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

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**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.<sup>1–4</sup> 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).<sup>5</sup> 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.<sup>6</sup> 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,<sup>7,8</sup> 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.<sup>7</sup> 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

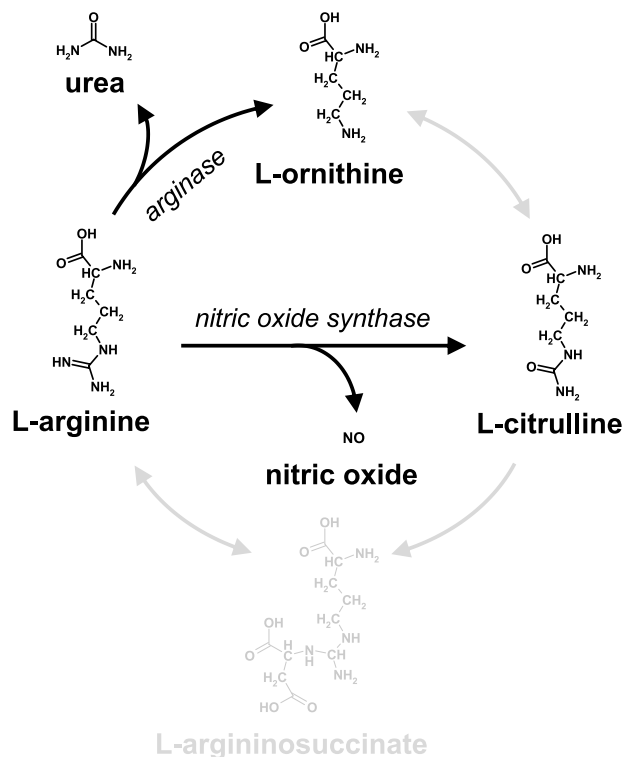
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**FIGURE 1.** Key enzymes involved in the metabolism of L-arginine. Nitric oxide synthase catalyzes the conversion of L-arginine to nitric oxide and L-citrulline. Arginase hydrolyzes L-arginine to L-ornithine and urea.

activity. All blood samples were centrifuged at 2500 g for 20 minutes, and the serum was stored at  $-25^{\circ}\text{C}$ .

### 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  $\text{N}_2$  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  $\text{MgSO}_4$ ). 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  $\text{CaCl}_2$ ) 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  $2 \times 30$  seconds. The homogenates were then centrifuged at 25,000 g for 30 minutes (at  $4^{\circ}\text{C}$ ), and the heat-activated ( $60^{\circ}\text{C}$ , 20 minutes) supernatants (their protein concentrations were measured by the method of Bradford<sup>14</sup>) 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.<sup>9</sup> Briefly, 20 mM L-arginine-HCl substrate (pH set to 9.7), 0.2 mM  $\text{MnCl}_2$ , and the sample were mixed in 250  $\mu\text{L}$  and incubated at  $37^{\circ}\text{C}$  for 5 to 15 minutes. The reaction was stopped by adding 250  $\mu\text{L}$  1 N  $\text{HClO}_4$  and centrifuged, and 140  $\mu\text{L}$  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.<sup>10</sup> After heating at  $100^{\circ}\text{C}$  (20 minutes) and cooling, optical densities were read at 535 nm using urea standards.

The inhibitory effect of 0 to 120  $\mu\text{M}$  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  $\mu\text{M}$  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.<sup>11</sup>

### 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.<sup>12</sup>

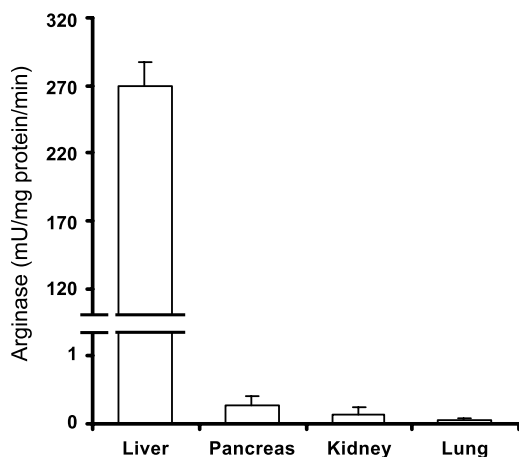
### Expression of Pancreatic Heat Shock Proteins 27 and 72

