Original Paper

Pancreatology

Pancreatology 2011;11:57–67
DOI: 10.1159/000325972

Received: November 23, 2010
Accepted after revision: February 15, 2011
Published online: April 8, 2011

Prophylactic Glycine Administration Attenuates Pancreatic Damage and Inflammation in Experimental Acute Pancreatitis

G.O. Ceyhan[a, b] A.-K. Timm[b] F. Bergmann[c] A. Günther[d] A.A. Aghdassi[d] I.E. Demir[a] J. Mayerle[d] M. Kern[c] M.M. Lerch[d] M.W. Büchler[b] H. Friess[a] P. Schemmer[b]

[a]Department of Surgery, Technische Universität München, Munich, Departments of [b]General Surgery and [c]Pathology, University of Heidelberg, Heidelberg, and [d]Department of Gastroenterology, Endocrinology and Nutrition, University of Greifswald, Greifswald, Germany

Key Words
Glycine · Acute pancreatitis · Cerulein · Taurocholate

Abstract
Background/Aims: Acute pancreatitis (AP) is characterized by premature zymogen activation, systemic inflammatory response resulting in inflammatory infiltrates, sustained intracellular calcium, neurogenic inflammation and pain. The inhibitory neurotransmitter and cytoprotective amino acid glycine exerts a direct inhibitory effect on inflammatory cells, inhibits calcium influx and neuronal activation and therefore represents a putative therapeutic agent in AP. ***Methods:*** To explore the impact of glycine, mild AP was induced in rats by supramaximal cerulein stimulation (10 μg/kg BW/h) and severe AP by retrograde injection of sodium taurocholate solution (3%) into the common biliopancreatic duct. 100/300 mmol glycine was administered intravenously before induction of AP. To elucidate the effect of glycine on AP, we determined pathomorphology, pancreatic cytokines as well as proteases, serum lipase and amylase, pancreatic and lung MPO activity and pain sensation. ***Results:*** Glycine administration resulted in a noticeable improvement of pathomorphological alterations in AP, such as a reduction of necrosis, inflammatory infiltrates and cytoplasmic vacuoles in cerulein pancreatitis. In taurocholate pancreatitis, glycine additionally diminished pancreatic cytokines and MPO activity, as well as serum lipase and amylase levels. ***Conclusions:*** Glycine reduced the severity of mild and much more of severe AP by attenuating the intrapancreatic and systemic inflammatory response. Therefore, glycine seems to be a promising tool for prophylactic treatment of AP.

Introduction

Acute pancreatitis is characterized by an 'enigmatic' clinical course and a very complex pathophysiology. Although the course of the disease is mild in the majority of patients, about 25% of them suffer a severe attack. Unfortunately, up to 50% of the severe cases are lethal due to multiple organ dysfunction and/or sepsis [1].

Efforts to elucidate the pathophysiology behind this unpredictable disease expanded our understanding of the nature of the disease considerably; however, there is still no consensus over a single a pathomechanism sug-

G.O. Ceyhan and A.-K. Timm contributed equally.

KARGER

Fax +41 61 306 12 34
E-Mail karger@karger.ch
www.karger.com

1424–3903/11/0111–0057$38.00/0

Accessible online at:
www.karger.com/pan

Güralp O. Ceyhan, MD
Department of Surgery
Klinikum Rechts der Isar, Technische Universität München
Ismaninger Strasse 22, DE–81675 München (Germany)
Tel. +49 89 4140 5091, E-Mail ceyhan@chir.med.tu-muenchen.de

gesting that therapeutic double hits might be desirable. It is known that bile acids or nonoxidative alcohol metabolites can trigger a sustained abnormal cytosolic ionized calcium (Ca^{2+}) signaling. This abnormal calcium activity is accompanied by premature activation of zymogens within pancreatic acinar cells, leading to autodigestion of the gland and local inflammation [2]. Subsequently, neutrophils, macrophages, and lymphocytes release pro-inflammatory cytokines [3] such as interleukin-1 (IL-1), IL-6, IL-8 and tumor necrosis factor-α (TNFα), generating a pronounced systemic inflammatory response with potential subsequent organ failure [4, 5].

As one can expect, several studies aimed at targeting specific leukocyte subsets such as neutrophils [6–9], lymphocytes [10, 11], or monocytes [12–14] and indeed demonstrated a significant improvement of pancreatic damage and mortality in various experimental pancreatitis models. However, a clinical translation of these achievements has yet to take place.

One of the most interesting pathomechanistic concepts to emerge in recent years was the contribution of the 'neurogenic inflammation' to the generation of acute pancreatitis. It has been shown that in acute pancreatitis, activated sensory neurons projecting into the pancreas can secrete inflammatory neuromediators like substance P and bradykinin in an antidromic fashion, leading to vasodilatation and increased leukocyte extravasation [15]. Furthermore, inhibition of these sensory neurons via antagonists of the transient receptor potential vanilloid 1 (TRPV1) channel was shown to significantly reduce the severity of caerulein-induced acute pancreatitis in mice [16].

Looking at the impressive intersection of the nervous system activation and inflammatory cell recruitment in the induction of acute pancreatitis, we aimed at investigating the role of a molecule in acute pancreatitis that seems to well fit into this intersection: L-glycine. L-Glycine is a nonessential amino acid which acts as an inhibitory neurotransmitter in the central nervous system and mediates a chloride influx and thus neuronal hyperpolarization via a glycine-gated chloride-sensitive transmembrane channel (GlyR) [17]. However, the critical finding that L-glycine could act as an anti-inflammatory agent was based on the expression of GlyR on leukocytes including neutrophils, Kupffer cells, and splenic and alveolar macrophages [19]. Activation of the GlyR on these cells can, as known for neurons, result in hyperpolarization of the plasma membrane and hereby blunting increased intracellular calcium concentrations. As a result, production of cytokines [18] and the subsequent inflammatory cell activation is inhibited, and the formation of free radicals is decreased [19, 20]. Over these mechanisms, glycine was shown to have a protective effect in several experimental disease models such as ischemia-reperfusion injury [21, 22], shock [23], liver transplantation [24–26], arthritis [27] and alcoholic hepatitis [28].

For these reasons, we tested the potential of the neuroimmune linking molecule glycine as a prophylactic agent in two rat models of acute pancreatitis. For this purpose, we administered glycine intravenously before induction of a mild-edematous (cerulein) or severe-hemorrhagic (sodium taurocholate) pancreatitis in rats and examined the degree of pancreatic damage, intrapancreatic inflammation and pain under the influence of glycine.

Material and Methods

Pancreatitis Models and Glycine Treatment

All animal procedures were performed in accordance with ethical guidelines as specified by the local governing body. Male Sprague-Dawley rats (200–250 g), obtained from Charles River Laboratories, were maintained on a 12-hour light/dark cycle at 22 ± 2°C and relative humidity of 50 ± 5%. Animals were fed ad libitum on a standard diet and had free access to water. All animals were anaesthetized by intraperitoneal injection of Narcoren® (17 mg/kg BW) and intramuscular injection of Ketanest S® 25 mg (100 mg/kg BW). Mild edematous and necrotizing acute pancreatitis were both induced, as described previously [29]. Severe acute necrotizing pancreatitis was performed by retrograde injection of sodium taurocholate (3%) in the common biliopancreatic duct with a constant pressure of 30 cm H_2O/min using an infusion pump (Harvard Apparatus, Inc., Holliston, Mass., USA). Acute edematous pancreatitis was induced by a continuous infusion of supramaximal concentrations of cerulein (10 μg/kg BW/h) via a central venous line for 6 h. Ten minutes prior to induction of the taurocholate pancreatitis/cerulein pancreatitis or sham treatment, the glycine groups (n = 8) received a single intravenous bolus of 100 or 300 mmol glycine, respectively. The sham groups (n = 6, each group) received either a retrograde infusion of an equivalent volume of NaCl into the common bilio-pancreatic duct, or NaCl was given intravenously instead of cerulein. After the observation period of 6 h in the cerulein and after 24 h in the taurocholate pancreatitis, exsanguination was performed under anesthesia, as mentioned above. The pancreas was rapidly removed and trimmed of fat; pancreatic tissue including the head and tail were divided into several parts, and the aliquots were frozen in liquid nitrogen and stored at –80°C for protein extraction and fixed in 5% paraformaldehyde and later embedded in paraffin for histological analysis.

