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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).

- Acute pancreatitis + oxypurinol (O): Infusion of 3.5% sodium taurocholate into the biliopancreatic duct and 5 mM oxypurinol into the femoral vein (0.066 ml/min for 30 min).
- Acute pancreatitis + pentoxifylline (P): Infusion of 3.5% sodium taurocholate into the biliopancreatic duct and pentoxifylline (12 mg/kg b.w.) into the femoral vein (0.066 ml/min for 30 min).
- Acute pancreatitis + oxypurinol + pentoxifylline (OP): Infusion of 3.5% sodium taurocholate into the biliopancreatic duct and 5 mM oxypurinol and pentoxifylline (12 mg/kg b.w.) into the femoral vein (0.066 ml/min for 30 min).

Reduced and oxidized glutathione levels in the pancreas and in the lung were measured at the selected time point after induction of pancreatitis and GSSG/GSH ratio was calculated.

In the third series of experiments, we measured pancreatic glutathione levels in wild-type mice, TNF-α receptor 1 or 2 knock-out mice, and TNF-α knock-out mice. All these mice were divided into two groups: a control group and a group with caerulein-induced acute pancreatitis.

In the fourth series of experiments, the effects of pentoxifylline, oxypurinol and other antioxidants, such as trolox and glutathione monoethyl ester, on the up-regulation of pro-inflammatory genes were assessed *in vitro* in pancreatic AR42J acinar cells.

2.5. Assays

2.5.1. Lipase activity

Serum lipase activity was determined by the LIPASE-PS™ kit (Sigma Diagnostics, Lyon, France) according to the supplier's specifications.

2.5.2. Reduced and oxidized glutathione

Reduced glutathione (GSH) levels were determined spectrophotometrically at 340 nm using glutathione-S-transferase (Sigma, St. Louis, Missouri, USA) and 1-chloro-2,4-dinitrobencene (Sigma, St. Louis, Missouri, USA) [32]. Oxidized glutathione (GSSG) levels were measured by HPLC with detection at 365 nm using *N*-ethylmaleimide (Sigma, St. Louis, Missouri, USA) as a chelating agent to prevent GSH auto-oxidation during sample processing [33]. This latter method was developed to measure GSSG accurately in the presence of a large excess of GSH, as occurs in biological samples [34]. For GSH measurement, tissue samples were homogenized with 6% perchloric acid (Panreac, Barcelona, Spain) containing 1 mM EDTA (Fluka, Buchs St. Gallen, Switzerland), whereas for GSSG, samples were homogenized with 6% perchloric acid containing 40 mM *N*-ethylmaleimide and 1 mM BPDS (Fluka, Buchs St. Gallen, Switzerland). Then homogenates were centrifuged at 15,000 × *g* for 10 min at 4 °C. Acidic supernatants were used for these assays.

2.5.3. RT-PCR

A small piece of the pancreas was excised and immediately immersed in 1 ml RNA-later solution (Ambion, Foster City, California, USA) to stabilize the RNA. Total RNA was isolated from pancreas and from cultured AR42J cells by the guanidinium thyocianate (Ambion, Foster City, California, USA) method [35]. The isolated RNA was size-fractioned by electrophoresis (2 μg/lane) in a 1% agarose/formalin gel and stained with ethidium bromide (Sigma, St. Louis, Missouri, USA) to assess the quality of the RNA. The cDNA used as template for amplification in the PCR assay was constructed by reverse transcription reaction using SuperScript II (Invitrogen, Paisley, UK) with random hexamers as primers starting with 1 μg of RNA. As a PCR internal control, 18S *rRNA* was simultaneously amplified. Real time-PCR was performed using the double-stranded DNA binding dye Syber Green PCR Master mix (Applied Biosystems, Paisley, UK) in an ABI GeneAmp 7000 Sequence Detection System. Each reaction was performed in triplicate and the melting curves were constructed using Dissociation Curves Software (Applied Biosystems, Paisley, UK) to ensure that only a single product was amplified. 18S *rRNA* was also analyzed as real time RT-PCR control. The following specific primers were used: *Icam-1*: forward 5′-TGTCGGTGCTCAGGTATCCA-3′ and reverse 5′-TTCACCTGCACGGATCCA-3′; *iNos*: forward 5′-AGCGGCTCCATGACTCTCA-3′ and reverse 5′-TGCACCC AAACACCAAGGT-3′; *Tnf-α*: forward 5′- CAGCCGATTTGCCATTTCAT-3′ and reverse 5′-TCCTTAGGGCAAGGGCTCTT-3′ *rRNA* 18S: forward 5′-AGTCCCTGCCCTTTGTACACA-3′, reverse 5′- GATCCG AGG GCCTCACTAAAC-3′. The threshold cycle (CT) was determined and the relative gene expression was expressed as follows: fold change = $2^{-\Delta(\Delta CT)}$, where $\Delta CT = CT_{target} - CT_{housekeeping}$, and $\Delta(\Delta CT) = \Delta CT_{treated} - \Delta CT_{control}$.