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.<sup>13</sup> The protein concentration of the homogenate was determined by the method of Bradford.<sup>14</sup> 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.<sup>15</sup> 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.<sup>16</sup>) 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

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**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.<sup>9</sup>

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- $\mu$ 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.<sup>7</sup> 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%.<sup>[F3]</sup>

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

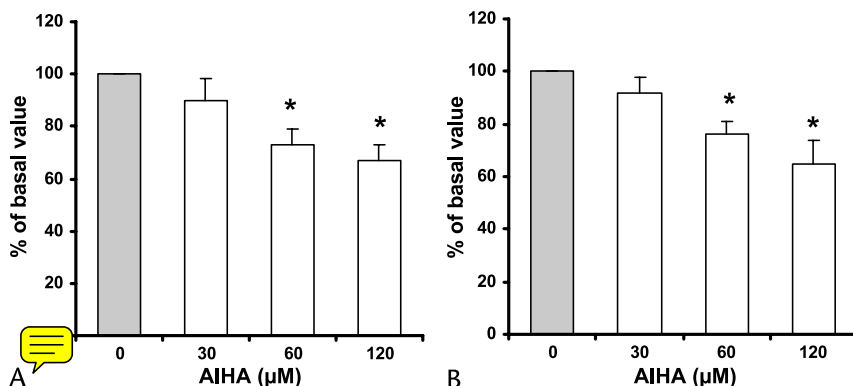
Pancreatic weight/body weight ratio was significantly increased in response to administration of L-arginine (Fig. 4A). Pretreatment with AIHA significantly ameliorated this increase of PW/BW ratio. Serum amylase activity was not significantly altered in any of the groups (Fig. 4B). Pancreatic contents 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

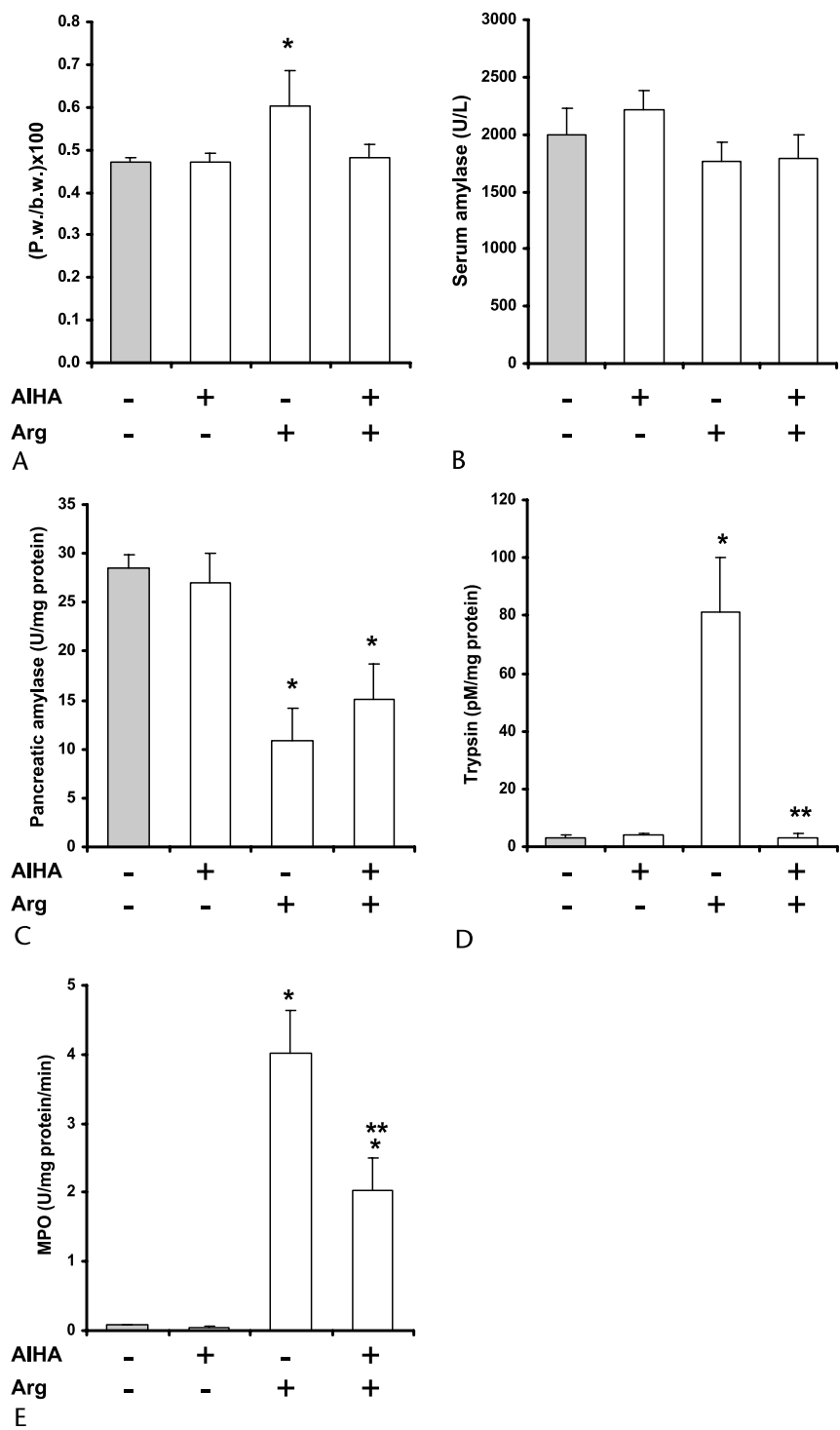
Pancreatic MPO activity was significantly increased at 24 hours after L-arginine injection (Fig. 4E). Pretreatment with AIHA significantly decreased MPO activity in the L-arginine-induced pancreatitis group.

