Pancreatitis-associated Protein Protects the Lung from Leukocyte-induced Injury

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Background: **Severe pancreatitis is often complicated by shock and acute lung failure. Little is known about the pathophysiologic impact of the 16.6-kD lectine, named pancreatitis-associated protein (PAP), which is expressed during pancreatitis and which reduces mortality in a rat model with severe pancreatitis. Therefore, the aim of this study was to investigate the effects of PAP on the pulmonary vasculature after leukocyte activation with *N*-formyl-Met-Leu-Phe (fMLP).**

Methods: **The experiments were performed in buffer-perfused isolated rabbit lungs. Mean pulmonary artery pressure, weight gain, and thromboxane A_2 synthesis of the lungs were monitored. PAP was obtained by affinity chromatography of pancreas juice from pancreatitic rats. The authors tested whether treatment with PAP (260 μg/l, n = 9; or 500 μg/l, n = 6) before fMLP injection (10^{-6} M) influences mean pulmonary artery pressure and edema formation. Lungs that were treated only with fMLP (n = 6) served as controls. Additional experiments in which PAP was applied were performed to study whether PAP (260 μg/l, n = 3; 500 μg/l, n = 3; 1,000 μg/l, n = 3) itself effects lung vasculature.**

Results: **Application of fMLP resulted in an increase of mean pulmonary artery pressure (± SD) from 8 ± 2 mmHg up to 26 ± 13 mmHg ($P < 0.01$) at a flow of 150 ml/min. Pretreatment with PAP reduced the peak pressure developed after fMLP to 15 ± 7 mmHg (PAP 260 μg/l; $P < 0.05$) and to 9 ± 4 mmHg (PAP 500 μg/l), respectively. In addition, the fMLP-induced lung weight gain of 9 ± 7 g in the controls was prevented by pretreatment with PAP after 150 min in either concentration. In parallel to the attenuated pressure increase, thromboxane A_2 release was significantly suppressed in the 260-μg/l (200 ± 220 pmol · ml^{-1} · min^{-1}; $P < 0.01$) and 500-μg/l (285 ± 70 pmol · ml^{-1} · min^{-1}; $P < 0.05$) PAP groups compared with controls (1,138 ± 800 pmol · ml^{-1} · min^{-1}). Treatment with PAP alone in either concentration did not induce any changes in mean pulmonary artery pressure, weight gain, or thromboxane A_2 release.**

Conclusion: **Clinically relevant concentrations of PAP prevented fMLP-induced vasoconstriction and edema formation in the lung. These findings point toward a protective effect of PAP on polymorphonuclear neutrophil leukocyte–mediated lung injury. (Key words: Acute phase response; acute respiratory distress syndrome; neutrophils.)**

SEVERE pancreatitis is often complicated by acute lung failure, which increases mortality.[1] During severe pancreatitis, the cascade of inflammation is activated, resulting in the release of cytokines, *e.g.*, tumor necrosis factor-α and interleukin-1 and -6 from macrophages and monocytes.[2,3] These early inflammatory cytokines have pleiotropic activity and act both locally and distally, causing the release of secondary wave cytokines, the expression of adhesion molecules, chemotaxis of leukocytes, and release of final mediators such as lipid mediators and reactive oxygen species.[4,5] The resulting increased capillary permeability is followed by edema formation.[6]

During pancreatitis, digestive enzymes such as amylase and lipase are downregulated,[7] and newly synthesized proteins are overexpressed.[8] In 1984, Keim *et al.*[9,10] identified a unique 16.6-kD secretory protein that was not detectable in the healthy gland but appeared 6 h after induction of experimental pancreatitis, reaching peak values after 48 h.[11] Therefore, the protein was called pancreatitis-associated protein (PAP). PAP may be of clinical relevance as a prognostic marker because a direct relationship between severity of pancreatitis and

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increased serum levels of PAP has been shown in rats[12] and humans.[11] Peak values ranged between 240 μg/l and 1,400 μg/l in patients with acute pancreatitis.[11,13] Little is known about the pathophysiologic impact of PAP. Recently, Fiedler *et al.*[14] demonstrated improved survival in animals with elevated levels of PAP in a rat model of necrotizing pancreatitis.

