

**PATHOGENESIS OF BASIC AMINO ACID-
INDUCED EXPERIMENTAL ACUTE
PANCREATITIS: THE ROLE OF UREA CYCLE
METABOLITES AND POLYAMINES**

Ph.D. Thesis

György Biczó

First Department of Medicine

University of Szeged

Szeged

2010

TABLE OF CONTENTS

LIST OF FULL PAPERS CITED IN THE THESIS.....	III
ABBREVIATIONS	IV
SUMMARY	V
1 INTRODUCTION	1
2 MATERIALS AND METHODS.....	5
2.1 Materials.....	5
2.2 Methods	5
2.2.1 <i>Animals</i>	<i>5</i>
2.2.2 <i>Experimental Protocols</i>	<i>5</i>
2.2.2.1 <i>Pilot Study with the Main Direct Downstream Metabolites of L-arginine.....</i>	<i>5</i>
2.2.2.2 <i>Dose–response and Time Course Changes of L-ornithine Injection.....</i>	<i>5</i>
2.2.2.3 <i>Effect of Irreversible Arginase Inhibition on L-arginine-induced Pancreatitis .</i>	<i>6</i>
2.2.2.4 <i>Characterization of Polyamine Homeostasis in L-ornithine-induced Acute Pancreatitis.....</i>	<i>6</i>
2.2.2.5 <i>Effect of 1-methylspermidine on L-ornithine-induced Acute Pancreatitis</i>	<i>6</i>
2.2.2.6 <i>Anaesthesia and Preparation of Tissue Samples.....</i>	<i>6</i>
2.2.3 <i>Assays.....</i>	<i>7</i>
2.2.3.1 <i>Pancreatic Weight/Body Weight Ratio</i>	<i>7</i>
2.2.3.2 <i>Serum, Pancreatic and Ascitic Amylase Activity, Serum Lipase Activity, Serum Aspartate Aminotransferase Activity, and Concentrations of Glucose, Calcium, Triglyceride, Urea, Creatinine, Arginine, Ornithine, and Citrulline</i>	<i>7</i>
2.2.3.3 <i>Pancreatic Trypsin Activity</i>	<i>7</i>
2.2.3.4 <i>Arginase Activity.....</i>	<i>7</i>
2.2.3.5 <i>Polyamine Levels and Activities of Spermidine/Spermine N¹-Acetyltransferase and Ornithine Decarboxylase.....</i>	<i>8</i>
2.2.3.6 <i>Pancreatic Myeloperoxidase Activity</i>	<i>8</i>
2.2.3.7 <i>Expression of Pancreatic HSP72, HSP27, IκB-α, and IκB-β.....</i>	<i>8</i>
2.2.3.8 <i>Pancreatic Interleukin-1β Concentrations</i>	<i>8</i>
2.2.3.9 <i>Pancreatic Nonprotein Sulfhydryl Group Content and the Activities of Glutathione Peroxidase and Superoxide Dismutase</i>	<i>9</i>
2.2.4 <i>Detection of Apoptosis in the Pancreas.....</i>	<i>9</i>
2.2.4.1 <i>Pancreatic Genomic DNA Analysis.....</i>	<i>9</i>
2.2.4.2 <i>TdT-mediated dUTP Nick End-labeling Technique.....</i>	<i>9</i>
2.2.4.3 <i>Microscopy.....</i>	<i>10</i>
2.2.5 <i>Histologic Examination (Light Microscopy)</i>	<i>10</i>
2.2.6 <i>Statistical Analysis.....</i>	<i>10</i>
3 RESULTS.....	11
3.1 <i>In Vivo</i> Effects of Large Doses of the Main Direct Metabolites of L-arginine	11
3.2 Time Course Changes of Serum Arginine, Citrulline, and Ornithine Concentrations in Rats Injected Intraperitoneally with 3.5 g/kg L-arginine	11
3.3 Dose–response of Intraperitoneal Injection of L-ornithine.....	11

3.4	Time Course Studies After Intraperitoneal Injection of Rats with 3 g/kg L-ornithine....	14
3.4.1	<i>Macroscopic Observations</i>	14
3.4.2	<i>Histologic Examination of the Pancreas</i>	14
3.4.3	<i>Confirmation of Pancreatic Apoptosis Observed on Histologic Examination.....</i>	17
3.4.3.1	<i>Genomic DNA Analysis</i>	17
3.4.3.2	<i>TUNEL Technique</i>	17
3.4.4	<i>Activities of Serum, Pancreatic, and Ascitic Amylase</i>	18
3.4.5	<i>Pancreatic Trypsin Activity</i>	18
3.4.6	<i>Pancreatic Myeloperoxidase Activity</i>	18
3.4.7	<i>Induction of Pancreatic HSP72 Synthesis</i>	18
3.4.8	<i>Degradation of Pancreatic IκB-α and IκB-β and Induction of Interleukin-1β Synthesis.....</i>	18
3.4.9	<i>Pancreatic Nonprotein Sulfhydryl Group Content and the Activities of Glutathione Peroxidase and Superoxide Dismutase</i>	21
3.4.10	<i>Body Weight and Pancreatic Weight/Body Weight Ratio.....</i>	22
3.4.11	<i>Serum Aspartate Aminotransferase Activity and Concentrations of Glucose, Calcium, Triglyceride, Urea, and Creatinine.....</i>	23
3.5	Arginase Activity in Different Tissues and <i>In Vitro</i> Effect of AIHA on Arginase Activity.....	23
3.5.1	<i>Arginase Activity in Different Tissues</i>	23
3.5.2	<i>In Vitro Effect of AIHA on Arginase Activity.....</i>	23
3.6	Effect of AIHA on L-arginine-Induced Acute Pancreatitis in Rats	24
3.6.1	<i>Pancreatic Weight/Body Weight Ratio, Serum and Pancreatic Amylase Activity, and Pancreatic Trypsin and Myeloperoxidase Activities</i>	24
3.6.2	<i>Pancreatic Nonprotein Sulfhydryl Group Content and the Activities of Glutathione Peroxidase and Superoxide Dismutase</i>	26
3.6.3	<i>Pancreatic Heat Shock Protein Expression.....</i>	27
3.6.4	<i>Histological Examination</i>	27
3.7	Polyamine Homeostasis in L-ornithine-induced Acute Pancreatitis.....	28
3.7.1	<i>Pancreas</i>	28
3.7.2	<i>Liver.....</i>	29
3.7.3	<i>Lung</i>	29
3.8	Effect of the Synthetic Polyamine Analogue 1-methylspermidine on L-ornithine-induced Acute Pancreatitis	31
3.8.1	<i>Pancreatic SSAT Activity, Putrescine, Spermidine and Spermine Content.....</i>	31
3.8.2	<i>Histological Examination</i>	32
3.8.3	<i>Serum and Pancreatic Amylase Activities, Serum Lipase Activity, and Pancreatic Weight/Body Weight Ratio.....</i>	33
3.8.4	<i>Pancreatic HSP72 and IκB-α Expression.....</i>	33
3.8.5	<i>Pancreatic Myeloperoxidase Activity, Pancreatic Interleukin-1β Levels and Serum Concentrations of Creatinine and Aspartate Aminotransferase Activity..</i>	35
4	DISCUSSION.....	36
5	ACKNOWLEDGEMENTS.....	42
6	REFERENCES	43
7	ANNEX.....	50

LIST OF FULL PAPERS CITED IN THE THESIS

- I. Rakonczay Jr Z, Hegyi P, Dósa S, Iványi B, Jármay K, **Biczó G**, Hracskó Z, Varga IS, Karg E, Kaszaki J, Varró A, Lonovics J, Boros I, Gukovsky I, Gukovskaya AS, Pandol SJ, Takács T. A new severe acute necrotizing pancreatitis model induced by L-ornithine in rats. *Crit Care Med* 2008;36:2117-2127.
IF₂₀₀₈: 6.594
- II. **Biczó G**, Hegyi P, Berczi S, Dósa S, Hracskó Z, Varga IS, Iványi B, Venglovecz V, Wittmann T, Takács T, Rakonczay Jr Z. Inhibition of arginase activity ameliorates L-arginine-induced acute pancreatitis in rats. *Pancreas* 2010 (in press).
IF₂₀₀₉: 2.733
- III. **Biczó G**, Hegyi P, Sinervirta R, Berczi S, Dósa S, Siska A, Iványi B, Venglovecz V, Takács T, Alhonen L, Rakonczay Jr Z. Characterization of polyamine homeostasis in L-ornithine-induced acute pancreatitis in rats. *Pancreas* 2010 (Epub).
IF₂₀₀₉: 2.733

LIST OF FULL PAPERS RELATED TO THE SUBJECT OF THE THESIS

- IV. Szabolcs A, **Biczó G**, Rakonczay Jr Z, Tiszlavicz L, Halm G, Wittmann T, Takács T. Simultaneous proteasome inhibition and heat shock protein induction by bortezomib is beneficial in experimental pancreatitis. *Eur J Pharmacol* 2009;616:270-274.
IF₂₀₀₉: 2.585
- V. Takács T, Szabolcs A, **Biczó G**, Hegyi P, Rakonczay Jr Z. [The clinical relevance of experimental acute pancreatitis models]. *Orv Hetil* 2008;149:1981-1986. [Article in Hungarian]
IF₂₀₀₈: 0.0

Number of full publications:	5
Cumulative impact factor:	14.645

ABBREVIATIONS

AIHA	(+)-S-2-amino-6-iodoacetamidohexanoic acid
Arg	L-arginine
ASAT	aspartate aminotransferase
Citr	L-citrulline
DcSAM	decarboxylated SAM
GSH-Px	glutathione peroxidase
HSP	heat shock protein
IL-1α	interleukin-1 α
IP	intraperitoneal(ly)
IκB	inhibitor of kappa B
MeSpd	1-methylspermidine
MPO	myeloperoxidase
NF-κB	nuclear factor kappa B
NO	nitric oxide
NOS	nitric oxide synthase
NSG	nonprotein sulfhydryl group
ODC	ornithine decarboxylase
Orn	L-ornithine
p.w./b.w.	pancreatic weight/body weight ratio
PAO	polyamine oxidase
PS	physiological saline
SAM	S-adenosylmethionine
SAMDC	S-adenosylmethionine decarboxylase
SEM	standard error of the mean
SOD	superoxide dismutase
SSAT	spermidine/spermine N ¹ -acetyltransferase
TE	Tris-EDTA
TUNEL	TdT-mediated dUTP nick end-labeling

SUMMARY

Background & Aims. Acute pancreatitis is a sudden inflammatory disorder of the pancreas, the pathomechanism of which is not well understood. Although the overall mortality of patients with acute pancreatitis is approximately 5%, a great proportion of deaths is the result of the necrotizing form of the disease. Due to the rapid course of the disease and relative inaccessibility of human pancreatic tissue, a number of animal models have been developed to study the pathomechanism and to test possible treatment options. One noninvasive, reproducible model of necrotizing pancreatitis is the L-arginine-induced model. Its exact pathomechanism is unknown, therefore: **A)** We set out to determine whether the main metabolites of L-arginine (L-ornithine, L-citrulline, and nitric oxide) induce pancreatitis in rats. Since large doses of intraperitoneally (IP) injected L-ornithine induced severe acute necrotizing pancreatitis, we characterized the dose–response and time course changes of L-ornithine administration. **B)** L-arginine injection (3.5 g/kg, IP) resulted in much greater increases in serum ornithine versus citrulline concentration in rats. These data indicate a major role of arginase in the catabolism of L-arginine. Therefore, we tested the effects of the irreversible arginase inhibitor (+)-S-2-amino-6-iodoacetamidohexanoic acid (AIHA) on L-arginine-induced acute pancreatitis. **C)** L-ornithine is a precursor of polyamine biosynthesis, therefore, we investigated changes in pancreatic and extrapancreatic polyamine homeostasis after injection of L-ornithine. As we observed pancreatic spermidine catabolism in rats with L-ornithine-induced pancreatitis, we tested the effects of the metabolically stable polyamine analogue 1-methylspermidine (MeSpd) on L-ornithine-induced pancreatitis.

Methods. **A)** Male Wistar weighing 180-250 g were injected with L-ornithine, L-citrulline and/or sodium nitroprusside (nitric oxide donor) IP. Dose-response and time-course changes of laboratory and histological parameters of pancreatitis were determined. **B)** The inhibitory effect of AIHA on arginase activity was tested *in vitro* and *in vivo*. Fifteen mg/kg AIHA or its vehicle were administered IP 1 hour before the injection of physiological saline (PS) or 3.5 g/kg L-arginine IP. Laboratory and histological parameters of pancreatitis were determined 24 hours after the injection of PS or L-arginine. **C)** Rats were injected IP with 3 g/kg L-ornithine and/or PS and were untreated, pretreated or treated with 50 mg/kg MeSpd IP. The severity of pancreatitis was assessed by measuring standard laboratory and histological parameters.

Results. Intraperitoneal injection of 3 g/kg L-ornithine but not L-citrulline or sodium nitroprusside caused severe acute pancreatitis; 4 to 6 g/kg L-ornithine killed the animals within hours. Serum and ascitic amylase activities were significantly increased, whereas pancreatic amylase activity was decreased after IP injection of 3 g/kg L-ornithine. Morphologic examination of the pancreas showed massive interstitial edema, apoptosis, and necrosis of acinar cells and infiltration of neutrophil granulocytes and monocytes after 3 g/kg L-ornithine injection. One month after L-ornithine injection, the pancreas appeared almost normal; the destructed parenchyma was partly replaced by fat. L-ornithine concentration in the blood were increased 54-fold after IP administration of L-arginine. Sixty micromolars AIHA significantly inhibited arginase activity *in vitro* by about 25%. Pretreatment with AIHA significantly ameliorated pancreatic damage caused by L-arginine administration. It decreased pancreatic weight/body weight ratio, pancreatic glutathione peroxidase and myeloperoxidase activities, and histological damage. Administration of AIHA in itself significantly increased levels of pancreatic heat shock proteins. Injection of large doses of L-ornithine paradoxically induced pancreatic spermidine catabolism, possibly via activation of spermidine/spermine N¹-acetyltransferase (SSAT), after appearance of the first histological signs of acute pancreatitis. Polyamine levels generally increased in the lung and liver with the exception of lung spermidine levels, which decreased. 1-methylspermidine administration did not influence pancreatic polyamine levels and SSAT activity and failed to ameliorate the severity of L-ornithine-induced pancreatitis.

Conclusions. In conclusion, we have developed a simple, noninvasive, reproducible model of acute necrotizing pancreatitis in rats by IP injection of 3 g/kg L-ornithine and examined its time course. Large doses of L-arginine may also produce a toxic effect on the pancreas, at least in part, through L-ornithine. Pretreatment with AIHA reduced the severity of L-arginine-induced pancreatitis most likely by inhibiting arginase activity. L-ornithine-induced pancreatitis was associated with activation of pancreatic polyamine catabolism. However, administration of MeSpd did not affect disease severity. Further studies are needed to determine how basic amino acids produce pancreatic injury and to reveal the exact role of polyamine homeostatic processes in the maintenance of pancreatic integrity.

1 INTRODUCTION

Acute pancreatitis is an inflammatory disease of the pancreas, the severity of which varies widely from mild to severe (approximately 85% vs 15%, respectively). Its overall mortality is nearly 5%, however, it is approximately six times higher in patients with severe necrotizing pancreatitis compared with those with mild interstitial pancreatitis.^[1-4] Specific therapy for acute pancreatitis is not yet available which is partly due to the fact that the pathogenesis of acute pancreatitis is not well understood.^[2, 5]

The rapid course of the disease and relative inaccessibility of human pancreatic tissue emphasize the importance of animal models in studying the pathomechanism of acute pancreatitis.^[6-11] Many experimental acute pancreatitis models have been developed, however, the most thoroughly characterized and widely used is the secretagogue hyperstimulation model in rodents. The current dogmas and paradigms concerning the early events of acute pancreatitis basically arises from the investigation of the cholecystokinin/cerulein-induced model.^[3] Premature activation of trypsinogen and other zymogens^[12-15], as well as activation of the proinflammatory transcription factor nuclear factor- κ B (NF- κ B)^[16-20] and subsequent induction of cytokine synthesis^[21-26] have been considered as key early events in acute pancreatitis. The fact that the earliest molecular processes that lead to acinar cell injury cannot be investigated in humans underline the importance of investigation of the pathomechanism of acute pancreatitis in animal models.

In practice, most of the experimental models require invasive intervention (e.g. the retrograde ductal injection of bile acids and the closed duodenal loop method^[27-28]), or pancreatic damage is not that reproducible or homogeneous in distribution (like in choline-deficient ethionine-supplemented diet in female mice^[29]). However, the noninvasive nature, reproducibility, and homogeneity (e.g. the cholecystokinin/cerulein pancreatitis model^[30]) are preferential in an experimental setup. Intraperitoneal (IP) injection of large doses of L-arginine induce severe acute necrotizing pancreatitis in rats and mice, and this model fulfills the preferred criteria.^[31-34] Therefore, its use is becoming more popular with researchers investigating this challenging disease.

The pathomechanism of L-arginine-induced pancreatitis is unknown, especially concerning the early events leading to the disease.^[33] Changes in the levels of free radicals, including nitric oxide (NO), inflammatory mediators (NF- κ B, interleukins), and polyamines,

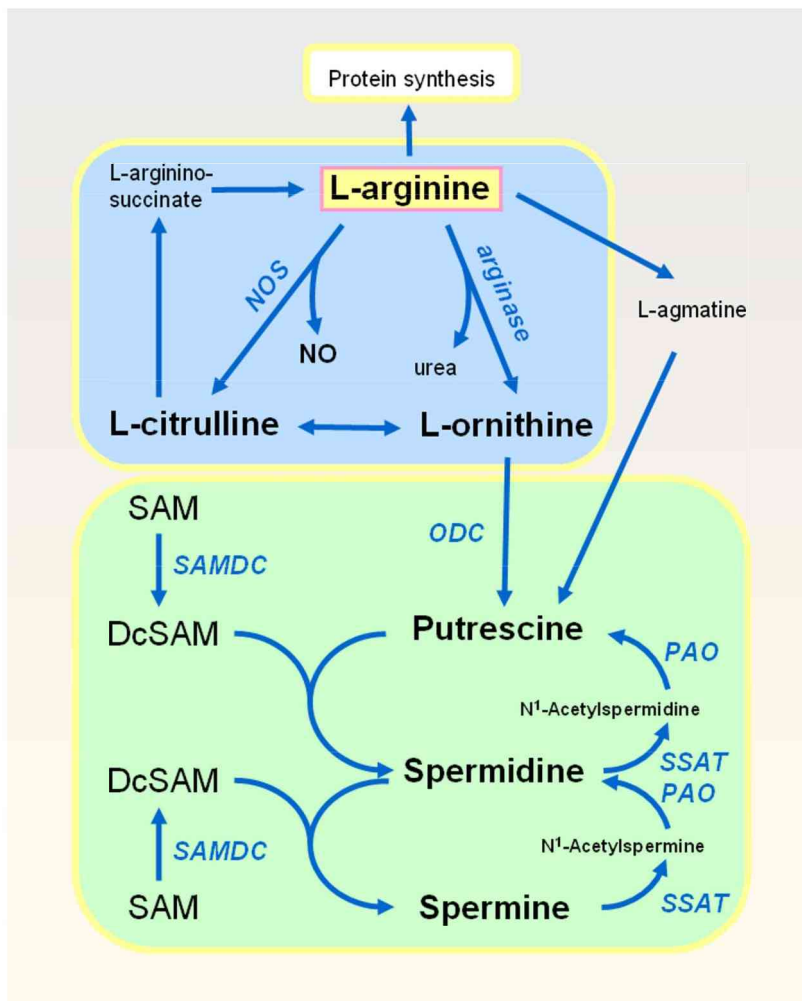


Figure 1. Main metabolic pathways of L-arginine. The main reactions involved in the metabolism of L-arginine are part of the urea cycle (highlighted in the blue textbox) and the polyamine biosynthesis (highlighted in the green textbox)

DcSAM: decarboxylated SAM; NO: nitric oxide; NOS: nitric oxide synthase; ODC: ornithine decarboxylase; PAO: polyamine oxidase; SAM: S-adenosylmethionine SAMDC: SAM decarboxylase; SSAT: spermidine/spermine N¹-acetyltransferase

as well as endoplasmic reticulum stress have all been thought to play critical role in the onset of this experimental pancreatitis.^[35-38] L-arginine can be metabolized via a number of different pathways (Fig. 1). Two key reactions that are involved in the catabolism of L-arginine are part of the urea cycle.^[39] Nitric oxide synthase (NOS) catalyzes the conversion of L-arginine to NO and L-citrulline. NOS has three isoforms: the constitutive endothelial and neuronal and an inducible form (iNOS). The other key enzyme arginase has two isoforms which hydrolyze L-arginine to L-ornithine and urea. The two arginase isoforms (types I and II) are

expressed from two different loci and show different molecular properties, tissue distribution, subcellular location, and regulation of expression. Arginase I is a cytosolic enzyme and is highly expressed together with other urea cycle enzymes in the liver and to a much less extent, without any other urea cycle enzymes, in a few other tissues. In contrast, arginase II is expressed in the mitochondria, and is widely distributed in extrahepatic tissues. The putative function of arginase II is to regulate the L-arginine/L-ornithine ratio in different tissues where it is always expressed without other urea cycle enzymes.

Polyamines (putrescine, spermidine, and spermine) are downstream metabolites of L-arginine (Fig. 1). Their tightly controlled cellular homeostasis is required for optimal growth and function of cells. Depletion of cellular polyamine levels has been associated with inhibition of growth and programmed cell death.^[40] Stabilization of DNA by polycationic polyamines has been long recognized, however, determinations of their exact physiological functions remain to be proven.^[41-42] Polyamine biosynthesis is regulated by the activities of ornithine and S-adenosylmethionine decarboxylases (ODC and SAMDC, respectively), whereas polyamine catabolism is controlled by the rate-limiting spermidine/spermine N¹-acetyltransferase (SSAT). The acetylated products of SSAT catalyzed reactions are oxidized further by polyamine oxidase (PAO) to spermidine and putrescine.

In attempts to elucidate the physiological roles of polyamines, a number of transgenic mouse and rat lines have been generated with genetically altered polyamine metabolism.^[40] The activation of polyamine biosynthesis through an overexpression of ODC brings about many interesting phenotypic changes, such as male infertility.^[43-44] Yet these studies are complicated by the fact that overexpression of ODC only expands tissue putrescine pools as the latter diamine is not further converted to spermidine and spermine.^[45-46] Overexpression of SSAT in transgenic rodents resulted in an immense induction of pancreatic SSAT activity, a profound depletion of spermidine, and spermine pools and acute pancreatitis.^[47-48] Induction of pancreatitis could not be prevented by inhibition of polyamine oxidase, which generates hydrogen peroxide and a reactive aldehyde. Therefore, pancreatic inflammation was causally related to the profound depletion of spermidine and spermine. It has been shown that pancreatic SSAT activation and subsequent polyamine catabolism are characteristic features of experimental acute pancreatitis models and also of human pancreatic tissue samples from patients with acute pancreatitis.^[38, 49-50] Furthermore, methylated polyamine analogues, which are supposed to fulfill the putative cellular functions of spermidine but are resistant to SSAT-dependent catabolism, prevented acute pancreatitis when administered before the induction of the SSAT overexpressing transgene.^[51] Polyamine analogue administration was also beneficial in other pancreatitis models including L-arginine-induced pancreatitis.^[38] The importance of polyamines in the maintenance of pancreatic integrity is further highlighted by the fact that the pancreas contains the highest concentration of spermidine in the mammalian body.^[52] However, the exact physiological function of spermidine in the pancreas is

unknown.^[42] Consequently, metabolites of the urea cycle and polyamine biosynthesis may play a crucial role in the maintenance of pancreatic integrity.

The main **aims** of this work were to determine whether equimolar doses of the L-arginine metabolites L-ornithine or L-citrulline and/or the NO donor sodium nitroprusside cause acute pancreatitis in rats. Large doses of IP injected L-ornithine induced severe acute necrotizing pancreatitis, therefore, we characterized the dose–response and time course changes of L-ornithine administration. Based on these results, we speculated that L-arginine produces a toxic effect on the pancreas, at least in part, via L-ornithine. Therefore, we tested the effects of the irreversible arginase inhibitor (+)-S-2-amino-6-iodoacetamidohexanoic acid (AIHA) on L-arginine-induced acute pancreatitis. As L-ornithine is a direct precursor of polyamines, the question arises how the biosynthesis and catabolism of polyamines change in L-ornithine-induced acute pancreatitis. Furthermore, as we observed pancreatic spermidine catabolism in rats with L-ornithine-induced pancreatitis, we also tested the effects of the metabolically stable polyamine analogue 1-methylspermidine (MeSpd) administration on the disease as treatment to compensate for the loss of natural spermidine.

2 MATERIALS AND METHODS

2.1 Materials

All chemicals were from Sigma-Aldrich (Munich, Germany) unless stated otherwise.

2.2 Methods

2.2.1 *Animals*

Male Wistar rats weighing 180 to 250 g were used. The animals were kept at a constant room temperature of 24°C with a 12-hour light–dark cycle and were allowed free access to water and standard laboratory chow (Biofarm, Zagyvaszántó, Hungary). All experiments performed in this study were approved by the Animal Care Committee of the University of Szeged.

2.2.2 *Experimental Protocols*

2.2.2.1 *Pilot Study with the Main Direct Downstream Metabolites of L-arginine*

Rats ($n = 3\text{--}5$) were injected IP with equimolar (11.7 mL/kg 1.424 M/L) L-arginine-HCl (cat.# A5131), L-citrulline (cat.# 27510) and/or the NO donor sodium nitroprusside (cat.# 71780), L-ornithine-HCl (cat.# O8305), or D-ornithine-HCl (cat.# 75480) (all chemicals dissolved in physiological saline (PS) and pH set to 7.4). The animals were killed 24 hours after the IP injection. To determine the serum concentrations of arginine, citrulline, and ornithine after injection of L-arginine, rats were killed at 2, 4, 6, and 12 hours.

2.2.2.2 *Dose–response and Time Course Changes of L-ornithine Injection*

To study the dose-response ($n = 6$) of L-ornithine administration, rats were injected IP with 1 to 6 g/kg body weight of L-ornithine-HCl (dissolved in PS at a concentration of 300 mg/mL, pH = 7.4) and were killed after 24 hours. For the time course studies ($n = 4\text{--}10$), rats were injected with 3 g/kg L-ornithine and were killed 2 to 72 hours, 1 week, or 1 month after the injection. The control animals received PS IP and were killed 24 hours after the injection.

2.2.2.3 *Effect of Irreversible Arginase Inhibition on L-arginine-induced Pancreatitis*

In each experimental group, five to eight rats were used. Rats were pretreated with 15 mg/kg AIHA (Alexis Biochemicals, Lausen, Switzerland) (dissolved in 6 M HCl and pH set to 7.4 with NaOH in phosphate-buffered saline) or its vehicle IP 1 hour before injection with PS or 3.5 g/kg L-arginine-HCl (350 mg/mL, pH: 7.4) IP. Rats were sacrificed 24 hours after the L-arginine or PS injection. Pancreas, liver, kidney, and lung tissue were frozen from control animals for determination of arginase activity.

2.2.2.4 *Characterization of Polyamine Homeostasis in L-ornithine-induced Acute Pancreatitis*

Pancreatitis was induced by IP injection with 3 g/kg L-ornithine-HCl. Polyamine homeostasis was studied at 6, 24, 72, and 168 hours after the induction of pancreatitis (n = 5-6). The control animals received PS IP and were killed 24 hours after the injection (n = 5).

2.2.2.5 *Effect of 1-methylspermidine on L-ornithine-induced Acute Pancreatitis*

The polyamine analogue MeSpd was synthesized from 3-aminobutanol as described previously^[53] and was dissolved in PS (25 mg/mL, pH 7.4). Rats were divided randomly into 6 groups. In the O24 group (n = 8), rats were injected with 3 g/kg L-ornithine-HCl IP and received PS IP 4 hours before (n = 4) or 4 hours after (n = 4) the L-ornithine treatment. In the MO24 group (n = 6), rats were pretreated with 50 mg/kg MeSpd IP 4 hours before injection with 3 g/kg L-ornithine IP. In the OM24 (n = 6) group, rats received 50 mg/kg MeSpd IP 4 hours after the L-ornithine (3 g/kg) injection. In the O48 (n = 5) group, rats were injected with 3 g/kg L-ornithine IP and received PS IP 4 and 24 hours thereafter. In the OM48 group (n = 5), rats were treated with 50 mg/kg MeSpd IP 4 and 24 hours after the L-ornithine (3 g/kg) treatment. In the control group (n = 5), rats received PS IP instead of L-ornithine and MeSpd. The „24” and „48” labels in the group names indicate the time points in hours at which rats were sacrificed after the injection of L-ornithine.

2.2.2.6 *Anaesthesia and Preparation of Tissue Samples*

In all experimental protocols, rats were killed by exsanguination through the abdominal aorta after anaesthetization with 44 mg/kg pentobarbital IP. Tissue samples from

the pancreas (cleaned from fat and lymph nodes), liver, and lung were frozen in liquid nitrogen and stored at -80°C until use. All blood samples were centrifuged at 2500g for 20 minutes, and the serum was stored at -25°C.

2.2.3 Assays

2.2.3.1 Pancreatic Weight/Body Weight Ratio

The pancreatic weight/body weight (p.w./b.w.) ratio was used to evaluate the degree of pancreatic edema.

2.2.3.2 Serum, Pancreatic and Ascitic Amylase Activity, Serum Lipase Activity, Serum Aspartate Aminotransferase Activity, and Concentrations of Glucose, Calcium, Triglyceride, Urea, Creatinine, Arginine, Ornithine, and Citrulline

Serum, pancreatic and ascitic amylase activities were determined by an enzymatic colorimetric assay standardized by the International Federation of Clinical Chemistry (Diagnosticum Ltd., Budapest, Hungary). Serum lipase activity was determined by an enzymatic colorimetric method and serum concentration of creatinine by the kinetic colorimetric compensated Jaffé method of Roche Diagnostics GmbH (Mannheim, Germany). Aspartate aminotransferase (ASAT) was measured by an IFCC UV kinetic method (Human GmbH, Wiesbaden, Germany). Concentrations of glucose, calcium, triglyceride and urea were determined by standard kits from Dialab (Vienna, Austria). The amino acid concentrations were assayed in dried serum specimens according to the method of Chace et al.^[54]

2.2.3.3 Pancreatic Trypsin Activity

Active trypsin in pancreatic tissue homogenates was measured as described previously.^[55]

2.2.3.4 Arginase Activity

Arginase activities in the liver, pancreas, kidney, and lung were measured as described previously.^[56]

2.2.3.5 *Polyamine Levels and Activities of Spermidine/Spermine N¹-Acetyltransferase and Ornithine Decarboxylase*

The levels of the natural polyamines (spermidine, spermine, and putrescine) and the polyamine analogue MeSpd were determined by high-performance liquid chromatography according to the method of Hyvönen et al.^[57] Pancreatic ODC and SSAT activities were assayed according to Jänne and Williams-Ashman^[58] and Bernacki et al.^[59], respectively.

2.2.3.6 *Pancreatic Myeloperoxidase Activity*

Pancreatic myeloperoxidase (MPO) activity, as a marker of tissue leukocyte infiltration, was assessed by the method of Kuebler et al.^[60]

2.2.3.7 *Expression of Pancreatic HSP72, HSP27, IκB-α, and IκB-β*

Western blot analysis of pancreatic heat shock protein 27 (HSP27) and HSP72, as well as IκB-α and IκB-β expression was performed as described previously.^[61] The protein concentration of the pancreatic cytosolic fraction was determined by the method of Bradford.^[62] Samples were electrophoresed on a 10% sodium dodecyl sulfate-polyacrylamide gel according to the method of Laemmli.^[63] Membranes were incubated with rabbit anti-HSP72 (1:10,000 dilution, characterized previously^[64]), or goat anti-HSP27 (1:500 dilution) or rabbit anti-IκB-α (1:500 dilution) or rabbit anti-IκB-β (1:500 dilution) (Santa Cruz Biotechnology, Santa Cruz, CA) antibody for an additional 1-3 hours at room temperature. The immunoreactive protein was visualized by enhanced chemiluminescence using horseradish peroxidase-coupled anti-rabbit or anti-goat immunoglobulin (Dako, Glostrup, Denmark) at 1:10,000 dilution for 1 hour.

2.2.3.8 *Pancreatic Interleukin-1β Concentrations*

The proinflammatory interleukin-1β (IL-1β) concentrations were measured in the pancreatic cytosolic fractions with an enzyme-linked immunosorbent assay kit (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions.

2.2.3.9 Pancreatic Nonprotein Sulphydryl Group Content and the Activities of Glutathione Peroxidase and Superoxide Dismutase

To determine nonprotein sulphydryl group (NSG) content and activities of glutathione peroxidase (GSH-Px), Mn-, and Cu/Zn-superoxide dismutase (SOD), a part of the pancreas was homogenized, the homogenates centrifuged at 3000 g for 10 minutes, and the supernatants were used for measurements as described previously.^[65]

2.2.4 Detection of Apoptosis in the Pancreas

2.2.4.1 Pancreatic Genomic DNA Analysis

A biochemical hallmark of apoptosis is a characteristic form of DNA degradation in which the genome is cleaved at internucleosomal sites, generating a ladder-like pattern of DNA fragments (multiples of 180 bp) when analyzed by agarose gel electrophoresis. For a qualitative assessment of genomic DNA fragmentation/degradation, rat pancreata were ground to a powder with a mortar and pestle under liquid nitrogen and were then homogenized by five strokes in a glass Dounce homogenizer with 1.5- to four-fold excess of extraction buffer (50 mmol/L Tris-HCl [pH = 8.0], 50 mmol/L EDTA, 0.5% sodium dodecyl sulfate, and 0.2 mg/mL proteinase K). The homogenates were transferred to Eppendorf tubes and rotated at 55°C overnight. The DNA solution was extracted twice with Tris-EDTA (TE)-saturated phenol, once with 1:1 TE-saturated phenol : chloroform and once with chloroform. The DNA was precipitated by adding 0.1 vol 3 mol/L sodium acetate (pH = 5.5) and 2 vol 96% ethanol. DNA precipitate was collected by centrifugation at 13,000 g for 10 mins, rinsed with 70% ethanol, vacuum-dried, resuspended in 100 to 200 µL TE buffer, and finally digested for 1 hour at 37°C with DNase-free RNase. Fifteen to 20 µg of DNA was electrophoretically fractionated on a 1.8% agarose gel with 0.5 µg/mL ethidium bromide.

2.2.4.2 TdT-mediated dUTP Nick End-labeling Technique

Apoptotic cells were quantitated by TdT-mediated dUTP nick end-labeling (TUNEL) assay using an In Situ Cell Death Detection Kit from Roche Diagnostics (Mannheim, Germany) according to the manufacturer's instructions. The number of apoptotic cells was counted in 0.5 mm² of pancreatic tissue. Results are expressed as percentage of the number of

cell nuclei in the same area in control tissue. Note that this method of calculation will underestimate the rates of apoptosis in edematous pancreata.

2.2.4.3 Microscopy

Cells showing characteristic changes of apoptosis were also identified by light microscopic technique (see subsequently).

2.2.5 Histologic Examination (Light Microscopy)

A portion of the pancreas, liver, kidney, and lungs was fixed in 6% neutral formaldehyde solution and subsequently embedded in paraffin. Sections were cut at 4 μ m thickness and stained with hematoxylin and eosin. The slides were coded and read by two independent observers who were blind to the experimental protocol. Pancreatic tissue injury was evaluated as follows: semiquantitative grading of interstitial edema (0–3), vascular congestion (0 –1), leukocyte adhesion (0 –3) and infiltration (0 – 4), apoptosis (0 –3) and necrosis (0–4) of acinar cells was determined in each animal (described in more detail in Table 1). Signs indicative of regeneration: mitotic figures, ductuloacinar structures, and basophilia of acinar cells were recorded.

2.2.6 Statistical Analysis

Results are expressed as means \pm SEM. Experiments were evaluated by using the analysis of variance followed by Dunnett's multiple comparison *post hoc* test. Values of $p < 0.05$ were accepted as significant.

Table 1. Histological scoring system for the evaluation of pancreatic injury in rats. (The slides were coded and read for the traditional histological markers of pancreatic tissue injury by two independent observers who were blind to the experimental protocol.)

	Scores				
	0	1	2	3	4
Interstitial edema	0	mild	moderate	severe	-
Vascular congestion	0	present	-	-	-
Leukocyte adherence to vessel walls	0	mild	moderate	extensive	-
Leukocyte infiltration	0	focal	diffuse/mild	diffuse/moderate	diffuse/severe
Vacuolization (% of total acinar cells)	0	focal (<10)	11-25	26-50	51-75
Necrosis (% of total acinar cells)	0	focal (<10)	11-25	26-50	51-75
Number of apoptotic bodies (% of total acinar cells)	0	<5	6-10	11-20	-
Regeneration	0	present	-	-	-

3 RESULTS

3.1 *In Vivo* Effects of Large Doses of the Main Direct Metabolites of L-arginine

We first tested the effects of 3.5 g/kg L-arginine and its catabolites (administered at equimolar doses) on serum amylase activity, p.w./b.w. ratio, pancreatic MPO activity, and pancreatic histology 24 hours after the injection (n = 3). Interestingly, the IP injection of 2.8 g/kg L-ornithine caused a more severe pancreatitis compared with the L-arginine group (Fig. 2; Table 2). Similar effect was seen with the 3 g/kg L-ornithine dose, so this was used throughout the rest of the study. In contrast, IP administration of 3 g/kg D-ornithine did not result in pancreatic injury (n = 5, results not shown). A dose of 2.9 g/kg L-citrulline did not cause an alteration in any of the measured parameters and the pancreas seemed normal in histology (Fig. 2; Table 2). The animals that received sodium nitroprusside (4.95 g/kg) alone or in combination with L-citrulline became lethargic soon after the injection and died by the next morning. Autopsy did not show pancreatitis in these animals. Most likely they died as a result of vascular complications, that is, hypotension.

3.2 Time Course Changes of Serum Arginine, Citrulline, and Ornithine Concentrations in Rats Injected Intraperitoneally with 3.5 g/kg L-arginine

Serum arginine, citrulline, and ornithine concentrations (n = 5) were all significantly increased after the injection of L-arginine (Fig. 3). Importantly, there were much greater increases in serum ornithine versus citrulline levels after L-arginine injection.

3.3 Dose-response of Intraperitoneal Injection of L-ornithine

The rats injected with 1 or 2 g/kg L-ornithine did not develop any pancreatic lesions (n = 6, results not shown). However, 3 g/kg of this basic amino acid caused a severe acute pancreatitis as described subsequently. A dose of 4 to 6 g/kg (n = 6) killed the animals within a couple of hours after the injection after developing lethargy, neurologic and neuromuscular symptoms (tremor, twitching, convulsion, and in some cases jumping all over the cage).

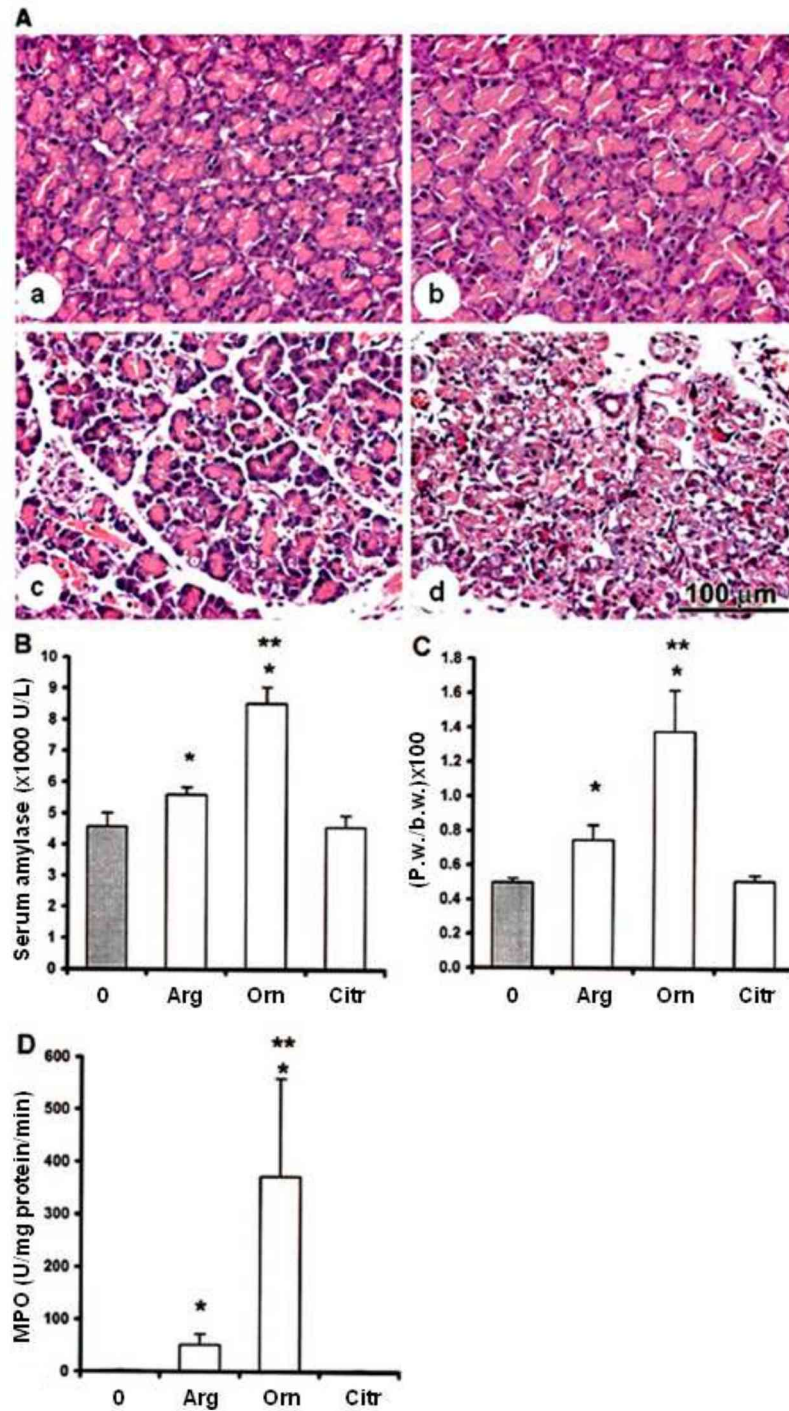


Figure 2. Intraperitoneal administration of L-ornithine to rats resulted in necrotizing acute pancreatitis, which was more severe than that induced with L-arginine. Rats were injected intraperitoneally (IP) with physiological saline (PS) (control, 0) or equimolar L-arginine-HCl (Arg, 3.5 g/kg), L-ornithine-HCl (Orn, 2.8 g/kg), L-citrulline (Citr, 2.9 g/kg), and/or the nitric oxide donor sodium nitroprusside (4.95 g/kg). The animals that received sodium nitroprusside alone or in combination with L-citrulline died by the next morning. The rest of the rats survived the treatment and were killed by exsanguination through the abdominal aorta 24 hours after the IP injection. (A) The diagrams show light micrographs (hematoxylin and eosin staining) of the pancreata of (a) control, (b) *Citr*, (c) *Arg*, or (d) *Orn*-treated rats. The bar diagrams show the (B) serum amylase activity, (C) pancreatic weight (*p.w.*) / body weight (*b.w.*) ratio, and (D) pancreatic myeloperoxidase (MPO) activity. Data are shown as means \pm SEM, $n = 3-5$. *Significant difference ($p < 0.05$) vs. the control group (0 hour). **Significant difference ($p < 0.05$) vs. the Arg group.