Histological Examination

Paraffin-embedded tissue sections (3 μm) were stained with hematoxylin and eosin. Histopathological analysis was performed by 2 independent observers (F.B., M.K.) blinded to diagnosis and treatment, followed by resolution of any differences by joint review

and consultation with a third observer (G.O.C.). Histomorphological evaluation of the specimen included the quantification of the morphological severity of the two acute pancreatitis forms according to the point score system of Spormann, as previously described [30, 31]. Potential systemic effects of glycine were investigated in the right middle lung lobe. Morphological changes were evaluated, and digital imaging was performed with the Zeiss AxioCam HR system (Carl Zeiss AG, Oberkochen, Germany).

An observer blinded to glycine treatment performed grading of intrapancreatic vacuolization in the cerulein pancreatitis. In tissue sections of the pancreatic head and tail, 5 randomly selected areas were photographed, and the total number of vacuoles was counted. The mean numbers of the vacuoles in the analyzed 5 areas were regarded as the representative figure for each animal.

Trypsin and Elastase Activity in Pancreatic Homogenates

Tissue was homogenized on ice in 100 mmol/l Tris (pH 8) and 5 mmol/l $CaCl_2$ and centrifuged for 10 min at 20,000 *g* at 4°C. Protein content was determined according to the method of Bradford. 10 μmol/l of elastase substrate R110-(CBZ-Ala4)2, 10 μmol/l of trypsin substrate R110-(CBZ-Ile-Pro-Arg)2, and 1 μg protein, respectively, were incubated in 150 μl final volume at an excitation wavelength of 485 nm and an emission wavelength of 530 nm at 37°C. Initial rates of substrate hydrolysis were measured in arbitrary fluorescence units per minute. Enzyme activity was calculated as units per milligram with purified elastase and trypsin as an internal standard, and activity was set in relation to caerulein- and taurocholate-treated animals, as demonstrated previously [32].

Measurement of Serum Amylase and Lipase

Serum amylase and lipase were detected using the automatic chemical analyzer ADVIA2400. The analyses were performed according to the IFCC Method (International Federation of Clinical Chemistry and Laboratory Medicine).

Myeloperoxidase Activity in Pancreatic and Lung Homogenates

Tissue processing was performed as demonstrated recently [32]. Tissue was homogenized on ice in 20 mmol/l potassium phosphate buffer (pH 7.4) and centrifuged for 10 min at 20,000 *g* at 4°C. The pellet was resuspended in 50 mmol/l potassium phosphate buffer (pH 6.0) containing 0.5% cetyltrimethylammonium bromide. The suspension was frozen/thawed 4 times, sonicated twice for 10 s, and centrifuged at 20,000 *g* for 10 min at 4°C. Myeloperoxidase (MPO) activity was assayed after mixing 50 μl supernatant in 200 μl of 50 mmol/l potassium phosphate buffer (pH 6) containing 0.53 mmol/l O-dianisidine and 0.15 mmol/l H_2O_2. The initial increase in absorbance at 460 nm was measured at room temperature with a Dynatech MR 5000 Elisa reader (Eningen, Germany). The results are expressed in units of MPO activity on the basis of 1 unit being able to oxidize 1 μmol H_2O_2 per minute per milligram pancreatic protein. Bars indicate mean values in mU MPO activity/mg pancreatic protein ± SEM per time point.

ELISA Analysis of IL-1, IL-6, IL-10 and TNFα in Pancreatic Homogenates

For ELISA-analysis, pancreatic homogenates were processed as described previously [33]. IL-1, IL-6 and IL-10 and TNFα levels were quantified using specific rat ELISA kits obtained by R&D Biotechniques (DY501, DY506, DY522, DY510). ELISA analyses were performed according to the manufacturers' instructions (R&D Biotechniques).

Behavioral Pain Testing

Behavioral measurements were carried out in awake, unrestrained rats in a blinded manner using the standardized open-field test [34]. Behavioral activity was measured for periods of 5 min before the induction of pancreatitis, 12 and 24 h after taurocholate and 6 h after caerulein pancreatitis. The behavior was analyzed for urination, feces, straighten up (vertical activity), crossing the lines (inner rows) and cleaning. The open-field apparatus was a square wooden box (70 × 70 × 60 cm), painted white. The floor was divided into 16 equal-sized squares (17.5 × 17.5 cm). Each activity chamber was cleaned with alcohol between tests to eliminate urine and olfactory cues from previous subjects.

Statistical Analysis

Statistical analysis was performed using the GraphPad Prism 4 Software. The Shapiro-Wilk test was used to evaluate data distribution. Results are expressed as mean ± SEM. Comparisons of histological changes, MPO activity, tissue homogenate analysis and behavioral testing were performed using analysis of variance for random measures followed by Bonferroni's post hoc test. Two-sided p values were always computed, and an effect was considered statistically significant at $p \leq 0.05$.

Results

Glycine Improves Pathomorphological Changes in Experimental Pancreatitis

Cerulein Pancreatitis

In rats with cerulein pancreatitis, all typical changes with immense inter- and intralobular edema, immune cell infiltration and cytoplasmatic vacuoles in the acini were evident (fig. 1a, b). Treatment with 100 mmol (2.3 ± 0.3; $p < 0.001$) and 300 mmol (3.0 ± 0.2; $p < 0.01$) glycine resulted in a remarkable reduction of the entire pancreatic damage (Spormann score) compared to nontreated animals (4.2 ± 0.2) (fig. 1a–g). In detail, compared to the cerulein animals (2.2 ± 0.1) the inflammatory cell infiltration was markedly reduced in the 100 mol glycine-treated animals (1.2 ± 0.2; $p < 0.01$) and also tended to decrease at higher concentrations of glycine (300 mmol, 1.7 ± 0.2) but failed to reach statistical significance (fig. 1h). Furthermore, pretreatment with 100 mmol (1.3 ± 0.2; $p < 0.05$) and 300 mmol (1.3 ± 0.1; $p < 0.001$) glycine noticeably reduced pancreatic edema compared to nontreated animals (1.9 ± 0.1) (fig. 1a–f, i). All cerulein pancreatitis animals with or without glycine treatment did not show up any fat or parenchymal necrosis. Both sham groups did not show any pathomorphological changes in the pancreas at all.