Pancreatitis-induced pulmonary distress has been implicated to be caused by cytokine-derived activation of leukocytes in the pulmonary vasculature.[15,16] Thus, effects of leukocyte activation in the lung are examined in the current study. To provoke respiratory burst, the well-established standardized method of receptor stimulation of leukocytes with *N*-formyl-Met-Leu-Phe (fMLP) was used.[17]

The aim of the current study was to evaluate the effect of PAP in leukocyte-associated lung failure. The effects of PAP on pulmonary vascular resistance and edema formation were investigated, and synthesis of thromboxane (TX) A_2 was measured by analyzing its stable metabolite, TXB_2.

Materials and Methods

Isolated Rabbit Lung

The techniques of preparing and perfusing isolated rabbit lungs have been previously described in detail.[18,19] After approval by the local board for animal protection, female chinchilla rabbits (*Orticolagus caniculus*) weighing 2,100 ± 196 g (mean ± SD) were anesthetized with ketamine (50 mg/kg) and xylazine (4 mg/kg) and anticoagulated with heparin-sodium 1,000 U/kg injected in the ear vein. After placement of a tracheostomy tube, the rabbits were mechanically ventilated with room air. The thorax was opened *via* the diaphragm, and after a median sternotomy, a catheter was inserted into the pulmonary artery. The lung organ preparation was isolated and suspended from a weight transducer in a temperature-controlled (37°C) and humidified chamber. After the cannulation procedure, the lungs were perfused with 200 ml Krebs Henseleit hydroxy-ethyl-starch buffer solution (KHB) by a roller pump at a constant volume inflow of 150 ml/min in a closed recirculatory system with an open reservoir to collect outflow from the left atrium. Pulmonary embolism by circulating particles or air was prevented by a 40-μm filter and a bubbletrap placed right before the pulmonary artery catheter. The lungs were ventilated with 4% CO_2 in air (frequency, 25 breaths/min; tidal volume, 30 ml; positive end-expiratory pressure, 0.5–1.0 cm H_2O) and, to avoid atelectasis formation, intermittently expanded by increasing the expiratory pressure up to 3 cm H_2O for three inspirations. The mean pulmonary arterial pressure (mpap) and airway pressures were continuously recorded *via* Statham strain-gauge transducers. Because oxygenation of the lung by ventilation was satisfactory and previous experiments did not show any hypoxic cellular damage,[20] no additional oxygenator unit in the perfusion circuit was required. Because of a constant perfusion flow, alterations of perfusion pressure directly reflect alterations of pulmonary vascular resistance. Samples of perfusate were taken intermittently from a catheter that collects the effluent from the pulmonary veins, for measurements of *p*H, oxygen and carbon dioxide partial pressure, oxygen saturation (blood gas analysis system 288; Ciba Corning, Fernwald, Germany), oncotic pressure (Onkometer BMT 921, Dr. Karl Thomae GmbH, Berlin, Germany), and determination of TXB_2 concentrations. The lungs were initially perfused with KHB solution using low flow rates in an open circulatory system to remove remaining blood from the vascular bed. The perfusion fluid was then exchanged for fresh buffer *via* two separate perfusion circuits 2 min after the beginning of the extracorporeal circulation and 15 min later, after the flow was increased to 150 ml/min. After another 30-min steady-state period, these lungs had a constant mean mpap of 8–9 mmHg (zero-referenced at the hilum). The only lungs selected for the study were those that showed a homogenous white appearance with no signs of hemostasis or edema formation and that had no changes in weight during the steady-state period. In previous experiments, perfusion with KHB has been documented to maintain integrity of the microcirculation for more than 5 h in our model, which was assessed by measurements of mpap and weight gain, by biochemical analysis (lactate dehydrogenase, arachidonic acid metabolites, histamine), and by ultrastructural studies.[20]

Preparation and Purification of Rat PAP

Male Sprague Dawley rats (250–300 g) were housed with free access to food and water. The main pancreatic duct was cannulated under ether anesthesia, and edematous pancreatitis was induced by intravenous infusion of cerulein (5 $\mu g \cdot kg^{-1} \cdot h^{-1}$) for 6 h.[21] Pancreatic juice was collected on ice for several hours as previously described[22] and immediately frozen, lyophilized, and stored at −70°C.