Table 2. Evaluation of pancreatic injury 24 hours after the intraperitoneal injection of physiological saline (control, 0), equimolar L-citrulline (Citr, 2.9 g/kg), L-arginine (Arg, 3.5 g/kg) or L-ornithine (Orn, 2.8 g/kg). Scores are shown for an average of three animals.

	Control, 0	Citr	Arg	Orn
Interstitial edema	0	0	1	3
Vascular congestion	0	0	1	1
Leukocyte adherence	0	0	1	3
Leukocyte infiltration	0	0	1	3
Vacuolization	0	0	1	1
Apoptosis	0	0	1	2
Necrosis	0	0	3	4

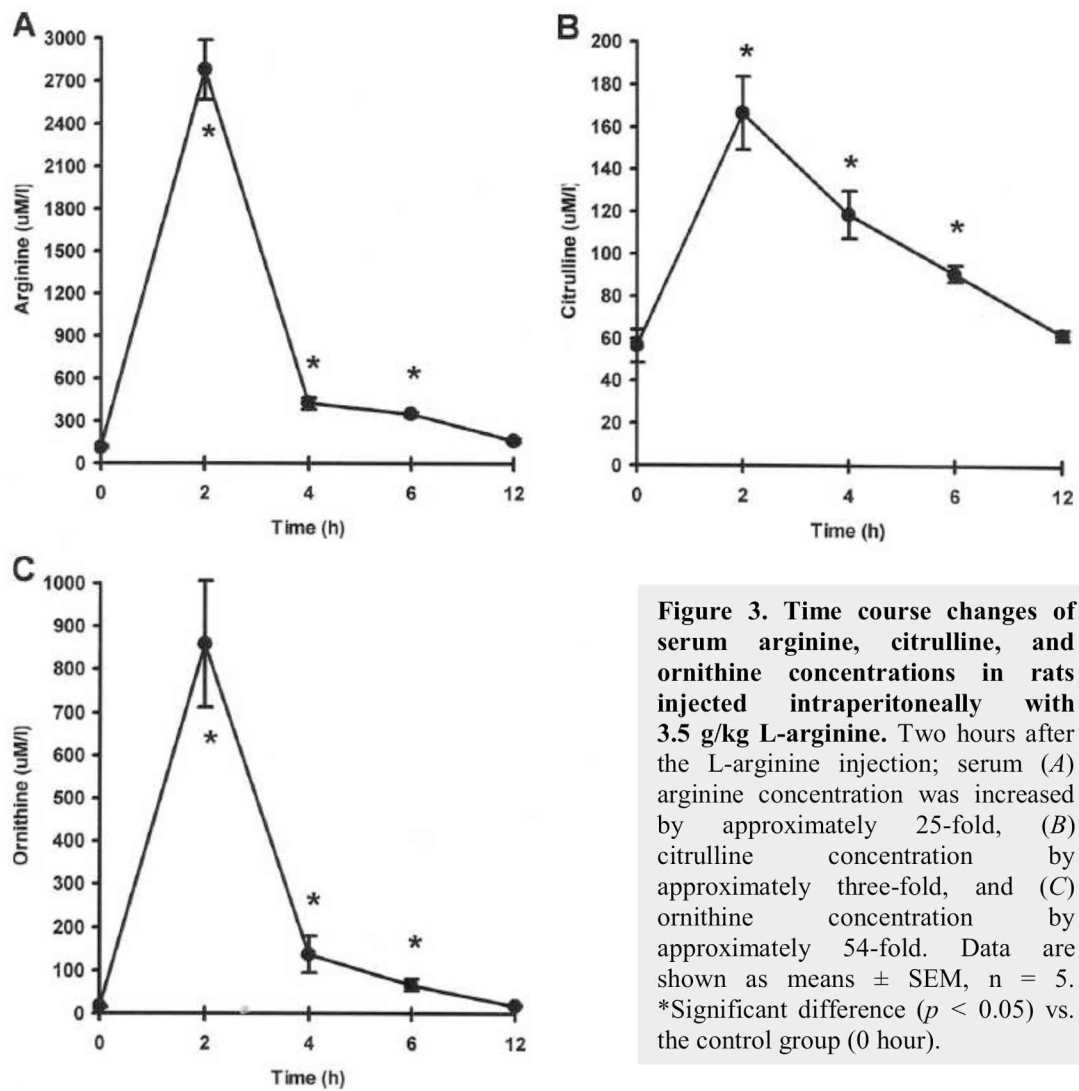


Figure 3. Time course changes of serum arginine, citrulline, and ornithine concentrations in rats injected intraperitoneally with 3.5 g/kg L-arginine. Two hours after the L-arginine injection; serum (A) arginine concentration was increased by approximately 25-fold, (B) citrulline concentration by approximately three-fold, and (C) ornithine concentration by approximately 54-fold. Data are shown as means \pm SEM, $n = 5$. *Significant difference ($p < 0.05$) vs. the control group (0 hour).

3.4 Time Course Studies After Intraperitoneal Injection of Rats with 3 g/kg L-ornithine

3.4.1 *Macroscopic Observations*

The pancreas appeared edematous from 18 to 36 hours, its peak being at 24 hours. Ascites and adhesions of organs were seen from 4 to 6 hours (peaking at 24 hours). Occasionally, yellow–white foci indicative of chalky fat necrosis was detected in the mesentery of the bowels and retroperitoneum at 24 to 72 hours. Dilated small and large bowels suggesting functional ileus was apparent at 72 hours to 1 week after L-ornithine injection.

3.4.2 *Histologic Examination of the Pancreas*

The pancreas appeared normal 2 hours after L-ornithine (3 g/kg) injection. At 4 hours, mild interstitial edema and foamy vacuolization of the acini and vascular congestion were observed. At 6 hours, the number of apoptotic bodies was greatly increased and we could also observe focal necrosis (<10%) of acini (Fig. 4). At 9 hours, there was interstitial edema, neutrophilic and monocytic adherence, and focal inflammatory infiltration. There were great numbers of apoptotic bodies. The extent of acinar cell necrosis was 15% to 25%. At 12 hours, there was diffuse moderate infiltrate of monocytes and neutrophils, and the necrosis of acinar cells was 26% to 50%. Eighteen hours after the L-ornithine injection, the extent of pancreatic edema was greatly increased. Necrosis of acinar cells was increased to 51% to 75%. The most severe interstitial edema was observed at 24 hours. Large numbers of neutrophils and monocytes could be observed in the interstitial space. At 36 hours, pancreatic edema was still severe, but was decreased versus 24 hours. There was also a decrease in monocytic and neutrophilic adherence, neutrophilic infiltration, and necrosis of acinar cells (26–50%). Lobular architecture was distorted. Fibroblasts also appeared in the inflamed interstitium. Regeneration starting with undifferentiated, ductuloacinar structures with scanty cytoplasm and densely basophilic nuclei in the peripheral zone of the pancreas appeared. At 48 hours, there was diffuse severe infiltrates of macrophages/monocytes, fibroblasts, and neutrophils. At 72 hours, there was no pancreatic edema, but there was a diffuse severe infiltrate of fibroblasts, macrophages/monocytes, eosinophils, and neutrophils (Fig. 4D). There was a mild

degree of collagen deposition within the lobules and around ductuloacinar structures. Ductuloacinar structures were budding from tubular lumina. Mitotic figures and a decrease in the number of apoptotic bodies were evident. At 1 week, diffuse moderate infiltrates of fibroblasts and macrophages and relatively smaller number of eosinophils and neutrophils were observed (Fig. 4E). There was a mild deposition of collagen within the lobules and around ductuloacinar structures. Adipose tissue replaced some of the destructed lobules. Budding ductuloacinar structures started to form ductules and acini and a few newly formed acinar cells displayed zymogen granules. Dilation of small ducts and ductules were observed; in some cases, they contained eosinophilic material. One month after injection, the pancreas appeared normal, except that part of the parenchyma was replaced by fat (Fig. 4F). The acini showed numerous mitotic figures and regenerative atypia. Fibroblasts and macrophages were no longer present. One of four animals had focal periductal infiltrate composed of lymphocytes, macrophages, and eosinophils.

Overall, there were no major pathologic alterations of the pancreatic duct cells, islets of Langerhans, and the liver in the hematoxylin and eosin sections. However, in one animal, striking Langerhans islet hyperplasia with mitotic figures was found at 1 month. At 9 to 36 hours, some eosinophilic cylinders in the tubular lumina were noted in the kidneys with attenuation and dilation of the proximal tubules. Occasional detachment of microvilli and mild peritubular capillaritis also were seen. These latter changes are consistent with mild acute tubular necrosis. At the same time, alveolar thickening with predominant neutrophil infiltration and occasional hemorrhage were seen in the lungs indicative of mild respiratory distress syndrome.

Table 3. Evaluation of pancreatic injury by histological examination 2–168 hours and 1 month after intraperitoneal injection of rats with 3 g/kg L-ornithine. Scores are shown for an average of four animals.

	2h	4h	6h	9h	12h	18h	24h	36h	48h	72h	168h	1mo
Interstitial edema	0	0	1	1	1	2	3	2	1	0	0	0
Vascular congestion	0	1	1	1	1	1	1	1	1	1	1	0
Leukocyte adherence	0	0	0	1	3	3	3	2	1	0	1	0
Leukocyte infiltration	0	0	0	1	3	3	3	4	4	4	3	0
Vacuolization	0	1	2	3	2	1	1	0	0	0	0	0
Apoptosis	0	1	3	3	3	3	2	2	1	1	0	0
Necrosis	0	0	1	2	3	4	4	3	3	0	0	0
Regeneration	0	0	0	0	0	0	0	1	1	1	1	1

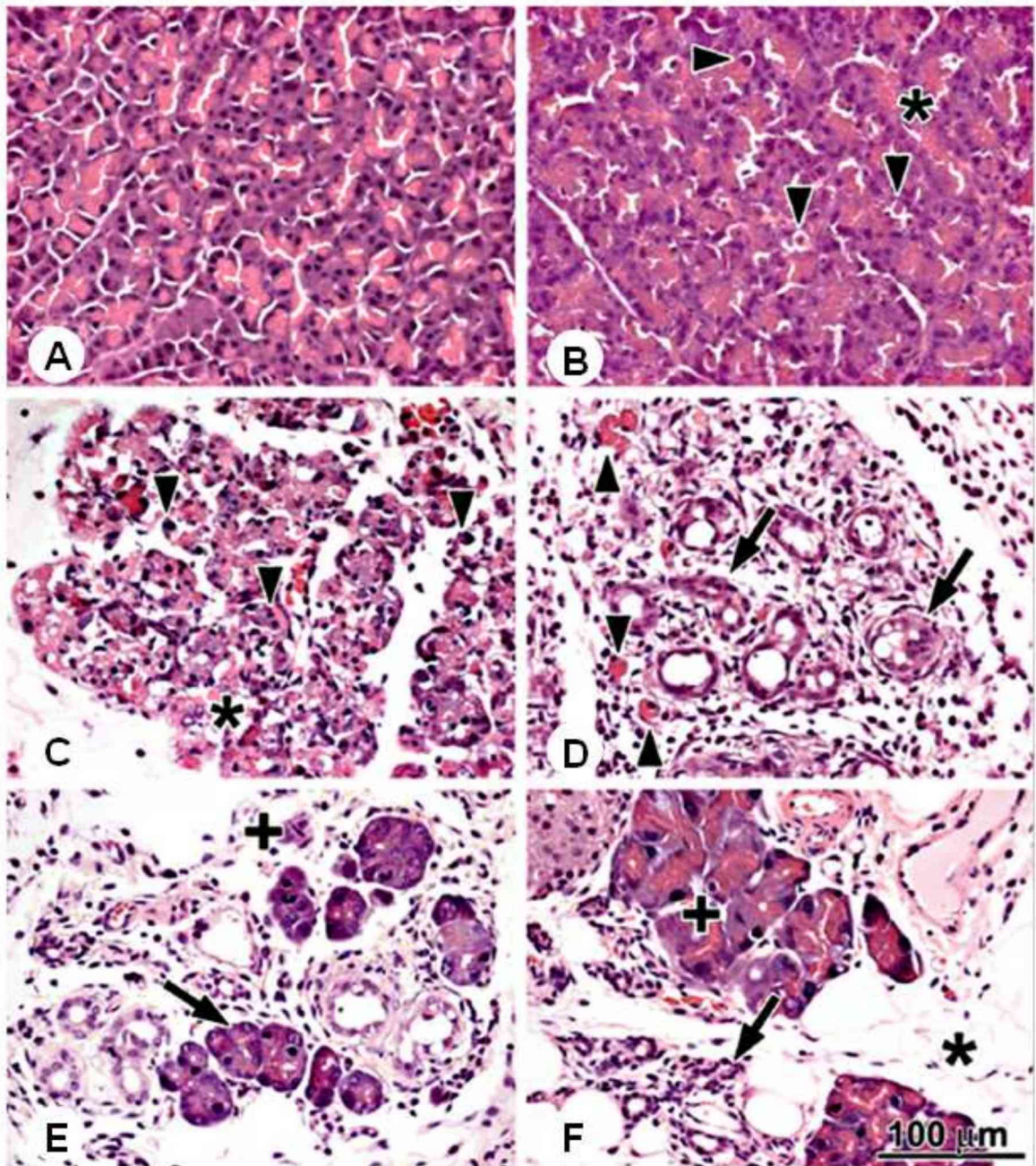


Figure 4. Histopathologic changes of the pancreas in response to intraperitoneal administration of 3 g/kg L-ornithine. (A) Two hours: no major histopathologic changes. (B) Six hours: foamy degeneration (*asterisk*) of acinar cells; arrowheads show apoptotic bodies. (C) Twenty-four hours: widespread necrosis of acinar cells (*asterisk*) and lots of apoptotic bodies. (D) Seventy-two hours: ductuloacinar structures (*arrow*) in place of disappeared acini; fibroblasts, macrophages, and some neutrophils in the interstitium. (E) One week: some acini have regenerated (*arrow*) with mitotic figures (+). (F) One month: acini show mitotic figures (+) and regenerative atypia; some ductuloacinar structures had undergone atrophy (*arrow*) with fatty ingrowth around them (*asterisk*).

3.4.3 Confirmation of Pancreatic Apoptosis Observed on Histologic Examination

3.4.3.1 Genomic DNA Analysis

Genomic DNA was extracted from pancreata 9 or 24 h after L-ornithine administration. Fig. 5/A shows that 9 h after L-ornithine injection we could detect a ladder pattern on agarose gel electrophoresis. On the other hand, DNA showed unspecific degradation (smear) 24 h after the administration of L-ornithine indicating severe necrosis. This is in accord with our histological findings as at this latter time point apoptosis was overcome by necrosis of the cells.

3.4.3.2 TUNEL Technique

According to the histological examination, the number of apoptotic cells was greatly elevated by 6 h after L-ornithine administration. To obtain quantitative data, the number of apoptotic cells was counted at selected time-points 0-48 h after L-ornithine administration. As shown in Fig. 5/B, the percentage of apoptotic cells was greatly increased in response to L-ornithine administration. The peak of apoptosis was around 6-9 h after the injection ($n = 4$).

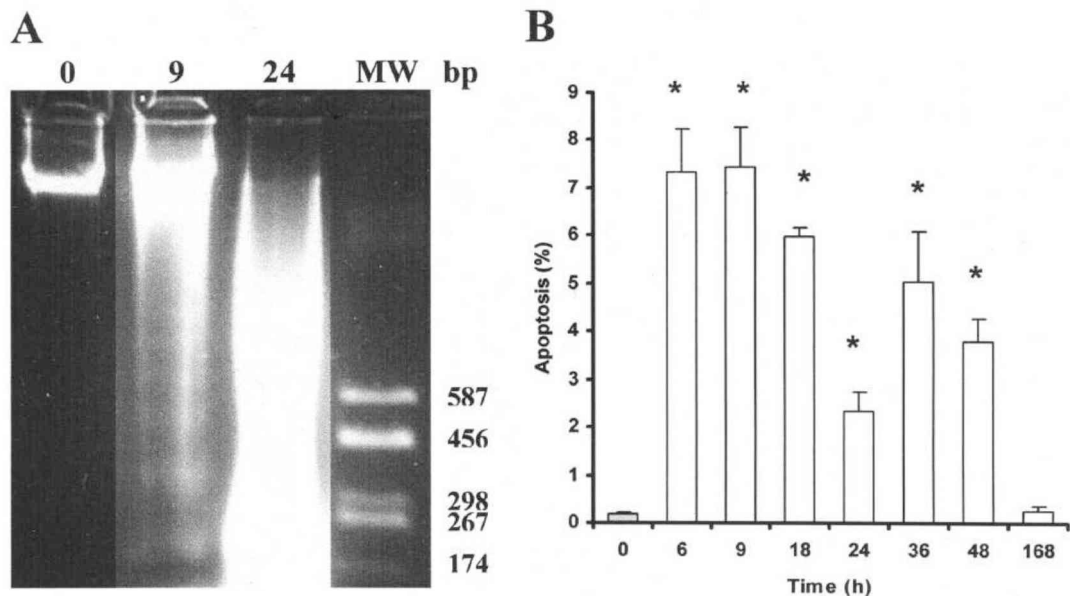


Figure 5. Pancreatic apoptosis is greatly increased in rats treated with 3 g/kg L-ornithine intraperitoneally. (A) Genomic DNA analysis of L-ornithine (3 g/kg) injected rats in agarose gel electrophoresis. Genomic DNA was isolated from the pancreata of rats 0, 9, 24 hours after L-ornithine injection. Fifteen to 20 μ g of DNA was electrophoretically fractionated on a 1.8% agarose gel with 0.5 μ g/mL ethidium bromide. Data presented are representative of three independent experiments. Mw, molecular weight marker; bp, base pairs. (B) The percentage of apoptotic cells in the pancreas was quantitated by using the TdT-mediated dUTP Nick end-labeling assay. Data are shown as means \pm SEM, $n = 4$. *Significant difference ($p < 0.05$) vs. the control group (0 hour).

3.4.4 Activities of Serum, Pancreatic, and Ascitic Amylase

The serum amylase activity significantly increased from 9 to 24 hours, but thereafter (at 48 hours) fell below control values ($n = 4-10$, Fig. 6A). Pancreatic amylase activity was significantly decreased from 24 hours to 1 month after L-ornithine injection and was just about detectable at 72 and 168 hours ($n = 4-8$, Fig. 6B). The ascites recovered from rats 24 hours after L-ornithine administration had a huge amylase activity ($98,096 \pm 25,590$ U/L, $n = 7$).

3.4.5 Pancreatic Trypsin Activity

Premature activation of trypsinogen is thought to play an important role in the development of acute pancreatitis. Pancreatic trypsin activity was significantly increased 9 to 48 hours after IP injection of 3 g/kg L-ornithine (Fig. 6C).

3.4.6 Pancreatic Myeloperoxidase Activity

The extent of neutrophil infiltration was also judged by the measurement of MPO activity (Fig. 6D). Interestingly, inflammatory infiltration had two phases; the first one coincided with the peak of amylase activity (9–36 hours) and the second one occurred much later (at 72 hours).

3.4.7 Induction of Pancreatic HSP72 Synthesis

Heat shock proteins are essential cytoprotective molecules that are known to be induced in acute pancreatitis. HSP72, the highly inducible form of the HSP70 family, could not be detected in the PS-treated control group (Fig. 7A). However, by 4 hours after the injection of 3 g/kg L-ornithine, the levels of HSP72 were significantly increased, peaked at 18 hours, and remained elevated until 1 month.

3.4.8 Degradation of Pancreatic I κ B- α and I κ B- β and Induction of Interleukin-1 β Synthesis

I κ B proteins are inhibitory proteins of the proinflammatory transcription factor NF- κ B. Pancreatic I κ B levels in response to L-ornithine injection were significantly decreased from 9 hours (Fig. 7B–C). I κ B- α levels (Fig. 7B) returned to normal by 36 hours; however,

I κ B- β level was significantly lower for up to 168 hours after injection (Fig. 7C). Corresponding to I κ B degradation, and consequently to activation of NF- κ B, pancreatic interleukin-1 β synthesis significantly increased from 9 hours (Fig. 7D).

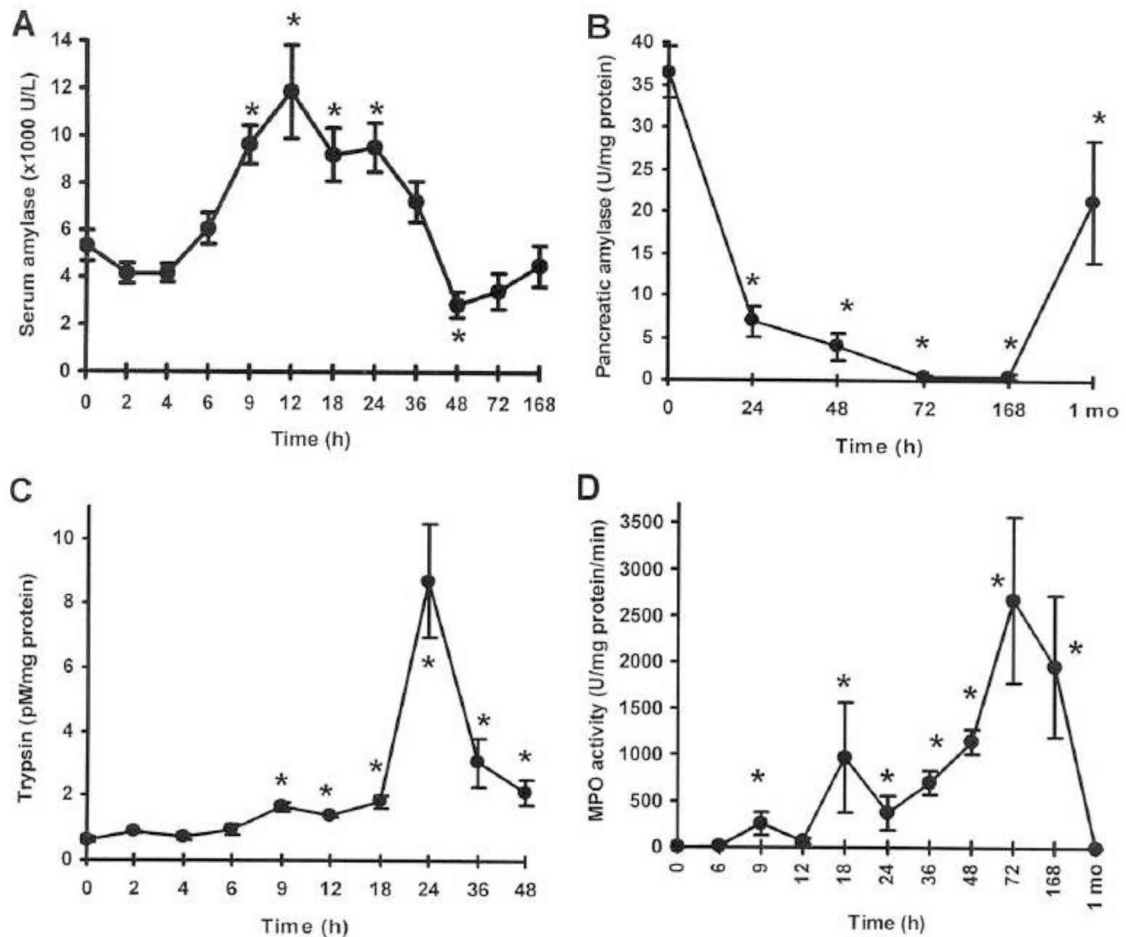


Figure 6. Time course of serum amylase and pancreatic amylase, trypsin and myeloperoxidase activities after intraperitoneal administration of 3 g/kg L-ornithine. (A) Serum amylase, (B) pancreatic amylase, (C) trypsin, and (D) MPO activities. The serum amylase activity significantly increased from 9 to 24 hours, but thereafter (at 48 hours) fell below control values. Pancreatic amylase activity was significantly decreased from 24 hours to 1 month after L-ornithine injection and was just about detectable at 72 and 168 hours. Trypsin activity was gradually increased from 9 hours and peaked at 24 hours after L-ornithine administration. MPO activity gradually increased during the course of the disease and was highest in the 72- and 168-hour groups. Data are shown as means \pm SEM, $n = 4-10$. *Significant difference ($p < 0.05$) vs. the control group (0 hour).

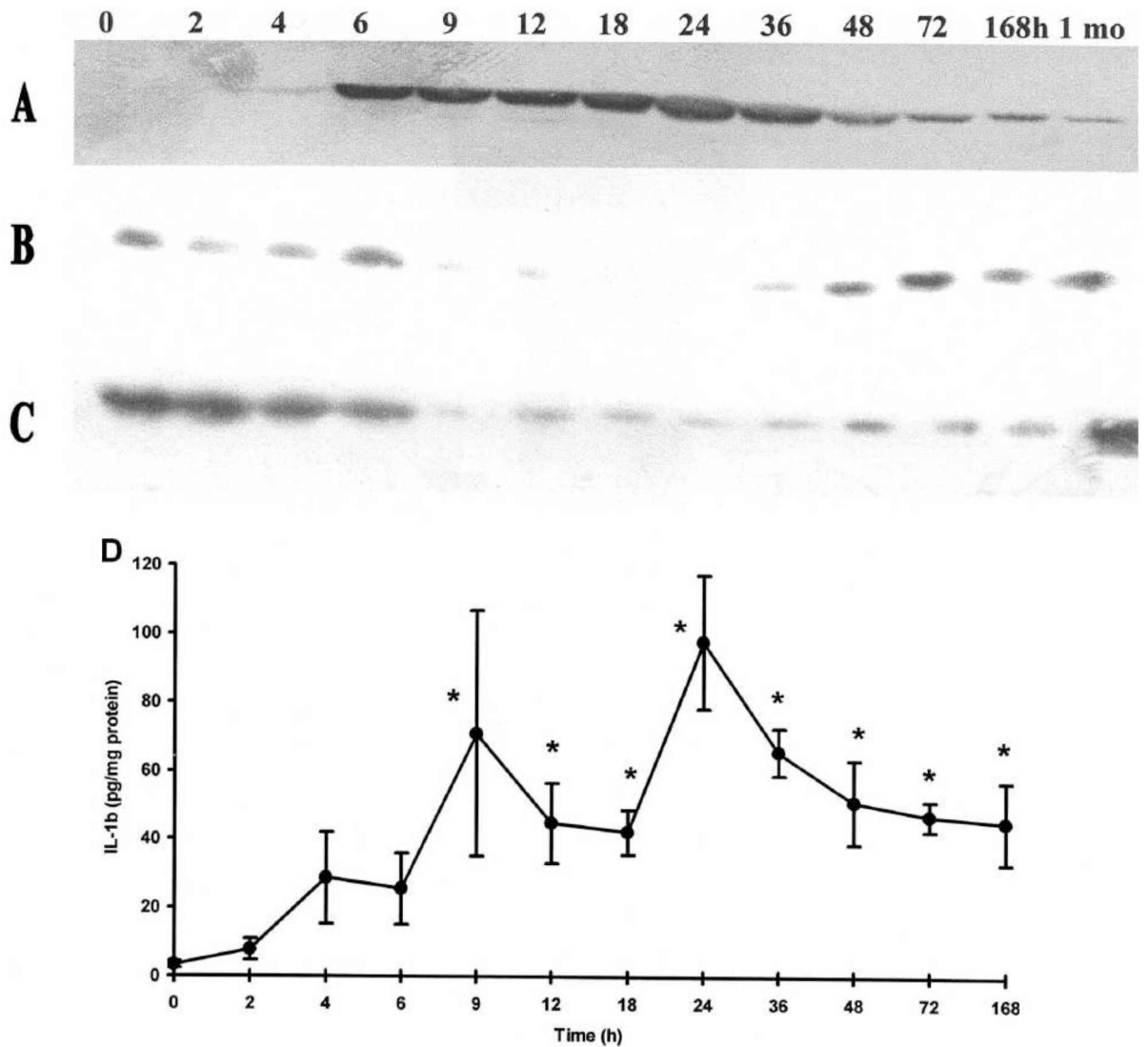


Figure 7. Effects of L-ornithine administration (3 g/kg intraperitoneally) on pancreatic HSP72, IκB-α, IκB-β, and interleukin-1β expression as a function of time. A–C, Representative Western immunoblot analysis of protein lysates (40 μg/lane) from the pancreata of rats 0 to 168 hours and 1 month after injection, showing the levels of (A) HSP72, (B) IκB-α, and (C) IκB-β as a function of time after 3 g/kg L-ornithine injection. (D) The graph shows the expression of the proinflammatory cytokine, interleukin-1β, as determined from the cytosolic fractions of pancreatic homogenates by enzyme-linked immunosorbent assay. Means ± SEM for four to six animals are shown. *Significant difference ($p < 0.05$) vs. the control group (0 hour).

3.4.9 Pancreatic Nonprotein Sulfhydryl Group Content and the Activities of Glutathione Peroxidase and Superoxide Dismutase

Nonprotein sulfhydryl group content was significantly increased at 6 hours and decreased thereafter in the ornithine-treated group compared with the control group (Fig. 8A). The activities of GSH-Px (Fig. 8B) and Cu/Zn-SOD (Fig. 8C) significantly increased from 24 hours. In contrast, Mn-SOD activity (Fig. 8D) was significantly decreased at 24 hours and significantly increased at 48 hours versus the control. Taken together these findings suggest the presence of oxidative stress in the pancreas of rats in response to L-ornithine treatment.

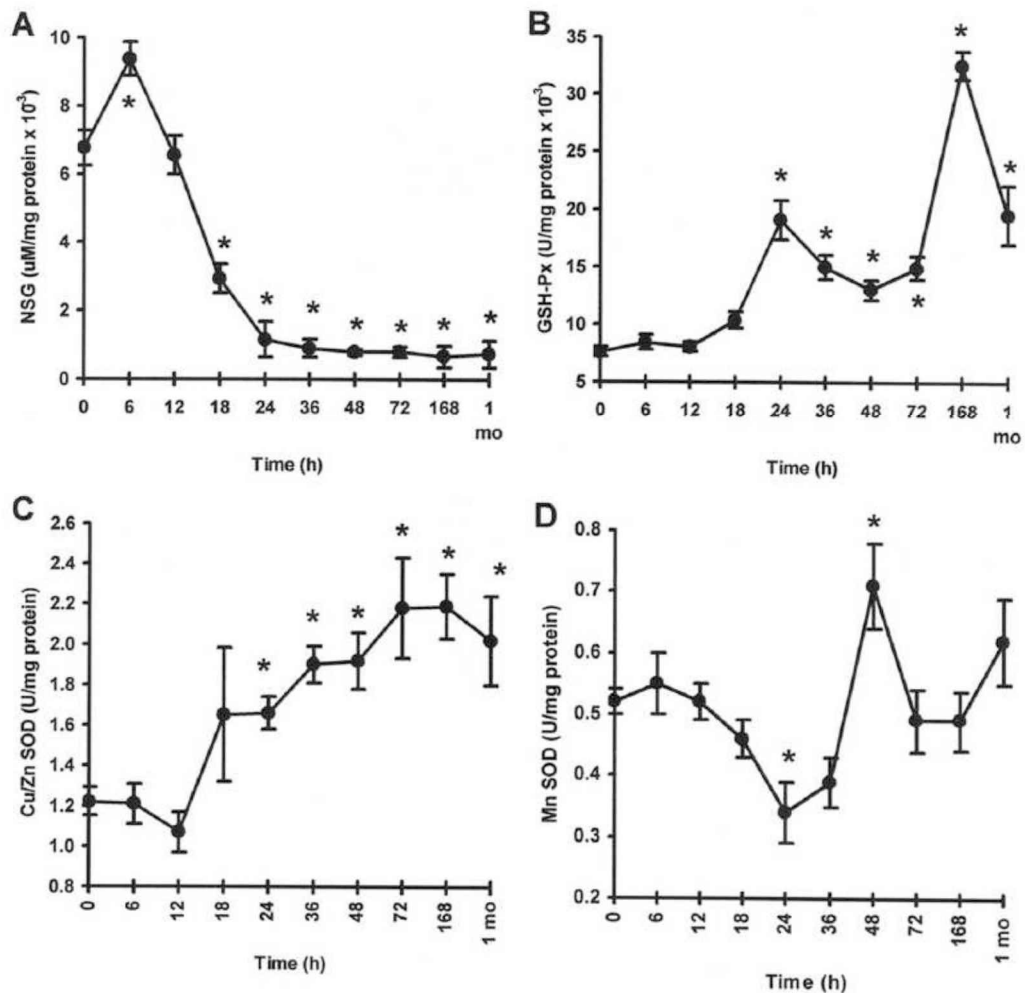


Figure 8. L-ornithine injection (3 g/kg intraperitoneally) induces pancreatic oxidative stress. Changes in pancreatic (A) nonprotein sulfhydryl group (NSG) content, (B) glutathione-peroxidase (GSH-Px), (C) Cu/Zn, and (D) Mn superoxide dismutase (SOD) are depicted. Means \pm SEM for five to six animals are shown. *Significant difference ($p < 0.05$) vs. the control group (0 hour).

3.4.10 Body Weight and Pancreatic Weight/Body Weight Ratio

The body weight of the rats was significantly decreased from 1 day to 1 month after the administration of 3 g/kg L-ornithine versus the PS-treated control ($n = 5-10$, Fig. 9A). Pancreatic weight/body weight ratio was significantly elevated at 18 to 48 hours and significantly decreased at 168 hours to 1 month after L-ornithine injection (Fig. 9B).

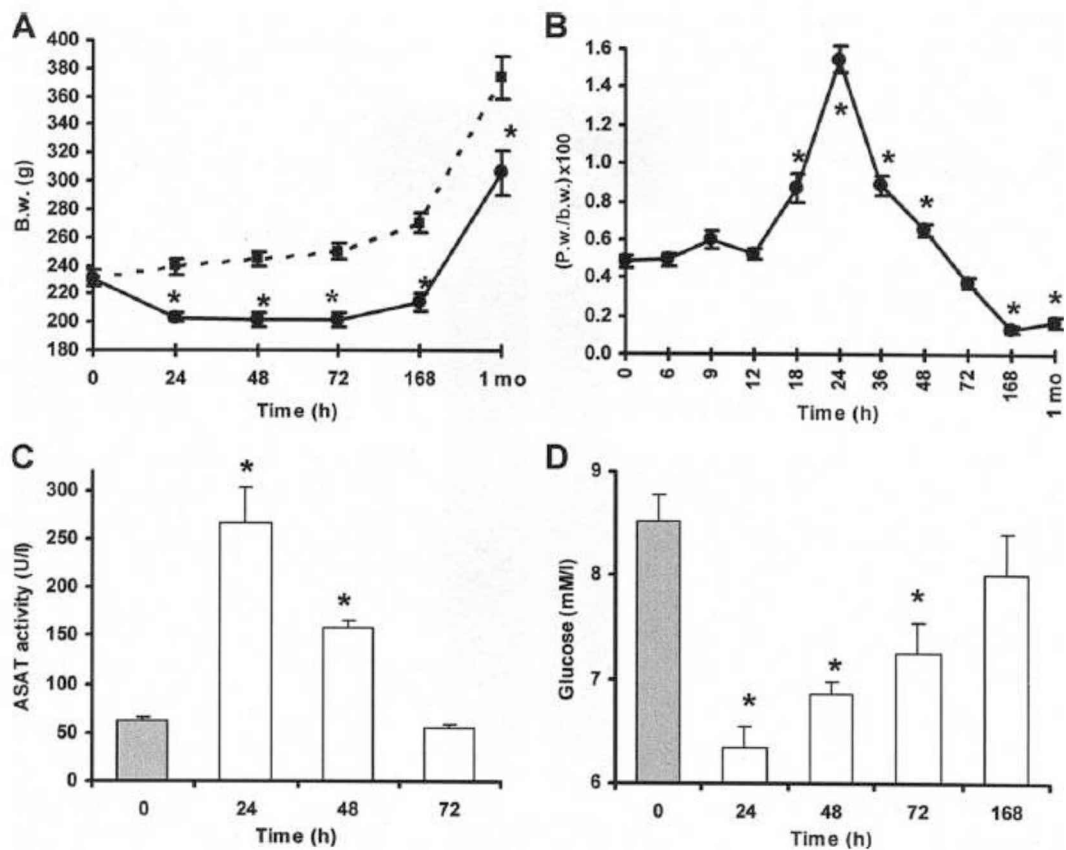


Figure 9. Time course of body weight (*b.w.*), pancreatic weight (*p.w.*)/*b.w.*, serum aspartate aminotransferase (*ASAT*) activity and glucose concentration after intraperitoneal administration of 3 g/kg L-ornithine. (A) *b.w.*, (B) *p.w./b.w.*, serum (C) *ASAT* activity, and (D) glucose concentration were determined as described in the Materials and Methods section. Data are shown as means \pm SEM, $n = 4-10$. *Significant difference ($p < 0.05$) vs. the control group (0 hour).

3.4.11 Serum Aspartate Aminotransferase Activity and Concentrations of Glucose, Calcium, Triglyceride, Urea, and Creatinine

Serum ASAT activity was significantly increased by approximately five-fold at 24 hours and three-fold at 48 hours after L-ornithine injection (Fig. 9C). Serum concentrations of glucose were significantly decreased from 24 to 72 hours and returned to normal by 1 week (Fig. 9D). Serum levels of triglyceride were only significantly effected at 72 hours (0.40 ± 0.03 mM/L vs. 0.71 ± 0.18 mM/L in the control group). Calcium, urea, and creatinine concentrations were not significantly different versus the control (results not shown).

3.5 Arginase Activity in Different Tissues and *In Vitro* Effect of AIHA on Arginase Activity

3.5.1 Arginase Activity in Different Tissues

Arginase activity was by far the highest in the liver (Fig. 10). Nevertheless, we could also detect arginase activity in the pancreas, lung, and kidney.

3.5.2 *In Vitro* Effect of AIHA on Arginase Activity

(+)-S-2-amino-6-iodoacetamido-hexanoic acid (AIHA) dose-dependently inhibited liver arginase activity of rat liver homogenate and purified bovine arginase *in vitro*

(Fig. 11). Sixty μ M AIHA (equimolar to an *in vivo* dose of 15 mg/kg) significantly inhibited liver arginase activity by about 25%.

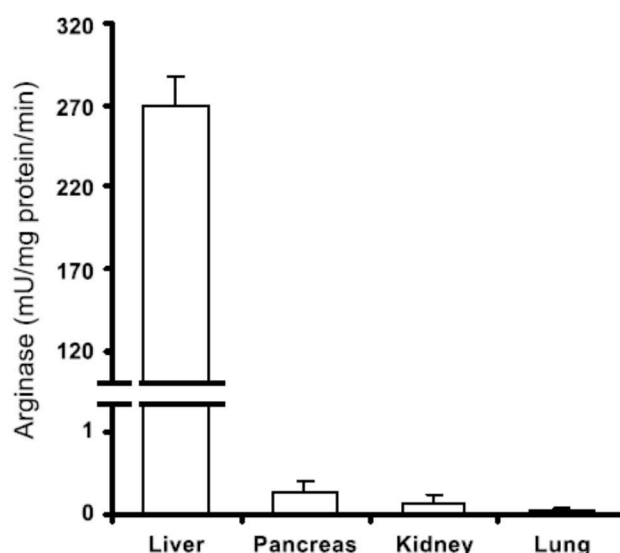


Figure 10. Arginase activity in the liver, pancreas, lung, and kidney of rats. Tissues were removed from control animals and then homogenized. Arginase activity was measured by exogenously added L-arginine using a colorimetric method, based on the determination of released urea.