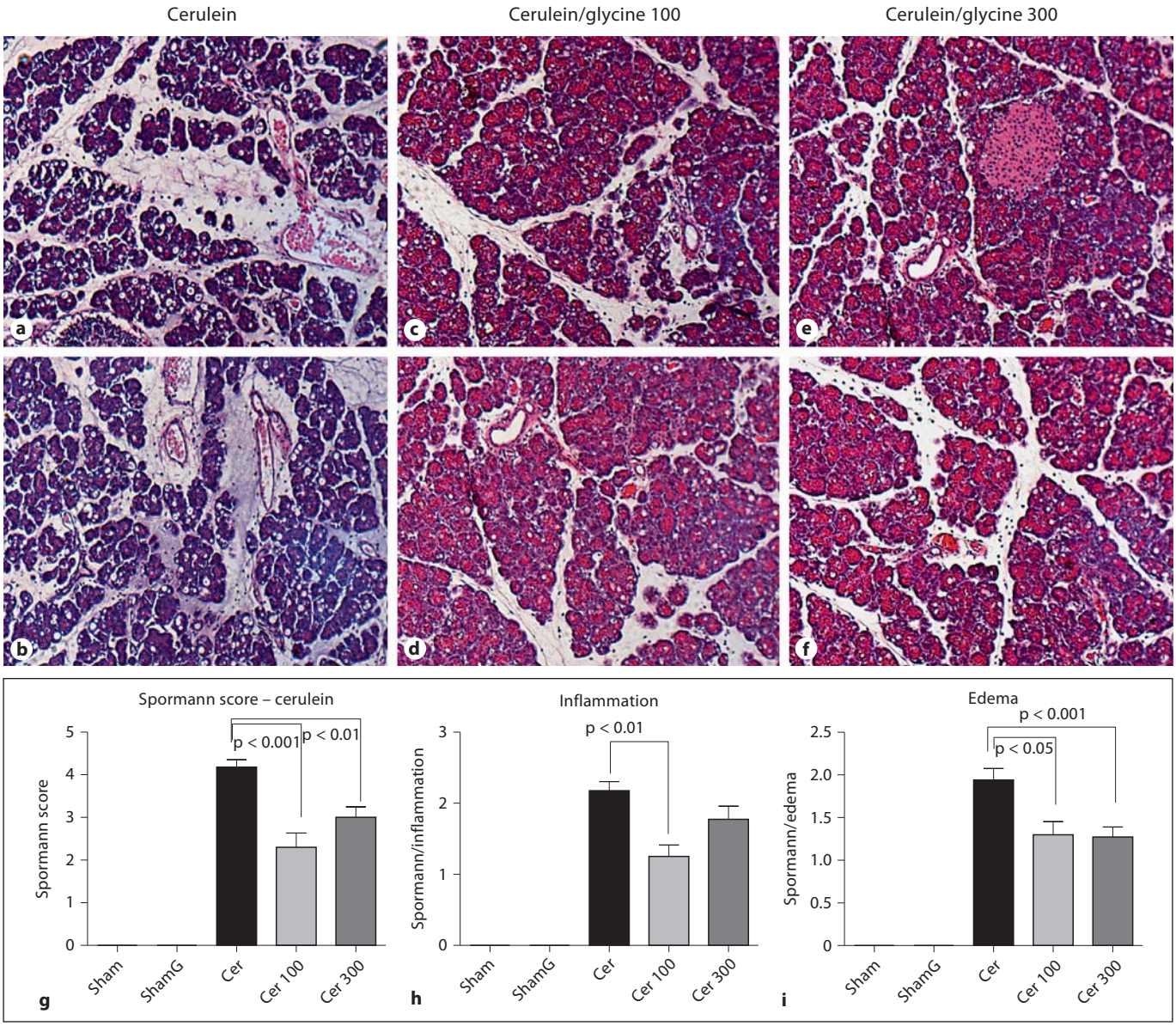

Fig. 1. Representative pictures demonstrating the effect of 100 mmol/Cer 100 (**c**, **d**) and 300 mmol/Cer 300 (**e**, **f**) glycine pretreatment on the course of cerulein pancreatitis/Cer. **a**, **b** Representative areas of damaged pancreatic parenchyma without glycine treatment. Glycine treatment improved pathomorphological changes in cerulein pancreatitis and noticeable reduced the Spormann score in the pretreated animals (**g**). Especially inflammation (**h**) and edema (**i**) were significantly attenuated in glycine-pretreated animals. Sham: NaCl is given intravenously instead of cerulein; shamG: NaCl is given intravenously instead of cerulein + 300 mmol glycine. Data are presented as mean ± SEM. **a–f** ×100.

The cytoplasmatic vacuoles, regarded as one the major characteristic changes of cerulein-induced pancreatitis, were markedly diminished by glycine pretreatment at the concentrations of 100 mmol (84 ± 13; $p < 0.01$) and 300 mmol (70 ± 11; $p < 0.001$), compared to nontreated animals (151 ± 6) (fig. 2a–d).

Taurocholate Pancreatitis

The untreated taurocholate animals showed severe pathomorphological changes (8.9 ± 0.7), including edema, massive immune cell infiltration, hemorrhage and aggravated fat and parenchymal necrosis (fig. 3a, b). This severe pancreatic damage was impressively reduced by

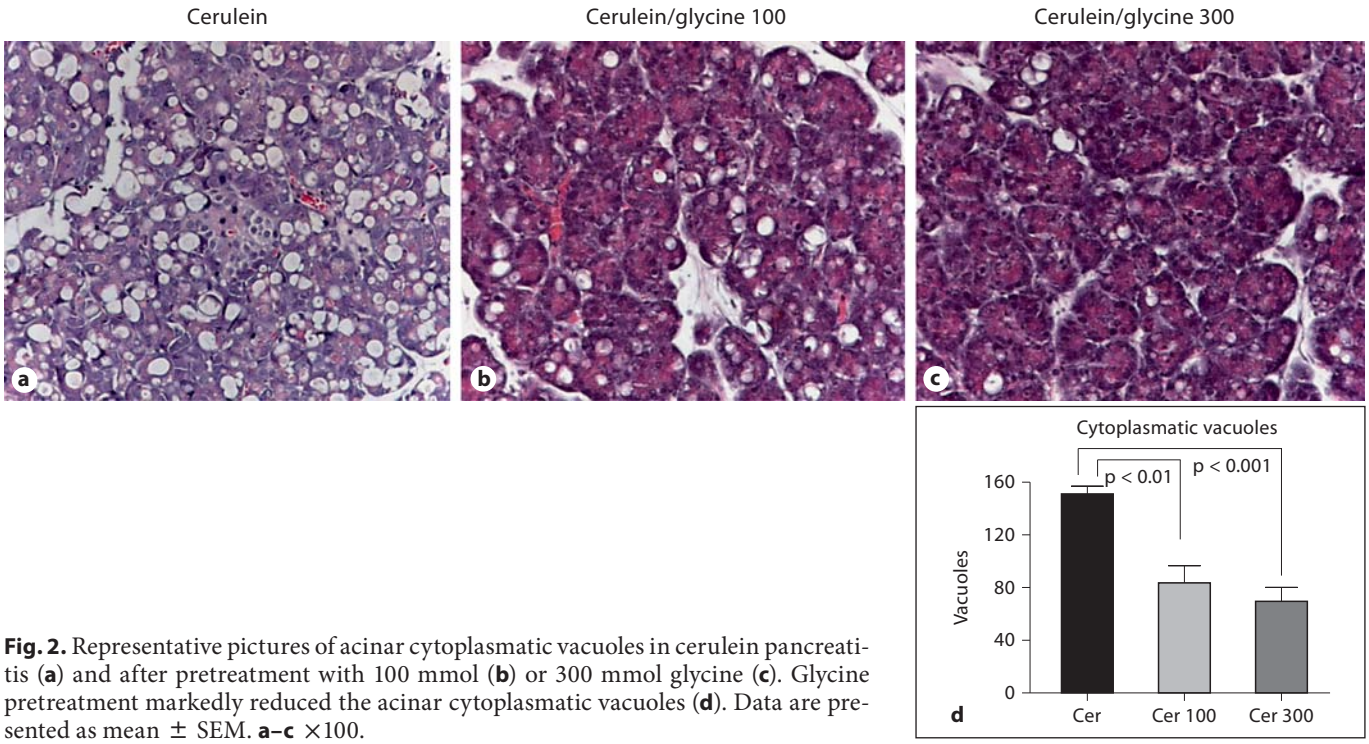

Fig. 2. Representative pictures of acinar cytoplasmatic vacuoles in cerulein pancreatitis (**a**) and after pretreatment with 100 mmol (**b**) or 300 mmol glycine (**c**). Glycine pretreatment markedly reduced the acinar cytoplasmatic vacuoles (**d**). Data are presented as mean ± SEM. **a–c** ×100.