Affinity Chromatography

A specific antirat-PAP antibody[23] was prepared according to the method described by Iovanna *et al.*[24] and was covalently bound to a solid matrix (Affi-Gel 10 gel; Bio-Rad, Ivry sur Seine, France) according to the manufacturers' specifications. The column was equilibrated with 2-[*N*-morpholino] ethane sulfonic acid buffer (20 mM; *p*H 6.5) with 150 mM NaCl. Lyophilized pancreatic juice was resuspended in 2-[*N*-morpholino] ethane sulfonic acid buffer (20 mM; *p*H 6.5), 150 mM NaCl, 5 mM benzamidine, 2 mM phenylmethylsulfonyl fluoride, and 5 mM *N*α-benzoyl-L-arginin. After centrifugation (3,000*g* for 15 min), the supernatant was applied to the affinity gel column and recirculated overnight at 4°C. Thereafter, the column was washed with 2-[*N*-morpholino] ethane sulfonic acid buffer to elute unbound contaminants. The bound protein was eluted with glycine buffer (200 mM; *p*H 2.8), dialyzed against phosphate-buffered saline buffer, and concentrated by centrifugation with Centrifugal Ultrafree-20-Kit (Millipore; Saint-Quentin, France).

Quality Control of Protein Isolation

Protein concentration was measured according to Bradford.[25] Part of the protein solution was submitted to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (15% acrylamide; 0.5% bisacrylamide), which was performed according to standard procedures.[26] The gels were either stained by a silver-staining method,[27] or the samples were blotted on nitrocellulose membranes, and Western blot analysis was performed with a specific antirat-PAP antibody.[23,24,26] Silver staining revealed a single protein band corresponding to the molecular weight of PAP, which crossreacts with the antirat-PAP antibody during Western blot analysis, indicating that the purified protein was indeed PAP.

Experimental Protocol

Thirty lung preparations were randomly assigned to the different groups. After a 30-min equilibration period, the first perfusate sample was drawn for measurements of baseline values. In pilot experiments, the effects of different concentrations of PAP (260 μg/l, n = 3; 500 μg/l, n = 3; 1,000 μg/l, n = 3) were investigated. Therefore, only PAP was administered after the steady-state phase, and mpap and weight gain of the lungs were monitored for 150 min. Samples of perfusate were taken at 5, 10, 30, 60, 120, 150 min to investigate TXB_2 release and to analyze blood gases.

As in pancreatitis-induced lung injury, which is likely to be leukocyte-mediated *via* inflammatory receptor stimulation, neutrophil activation was achieved in six lung preparations without further interventions. For this purpose, the chemotactic peptide fMLP was added after a 30-min steady-state period, yielding a final concentration of 10^{-6} M in the perfusion fluid, and monitoring was performed as described for the pilot experiments (control group). To study protective effects of PAP on polymorphonuclear neutrophil leukocyte-mediated lung injury, in the experimental groups PAP was added to the perfusion fluid in two different concentrations (260 μg/l, n = 9; or 500 μg/l, n = 6) 15 min before fMLP administration (10^{-6} M). These concentrations have been reported in moderate to severe pancreatitis.[11] The applied time schedule was chosen to guarantee a uniform distribution of PAP in the whole lung before inflammatory activation, which induces transient perfusion inhomogenities. Monitoring of mpap, weight gain, TXB_2, and blood gases was performed similar to the other groups.

Measurement of TXB_2

Thromboxane B_2 was assayed from 100 μl of recirculating KHB as stable hydrolysis product of TXA_2 by an enzyme immunoassay (TiterZyme TXB_2 EIA; PerSeptive Diagnostics Inc., Framingham, MA). The immunoassay and the photometric measurement (AR 2001 photometer; Anthos Labtech Instruments, Krefeld, Germany) were performed according to the manufacturers specifications. The crossreactivity of TXB_2-antiserum with 2,3-Dinor-TXB_2 was 55.8%, 1.5% with 11-Dehydro TXB_2, 1.0% with prostaglandin B_2, and 0.5% with prostaglandin D_2. Other eicosanoids exert cross reactivity less than 0.1%.

Because the lungs in the current study were perfused in a closed circuit, the inactive metabolite TXB_2 accumulated over the observation period in the perfusate. Thus, TXB_2 release was calculated per minute as the difference of TXB_2 to the previous value divided by the time in minutes.