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

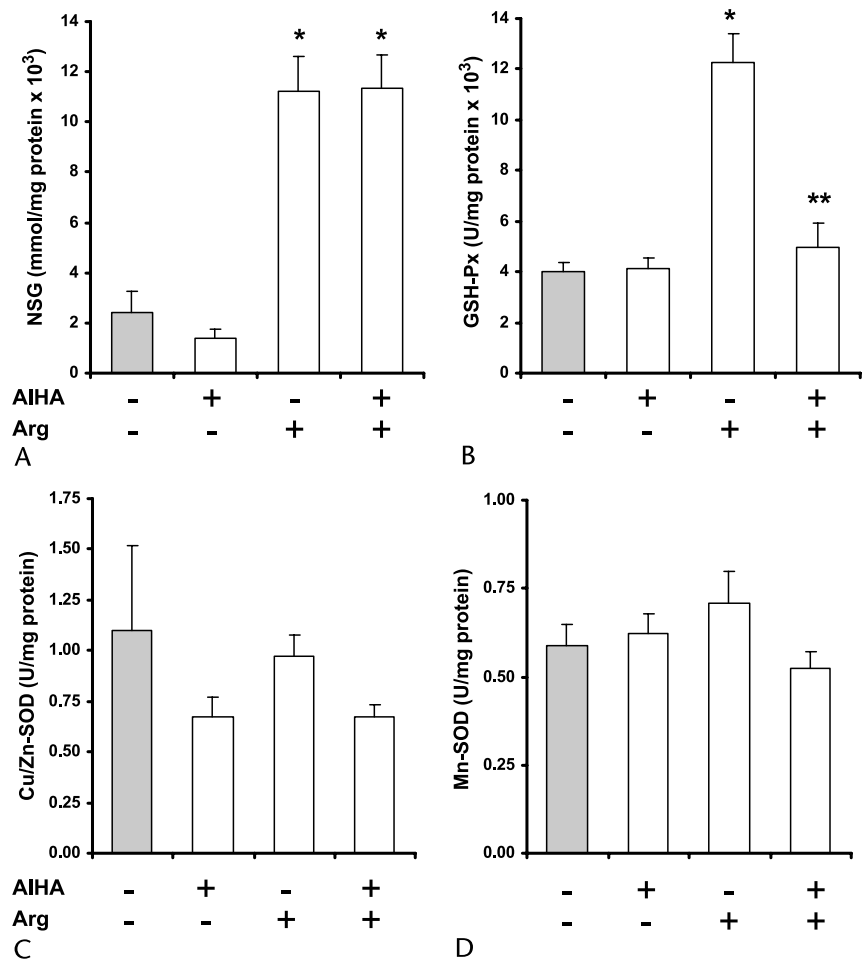
Pancreatic NSG content and GSH-Px activity were significantly increased 24 hours after the injection with L-arginine (Figs. 5A, B). Pretreatment with AIHA did not influence NSG<sup>[F5]</sup>