2.5.4. Western blotting

Pancreatic specimens were frozen at −80 °C until they were homogenized in extraction buffer (100 mg/ml) on ice. The extraction buffer contained 10 mM Tris–HCl (pH 7.5) (Sigma, St. Louis, Missouri, USA), 0.25 M sucrose (Sigma, St. Louis, Missouri, USA), 5 mM EDTA (Fluka, Buchs St. Gallen, Switzerland), 50 mM NaCl (Sigma, St. Louis, Missouri, USA), 30 mM sodium pyrophosphate (Sigma, St. Louis, Missouri, USA), 50 mM sodium fluoride (Sigma, St. Louis, Missouri, USA), 100 μM sodium orthovanadate (Merck, Darmstadt, Germany), and the protease inhibitor cocktail (Sigma, St. Louis, Missouri, USA). Protein nitration, phospho-MEK1/2, phospho-MKK3/6, MKK4, and tubuline as reference of protein loading were measured by western blotting and chemiluminescence detection using the Phototope™-HRP Detection kit (Cell Signaling Technology, Danvers, Massachusetts, USA). The following antibodies were used: anti-nitro-tyr (Hycult Biotechnology, PB Uden, Netherlands); phospho-MEK1/2 (Ser217/221), phospho-MKK3/6, and phospho SEK1/MKK4 (Cell Signaling Technology, Danvers, Massachusetts, USA); tubulin (Sigma, St. Louis, Missouri, USA).

2.5.5. Assay of MEK1/2 activity

MAPK Kinase (MEK1/2) activity was assayed using the MAPK Kinase (MEK1/2) Activity Assay Kit SGT440 from Chemicon International (Billerica, Massachusetts, USA) following the instructions from the manufacturer.

2.6. Statistical analysis

Results are expressed as mean ± standard deviation (S.D.) with the number of experiments given in parentheses. Statistical analysis was performed in two steps. One-way analysis of variance (ANOVA) was performed first. When the overall comparison of groups was significant, differences between individual groups were investigated by Scheffé method. Differences were considered to be significant at $P < 0.05$.

3. Results

3.1. GSH and GSSG levels and GSSG/GSH ratio in pancreas and lung in the course of acute pancreatitis in rats

Fig. 1 A and B shows that reduced glutathione (GSH) levels were already markedly depleted in pancreas at 1 h after induction of pancreatitis and were kept low till 9 h, whereas oxidized glutathione (GSSG) levels increased in pancreas only at 6 and 9 h after induction of acute pancreatitis. The GSSG/GSH ratio increased significantly at 3 h and thereafter (see Fig. 1C). Consequently, although glutathione depletion was established rapidly in the pancreas, pancreatic glutathione oxidation as a

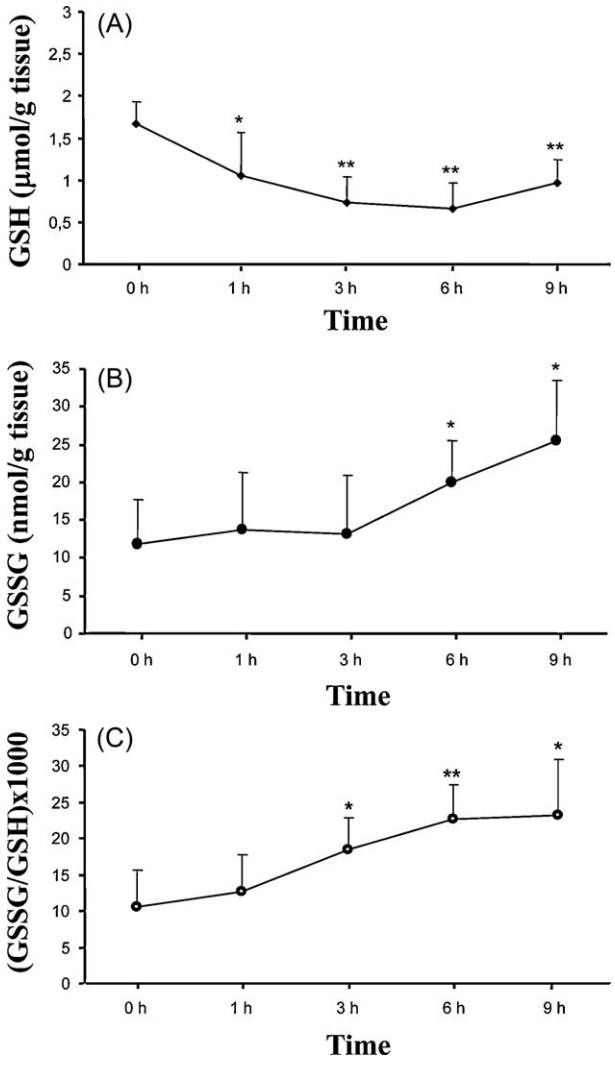

Fig. 1. Reduced glutathione (GSH) (A), oxidized glutathione (GSSG) (B), and GSH/GSSG ratio (C) in pancreas in the course of taurocholate-induced pancreatitis in rats. The number of rats per group was 4–7. The statistical difference is indicated as follows: $^{*}P < 0.05$, $^{**}P < 0.01$ vs. 0 h.