glycine pretreatment with 100 mmol (4.1 ± 0.9) and 300 mmol (5.2 ± 1.0) (fig. 3g). Fat (1.8 ± 0.3) and parenchymal necrosis (2.0 ± 0.3) significantly decreased under 300 mmol glycine treatment (0.5 ± 0.2/0.6 ± 0.3; $p < 0.05$) (fig. 3h, i). Pancreatic edema was attenuated with glycine treatment only at lower concentrations with 100 mmol (1.0 ± 0.01; $p < 0.05$) compared to taurocholate-only treated animals (2.0 ± 0.3) (fig. 3i). Both the severity of inflammatory cell infiltration and hemorrhage tended to be decreased with glycine pretreatment not reaching statistical significance (data not shown).

Animals with taurocholate-pancreatitis developed a significantly higher amount of ascites (12 ± 3 ml), whereas the animals with 100 mmol glycine pretreatment had nearly no ascites production at all (0.02 ± 0.01 ml; $p < 0.01$; fig. 3k). Treatment with glycine at a dosage of 300 mmol (2.6 ± 1.7 ml; $p < 0.05$) noticeably decreased ascites development during the course of acute necrotizing pancreatitis (fig. 3k).

Effect of Glycine on Pancreatitis-Specific Biomarkers

To study whether glycine pretreatment is affecting specific biomarkers for pancreatitis, serum lipase and amylase were determined. In the edematous cerulein pancreatitis, glycine treatment did not change serum levels for lipase or amylase (fig. 4a, b). In contrast, glycine application in animals prior to induction of severe necrotizing taurocholate pancreatitis noticeably reduced both lipase and amylase in a significant manner. Animals treated with 100 mmol (22 ± 9 U/l; $p < 0.01$) or 300 mmol (52 ± 22 U/l; $p < 0.01$) glycine revealed noticeable reduced lipase values when compared to non-treated animals (295 ± 130 U/l) (fig. 4c). In a similar way also amylase levels were significantly reduced by glycine treatment (100 mmol: 2,580 ± 775 U/l, $p < 0.05$; 300 mmol: 3,239 ± 525 U/l, $p < 0.05$) compared to taurocholate animals (7,904 ± 2,195 U/l) (fig. 4d).

Glycine and Intrapancreatic Activation of Trypsin and Elastase during Experimental Pancreatitis

In order to investigate if glycine pretreatment is affecting premature activation of pancreatic zymogens, we measured the activity of trypsin and elastase in pancreatic homogenates. Different concentrations of glycine did not alter intrapancreatic activation of trypsin and elastase neither in the mild cerulein nor in the severe taurocholate pancreatitis (data not shown). Trypsin activity tended to be attenuated by glycine pretreatment in the taurocholate pancreatitis but failed to reach statistical significance.

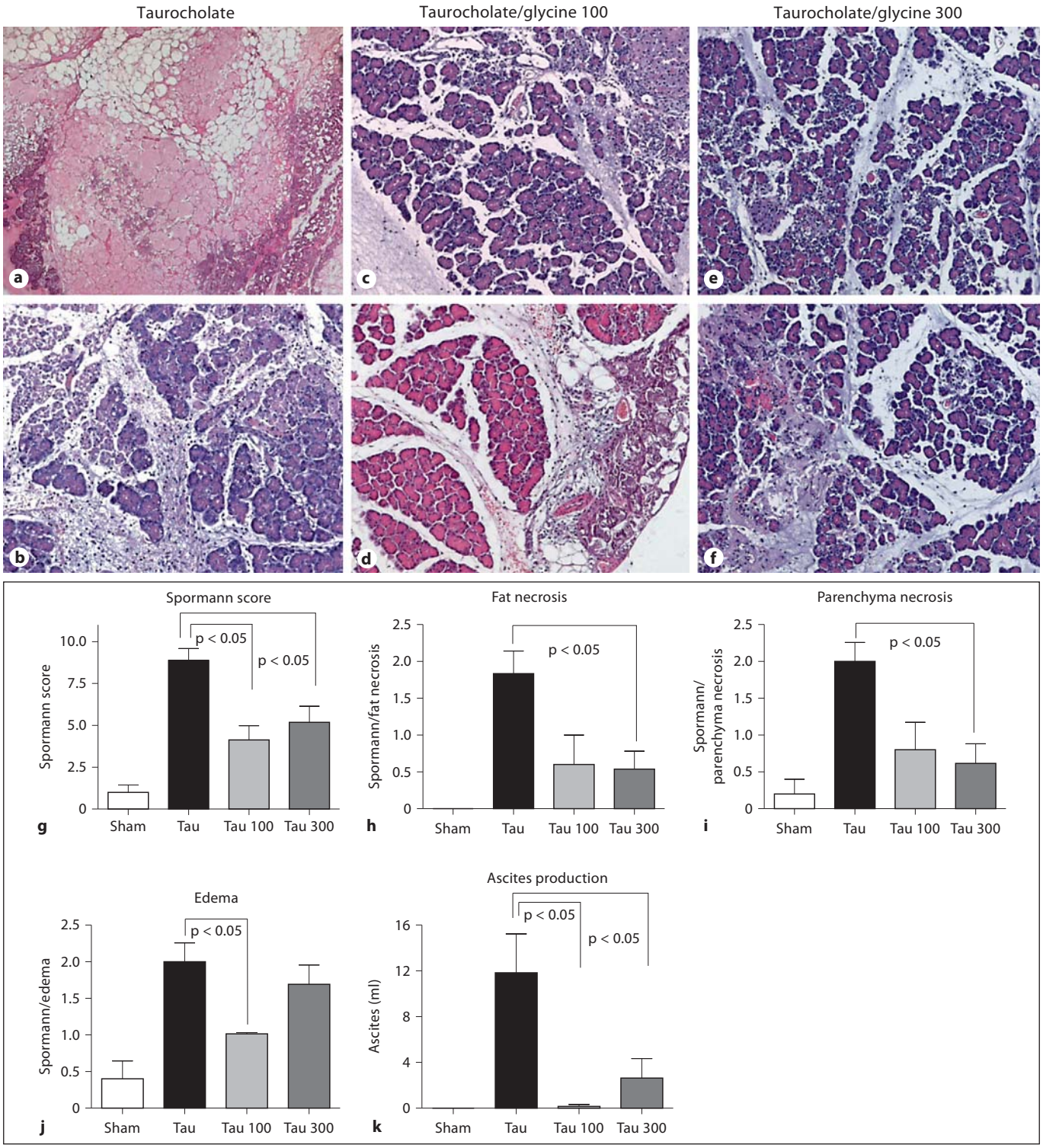

Fig. 3. Representative pictures demonstrating the effect of 100 mmol/Tau 100 (**c**, **d**) and 300 mmol/Tau 300 (**e**, **f**) glycine pretreatment on the course of severe taurocholate (3%) pancreatitis/Tau. **a**, **b** Areas of damaged pancreatic parenchyma without glycine treatment. Glycine treatment improved pathomorphological changes in taurocholate pancreatitis and noticeably reduced the Spormann score in the pretreated animals (**g**). Especially fat (**h**) and parenchyma (**i**), necrosis and edema (**j**) were significantly attenuated in glycine-pretreated animals. The amount of reactive ascites production was diminished by glycine pretreatment (**k**). Sham: retrograde infusion of an equivalent volume of NaCl into the common biliopancreatic duct. Data are presented as mean ± SEM. **a–f** ×100.

