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Oxidative and nitrosative stress in acute pancreatitis. Modulation by pentoxifylline and oxypurinol

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ABSTRACT

Reactive oxygen species are considered mediators of the inflammatory response and tissue damage in acute pancreatitis. We previously found that the combined treatment with oxypurinol – as inhibitor of xanthine oxidase- and pentoxifylline – as inhibitor of TNF-α production-restrained local and systemic inflammatory response and decreased mortality in experimental acute pancreatitis. Our aims were (1) to determine the time-course of glutathione depletion and oxidation in necrotizing pancreatitis in rats and its modulation by oxypurinol and pentoxifylline; (2) to determine whether TNF-α is responsible for glutathione depletion in acute pancreatitis; and (3) to elucidate the role of oxidative stress in the inflammatory cascade in pancreatic AR42J acinar cells.

We report here that oxidative stress and nitrosative stress occur in pancreas and lung in acute pancreatitis and the co-treatment with oxypurinol and pentoxifylline prevents oxidative stress in both tissues. Oxypurinol was effective in preventing glutathione oxidation, whereas pentoxifylline abrogated glutathione depletion. This latter effect was independent of TNF-α since glutathione depletion occurred in mice deficient in TNF-α or its receptors after induction of pancreatitis. The beneficial effects of oxypurinol in the inflammatory response may also be ascribed to a partial inhibition of MEK1/2 activity. Pentoxifylline markedly reduced the expression of *Icam1* and *iNos* induced by TNF-α *in vitro* in AR42J cells. Oxidative stress significantly contributes to the TNF-α-induced up-regulation of *Icam* and *iNos* in AR42J cells. These results provide new insights into the mechanism of action of oxypurinol and pentoxifylline as anti-inflammatory agents in acute pancreatitis.

1. Introduction

Acute pancreatitis (AP) is characterized by a local inflammation of the pancreas which may lead to a systemic response. In the severe forms of the disease the mortality rate is high (20%) due to multiple organ failure [1,2]. Several mechanisms seem to be involved in the development of the local and systemic response in AP, namely pro-inflammatory cytokines, chemokines, reactive oxygen species (ROS), Ca^{2+}, platelet activating factor, proteases, phospholipases, complement system, adenosine, as well as neuronal and vascular responses [3–17]. This inflammatory response is triggered not only by leukocytes, but also by pancreatic acinar cells. Indeed, acinar cells may act as inflammatory cells because they respond, synthesize, and release cytokines, chemokines, and adhesion molecules [18,19].

The involvement of oxidative stress in AP is evidenced by glutathione depletion and lipid peroxidation in the pancreas and it is supported by the beneficial effects of antioxidants in experimental AP [20,21]. Mice deficient in NADPH oxidase exhibited attenuation of cerulein-induced trypsin activation in the pancreas

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[22] and this ROS generating enzyme up-regulates IL-6 and mediates apoptosis in pancreatic AR42J acinar cells stimulated with caerulein [23]. In addition, xanthine oxidase triggers intracellular trypsinogen activation and zymogen granule damage in isolated pancreatic acini [24] and ROS generated by circulating xanthine oxidase contributes to leukocyte recruitment in the lung through up-regulation of P-selectin [25,26].

Nevertheless, ROS are considered mediators of the inflammatory response and tissue damage rather than the initiation event in AP. It seems to be a cross-talk between oxidative stress and pro-inflammatory cytokines, particularly TNF-α, that amplifies the inflammatory cascade through different mechanisms, such as the activation of mitogen activated protein kinases (MAPK) and nuclear factor-kappa B (NF-κB) and/or the inactivation of protein phosphatases [15,17,27,28]. We found that the combined treatment with oxypurinol – as inhibitor of xanthine oxidase- and pentoxifylline – as inhibitor of TNF-α production – led to simultaneous blockade of the three major MAPK extracellular signal-regulated kinase (ERK), p38, and c-Jun N-terminal kinase (JNK) in the pancreas in necrotizing pancreatitis, restrained local and systemic inflammatory response and decreased mortality [29]. However, the effects of this combined treatment on parameters of oxidative stress in acute pancreatitis and the contribution of these effects to their beneficial actions remains to be established. Pentoxifylline is a phosphodiesterase inhibitor that is beneficial early in the course of acute pancreatitis by maintaining serine/threonine protein phosphatase PP2A activity in pancreas and reducing the resulting up-regulation of inflammatory mediators.

The aims of the present study were (1) to determine the time-course of glutathione depletion and oxidation in necrotizing pancreatitis in rats and its modulation by oxypurinol and pentoxifylline; (2) to determine whether TNF-α is responsible for glutathione depletion in acute pancreatitis; and (3) to elucidate the role of oxidative and nitrosative stress in the inflammatory cascade in AR42J acinar cells.

2. Materials and methods

2.1. Animals

Young male Wistar rats and young male mice were used in the experiments. They received humane care and were handled in conformance with the European regulations (Council Directive 86/609/EEC) and the studies were approved by the Research Committee of the University of Valencia. Animals were fed on a standard laboratory diet and tap water *ad libitum* and were subjected to a 12 h light–dark cycle.