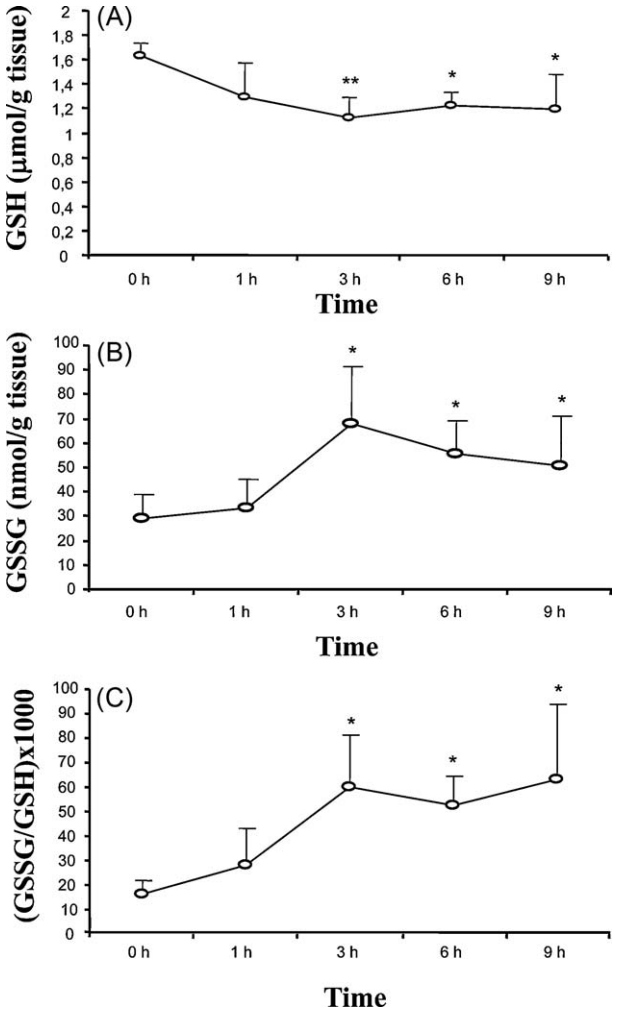

Fig. 2. Reduced glutathione (GSH) (A), oxidized glutathione (GSSG) (B), and GSH/GSSG ratio (C) in lung in the course of taurocholate-induced pancreatitis in rats. The number of rats per group was 4–7. The statistical difference is indicated as follows: $^{*}P < 0.05$, $^{**}P < 0.01$ vs. 0 h.

marker of oxidative stress occurred late in the course of acute pancreatitis, i.e. from 3 h after induction of the disease.

In the lung a moderate GSH depletion occurred at 3 h after induction of pancreatitis and it was maintained till 9 h (see Fig. 2A). It is noteworthy that in the case of the lung, GSH depletion was associated with a remarkable increase in GSSG levels that were more than 2-fold higher at 3 h than at 0 h and were kept high at 6 and 9 h (Fig. 2B). The profile of the GSSG/GSH ratio was parallel with that of GSSG, i.e. exhibiting a significant increase at 3, 6 and 9 h after pancreatitis induction (Fig. 2C).

3.2. Effects of oxypurinol and pentoxifylline on GSH and GSSG levels in pancreas and lung and on serum lipase activitiy in acute pancreatitis in rats

Fig. 3 A shows that the marked GSH depletion that occurred in the pancreas at 6 h post-induction was prevented by pentoxifylline treatment as well as with the combined treatment with oxypurinol plus pentoxifylline. Oxypurinol alone did not change pancreatic GSH levels in AP. Fig. 3B shows that oxypurinol, as well as the combined treatment significantly prevented the increase in pancreatic GSSG levels. Consequently, each of these three treatments abolished the rise in the GSSG/GSH ratio in pancreas (Fig. 3C).

Pentoxifylline and the combined treatment also prevented the moderate GSH depletion in lung observed 6 h after induction of pancreatitis (see Fig. 4A). However, oxypurinol treatment did not change GSH levels significantly in this tissue in pancreatitis. Fig. 4B shows that oxypurinol and the combined treatment significantly prevented the increase in lung GSSG levels. In addition, each of the three treatments abolished the rise in the GSSG/GSH ratio in lung (Fig. 4C).

Regarding serum lipase activity, it was 23 ± 2 U/L in control rats, 1094 ± 145 U/L in rats with AP without treatment, 950 ± 388 U/L in rats with AP treated with oxypurinol, 900 ± 218 U/L in rats with AP treated with pentoxifylline, and 405 ± 54 U/L in rats with AP that received both oxypurinol and pentoxifylline ($P < 0.01$ vs. rats with AP), for $n = 3$–7. The effects of oxypurinol or pentoxifylline on pancreatic and pulmonary GSH levels and serum lipase activity were also assessed in control rats and no significant differences were found (results now shown).

3.3. Effects of oxypurinol and pentoxifylline on protein nitration in pancreas and in lung in the course of acute pancreatitis

Fig. 5A and B shows that acute necrotizing pancreatitis in rats triggers protein nitration as an index of nitrosative stress in the

