg ip used in this study was not completely effective; however, this may not be the optimal dose. Although the leukocyte binding on the inflamed rat heart was inhibited by this dose (21), organ-to-organ and species differences contribute to different results. Further studies are required with higher feG doses and/or routes of administration and/or duration of administration during experimental AP. These additional studies are warranted by the observation that feG administered at the dose of 100 μg/kg ip reduces the percentage of abnormal acinar cells, as well as edema and ICAM-1 mRNA expression.

In summary, this study has demonstrated that feG can ameliorate AP. This observation is of particular clinical significance because of the mortality rate attributed to severe AP.

GRANTS

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REFERENCES