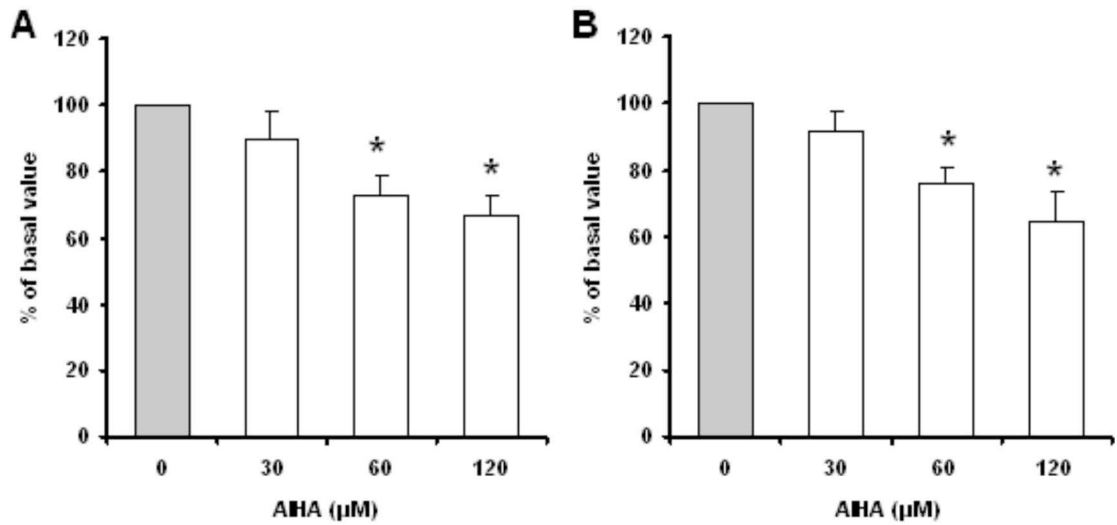
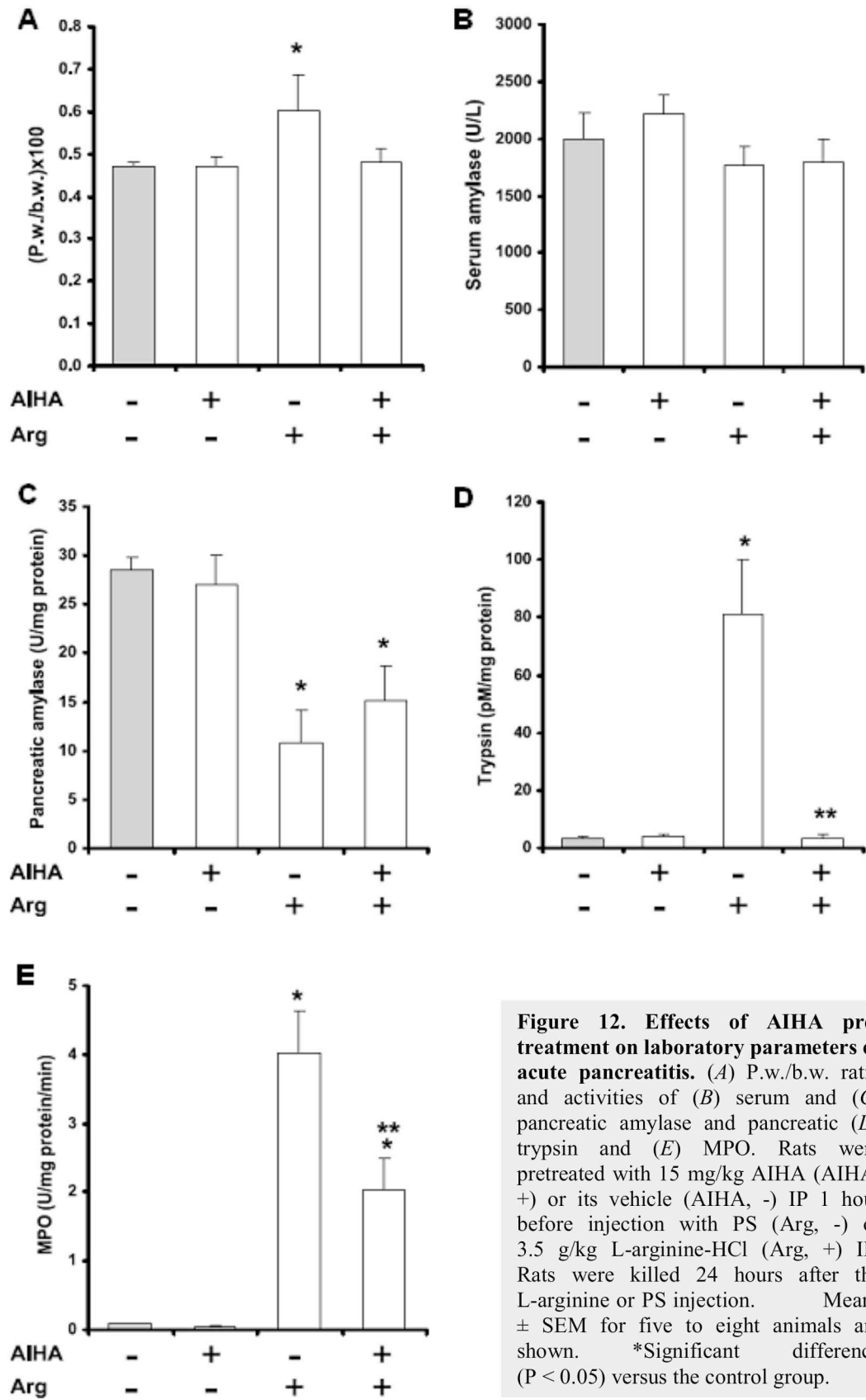


Figure 11. (+)-S-2-amino-6-iodoacetamidohexanoic acid (AIHA) inhibits arginase activity of rat liver homogenate and purified bovine liver arginase. The effect of 0 to 120 μ M AIHA was tested on (A) rat liver homogenate or (B) purified bovine liver arginase. Sixty micromolars of AIHA (equimolar to an *in vivo* dose of 15 mg/kg) significantly inhibited arginase activity by about 25%.

3.6 Effect of AIHA on L-arginine-Induced Acute Pancreatitis in Rats

3.6.1 Pancreatic Weight/Body Weight Ratio, Serum and Pancreatic Amylase Activity, and Pancreatic Trypsin and Myeloperoxidase Activities

Pancreatic weight/body weight ratio was significantly increased in response to IP administration of 3.5 g/kg L-arginine (Fig. 12A). Pretreatment with AIHA significantly ameliorated this increase of p.w./b.w. ratio. Serum amylase activity was not significantly altered in any of the groups (Fig. 12B). Pancreatic contents of amylase were significantly decreased in the L-arginine-treated groups (Fig. 12C). Pretreatment with AIHA did not influence pancreatic amylase activity in rats injected with L-arginine. Pancreatic trypsin activity was significantly increased by L-arginine administration (Fig. 12D). Pretreatment with AIHA significantly ameliorated this increased pancreatic trypsin activity. Myeloperoxidase activity was significantly increased at 24 hours after L-arginine injection (Fig. 12E). Pretreatment with AIHA significantly decreased MPO activity in the L-arginine-induced pancreatitis group.



3.6.2 Pancreatic Nonprotein Sulfhydryl Group Content and the Activities of Glutathione Peroxidase and Superoxide Dismutase

Nonprotein sulfhydryl group content and GSH-Px activity were significantly increased 24 hours after the injection with L-arginine (Figs. 13A, B). Pretreatment with AIHA did not influence NSG content, but significantly reduced GSH-Px activity. Activities of Cu/Zn and Mn SOD were unaltered by AIHA pretreatment in the L-arginine-pancreatitis group (Figs. 13C, D).

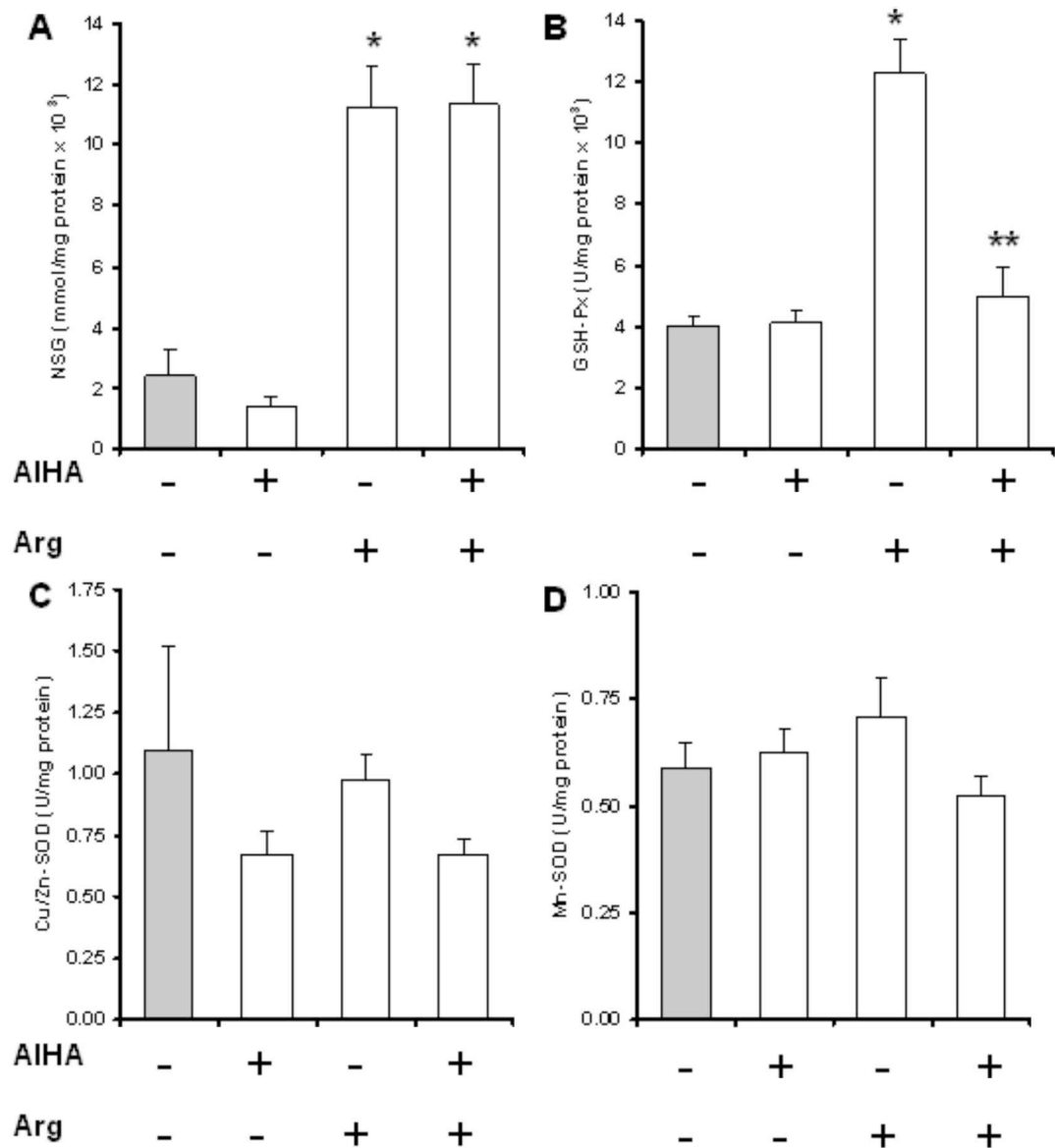


Figure 13. Effects of AIHA pretreatment on pancreatic markers of oxidative stress in L-arginine-induced acute pancreatitis. Changes in pancreatic (A) NSG content, (B) GSH-Px, and (C) Cu/Zn-SOD and (D) Mn-SOD activities are depicted. Rats were treated as indicated in the legend of Figure 12. Means \pm SEM for five to eight animals are shown. *Significant difference ($P < 0.05$) versus the control group.

3.6.3 Pancreatic Heat Shock Protein Expression

(+)-S-2-amino-6-iodoacetamidohexanoic acid and/or L-arginine administration resulted in up-regulation of pancreatic HSP27 and HSP72 synthesis versus the PS treated control group (Fig. 14). No significant difference was found at 24 hours between the AIHA-treated and untreated L-arginine-induced pancreatitis groups.

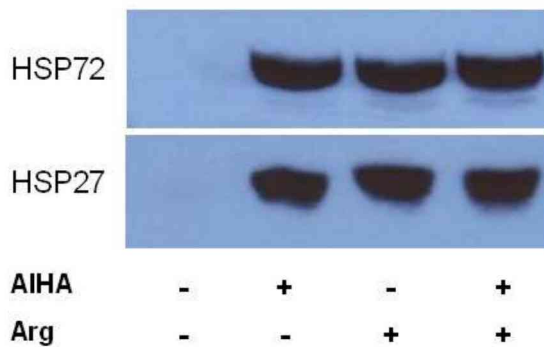


Figure 14. Effect of AIHA pretreatment on pancreatic heat shock protein synthesis in L-arginine-induced pancreatitis. Representative Western immunoblot analysis of protein lysates (40 µg/lane) from the pancreata of rats, showing the expression of HSP27 and HSP72. Rats were treated as indicated in the legend of Figure 12.

3.6.4 Histological Examination

The administration of 3.5 g/kg L-arginine caused severe necrotizing pancreatitis (Fig. 15; Table 4). Injection of AIHA in itself resulted in pancreatic hyperemia and mild inflammatory cell infiltration. However, AIHA pretreatment significantly reduced pancreatic damage in L-arginine-induced pancreatitis.

Table 4. Effects of AIHA pretreatment on the histological parameters in L-arginine-induced acute pancreatitis. Rats were treated as indicated in the legend of Figure 12. Data are means \pm SEM for five to eight animals. *Significant difference ($P < 0.05$) versus the control group. **Significant difference ($P < 0.05$) versus the L-arginine-treated group.

	control	AIHA	L-Arginine	AIHA+L-Arginine
Vascular congestion	0.0 \pm 0.0	0.0 \pm 0.0	1.0 \pm 0.0*	1.0 \pm 0.0*
Leukocyte adherence	0.0 \pm 0.0	0.0 \pm 0.0	2.5 \pm 0.3*	1.5 \pm 0.3*, **
Interstitial edema	0.0 \pm 0.0	0.0 \pm 0.0	2.3 \pm 0.5*	1.7 \pm 0.4*
Leukocyte infiltration	0.0 \pm 0.0	0.3 \pm 0.2	2.7 \pm 0.2*	1.7 \pm 0.2*, **
Vacuolisation	0.0 \pm 0.0	0.0 \pm 0.0	1.5 \pm 0.3*	0.5 \pm 0.4 **
Necrosis	0.0 \pm 0.0	0.3 \pm 0.2	3.5 \pm 0.4*	1.5 \pm 0.4*, **
Apoptosis	0.0 \pm 0.0	0.0 \pm 0.0	1.0 \pm 0.0*	1.0 \pm 0.0*
Regeneration	0.0 \pm 0.0	0.0 \pm 0.0	0.5 \pm 0.3	0.2 \pm 0.2
TOTAL	0.0 \pm 0.0	0.5 \pm 0.4	15.0 \pm 1.5*	9.0 \pm 1.3*, **

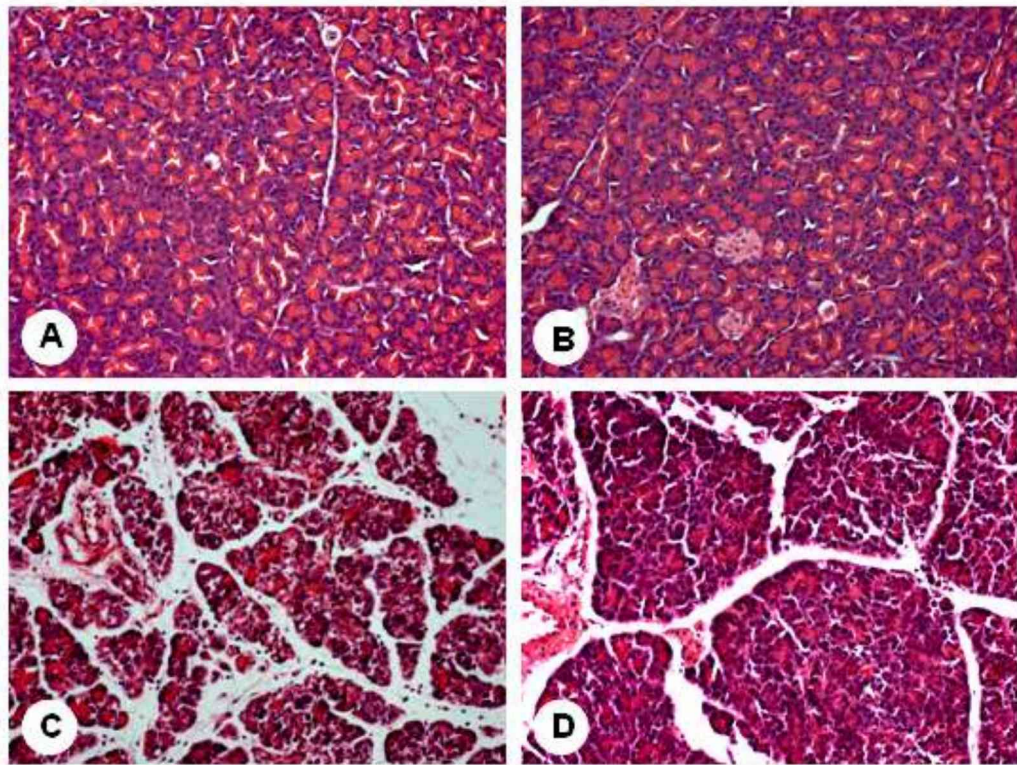


Figure 15. Effect of (+)-S-2-amino-6-iodoacetamidohexanoic acid pretreatment on the pancreatic morphological damage in L-arginine-induced pancreatitis. The histological pictures show hematoxylin and eosin staining images of the pancreas from (A) control, (B) AIHA (15 mg/kg IP), (C) L-arginine (3.5 g/kg IP), or (D) AIHA + L-arginine-treated rats. Original magnification x200. The point values for each of the scored parameters are shown in Table 4.

3.7 Polyamine Homeostasis in L-ornithine-induced Acute Pancreatitis

3.7.1 Pancreas

Pancreatic spermidine content significantly decreased 24 to 168 hours after the IP injection of 3 g/kg L-ornithine, whereas spermine content significantly increased (by 1.6-fold) only at 72 hours (Figs. 16A, B). Considering the different pool sizes of spermidine and spermine, the net effect was a significant depletion of the total polyamine pool beginning from 24 hours after injection of L-ornithine. Pancreatic ODC activity significantly increased at 24 hours (Fig. 16C); SSAT activity significantly increased from 24 hours and peaked at 72 hours with an almost 10-fold maximum elevation and remained significantly higher than the activity in the control group until 168 hours (Fig. 16D). Surprisingly, putrescine was not detectable in the pancreas, although putrescine accumulation should be an evident consequence of simultaneously increased activities of SSAT and ODC (if synthesis would not override catabolism).

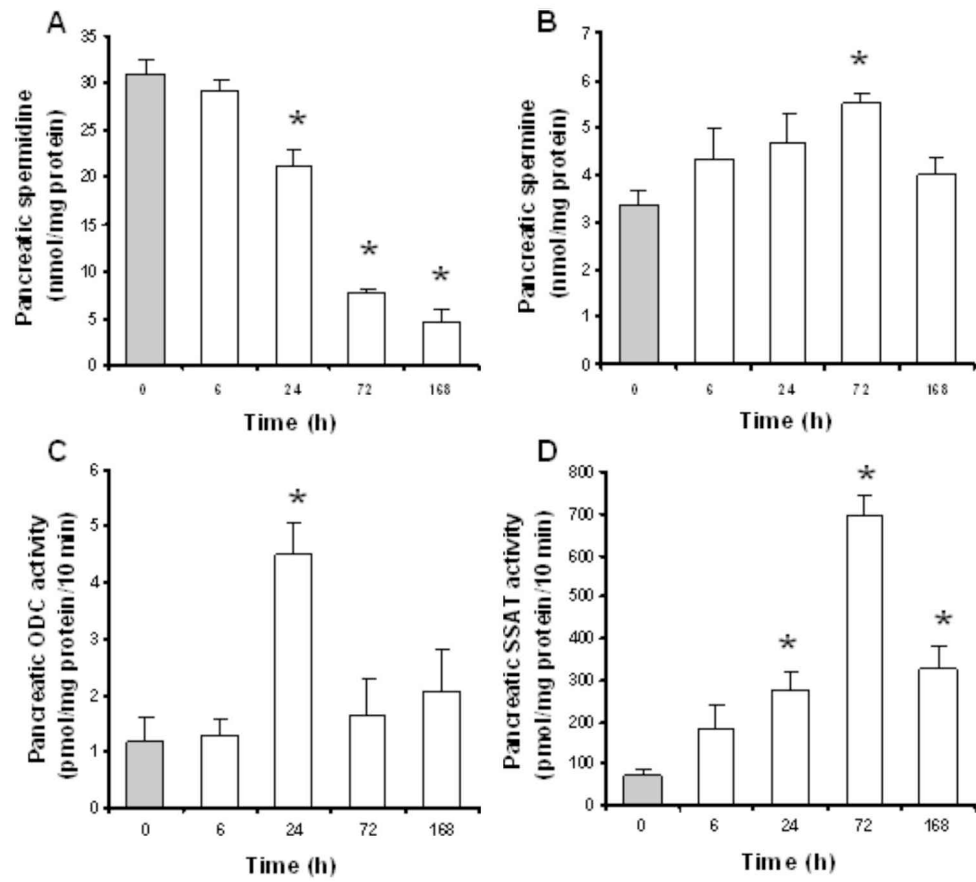


Figure 16. Time-course changes in pancreatic polyamine homeostasis after intraperitoneal administration of 3 g/kg L-ornithine. Pancreatic (A) spermidine and (B) spermine levels and activities of (C) ODC and (D) SSAT are shown. Data are presented as means \pm SEM, n = 5-6. *Significant difference ($P < 0.05$) versus the control group (0 hour, gray column).

3.7.2 Liver

Hepatic putrescine level showed a 26-fold elevation at 6 hours after L-ornithine injection, but thereafter fell back to control values (Fig. 17A). Hepatic spermidine level significantly increased from 6 to 72 hours (Fig. 17B), and spermine content also showed significant elevation from 24 to 72 hours (Fig. 17C).

3.7.3 Lung

Similarly to that observed in the liver, lung putrescine levels showed a significant peak at 6 hours after L-ornithine injection (Fig. 17D). In contrast to the changes observed in the hepatic polyamine pools, lung spermidine content showed significantly decreased levels at 6, 72, and 168 hours (Fig. 17E). Lung spermine content was not significantly altered at the investigated time points (Fig. 17F).

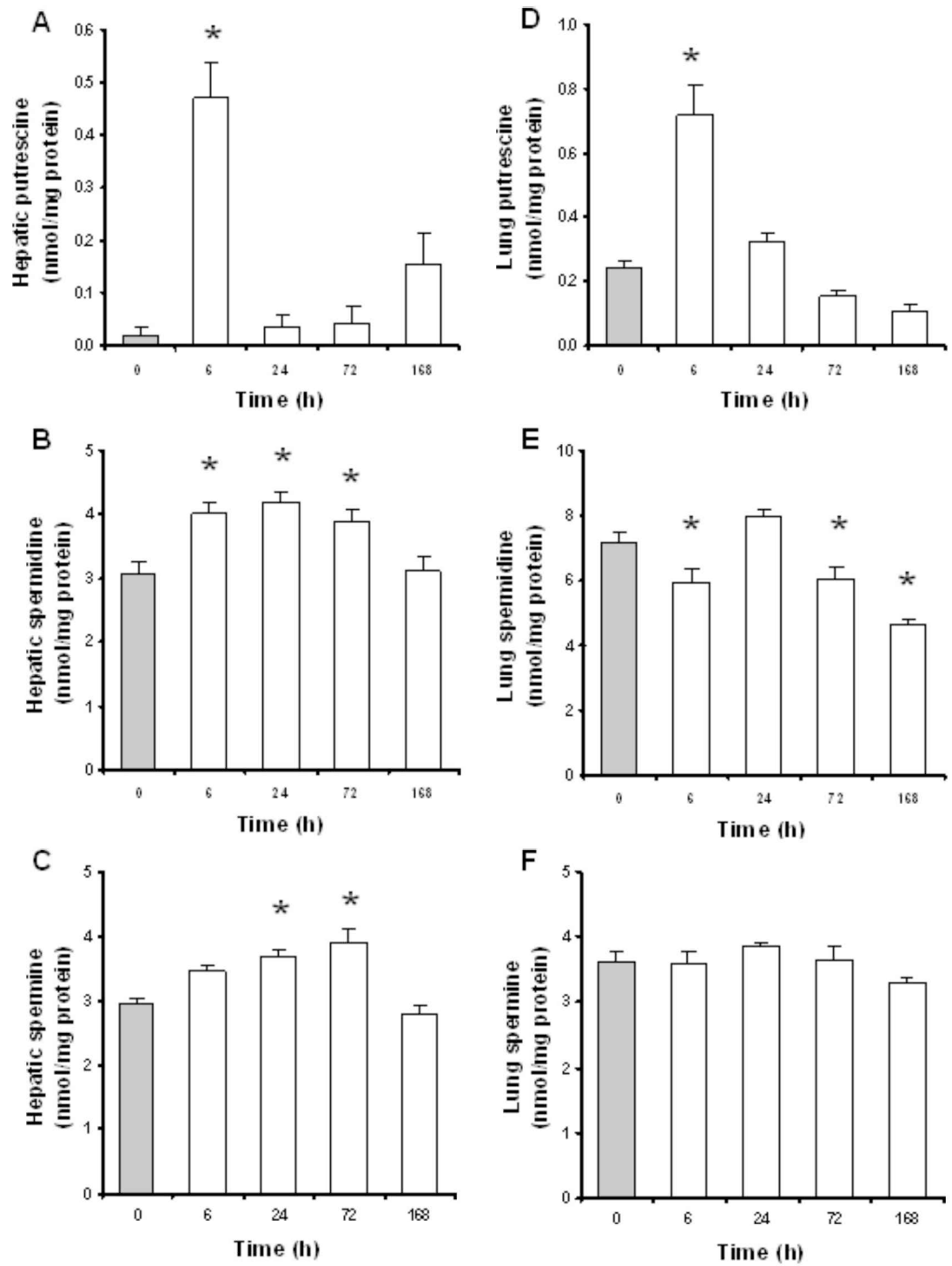


Figure 17. Time-course changes in hepatic and lung polyamine pools after intraperitoneal administration of 3 g/kg L-ornithine. The diagrams demonstrate hepatic (A) putrescine, (B) spermidine, and (C) spermine levels; and lung (D) putrescine, (E) spermidine, and (F) spermine levels. Data are shown as means \pm SEM, $n = 5-6$. *Significant difference ($P < 0.05$) versus the control group (0 hour, gray column).

3.8 Effect of the Synthetic Polyamine Analogue 1-methylspermidine on L-ornithine-induced Acute Pancreatitis

3.8.1 Pancreatic SSAT Activity, Putrescine, Spermidine and Spermine Content

1-methylspermidine accumulated in the pancreas as a result of both pretreatment (MO24: 4.53 ± 1.79 nmol/mg protein) and treatment (OM24: 10.42 ± 1.35 nmol/mg protein, OM48: 8.74 ± 1.18 nmol/mg protein). Pancreatic spermidine content significantly decreased in the L-ornithine-treated groups (Fig. 18A), whereas spermine contents did not show any alteration (Fig. 18B). Pancreatic SSAT activity significantly increased in response to L-ornithine injection by more than 4-fold at 24 hours and more than 7-fold at 48 hours (Fig. 18C). Putrescine was not present in detectable amounts in any of the groups (results not shown). 1-methylspermidine administration did not affect any of these parameters.

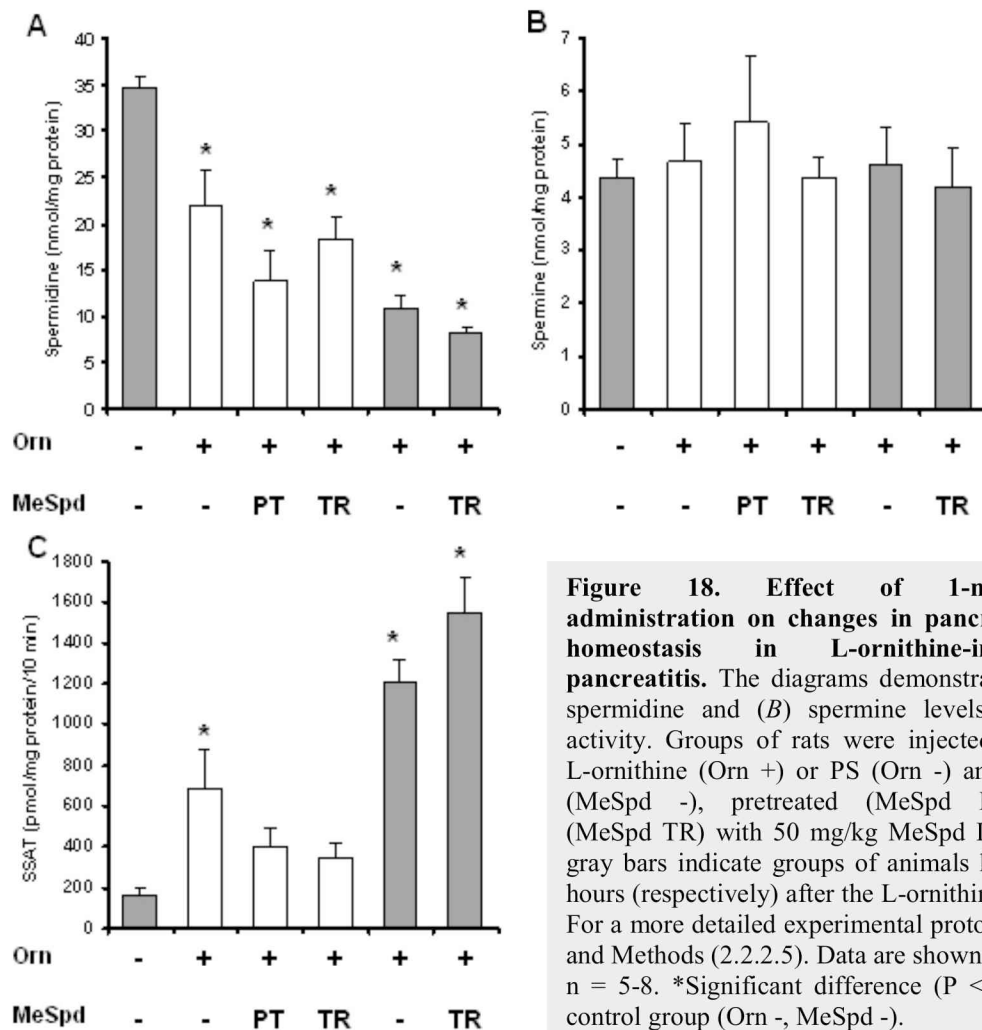


Figure 18. Effect of 1-methylspermidine administration on changes in pancreatic polyamine homeostasis in L-ornithine-induced acute pancreatitis. The diagrams demonstrate pancreatic (A) spermidine and (B) spermine levels and (C) SSAT activity. Groups of rats were injected IP with 3 g/kg L-ornithine (Orn +) or PS (Orn -) and were untreated (MeSpd -), pretreated (MeSpd PT), or treated (MeSpd TR) with 50 mg/kg MeSpd IP. The white and gray bars indicate groups of animals killed at 24 or 48 hours (respectively) after the L-ornithine or PS injection. For a more detailed experimental protocol, see Materials and Methods (2.2.2.5). Data are shown as means \pm SEM, $n = 5-8$. *Significant difference ($P < 0.05$) versus the control group (Orn -, MeSpd -).

3.8.2 Histological Examination

Interstitial edema, vascular congestion, leukocyte adherence and infiltration and necrosis of acinar cells greatly increased at 24 and 48 hours in response to L-ornithine injection (Fig. 19). Apoptosis of acinar cells was detected only at 24 hours. 1-methylspermidine administration did not ameliorate any of the investigated histological parameters (Fig. 19; Table 5).

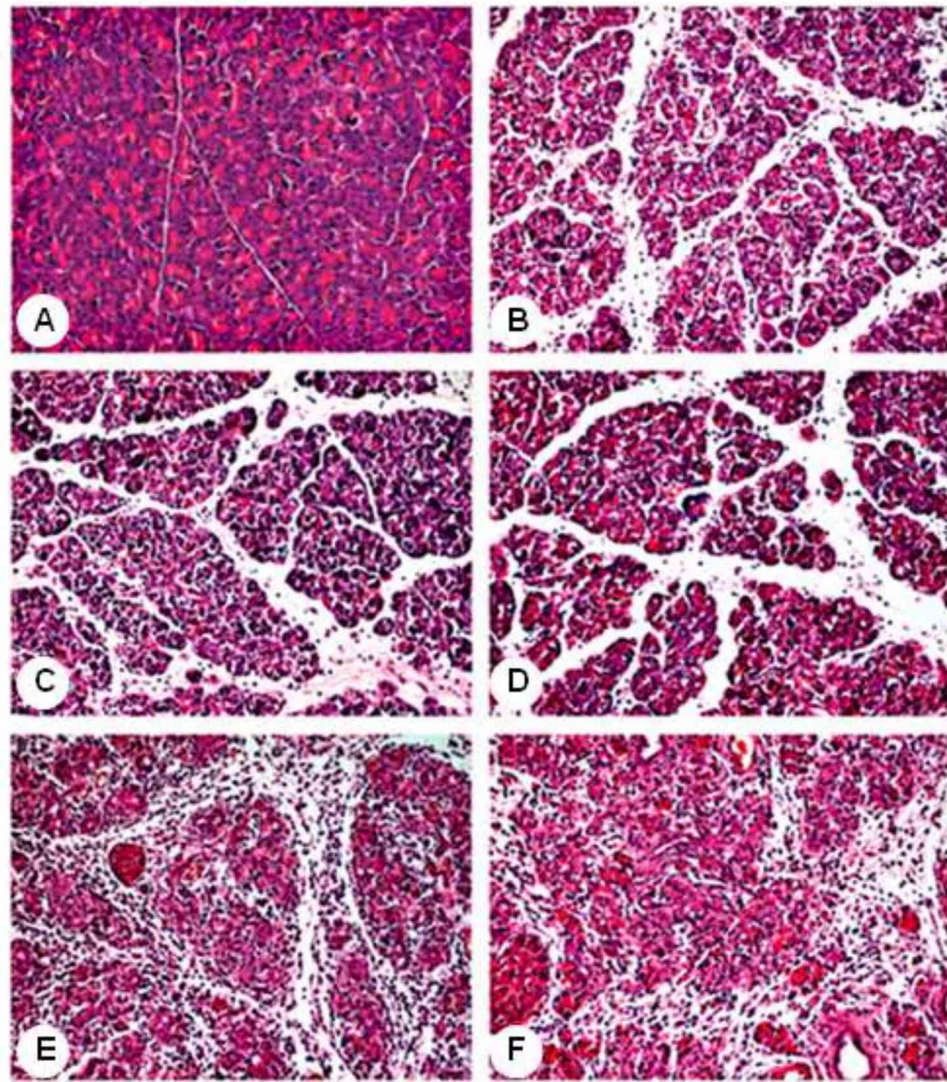


Figure 19. Effect of 1-methylspermidine on pancreatic morphological damage in L-ornithine-induced acute pancreatitis. The images show representative hematoxylin and eosin images of pancreata of rats injected IP with (A) PS or (B-F) 3 g/kg L-ornithine, which were (A, B, E) untreated, (C) pretreated, or (D, F) treated (twice in case of F) with 50 mg/kg MeSpd IP. Animals were killed at (A-D) 24 hours or (E-F) 48 hours after the injection of L-ornithine or PS. Original magnification x200.

Table 5. Effect of 1-methylspermidine on histological parameters in L-ornithine-induced acute pancreatitis. Rats were injected IP with 3 g/kg L-ornithine and were untreated (MeSpd -), pretreated (MeSpd PT), or treated (MeSpd TR, twice in case of the animals killed at 48 hours) with 50 mg/kg MeSpd IP. Animals were killed at 24 or 48 hours after the injection of L-ornithine. Values for control animals (injected IP with PS instead of L-ornithine and MeSpd) were 0.0 ± 0.0 . *Significant difference ($P < 0.05$) versus the control group.

MeSpd	-	PT	TR	-	TR
Time of sacrifice (h)		24		48	
Hyperaemia	$0.6 \pm 0.2^*$	$1.0 \pm 0.0^*$	$0.8 \pm 0.2^*$	$0.8 \pm 0.2^*$	$1.0 \pm 0.0^*$
Leukocyte adherence	$3.0 \pm 0.0^*$	$3.0 \pm 0.0^*$	$2.7 \pm 0.2^*$	$2.8 \pm 0.2^*$	$2.8 \pm 0.2^*$
Interstitialis edema	$2.8 \pm 0.2^*$	$3.0 \pm 0.0^*$	$2.8 \pm 0.2^*$	$3.0 \pm 0.0^*$	$2.8 \pm 0.2^*$
Leukocyte infiltration	$2.9 \pm 0.5^*$	$2.8 \pm 0.3^*$	$3.5 \pm 0.3^*$	$4.0 \pm 0.0^*$	$3.4 \pm 0.2^*$
Vacuolization	0.3 ± 0.2	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.2 ± 0.2
Necrosis	$4.0 \pm 0.0^*$	$4.0 \pm 0.0^*$	$3.8 \pm 0.2^*$	0.0 ± 0.0	0.0 ± 0.0
Apoptosis	$1.8 \pm 0.5^*$	$1.7 \pm 0.4^*$	$1.3 \pm 0.5^*$	0.0 ± 0.0	0.2 ± 0.2
Regeneration	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	$1.8 \pm 0.2^*$	$2.0 \pm 0.0^*$
TOTAL	$15.3 \pm 0.5^*$	$15.5 \pm 0.6^*$	$15.0 \pm 0.8^*$	$12.4 \pm 0.2^*$	$12.4 \pm 0.5^*$

3.8.3 Serum and Pancreatic Amylase Activities, Serum Lipase Activity, and Pancreatic Weight/Body Weight Ratio

Serum amylase activities did not increase significantly in response to L-ornithine injection. However, MeSpd treatment in the OM24 group significantly increased serum amylase activity, and it was also significant versus the O24 group (Fig. 20A). Pancreatic contents of amylase significantly decreased in the L-ornithine-treated groups versus the control group except in the OM24 group (Fig. 20B). Serum lipase activity significantly increased in the L-ornithine-treated groups at 24 hours versus the control group (Fig. 20C). Serum lipase activity significantly increased in the OM24 group versus the O24 group (Fig. 19C). Pancreatic weight/body weight ratio significantly increased at 24 hours in response to 3 g/kg L-ornithine (Fig. 20D). 1-methylspermidine administration did not influence p.w./b.w., either at 24 hours or at 48 hours.

3.8.4 Pancreatic HSP72 and I κ B- α Expression

Injection of 3 g/kg L-ornithine induced pancreatic HSP72 synthesis at 24 hours (Fig. 21). Pancreatic I κ B- α levels decreased significantly 24 and 48 hours after the L-ornithine injection (Fig. 21). 1-methylspermidine administration did not affect HSP72 and I κ B- α levels.

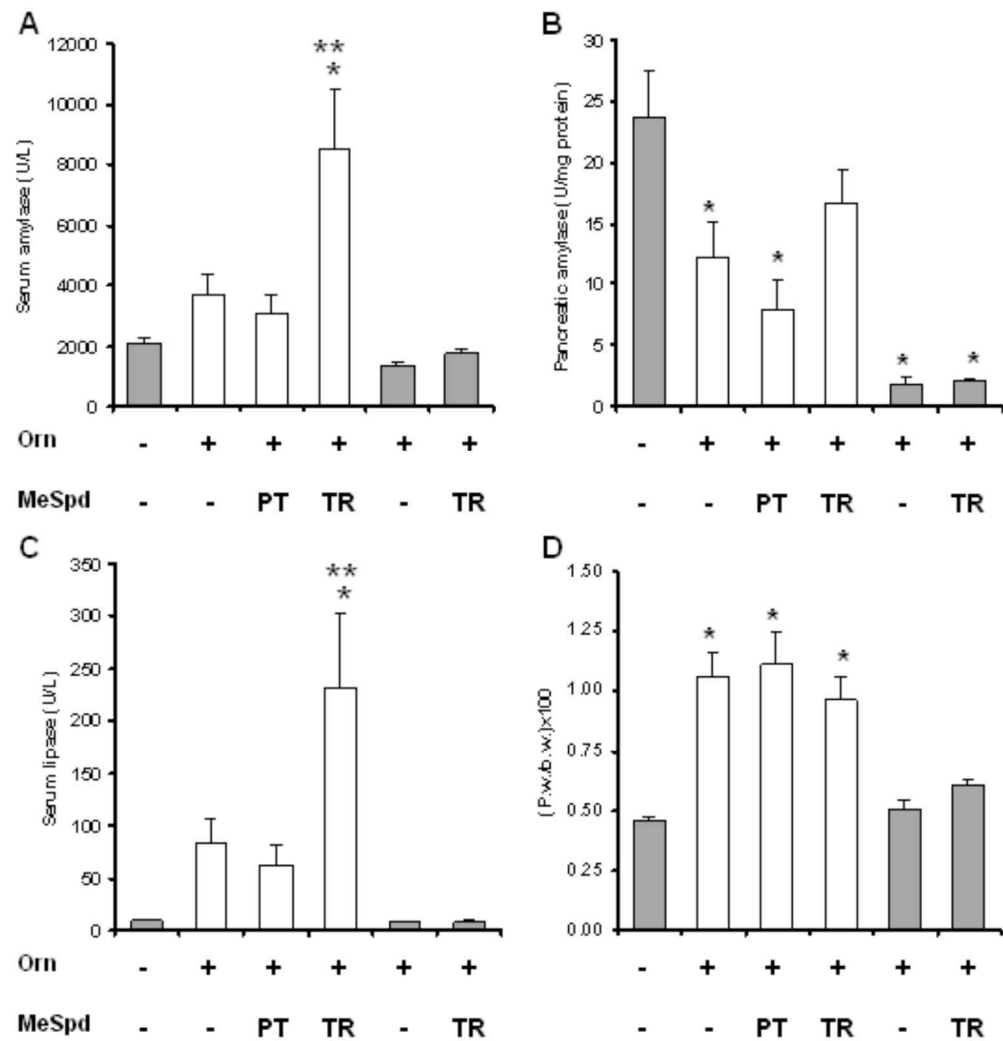


Figure 20. Effect of 1-methylspermidine on the activities of serum and pancreatic amylase, serum lipase and p.w./b.w. ratio in L-ornithine-induced acute pancreatitis. The bar charts demonstrate activities of (A) serum and (B) pancreatic amylase, (C) serum lipase, and (D) p.w./b.w. ratio. See legend of Figure 18 for the labeling of the bars. Data are shown as means \pm SEM, $n = 5-8$. Significant difference ($P < 0.05$) versus the *control (Orn -, MeSpd -) or **Orn 24 hours (Orn +, MeSpd -, white bar) group.