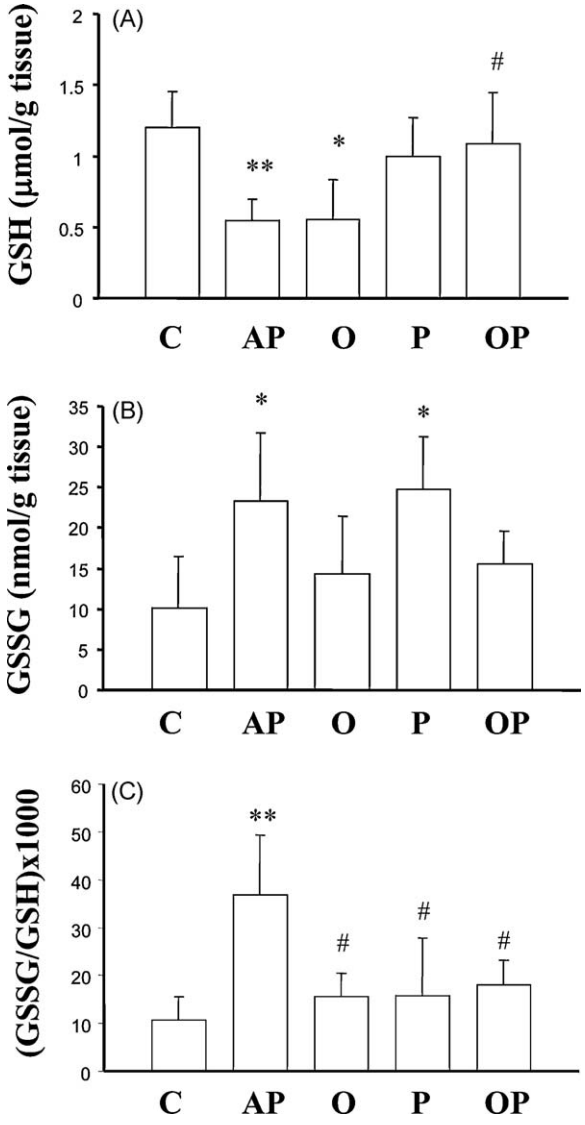

Fig. 3. Effects of treatment with oxypurinol and pentoxifylline on pancreatic levels of reduced (A) and oxidized (B) glutathione and the ratio of oxidized to reduced glutathione (GSSG/GSH) (C) 6 h after induction of acute pancreatitis in rats. Abbreviations used: C = control rats; AP = rats with taurocholate-induced acute pancreatitis; O = rats with acute pancreatitis treated with oxypurinol; P = rats with acute pancreatitis treated with pentoxifylline; OP = rats with acute pancreatitis receiving the combined treatment with oxypurinol and pentoxifylline. The number of experiments was 4–6. Results are expressed as mean ± SD. Statistical difference is indicated as follows: $^{*}P < 0.05$, $^{**}P < 0.01$ vs. control group; $^{\#}P < 0.05$ vs. taurocholate group.

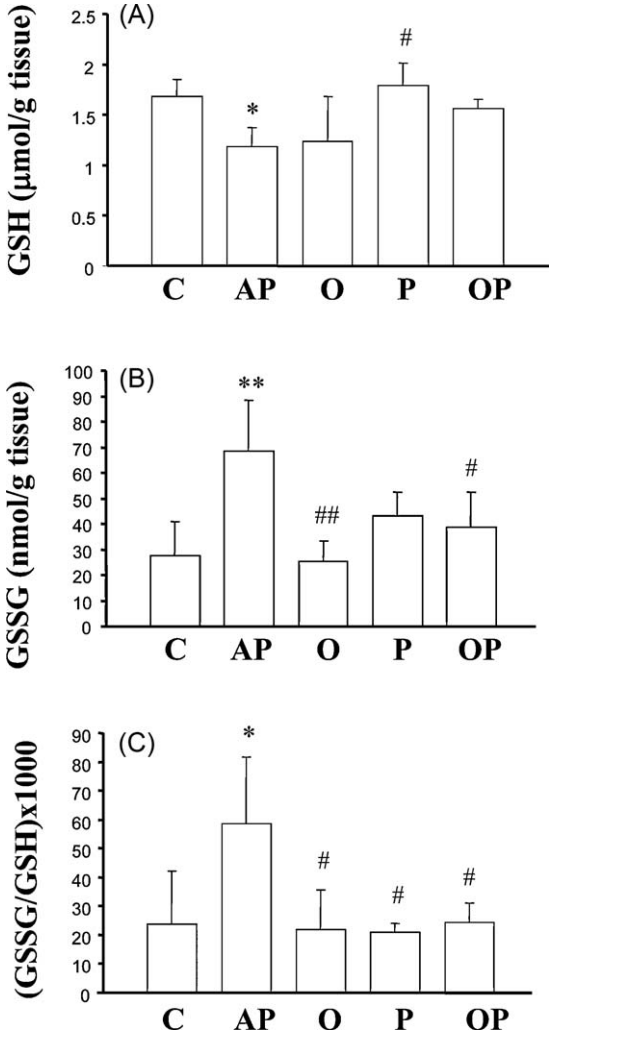

Fig. 4. Effects of treatment with oxypurinol and pentoxifylline on lung levels of reduced (A) and oxidized (B) glutathione and the ratio of oxidized to reduced glutathione (GSSG/GSH) (C) 6 h after induction of acute pancreatitis in rats. Abbreviations used: C = control rats; AP = rats with taurocholate-induced acute pancreatitis; O = rats with acute pancreatitis treated with oxypurinol; P = rats with acute pancreatitis treated with pentoxifylline; OP = rats with acute pancreatitis receiving the combined treatment with oxypurinol and pentoxifylline. The number of experiments was 3–6. Results are expressed as mean ± SD. Statistical difference is indicated as follows: $^{*}P < 0.05$, vs. control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. taurocholate group.

pancreas and in the lung in the course of acute pancreatitis, starting from the first hour. The combined treatment with pentoxifylline and oxypurinol abrogated the increase in protein nitration in the pancreas upon pancreatitis, although the single treatments with oxypurinol or pentoxifylline did not affect pancreatitis-associated protein nitration (see Fig. 5A). However, this combined treatment or the single treatments did not prevent the enhanced protein nitration in the lung (see Fig. 5B).

3.4. Effect of oxypurinol on the up-regulation of pro-inflammatory genes in acute pancreatitis in rats

The effect of oxypurinol on the up-regulation of *Tnf-α*, *Icam-1*, and *iNos* in taurocholate-induced acute pancreatitis in rats has been assessed. Fig. 6 shows that oxypurinol treatment partially prevented the increase in *Icam-1* gene expression, but it did not affect the expression of *Tnf-α* and *iNos*. The effect of pentoxifylline on the up-regulation of pro-inflammatory genes has been previously reported by our group [36].