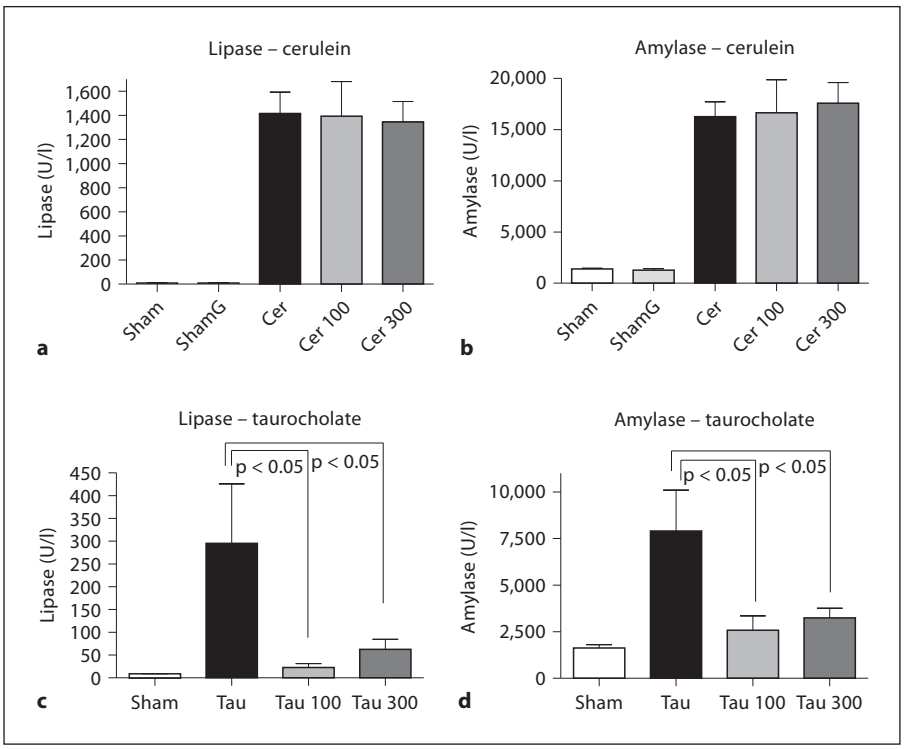

Fig. 4. Effect of glycine on serum pancreatitis markers. Glycine pretreatment did not influence lipase (**a**) and amylase levels (**b**) during the course of cerulein pancreatitis. In taurocholate pancreatitis, lipase (**c**) as well as amylase levels (**d**) were noticeably attenuated after 24 h of taurocholate pancreatitis. Data are presented as mean ± SEM.

Influence of Glycine on Leukocyte Transmigration into the Pancreas and Lung during Experimental Pancreatitis

To study whether tissue leukocyte infiltration is influenced by glycine pretreatment, we measured the myeloperoxidase (MPO) activity in pancreatic and lung tissue homogenates. The pancreatic MPO activity was not affected by glycine pretreatment in mild cerulein-induced pancreatitis, whereas in taurocholate-induced pancreatitis (300 ± 76), glycine treatment with 100 mmol (83 ± 20; $p < 0.01$) and 300 mmol (126 ± 30; $p < 0.05$) markedly reduced the pancreatic MPO activity (fig. 5a, b). Glycine pretreatment had no impact on the MPO activity in the lungs during severe taurocholate acute necrotizing pancreatitis (fig. 5c).

Glycine Ameliorates Pancreatic Cytokine Activation in Experimental Pancreatitis

To study whether glycine pretreatment can interfere in the release of the pro-inflammatory cytokines during the course of acute pancreatitis, IL-1, IL-6, TNFα and the anti-inflammatory cytokine IL-10 were measured via ELISA in pancreatic homogenates. In mild cerulein pancreatitis, none of the investigated cytokines were affected by glycine treatment (data not shown). But again in the severe taurocholate pancreatitis, the release of IL-1 and TNFα within the damaged pancreatic tissue was markedly reduced compared to the sham animals without glycine treatment (fig. 6a, c). IL-6 tended to be diminished in animals with glycine pretreatment, but this finding did not reach statistical significance (fig. 6b). Interestingly, also IL-10 was significantly reduced in the glycine-pretreated groups (fig. 6d).

Glycine Has No Impact on Pancreatic Pain Sensation in Acute Pancreatitis

Glycine receptors are currently considered to be new targets for pain therapies [35]. As abdominal pain represents the most common symptom in patients with acute necrotizing pancreatitis, potential effects of glycine pretreatment on analgesia were investigated in a standardized open-field test. The behavior was analyzed for 'straighten up' (vertical activity), 'crossing the lines' (inner rows) and 'cleaning' (horizontal activity) during the course of taurocholate pancreatitis at the time points 0, 12 and 24 h. In none of the time points did pretreatment with 100 or 300 mmol glycine have an analgesic effect on the taurocholate pancreatitis animals. Vertical activity tented to be increased in animals treated with 300 mmol glycine, but the results did not reach statistical significance (data not shown).

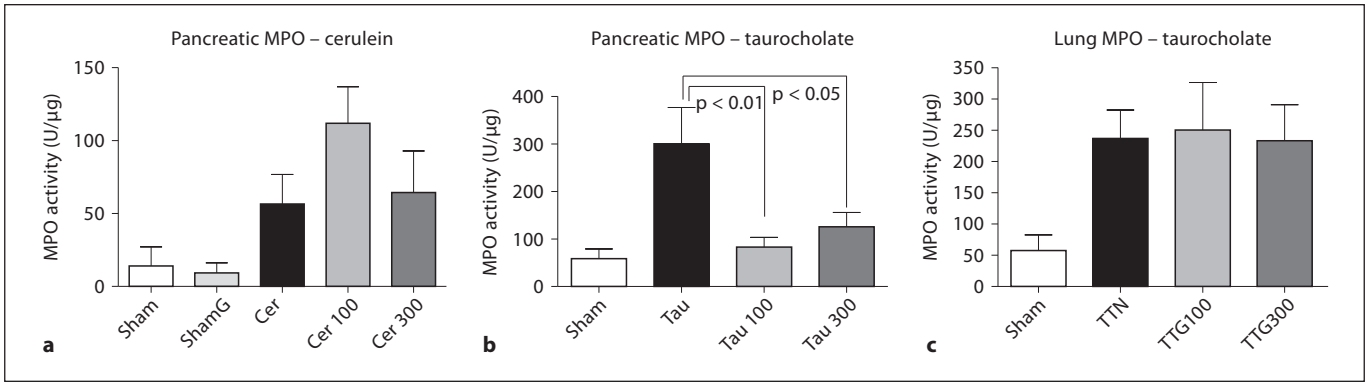

Fig. 5. Pancreatic MPO activity was not affected by glycine pretreatment in mild cerulein pancreatitis (**a**). On the contrary, pretreatment with 100 and 300 mmol glycine significantly reduced MPO activity in pancreatic tissue homogenates (**b**). MPO activity in lung tissue homogenates was not altered by glycine treatment (**c**). Data are presented as mean ± SEM.