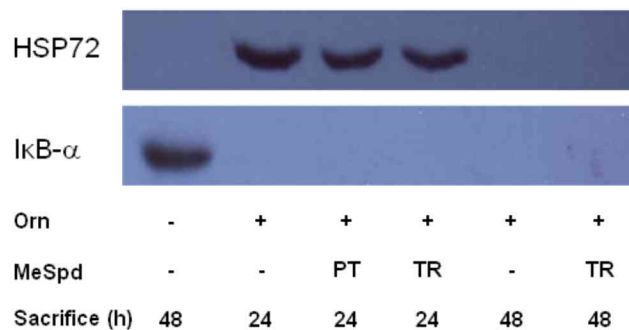


Figure 21. Effect of 1-methylspermidine on pancreatic HSP72 and I κ B- α levels in L-ornithine-induced acute pancreatitis. The figure shows representative Western immunoblot analysis of protein lysates (40 μ g/lane) from the pancreata of rats administered PS, L-ornithine, and/or MeSpd IP. See legend of Figure 18 for the labeling of groups. Rats were sacrificed, as indicated, at 24 or 48 hours after injection with PS or L-ornithine.

3.8.5 Pancreatic Myeloperoxidase Activity, Pancreatic Interleukin-1 β Levels and Serum Concentrations of Creatinine and Aspartate Aminotransferase Activity

Pancreatic MPO activity significantly increased in the L-ornithine-treated groups versus the control group (Fig. 22A). Administration of MeSpd did not influence MPO activity in any of the groups. Corresponding to I κ B degradation, and consequently to activation of NF- κ B, pancreatic IL-1 β synthesis showed significantly elevated levels at 24 and 48 hours after the L-ornithine injection (Fig. 22B). 1-methylspermidine treatments had no significant effects on proinflammatory cytokine levels, although the analogue treatment seemed to partially prevent the elevation of IL-1 β level. Significant elevations of serum creatinine concentrations were detected in the O24 and OM24 groups versus the control group (Fig. 22C). Serum ASAT activities significantly increased 24 and 48 hours after L-ornithine injection (Fig. 22D). 1-methylspermidine administration did not affect serum ASAT activity in any of the groups.

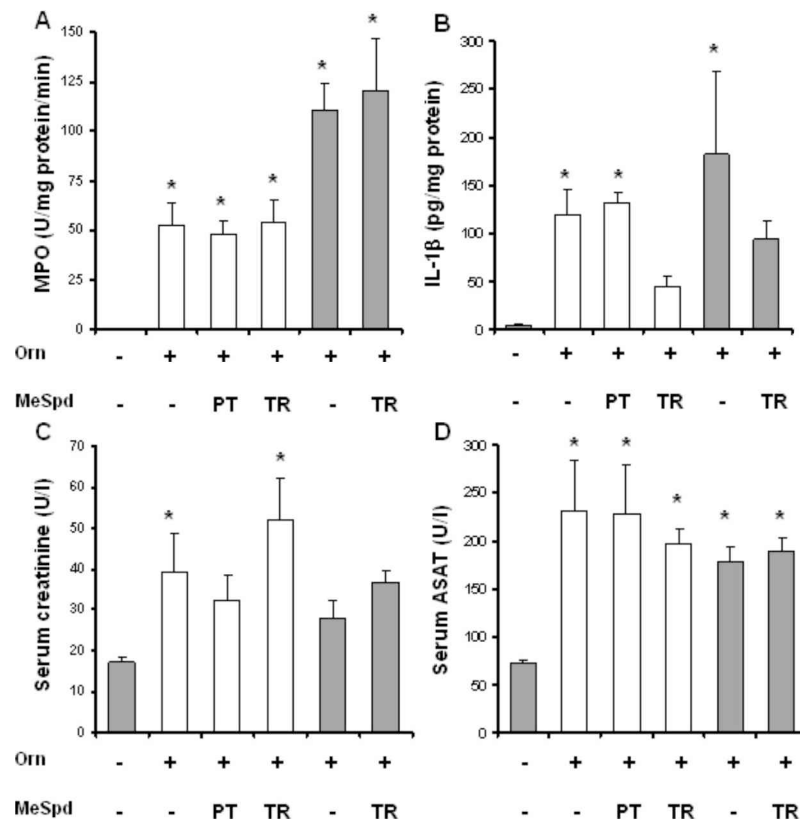


Figure 22. Effect of 1-methylspermidine on pancreatic parameters reflecting inflammation and extra-pancreatic organ involvement in L-ornithine-induced acute pancreatitis. The bar charts demonstrate pancreatic (A) MPO activities, (B) IL-1 β levels and (C) serum creatinine concentrations, and (D) ASAT activities. See legend of Figure 18 for the labeling of the bars. Data are shown as means \pm SEM, n = 5-8. *Significant difference ($P < 0.05$) versus the control group (Orn -, MeSpd -).

4 DISCUSSION

This thesis characterizes a novel model of severe acute necrotizing pancreatitis induced by IP injection of 3 g/kg L-ornithine in rats showing typical laboratory and morphologic signs of that observed in the human disease. This novel pancreatitis model is noninvasive, more diffuse, and reproducible compared with those induced by retrograde ductal injection of bile acids, the closed duodenal loop method, or choline-deficient ethionine diet. L-ornithine-induced pancreatitis is superior to the L-arginine-induced model in that it produces a much severe disease with massive edema and without the confounding effects of possible excessive NO synthesis (at least in the initial phase of pancreatitis induction). The fact that 3 g/kg L-ornithine administration can induce acute pancreatitis in rats was recently confirmed by Zhou et al.^[67] Intraperitoneal administration of 4 to 6 g/kg L-ornithine killed the rats within hours (before pancreatitis could develop). The death of these animals may be the result of effects on the central nervous system.

In response to 3 g/kg L-ornithine the pancreas showed massive interstitial edema, apoptosis, and necrosis of acinar cells and infiltration of neutrophil granulocytes and monocytes. The necrotic process lacked morphologic signs indicative of lytic and ischemic damage to the cells and was evidently the result of toxic injury to the acini. The acini eventually regenerated. Acini that did not regenerate were replaced by fat tissue. The rate of pancreatic apoptosis strongly increased at 16 to 24 hours after the administration of 2.5 g/kg or 4 g/kg L-arginine.^[37, 66] In fact, Kubish *et al.* found that endoplasmic reticulum stress-regulated mechanisms are likely to be involved in the apoptosis observed in this model of the disease.^[37] Notably, in our study, apoptosis was already at its highest level at 6 to 9 hours. A possible explanation for this is that in fact L-ornithine is mediating the apoptotic process (that is, L-arginine has been metabolized).

Several molecular features that have been observed in other models of pancreatitis were observed with administration of L-ornithine. These included intrapancreatic activation of the digestive enzyme trypsinogen^[68-70], degradation of I κ B proteins associated with activation of the transcription factor, NF- κ B^[19], increased IL-1 β production, increased HSP72 content^[71-72], and signs of oxidative stress^[73]. The time course changes of these laboratory parameters were after appearance of first histological signs of acute pancreatitis (4 hours) and were considerably delayed compared with the cerulein-induced pancreatitis model.^[33] These

findings are further supported by that observed in the L-arginine-model, and by our most recent results with another basic amino acid; in L-lysine-induced pancreatitis.^[74] Since premature trypsinogen and NF- κ B activation are considered as key early intra-acinar events in the onset of acute pancreatitis, these results suggest a quite different pathophysiology for basic amino acid-induced compared to cerulein-induced experimental pancreatitis.

There has been longstanding debate on whether NO is involved in the pathogenesis of acute pancreatitis.^[75] In arginine-induced pancreatitis, pancreatic constitutive NOS activity was depleted 6 hours after L-arginine administration, then gradually increased to significantly higher level than the control at 24 hours.^[76] The activity of pancreatic iNOS was significantly increased at 24 hours after L-arginine injection. A number of studies have shown a protective^[77-79], no^[80], or detrimental^[81-83] effect of NO on experimental pancreatitis. A very important finding deduced from the present work is that NO is unlikely to be a primary factor in inducing L-ornithine (or L-arginine) pancreatitis. Serum citrulline levels were only increased by approximately three-fold after injection of rats with 3.5 g/kg L-arginine; L-ornithine levels were up by approximately 54-fold. In fact, this fits in well with previous findings, which have shown that inhibition of NO formation by N-nitro-L-arginine methyl ester in L-arginine-induced pancreatitis only ameliorated laboratory parameters and did not influence pancreatic damage on histology.^[76] In contrast, others have found that aminoguanidine, an isoform-specific inhibitor of iNOS, ameliorated L-arginine induced pancreatic injury.^[82] Anyway, NO is unlikely to be involved in the early events (development) of pancreatitis given the time course of NOS induction. In the L-arginine-induced pancreatitis model, pancreatic cNOS activity was found to be depleted at 6 hrs followed by a gradually increasing level to a peak as observed at 24 hours.^[76] The activity of iNOS was not increased until 24 hours (peak of injury). These results also suggest that L-arginine-induced pancreatitis is due to metabolism of L-arginine to L-ornithine by arginase and is not caused by its metabolism to L-citrulline (and NO) by NOS. That is, L-arginine administration results in a greater increase in blood concentration of ornithine than citrulline, and administration of L-ornithine but not L-citrulline causes severe pancreatitis. This finding suggested a major role for arginase (rather than NOS) in the catabolism of L-arginine.

In this thesis, we have shown that pretreatment with the irreversible arginase inhibitor AIHA significantly reduced pancreatic damage caused by the administration of 3.5 g/kg L-arginine. Arginase is a key enzyme of the hepatic urea cycle, so it was not surprising that

the highest enzyme activity was found in the liver of rats. In accordance with values reported elsewhere^[84], arginase activity was several-order magnitudes higher in the liver compared with other tissues (pancreas, lung, and kidney). Therefore, most likely, large doses of L-arginine are metabolized mainly by the liver. In fact, serum L-arginine concentration was significantly reduced below the control value 24 hours after injection.^[66] Two studies have investigated the changes in serum arginase activity in patients with acute pancreatitis. Serum arginase activity was unaltered in the early phases of acute pancreatitis.^[85] On the contrary, Scibior et al. have found that serum arginase activity was significantly reduced by treatment of the disease.^[86]

(+)-S-2-amino-6-iodoacetamidohexanoic acid is an irreversible inhibitor of the ODC and arginase and has been reported to have antifertility and antitumor effects.^[87-88] The dosing of AIHA was based on literature data.^[87-88] Sixty micromolars of AIHA (which corresponds to an *in vivo* dose of 15 mg/kg) inhibited arginase activity by about 25%. Although this rate of arginase inhibition is not great, even applying 120 μ M AIHA *in vitro* did not result in significantly lower levels of arginase activity versus 60 μ M (Fig. 11). Furthermore, since arginase is a critical enzyme of the urea cycle, most likely we have a limit to what we could decrease enzyme activity without seeing any detrimental effects. Because AIHA also inhibits ODC and ornithine per se can induce acute pancreatitis, the action of AIHA in modifying the acute pancreatitis response to arginine might be due to the inhibition of the latter step, which is the formation of precursor for polyamine synthesis.

Interestingly, administration of AIHA in itself caused a stress response in the pancreas characterized by increased expression of HSP27 and HSP72. This may be due to the fact that inhibition of arginase activity will interfere with the urea cycle and increase levels of toxic ammonia. Preinduction of HSPs has been shown to protect against L-arginine-induced acute pancreatitis^[89], although Takács et al. found only reductions in proinflammatory cytokine synthesis.^[90] Nevertheless, given these contrasting results, we cannot exclude the beneficial effect of these chaperones on disease progression, providing their levels are increased before the initiation of L-arginine pancreatitis.^[71] The administration of AIHA 1 hour before L-arginine injection ameliorated the degree of pancreatic edema, but it did not influence serum amylase activity in L-arginine-induced pancreatitis. Serum amylase activity is not a good marker of disease severity in this pancreatitis model; it is usually only mildly increased in the early phases of the disease. However, AIHA pretreatment also significantly reduced

parameters of pancreatic oxidative stress, trypsin and MPO activity, and histological damage. Taken together, our results suggest that AIHA pretreatment reduces the severity of L-arginine-induced acute pancreatitis most likely by inhibiting arginase activity. Conversion of L-arginine to L-ornithine by arginase seems to be detrimental in L-arginine-induced acute pancreatitis.

L-ornithine can serve as substrate for ODC, the initial and rate-limiting enzyme in the polyamine biosynthetic pathway. A large dose of L-ornithine/L-arginine will inevitably influence polyamine levels. The involvement of polyamines in the pathogenesis of acute pancreatitis was first found in transgenic rats overexpressing SSAT.^[47] Later studies have further supported that polyamine catabolism seems to be a general feature of acute pancreatitis.^[38, 49-50] Interestingly, we have also found increased pancreatic spermidine catabolism (possibly mediated via activation of SSAT) in L-ornithine-induced acute pancreatitis. In contrast, polyamine levels generally increased in the lung and liver with the exception of lung spermidine levels, which decreased. Assuming that the direct transformation of L-ornithine into putrescine may secure specific targeting of the polyamine metabolism but consists of a partial utilization of L-ornithine, „detoxification” of the excess amino acid load may also include conversion of L-ornithine to citrulline or glutamic semialdehyde. The influence of these metabolic routes cannot be excluded. In this thesis on L-ornithine-induced acute pancreatitis, we have shown that polyamine levels are altered only after the first signs of histological damage. Whereas the earliest events (vascular congestion, vacuolization, and apoptosis of acinar cells) in L-ornithine-induced acute pancreatitis pancreas can already be detected at 4 hours, polyamine catabolism started only after 6 hours. The belated elevations in the activity of SSAT and spermidine catabolism suggest that these mechanisms do not take part in the initiation of L-ornithine-induced acute pancreatitis. Nevertheless, polyamine catabolism was apparent at 24 hours after the injection of L-ornithine when the highest degree of pancreatic injury was detected. The earliest time point at which pancreatic polyamine pools and SSAT activity were investigated in the similar L-arginine-induced model was 24 hours.^[38] However, in taurocholate-induced pancreatitis, pancreatic SSAT activity already increased from 3 to 6 hours.^[50] An interesting difference between the taurocholate-induced, L-arginine-induced, and the L-ornithine-induced models is that neither spermine catabolism nor putrescine accumulation could be detected in the pancreas despite the elevations in SSAT and ODC activities in the L-ornithine-induced

model. As polyamine homeostasis is controlled not only by their metabolism, but also by their secretion and uptake, these findings might be a consequence of altered polyamine transport.^[91-92] They may also be an indication of accelerated polyamine metabolic cycle, as described for SSAT transgenic mice^[93], which could in part explain the lack of spermine depletion in L-ornithine-induced pancreatitis where the synthesis dominates over the catabolism. As acute pancreatitis will affect extrapancreatic organs such as the liver and lung, we also investigated changes of polyamine pools in these tissues. Depletion of spermidine and spermine levels has been reported in red blood cells of rats with taurocholate-induced pancreatitis, and these changes correlated with the extent of pancreatic necrosis.^[94] We have demonstrated that changes in polyamine pools in response to L-ornithine injection occur not only in the pancreas and blood, but also in the liver and lung. Although a marked accumulation of putrescine was observed in these tissues at 6 hours, subsequent activation of polyamine catabolism was not detected in these organs. Only a moderate decrease was found in lung spermidine levels.

Synthetic α -methylated polyamine analogues, such as MeSpd, are metabolically more stable than natural polyamines as they are not substrates for SSAT and are poor substrates for spermine synthase.^[95] Nevertheless, they are supposed to fulfill most of the putative cellular functions of natural spermidine and spermine. Methylated derivatives of polyamines are substrates of proteins involved in polyamine transport and accumulate in the pancreas after IP administration. Moreover, they act as substitutes of natural polyamines.^[96] 1-methylspermidine pretreatment totally prevented zinc-induced pancreatitis in transgenic rats overexpressing SSAT as judged by plasma α -amylase activity and histopathology.^[51] We administered MeSpd (to compensate for depleted pancreatic spermidine levels) as a single-injection pretreatment before the induction of L-ornithine-induced pancreatitis or twice as treatment after the induction of pancreatitis. Pretreatment or treatment with MeSpd did not influence the levels of natural polyamines and SSAT activity. In both cases, MeSpd was ineffective at reducing the severity of the disease. This was demonstrated by measuring several laboratory and histological parameters of acute pancreatitis. Both the pretreatment and treatment with MeSpd led to decreased spermidine levels in the L-ornithine-induced pancreatitic groups, which suggest that catabolism of the natural polyamines further increased in the presence of synthetic polyamine analogue or the analogue replaces the natural spermidine in its binding site in the tissue. This may be explained by the finding that the

analogue did not accumulate in the pancreas to the level compensating for the most of spermidine depletion with the exception of the treatment group at 24 hours. The dosing of MeSpd was based on literature data, we applied the same doses as Hyvönen et al. with the L-arginine model.^[38] The current results are partly in contrast with the earlier studies on L-arginine- and taurocholate-induced pancreatitis. 1-methylspermidine pretreatment was shown to reduce the severity of L-arginine-induced (2.5 g/kg) pancreatitis without preventing SSAT activation and subsequent polyamine catabolism.^[38] In contrast (and in accordance with our results), the severity of cerulein-induced (7 x 50 µg/kg) pancreatitis was not affected by MeSpd pretreatment.^[38] The common feature is that neither in cerulein-induced pancreatitis nor in L-ornithine-induced pancreatitis did the analogue accumulate to the levels compensating for the depleted polyamine pools. Bismethylspermine treatment (but not pretreatment) ameliorated pancreatic injury at 24 hours after the induction of taurocholate-induced pancreatitis, but it did not ameliorate the late progression of the pancreatic necrosis at 72 hours.^[94] Moreover, bismethylspermine treatment resulted in lethal toxicity at 72 hours after induction of pancreatitis.^[94] We must note that the infusion of sodium taurodeoxycholate (2%) into the pancreatic duct or IP injection of 2.5 g/kg L-arginine will result in milder pancreatitis versus 3 g/kg L-ornithine. In the present work, it was shown that increased SSAT activity and consequent spermidine catabolism in the pancreas are apparent phenomena in L-ornithine-induced acute pancreatitis in rats. However, the fact that these changes occur belatedly and that MeSpd administration did not ameliorate any of the parameters, either in the pretreatment or in treatment group, suggests that activated polyamine catabolism does not play a role in the initiation of pancreatic injury in L-ornithine-induced experimental pancreatitis. The organ-specific responses to L-ornithine and the method of pancreatic damage induction by the basic amino acid need to be investigated.

In summary, we have developed a simple, noninvasive, reproducible model of acute necrotizing pancreatitis in rats by intraperitoneal injection of 3 g/kg L-ornithine and examined its time course. Large doses of L-arginine (known to induce acute pancreatitis) may also produce a toxic effect on the pancreas, at least in part, through L-ornithine. Further studies are needed to determine how these basic amino acids produce pancreatic injury and to reveal the exact role of polyamine homeostatic processes in the maintenance of pancreatic integrity.

5 ACKNOWLEDGEMENTS

I would like to thank the people who have helped and inspired me during my doctoral studies.

I am grateful to **Prof. János Lonovics** and **Prof. Tibor Wittmann**, past and present head of the First Department of Medicine, who gave me the opportunity to work in the department.

I would like express my deep and sincere gratitude to my supervisors **Dr. Zoltán Rakonczay Jr.** and **Dr. Péter Hegyi** who provided me the opportunity to work in their laboratory. Their wide knowledge and their logical way of thinking have been of great value for me. Their understanding and encouragement provided a good basis for the present thesis.

I would also like to thank my colleagues, **Dr. Mátyás Czepán**, **Dr. Klaudia Farkas**, **Imre Ignáth**, **Linda Judák**, **Éva Kunstár**, **Dr. József Maléth**, **Petra Pallagi**, **Andrea Schnúr** and **Dr. Viktória Venglovecz** for all the emotional support, entertainment and care they provided.

This work would not have been possible to accomplish without the assistance of **Miklósne Árva**, **Zoltánné Fuksz**, **Bélané Horesnyi**, **Zoltán Kocsispéter** and **Edit Magyarné Pálfi**.

I would also like to thank my **co-authors** for their help. Our research was supported by grants from **OTKA**, **MTA**, **NKTH** and **Academy of Finland**.

My deepest gratitude goes to **my family** for their unflagging love and support throughout my life; this dissertation would have been impossible to accomplish without their help. I dedicate this thesis to them.

6 REFERENCES

1. Pandol SJ, Saluja AK, Imrie CW, et al. Acute pancreatitis: bench to the bedside. *Gastroenterology* 2007;132:1127-1151.
2. Frossard JL, Steer ML, Pastor CM. Acute pancreatitis. *Lancet* 2008;371:143-152.
3. Saluja AK, Lerch MM, Phillips PA, et al. Why does pancreatic overstimulation cause pancreatitis? *Annu Rev Physiol* 2007;69:249–269.
4. Munsell MA, Buscaglia JM. Acute pancreatitis. *J Hosp Med* 2010;5:241-250.
5. Pezzilli R. Pharmacotherapy for acute pancreatitis. *Expert Opin Pharmacother* 2009;10:2999-3014.
6. Foitzik T, Hotz HG, Eibl G, et al. Experimental models of acute pancreatitis: are they suitable for evaluating therapy? *Int J Colorectal Dis* 2000;15:127–135.
7. Pastor CM, Frossard JL. Are genetically modified mice useful for the understanding of acute pancreatitis? *FASEB J* 2001;15: 893–897.
8. Pandol SJ, Gukovsky I, Satoh A, et al. Emerging concepts for the mechanism of alcoholic pancreatitis from experimental models. *J Gastroenterol* 2003;38:623–628.
9. Schneider A, Whitcomb DC, Singer MV. Animal models in alcoholic pancreatitis—what can we learn? *Pancreatology* 2002;2:189–203.
10. Chan YC, Leung PS. Acute pancreatitis – Animal models and recent advances in basic research. *Pancreas* 2007;34:1-14.
11. Takács T, Szabolcs A, Biczó G, et al. [The clinical relevance of experimental acute pancreatitis models] *Orv Hetil* 2008;149:1981-1986. [Article in Hungarian]
12. Grady T, Saluja A, Kaiser A, et al. Edema and intrapancreatic trypsinogen activation precede glutathione depletion during caerulein pancreatitis. *Am J Physiol* 1996;271:G20–G26.
13. Luthen R, Niederau C, Grendell JH. Intrapancreatic zymogen activation and levels of ATP and glutathione during caerulein pancreatitis in rats. *Am J Physiol* 1995;268:G592–G604.
14. Grady T, Mah’Moud M, Otani T, et al. Zymogen proteolysis within the pancreatic acinar cell is associated with cellular injury. *Am J Physiol* 1998;275:G1010– G1017.

15. Saluja AK, Bhagat L, Lee HS, et al. Secretagogue-induced digestive enzyme activation and cell injury in rat pancreatic acini. *Am J Physiol* 1999;276:G835–G842.
16. Wulczyn FG, Krappmann D, Scheidereit C. The NF- κ B/Rel and I κ B gene families: mediators of immune response and inflammation. *J Mol Med* 1996;74:749–769.
17. Chen X, Ji B, Han B, et al. NF- κ B activation in pancreas induces pancreatic and systemic inflammatory response. *Gastroenterology* 2002;122:448–457.
18. Han B, Logsdon CD. Cholecystokinin induction of mob-1 chemokine expression in pancreatic acinar cells requires NF- κ B activation. *Am J Physiol* 1999;277:C74–82.
19. Rakonczay Z Jr, Hegyi P, Takács T, et al. The role of NF-kappaB activation in the pathogenesis of acute pancreatitis. *Gut* 2008;57:259–267.
20. Rakonczay Z Jr, Duda E, Kaszaki J, et al. The anti-inflammatory effect of methylprednisolone occurs down-stream of nuclear factor- κ B DNA binding in acute pancreatitis. *Eur J Pharmacol* 2003;464:217–227.
21. Makhija R, Kingsnorth AN. Cytokine storm in acute pancreatitis. *J Hepatobiliary Pancreat Surg* 2002;9:401–410.
22. Denham W, Yang J, Fink G, et al. Gene targeting demonstrates additive detrimental effects of interleukin 1 and tumor necrosis factor during pancreatitis. *Gastroenterology* 1997;113:1741–1746.
23. Blinman TA, Gukovsky I, Mouria M, et al. Activation of pancreatic acinar cells on isolation from tissue: cytokine upregulation via p38 MAP kinase. *Am J Physiol Cell Physiol* 2000;279:C1993–2003.
24. Gukovskaya AS, Gukovsky I, Zaninovic V, et al. Pancreatic acinar cells produce, release, and respond to tumor necrosis factor- α . Role in regulating cell death and pancreatitis. *J Clin Invest* 1997;100:1853–1862.
25. Yu JH, Lim JW, Namkung W, et al. Suppression of cerulein-induced cytokine expression by antioxidants in pancreatic acinar cells. *Lab Invest* 2002;82:1359–1368.
26. Grady T, Liang P, Ernst SA, et al. Chemokine gene expression in rat pancreatic acinar cells is an early event associated with acute pancreatitis. *Gastroenterology* 1997;113:1966–1975.
27. Nevalainen TJ, Seppä A. Acute pancreatitis caused by closed duodenal loop in the rat. *Scand J Gastroenterol* 1975;10:521–527.
28. Aho HJ, Koskensalo SM, Nevalainen TJ. Experimental pancreatitis in the rat. Sodium taurocholate-induced acute haemorrhagic pancreatitis. *Scand J Gastroenterol* 1980;15:411–416.

29. Lombardi B, Estes LW, Longnecker DS. Acute hemorrhagic pancreatitis (massive necrosis) with fat necrosis induced in mice by DL-ethionine fed with a choline-deficient diet. *Am J Pathol* 1975;79:465–480.
30. Lampel M, Kern HF. Acute interstitial pancreatitis in the rat induced by excessive doses of a pancreatic secretagogue. *Virchows Arch A Pathol Anat Histol* 1977;373:97–117.
31. Mizunuma T, Kawamura S, Kishino Y. Effects of injecting excess arginine on rat pancreas. *J Nutr* 1984;114:467–471.
32. Tani S, Itoh H, Okabayashi Y, et al. New model of acute necrotizing pancreatitis induced by excessive doses of arginine in rats. *Dig Dis Sci* 1990;35:367–374.
33. Hegyi P, Rakonczay Z Jr, Sári R, et al. L-arginine-induced experimental pancreatitis. *World J Gastroenterol* 2004;10:2003–2009.
34. Dawra R, Sherif R, Phillips PA, et al. Development of a new mouse model of acute pancreatitis induced by administration of Larginine. *Am J Physiol Gastrointest Liver Physiol* 2007;292:G1009–1018.
35. Szabolcs A, Reiter RJ, Letoha T, et al. The effect of melatonin on the severity of L-arginine-induced experimental acute pancreatitis in rats. *World J Gastroenterol* 2006;12:251–258.
36. Szabolcs A, Biczó G, Rakonczay Z Jr, et al. Simultaneous proteasome inhibition and heat shock protein induction by bortezomib is beneficial in experimental pancreatitis. *Eur J Pharmacol* 2009;616:270–274.
37. Kubisch CH, Sans MD, Arumugam T, et al. Early activation of endoplasmic reticulum stress is associated with arginine-induced acute pancreatitis. *Am J Physiol Gastrointest Liver Physiol* 2006;291:G238–245.
38. Hyvönen MT, Herzig KH, Sinervirta R, et al. Activated polyamine catabolism in acute pancreatitis: alpha-methylated polyamine analogues prevent trypsinogen activation and pancreatitis-associated mortality. *Am J Pathol* 2006;168:115–122.
39. Morris SM. Enzymes of arginine metabolism. *J Nutr* 2004;134:2743S–2747S.
40. Alhonen L, Uimari A, Pietilä M, et al. Transgenic animals modelling polyamine metabolism-related diseases. *Essays Biochem* 2009;4:125–144.
41. Basu HS, Schwietert HC, Feuerstein BG, et al. Effects of variation in the structure of spermine on the association with DNA and the induction of DNA conformational changes. *Biochem J* 1990;269:329–334.

42. Jänne J, Alhonen L, Pietilä M, et al. Genetic approaches to the cellular functions of polyamines in mammals. *Eur J Biochem* 2004;271:877–894.
43. Hakovirta, H, Keiski A, Toppari J, et al. Polyamines and regulation of spermatogenesis: selective stimulation of late spermatogonia in transgenic mice overexpressing the human ornithine decarboxylase gene. *Mol Endocrinol* 1993;7:1430–1436.
44. Halmekytö M, Hyttinen JM, Sinervirta R, et al. Transgenic mice aberrantly expressing human ornithine decarboxylase gene. *J Biol Chem* 1991;266:19746–19751.
45. Halmekytö M, Alhonen L, Alakuijala L, et al. Transgenic mice over-producing putrescine in their tissues do not convert the diamine into higher polyamines. *Biochem J* 1993;291:505–508.
46. Suppola S, Heikkinen S, Parkkinen JJ, et al. Concurrent overexpression of ornithine decarboxylase and spermidine/spermine N(1)-acetyltransferase further accelerates the catabolism of hepatic polyamines in transgenic mice. *Biochem J* 2001;358:343–348.
47. Alhonen L, Parkkinen JJ, Keinänen T, et al. Activation of polyamine catabolism in transgenic rats induces acute pancreatitis. *Proc Natl Acad Sci USA* 2000;97:8290–8295.
48. Merentie M, Uimari A, Pietilä M. Oxidative stress and inflammation in the pathogenesis of activated polyamine catabolism-induced acute pancreatitis. *Amino Acids* 2007;33:323–330.
49. Hyvönen MT, Merentie M, Uimari A, et al. Mechanisms of polyamine catabolism-induced acute pancreatitis. *Biochem Soc Trans* 2007;35:326–330.
50. Jin HT, Lämsä T, Merentie M, et al. Polyamine levels in the pancreas and the blood change according to the severity of pancreatitis. *Pancreatology* 2008;8:15–24.
51. Räsänen TL, Alhonen L, Sinervirta R, et al. A polyamine analogue prevents acute pancreatitis and restores early liver regeneration in transgenic rats with activated polyamine catabolism. *J Biol Chem* 2002;277:39867–39872.
52. Löser C, Fölsch UR, Cleffmann U, et al. Role of ornithine decarboxylase and polyamines in camostate (Foy-305)-induced pancreatic growth in rats. *Digestion* 1989;43:98–112.
53. Grigorenko NA, Vepsäläinen J, Järvinen A, et al. A new synthesis of alpha-methylspermidine. *Bioorgan Khim (Moscow)* 2004;30:441–445.
54. Chace DH, Millington DS, Terada N, et al. Rapid diagnosis of phenylketonuria by quantitative analysis for phenylalanine and tyrosine in neonatal blood spots by tandem mass spectrometry. *Clin Chem* 1993;39:66–71.
55. Gukovsky I, Gukovskaya AS, Blinman TA, et al. Early NF-kappaB activation is associated with hormone-induced pancreatitis. *Am J Physiol* 1998;275:G1402–1414.

56. Hrabák A, Bajor T, Mészáros G. The inhibitory effect of various indolyl amino acid derivatives on arginase activity in macrophages. *Amino Acids* 2008;34:293-300.
57. Hyvönen T, Keinänen TA, Khomutov AR, et al. Monitoring of the uptake and metabolism of aminooxy analogues of polyamines in cultured cells by high-performance liquid chromatography. *J Chromatogr* 1992;574:17-21.
58. Jänne J, Williams-Ashman HG. On the purification of L-ornithine decarboxylase from rat prostate and effects of thiol compounds on the enzyme. *J Biol Chem* 1971;246:1725-1732.
59. Bernacki RJ, Bergeron RJ, Porter CW. N,N'-bis(ethyl)spermine homologues against human MALME-3 melanoma xenografts. *Cancer Res* 1992;52:2424-2430.
60. Kuebler WM, Abels C, Schuerer L, et al. Measurement of neutrophil content in brain and lung tissue by a modified myeloperoxidase assay. *Int J Microcirc Clin Exp* 1996;16:89-97.
61. Rakonczay Z Jr, Takács T, Boros I, et al. NF- κ B activation is detrimental in arginine-induced acute pancreatitis. *Free Radic Biol Med* 2003;34:696-709.
62. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 1976;72:248-254.
63. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970;227:680-685.
64. Kurucz I, Tombor B, Prechl J, et al. Ultrastructural localization of HSP-72 examined with a new polyclonal antibody raised against the truncated variable domain of the heat shock protein. *Cell Stress Chaperones* 1999;4:139-152.
65. Czakó L, Takács T, Varga IS, et al. Involvement of oxygen-derived free radicals in L-arginine-induced acute pancreatitis. *Dig Dis Sci* 1998;43:1770-1777.
66. Trulsson L, Sandström P, Sundqvist T, et al. The influence of a load of L-arginine on serum amino acids and pancreatic apoptosis/proliferation and ATP levels in the rat. *Pancreas* 2004;29:e113-120.
67. Zhou H, Liu L, Bai Y, et al. Damage of the interstitial cells of Cajal and myenteric neurons causing ileus in acute necrotizing pancreatitis rats. *Surgery* 2010 (EPUB).
68. Mithofer K, Fernandez-del Castillo C, Rattner D, et al. Subcellular kinetics of early trypsinogen activation in acute rodent pancreatitis. *Am J Physiol* 1998;274:G71-79.
69. Bialek R, Willemer S, Arnold R, et al. Evidence of intracellular activation of serine proteases in acute cerulein-induced pancreatitis in rats. *Scand J Gastroenterol* 1991;26:190-196.

70. Saluja AK, Donovan EA, Yamanaka K, et al. Cerulein-induced in vitro activation of trypsinogen in rat pancreatic acini is mediated by cathepsin B. *Gastroenterology* 1997;113:304–310.
71. Rakonczay Z Jr, Takács T, Boros I, et al. Heat shock proteins and the pancreas. *J Cell Physiol* 2003;195:383–391.
72. Saluja A, Dudeja V. Heat shock proteins in pancreatic diseases. *J Gastroenterol Hepatol* 2008;23:S42–45.
73. Schulz HU, Niederau C, Klonowski-Stumpe H, et al. Oxidative stress in acute pancreatitis. *Hepatogastroenterology* 1999;46:2736–2750.
74. Biczó G, Hegyi P, Dósa S, et al. The crucial role of early mitochondrial injury in L-lysine-induced acute pancreatitis. *Antioxid Redox Signal* (under revision).
75. Hegyi P, Rakonczay Z Jr. The role of nitric oxide in the physiology and pathophysiology of the exocrine pancreas. *Antioxid Redox Signal* (under revision).
76. Takács T, Czakó L, Morschl É, et al. The role of nitric oxide in edema formation in L-arginine induced acute pancreatitis. *Pancreas* 2002;25:31–38.
77. Molero X, Guarner F, Salas A, et al. Nitric oxide modulates pancreatic basal secretion and response to cerulein in the rat: effects in acute pancreatitis. *Gastroenterology* 1995;108:1855–1862.
78. Werner J, Fernández-del Castillo C, Rivera JA, et al. On the protective mechanisms of nitric oxide in acute pancreatitis. *Gut* 1998;43:401–407.
79. Sugiyama Y, Kato S, Mitsufuji S, et al. Pathogenic role of endothelial nitric oxide synthase (eNOS/NOS-III) in cerulein-induced rat acute pancreatitis. *Dig Dis Sci* 2006;51:1396–1403.
80. Weidenbach H, Lerch MM, Gress TM, et al. Vasoactive mediators and the progression from edematous to necrotizing experimental acute pancreatitis. *Gut* 1995;37:434–440.
81. Dabrowski A, Gabryelewicz A. Nitric oxide contributes to multiorgan oxidative stress in acute experimental pancreatitis. *Scand J Gastroenterol* 1994;29:943–948.
82. Lomis TJ, Siffring CW, Chalasani S, et al. Nitric oxide synthase inhibitors N-monomethylarginine and aminoguanidine prevent the progressive and severe hypotension associated with a rat model of pancreatitis. *Am Surg* 1995;61:7–10.
83. Shields CJ, Delaney CP, Winter DC, et al. Induction of nitric oxide synthase is a key determinant of progression to pulmonary injury in experimental pancreatitis. *Surg Infect (Larchmt)* 2006;7:501–511.

84. Herzfeld A, Raper SM. The heterogeneity of arginases in rat tissues. *Biochem J* 1976;153:469-478.
85. Sandström P, Trulsson L, Gasslander T, et al. Serum amino acid profile in patients with acute pancreatitis. *Amino Acids* 2008;35:225-231.
86. Scibior D, Ashamiss F, Wierzbicki Z, et al. Arginase activity in blood serum of patients with acute and chronic pancreatitis. *Pol Merkuriusz Lek* 2006;21:522-524.
87. Trujillo-Ferrara J, Koizumi G, Muñoz O, et al. Antitumor effect and toxicity of two new active-site-directed irreversible ornithine decarboxylase and extrahepatic arginase inhibitors. *Cancer Lett* 1992;67:193-197.
88. Chamorro G, Salazar M, Salazar S, et al. Antifertility effects of (+)-S-2-amino-6-iodoacetamidohexanoic acid (2-AIHA) in female rats. *Contraception* 1996;53:247-251.
89. Tashiro M, Ernst SA, Edwards J, et al. Hyperthermia induces multiple pancreatic heat shock proteins and protects against subsequent arginine-induced acute pancreatitis in rats. *Digestion* 2002;65:118-126.
90. Takács T, Rakonczay Z Jr, Varga IS, et al. The comparative effects of water immersion pretreatment on three different acute pancreatitis models in rats. *Biochem Cell Biol* 2002;80:241-251.
91. Wallace HM, Fraser AV, Hughes A. A perspective of polyamine metabolism. *Biochem J* 2003;376:1-14.
92. Moinard C, Cynober L, de Bandt JP. Polyamines: metabolism and implications in human diseases. *Clin Nutr* 2005;24:184-197.
93. Pirinen E, Kuulasmaa T, Pietilä M, et al. Enhanced polyamine catabolism alters homeostatic control of white adipose tissue mass, energy expenditure, and glucose metabolism. *Mol Cell Biol* 2007;27:4953-4967.
94. Jin HT, Lämsä T, Hyvönen MT, et al. A polyamine analog bismethylspermine ameliorates severe pancreatitis induced by intraductal infusion of taurodeoxycholate. *Surgery* 2008;144:49-56.
95. Lakanen JR, Coward JK, Pegg AE. alpha-Methyl polyamines: metabolically stable spermidine and spermine mimics capable of supporting growth in cells depleted of polyamines. *J Med Chem* 1992;35:724-734.
96. Järvinen A, Grigorenko N, Khomutov AR, et al. Metabolic stability of alpha-methylated polyamine derivatives and their use as substitutes for the natural polyamines. *J Biol Chem* 2005;280:6595-6601.

I.

A new severe acute necrotizing pancreatitis model induced by L-ornithine in rats

Zoltán Rakonczay Jr, MD, PhD; Péter Hegyi, MD, PhD; Sándor Dósa, MD; Béla Iványi, MD, PhD, DSc; Katalin Jármay, MD, PhD; György Biczó, MSc; Zsuzsanna Hracskó, MSc; Ilona S. Varga, MSc, PhD; Eszter Karg, MD, PhD; József Kaszaki, MSc, PhD; András Varró, MD, PhD, DSc; János Lonovics, MD, PhD, DSc; Imre Boros, MSc, PhD, DSc; Ilya Gukovsky, MSc, PhD; Anna S. Gukovskaya, MSc, PhD, DSc; Stephen J. Pandol, MD; Tamás Takács, MD, PhD, DSc

Objective: Intraperitoneal administration of large doses of L-arginine is known to induce severe acute pancreatitis in rats. We therefore set out to determine whether metabolites of L-arginine (L-ornithine, L-citrulline, and nitric oxide) cause pancreatitis.