3.5. Effect of oxypurinol and pentoxifylline on upstream MAPK

The effects of oxypurinol and pentoxifylline on the phosphorylation of upstream MAPK MEK1/2, MKK3/6 and MKK4 as an index of their activation were studied to provide new insights into the mechanism of action of these drugs. Fig. 7A shows that oxypurinol alone or in combination with pentoxifylline reduced significantly MEK1/2 phosphorylation. Pentoxifylline had no significant effect in this regard. Neither oxypurinol or pentoxifylline affected the phosphorylation of MKK3/6 or MKK4 in the course of pancreatitis.

Since the effect of oxypurinol on pancreatic phospho-MEK1/2 was found *in vivo* in rats with pancreatitis and it might be affected by the effect of oxypurinol on the inflammatory cascade, we decided to test the effect of oxypurinol on MEK1/2 activity *in vitro* in a pancreatic homogenate. Thus, MEK1/2 activity was measured

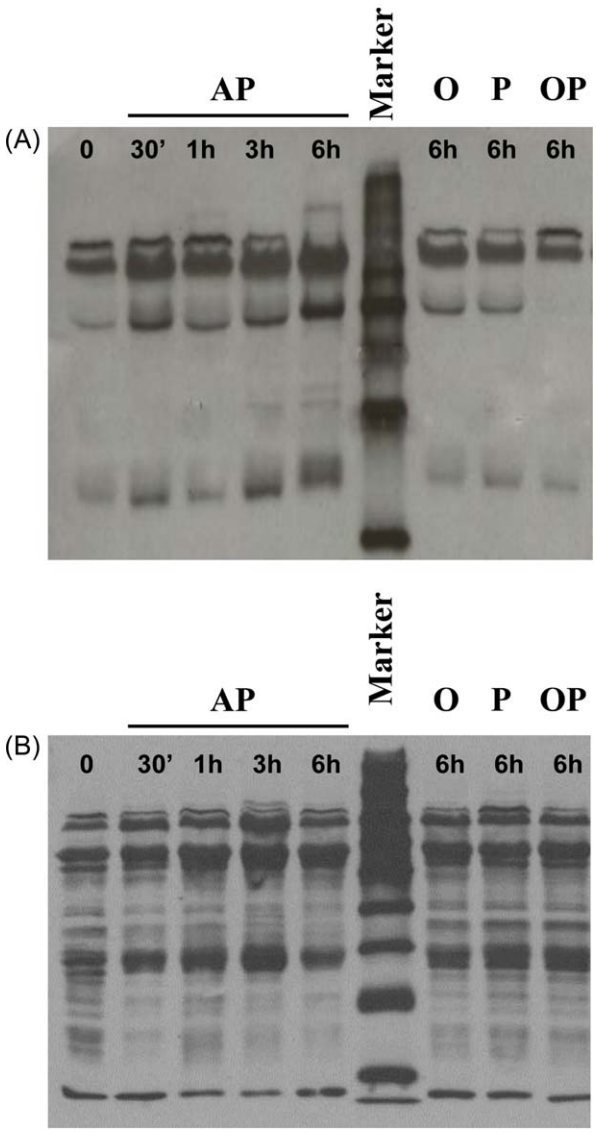

Fig. 5. Protein nitration in the pancreas (A) and lung (B) in the course of taurocholate-induced acute pancreatitis. Effects of oxypurinol and/or pentoxifylline. Nitrotyrosine was measured by western blotting as an index of protein nitration. This figure shows a representative western from three experiments.

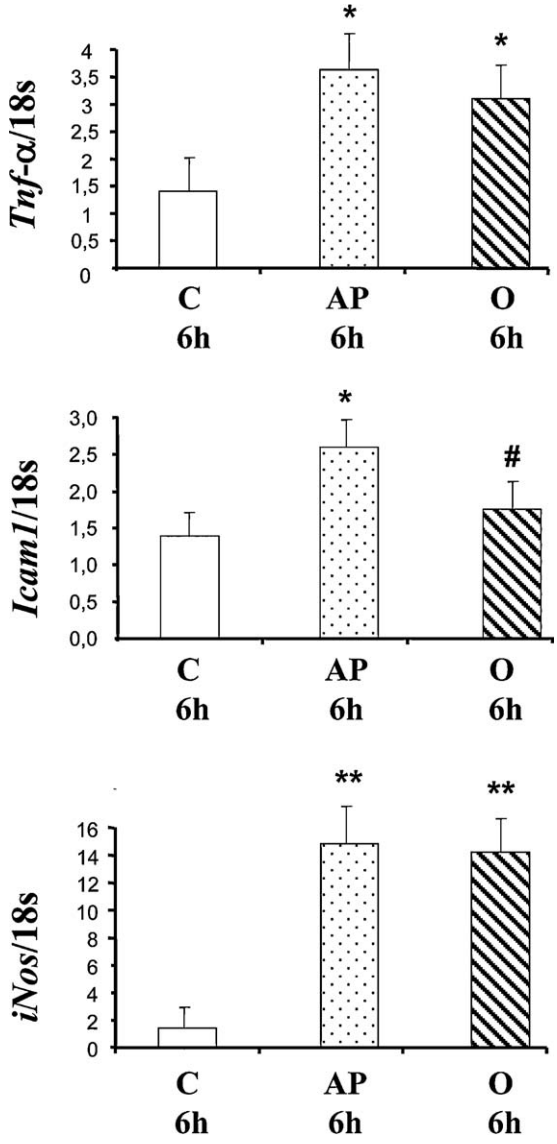

Fig. 6. Up-regulation of *Tnf-α*, *Icam-1*, and *iNos* mRNAs in pancreas in acute pancreatitis. Effect of oxypurinol. mRNA expression was measured by real time RT-PCR (see Section 2). The number of rats per group was 3–4. The statistical difference is indicated as follows: $^{*}P < 0.05$, $^{**}P < 0.01$ vs. control; $^{\#}P < 0.05$ vs. taurocholate.

in pancreatic homogenates incubated for 30 min with 1 mM oxypurinol. The effect of 0.4 mg/ml pentoxifylline was also tested. Fig. 7B shows that oxypurinol reduced by 1/3 MEK1/2 activity, whereas pentoxifylline exhibited no significant effect on this kinase.