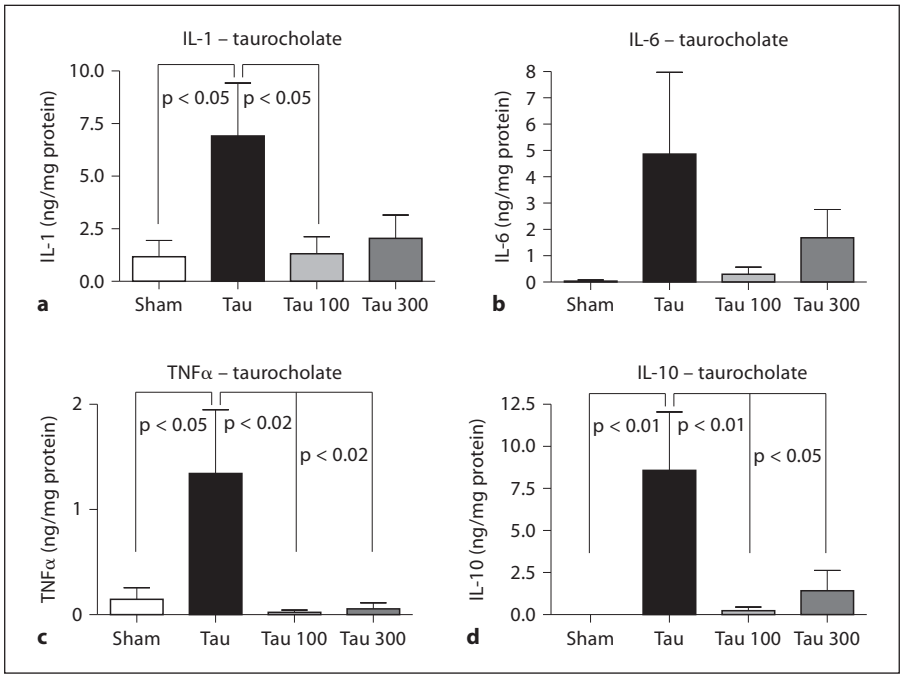

Fig. 6. Impact of glycine treatment on pancreatic cytokine activation in severe taurocholate pancreatitis: IL-1 (**a**), IL-6 (**b**), TNFα (**c**) and the anti-inflammatory cytokine IL-10 (**d**) were all decreased in the pancreas of glycine- (100 and 300 mmol) pretreated animals. Data are presented as mean ± SEM.

Discussion

To analyze the potential therapeutic impact of glycine on the course of acute pancreatitis, the cerulein-induced mild and the taurocholate-induced severe models of pancreatitis were chosen. Here, we could demonstrate that glycine significantly attenuated pancreatic tissue damage in acute pancreatitis when given prophylactically. Especially in severe taurocholate pancreatitis, glycine reduced pancreatic enzymes, cytokine production and MPO activity.

Considering the current knowledge on the pathophysiology of acute pancreatitis, there seems to be several point of action, which explains the protective effects of glycine in acute pancreatitis. First, as abnormal calcium influx is a crucial event in acute pancreatitis leading to premature zymogen activation, glycine might act via the glycine-gated chloride channels and cause a hyperpolarization of the acinar plasma membrane [36]. As a result, the opening of the voltage-gated calcium channels and a subsequent increase of intracellular calcium concentra-

tion would be prevented and hereby premature zymogen activation [37]. In this regard, our results showing decreased vacuole formation (which contain trypsinogen and cathepsin B) in the glycine pretreatment group seem to confirm this mechanism.

Another possibility is the stabilization of the acinar membrane by glycine. During acute pancreatitis, the conversion of trypsinogen into active trypsin within acinar cells is followed by an activation of several enzymes, such as elastase, phospholipase A_2 and NADPH oxidase [38]. Through the increase of intracellular calcium concentration, elastase and ROS (H_2O_2), NADPH oxidase and phospholipase A_2 are activated [39, 40]. Here, it should be considered that glycine inhibits phospholipase A_2, an event which leads to stabilization of the cell membrane [41]. As a result, the cell is less sensitive towards oxidative stress and radicals, which usually result in apoptosis and necrotic cell death. In accordance with this notion, we observed less parenchymal and fat tissue necrosis in the glycine-pretreated groups when compared to the control groups.

In addition to its potential direct protective effects on acinar cells, a major site of action for glycine would be the systemic inflammatory response. In acute pancreatitis, a striking inflammatory response and an activation of other inflammatory cascades like the kinin and complement pathways follows premature activation of enzymes [42]. Furthermore, the activation of the above-mentioned oxidizing enzymes increases the amount of oxygen radicals and thus oxidative stress. A glycine-mediated protection from oxidative stress was not observed on hepatocytes [43]. Nevertheless, the damage mediated by oxygen radicals results in increased NF-κB expression [44]. Subsequently, NF-κB activates the proinflammatory cytokine production within the pancreas, including the production of the TNFα [45], Il-6 [46] and IL-1β [47], platelet activating factor and leukotriene B_4 [48] which lead to enhanced recruitment of neutrophils, macrophages, and lymphocytes to the site of inflammation. These cells further produce cytokines like IL-1, IL-6, and IL-8 [49]. Since the glycine receptor GlyR is expressed on inflammatory cells [50], these cells might be another possible spot for glycine interference. In this context, glycine may be exerting an inhibitory action on these inflammatory cells as it otherwise does on neurons, or it may be rendering the leukocytes less sensitive to inflammatory stimuli, as previously shown for glycine-mediated endotoxin desensitization [51, 52]. In our study, we could show less inflammation and also decreased interleukin levels in the glycine pretreatment groups, e.g., IL-1 and TNFα were significantly decreased in severe pancreatitis. Interestingly, this inhibitory effect upon leukocyte activity and sequestration was mainly present within the pancreas but not in the lungs, as evidenced by the differences in the MPO activity of the control and pretreatment groups. The accompanying decrease in the anti-inflammatory cytokine IL-10 level is a finding in support of this protective effect of glycine since IL-10 is known to be increased in experimental acute pancreatitis in parallel with TNFα production [53]. However, the absence of any alterations in the pancreatic enzyme activation and the reduction of pro-inflammatory cytokines after glycine pretreatment strongly suggests that glycine is predominantly inhibitory on the systemic inflammatory response but not on the local damage in the pancreas. This specific action of glycine upon systemic but not local inflammation is not genuinely specific for glycine. In a previous study, it was demonstrated that also other agents, i.e. statins, also reduce systemic inflammatory cytokines but not pancreatic vascular permeability, tissue water content, histological lesions in the pancreas. This differential responsiveness of these two arms of mechanisms implies that combination therapies targeting at both the pancreatic and systemic inflammation may be more beneficial than therapies targeting either of the two.

At this point, it is difficult to explain why glycine exerted a more prominent inhibitory effect in the more severe form of taurocholate pancreatitis. The more prominent inhibition of cytokine production might be due to the fact that severe forms of pancreatitis are associated with a naturally higher degree of inflammatory cell recruitment. Indeed, neutrophils, as cytokine-secreting inflammatory cells, have been shown to determine the severity of the injury to the gland as well as the distant organs. Furthermore, a comparison of these models previously revealed that these two models are characterized by differences in origin and evolution of tissue injury. For example, in taurocholate pancreatitis, oxygen radicals produced by xanthine oxidase activity were shown to be probably induced by cell death, whereas in cerulein pancreatitis, other sources of oxygen free radicals, such as inflammatory cells, were considered to be more likely [54]. Overall, it is plausible that these two models may be marked by two different patterns of generation of inflammation and thus by different glycine responsiveness.

The lack of an analgesic effect for glycine in our study should not restrain researchers from studying its possibly analgesic effects in pancreatitis. In fact, most studies in the literature aiming at modulating glycine activity or its receptor involve chronic or neuropathic rather than acute pain conditions as seen in acute pancreatitis [55]. Hence,

models containing longer-lasting acute pancreatitis symptoms or experimental models of chronic pancreatitis may certainly be more suitable to discover a possible analgesic role for glycine in pancreatic disease.