Design: The authors conducted an *in vivo* animal study.

Setting: This study was conducted at a university research laboratory.

Subjects: Study subjects were male Wistar rats.

Interventions: Dose-response and time course changes of laboratory and histologic parameters of pancreatitis were determined after L-arginine, L-ornithine, L-citrulline, or sodium nitroprusside (nitric oxide donor) injection.

Measurements and Main Results: Intraperitoneal injection of 3 g/kg L-ornithine but not L-citrulline or nitroprusside caused severe acute pancreatitis; 4 to 6 g/kg L-ornithine killed the animals within hours. Serum and ascitic amylase activities were significantly increased, whereas pancreatic amylase activity was decreased after intraperitoneal injection of 3 g/kg L-ornithine. The increase in pancreatic trypsin activity (9–48 hrs) correlated with the degradation of I κ B proteins and elevated interleukin-1 β levels. Oxidative stress in the pancreas was evident from 6 hrs;

HSP72 synthesis was increased from 4 hrs after L-ornithine administration. Morphologic examination of the pancreas showed massive interstitial edema, apoptosis, and necrosis of acinar cells and infiltration of neutrophil granulocytes and monocytes 18 to 36 hrs after 3 g/kg L-ornithine injection. One month after L-ornithine injection, the pancreas appeared almost normal; the destructed parenchyma was partly replaced by fat. Equimolar administration of L-arginine resulted in lower pancreatic weight/body weight ratio, pancreatic myeloperoxidase activity, and histologic damage compared with the L-ornithine-treated group. L-ornithine levels in the blood were increased 54-fold after intraperitoneal administration of L-arginine.

Conclusions: We have developed a simple, noninvasive model of acute necrotizing pancreatitis in rats by intraperitoneal injection of 3 g/kg L-ornithine. Interestingly, we found that, compared with L-arginine, L-ornithine was even more effective at inducing pancreatitis. Large doses of L-arginine produce a toxic effect on the pancreas, at least in part, through L-ornithine. (Crit Care Med 2008; 36:2117–2127)

KEY WORDS: L-ornithine; L-arginine; acute pancreatitis

Acute pancreatitis is a sudden inflammatory disorder of the pancreas (1). The disease can present in a mild or severe form. Although the overall mortality of patients with acute pancreatitis is approximately 5%, a great proportion of deaths is the result of the necrotizing form of the disease. To study the pathomecha-

nism and to test possible treatment options of severe acute pancreatitis, a number of experimental models have been developed. However, most of these models are invasive (such as the retrograde ductal injection of bile acids and the closed duodenal loop method) (2, 3), or pancreatic damage is not that reproducible or homogeneous in distribution (like

in choline-deficient ethionine-supplemented diet) (4). However, the noninvasive nature, reproducibility, and homogeneity (as found with the edematous cerulein pancreatitis model [5]) are preferential in an experimental setup. Severe acute pancreatitis induced by intraperitoneal (IP) injection of rats and mice with L-arginine fulfills these criteria and its use is getting more popular with researchers investigating this challenging disease (6–9). The mechanism by which L-arginine produces pancreatic injury is unknown. Free radicals, including nitric oxide (NO), inflammatory mediators (nuclear factor- κ B, interleukins), endoplasmic reticulum stress, and polyamines, have all been implicated in this response (8, 10, 11).

The metabolism of L-arginine can occur through a number of different pathways. Two key enzymes that are involved

*See also p. 2208.

From the First Department of Medicine (ZR, PH, KJ, GB, JL, TT), the Department of Pathology (SD, BI), the Department of Biochemistry and Molecular Biology (ZH, ISV, IB), the Department of Pediatrics (EK), the Institute of Experimental Surgery (JK), and the Department of Pharmacology and Pharmacotherapy (AV), University of Szeged, Szeged, Hungary; and the Veterans Affairs Greater Los Angeles Healthcare System (IG, ASG, SJP), University of California, Los Angeles, CA.

Supported by the Hungarian Scientific Research Fund (K60406 to TT and PF63951 to ZR), the Hungarian Academy of Sciences (BO 00276/04 to PH and BO 00218/06 to ZR),

and The Physiological Society, UK (Junior Fellowship to ZR).

The rabbit anti-HSP72 antibody was a generous gift from Dr. István Kurucz (IVAX Drug Research Institute, Budapest, Hungary) (33). We thank the Rosztóczy Foundation for helping to establish contact between the University of Szeged and UCLA through their generous scholarship awarded to ZR.

For information regarding this article, E-mail: raz@in1st.szote.u-szeged.hu

Copyright © 2008 by the Society of Critical Care Medicine and Lippincott Williams & Wilkins

DOI: 10.1097/CCM.0b013e31817d7f5c

in this process are NO synthase (NOS) and arginase. NOS (which has three isoforms: the constitutive endothelial and neuronal, and an inducible form [iNOS]) catalyze the conversion of L-arginine into NO and L-citrulline, whereas arginase hydrolyzes L-arginine to L-ornithine and urea (12). Therefore, we set out to determine whether equimolar doses of the L-arginine metabolites L-ornithine or L-citrulline and/or the NO donor sodium nitroprusside cause pancreatitis in rats. Intraperitoneally injected L-ornithine but not L-citrulline or nitroprusside induced severe acute necrotizing pancreatitis. The main aim of this study was to characterize the dose-response and time course changes of L-ornithine administration.

MATERIALS AND METHODS

Materials

Chemicals were from Sigma-Aldrich (Munich, Germany) unless stated otherwise.

Experimental Protocol

Animals. Male Wistar rats weighing 200 to 240 g were used. The animals were kept at a constant room temperature of 24°C with a 12-hr light-dark cycle and were allowed free access to water and standard laboratory chow (Biofarm, Zagyvaszántó, Hungary). In each experimental group, four to ten rats were used. The experiments performed in this study were approved by the Animal Care Committee of the university.

Pilot study. In a pilot study, rats ($n = 3-5$) were injected IP with equimolar (11.7 mL/kg 1.424 M/L) L-arginine (cat. A5131), L-citrulline (cat. 27510), and/or the NO donor sodium nitroprusside (cat. 71780), L-ornithine (cat. O8305), or D-ornithine (cat. 75480). The animals were killed by exsanguination through the abdominal aorta 24 hrs after the IP injection. To determine the serum concentrations

of arginine, citrulline, and ornithine after injection of L-arginine, rats were killed at 2, 4, 6, and 12 hrs. The pancreas was quickly removed, cleaned from fat and lymph nodes, weighed, and frozen in liquid nitrogen and stored at -80°C until use. All blood samples were centrifuged at 2500 g for 20 mins and the serum was stored at -25°C .

Dose-response and Time Course Changes of L-ornithine Injection. To study the dose-response ($n = 6$) of L-ornithine, rats were injected IP with 1 to 6 g/kg body weight of L-ornithine (dissolved in physiological saline at a concentration of 300 mg/mL, pH = 7.4) and were killed after 24 hrs as described previously. For the time course studies ($n = 4-10$), the rats were injected with 3 g/kg L-ornithine and were killed 2 to 72 hrs, 1 wk, or 1 month after the injection. The control animals received physiological saline IP and were killed 24 hrs after the injection.

Assays

Serum, Pancreatic and Ascites Amylase Activity, Serum Aspartate Aminotransferase Activity, and Concentrations of Glucose, Calcium, Triglyceride, Urea, Creatinine, Arginine, Ornithine, and Citrulline. Laboratory parameters, excluding the amino acids, were determined by standard kits from Dialab (Vienna, Austria). The amino acids were assayed in dried serum specimens according to the method of Chace et al (13).

Pancreatic Weight/Body Weight Ratio. This ratio was used to evaluate the degree of pancreatic edema.

Pancreatic Trypsin Activity. Active trypsin in pancreatic tissue homogenates was measured as described previously (14).

Expression of Pancreatic HSP72, I κ B- α , and I κ B- β . Western blot analysis of pancreatic HSP72, I κ B- α , and I κ B- β expression was performed from the cytosolic fraction of the pancreas homogenate as described previously (15).

Pancreatic Interleukin-1 β Concentrations. The proinflammatory interleukin-1 β concentrations were measured in the pancreatic cytosolic fractions with an enzyme-linked im-

munosorbent assay kit (R&D Systems, Minneapolis, MN) according to the manufacturers' instructions.

Pancreatic Nonprotein Sulfhydryl Group Content and the Activities of Glutathione Peroxidase and Superoxide Dismutase. To determine nonprotein sulfhydryl group content and activities of glutathione peroxidase, Mn-, and Cu/Zn-superoxide dismutase (SOD), a part of the pancreas was homogenized, the homogenates centrifuged at 3000 g for 10 mins, and the supernatants were used for measurements as described previously (16).

Pancreatic Myeloperoxidase Activity. Pancreatic myeloperoxidase activity, as a marker of tissue leukocyte infiltration, was assessed by the method of Kuebler et al (17).

Detection of Apoptosis in the Pancreas

Pancreatic Genomic DNA Analysis. A biochemical hallmark of apoptosis is a characteristic form of DNA degradation in which the genome is cleaved at internucleosomal sites, generating a ladder-like pattern of DNA fragments (multiples of 180 bp) when analyzed by agarose gel electrophoresis. For a qualitative assessment of genomic DNA fragmentation/degradation, rat pancreata were ground to a powder with a mortar and pestle under liquid nitrogen and were then homogenized by five strokes in a glass Dounce homogenizer with 1.5- to four-fold excess of extraction buffer (50 mmol/L Tris-HCl [pH = 8.0], 50 mmol/L EDTA, .5% sodium dodecyl sulfate, and .2 mg/mL proteinase K). The homogenates were transferred to Eppendorf tubes and rotated at 55°C overnight. The DNA solution was extracted twice with TE-saturated phenol, once with 1:1 Tris-EDTA-saturated phenol:chloroform and once with chloroform. The DNA was precipitated by adding .1 vol 3 mol/L sodium acetate (pH = 5.5) and 2 vol 96% ethanol. DNA precipitate was collected by centrifugation at 13,000 g for 10 mins, rinsed with 70% ethanol, vacuum-dried, resuspended in 100 to 200 μL Tris-EDTA

Table 1. Histological scoring system for the evaluation of pancreatic injury in rats

	Scores				
	0	1	2	3	4
Interstitial edema	0	Mild	Moderate	Severe	—
Vascular congestion	0	Present	—	—	—
Leukocyte adherence to vessel walls	0	Mild	Moderate	Extensive	—
Leukocyte infiltration	0	Focal	Diffuse/mild	Diffuse/moderate	Diffuse/severe
Vacuolization (% of total acinar cells)	0	Focal (<10)	11-25	26-50	51-75
Necrosis (% of total acinar cells)	0	Focal (<10)	11-25	26-50	51-75
Number of apoptotic bodies (% of total acinar cells)	0	<5	6-10	11-20	—
Regeneration	0	Present	—	—	—

The slides were coded and read for the traditional histological markers of pancreatic tissue injury by two independent observers who were blind to the experimental protocol.

buffer, and finally digested for 1 hr at 37°C with DNase-free RNase. Fifteen to 20 µg of DNA was electrophoretically fractionated on a 1.8% agarose gel with .5 µg/mL ethidium bromide.

TdT-mediated dUTP Nick End-labeling Technique. Apoptotic cells were quantitated by TdT-mediated dUTP nick end-labeling (TUNEL) assay using an In Situ Cell Death Detection Kit from Roche Diagnostics (Mannheim, Germany) according to the manufacturer's instructions. The number of apoptotic cells was counted in .5 mm² of pancreatic tissue. Results are expressed as percentage of the number of cell nuclei in the same area in control tissue. Note that this method of calculation will underestimate the rates of apoptosis in edematous pancreata.

Microscopy. Cells showing characteristic changes of apoptosis were also recognized by light and electron microscopic techniques (see subsequently).

Histologic Examination

Light Microscopy. A portion of the pancreas, liver, kidney, and lungs was fixed in 6% neutral formaldehyde solution and subsequently embedded in paraffin. Sections were cut at 4 µm thickness and stained with hematoxylin and eosin. The slides were coded and read by two independent observers who were blind to the experimental protocol. Pancreatic tissue injury was evaluated as follows: semiquantitative grading of interstitial edema (0–3), vascular congestion (0–1), leukocyte adhesion (0–3) and infiltration (0–4), and apoptosis (0–3) and necrosis (0–4) of acinar cells was determined in each animal (described in more detail in Table 1). Signs indicative of regeneration: mitotic figures, ductuloacinar structures, and basophilia of acinar cells were recorded.

Transmission Electron Microscopy. Small pieces of pancreata were prepared for electron microscopy 0, 6, and 24 hrs and 1 wk after L-ornithine treatment as described previously (18).

Statistical Analysis

Results are expressed as means ± SEM. Experiments were evaluated by using the analysis of variance followed by Dunnett's multiple comparison *post hoc* test. Values of *p* < .05 were accepted as significant.

RESULTS

Pilot Study

We first tested the effects of 3.5 g/kg L-arginine and its metabolites (administered at equimolar doses) on serum amylase activity, pancreatic weight/body weight ratio, pancreatic myeloperoxidase activity, and pancreatic histology 24 hrs

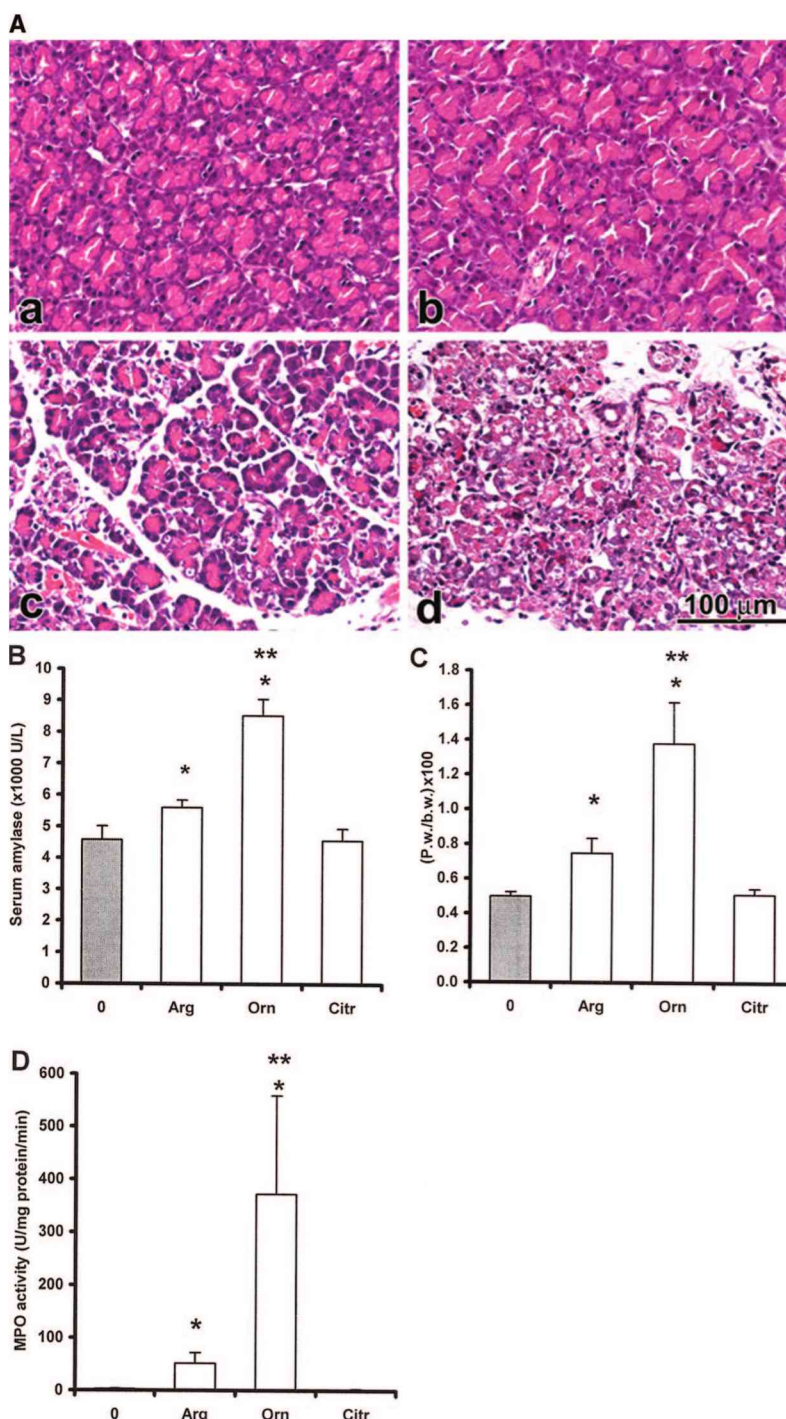


Figure 1. Intraperitoneal (IP) administration of L-ornithine to rats resulted in necrotizing acute pancreatitis, which was more severe than that induced with L-arginine. Rats were injected intraperitoneally with physiologic saline (control, 0) or equimolar L-arginine (Arg, 3.5 g/kg), L-ornithine (Orn, 2.8 g/kg), L-citrulline (Citr, 2.9 g/kg), and/or the nitric oxide donor sodium nitroprusside (4.95 g/kg). The animals that received sodium nitroprusside alone or in combination with L-citrulline died by the next morning. The rest of the rats survived the treatment and were killed by exsanguination through the abdominal aorta 24 hrs after the IP injection. A, The diagrams show light micrographs (hematoxylin and eosin staining) of the pancreata of (a) control, (b) citrulline (Citr.), (c) arginine (Arg), or (d) ornithine (Orn)-treated rats. The bar diagrams show the (B) serum amylase activity, (C) pancreatic weight (Pw) body weight (b.w.) ratio, and (D) pancreatic myeloperoxidase activity. Data are shown as means ± SEM, n = 3–5. *Significant difference (*p* < .05) vs. the control group (0 hr). **Significant difference (*p* < .05) vs. the Arg group. MPO, myeloperoxidase.

after the injection ($n = 3$). Interestingly, the IP injection of 2.8 g/kg L-ornithine caused a more severe pancreatitis compared with the L-arginine group (Fig. 1; Table 2). Similar effect was seen with the 3 g/kg L-ornithine dose, so this was used throughout the rest of the study. In contrast, IP administration of 3 g/kg D-ornithine did not result in pancreatic injury ($n = 5$, results not shown). A dose of 2.9 g/kg L-citrulline did not cause an alteration in any of the measured parameters and the pancreas seemed normal in histology (Fig. 1; Table 2). The animals that received sodium nitroprusside (4.95 g/kg) alone or in combination with L-citrulline became lethargic soon after the injection and died by the next morning. This is in accord with the material safety data sheet provided with nitroprusside by Sigma-Aldrich, who report the IP LD50 for this compound at only 7 mg/kg. Autopsy did not show pancreatitis in these animals. Most likely they died as a result of vascular complications, that is, hypotension.

Time Course Changes of Serum Arginine, Citrulline, and Ornithine Concentrations in Rats Injected Intraperitoneally with 3.5 g/kg L-arginine. Serum arginine, citrulline, and ornithine concentrations ($n = 5$) were all significantly increased after the injection of L-arginine (Fig. 2). Importantly, there were much greater increases in serum ornithine versus citrulline levels after L-arginine injection.

Dose-response of Intraperitoneal Injection of 1 to 6 g/kg L-ornithine

The rats injected with 1 or 2 g/kg L-ornithine did not develop any pancreatic lesions ($n = 6$, results not shown). However, 3 g/kg of this basic amino acid caused a severe acute pancreatitis as described subsequently. A dose of 4 to 6 g/kg ($n = 6$) killed the animals within a couple of hours after the injection after developing lethargy, neurologic and neuromuscular symptoms (tremor, twitching, convulsion, and in some cases jumping all over the cage).

Time Course Studies After Intraperitoneal Injection of Rats with 3 g/kg L-ornithine

Macroscopic Observations. The pancreas appeared edematous from 18 to 36 hrs, its peak being at 24 hrs. Ascites and adhesions of organs were seen from 4 to 6 hrs (peaking at

24 hrs). Occasionally, yellow-white foci indicative of chalky fat necrosis was detected in the mesentery of the bowels and retroperitoneum at 24 to 72 hrs. Dilated small and large bowels suggesting functional ileus was apparent at 72 hrs to 1 wk after L-ornithine injection.

Histologic Examination of the Pancreas (Table 3)

0 to 6 Hrs. The pancreas appeared normal 2 hrs after L-ornithine (3 g/kg)

injection. At 4 hrs, mild interstitial edema and foamy vacuolization of the acini and vascular congestion were observed. At 6 hrs, the number of apoptotic bodies was greatly increased and we could also observe focal necrosis (<10%) of acini (Fig. 3). Electron microscopy revealed the appearance of large autophagic vacuoles containing zymogen granules in varying stages of degradation, dense material, myelin figures, and mitochondrial

Table 2. Evaluation of pancreatic injury 24 hrs after the intraperitoneal injection of physiological saline (control, 0), equimolar L-citrulline (Citr, 2.9 g/kg), L-arginine (Arg, 3.5 g/kg) or L-ornithine (Orn, 2.8 g/kg)

	Control, 0	Citr	Arg	Orn
Interstitial edema	0	0	1	3
Vascular congestion	0	0	1	1
Leukocyte adherence	0	0	1	3
Leukocyte infiltration	0	0	1	3
Vacuolization	0	0	1	1
Apoptosis	0	0	1	2
Necrosis	0	0	3	4

Scores are shown for an average of three animals.

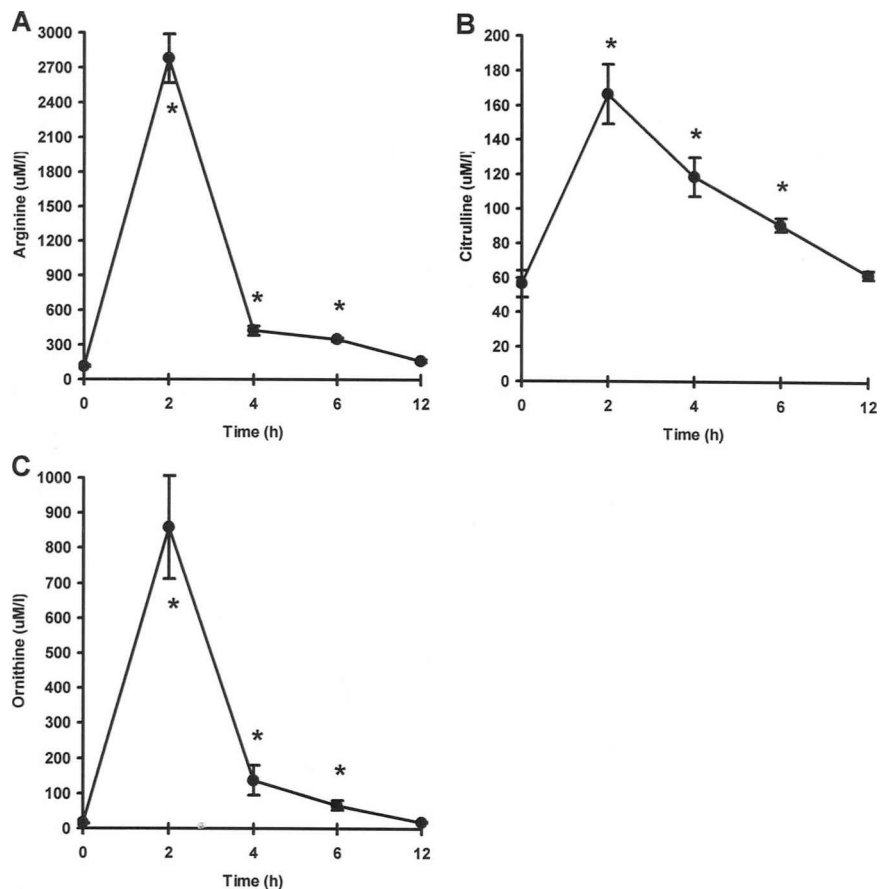


Figure 2. Time course changes of serum arginine, citrulline, and ornithine concentrations in rats injected intraperitoneally with 3.5 g/kg L-arginine. 2 hrs after the L-arginine injection; serum (A) arginine concentration was increased by approximately 25-fold, (B) citrulline concentration by approximately three-fold, and (C) ornithine concentration by approximately 54-fold. Data are shown as means \pm SEM, $n = 5$. *Significant difference ($p < .05$) vs. the control group (0 hrs).

Table 3. Evaluation of pancreatic injury by histological examination 2–168 hrs and 1 month after intraperitoneal injection of rats with 3 g/kg L-ornithine

	2 hrs	4 hrs	6 hrs	9 hrs	12 hrs	18 hrs	24 hrs	36 hrs	48 hrs	72 hrs	168 hrs	1 Month
Interstitial edema	0	0	1	1	1	2	3	2	1	0	0	0
Vascular congestion	0	1	1	1	1	1	1	1	1	1	1	0
Leukocyte adherence	0	0	0	1	3	3	3	2	1	0	1	0
Leukocyte infiltration	0	0	0	1	3	3	3	4	4	4	3	0
Vacuolization	0	1	2	3	2	1	1	0	0	0	0	0
Apoptosis	0	1	3	3	3	3	2	2	1	1	0	0
Necrosis	0	0	1	2	3	4	4	3	3	0	0	0
Regeneration	0	0	0	0	0	0	0	1	1	1	1	1

Scores are shown for an average of four animals.

remnants in acinar cells at 6 hrs (Fig. 4). The foamy vacuolization corresponded to accumulation of lipid droplets of medium density in the basal compartment of the cells. Overall, tight junctions, mitochondria, and endoplasmic reticulum were well preserved. In some acinar cells, condensation of the chromatin under the nuclear membrane and disappearance of the nucleoli with or without shrinkage of the cytoplasm (ongoing apoptosis) were detected. In these cells, the mitochondrial cristae and the rough endoplasmic reticulum were mildly dilated with disengagement of ribosomes from the latter.

9 to 12 Hrs. At 9 hrs, there was interstitial edema, neutrophilic and monocytic adherence, and focal infiltration. There were great numbers of large autophagic vacuoles and apoptotic bodies. The extent of acinar cell necrosis was 15% to 25%. At 12 hrs, there was diffuse moderate infiltrate of monocytes and neutrophils, and the necrosis of acinar cells was 26% to 50%.

18 to 24 Hrs. Eighteen hours after the L-ornithine injection, the extent of pancreatic edema was greatly increased and there was a decrease in the number of autophagic vacuoles in the acinar cells. Necrosis of acinar cells was increased to 51% to 75%. The most severe interstitial edema was observed at 24 hrs. Electron microscopy of tissue at 24 hrs showed severe but zonal damage to the acini as a whole and several types of cell death (Fig. 4). Damaged, necrotic cells predominantly localized to the center of the acini, and the less severely injured cells were observed in the basal part of the acini. There were large autophagic vacuoles containing zymogen granules in varying stages of degradation, myelin figures, and/or granular electron-dense material. There was a marked reduction or complete disappearance of zymogen granules. Two main types of nuclear damage were

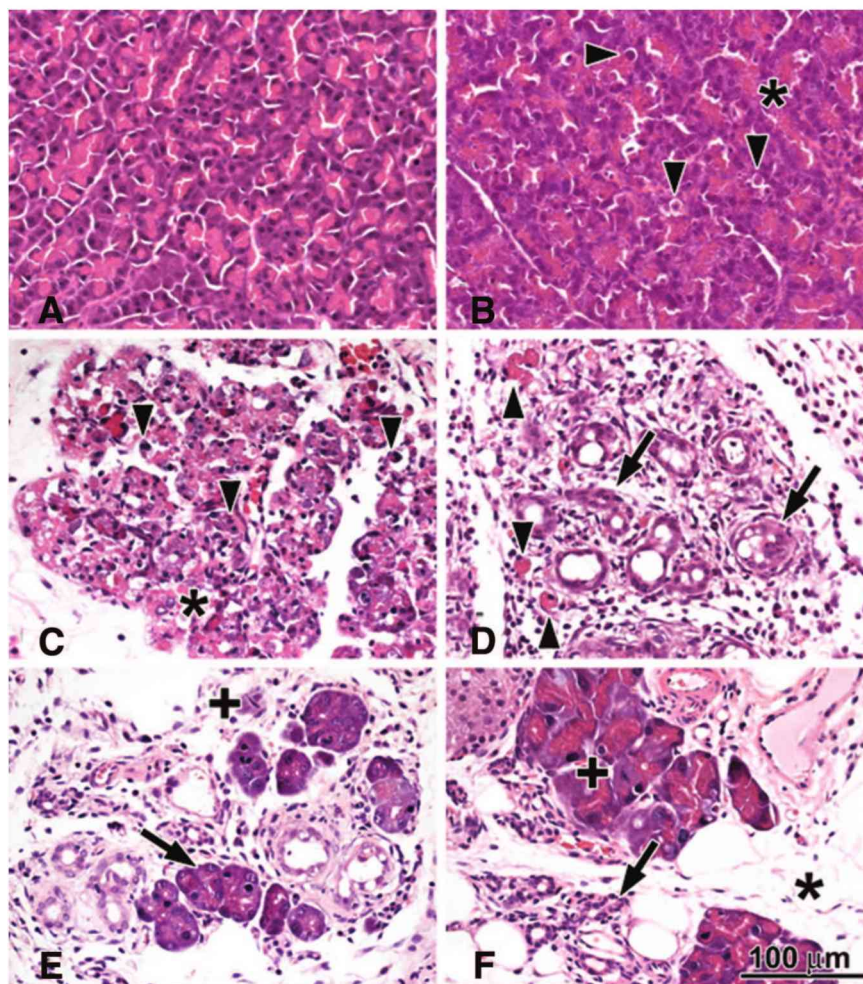


Figure 3. Histopathologic changes of the pancreas in response to intraperitoneal administration of 3 g/kg L-ornithine. *A*, Two hours: no major histopathologic changes. *B*, Six hours: foamy degeneration (asterisk) of acinar cells; arrowheads show apoptotic bodies. *C*, Twenty-four hours: widespread necrosis of acinar cells (asterisk) and lots of apoptotic bodies. *D*, Seventy-two hours: ductuloacinar structures (arrow) in place of disappeared acini; fibroblasts, macrophages, and some neutrophils in the interstitium. *E*, One week: some acini have regenerated (arrow) with mitotic figures (+). *F*, One month: acini show mitotic figures (+) and regenerative atypia; some ductuloacinar structure had undergone atrophy (arrow) with fatty ingrowth around them (asterisk).

observed: the disappearance of the nuclear membrane with entire lysis of the nucleus or the condensation of the chromatin beneath the nuclear membrane (the extent of the latter was lower). Dis-

appearance of the nucleoli with or without shrinkage of the cytoplasm was extensively seen. Lipid droplets of medium density persisted in the basal compartment of the acinar cells. The mitochondria

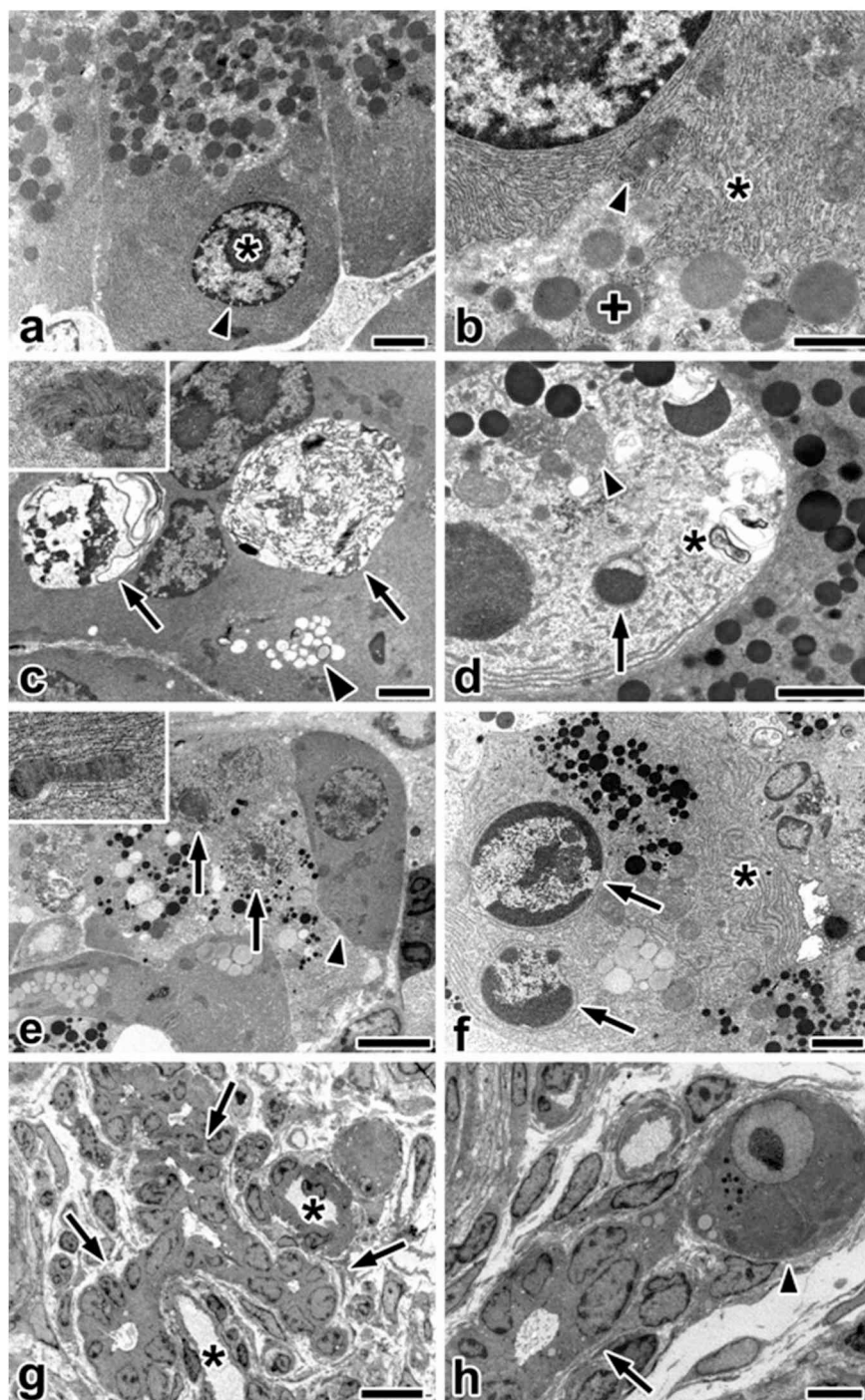


Figure 4. Electron micrographs of the rat pancreas 0 hrs (a–b), 6 hrs (c–d), 24 hrs (e–f), and 1 wk (g–h) after L-ornithine administration (3 g/kg intraperitoneally). A, Cell nucleus (arrowhead) and nucleolus (asterisk). B, Mitochondrion (arrowhead), rough endoplasmic reticulum (asterisk), and zymogen granules (cross) (scale bar = 1 μ m). C, Mitochondria seemed unaltered (inset); autophagic vacuoles (arrows) and lipid droplets (arrowhead) appeared. D, Autophagic vacuole with zymogen granules (arrow), myelin figures (asterisk), and mitochondrial remnants (arrowhead). E, Lysis of nuclei (arrows); mitochondria seemed unaltered (inset) and occasional condensation of chromatin with dense shrinkage of the cytoplasm (arrowhead) were observed (scale bar = 5 μ m). F, Dilated rough endoplasmic reticulum (asterisk); peripheral crescents (arrow) of compacted chromatin. G, Ductuloacinar structures (arrows) between capillaries (asterisk) and fibroblasts (scale bar = 10 μ m). H, Ductuloacinar structure (arrow) differentiated into acinar cell (arrowhead) with few zymogen granules (scale bar = 5 μ m). Scale bars = 2 μ m, except indicated otherwise.

seemed not primarily altered, but the rough endoplasmic reticulum was dilated with the separation of ribosomes. Large numbers of neutrophils and monocytes could be observed in the interstitial space.

36 to 48 Hrs. At 36 hrs, pancreatic edema was still severe, but was decreased versus 24 hrs. There was also a decrease in monocytic and neutrophilic adherence, neutrophilic infiltration, and necrosis of acinar cells (26%–50%). Lobular architecture was distorted. Fibroblasts also appeared in the inflamed interstitium. Regeneration starting with undifferentiated, ductuloacinar structures with scanty cytoplasm and densely basophilic nuclei in the peripheral zone of the pancreas appeared. At 48 hrs, there was diffuse severe infiltrates of macrophages/monocytes, fibroblasts, and neutrophils.

At 72 hrs, there was no pancreatic edema, but there was a diffuse severe infiltrate of fibroblasts, macrophages/monocytes, eosinophils, and neutrophils (Fig. 3D). There was a mild degree of collagen deposition within the lobules and around ductuloacinar structures. Ductuloacinar structures were budding from tubular lumina. Mitotic figures and a decrease in the number of apoptotic bodies were evident.

At 1 wk, diffuse moderate infiltrates of fibroblasts and macrophages and relatively smaller number of eosinophils and neutrophils were observed (Fig. 3E). There was a mild deposition of collagen within the lobules and around ductuloacinar structures. Adipose tissue replaced some of the destroyed lobules. Budding ductuloacinar structures started to form ductules and acini and a few newly formed acinar cells displayed zymogen granules. Dilation of small ducts and ductules were observed; in some cases, they contained eosinophilic material. Low-power electron micrographs revealed several ductuloacinar structures, an increased number of capillaries, mononuclear cells, and collagen in the interstitial space (Fig. 4). Some cells were enlarged and contained huge number of small electron-dense granules, indicating ductoendocrine proliferation.

One month after injection, the pancreas appeared normal, except that part of the parenchyma was replaced by fat (Fig. 3F). The acini showed numerous mitotic figures and regenerative atypia. Fibroblasts and macrophages were no longer present. One of four animals had focal periductal infiltrate composed of

lymphocytes, macrophages, and eosinophils.

Overall, there were no major pathologic alterations of the pancreatic duct cells, islets of Langerhans, and the liver in the hematoxylin and eosin sections. However, in one animal, striking Langerhans islet hyperplasia with mitotic figures was found at 1 mo. At 9 to 36 hrs, some eosinophilic cylinders in the tubular lumina were noted in the kidneys with attenuation and dilation of the proximal tubules. Occasional detachment of microvilli and mild peritubular capillaritis also were seen. These latter changes are consistent with mild acute tubular necrosis. At the same time, alveolar thickening with predominant neutrophil infiltration and occasional hemorrhage were seen in the lungs indicative of mild respiratory distress syndrome.

Activities of Serum, Pancreatic, and Ascitic Amylase

The serum amylase activity significantly increased from 9 to 24 hrs, but thereafter (at 48 hrs) fell below control values ($n = 4-10$, Fig. 5A). Pancreatic amylase activity was significantly decreased from 24 hrs to 1 mo after L-ornithine injection and was just about detectable at 72 and 168 hrs ($n = 4-8$, Fig. 5B). The ascites recovered from rats 24 hrs after L-ornithine administration had a huge amylase activity ($98,096 \pm 25,590$ U/L, $n = 7$).

Pancreatic Trypsin Activity

Premature activation of trypsinogen is thought to play an important role in the development of acute pancreatitis. Pancreatic trypsin activity was significantly increased 9 to 48 hrs after IP injection of 3 g/kg L-ornithine (Fig. 5C).

Pancreatic Myeloperoxidase Activity

The extent of neutrophil infiltration was also judged by the measurement of myeloperoxidase activity (Fig. 5D). Interestingly, inflammatory infiltration had two phases; the first one coincided with the peak of amylase activity (9–36 hrs) and the second one occurred much later (at 72 hrs).

Induction of Pancreatic HSP72 Synthesis

HSP72, the highly inducible form of the HSP70 family, could not be detected in the physiological saline-treated control

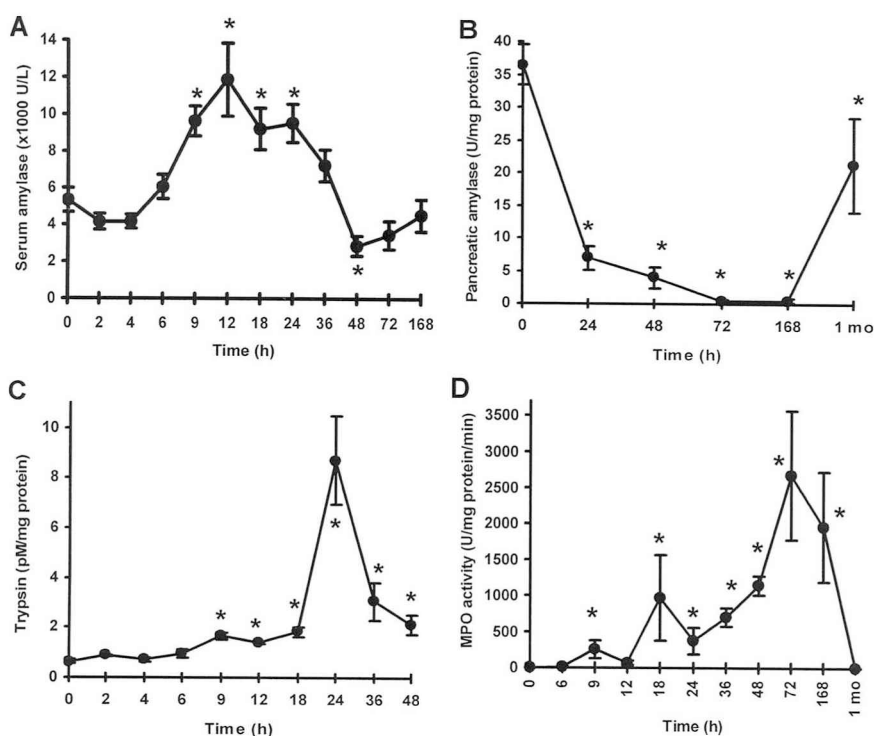


Figure 5. Time course of serum amylase and pancreatic amylase, trypsin and myeloperoxidase activities after intraperitoneal administration of 3 g/kg L-ornithine. A, Serum amylase and pancreatic; (B) amylase, (C) trypsin, and (D) myeloperoxidase (MPO) activities. The serum amylase activity significantly increased from 9 to 24 hrs, but thereafter (at 48 hrs) fell below control values. Pancreatic amylase activity was significantly decreased from 24 hrs to 1 mo after L-ornithine injection and was just about detectable at 72 and 168 hrs. Trypsin activity was gradually increased from 9 hrs and peaked at 24 hrs after L-ornithine administration. MPO activity gradually increased during the course of the disease and was highest in the 72- and 168-hr groups. Data are shown as means \pm SEM, $n = 4-10$. *Significant difference ($p < .05$) vs. the control group (0 hrs).

group (Fig. 6A). However, by 4 hrs after the injection of 3 g/kg L-ornithine, the levels of HSP72 were significantly increased, peaked at 18 hrs, and remained elevated until 1 mo.