Materials

A cell- and plasma-free perfusion medium was used to avoid the complex interactions with different circulating cells, which may mask direct effects on vascular tone and mediator release. The perfusate consisted of a Krebs-Henseleit buffer solution with a colloid oncotic pressure between 23 and 25 mmHg, yielding the following final concentrations: Na^+, 138 mM; K^+, 4.5 mM; Mg^{++}, 1.33 mM; Cl^-, 135 mM; Ca^{++}, 2.38 mM; glucose, 12 mM; HCO_3^-, 12 mM. The osmolality was approximately 330

mOsm/kg (Mikro-Osmometer; Roebling Meßtechnik, Berlin, Germany). The *p*H of the buffer solution was adjusted to 7.4 with 1 M $NaHCO_3$. Effects caused by endotoxin contamination of the plasma-free perfusate can be excluded in our model, as assessed in previous experiments.[28] No hemodynamic reactions, thromboxane generation, or histamine release was observed after endotoxin addition to the perfusate in the absence of plasma complement components.[28]

fMLP

N-formyl-Met-Leu-Phe was purchased from Sigma Chemicals (Deisenhofen, Germany), dissolved in dimethyl sulfoxide, and stored deep-frozen until use. Final concentrations of fMLP in the pulmonary circuit were 10^{-6} M.

Statistical Analysis and Data Presentation

Data are presented as mean ± SD. Differences between groups were tested by one-way analysis of variance followed by a Student Newman-Keuls multiple-comparison procedure. Significance was accepted at $P < 0.05$.

This study was approved by the Animal Subject Protection Committee of the local government. The care and handling of animals were in accordance with the principles expressed in the Helsinki Declaration.

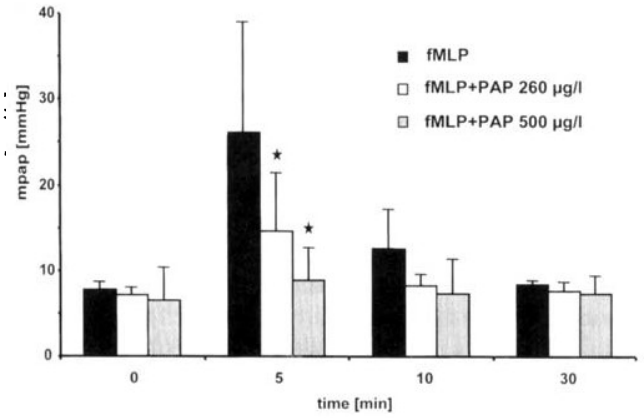

Fig. 1. Mean pulmonary artery pressure (mpap; ± SD) of lungs in which leukocyte activation was performed with 10^{-6} M *N*-formyl-Met-Leu-Phe (fMLP) in the absence or in presence of different concentrations of pancreatitis-associated protein (PAP; 260 µg/l, n = 9; 500 µg/l, n = 6). *$P < 0.05$ *vs.* fMLP, analysis of variance. PAP reduced mpap in a dose-dependent manner. Baseline values before fMLP administration at time point 0.

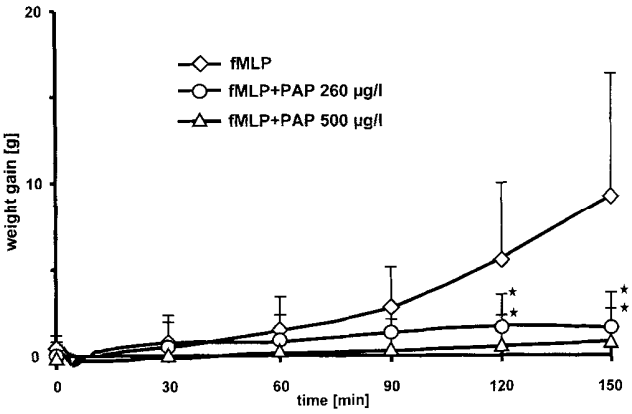

Fig. 2. Weight gain of lungs (± SD) in which leukocyte activation was performed with 10^{-6} M *N*-formyl-Met-Leu-Phe (fMLP) in the absence or in presence of pancreatitis-associated protein (PAP; 260 µg/l, n = 9; 500 µg/l, n = 6). *$P < 0.05$ *vs.* fMLP, analysis of variance. PAP reduced weight gain in a dose-dependent manner.

Results

After an equilibration period of 30 min, baseline values of mpap (8 ± 2 mmHg) and airway pressure (6-8 cm H_2O) were similar in all groups and in agreement with previous studies reported by our group.[18] Because effects of PAP on the pulmonary vasculature are unknown, administration of PAP was investigated to determine its properties in our lung preparation. During perfusion with any concentration of PAP (260 µg/l, n = 3; 500 µg/l, n = 3; 1,000 µg/l, n = 3), no changes in mpap, weight gain, or TXB_2 release were observed, and the lung preparations were stable until the end of the 150-min observation period. The leukocyte activation with fMLP in the lungs resulted in an acute increase of mpap up to 26 ± 13 mmHg ($P < 0.01$) within 5 min (fig. 1). Baseline levels of mpap were regained 30 min after fMLP addition and were then maintained for 120 min. A dose-dependent effect on mpap was observed after treatment with PAP. The pressure response was reduced significantly to 15 ± 7 mmHg (PAP 260 µg/l; $P < 0.05$) and 9 ± 4 mmHg (PAP 500 µg/l), respectively. Mean pulmonary artery pressure returned to baseline 10 min after fMLP and remained almost constant until the end of the experiment. fMLP induced lung weight gain of 9 ± 7 g during the 150-min observation period, whereas pretreatment with PAP in either concentration significantly ($P < 0.05$) prevented pulmonary edema formation (PAP 260 µg/l, 2 ± 2 g; 500 µg/l, 1 ± 2 g; fig. 2).