**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  $\mu$ 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).

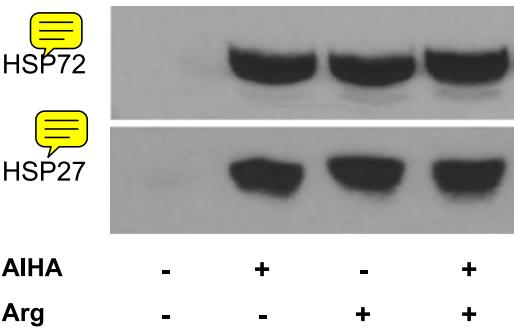
Pancreatic HSP Expression

(+)-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.

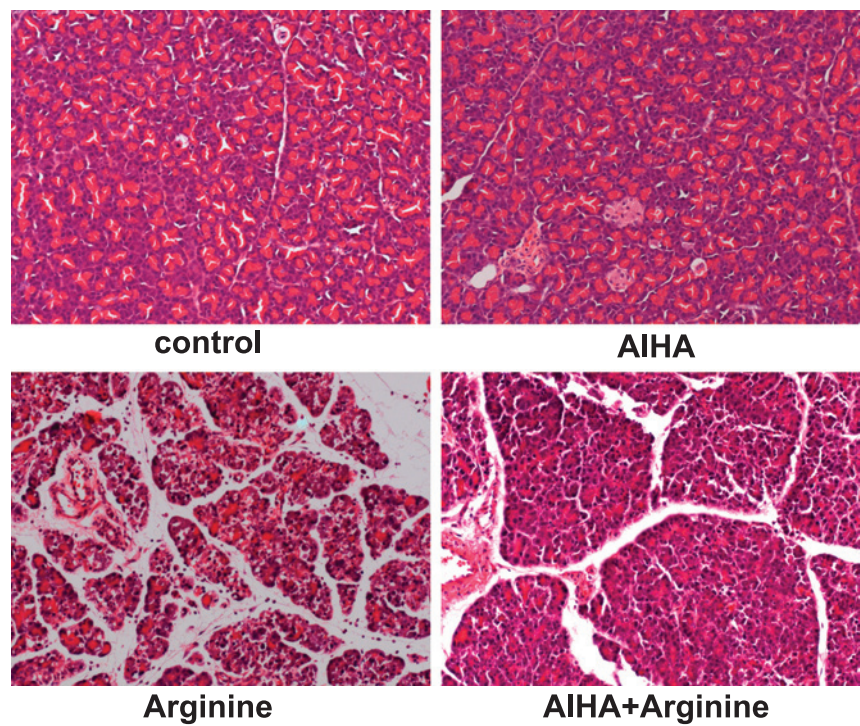
ornithine versus citrulline concentration.<sup>7</sup> This finding suggested a major role for arginase (rather than NOS) in the catabolism of L-arginine. In the present study, we have shown that pretreatment



**FIGURE 6.** Effects of AIHA pretreatment on pancreatic HSP synthesis in L-arginine-induced pancreatitis. Representative Western immunoblot analysis of protein lysates (40  $\mu$ 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.



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,<sup>17</sup> 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.<sup>8</sup>

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.<sup>18</sup> On the contrary, Scibior et al<sup>19</sup> 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.<sup>20,21</sup> The dosing of AIHA was based on literature data.<sup>20,21</sup> 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  $\mu$ M AIHA in vitro did not result in significantly lower levels of arginase activity versus 60  $\mu$ 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,<sup>22</sup> although in our studies we found only reductions in proinflammatory cytokine synthesis.<sup>23</sup> 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.<sup>24</sup>

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.

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