Mice were either wild-type, TNF-α receptor 1 (TNFR1) knock-out (KO), TNF-α receptor 2 (TNFR2) KO or TNF-α KO (KO). Breeding pairs of homozygous TNFR1 and TNFR2 null mice (TNFR1 and TNFR2 KO, respectively) were kindly provided by Horst Bluethmann (Hoffmann-la Roche), and maintained on the inbred C57BL/6 genetic background.

2.2. Experimental models of acute pancreatitis

2.2.1. Acute pancreatitis in rats

Male Wistar rats (250–300 g body weight (b.w.)) were anesthetized with ketamine (Merial, Lyon, France) (80 mg/kg b.w.) and acepromazine (Pfizer, USA) (2.5 mg/kg b.w.) i.p. Then, the biliopancreatic duct was cannulated through the duodenum and the hepatic duct was closed by a small bulldog clamp. Acute necrotizing pancreatitis was induced by retrograde injection into the biliopancreatic duct of sodium taurocholate (3.5%) (Sigma, St. Louis, Missouri, USA) in a volume of 0.1 ml/100 g b.w. using an infusion pump (Harvard Instruments) [29]. Serum lipase activity was measured to confirm the appropriate induction of pancreatitis.

2.2.2. Acute pancreatitis in mice

Mice were treated with the cholecystokinin analogue caerulein to induce acute pancreatitis [30]. Caerulein (Sigma, St. Louis, Missouri, USA) was administered in seven intraperitoneal injections at hourly intervals, each injection containing 50 μg/kg body weight. The control group received seven i.p. injections of 0.9% saline at hourly intervals. Mice were sacrificed 1 h after the last injection of caerulein or saline, and were anaesthetized with i.p. administration of ketamine (80 mg/kg b.w.) and acepromazine (2.5 mg/kg b.w.). Serum lipase activity was measured to confirm the appropriate induction of pancreatitis.

2.3. Culture of rat pancreatic AR42J acinar cells

The AR42J cell line, derived from an exocrine pancreatic tumour (ATCC CRL 1492), was grown in Dulbecco's modified Eagle's medium (DMEM) (Gibco BRL, Paisley, UK) containing 25 mmol/L glucose (Gibco BRL, Paisley, UK), 100 μg/ml penicillin (Gibco BRL, Paisley, UK), 100 μg/ml streptomycin (Gibco BRL, Paisley, UK) and 25 μg/ml fungizone (Gibco BRL, Paisley, UK), supplemented with 10% foetal bovine serum (FBS) (Gibco BRL, Paisley, UK). AR42J cells were differentiated into secretory cells by incubation with 100 nM dexamethasone (Sigma, St. Louis, Missouri, USA) for 72 h [31]. Amylase activity increased 7-fold at 72 h after the treatment with dexamethasone [840 ± 375 mIU/mg vs. 119 ± 18 mIU/mg protein (n = 4)]. The increase in amylase content was confirmed by western blotting (results not shown). When indicated, AR42J cells were incubated with 10 ng/ml of TNF-α (Sigma, St. Louis, Missouri, USA) for 3 h in presence or absence of 100 μM oxypurinol (Sigma, St. Louis, Missouri, USA), 12 mg/L pentoxifylline (Robert, Barcelona, Spain), or antioxidants [5 mM GSH monoethyl ester (Sigma, St. Louis, Missouri, USA) or 500 μM trolox (Fluka, Buchs St. Gallen, Switzerland). These agents were added 30 min prior to the incubation with TNF-α.

2.4. Study design

In a first series of experiments, the time-course of glutathione depletion and oxidation in pancreas and lung during acute necrotizing pancreatitis in rats was investigated in order to determine a single time-point for assessing the effects of treatments. Samples were obtained at 0, 1, 3, 6 and 9 h after intraductal infusion of taurocholate, immediately frozen and maintained at −80 °C until assayed. An additional group was obtained at 30 min after induction of pancreatitis when protein nitration and MAPK kinase phosphorylation were assessed. Serum lipase activity was measured to confirm the appropriate induction of pancreatitis.

In the second series of experiments, we assessed the effects of treatment with oxypurinol – as inhibitor of xanthine oxidase- and pentoxifylline – as inhibitor of TNF-α production – at a time-point when glutathione depletion and oxidation were firmly established in both pancreas and lung. This time-point was selected according to the results of the first series of experiments. Treatments were administered immediately after taurocholate infusion. Animals in this second series of experiments were distributed in the following groups:

- Control (C): Infusion of 0.9% NaCl (Sigma, St. Louis, Missouri, USA) into the biliopancreatic duct (0.1 ml/100 g) and into the femoral vein (0.066 ml/min for 30 min).
- Acute pancreatitis (AP): Infusion of 3.5% sodium taurocholate into the biliopancreatic duct and saline solution into the femoral vein (0.066 ml/min for 30 min).