Analysis of perfusate samples revealed a baseline concentration of circulating TXB_2 of 2.63 ± 2.60 nmol/ml

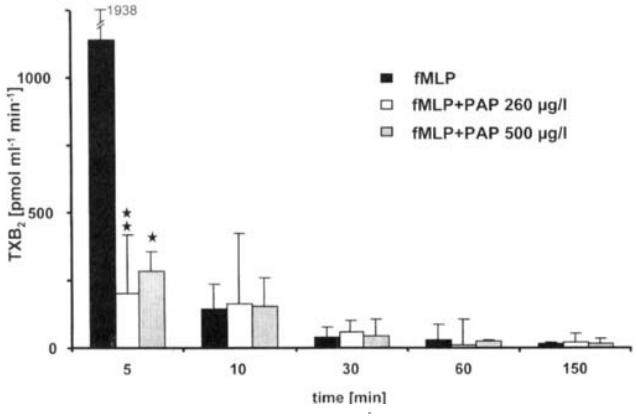

Fig. 3. Release of thromboxane (TX) B_2 (± SD), a stable metabolite of TXA_2, into the perfusate of isolated rabbit lungs in which leukocyte activation was performed with 10^{-6} M *N*-formyl-Met-Leu-Phe (fMLP) in the absence or in presence of pancreatitis-associated protein (PAP; 260 µg/l, n = 9; 500 µg/l, n = 6). *$P < 0.05$ *vs.* fMLP, **$P < 0.01$ *vs.* fMLP, analysis of variance. PAP reduced TXB_2 generation in a dose-dependent manner.

and an immediate increase ($P < 0.005$) after fMLP injection. PAP significantly suppressed TXB_2 formation caused by fMLP injection compared with controls. Although mean values in controls reached 1,138 ± 800 pmol · ml^{-1} · min^{-1}, TXB_2 detected in the PAP groups were 200 ± 220 pmol · ml^{-1} · min^{-1} ($P < 0.01$; PAP 260 µg/l) and 285 ± 70 pmol · ml^{-1} · min^{-1} ($P < 0.05$; PAP 500 µg/l) 5 min after fMLP injection (fig. 3).

Oxygen saturation, oxygen and carbon dioxide partial pressures, and *p*H and base excess did not significantly change throughout the observation period in all experiments.

Discussion

Pulmonary dysfunction such as pulmonary edema and hypoxemia is one of the main systemic complications in acute pancreatitis.[1,6] Experimental studies provided evidence that pancreatitis induces cytokines that activate granulocytes in the lung vasculature, initiating pulmonary damage[15] by elastase and phospholipase A_2 release.[29] Despite intensive research efforts, until now the effects of potential modifiers of this inflammatory response, such as heat shock proteins and PAP, are not well understood. Recently, characteristic alterations of protein synthesis at translational and transcriptional levels were detected during acute pancreatitis.[8] The most prominent finding was the overexpression of an unique c-lectin protein in the pancreas of animals[9] and humans.[10] This protein is absent in the normal gland but synthesized in high amounts in the inflamed pancreas.[12] During inflammation, evidence accumulated that the acute-phase protein response, including PAP, exerts beneficial effects in different species and in distinct experimental settings.[14] After induction of acute pancreatitis in rats, PAP was first detected after 6 h. Maximum levels corresponding to a 400–1,000-fold increase of PAP were measured within 48 h.[11,30] Considering that high amounts of this unique protein are expressed during acute pancreatitis correlating with the severity of disease,[11] but also correlating with increased survival in rats,[14] our interest was focused on whether PAP might show organ protective properties in a model of pancreatitis-associated lung failure. Because alterations in vascular tone and a capillary leak are hallmarks of acute respiratory distress syndrome, the study was designed to investigate the pathophysiologic effects of this protein on fMLP-induced leukocyte activation and on subsequent pulmonary vascular resistance and edema formation. For this purpose, highly purified PAP from rat pancreatic juice was added to the perfusion fluid of the isolated lung. The applied dosage in our model was comparable to serum levels measured in patients with mild to severe pancreatitis.[11] To guarantee homologous distribution of PAP in the lung, PAP was administered before the inflammatory fMLP stimulus.