Finally, it is yet to be determined if the anti-inflammatory effect of glycine we observed in these models may be related to an interference with neurogenic inflammation. Overall, it is conceivable that glycine might be inhibiting the activated sensory neurons due to its main attribute as an inhibitory neurotransmitter. However, it should be considered that this inhibition is less likely to be a direct inhibition since dorsal root ganglia neurons (DRG) that receive the afferent signals from visceral organs normally do not express glycine receptors [56], although they were shown to express its beta subunit [57]. However, it is possible that glycine inhibits spinal or brainstem neurons that express the GlyR and possibly get involved in the modulation of pain signals received from the periphery [58]. Overall, it remains to be determined if GlyR can be upregulated in DRG and/or in the central nervous system in acute pancreatitis and thus neurogenic inflammation indeed represents a definite target for exogenous glycine.

In summary, this study shows for the first time the protective effect of amino acid glycine in both mild and severe experimental acute pancreatitis. It seems that glycine can exert its anti-inflammatory, cytoprotective action via several mechanisms, through pancreatic acinar membrane stabilization, via its direct inhibitory effect on inflammatory cells and/or due to an interference with the neurogenic inflammation in pancreatitis. Future studies should, therefore, shed light on the exact pathomechanism of glycine's promising protective action and the possible use of glycine as a therapeutic agent in acute pancreatitis.

Acknowledgement

This work is part of Anna-Katharina Timm's MD thesis.

Disclosure Statement

No conflicts of interest are declared by the author(s).

References

1 Bhatia M, Brady M, Shokuhi S, Christmas S, Neoptolemos JP, Slavin J: Inflammatory mediators in acute pancreatitis. J Pathol 2000; 190:117–125.

2 Saluja AK, Bhagat L, Lee HS, Bhatia M, Frossard JL, Steer ML: Secretagogue-induced digestive enzyme activation and cell injury in rat pancreatic acini. Am J Physiol 1999;276:G835–G842.

3 Lupia E, Goffi A, De Giuli P, Azzolino O, Bosco O, Patrucco E, Vivaldo MC, Ricca M, Wymann MP, Hirsch E, Montrucchio G, Emanuelli G: Ablation of phosphoinositide 3-kinase-gamma reduces the severity of acute pancreatitis. Am J Pathol 2004;165: 2003–2011.

4 Norman J: The role of cytokines in the pathogenesis of acute pancreatitis. Am J Surg 1998;175:76–83.

5 McKay CJ, Gallagher G, Brooks B, Imrie CW, Baxter JN: Increased monocyte cytokine production in association with systemic complications in acute pancreatitis. Br J Surg 1996;83:919–923.

6 Sandoval D, Gukovskaya A, Reavey P, Gukovsky S, Sisk A, Braquet P, Pandol SJ, Poucell-Hatton S: The role of neutrophils and platelet-activating factor in mediating experimental pancreatitis. Gastroenterology 1996;111:1081–1091.

7 Frossard JL, Saluja A, Bhagat L, Lee HS, Bhatia M, Hofbauer B, Steer ML: The role of intercellular adhesion molecule 1 and neutrophils in acute pancreatitis and pancreatitis-associated lung injury. Gastroenterology 1999;116:694–701.

8 Bhatia M, Saluja AK, Hofbauer B, Lee HS, Frossard JL, Steer ML: The effects of neutrophil depletion on a completely noninvasive model of acute pancreatitis-associated lung injury. Int J Pancreatol 1998;24:77–83.

9 Kyriakides C, Jasleen J, Wang Y, Moore FD Jr, Ashley SW, Hechtman HB: Neutrophils, not complement, mediate the mortality of experimental hemorrhagic pancreatitis. Pancreas 2001;22:40–46.

10 Demols A, Le MO, Desalle F, Quertinmont E, Van Laethem JL, Deviere J: CD4(+)T cells play an important role in acute experimental pancreatitis in mice. Gastroenterology 2000; 118:582–590.

11 Mayer J, Laine VJ, Rau B, Hotz HG, Foitzik T, Nevalainen TJ, Beger HG: Systemic lymphocyte activation modulates the severity of diet-induced acute pancreatitis in mice. Pancreas 1999;19:62–68.

12 Yang J, Denham W, Tracey KJ, Wang H, Kramer AA, Salhab KF, Norman J: The physiologic consequences of macrophage pacification during severe acute pancreatitis. Shock 1998;10:169–175.

13 Mikami Y, Takeda K, Shibuya K, Qiu-Feng H, Shimamura H, Yamauchi J, Egawa S, Sunamura M, Yagi H, Endo Y, Matsuno S: Do peritoneal macrophages play an essential role in the progression of acute pancreatitis in rats? Pancreas 2003;27:253–260.

14 Shifrin AL, Chirmule N, Zhang Y, Raper SE: Macrophage ablation attenuates adenoviral vector-induced pancreatitis. Surgery 2005; 137:545–551.

15 Liddle RA, Nathan JD: Neurogenic inflammation and pancreatitis. Pancreatology 2004;4:551–559.

16 Nathan JD, Patel AA, McVey DC, Thomas JE, Prpic V, Vigna SR, Liddle RA: Capsaicin vanilloid receptor-1 mediates substance P release in experimental pancreatitis. Am J Physiol Gastrointest Liver Physiol 2001; 281:G1322–G1328.

17 Colquhoun D, Sivilotti LG: Function and structure in glycine receptors and some of their relatives. Trends Neurosci 2004;27: 337–344.

18 Hijioka T, Rosenberg RL, Lemasters JJ, Thurman RG: Kupffer cells contain voltage-dependent calcium channels. Mol Pharmacol 1992;41:435–440.

19 Wheeler M, Stachlewitz RF, Yamashina S, Ikejima K, Morrow AL, Thurman RG: Glycine-gated chloride channels in neutrophils attenuate calcium influx and superoxide production. FASEB J 2000;14:476–484.