3.6. Pancreatic GSH levels in acute pancreatitis in mice deficient in TNF-α or TNF-α receptors

GSH levels were measured in the pancreas of wild type, TNF-α receptor 1 or 2 KO and TNF-α KO mice after induction of caerulein-induced pancreatitis. Fig. 8 shows that pancreatic GSH depletion markedly occurred in all the three KO mice at 1 h post-induction similarly to wild type mice.

3.7. Oxidative stress modulate TNF-α-induced up-regulation of pro-inflammatory genes in AR42J acinar cells

Fig. 9 shows the effect of 12 mg/L pentoxifylline, 100 μM oxypurinol, and some antioxidants (500 μM Trolox or 5 mM GSH monoethyl ester) on the up-regulation of *Icam-1* and *iNOS* induced by 10 ng/ml TNF-α in AR42J cells. Pentoxifylline reduced the induction of *Icam-1* and *iNos* by 54% and 65%, respectively, whereas oxypurinol diminished the induction of these genes by 38% and 53%, respectively. GSH ester lowered the expression of these pro-inflammatory genes with a similar pattern to oxypurinol, while trolox was effective decreasing the expression of both genes by 66%. Hence, pentoxifylline, oxypurinol and other antioxidants down-regulate the TNF-α-dependent expression of pro-inflammatory genes in pancreatic AR42J cells.

4. Discussion

Pancreatic glutathione depletion is a hallmark of acute pancreatitis (AP) and restoration of intracellular glutathione levels ameliorates caerulein-induced pancreatitis [37–39]. Previously we reported that recovery of glutathione levels in the pancreas was rapid in mild acute pancreatitis, whereas marked glutathione depletion was maintained for at least 9 h in severe acute pancreatitis [40]. We report here that glutathione depletion is

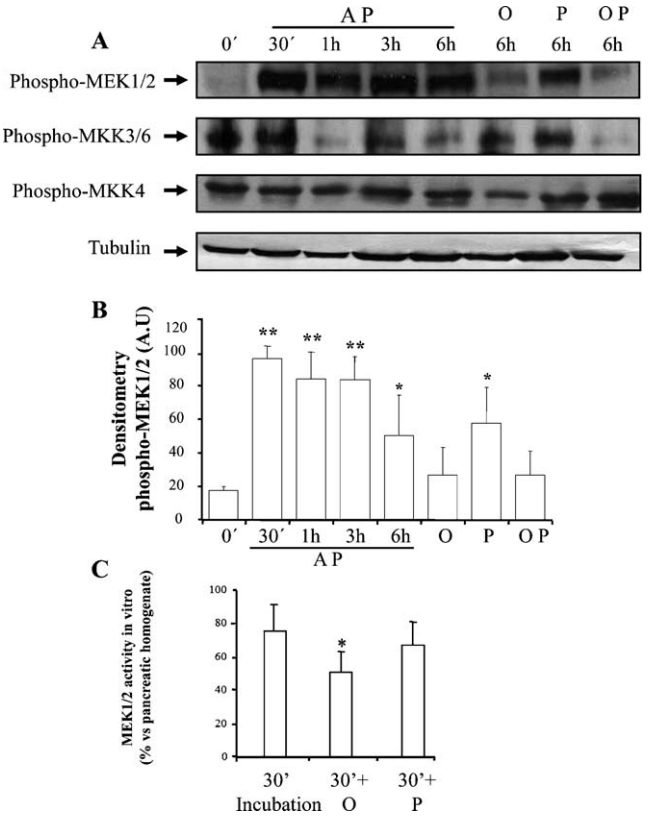

Fig. 7. Effect of oxypurinol and pentoxifylline on MAPK kinase phosphorylation and MEK1/2 activity. (A) Phosphorylation of MEK1/2, MKK3/6, and MKK4 was measured in pancreas in the course of taurocholate-induced necrotizing pancreatitis in rats untreated and treated with oxypurinol and/or pentoxifylline. The number of rats per group was 3–4. (B) Densitometry of MEK1/2 phosphorylation in pancreas in the course of taurocholate-induced pancreatitis. The statistical difference is indicated as follows: $^{*}P < 0.05$, $^{**}P < 0.01$ vs. 0 h. (C) MEK1/2 activity in pancreatic homogenates incubated with 1 mM oxypurinol or 0.4 mg/ml pentoxifylline for 30 min. The number of experiments was 3–4.