Although the isolated-cell free perfused rabbit lung is a relatively simple model that allows the investigation of PAP effects under standardized conditions, there are particular limitations. It does not allow analysis of interactions with other organ systems or between the endothelium and blood components. Moreover, the observation period is limited to approximately 6 h. However, this experimental set-up allows selective measurements of alterations in pulmonary vascular resistance and permeability as well as the release of mediators into the perfusate. Therefore, conclusions on pathophysiologic mechanisms can be drawn from this model.[18–20] It has been shown that granulocyte activation by proinflammatory cytokines plays a crucial role in pulmonary damage during pancreatitis.[4,5] Thus, activation of granulocytes by fMLP in the lung vasculature was chosen in the present study. Although fMLP-induced lung injury may reproduce some features of injury produced by pancreatitis, inflammation *in vivo* is considerably more complex, characterized by a mounting cascade of a variety of mediators released from a variety of inflammatory cells. These additional factors may modulate the actual effect of PAP during clinical pancreatitis. The time course of pulmonary artery pressure, with maximum vasoconstric-

tion after 5 min and decreasing values until baseline at 30 min, is in agreement with that of other investigators[31] using fMLP in isolated lungs.

The current data on PAP-induced effects on lung vascular tone and edema formation demonstrate protective properties of PAP on leukocyte-induced pulmonary injury. Pulmonary artery pressure (fig. 1) as well as edema formation (fig. 2) in the lung were significantly suppressed after pretreatment with PAP, whereas application of PAP without leukocyte activation did not affect any measured parameter. In seeking to understand the protective effects of PAP concerning the acute pressure increase after polymorphonuclear neutrophil leukocyte activation, the role of TXA_2 as a potent vasoconstrictive mediator in the pulmonary circulation[32,33] was investigated in the presence and absence of PAP. The generation and release of this mediator is induced by a variety of factors, including tissue damage, allergic reaction, infection, and other inflammatory diseases. TXA_2 is supposed to be the predominant mediator of arachidonic acid-induced pressure responses in rabbit lungs, surpassing the vasodilatory properties of simultaneously generated prostacyclin. This was confirmed in our model in previous studies.[34] In the current study, the release of TXA_2 into the perfusate corresponded with an increase in pulmonary artery pressure in all experimental set-ups. Hence, PAP dose-dependently suppressed TXA_2 generation, which was associated with the modulation of the pulmonary pressure response (fig. 3).

In the present study, PAP was obtained from rats by affinity chromatography, which is highly selective for PAP, and its purity was checked carefully,[23,25-27] indicating that the isolated protein indeed was PAP. Thus, effects caused by impurities can be excluded, and the present data precisely reflects properties of PAP. Homology of human and rat PAP has been described[35] in terms of size, 71% amino acid identity, and the six half-cystines being in identical positions. Because rabbit PAP has not been sequenced, assumptions about the structure of rabbit PAP remain speculative. Nevertheless, the data obtained in this xeno-model indicate protective effects of the investigated protein without any signs of foreign protein reaction, which would evoke alterations in vascular tone and permeability in isolated lungs.[36] Because PAP has been described as a nonenzymatic protein without protease activity, its effects on fMLP-induced pulmonary damage are very unlikely to be caused by hydrolysis of fMLP, which would cause insufficient leukocyte activation. The distinct molecular mechanisms of action cannot be answered from the current study design.

Antiapoptotic properties of PAP have been reported[37] that protect AR4-2J cells from oxygen radical-induced cell injury. Although reactive oxygen species play a crucial role in pancreatitis-induced pulmonary damage and were released in our model after fMLP stimulation, protection from apoptosis might contribute to the observed effects. Whether the observed protective effects of PAP on acute lung injury play a significant role in human acute respiratory distress syndrome after acute pancreatitis remains to be elucidated.

In summary, PAP prevents polymorphonuclear neutrophil leukocyte-mediated pulmonary vasoconstriction and edema formation in the isolated rabbit lung in concentrations found during pancreatitis. These findings point toward a protective effect of PAP on pancreatitis-associated lung injury.

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