20 Wheeler MD, Thurman RG: Production of superoxide and TNF-alpha from alveolar macrophages is blunted by glycine. Am J Physiol 1999;277:L952–L959.
21 Lee MA, McCauley RD, Kong SE, Hall JC: Pretreatment with glycine reduces the severity of warm intestinal ischemic-reperfusion injury in the rat. Ann Plast Surg 2001;46: 320–326.
22 Thurman RG, Schemmer P, Zhong Z, Bunzendahl H, von Frankenberg M, Lemasters JJ: Kupffer cell-dependent reperfusion injury in liver transplantation: new clinically relevant use of glycine. Langenbecks Arch Chir Suppl Kongressbd 1998;115:185–190.
23 Chensue SW, Terebuh PD, Remick DG, Scales WE, Kunkel SL: In vivo biologic and immunohistochemical analysis of interleukin-1 alpha, beta and tumor necrosis factor during experimental endotoxemia: kinetics, Kupffer cell expression, and glucocorticoid effects. Am J Pathol 1991;138:395–402.
24 Schemmer P, Bradford BU, Rose ML, Bunzendahl H, Raleigh JA, Lemasters JJ, Thurman RG: Intravenous glycine improves survival in rat liver transplantation. Am J Physiol Gastrointest Liver Physiol 1999;276:G924–G932.
25 Schemmer P, Golling M, Kraus T, Mehrabi A, Mayatepek E, Herfarth C, Klar E: Extended experience with glycine for prevention of reperfusion injury after human liver transplantation. Transplant Proc 2002;34:2307–2309.
26 Bachmann S, Peng XX, Currin RT, Thurman RG, Lemasters JJ: Glycine in Carolina rinse solution reduces reperfusion injury, improves graft function, and increases graft survival after rat liver transplantation. Transplant Proc 1995;27:741–742.
27 Zhong Z, Wheeler MD, Li X, Froh M, Schemmer P, Yin M, Bunzendaul H, Bradford B, Lemasters JJ: L-Glycine: a novel anti-inflammatory, immunomodulatory, and cytoprotective agent. Curr Opin Clin Nutr Metab Care 2003;6:229–240.
28 Senthilkumar R, Viswanathan P, Nalini N: Effect of glycine on oxidative stress in rats with alcohol induced liver injury. Pharmazie 2004;59:55–60.
29 Martignoni ME, Ceyhan GO, Ayuni E, Kondo Y, Zimmermann A, Büchler MW, Friess H: Endothelin receptor antagonists are not beneficial in the therapy of acute experimental pancreatitis. Langenbecks Arch Surg 2004;389:184–192.
30 Martignoni ME, Ceyhan GO, Ayuni E, Kondo Y, Zimmermann A, Büchler MW, Friess H: Endothelin receptor antagonists are not beneficial in the therapy of acute experimental pancreatitis. Langenbecks Arch Surg 2004;389:184–192.
31 Spormann H, Sokolowski A, Letko G: Effect of temporary ischemia upon development and histological patterns of acute pancreatitis in the rat. Pathol Res Pract 1989;184:507–513.
32 Mayerle J, Schnekenburger J, Kruger B, Kellermann J, Ruthenburger M, Weiss FU, Nalli A, Domschke W, Lerch MM: Extracellular cleavage of E-cadherin by leukocyte elastase during acute experimental pancreatitis in rats. Gastroenterology 2005;129:1251–1267.
33 Koninger J, Balaz P, Wagner M, Shi X, Cima I, Zimmermann A, Di Sebastiano P, Büchler MW, Friess H: Phosphatidylserine receptor in chronic pancreatitis: evidence for a macrophage independent role. Ann Surg 2005; 241:144–151.
34 Michalski CW, Laukert T, Sauliunaite D, Pacher P, Bergmann F, Agarwal N, Su Y, Giese T, Giese NA, Batkai S, Friess H, Kuner R: Cannabinoids ameliorate pain and reduce disease pathology in cerulein-induced acute pancreatitis. Gastroenterology 2007;132:1968–1978.
35 Kirsch J: Glycinergic transmission. Cell Tissue Res 2006;326:535–540.
36 Gundersen RY, Vaagenes P, Breivik T, Fonnum F, Opstad PK: Glycine – an important neurotransmitter and cytoprotective agent. Acta Anaesthesiol Scand 2005;49:1108–1116.
37 Wheeler M, Stachlewitz RF, Yamashina S, Ikejima K, Morrow AL, Thurman RG: Glycine-gated chloride channels in neutrophils attenuate calcium influx and superoxide production. FASEB J 2000;14:476–484.
38 Frossard JL, Hadengue A: Acute pancreatitis: new physiopathological concepts. Gastroenterol Clin Biol 2001;25:164–176.
39 Gukovskaya AS, Vaquero E, Zaninovic V, Gorelick FS, Lusis AJ, Brennan ML, Holland S, Pandol SJ: Neutrophils and NADPH oxidase mediate intrapancreatic trypsin activation in murine experimental acute pancreatitis. Gastroenterology 2002;122:974–984.
40 Mayer JM, Rau B, Siech M, Beger HG: Local and systemic zymogen activation in human acute pancreatitis. Digestion 2000;62:164–170.
41 Schilling M, den Butter G, Marsh DC, Belzer FO, Southard JH: Glycine inhibits phospholipolysis of hypoxic membranes. Transplant Med 1994;6:140–143.
42 Elfar M, Gaber LW, Sabek O, Fischer CP, Gaber AO: The inflammatory cascade in acute pancreatitis: relevance to clinical disease. Surg Clin North Am 2007;87:1325–1340, vii.
43 Marsh DC, Vreugdenhil PK, Mack VE, Belzer FO, Southard JH: Glycine protects hepatocytes from injury caused by anoxia, cold ischemia and mitochondrial inhibitors, but not injury caused by calcium ionophores or oxidative stress. Hepatology 1993;17:91–98.
44 Algul H, Tando Y, Beil M, Weber CK, Von WC, Schneider G, Adler G, Schmid RM: Different modes of NF-kappaB/Rel activation in pancreatic lobules. Am J Physiol Gastrointest Liver Physiol 2002;283:G270–G281.
45 Gukovskaya AS, Gukovsky I, Zaninovic V, Song M, Sandoval D, Gukovsky S, Pandol SJ: Pancreatic acinar cells produce, release, and respond to tumor necrosis factor-alpha: role in regulating cell death and pancreatitis. J Clin Invest 1997;100:1853–1862.
46 Norman JG, Fink GW, Franz MG: Acute pancreatitis induces intrapancreatic tumor necrosis factor gene expression. Arch Surg 1995;130:966–970.
47 Vonlaufen A, Apte M, Imhof B, Frossard J: The role of inflammatory and parenchymal cells in acute pancreatitis. J Pathol 2007;213: 239–248.
48 Hotter G, Closa D, Prats N, Pi F, Gelpi E, Rosello-Catafau J: Free radical enhancement promotes leucocyte recruitment through a PAF and LTB4 dependent mechanism. Free Radic Biol Med 1997;22:947–954.
49 Lupia E, Goffi A, De Giuli P, Azzolino O, Bosco O, Patrucco E, Vivaldo MC, Ricca M, Wymann MP, Hirsch E, Montrucchio G, Emanuelli G: Ablation of phosphoinositide 3-kinase-gamma reduces the severity of acute pancreatitis. Am J Pathol 2004;165: 2003–2011.
50 Froh M, Thurman RG, Wheeler MD: Molecular evidence for a glycine-gated chloride channel in macrophages and leukocytes. Am J Physiol Gastrointest Liver Physiol 2002; 283:G856–G863.
51 Wheeler MD, Ikejema K, Enomoto N, Stacklewitz RF, Seabra V, Zhong Z, Yin M, Schemmer P, Rose ML, Rusyn I, Bradford B, Thurman RG: Glycine: a new anti-inflammatory immunonutrient. Cell Mol Life Sci 1999;56: 843–856.
52 Wheeler MD, Rose ML, Yamashima S, Enomoto N, Seabra V, Madren J, Thurman RG: Dietary glycine blunts lung inflammatory cell influx following acute endotoxin. Am J Physiol Lung Cell Mol Physiol 2000; 279:L390–L398.
53 Van Laethem JL, Eskinazi R, Louis H, Rickaert F, Robberecht P, Deviere J: Multisystemic production of interleukin 10 limits the severity of acute pancreatitis in mice. Gut 1998; 43:408–413.
54 Closa D, Bulbena O, Hotter G, Rosello-Catafau J, Fernandez-Cruz L, Gelpi E: Xanthine oxidase activation in cerulein- and taurocholate-induced acute pancreatitis in rats. Arch Int Physiol Biochim Biophys 1994;102: 167–170.
55 Zeilhofer HU: Loss of glycinergic and GABAergic inhibition in chronic pain – contributions of inflammation and microglia. Int Immunopharmacol 2008;8:182–187.
56 Kung AY, Rick C, O'Shea S, Harrison NL, McGehee DS: Expression of glycine receptors in rat sensory neurons vs. HEK293 cells yields different functional properties. Neurosci Lett 2001;309:202–206.
57 Furuyama T, Sato M, Sato K, Araki T, Inagaki S, Takagi H, Tohyama M: Co-expression of glycine receptor beta subunit and GABAA receptor gamma subunit mRNA in the rat dorsal root ganglion cells. Brain Res Mol Brain Res 1992;12:335–338.
58 Zafra F, Aragon C, Gimenez C: Molecular biology of glycinergic neurotransmission. Mol Neurobiol 1997;14:117–142.