Degradation of Pancreatic I κ B- α and I κ B- β and Induction of Interleukin-1 β Synthesis

Pancreatic I κ B levels in response to L-ornithine injection were significantly decreased from 9 hrs (Fig. 6B–C). I κ B- α levels (Fig. 6B) returned to normal by 36 hrs; however, I κ B- β level was significantly lower for up to 168 hrs after injection (Fig. 6C). Corresponding to I κ B degradation, and consequently to activation of NF- κ B, pancreatic interleukin-1 β synthesis significantly increased from 9 hrs (Fig. 6D).

Confirmation of Pancreatic Apoptosis Observed on Histologic Examination

Genomic DNA Analysis. Genomic DNA was extracted from pancreata 9 or

24 hrs after L-ornithine administration. Figure 7A shows that 9 hrs after L-ornithine injection, we could detect a ladder pattern on agarose gel electrophoresis. On the other hand, DNA showed unspecific degradation (smear) 24 hrs after the administration of L-ornithine indicating severe necrosis. This is in accordance with our histologic findings because at this latter time point, apoptosis was overcome by necrosis of the cells.

TdT-mediated dUTP Nick End-labeling Technique. According to the histologic examination, the number of apoptotic cells was greatly elevated by 6 hrs after L-ornithine administration. To obtain quantitative data, the number of apoptotic cells was counted at selected time points 0 to 168 hrs after L-ornithine administration. As shown in Figure 7B, the percentage of apoptotic cells was greatly increased in response to L-ornithine administration. The peak of apoptosis was around 6 to 9 hrs after the injection ($n = 4$).

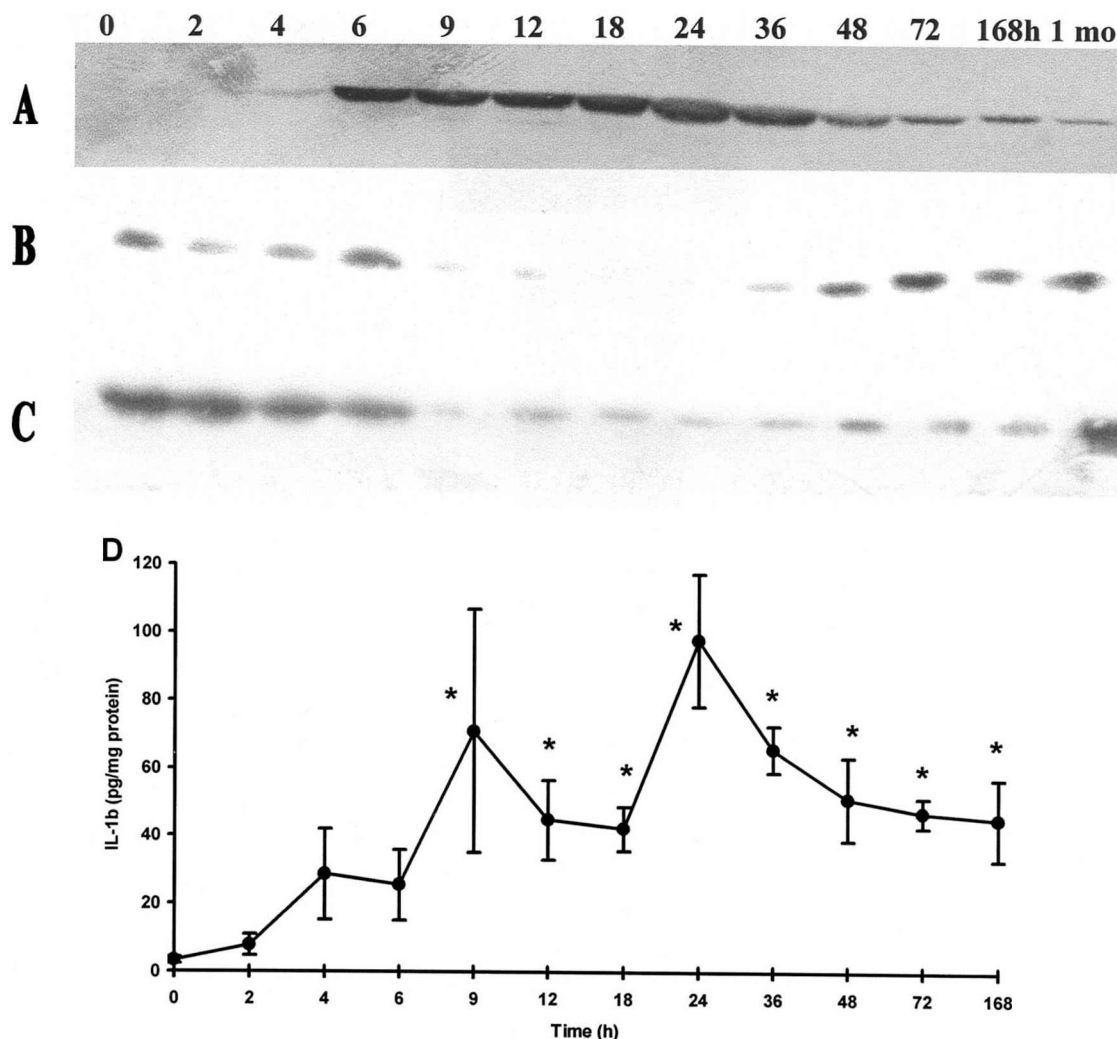


Figure 6. Effects of L-ornithine administration (3 g/kg intraperitoneally) on pancreatic HSP72, IκB-α, IκB-β, and interleukin-1β expression as a function of time. A–C, Representative Western immunoblot analysis of protein lysates (50 μg/lane) from the pancreata of rats 0 to 168 hrs and 1 mo after injection, showing the levels of (A) HSP72, (B) IκB-α, and (C) IκB-β as a function of time after 3 g/kg L-ornithine injection. D, The graph shows the expression of the proinflammatory cytokine, interleukin-1β, as determined from the cytosolic fractions of pancreatic homogenates by enzyme-linked immunosorbent assay. Means ± SEM for four to six animals are shown. *Significant difference ($p < .05$) vs. the control group (0 hrs).

Pancreatic Nonprotein Sulfhydryl Group Content and the Activities of Glutathione Peroxidase and Superoxide Dismutase

Nonprotein sulfhydryl group content was significantly increased at 6 hrs and decreased thereafter in the ornithine-treated group compared with the control group (Fig. 8A). The activities of glutathione peroxidase (Fig. 8B) and Cu/Zn-SOD (Fig. 8C) significantly increased from 24 hrs. In contrast, Mn-SOD activity (Fig. 8D) was significantly decreased at 24 hrs and significantly increased at 48 hrs versus the control. Taken together these findings suggest the presence of oxidative stress in the pancreas of rats in response to L-ornithine treatment.

Body Weight and Pancreatic Weight/Body Weight Ratio

The body weight of the rats was significantly decreased from 1 day to 1 mo after the administration of 3 g/kg L-ornithine versus the physiological saline-treated control ($n = 5-10$, Fig. 9A). Pancreatic weight/body weight ratio was significantly elevated at 18 to 48 hrs and significantly decreased at 168 hrs to 1 mo after L-ornithine injection (Fig. 9B).

Serum Aspartate Aminotransferase Activity and Concentrations of Glucose, Calcium, Triglyceride, Urea, and Creatinine

Serum ASAT activity was significantly increased by approximately five-fold at 24

hrs and three-fold at 48 hrs after L-ornithine injection (Fig. 9C). Serum concentrations of glucose were significantly decreased from 24 to 72 hrs and returned to normal by 1 wk (Fig. 9D). Serum levels of triglyceride were only significantly affected at 72 hrs ($.40 \pm .03$ mM/L vs. $.71 \pm .18$ mM/L in the control group). Calcium, urea, and creatinine concentrations were not significantly different versus the control (results not shown).

DISCUSSION

The present study characterizes a novel model of severe acute necrotizing pancreatitis induced by IP injection of 3 g/kg L-ornithine showing typical laboratory and morphologic signs of that ob-

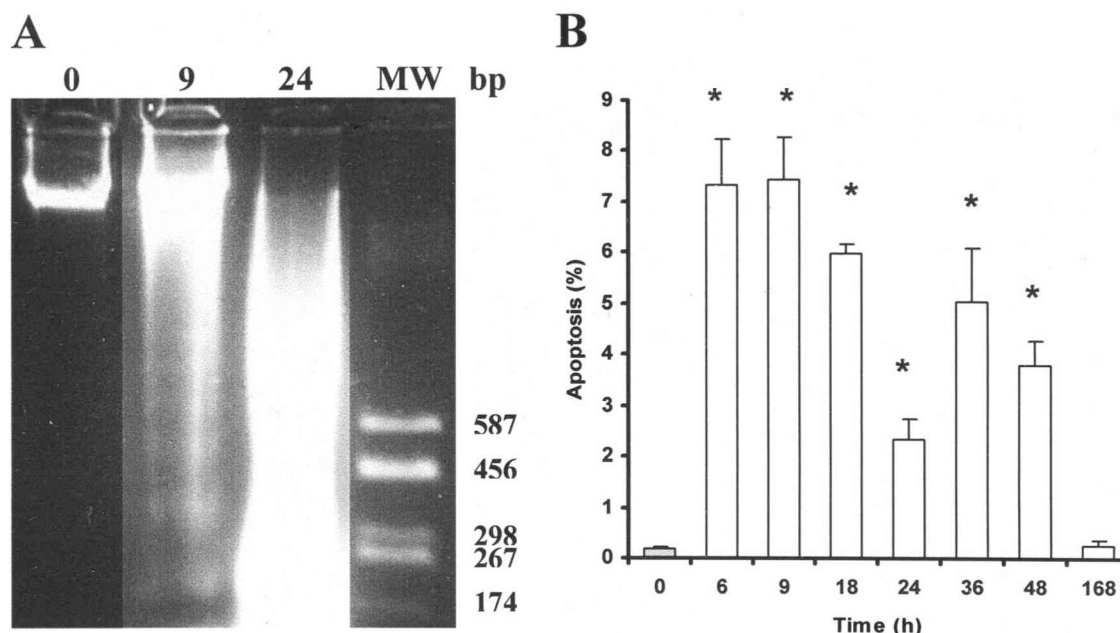


Figure 7. Pancreatic apoptosis is greatly increased in rats treated with 3 g/kg L-ornithine intraperitoneally. **A**, Genomic DNA analysis of L-ornithine (3 g/kg) injected rats in agarose gel electrophoresis. Genomic DNA was isolated from the pancreata of rats 0, 9, and 24 hrs after L-ornithine injection. Fifteen to 20 μ g of DNA was electrophoretically fractionated on a 1.8% agarose gel with .5 μ g/mL ethidium bromide. Data presented are representative of three independent experiments. *MW*, molecular weight marker; *bp*, base pairs. **B**, The percentage of apoptotic cells in the pancreas was quantitated by using the TdT-mediated dUTP Nick end-labeling assay. Data are shown as means \pm SEM, $n = 4$. *Significant difference ($p < .05$) vs. the control group (0 hrs).

served in the human disease. The present pancreatitis model is noninvasive, more diffuse, and reproducible compared with those induced by retrograde ductal injection of bile acids, the closed duodenal loop method, or choline-deficient ethionine diet. L-ornithine-induced pancreatitis is superior to the L-arginine-induced model in that it produces a much severe disease with massive edema and without the confounding effects of possible excessive NO synthesis (at least in the initial phase of pancreatitis induction). IP administration of 4 to 6 g/kg L-ornithine killed the rats within hours (before pancreatitis could develop). The death of these animals may be the result of effects on the central nervous system. Our results also suggest that L-arginine-induced pancreatitis is due to metabolism of L-arginine to L-ornithine by arginase and is not caused by its metabolism to L-citrulline (and NO) by NOS. That is, L-arginine administration results in a greater increase in blood concentration of ornithine than citrulline, and administration of L-ornithine but not L-citrulline causes severe pancreatitis.

There has been longstanding debate on whether NO is involved in the pathogenesis of acute pancreatitis. Studies have shown a protective (19–21), no (22), or detrimental (23–25) effect of NO on experimental pancreatitis. One very im-

portant finding deduced from the present study is that NO is unlikely to be a primary factor in inducing L-ornithine (or L-arginine) pancreatitis. Serum citrulline levels were only increased by approximately three-fold after injection of rats with 3.5 g/kg L-arginine; L-ornithine levels were up by approximately 54-fold. In fact, this fits in well with our previous findings, which have shown that inhibition of NO formation by N-nitro-L-arginine methyl ester in L-arginine-induced pancreatitis only ameliorated laboratory parameters and did not influence pancreatic damage on histology (26). In contrast, others have found that aminoguanidine, an isoform-specific inhibitor of iNOS, ameliorated L-arginine induced pancreatic injury (24). Anyway, NO is unlikely to be involved in the early events (development) of pancreatitis given the time course of NOS induction. In the L-arginine-induced pancreatitis model, pancreatic cNOS activity was found to be depleted at 6 hrs followed by a gradually increasing level to a peak as observed at 24 hrs (26). The activity of iNOS was not increased until 24 hrs (peak of injury).

Morphologic examination of the pancreas showed the typical signs of severe acute necrotizing pancreatitis with massive interstitial edema, apoptosis, and necrosis of acinar cells and infiltration of

neutrophil granulocytes (also confirmed by myeloperoxidase activity measurements) and monocytes. The necrotic process lacked morphologic signs indicative of lytic and ischemic damage to the cells and was evidently the result of toxic injury to the acini. The acini eventually regenerated. Acini that did not regenerate were replaced by fat tissue. Electron microscopy of pancreatic tissue revealed large autophagic vacuoles containing zymogen granules, lipid droplets, severe nuclear damage, and dilated endoplasmic reticulum in acinar cells. Zymogen granules completely disappeared by 72 to 168 hrs, which are in accord with our pancreatic amylase activity measurements.

Interestingly, apoptosis of pancreatic acini (confirmed by light microscopic, electron microscopy, TUNEL, and DNA ladder analysis) was greatly increased within a few hours after L-ornithine administration. The rate of pancreatic apoptosis strongly increased at 16 to 24 hrs after the administration of 2.5 g/kg or 4 g/kg L-arginine (10, 27). In fact, Kubish et al. found that endoplasmic reticulum stress-regulated mechanisms are likely to be involved in the apoptosis observed in this model of the disease (10). Notably, in our study, apoptosis was already at its highest level at 6 to 9 hrs. A possible explanation for this is that in fact L-ornithine is mediating the apoptotic pro-

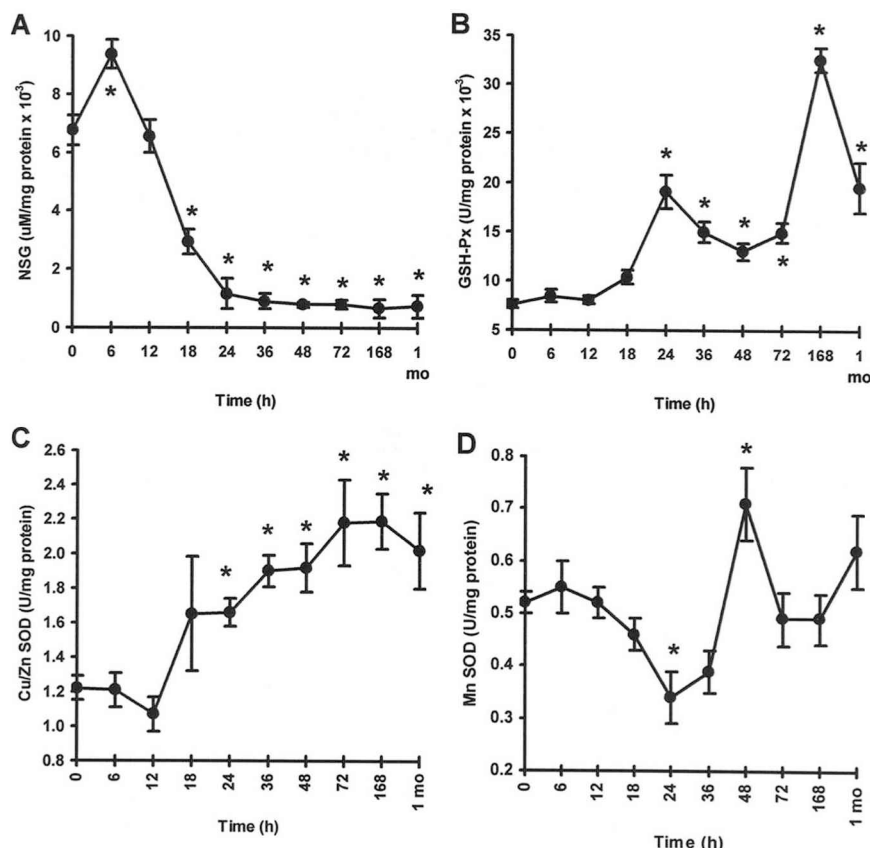


Figure 8. L-ornithine injection (3 g/kg intraperitoneally) induces pancreatic oxidative stress. Changes in pancreatic (A) nonprotein sulphydryl group (NSG) content, (B) glutathione-peroxidase (GSH-Px), (C) Cu/Zn, and (D) Mn superoxide dismutase (SOD) are depicted. Means \pm SEM for five to six animals are shown. *Significant difference ($p < .05$) vs. the control group (0 hrs).

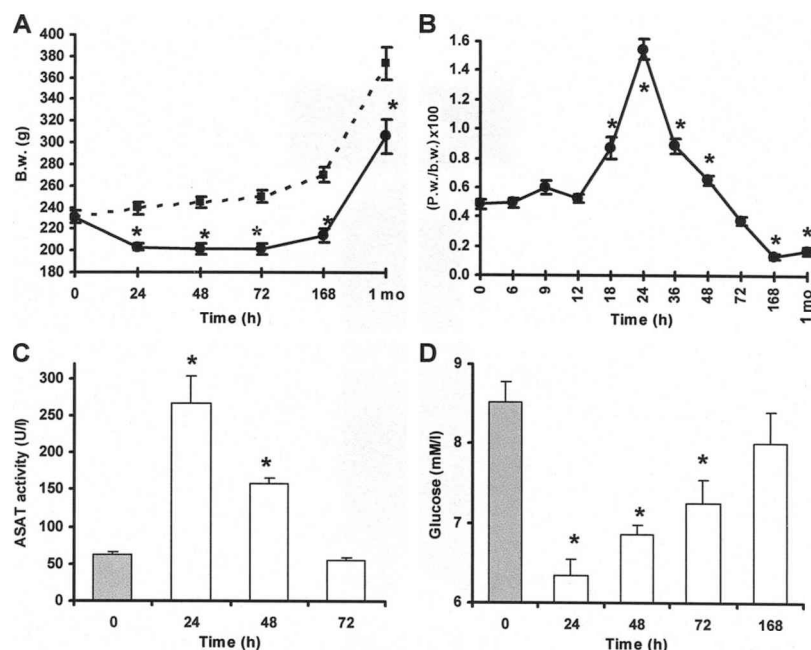


Figure 9. Time course of body weight (b.w.), pancreatic weight (p.w.)/b.w., serum aspartate aminotransferase (ASAT) activity and glucose concentration after intraperitoneal administration of 3 g/kg L-ornithine. A, B.w., (B) p.w./b.w., serum (C) ASAT activity, and (D) glucose concentration were determined as described in the Materials and Methods section. Data are shown as means \pm SEM, $n = 4-10$. *Significant difference ($p < .05$) vs. the control group (0 hrs).

cess (that is, L-arginine has been metabolized).

Several molecular features that have been observed in other models of pancreatitis were observed with administration of L-ornithine. These included intrapancreatic activation of the digestive enzyme trypsinogen (28), degradation of I κ B proteins associated with activation of the transcription factor, NF- κ B (29), increased interleukin-1 β production, increased HSP72 content (30), and signs of oxidative stress (31). The time course changes of these laboratory parameters were similar to that observed in the L-arginine-induced pancreatitis model and were delayed compared with the cerulein-induced pancreatitis model (8). These point to possible differences in pathophysiology.

The mechanism underlying L-ornithine-induced pancreatitis is not clear. It is known that L-ornithine can serve as substrate for ornithine decarboxylase, the initial and rate-limiting enzyme in the polyamine biosynthetic pathway. Polyamines are essential for normal cell growth and development. A large dose of L-ornithine/L-arginine will inevitably influence their levels. Both increased and decreased levels of polyamines have been implicated in mediating apoptosis (32). Although one would probably expect to find an increase in polyamine levels, large doses of L-arginine have been shown to paradoxically reduce spermine and spermidine and increase putrescine levels by elevating polyamine catabolism (11). Spermine and spermidine depletion are also associated with human acute necrotizing pancreatitis (11). A decrease in pancreatic polyamine levels is likely to result in an inhibition of DNA and protein synthesis, which will result in the death of acini.

In conclusion, we have developed a simple, noninvasive, reproducible model of acute necrotizing pancreatitis by intraperitoneal injection of 3 g/kg L-ornithine and examined its time course in rats. Large doses of L-arginine (known to induce acute pancreatitis) may also produce a toxic effect on the pancreas, at least in part, through L-ornithine. Further studies are needed to investigate the mechanism of L-ornithine-induced pancreatitis.

REFERENCES

1. Pandolfi SJ, Saluja AK, Imrie CW, et al: Acute pancreatitis: Bench to the bedside. *Gastroenterology* 2007; 132:1127-1151

2. Nevalainen TJ, Seppä A: Acute pancreatitis caused by closed duodenal loop in the rat. *Scand J Gastroenterol* 1975; 10:521–527
3. Aho HJ, Koskensalo SM, Nevalainen TJ: Experimental pancreatitis in the rat. Sodium taurocholate-induced acute haemorrhagic pancreatitis. *Scand J Gastroenterol* 1980; 15: 411–416
4. Lombardi B, Estes LW, Longnecker DS: Acute hemorrhagic pancreatitis (massive necrosis) with fat necrosis induced in mice by DL-ethionine fed with a choline-deficient diet. *Am J Pathol* 1975; 79:465–480
5. Lampel M, Kern HF: Acute interstitial pancreatitis in the rat induced by excessive doses of a pancreatic secretagogue. *Virchows Arch A Pathol Anat Histol* 1977; 373:97–117
6. Mizunuma T, Kawamura S, Kishino Y: Effects of injecting excess arginine on rat pancreas. *J Nutr* 1984; 114:467–471
7. Tani S, Itoh H, Okabayashi Y, et al: New model of acute necrotizing pancreatitis induced by excessive doses of arginine in rats. *Dig Dis Sci* 1990; 35:367–374
8. Hegyi P, Rakonczay Z Jr, Sári R, et al: L-arginine-induced experimental pancreatitis. *World J Gastroenterol* 2004; 10:2003–2009
9. Dawra R, Sherif R, Phillips PA, et al: Development of a new mouse model of acute pancreatitis induced by administration of L-arginine. *Am J Physiol Gastrointest Liver Physiol* 2007; 292:G1009–1018
10. Kubisch CH, Sans MD, Arumugam T, et al: Early activation of endoplasmic reticulum stress is associated with arginine-induced acute pancreatitis. *Am J Physiol Gastrointest Liver Physiol* 2006; 291:G238–245
11. Hyvönen MT, Herzig KH, Sinervirta R, et al: Activated polyamine catabolism in acute pancreatitis: Alpha-methylated polyamine analogues prevent trypsinogen activation and pancreatitis-associated mortality. *Am J Pathol* 2006; 168:115–122
12. Morris SM: Enzymes of arginine metabolism. *J Nutr* 2004; 134:2743S–2747S
13. Chace DH, Millington DS, Terada N, et al: Rapid diagnosis of phenylketonuria by quantitative analysis for phenylalanine and tyrosine in neonatal blood spots by tandem mass spectrometry. *Clin Chem* 1993; 39: 66–71
14. Gukovsky I, Gukovskaya AS, Blinman TA, et al: Early NF- κ B activation is associated with hormone-induced pancreatitis. *Am J Physiol* 1998; 275:G1402–1414
15. Rakonczay Z Jr, Takács T, Boros I, et al: NF- κ B activation is detrimental in arginine-induced acute pancreatitis. *Free Radic Biol Med* 2003; 34:696–709
16. Czakó L, Takács T, Varga IS, et al: Involvement of oxygen-derived free radicals in L-arginine-induced acute pancreatitis. *Dig Dis Sci* 1998; 43:1770–1777
17. Kuebler WM, Abels C, Schuerer L, et al: Measurement of neutrophil content in brain and lung tissue by a modified myeloperoxidase assay. *Int J Microcirc Clin Exp* 1996; 16: 89–97
18. Rakonczay Z Jr, Takács T, Mándi Y, et al: Water immersion pretreatment decreases pro-inflammatory cytokine production in cholecystokinin-octapeptide-induced acute pancreatitis in rats: Possible role of HSP72. *Int J Hyperthermia* 2001; 17:520–535
19. Molero X, Guarner F, Salas A, et al: Nitric oxide modulates pancreatic basal secretion and response to cerulein in the rat: effects in acute pancreatitis. *Gastroenterology* 1995; 108:1855–1862
20. Werner J, Fernández-del Castillo C, Rivera JA, et al: On the protective mechanisms of nitric oxide in acute pancreatitis. *Gut* 1998; 43:401–407
21. Sugiyama Y, Kato S, Mitsufuji S, et al: Pathogenic role of endothelial nitric oxide synthase (eNOS/NOS-III) in cerulein-induced rat acute pancreatitis. *Dig Dis Sci* 2006; 51: 1396–1403
22. Weidenbach H, Lerch MM, Gress TM, et al: Vasoactive mediators and the progression from edematous to necrotizing experimental acute pancreatitis. *Gut* 1995; 37:434–440
23. Dabrowski A, Gabryelewicz A: Nitric oxide contributes to multiorgan oxidative stress in acute experimental pancreatitis. *Scand J Gastroenterol* 1994; 29:943–948
24. Lomis TJ, Siffring CW, Chalasani S, et al: Nitric oxide synthase inhibitors N-monomethylarginine and aminoguanidine prevent the progressive and severe hypotension associated with a rat model of pancreatitis. *Am Surg* 1995; 61:7–10
25. Shields CJ, Delaney CP, Winter DC, et al: Induction of nitric oxide synthase is a key determinant of progression to pulmonary injury in experimental pancreatitis. *Surg Infect (Larchmt)* 2006; 7:501–511
26. Takács T, Czakó L, Morschl É, et al: The role of nitric oxide in edema formation in L-arginine induced acute pancreatitis. *Pancreas* 2002; 25:31–38
27. Trulsson L, Sandström P, Sundqvist T, et al: The influence of a load of L-arginine on serum amino acids and pancreatic apoptosis/proliferation and ATP levels in the rat. *Pancreas* 2004; 29:e113–120
28. Saluja AK, Donovan EA, Yamanaka K, et al: Cerulein-induced in vitro activation of trypsinogen in rat pancreatic acini is mediated by cathepsin B. *Gastroenterology* 1997; 113:304–310
29. Rakonczay Z Jr, Hegyi P, Takács T, et al: The role of NF- κ B activation in the pathogenesis of acute pancreatitis. *Gut* 2008; 57:259–267
30. Rakonczay Z Jr, Takács T, Boros I, et al: Heat shock proteins and the pancreas. *J Cell Physiol* 2003; 195:383–391
31. Schulz HU, Niederau C, Klonowski-Stumpe H, et al: Oxidative stress in acute pancreatitis. *Hepatogastroenterology* 1999; 46: 2736–2750
32. Schipper RG, Penning LC, Verhofstad AA: Involvement of polyamines in apoptosis. Facts and controversies: Effectors or protectors? *Semin Cancer Biol* 2000; 10:55–68
33. Kurucz I, Tombor B, Prechl J, et al: Ultrastructural localization of HSP-72 examined with a new polyclonal antibody raised against the truncated variable domain of the heat shock protein. *Cell Stress Chaperones* 1999; 4:139–152

II.

Inhibition of Arginase Activity Ameliorates L-Arginine–Induced Acute Pancreatitis in Rats

György Biczó, MSc,* Péter Hegyi, MD, PhD,* Sándor Berczi, MD,† Sándor Dósa, MD,‡ Zsuzsanna Hracskó, MSc,‡ Ilona S. Varga, PhD,‡ Béla Iványi, MD, PhD, DSc,† Viktória Venglovecz, PhD,§ Tibor Wittmann, MD, PhD,* Tamás Takács, MD, PhD, DSc,* and Zoltán Rakonczay Jr, MD, PhD*

Objectives: Intraperitoneal (IP) injection of 3.5 g/kg L-arginine (known to induce acute pancreatitis) in rats will result in much greater increases in serum ornithine versus citrulline concentration (*Crit Care Med.* 2008; 36:2117–2127). These data indicate a major role of arginase in the catabolism of L-arginine. Therefore, we tested the effects of the irreversible arginase inhibitor (+)-S-2-amino-6-iodoacetamidohexanoic acid (AIHA) on L-arginine–induced acute pancreatitis.

Methods: The inhibitory effect of AIHA on arginase activity was tested on rat liver homogenate and purified bovine arginase. Male Wistar rats were administered 15 mg/kg AIHA or its vehicle IP 1 hour before the injection of physiological saline or 3.5 g/kg L-arginine IP. Laboratory and histological parameters of pancreatitis were determined 24 hours after the last injection.

Results: Sixty micromolars of AIHA (equimolar to an in vivo dose of 15 mg/kg) significantly inhibited arginase activity by about 25%. Pretreatment with AIHA significantly ameliorated pancreatic damage caused by L-arginine administration. It decreased pancreatic weight/body weight ratio, pancreatic glutathione peroxidase and myeloperoxidase activities, and histological damage. Administration of AIHA in itself significantly increased levels of pancreatic heat shock proteins.

Conclusions: Pretreatment with AIHA reduces the severity of L-arginine–induced pancreatitis most likely by inhibiting arginase activity.

Key Words: arginase, L-arginine, (+)-S-2-amino-6-iodoacetamidohexanoic acid, acute pancreatitis (*Pancreas* 2010;00: 00–00)

Large doses (2.5–5 g/kg) of intraperitoneally (IP) injected L-arginine are known to induce acute necrotizing pancreatitis in rats and mice.^{1–4} The pathomechanism of L-arginine pancreatitis is unknown, especially concerning the early events leading to the disease.

Two key enzymes that are involved in the metabolism of L-arginine are nitric oxide synthase (NOS) and arginase (Fig. 1).⁵ Nitric oxide synthase has 3 isoforms: the constitutive endothelial (eNOS) and neuronal (nNOS) and an inducible form

(iNOS). They catalyze the conversion of L-arginine to nitric oxide and L-citrulline. Arginase, which has 2 isoforms (types I and II), hydrolyzes L-arginine to L-ornithine and urea. These 2 arginase isoforms are encoded by 2 different genes and differ in molecular properties, tissue distribution, subcellular location, and regulation of expression. Arginase I is localized in the cytosol and is highly expressed in the liver and to a much less extent in a few other tissues. Arginase II is a mitochondrial enzyme, which is widely distributed in extrahepatic tissues.

In arginine-induced pancreatitis, pancreatic constitutive NOS activity was depleted at 6 hours then gradually increased to significantly higher level than the control at 24 hours.⁶ The activity of pancreatic iNOS was significantly increased at 24 hours after L-arginine injection.

Most of the IP injected L-arginine is converted to L-ornithine rather than L-citrulline,^{7,8} therefore indicating a major role of arginase in the catabolism of this basic amino acid. Furthermore, we have recently shown that administration of L-ornithine induces a more severe pancreatitis compared with L-arginine.⁷ Therefore, we speculated that L-arginine produces a toxic effect on the pancreas, at least in part, via L-ornithine. The aim of this study was to test the effects of the irreversible arginase inhibitor (+)-S-2-amino-6-iodoacetamidohexanoic acid (AIHA) on L-arginine–induced acute pancreatitis.

MATERIALS AND METHODS

Materials

(+)-S-2-amino-6-iodoacetamidohexanoic acid was purchased from Alexis Biochemicals. All other chemicals were obtained from Sigma-Aldrich, unless indicated otherwise.

Animals

Male Wistar rats weighing 220 to 250 g were used. The animals were kept at a constant room temperature of 23°C with a 12-hour light-dark cycle and were allowed free access to water and standard laboratory chow (Biofarm, Zagyvaszántó, Hungary). In each experimental group, 5 to 8 rats were used. The experiments performed in this study were approved by the Animal Care Committee of the University.

Experimental Protocol

Rats were pretreated with 15 mg/kg AIHA (dissolved in 6 M HCl and pH set to 7.4 with NaOH in phosphate-buffered saline) or its vehicle IP 1 hour before injection with physiological saline or 3.5 g/kg L-arginine–HCl (350 mg/mL, pH: 7.4) IP. Rats were killed by exsanguination through the abdominal aorta after anesthetization with 44 mg/kg pentobarbital IP 24 hours after the L-arginine or physiological saline injection. The pancreas was quickly removed, cleaned from fat and lymph nodes, weighed, and frozen in liquid nitrogen and stored at –80°C until use. Furthermore, liver, kidney, and lung tissue were frozen from control animals for determination of arginase

FIG 1

From the *First Department of Medicine and Departments of †Pathology, ‡Biochemistry and Molecular Biology, and §Pharmacology and Pharmacotherapy, University of Szeged, Szeged, Hungary.

Received for publication July 7, 2009; accepted January 9, 2010.

Reprints: Zoltán Rakonczay Jr, MD, PhD, First Department of Medicine, University of Szeged, PO Box 427, H-6701 Szeged, Hungary (e-mail: raz@in1st.szote.u-szeged.hu).

This study was supported by the Hungarian Scientific Research Fund (K78311 to Z.R., NNF78851 to P.H., and PD78087 to V.V.) and the Hungarian Academy of Sciences (BO 00334/08/5 to P.H. and BO 00218/06 to Z.R.).

Aprotinin was a generous gift from Gedeon Richter Plc (Budapest, Hungary). Copyright © 2010 by Lippincott Williams & Wilkins

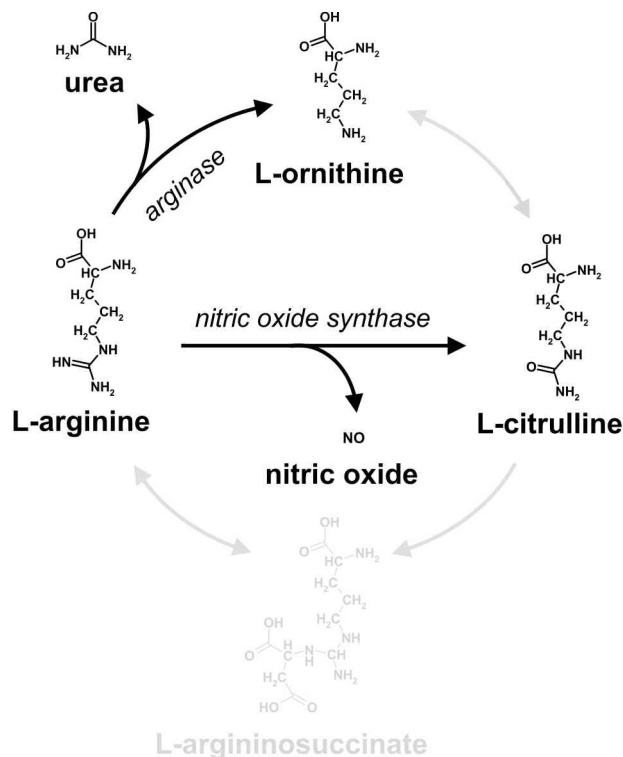


FIGURE 1. Key enzymes involved in the metabolism of L-arginine. Nitric oxide synthase catalyzes the conversion of L-arginine to nitric oxide and L-citrulline. Arginase hydrolyzes L-arginine to L-ornithine and urea.

activity. All blood samples were centrifuged at 2500 g for 20 minutes, and the serum was stored at -25°C .

Pancreatic Weight/Body Weight Ratio

The pancreatic weight/body weight (PW/BW) ratio was used to evaluate the degree of pancreatic edema.

Serum and Pancreatic Amylase Activity

The serum and pancreatic activities of amylase were determined by a colorimetric kinetic method (Dialab, Vienna, Austria).

Pancreatic Trypsin Activity

Pancreas samples were pulverized under liquid N_2 using a mortar and pestle. Pulverized tissue was suspended in ice-cold MOPS (3-[*N*-morpholino]propanesulfonic acid) buffer (5 mM MOPS, pH 6.5, 250 mM sucrose, 1 mM MgSO_4). The resulting homogenate was centrifuged (50 g for 5 minutes), and the supernatant was used for the enzyme assay. Trypsin activity was measured in Tris buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1 mM CaCl_2) by using the colorimetric substrate *N*-CBZ-Gly-Pro-Arg-*p*-nitroanilide (0.1 mM final concentration; Bachem GmbH, Weil am Rhein, Germany). Three-minute time courses of *p*-nitroaniline release were followed at 405 nm at room temperature with a Hitachi U-2900 spectrophotometer. Trypsin activity in the homogenate was estimated using a standard curve for purified bovine trypsin.

Arginase Activity

Parts of the liver, pancreas, kidney, and lung were homogenized in 9-fold excess (wt/vol) of ice-cold buffer containing 50 mM Tris-HCl (pH 7.5) supplemented with 1 mM phenyl methyl sulfonyl fluoride, 4 mM benzamidine, and 100 U/mL aprotinin using an Ultra-Turrax homogenizer for 2×30 seconds. The homogenates were then centrifuged at 25,000 g for 30 minutes (at 4°C), and the heat-activated (60°C , 20 minutes) supernatants (their protein concentrations were measured by the method of Bradford¹⁴) were used for determination of arginase activity. Arginase activity was measured by exogenously added L-arginine using a colorimetric method, based on the determination of released urea.⁹ Briefly, 20 mM L-arginine-HCl substrate (pH set to 9.7), 0.2 mM MnCl_2 , and the sample were mixed in 250 μL and incubated at 37°C for 5 to 15 minutes. The reaction was stopped by adding 250 μL 1 N HClO_4 and centrifuged, and 140 μL supernatant was used for urea determination by adding 1.36 mL diacetylmonoximethiosemicarbazide reagent after centrifugation; an aliquot was tested for released urea according to Coulombe and Favreau.¹⁰ After heating at 100°C (20 minutes) and cooling, optical densities were read at 535 nm using urea standards.

The inhibitory effect of 0 to 120 μM AIHA on arginase activity was tested on rat liver homogenate and purified bovine liver arginase (Serva). Considering that the total water content of the rat is about 80%, 60 μM AIHA is equimolar to an in vivo dose of 15 mg/kg.

Pancreatic Myeloperoxidase Activity

Pancreatic myeloperoxidase (MPO) activity, as a marker of tissue leukocyte infiltration, was assessed using the method of Kuebler et al.¹¹

Pancreatic Nonprotein Sulfhydryl Group Content and the Activities of Glutathione Peroxidase and Superoxide Dismutase

To determine nonprotein sulfhydryl group (NSG) content and activities of glutathione peroxidase (GSH-Px), Mn- and Cu/Zn-superoxide dismutase (SOD), a part of the pancreas was homogenized, the homogenates centrifuged at 3000 g for 10 minutes, and the supernatants were used for measurements as described previously.¹²

Expression of Pancreatic Heat Shock Proteins 27 and 72

Heat shock proteins (HSPs) are stress-inducible chaperones. Western blot analysis of pancreatic HSP27 and HSP72 expression was performed from the cytosolic fraction of the pancreas homogenate. Pancreatic cytosolic fractions were prepared as described previously.¹³ The protein concentration of the homogenate was determined by the method of Bradford.¹⁴ Forty micrograms of protein was loaded per lane. Samples were electrophoresed on a 10% sodium dodecyl sulfate-polyacrylamide gel according to the method of Laemmli.¹⁵ The proteins on the gels were either stained with Coomassie brilliant blue (to demonstrate equal loading of proteins for Western blot analysis) or transferred to a nitrocellulose membrane for 1 hour at 100 V. Equal transfer of proteins was verified by ponceau S staining. Membranes were blocked in 5% nonfat dry milk (Bio-Rad) for 1 hour and incubated with rabbit anti-HSP72 (1:10,000 dilution, characterized previously by Kurucz et al.¹⁶) or goat anti-HSP27 (1:500 dilution; Santa Cruz Biotechnology, Santa Cruz, Calif) antibody for an additional 1 or 3 hours (respectively) at room temperature. The

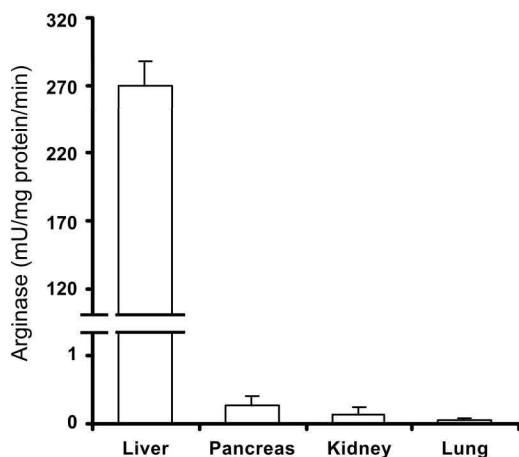


FIGURE 2. Arginase activity in the liver, pancreas, lung, and kidney of rats. Tissues were removed from control animals and then homogenized. Arginase activity was measured by exogenously added L-arginine using a colorimetric method, based on the determination of released urea.⁹

immunoreactive protein was visualized by enhanced chemiluminescence using horseradish peroxidase-coupled anti-rabbit or anti-goat immunoglobulin (Dako, Glostrup, Denmark) at 1:10,000 dilution.