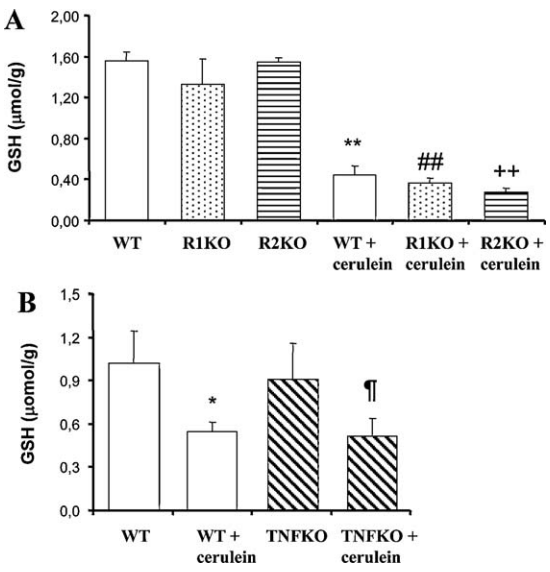

Fig. 8. GSH in pancreas from wild type (WT) mice and mice deficient in TNF-α (TNFKO) or its receptors 1 (KOR1) or 2 (KOR2) subjected to caerulein-induced pancreatitis. The statistical difference is indicated as follows: $^{*}P < 0.05$, $^{**}P < 0.01$ vs. WT; $^{\#\#}P < 0.01$ vs. R1KO; $^{++}P < 0.01$ vs. R2KO; $^{\P}P < 0.05$ vs. TNFKO. The number of mice per group was 3–4.

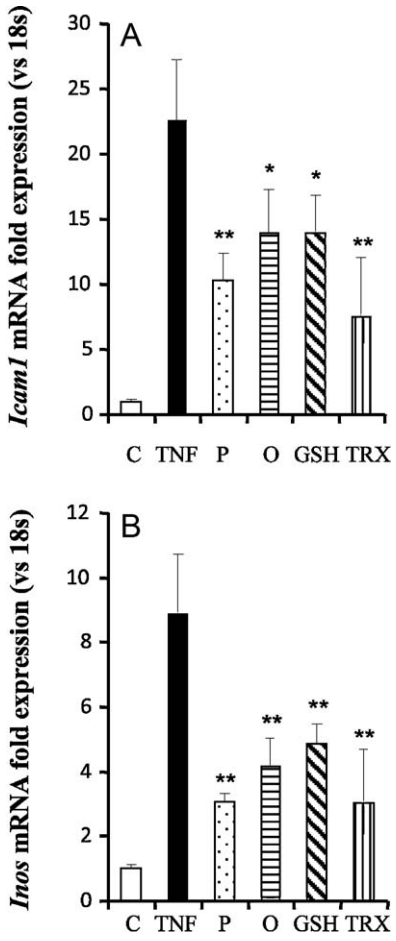

Fig. 9. Up-regulation of *Icam1* and *iNos* in pancreatic AR42J cells incubated with TNF-α. Prior to the experiment, AR42J cells were differentiated with dexamethasone (see Section 2). Cells were incubated for 3 h without additions or with 10 ng/ml TNF-α. When indicated, the following agents were added 30 min prior to the incubation with TNF-α: 12 mg/L pentoxifylline (P), 100 μM oxypurinol (O), 5 mM GSH monoethyl ester (GSH), or 500 μM trolox (TRX). The statistical difference is indicated as follows: $^{*}P < 0.05$, $^{**}P < 0.01$ vs. TNF. The number of experiments was 3.

not associated with glutathione oxidation in the pancreas and lung early –i.e. at 1 h- in acute pancreatitis. However, glutathione oxidation and hence oxidative stress takes place later – at 3 h and thereafter- in these tissues. The abrogation of pancreatitis-associated glutathione oxidation by oxypurinol suggests that xanthine oxidase is mainly responsible for the glutathione oxidation that occurs late in the course of acute pancreatitis. It is noteworthy that reactive oxygen species (ROS) generated by xanthine oxidase mediate the up-regulation of P-selectin in the lung during AP [26] which triggers leukocyte recruitment. Hence, glutathione oxidation is likely a consequence of the inflammatory infiltrate that evolves in course of the disease.

The role of ROS in the pathogenesis of acute pancreatitis has been the subject of numerous studies [17,20,21,28]. It was postulated that generation of ROS may play a pivotal role in the progression of AP [20]. ROS promote NF-κB activation in the course of the disease [41]. Furthermore, the antioxidant N-acetyl cysteine (NAC) inhibited NF-κB activation in acinar cells in response to caerulein hyperstimulation [42] and pre-treatment with NAC abrogated NF-κB activation in mild and severe experimental models of acute pancreatitis [27,43,44]. However, ROS seem to act as mediators of tissue damage rather than being the initiation event in AP [12]. Thus, generation of ROS did not induce the

enzymatic and morphological changes characteristic of acute pancreatitis [12].

In acute pancreatitis a cross-talk between oxidative stress and pro-inflammatory cytokines arises decisively contributing to tissue injury [17]. In this regard, we previously found that the combined treatment of taurocholate-induced acute pancreatitis with pentoxifylline, which inhibits TNF-α production, and oxypurinol led to a remarkable reduction in the inflammatory response [29]. The co-treatment with oxypurinol and pentoxifylline led to a marked reduction in the interstitial edema and in the infiltrate of inflammatory cells into the pancreas as well as to a significant decrease in serum lipase activity in acute pancreatitis [29].

The cross-talk between oxidative stress and TNF-α has been further investigated in the present work. We report here that simultaneous inhibition of xanthine oxidase and TNF-α production with oxypurinol and pentoxifylline abolished the oxidative stress changes associated with taurocholate-induced pancreatitis. This combined treatment completely prevented glutathione depletion and oxidation in pancreas and lung. This co-treatment also prevented the pancreatic nitrosative stress triggered by peroxynitrite in pancreatitis, as evidenced by the abrogation of nitrotyrosine formation in pancreas. The formation of peroxynitrite would be blocked since pentoxifylline would abrogate to a great extent TNF-α-dependent iNos induction, whereas oxypurinol would prevent superoxide formation by xanthine oxidase. The sole treatment with oxypurinol or pentoxyfylline reduced only partially protein nitration. In our opinion, the formation of peroxynitrite in acute pancreaitits would be abrogated by simultaneous blockade of superoxide generation by xanthine oxidase and of nitric oxide generation by TNF-dependent *iNos*. If only one of these mechanisms is blocked then a partial effect is achieved. When using only oxypurinol, the superoxide generated by other sources apart from xanthine oxidase may still yield peroxynitrite when *iNos* is induced. On the other hand, when using only pentoxifylline the increase in TNF levels is abrogated but normal TNF levels are present, and the up-regulation of *iNos* is markedly reduced but not completely avoided [36]. Consequently, the formation of peroxynitrite was significantly reduced in presence of pentoxyfylline, but it may still be present due to superoxide generation and still slight induction of *iNos*.