Histological Examination

A portion of the pancreas was fixed in 6% neutral formaldehyde solution and subsequently embedded in paraffin. Sections were cut at 4- μ m thickness and stained with hematoxylin and eosin. The slides were coded and read by 2 independent observers who were blind to the experimental protocol. Pancreatic tissue injury was evaluated as described previously.⁷ Briefly, semiquantitative grading of interstitial edema (0–3), vascular congestion (0–1), leukocyte adhesion (0–3) and infiltration (0–4), and apoptosis (0–3) and necrosis (0–4) of acinar cells was determined in each animal.

Statistical Analysis

Results are expressed as means \pm SEM. Experiments were evaluated by using the analysis of variance followed by Dunnett

multiple-comparisons post hoc test. Values of $P < 0.05$ were considered as significant.

RESULTS

Arginase Activity in the Liver, Pancreas, Lung, and Kidney

Arginase activity was by far the highest in the liver (Fig. 2). Nevertheless, we could also detect arginase activity in the pancreas, lung, and kidney.

Effect of AIHA on Arginase Activity

(+)-S-2-amino-6-iodoacetamidohexanoic acid dose-dependently inhibited liver arginase activity of rat liver homogenate and purified bovine arginase in vitro (Fig. 3). Sixty micromolars of AIHA (equimolar to an in vivo dose of 15 mg/kg) significantly inhibited liver arginase activity by about 25%.^[F3]

PW/BW Ratio, Serum and Pancreatic Amylase Activity, and Pancreatic Trypsin Activity

Pancreatic weight/body weight ratio was significantly increased in response to administration of L-arginine (Fig. 4A). Pretreatment with AIHA significantly ameliorated this increase of PW/BW ratio. Serum amylase activity was not significantly altered in any of the groups (Fig. 4B). Pancreatic contents of amylase were significantly decreased in the L-arginine-treated groups (Fig. 4C). Pretreatment with AIHA did not influence pancreatic amylase activity in rats injected with L-arginine. Pancreatic trypsin activity was significantly increased by L-arginine administration (Fig. 4D). Pretreatment with AIHA significantly ameliorated this increased pancreatic trypsin activity.

Pancreatic MPO Activity

Pancreatic MPO activity was significantly increased at 24 hours after L-arginine injection (Fig. 4E). Pretreatment with AIHA significantly decreased MPO activity in the L-arginine-induced pancreatitis group.

Pancreatic Nonprotein Sulfhydryl Group Content and the Activities of GSH-Px and SOD

Pancreatic NSG content and GSH-Px activity were significantly increased 24 hours after the injection with L-arginine (Figs. 5A, B). Pretreatment with AIHA did not influence NSG^[F5]

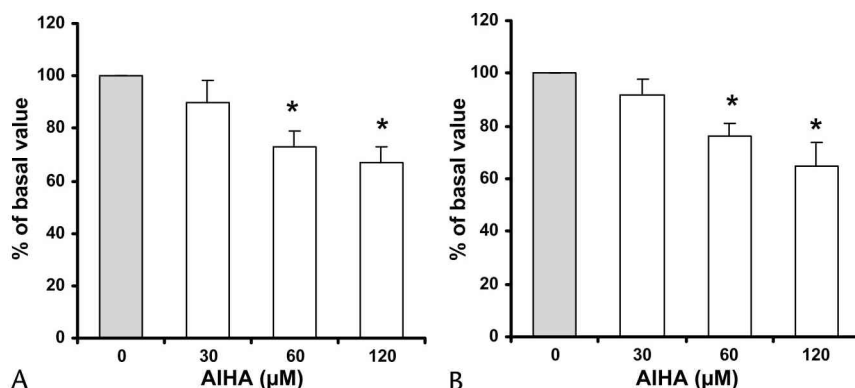


FIGURE 3. (+)-S-2-amino-6-iodoacetamidohexanoic acid inhibits arginase activity of rat liver homogenate and purified bovine liver arginase. The effect of 0 to 120 μ M AIHA was tested on (A) rat liver homogenate or (B) purified bovine liver arginase. Sixty micromolars of AIHA (equimolar to an in vivo dose of 15 mg/kg) significantly inhibited arginase activity by about 25%.

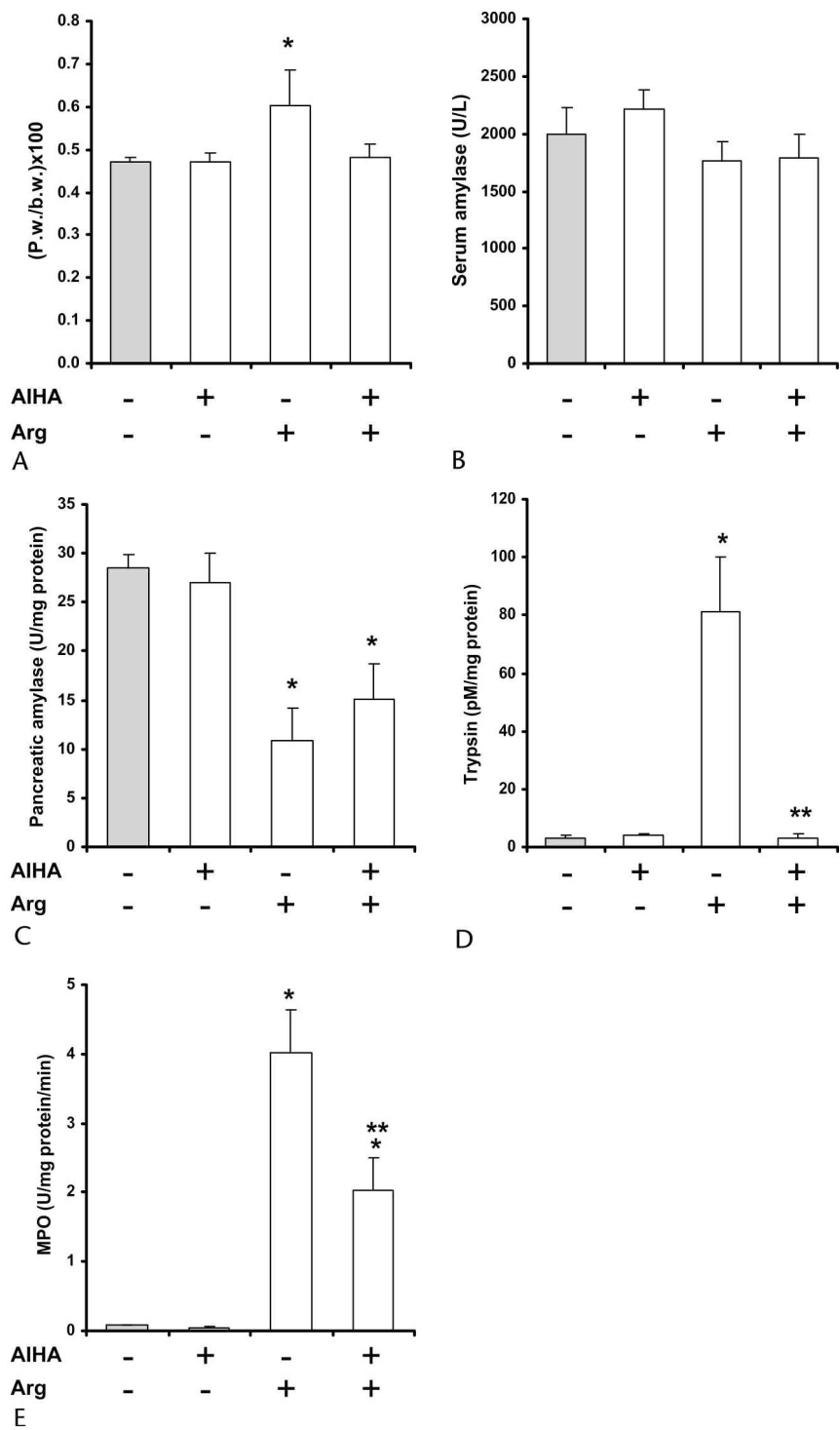


FIGURE 4. Effects of AIHA pretreatment on laboratory parameters of acute pancreatitis. A, PW/BW ratio and activities of (B) serum and (C) pancreatic amylase and pancreatic (D) trypsin and (E) MPO. Rats were pretreated with 15 mg/kg AIHA (AIHA, +) or its vehicle (AIHA, -) IP 1 hour before injection with physiological saline (Arg, -) or 3.5 g/kg L-arginine-HCl (Arg, +) IP. Rats were killed 24 hours after the L-arginine or physiological saline injection. Means \pm SEM for 5 to 8 animals are shown. *Significant difference ($P < 0.05$) versus the control group.

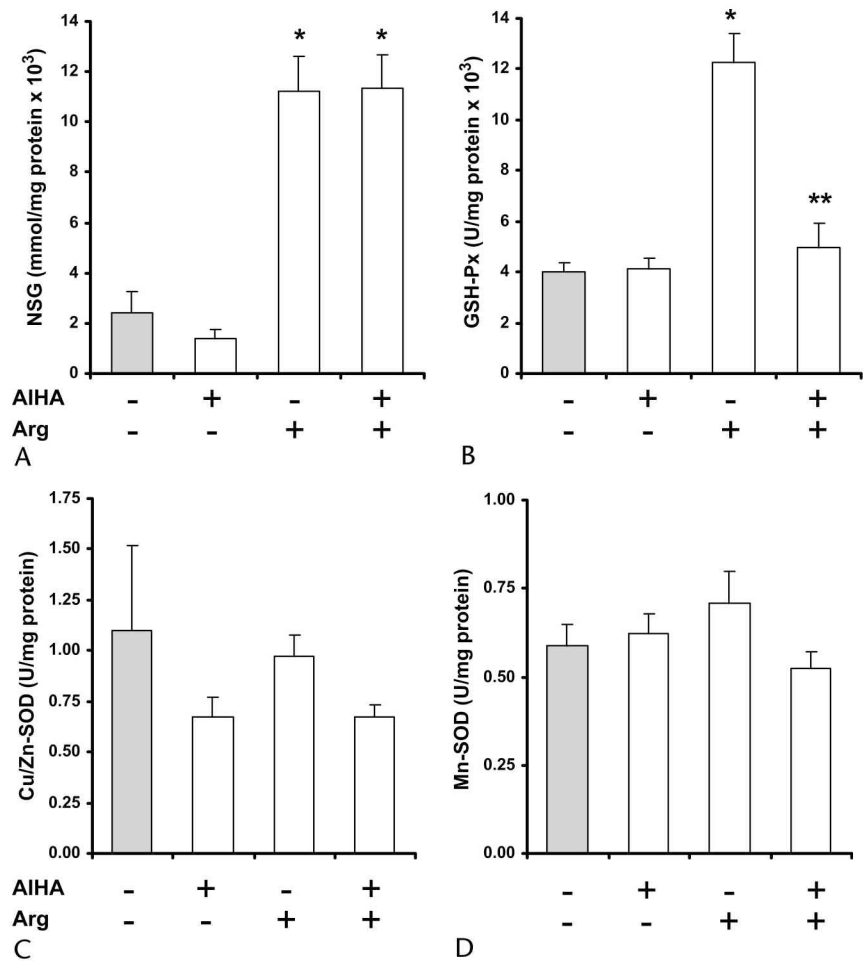


FIGURE 5. Effects of AIHA pretreatment on pancreatic markers of oxidative stress in L-arginine-induced acute pancreatitis. Changes in pancreatic (A) NSG content, (B) GSH-Px, and (C) Cu/Zn-SOD and (D) Mn-SOD activities are depicted. Rats were treated as indicated in the legend of Figure 3. Means \pm SEM for 5 to 8 animals are shown. *Significant difference ($P < 0.05$) versus the control group.

content, but significantly reduced GSH-Px activity. Activities of Cu/Zn and Mn SOD were unaltered by AIHA pretreatment in the L-arginine-pancreatitis group (Figs. 5C, D).

Pancreatic HSP Expression

(+)-S-2-amino-6-iodoacetamidohexanoic acid and/or L-arginine administration resulted in up-regulation of pancreatic HSP27 and HSP72 synthesis versus the physiological saline-treated control group (Fig. 6). No significant difference was found at 24 hours between the AIHA-treated and untreated L-arginine-induced pancreatitis groups.

Histological Examination

The administration of 3.5 g/kg L-arginine caused severe necrotizing pancreatitis (Fig. 7; Table 1). Injection of AIHA in itself resulted in pancreatic hyperemia and mild inflammatory cell infiltration. However, AIHA pretreatment significantly reduced pancreatic damage in L-arginine-induced pancreatitis.

ornithine versus citrulline concentration.⁷ This finding suggested a major role for arginase (rather than NOS) in the catabolism of L-arginine. In the present study, we have shown that pretreatment

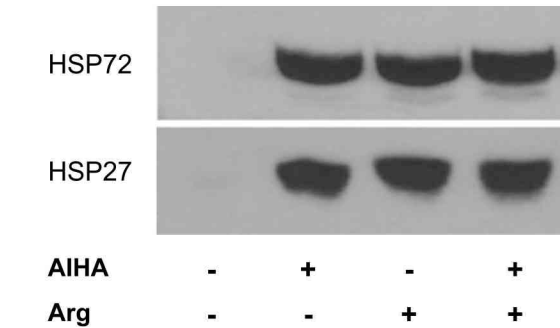


FIGURE 6. Effects of AIHA pretreatment on pancreatic HSP synthesis in L-arginine-induced pancreatitis. Representative Western immunoblot analysis of protein lysates (40 μ g/lane) from the pancreata of rats, showing the expression of HSP27 and HSP72. Rats were treated as indicated in the legend of Figure 3.

DISCUSSION

We have previously demonstrated that IP injection of 3.5 g/kg L-arginine results in much greater increases in serum

Fig 7 4/C

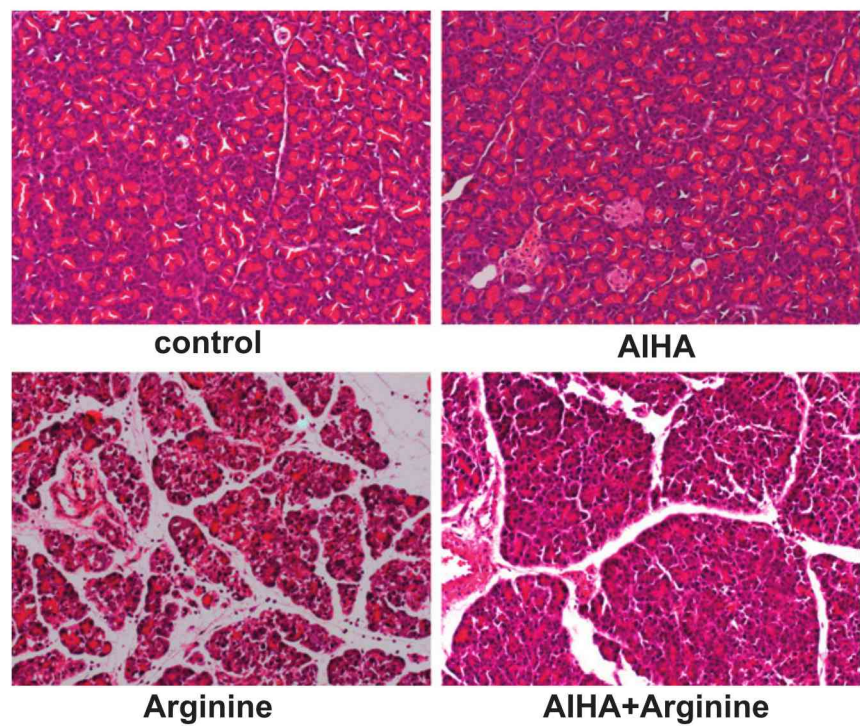


FIGURE 7. Effects of AIHA pretreatment on the pancreatic morphological damage in L-arginine–induced pancreatitis. The histological pictures show hematoxylin-and-eosin staining images of the pancreas from control, AIHA (15 mg/kg IP), L-arginine (3.5 g/kg IP), or AIHA + L-arginine–treated rats. Original magnification ×200. The point values for each of the scored parameters are shown in Table 1.

with the irreversible arginase inhibitor AIHA significantly reduced pancreatic damage caused by the administration of 3.5 g/kg L-arginine.

Arginase is a key enzyme of the hepatic urea cycle, so it was not surprising that the highest enzyme activity was found in the liver of rats. In accordance with values reported elsewhere,¹⁷ arginase activity was several-order magnitudes higher in the liver compared with other tissues (pancreas, lung, and kidney). Therefore, most likely, large doses of L-arginine are metabolized mainly by the liver. In fact, serum L-arginine concentration was significantly reduced below the control value 24 hours after injection.⁸

Two studies have investigated the changes in serum arginase activity in patients with acute pancreatitis. Serum arginase activity was unaltered in the early phases of acute pancreatitis.¹⁸ On the contrary, Scibior et al¹⁹ have found that serum arginase activity was significantly reduced by treatment of the disease.

(+)-S-2-amino-6-iodoacetamidohexanoic acid is an irreversible inhibitor of the ornithine decarboxylase and arginase and has been reported to have antifertility and antitumor effects.^{20,21} The dosing of AIHA was based on literature data.^{20,21} Sixty micromolars of AIHA (which corresponds to an in vivo dose of 15 mg/kg) inhibited arginase activity by about 25%. Although this rate of arginase inhibition is not great, even

TABLE 1. Effects of AIHA Pretreatment on the Histological Parameters in Arginine-Induced Acute Pancreatitis

	Control	AIHA	L-Arginine	AIHA + L-Arginine
Vascular congestion	0.0 ± 0.0	0.0 ± 0.0	1.0 ± 0.0*	1.0 ± 0.0*
Leukocyte adherence	0.0 ± 0.0	0.0 ± 0.0	2.5 ± 0.3*	1.5 ± 0.3*†
Interstitial edema	0.0 ± 0.0	0.0 ± 0.0	2.3 ± 0.5*	1.7 ± 0.4*
Leukocyte infiltration	0.0 ± 0.0	0.3 ± 0.2	2.7 ± 0.2*	1.7 ± 0.2*†
Vacuolization	0.0 ± 0.0	0.0 ± 0.0	1.5 ± 0.3*	0.5 ± 0.4†
Necrosis	0.0 ± 0.0	0.3 ± 0.2	3.5 ± 0.4*	1.5 ± 0.4*†
Apoptosis	0.0 ± 0.0	0.0 ± 0.0	1.0 ± 0.0*	1.0 ± 0.0*
Regeneration	0.0 ± 0.0	0.0 ± 0.0	0.5 ± 0.3	0.2 ± 0.2
Total	0.0 ± 0.0	0.5 ± 0.4	15.0 ± 1.5*	9.0 ± 1.3*†

Rats were treated as indicated in the legend of Figure 3. Data are means ± SEM for 5 to 8 animals.

*Significant difference ($P < 0.05$) versus the control group.

†Significant difference ($P < 0.05$) versus the L-arginine–treated group.

applying 120 μ M AIHA in vitro did not result in significantly lower levels of arginase activity versus 60 μ M (Fig. 3). Furthermore, because arginase is a critical enzyme of the urea cycle, most likely we have a limit to what we could decrease enzyme activity without seeing any detrimental effects. Because AIHA also inhibits ornithine decarboxylase and ornithine per se can induce acute pancreatitis, the action of AIHA in modifying the acute pancreatitis response to arginine might be due to the inhibition of the latter step, which is the formation of precursor for polyamine synthesis.

Interestingly, administration of AIHA in itself caused a stress response in the pancreas characterized by increased expression of HSP27 and HSP72. This may be due to the fact that inhibition of arginase activity will interfere with the urea cycle and increase levels of toxic ammonia. Preinduction of HSPs has been shown to protect against L-arginine-induced acute pancreatitis,²² although in our studies we found only reductions in proinflammatory cytokine synthesis.²³ Nevertheless, given these contrasting results, we cannot exclude the beneficial effect of these chaperones on disease progression, providing their levels are increased before the initiation of arginine pancreatitis.²⁴

The administration of AIHA 1 hour before L-arginine injection ameliorated the degree of pancreatic edema, but it did not influence serum amylase activity in L-arginine-induced pancreatitis. Serum amylase activity is not a good marker of disease severity in this pancreatitis model; it is usually only mildly increased in the early phases of the disease. However, AIHA pretreatment also significantly reduced parameters of pancreatic oxidative stress, trypsin and MPO activity, and histological damage.

Taken together, our results suggest that AIHA pretreatment reduces the severity of L-arginine-induced acute pancreatitis most likely by inhibiting arginase activity. Conversion of L-arginine to L-ornithine by arginase seems to be detrimental in L-arginine-induced acute pancreatitis. Further studies are needed to determine how these basic amino acids produce pancreatic injury.

ACKNOWLEDGMENTS

The authors thank Dr András Hrabák (Semmelweis University, Department of Medical Chemistry, Molecular Biology and Pathobiochemistry) for the helpful advice in setting up the arginase assay. The authors also thank Dr István Kurucz for providing the HSP72 antibody.

REFERENCES

- Mizunuma T, Kawamura S, Kishino Y. Effects of injecting excess arginine on rat pancreas. *J Nutr*. 1984;114:467–471.
- Tani S, Itoh H, Okabayashi Y, et al. New model of acute necrotizing pancreatitis induced by excessive doses of arginine in rats. *Dig Dis Sci*. 1990;35:367–374.
- Dawra R, Sherif R, Phillips PA, et al. Development of a new mouse model of acute pancreatitis induced by administration of L-arginine. *Am J Physiol Gastrointest Liver Physiol*. 2007;292:G1009–G1018.
- Hegyi P, Rakonczay Z Jr, Sári R, et al. L-Arginine-induced experimental pancreatitis. *World J Gastroenterol*. 2004;10:2003–2009.
- Morris SM. Enzymes of arginine metabolism. *J Nutr*. 2004;134:2743S–2747S.
- Takács T, Czákó L, Morschl É, et al. The role of nitric oxide in edema formation in L-arginine induced acute pancreatitis. *Pancreas*. 2002;25:31–38.
- Rakonczay Z Jr, Hegyi P, Dósa S, et al. A new severe acute necrotizing pancreatitis model induced by L-ornithine in rats. *Crit Care Med*. 2008;36:2117–2127.
- Trulsson L, Sandström P, Sundqvist T, et al. The influence of a load of L-arginine on serum amino acids and pancreatic apoptosis/proliferation and ATP levels in the rat. *Pancreas*. 2004;29:e113–e120.
- Hrabák A, Bajor T, Mészáros G. The inhibitory effect of various indolyl amino acid derivatives on arginase activity in macrophages. *Amino Acids*. 2008;34:293–300.
- Coulombe JJ, Favreau L. A new simple semimicro method for colorimetric determination of urea. *Clin Chem*. 1963;9:102–108.
- Kuebler WM, Abels C, Schuerer L, et al. Measurement of neutrophil content in brain and lung tissue by a modified myeloperoxidase assay. *Int J Microcirc Clin Exp*. 1996;16:89–97.
- Czákó L, Takács T, Varga IS, et al. Involvement of oxygen-derived free radicals in L-arginine-induced acute pancreatitis. *Dig Dis Sci*. 1998;43:1770–1777.
- Rakonczay Z Jr, Takács T, Boros I, et al. NF- κ B activation is detrimental in arginine-induced acute pancreatitis. *Free Radic Biol Med*. 2003;34:696–709.
- Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem*. 1976;72:248–254.
- Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*. 1970;227:680–685.
- Kurucz I, Tombor B, Prechl J, et al. Ultrastructural localization of HSP-72 examined with a new polyclonal antibody raised against the truncated variable domain of the heat shock protein. *Cell Stress Chaperones*. 1999;4:139–152.
- Herzfeld A, Raper SM. The heterogeneity of arginases in rat tissues. *Biochem J*. 1976;153:469–478.
- Sandström P, Trulsson L, Gasslander T, et al. Serum amino acid profile in patients with acute pancreatitis. *Amino Acids*. 2008;35:225–231.
- Scibior D, Ashamiss F, Wierzbicki Z, et al. Arginase activity in blood serum of patients with acute and chronic pancreatitis. *Pol Merkuriusz Lek*. 2006;21:522–524.
- Chamorro G, Salazar M, Salazar S, et al. Antifertility effects of (+)-S-2-amino-6-iodoacetamidohexanoic acid (2-AIHA) in female rats. *Contraception*. 1996;53:247–251.
- Trujillo-Ferrara J, Koizumi G, Muñoz O, et al. Antitumor effect and toxicity of two new active-site-directed irreversible ornithine decarboxylase and extrahepatic arginase inhibitors. *Cancer Lett*. 1992;67:193–197.
- Tashiro M, Ernst SA, Edwards J, et al. Hyperthermia induces multiple pancreatic heat shock proteins and protects against subsequent arginine-induced acute pancreatitis in rats. *Digestion*. 2002;65:118–126.
- Takács T, Rakonczay Z Jr, Varga IS, et al. The comparative effects of water immersion pretreatment on three different acute pancreatitis models in rats. *Biochem Cell Biol*. 2002;80:241–251.
- Rakonczay Z Jr, Takács T, Boros I, et al. Heat shock proteins and the pancreas. *J Cell Physiol*. 2003;195:383–391.

III.

Characterization of Polyamine Homeostasis in L-Ornithine–Induced Acute Pancreatitis in Rats

AQ1

György Biczó, MSc,* Péter Hegyi, MD, PhD,* Riitta Sinervirta,† Sándor Berczi, MD,‡ Sándor Dósa, MD,‡ Andrea Siska, PhD,§ Béla Iványi, MD, PhD, DSc,‡ Viktória Venglovecz, PhD,|| Tamás Takács, MD, PhD, DSc,* Leena Alhonen, PhD,† and Zoltán Rakonczay Jr, MD, PhD*

Objectives: L-Ornithine is a precursor of polyamine synthesis that is essential for cell survival. In contrast, intraperitoneal (IP) administration of a large dose of L-ornithine results in death of pancreatic acinar cells in rats. We investigated changes in pancreatic and extrapancreatic polyamine homeostasis after injection of L-ornithine and tested the effects of the stable polyamine analogue methylspermidine (MeSpd) on L-ornithine–induced pancreatitis.

Methods: Male Wistar rats were injected IP with 3 g/kg L-ornithine and were untreated, pretreated, or treated with 50 mg/kg MeSpd IP. Rats were killed after 0 to 168 hours for determinations of polyamines and activities of ornithine decarboxylase and spermidine/spermine N^1 -acetyltransferase (SSAT). Pancreatitis severity was assessed by measuring standard laboratory and histological parameters.

Results: Injection of L-ornithine paradoxically induced pancreatic spermidine catabolism, possibly via activation of SSAT, after (>6 hours) appearance of the first histological signs of acute pancreatitis. Polyamine levels generally increased in the lung and liver with the exception of lung spermidine levels, which decreased. Methylspermidine did not influence polyamine levels and SSAT activity and did not ameliorate the severity of L-ornithine–induced pancreatitis.

Conclusions: L-Ornithine–induced pancreatitis was associated with activation of pancreatic polyamine catabolism. However, administration of a metabolically stable polyamine analogue did not affect disease severity.

Key Words: polyamines, pancreatitis, L-ornithine

Abbreviations: ASAT - aspartate aminotransferase, HSP72 - heat shock protein 72, IL-1 β - interleukin 1 β , IP - intraperitoneal(ly), MPO - myeloperoxidase, SSAT - spermidine/spermine N^1 -acetyltransferase, ODC - ornithine decarboxylase, MeSpd - 1-methylspermidine, PS - physiological saline

(*Pancreas* 2010;00: 00–00)

Polyamines (putrescine, spermidine, and spermine) are essential for normal function and survival of cells.¹ They are positively charged molecules and have been shown to interact with DNA, RNA, proteins, and acidic phospholipids in

membranes. Polyamines have been implicated in the regulation of several membrane-bound enzymes, such as adenylate cyclase, and ion channels. Furthermore, they may also have antioxidant properties. Physiologically, polyamines are synthesized from L-arginine (via L-ornithine) and L-methionine (via S-adenosylmethionine [SAM]). Polyamine metabolism is highly regulated by 3 key enzymes (Fig. 1).² Ornithine decarboxylase (ODC) and SAM decarboxylase mediate polyamine biosynthesis, whereas polyamine catabolism is controlled by the rate-limiting spermidine/spermine N^1 -acetyltransferase (SSAT).

The pancreas contains the highest concentration of spermidine in the mammalian body, but the exact mechanism how spermidine contributes to the maintenance of pancreatic integrity is unclear.^{3–4} In addition, the importance of polyamines in the pancreas is highlighted by the fact that activation of polyamine catabolism through an overexpression of SSAT in a transgenic rat line resulted in severe acute pancreatitis.⁵ It has been shown that pancreatic SSAT activation and subsequent polyamine catabolism are characteristic features of experimental acute pancreatitis models and also of human pancreatic tissue samples from patients with acute pancreatitis.^{6–8}

We have recently described that intraperitoneal (IP) injection of large doses of L-ornithine results in severe acute necrotizing pancreatitis in rats.⁹ As L-ornithine is a precursor of polyamines, the question arises how the biosynthesis and catabolism of polyamines change in L-ornithine–induced acute pancreatitis. Furthermore, as we observed pancreatic spermidine catabolism in rats with L-ornithine–induced pancreatitis, we also tested the effects of the metabolically stable polyamine analogue 1-methylspermidine (MeSpd) administration on the disease as a treatment to compensate for the loss natural spermidine.

MATERIALS AND METHODS

Materials

MeSpd was synthesized from 3-aminobutanol as described earlier.¹⁰ The polyamine analogue was dissolved in physiological saline (PS; 25 mg/mL, pH 7.4). All other chemicals were from Sigma-Aldrich (Munich, Germany) unless stated otherwise.

Experimental Protocols

Animals

Male Wistar rats weighing 180 to 220 g were used. The animals were kept at a constant room temperature of 23°C with a 12-hour light-dark cycle and were allowed free access to water and standard laboratory chow (Biofarm, Zagyvaszántó, Hungary). In each experimental group, 5 to 8 rats were used. The experiments performed in this study were approved by the Animal Care Committee of the University of Szeged.

Characterization of Polyamine Homeostasis in L-Ornithine–Induced Acute Pancreatitis

Pancreatitis was induced by IP injection with 3 g/kg L-ornithine. Polyamine homeostasis was studied at 6, 24, 72, and

From the *First Department of Medicine, University of Szeged, Szeged, Hungary; †Department of Biotechnology and Molecular Medicine, A. I. Virtanen Institute for Molecular Sciences, Biocenter Kuopio, University of Kuopio, Kuopio, Finland; and Departments of ‡Pathology, §Clinical Chemistry, and ||Pharmacology and Pharmacotherapy, University of Szeged, Szeged, Hungary.

Received for publication July 7, 2009; accepted January 11, 2010.
Reprints: Zoltán Rakonczay Jr, MD, PhD, First Department of Medicine, University of Szeged, PO Box 427, H-6701 Szeged, Hungary (e-mail: raz@in1st.szote.u-szeged.hu).

This study was supported by the Hungarian Scientific Research Fund (K78311 to Z.R., NNF78851 to P.H., and PD78087 to V.V.), the Hungarian Academy of Sciences (BO 00334/08/5 to P.H. and BO 00218/06 to Z.R.), and the Academy of Finland.
Methylspermidine was synthesized by Dr Nikolay Grigorenko (Engelhardt Institute of Molecular Biology, Russian Academy of Sciences, Moscow, Russia).

Copyright © 2010 by Lippincott Williams & Wilkins

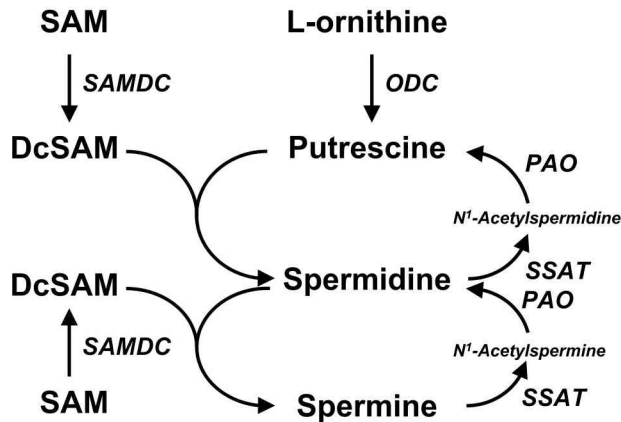


FIGURE 1. Rate-limiting enzymes of polyamine metabolism. Ornithine decarboxylase and SAM decarboxylase mediate polyamine biosynthesis, whereas polyamine catabolism is controlled by SSAT. DcSAM indicates decarboxylated SAM; PAO, polyamine oxidase.

168 hours after the initiation of pancreatitis ($n = 5-6$). The control animals received PS IP and were killed 24 hours after the injection ($n = 5$).

Effects of MeSpd on L-Ornithine-Induced Acute Pancreatitis

Rats were divided randomly into 6 groups. In the O24 group ($n = 8$), rats were injected with 3 g/kg L-ornithine IP and received PS IP 4 hours before ($n = 4$) or 4 hours after ($n = 4$) the L-ornithine treatment. In the OM24 group ($n = 6$), rats were pretreated with 50 mg/kg MeSpd IP 4 hours before injection with 3 g/kg L-ornithine IP. In the OM24 ($n = 6$) group, rats received 50 mg/kg MeSpd IP 4 hours after the L-ornithine (3 g/kg) injection. In the O48 ($n = 5$) group, rats were injected with 3 g/kg L-ornithine IP and received PS IP 4 and 24 hours thereafter. In the OM48 group ($n = 5$), rats were treated with 50 mg/kg MeSpd IP 4 and 24 hours after the L-ornithine (3 g/kg) treatment. In the control group ($n = 5$), rats received PS IP instead of L-ornithine and MeSpd. The “24” and “48” labels in the group names indicate the time points in hours at which rats were killed after the injection of L-ornithine.

In both protocols, rats were killed by exsanguination through the abdominal aorta after anaesthetization with 44 mg/kg pentobarbital IP. Tissue samples from pancreas (cleaned from fat and lymph nodes), liver, and lung were frozen in liquid nitrogen and stored at -80°C until use. All blood samples were centrifuged at 2500g for 20 minutes, and the serum was stored at -25°C .

Assays

Polyamine Levels and Activities of SSAT and ODC

The levels of the natural polyamines (spermidine, spermine, and putrescine) and the polyamine analogue MeSpd were determined by high-performance liquid chromatography according to the method of Hyvönen et al.¹¹ Pancreatic ODC and SSAT activities were assayed according to Jänne and Williams-Ashman¹² and Bernacki et al.,¹³ respectively.

Pancreatic Weight–Body Weight Ratio

The pancreatic weight–body weight (PW/BW) ratio was used to evaluate the degree of pancreatic edema.

Activities of Serum and Pancreatic Amylase, Serum Lipase, Aspartate Aminotransferase, and Serum Concentrations of Creatinine

Laboratory parameters were determined as described previously.⁹ Serum and pancreatic amylase activities were determined by an enzymatic colorimetric assay standardized by the International Federation of Clinical Chemistry (Diagnosticum Ltd, Budapest, Hungary). Serum lipase activity was determined by an enzymatic colorimetric method and serum concentration of creatinine by the kinetic colorimetric compensated Jaffé method of Roche Diagnostics GmbH (Mannheim, Germany). Aspartate aminotransferase (ASAT) was measured by an IFCC UV kinetic method (Human GmbH, Wiesbaden, Germany).

Pancreatic Myeloperoxidase Activity

Pancreatic myeloperoxidase (MPO) activity, as a marker of tissue leukocyte infiltration, was assessed using the method of Kuebler et al.¹⁴

Expression of Pancreatic HSP72 and IκB-α

Western blot analysis of pancreatic heat shock protein 72 (HSP72) and IκB-α expression was performed from the cytosolic fraction of the pancreas homogenate. Pancreatic cytosolic fractions were prepared as described previously.¹⁵ The protein concentration of the homogenate was determined by the method of Bradford.¹⁶ Forty micrograms of protein was loaded per lane. Samples were electrophoresed on an 10% sodium dodecyl sulfate–polyacrylamide gel according to the method of Laemmli.¹⁷ The proteins on the gels were either stained with Coomassie brilliant blue (to demonstrate equal loading of proteins for Western blot analysis) or transferred to a nitrocellulose membrane for 1 hour at 100 V. Equal transfer of proteins was verified by ponceau S staining. Membranes were blocked in 5% nonfat dry milk (Bio-Rad, Hercules, Calif) for 1 hour and incubated with rabbit anti-HSP72 (1:10,000 dilution, characterized previously by Kurucz et al.¹⁸) or rabbit anti-IκB-α (1:200 dilution; Santa Cruz Biotechnology, Santa Cruz, Calif) antibody for an additional 1 or 4 hours (respectively) at room temperature. The immunoreactive protein was visualized by enhanced chemiluminescence, using horseradish peroxidase–coupled anti-rabbit immunoglobulin (Dako, Glostrup, Denmark) at 1:10,000 dilution.

Pancreatic Interleukin 1β Concentration

The proinflammatory interleukin 1β (IL-1β) concentrations were measured in the pancreatic cytosolic fractions with an enzyme-linked immunosorbent assay kit (R&D Systems, Minneapolis, Minn) according to the manufacturer's instructions.

Histological Examination

A portion of the pancreas was fixed in 6% neutral formaldehyde solution and subsequently embedded in paraffin. Sections were cut at 4-μm thickness and stained with hematoxylin and eosin. The slides were coded and read by 2 independent observers who were blind to the experimental protocol. Pancreatic tissue injury was evaluated as described previously.⁹ Briefly, semiquantitative grading of interstitial edema (0–3), vascular congestion (0–1), leukocyte adhesion (0–3), and infiltration (0–4), apoptosis (0–3), necrosis (0–4), and regeneration (0–2) of acinar cells was determined in each animal.

Statistical Analysis

Results are expressed as means \pm SEM. Experiments were evaluated by using the analysis of variance followed by Dunnett

multiple-comparisons post hoc test. Values of $P < 0.05$ were considered significant.

RESULTS

Polyamine Homeostasis in L-Ornithine-Induced Acute Pancreatitis

Pancreas

F2 Pancreatic spermidine content significantly decreased 24 to 168 hours after the injection of 3 g/kg L-ornithine, whereas spermine content significantly increased (by 1.6-fold) only at 72 hours (Figs. 2A, B). Considering the different pool sizes of spermidine and spermine, the net effect was a significant depletion of the total polyamine pool beginning from 24 hours after injection of L-ornithine. Pancreatic ODC activity signifi-

cantly increased at 24 hours (Fig. 2C); SSAT activity significantly increased from 24 hours and peaked at 72 hours with an almost 10-fold maximum elevation and remained significantly higher than the activity in the control group until 168 hours (Fig. 2D). Surprisingly, putrescine was not detectable in the pancreas, although putrescine accumulation should be an evident consequence of simultaneously increased activities of SSAT and ODC (if synthesis would not override catabolism).