The cross-talk between oxidative stress and pro-inflammatory cytokines seems to occur through MAPK that amplify the inflammatory cascade contributing to tissue injury in acute pancreatitis [17]. We previously found that the combined treatment of taurocholate-induced acute pancreatitis with pentoxifylline and oxypurinol led to simultaneous blockade of the three major MAPK, i.e. p38, JNK, and ERK1/2, in the pancreas, which was associated with a remarkable reduction in the inflammatory infiltrate [29]. Oxypurinol blocked p38 phosphorylation and diminished the phosphorylation of ERK and JNK in the pancreas, whereas pentoxifylline blocked ERK and JNK phosphorylation without affecting p38 [29]. In the present work we have investigated the effects of these agents on the activation of MAPK kinases, such as MEK1/2,MKK3/6 and MKK4, and we have found that oxypurinol reduced significantly MEK1/2 phosphorylation and activity, while pentoxifylline did not exhibit any significant effect in this regard. It is worth noting that oxypurinol reduced MEK1/2 activity *in vitro* in a short incubation (i.e. 30 min). This result suggests that oxypurinol may have a direct inhibitory effect on MEK1/2 activity. Therefore, at least part of the beneficial effects of oxypurinol might be ascribed to a modulation of MAPK kinase activity in addition to its effect on xanthine oxidase.

In order to assess whether oxidative stress modulate the inflammatory cascade, we have determined the effect of antioxidants, such as trolox or GSH monoethyl ester, on the induction of pro-inflammatory genes by TNF-α in pancreatic AR42J acinar cells. We did not test the effect of these agents on taurocholate-treated AR42J acinar cells because taurocholate did not induce any significant up-regulation in *Tnf-α*, *iNos* or *Icam* expression (results not shown). In the present work we show that oxidative stress significantly contributes to the up-regulation of pro-inflammatory genes and hence to the uncontrolled inflammatory cascade in pancreatic acinar cells. Accordingly, the beneficial effect ascribed to oxypurinol may be due, at least in part, to its direct or indirect antioxidant properties, the latter through xanthine oxidase inhibition.

We report here that pentoxifylline prevented GSH depletion in pancreas and lung after induction of acute pancreatitis. Accordingly, we hypothesized that a protease activated by TNF-α might be involved in pancreatic glutathione depletion. Hence, we tested whether mice deficient in TNF-α or its receptors suffer GSH depletion in the pancreas upon induction of acute pancreatitis. Unexpectedly, pancreatic GSH depletion did occur in all of these mice (TNF-α KO, TNF-α R1 KO, or TNF-α R2 KO) after induction of caerulein-induced pancreatitis. Consequently, TNF-α is not responsible for pancreatic GSH depletion in acute pancreatitis. Furthermore, our results suggest that pentoxifylline may prevent glutathione depletion through a mechanism independent of inhibition of TNF-α production.

Pentoxifylline markedly lowered the induction of pro-inflammatory genes triggered by TNF-α *in vitro* in AR42J cells. Recently, we found that pentoxifylline prevented the decrease in PP2A activity that occurs in pancreas early in pancreatitis [36] and this may provide the mechanism for blockade of the inflammatory cascade by this drug. The action of pentoxifylline as phosphodiesterase inhibitor seems to account for this anti-inflammatory effect and further studies are needed to elucidate the mechanism responsible for the link between cAMP and GSH levels suggested by the present work.

TNF-α is considered initiator of the inflammatory cascade that activates leukocytes and induces the release of other cytokines which propagate the inflammatory cascade in pancreatitis. In knockout mice deficient in TNF-α receptors, the rate of mortality due to necrotizing AP decreased significantly because the systemic response was restrained although there was no reduction in the severity of the pancreatic damage [14,45,46]. The finding that marked glutathione depletion occurs in pancreatitis in mice deficient in TNF-α receptors would explain, at last in part, the previously reported effect on pancreatic damage in these mice.

In conclusion, we have shown that oxidative stress occurs in pancreas and lung in acute pancreatitis and the combined treatment with oxypurinol and pentoxifylline prevents oxidative stress in both tissues. Oxypurinol was effective in preventing glutathione oxidation, whereas pentoxyfilline abrogated glutathione depletion. Unexpectedly, this latter effect was independent of TNF-α levels since glutathione depletion occurred in mice deficient in TNF-α or its receptors after induction of pancreatitis. In addition, part of the beneficial effects of oxypurinol may be ascribed to a partial inhibition of MEK1/2 activity. Pentoxifylline and oxypurinol as well as antioxidants down-regulate the induction of pro-inflammatory genes by TNF-α in pancreatic AR42J acinar cells. Consequently, oxidative stress contributes to the inflammatory cascade in pancreatic acinar cells. These results provide new insights into the mechanism of action of oxypurinol and pentoxifylline as anti-inflammatory agents.

Acknowledgments

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