Liver

Hepatic putrescine level showed a 26-fold elevation at 6 hours after L-ornithine injection, but thereafter fell back to control values (Fig. 3A). Hepatic spermidine level significantly increased from 6 to 72 hours (Fig. 3B), and spermine content also showed significant elevation from 24 to 72 hours (Fig. 3C). **F3**

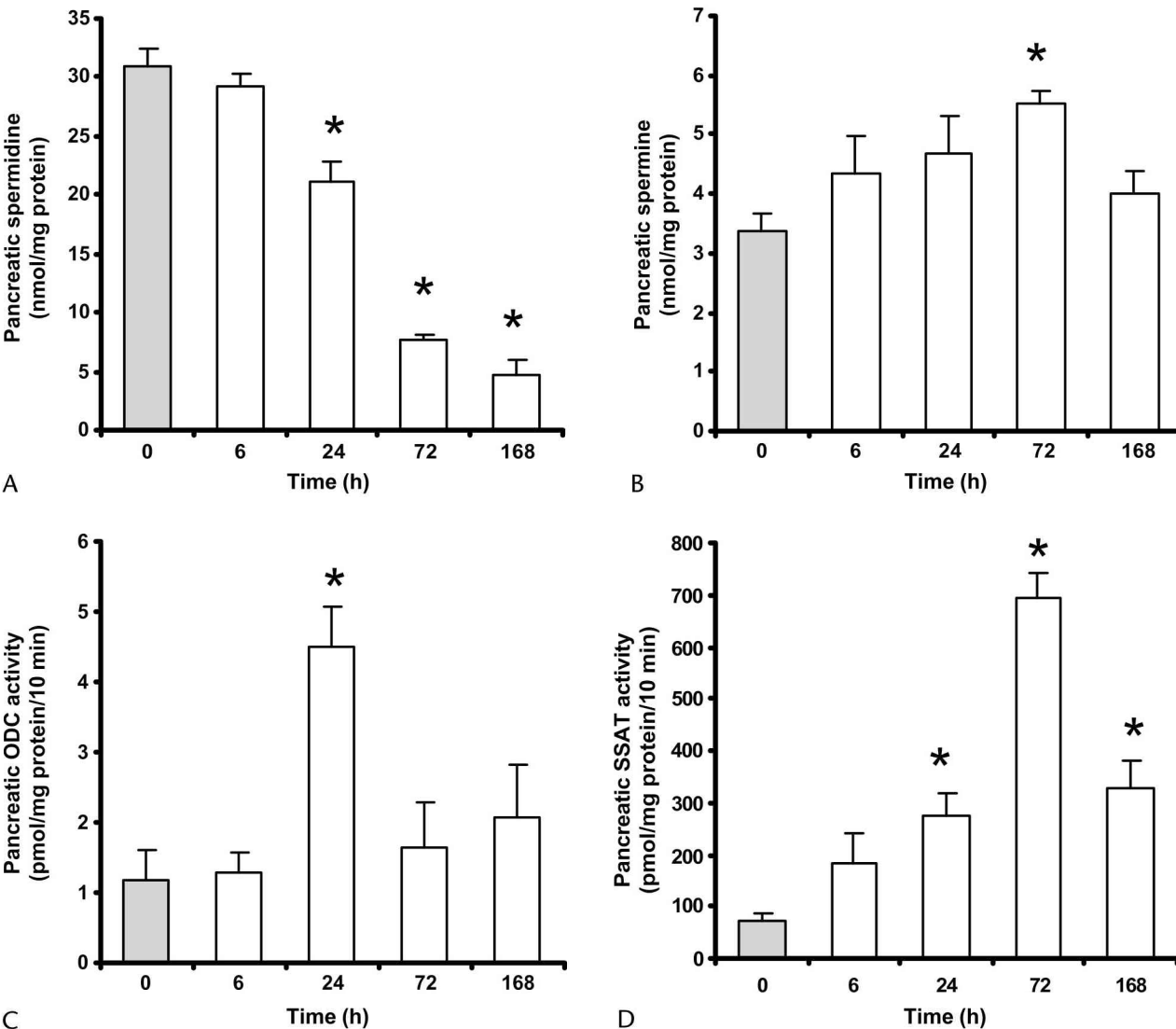


FIGURE 2. Time-course changes in pancreatic polyamine homeostasis after IP administration of 3 g/kg L-ornithine. Pancreatic (A) spermidine and (B) spermine levels and activities of (C) ODC and (D) SSAT are shown. Data are presented as means \pm SEM, $n = 5-6$. *Significant difference ($P < 0.05$) versus the control group (0 hour, gray column).

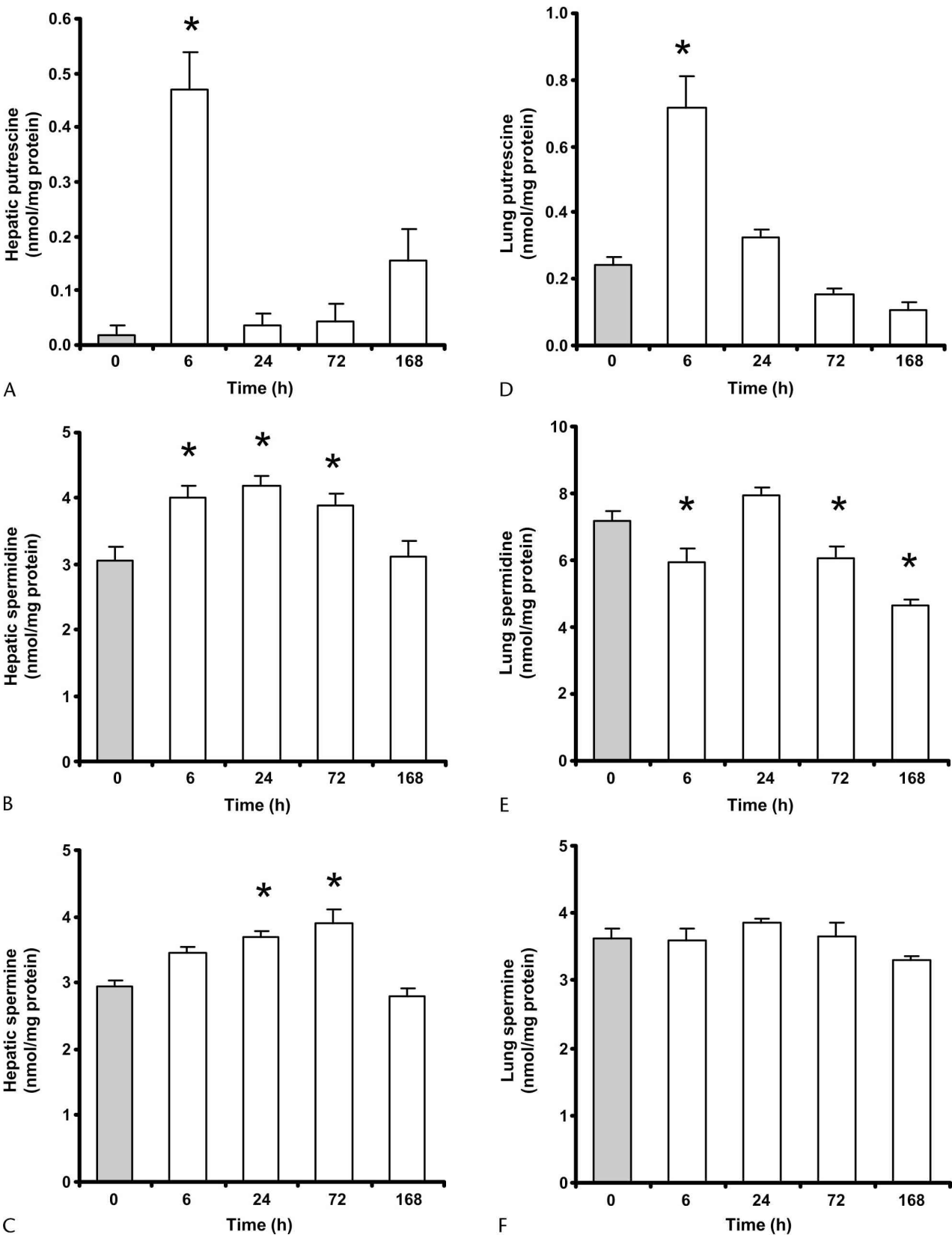


FIGURE 3. Time-course changes in hepatic and lung polyamine pools after IP administration of 3 g/kg L-ornithine. The diagrams demonstrate hepatic (A) putrescine, (B) spermidine, and (C) spermine levels; and lung (D) putrescine, (E) spermidine, and (F) spermine levels. Data are shown as means \pm SEM, $n = 5-6$. *Significant difference ($P < 0.05$) versus the control group (0 hour, gray column).

Lung

Similarly to that observed in the liver, lung putrescine levels showed a significant peak at 6 hours after L-ornithine injection (Fig. 3D). In contrast to the changes observed in the hepatic polyamine pools, lung spermidine content showed significantly decreased levels at 6, 72, and 168 hours (Fig. 3E). Lung spermine content was not significantly altered at the investigated time points (Fig. 3F).

Effects of the Synthetic Polyamine Analogue 1-MeSpd on L-Ornithine-Induced Acute Pancreatitis

Pancreatic SSAT Activity, Putrescine, Spermidine, MeSpd, and Spermine Content

Methylspermidine accumulated in the pancreas as a result of both pretreatment (MO24: 4.53 ± 1.79 nmol/mg protein) and

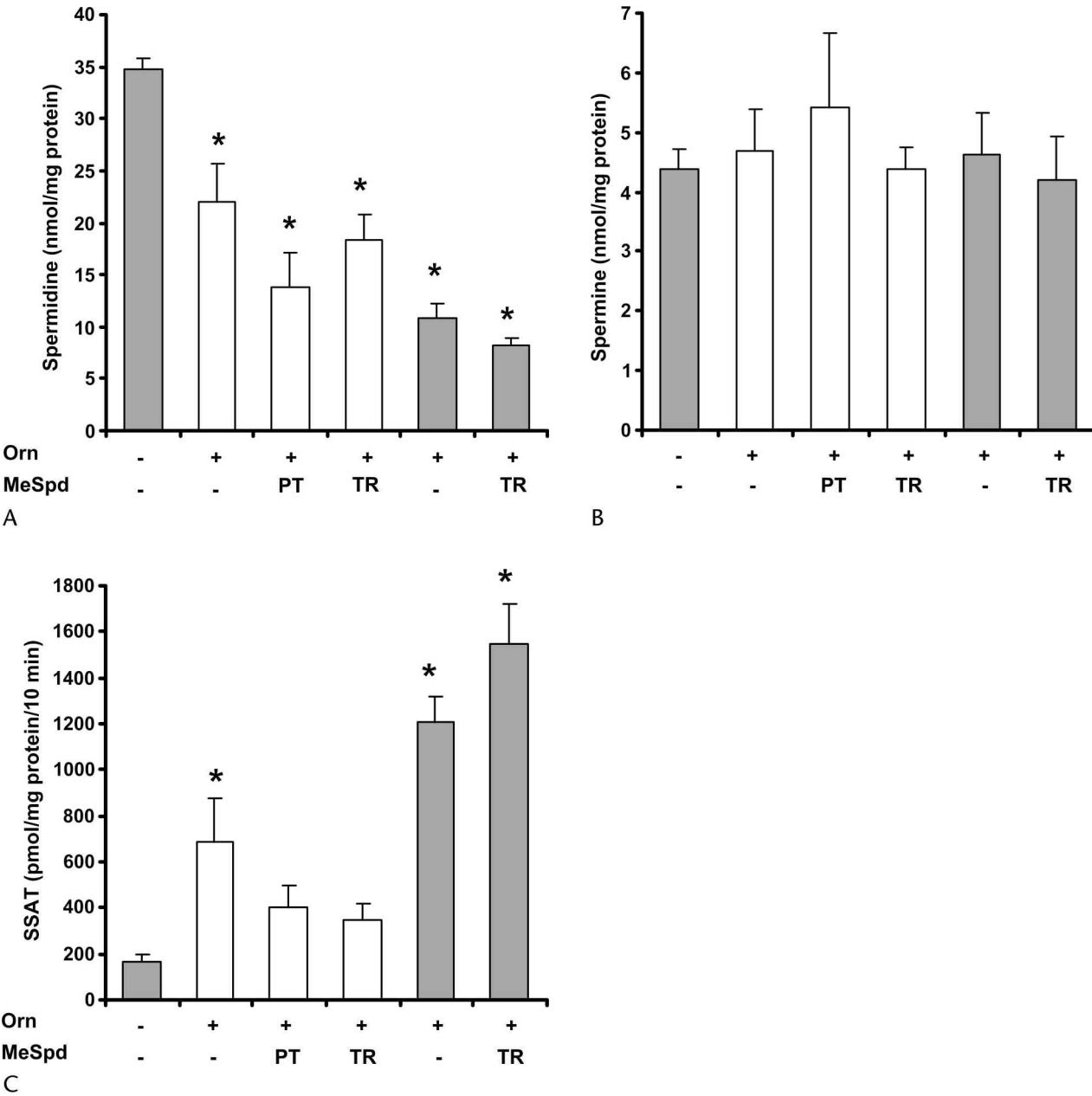


FIGURE 4. Effects of 1-MeSpd (MeSpd) administration on changes in pancreatic polyamine homeostasis in L-ornithine-induced acute pancreatitis. The diagrams demonstrate pancreatic (A) spermidine and (B) spermine levels and (C) SSAT activity. Groups of rats were injected IP with 3 g/kg L-ornithine (Orn +) or PS (Orn -) and were untreated (MeSpd -), pretreated (MeSpd PT), or treated (MeSpd TR) with 50 mg/kg MeSpd IP. The white and gray bars indicate groups of animals killed at 24 or 48 hours (respectively) after the L-ornithine or PS injection. For a more detailed experimental protocol, see Materials and Methods. Data are shown as means \pm SEM, n = 5–8. *Significant difference ($P < 0.05$) versus the control group (Orn -, MeSpd -).

Fig 5 4/C

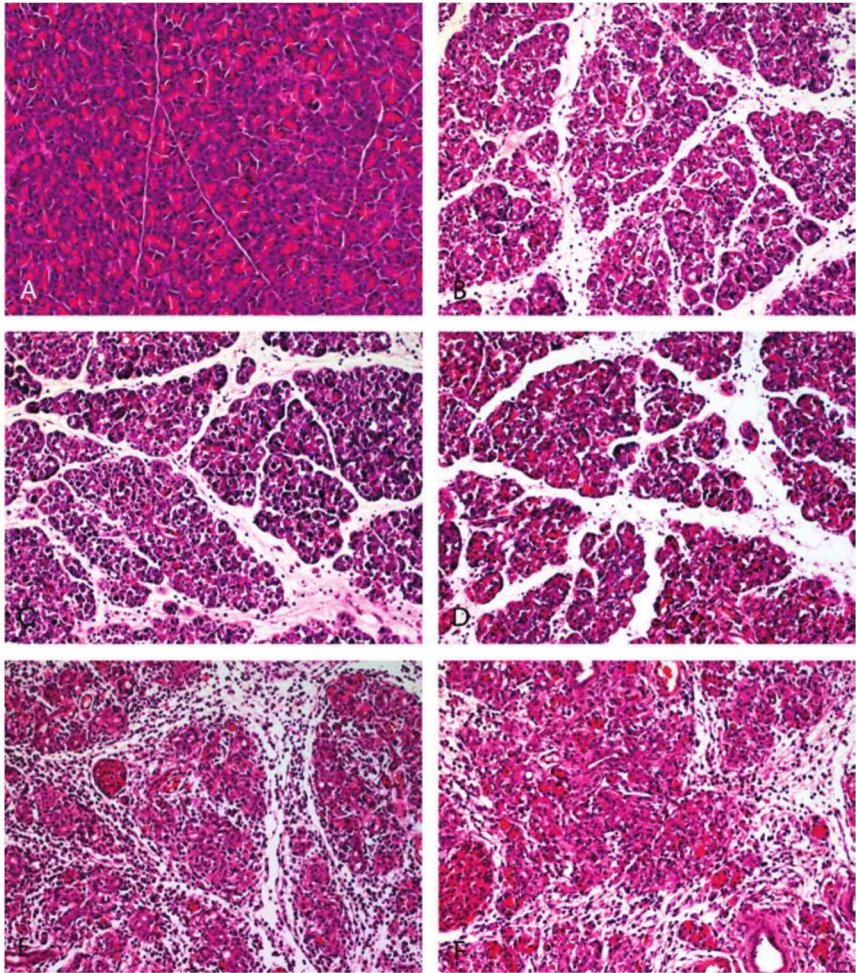


FIGURE 5. Effects of MeSpd on pancreatic morphological damage in L-ornithine–induced acute pancreatitis. The images show representative hematoxylin and eosin images of pancreata of rats injected IP with (A) PS or (B–F) 3 g/kg L-ornithine, which were (A, B, E) untreated, (C) pretreated, or (D, F) treated (twice in case of F) with 50 mg/kg MeSpd IP. Animals were killed at (A–D) 24 hours or (E–F) 48 hours after the injection of L-ornithine or PS. Original magnification $\times 200$.

TABLE 1. Effects of 1-MeSpd on Histological Parameters in L-Ornithine–Induced Acute Pancreatitis					
MeSpd Time of Sacrifice, h	—	PT	TR	—	TR
		24		48	
Vascular congestion	0.6 \pm 0.2*	1.0 \pm 0.0*	0.8 \pm 0.2*	0.8 \pm 0.2*	1.0 \pm 0.0*
Leukocyte adherence	3.0 \pm 0.0*	3.0 \pm 0.0*	2.7 \pm 0.2*	2.8 \pm 0.2*	2.8 \pm 0.2*
Interstitial edema	2.8 \pm 0.2*	3.0 \pm 0.0*	2.8 \pm 0.2*	3.0 \pm 0.0*	2.8 \pm 0.2*
Leukocyte infiltration	2.9 \pm 0.5*	2.8 \pm 0.3*	3.5 \pm 0.3*	4.0 \pm 0.0*	3.4 \pm 0.2*
Vacuolization	0.3 \pm 0.2	0.0 \pm 0.0	0.0 \pm 0.0	0.0 \pm 0.0	0.2 \pm 0.2
Necrosis	4.0 \pm 0.0*	4.0 \pm 0.0*	3.8 \pm 0.2*	0.0 \pm 0.0	0.0 \pm 0.0
Apoptosis	1.8 \pm 0.5*	1.7 \pm 0.4*	1.3 \pm 0.5*	0.0 \pm 0.0	0.2 \pm 0.2
Regeneration	0.0 \pm 0.0	0.0 \pm 0.0	0.0 \pm 0.0	1.8 \pm 0.2*	2.0 \pm 0.0*
Total	15.3 \pm 0.5*	15.5 \pm 0.6*	15.0 \pm 0.8*	12.4 \pm 0.2*	12.4 \pm 0.5*

Rats were injected IP with 3 g/kg L-ornithine and were untreated (MeSpd —), pretreated (MeSpd PT), or treated (MeSpd TR, twice in case of the animals killed at 48 hours) with 50 mg/kg MeSpd IP. Animals were killed at 24 or 48 hours after the injection of L-ornithine. Values for control animals (injected IP with PS instead of L-ornithine and MeSpd) were 0.0 \pm 0.0.

*Significant difference ($P < 0.05$) versus the control group.

treatment (OM24: 10.42 ± 1.35 nmol/mg protein, OM48: 8.74 ± 1.18 nmol/mg protein). Pancreatic spermidine content significantly decreased in the L-ornithine-treated groups (Fig. 4A), whereas spermine contents did not show any alteration (Fig. 4B). Pancreatic SSAT activity significantly increased in response to L-ornithine injection by more than 4-fold at 24 hours and more than 7-fold at 48 hours (Fig. 4C). Putrescine was not present in detectable amounts in any of the groups (results not shown). Methylspermidine administration did not affect any of these parameters.

Histological Examination

Interstitial edema, vascular congestion, leukocyte adherence, and infiltration and necrosis of acinar cells greatly increased at 24

and 48 hours in response to L-ornithine injection (Fig. 5). Apoptosis of acinar cells was detected only at 24 hours. Methylspermidine administration did not ameliorate any of the investigated histological parameters (Fig. 5; Table 1).

Serum and Pancreatic Amylase Activities, Serum Lipase Activity, and PW/BW Ratio

Serum amylase activities did not increase significantly in response to L-ornithine injection. However, MeSpd treatment in the OM24 group significantly increased serum amylase activity, and it was also significant versus the O24 group (Fig. 6A). Pancreatic contents of amylase significantly decreased in the L-ornithine-treated groups versus the control group except in the

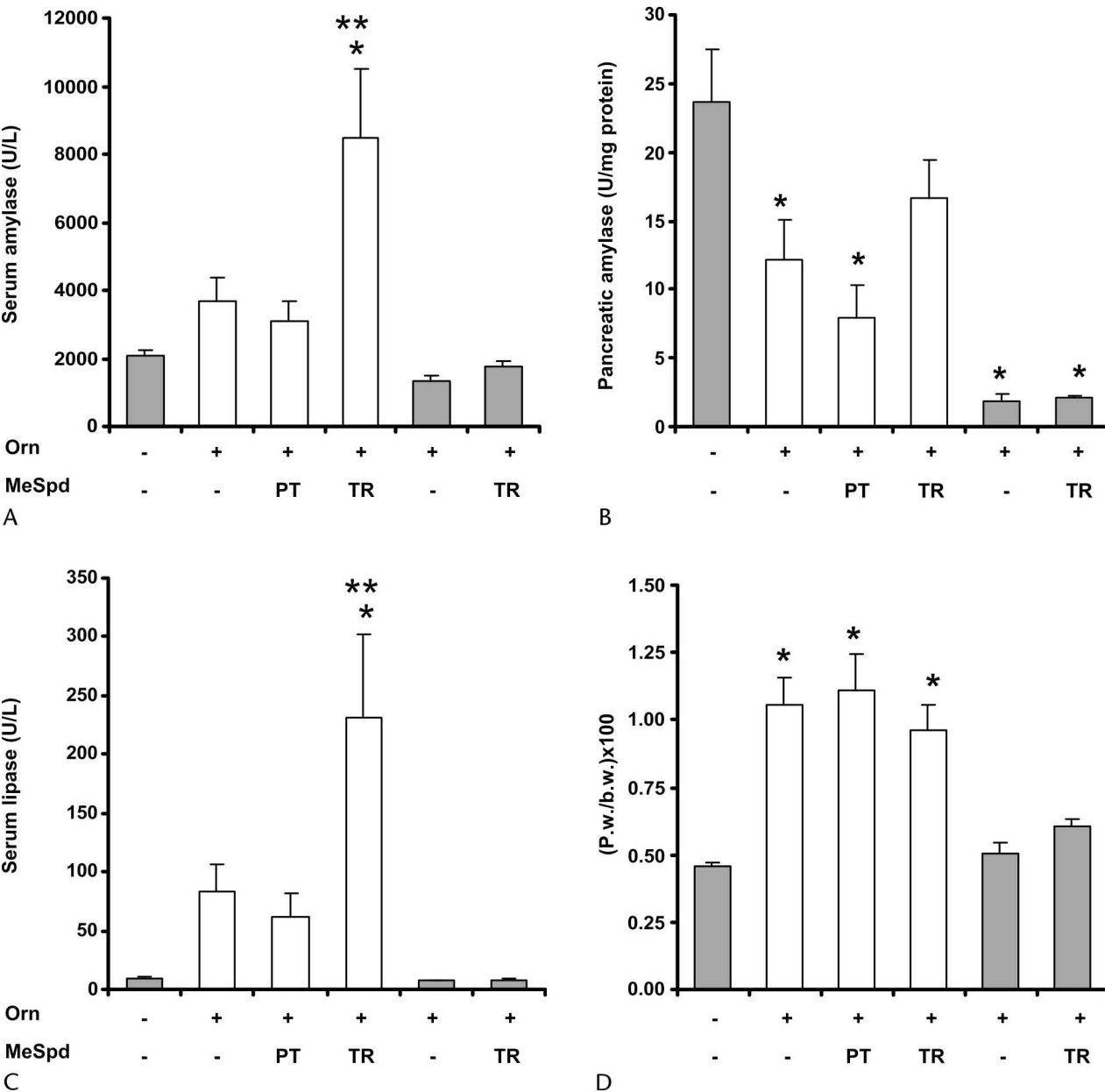


FIGURE 6. Effects of MeSpd on the activities of serum and pancreatic digestive enzymes and PW/BW ratio in L-ornithine-induced acute pancreatitis. The bar charts demonstrate activities of (A) serum and (B) pancreatic amylase, (C) serum lipase, and (D) PW/BW ratio. See legend of Figure 4 for the labeling of the bars. Data are shown as means \pm SEM, $n = 5-8$. Significant difference ($P < 0.05$) versus the *control (Orn -, MeSpd -) or **Orn 24 hours (Orn +, MeSpd -, white bar) group.

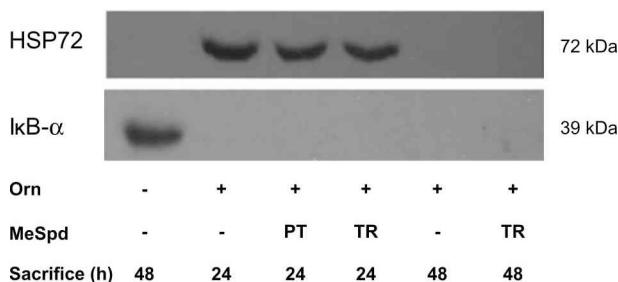


FIGURE 7. Effects of MeSpd on pancreatic HSP72 and IκB-α levels in L-ornithine-induced acute pancreatitis. The figure shows representative Western immunoblot analysis of protein lysates (40 μg/lane) from the pancreata of rats administered PS, L-ornithine, and/or MeSpd. See legend of Figure 4 for the labeling of groups. Rats were killed, as indicated, at 24 or 48 hours after injection with PS or L-ornithine.

OM24 group (Fig. 6B). Serum lipase activity significantly increased in the L-ornithine-treated groups at 24 hours versus the control group (Fig. 6C). Serum lipase activity significantly increased in the OM24 group versus the O24 group (Fig. 6C). Pancreatic weight–body weight ratio significantly increased at 24 hours in response to 3 g/kg L-ornithine (Fig. 6D). Methylspermidine administration did not influence PW/BW, either at 24 hours or at 48 hours.

Pancreatic HSP72 and IκB-α Expression

Heat shock proteins are essential cytoprotective molecules that are known to be induced in acute pancreatitis.¹⁸ IκB-α is an inhibitory protein of the proinflammatory transcription factor, nuclear factor κB (NF-κB).¹⁹ Injection of 3 g/kg L-ornithine induced pancreatic HSP72 synthesis at 24 hours (Fig. 7). Pancreatic IκB-α levels decreased significantly 24 and 48 hours after the L-ornithine injection (Fig. 7). Methylspermidine administration did not affect HSP72 and IκB-α levels.

Pancreatic MPO Activity

Pancreatic MPO activity significantly increased in the L-ornithine-treated groups versus the control group (Fig. 8A). Methylspermidine administration did not influence MPO activity in any of the groups.

Pancreatic IL-1β levels

Corresponding to IκB degradation, and consequently to activation of NF-κB, pancreatic IL-1β synthesis showed significantly elevated levels at 24 and 48 hours after the L-ornithine injection (Fig. 8B). Methylspermidine treatments had no significant effects on proinflammatory cytokine levels, although the analogue treatment seemed to partially prevent the elevation of IL-1β level.

Serum Concentrations of Creatinine and ASAT Activity

Significant elevations of serum creatinine concentrations were detected in the O24 and OM24 groups versus the control group (Fig. 8C). Serum ASAT activities significantly increased 24 and 48 hours after L-ornithine injection (Fig. 8D). Methylspermidine administration did not affect serum ASAT activity in any of the groups.

DISCUSSION

The involvement of polyamines in the pathogenesis of acute pancreatitis was first found in transgenic rats over-

expressing SSAT.⁵ Interestingly, we have also found increased pancreatic spermidine catabolism (possibly mediated via activation of SSAT) in L-ornithine-induced acute pancreatitis. In contrast, polyamine levels generally increased in the lung and liver with the exception of lung spermidine levels, which decreased. Pretreatment or treatment with the stable polyamine analogue MeSpd did not influence the levels of natural polyamines and SSAT activity and did not ameliorate the severity of L-ornithine-induced acute pancreatitis. This may be explained by the finding that the analogue did not accumulate in the pancreas to the level compensating for the most of spermidine depletion with the exception of the treatment group at 24 hours.

Assuming that the direct transformation of L-ornithine into putrescine may secure specific targeting of the polyamine metabolism but consists of a partial utilization of L-ornithine, “detoxification” of the excess amino acid load may also include conversion of L-ornithine to citrulline or glutamic semialdehyde. The influence of these metabolic routes cannot be excluded.

Pancreatic polyamine catabolism seems to be a general feature of acute pancreatitis.^{6–8} In the current study on L-ornithine-induced acute pancreatitis, we have shown that polyamine levels are altered only after the first signs of histological damage. Whereas the earliest events (vascular congestion, vacuolization, and apoptosis of acinar cells) in L-ornithine-induced acute pancreatitis pancreas can already be detected at 4 hours,⁹ polyamine catabolism started only after 6 hours. The belated elevations in the activity of SSAT and spermidine catabolism suggest that these mechanisms do not take part in the initiation of L-ornithine-induced acute pancreatitis. Nevertheless, polyamine catabolism was apparent at 24 hours after the injection of L-ornithine when the highest degree of pancreatic injury was detected. The earliest time point at which pancreatic polyamine pools and SSAT activity were investigated in the similar L-arginine-induced model was 24 hours.⁶ However, in taurocholate-induced pancreatitis, pancreatic SSAT activity already increased from 3 to 6 hours.⁸ An interesting difference between the taurocholate-induced, arginine-induced, and the L-ornithine-induced models is that neither spermine catabolism nor putrescine accumulation could be detected in the pancreas despite the elevations in SSAT and ODC activities in the L-ornithine-induced model. As polyamine homeostasis is controlled not only by their metabolism, but also by their secretion and uptake, these findings might be a consequence of altered polyamine transport.^{1–2} They may also be an indication of accelerated polyamine metabolic cycle, as described for SSAT transgenic mice,²⁰ which could in part explain the lack of spermine depletion in L-ornithine-induced pancreatitis where the synthesis dominates over the catabolism.

As acute pancreatitis will affect extrapancreatic organs such as the liver and lung, we also investigated changes of polyamine pools in these tissues. Depletion of spermidine and spermine levels has been reported in red blood cells of rats with taurocholate-induced pancreatitis, and these changes correlated with the extent of pancreatic necrosis.²¹ We have demonstrated that changes in polyamine pools in response to L-ornithine injection occur not only in the pancreas and blood, but also in the liver and lung. Interestingly, although a marked accumulation of putrescine was observed in these tissues at 6 hours, subsequent activation of polyamine catabolism was not detected in these organs. Only a moderate decrease was found in lung spermidine levels.

Synthetic α-methylated polyamine analogues, such as MeSpd, are metabolically more stable than natural polyamines as they are not substrates for SSAT and are poor substrates for spermine synthase.²² Nevertheless, they are supposed to fulfill

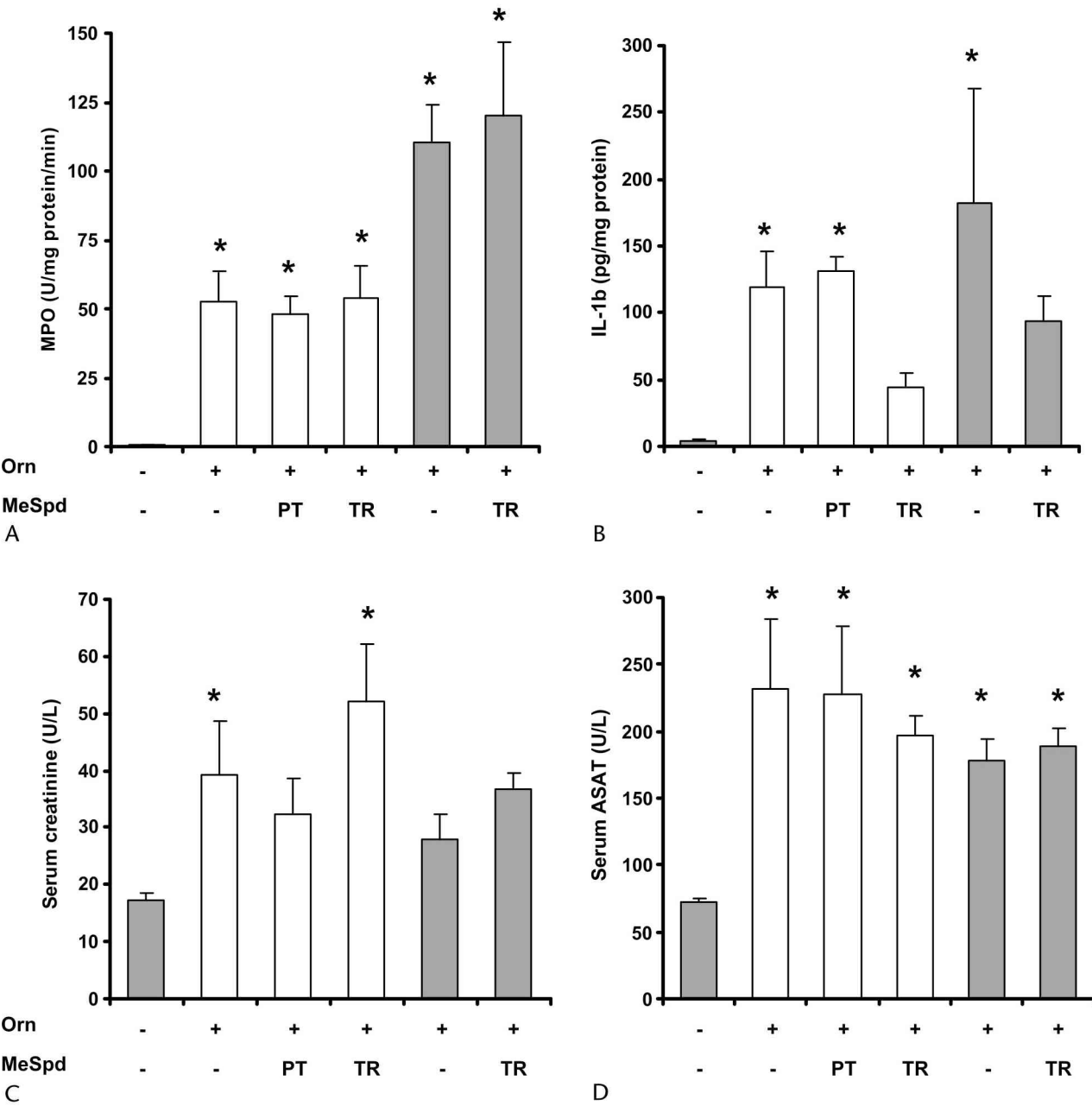


FIGURE 8. Effects of MeSpd on pancreatic parameters reflecting inflammation and extrapancreatic organ involvement in L-ornithine-induced acute pancreatitis. The bar charts demonstrate pancreatic (A) MPO activities, (B) IL-1β levels and (C) serum creatinine concentrations, and (D) ASAT activities. See legend of Figure 4 for the labeling of the bars. Data are shown as means ± SEM, n = 5–8. *Significant difference ($P < 0.05$) versus the control group (Orn −, MeSpd −).

most of the putative cellular functions of natural spermidine and spermine. Methylated derivatives of polyamines are substrates of proteins involved in polyamine transport and accumulate in the pancreas after IP administration. Moreover, they act as substitutes of natural polyamines.²³ Methylspermidine pretreatment totally prevented zinc-induced pancreatitis in transgenic rats overexpressing SSAT as judged by plasma α-amylase activity and histopathology.²⁴ In this study, we administered MeSpd (to compensate for depleted pancreatic spermidine levels) as a single-injection pretreatment before the induction of L-ornithine-induced pancreatitis or twice as

treatment after the induction of pancreatitis. In both cases, MeSpd was ineffective at reducing the severity of the disease. This was demonstrated by measuring several laboratory and histological parameters of acute pancreatitis. Both the pretreatment and treatment with MeSpd led to decreased spermidine levels in the L-ornithine-induced pancreatitic groups, which suggest that catabolism of the natural polyamines further increased in the presence of synthetic polyamine analogue or the analogue replaces the natural spermidine in its binding site in the tissue. The current results are partly in contrast with the earlier studies on L-arginine- and taurocholate-induced

pancreatitis. Methylspermidine pretreatment was shown to reduce the severity of L-arginine-induced (2.5 g/kg) pancreatitis without preventing SSAT activation and subsequent polyamine catabolism.⁶ In contrast (and in accordance with our results), the severity of cerulein-induced ($7 \times 50 \mu\text{g/kg}$) pancreatitis was not affected by MeSpd pretreatment.⁶ The common feature is that neither in cerulein-induced pancreatitis nor in L-ornithine-induced pancreatitis did the analogue accumulate to the levels compensating for the depleted polyamine pools. Bismethylspermine treatment (but not pretreatment) ameliorated pancreatic injury at 24 hours after the induction of taurocholate-induced pancreatitis, but it did not ameliorate the late progression of the pancreatic necrosis at 72 hours.²¹ Moreover, bismethylspermine treatment resulted in lethal toxicity at 72 hours after induction of pancreatitis.²¹ We must note that the infusion of sodium taurodeoxycholate (2%) into the pancreatic duct or IP injection of 2.5 g/kg L-arginine will result in milder pancreatitis versus 3 g/kg L-ornithine. The contrasting results may be attributed to the fact that stable polyamine analogues may be effective only at earlier time points and in less severe forms of necrotizing acute pancreatitis.

In the present study, we have shown that increased SSAT activity and consequent spermidine catabolism in the pancreas are apparent phenomena in L-ornithine-induced acute pancreatitis in rats. However, the fact that these changes occur belatedly and that MeSpd administration did not ameliorate any of the parameters, either in the pretreatment or in treatment group, suggests that activated polyamine catabolism does not play a role in the initiation of pancreatic injury in L-ornithine-induced experimental pancreatitis. The organ-specific responses to L-ornithine and the method of pancreatic damage induction by the basic amino acid need to be investigated. Further studies are needed to reveal the role of polyamine homeostatic processes in the maintenance of pancreatic integrity.

ACKNOWLEDGMENTS

The authors thank Dr István Kurucz for providing the HSP72 antibody and Ms Tuula Reponen for skillful technical assistance.

REFERENCES

- Wallace HM, Fraser AV, Hughes A. A perspective of polyamine metabolism. *Biochem J*. 2003;376:1–14.
- Moinard C, Cynober L, de Bandt JP. Polyamines: metabolism and implications in human diseases. *Clin Nutr*. 2005;24:184–197.
- Löser C, Fölsch UR, Cleffmann U, et al. Role of ornithine decarboxylase and polyamines in camostat (Foy-305)-induced pancreatic growth in rats. *Digestion*. 1989;43:98–112.
- Jänne J, Alhonen L, Pietilä M, et al. Genetic approaches to the cellular functions of polyamines in mammals. *Eur J Biochem*. 2004;271:877–894.
- Alhonen L, Parkkinen JJ, Keinänen T, et al. Activation of polyamine catabolism in transgenic rats induces acute pancreatitis. *Proc Natl Acad Sci U S A*. 2000;97:8290–8295.
- Hyvönen MT, Herzig KH, Sinervirta R, et al. Activated polyamine catabolism in acute pancreatitis: alpha-methylated polyamine analogues prevent trypsinogen activation and pancreatitis-associated mortality. *Am J Pathol*. 2006;168:115–122.
- Hyvönen MT, Merentie M, Uimari A, et al. Mechanisms of polyamine catabolism-induced acute pancreatitis. *Biochem Soc Trans*. 2007;35:326–330.
- Jin HT, Lämsä T, Merentie M, et al. Polyamine levels in the pancreas and the blood change according to the severity of pancreatitis. *Pancreatol*. 2008;8:15–24.
- Rakonczay Z Jr, Hegyi P, Dósa S, et al. A new severe acute necrotizing pancreatitis model induced by L-ornithine in rats. *Crit Care Med*. 2008;36:2117–2127.
- Grigorenko NA, Vepsäläinen J, Järvinen A, et al. A new synthesis of alpha-methylspermidine. *Bioorgan Khim (Moscow)*. 2004;30:441–445.
- Hyvönen T, Keinänen TA, Khomutov AR, et al. Monitoring of the uptake and metabolism of aminoxy analogues of polyamines in cultured cells by high-performance liquid chromatography. *J Chromatogr*. 1992;574:17–21.
- Jänne J, Williams-Ashman HG. On the purification of L-ornithine decarboxylase from rat prostate and effects of thiol compounds on the enzyme. *J Biol Chem*. 1971;246:1725–1732.
- Bernacki RJ, Bergeron RJ, Porter CW. *N,N'*-bis(ethyl)spermine homologues against human MALME-3 melanoma xenografts. *Cancer Res*. 1992;52:2424–2430.
- Kuebler WM, Abels C, Schuerer L, et al. Measurement of neutrophil content in brain and lung tissue by a modified myeloperoxidase assay. *Int J Microcirc Clin Exp*. 1996;16:89–97.
- Rakonczay Z Jr, Jármai K, Kaszaki J, et al. NF-kappaB activation is detrimental in arginine-induced acute pancreatitis. *Free Radic Biol Med*. 2003;34:696–709.
- Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem*. 1976;72:248–254.
- Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*. 1970;227:680–685.
- Kurucz I, Tombor B, Prechl J, et al. Ultrastructural localization of HSP-72 examined with a new polyclonal antibody raised against the truncated variable domain of the heat shock protein. *Cell Stress Chaperones*. 1999;4:139–152.
- Rakonczay Z Jr, Takács T, Boros I, et al. Heat shock proteins and the pancreas. *J Cell Physiol*. 2003;195:383–391.
- Rakonczay Z Jr, Hegyi P, Takács T, et al. The role of NF-kB activation in the pathogenesis of acute pancreatitis. *Gut*. 2008;57:259–267.
- Pirinen E, Kuulasmaa T, Pietilä M, et al. Enhanced polyamine catabolism alters homeostatic control of white adipose tissue mass, energy expenditure, and glucose metabolism. *Mol Cell Biol*. 2007;27:4953–4967.
- Jin HT, Lämsä T, Hyvönen MT, et al. A polyamine analog bismethylspermine ameliorates severe pancreatitis induced by intraductal infusion of taurodeoxycholate. *Surgery*. 2008;144:49–56.
- Lakanen JR, Coward JK, Pegg AE. alpha-Methyl polyamines: metabolically stable spermidine and spermine mimics capable of supporting growth in cells depleted of polyamines. *J Med Chem*. 1992;35:724–734.
- Järvinen A, Grigorenko N, Khomutov AR, et al. Metabolic stability of alpha-methylated polyamine derivatives and their use as substitutes for the natural polyamines. *J Biol Chem*. 2005;280:6595–6601.
- Räsänen TL, Alhonen L, Sinervirta R, et al. A polyamine analogue prevents acute pancreatitis and restores early liver regeneration in transgenic rats with activated polyamine catabolism. *J Biol Chem*. 2002;277:39867–39872.

AQ2