Pentoxifylline Ameliorates Cerulein-Induced Pancreatitis in Rats: Role of Glutathione and Nitric Oxide

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ABSTRACT

Reactive oxygen radicals, nitric oxide, and cytokines have been implicated in the initiation of pancreatic tissue damage and impairment of the pancreatic microcirculation in acute pancreatitis. Pentoxifylline is a methylxanthine derivative with rheologic and marked anti-inflammatory properties and inhibits the production of proinflammatory cytokines. We have examined whether pentoxifylline ameliorates interstitial edema, inflammatory infiltrate, and glutathione depletion associated with cerulein-induced pancreatitis. Cotreatment of animals with pentoxifylline significantly reduced cerulein-induced pancreatic inflammation and edema and attenuated the depletion of pancreatic glutathione and the increase in serum lipase activity, nitrate, and tumor necrosis factor-α levels. Pentoxifylline also prevented both mitochondrial swelling and damage to mitochondrial cristae caused by cerulein. Our findings provide an experimental basis for using pentoxifylline to attenuate inflammatory responses within the pancreas in acute pancreatitis and as an adjuvant in the treatment of acute pancreatitis.

Acute pancreatitis initially leads to interstitial edema and migration of neutrophils and macrophages into the pancreatic parenchyma, progressing to acinar cell damage and ultimately in hemorrhagic necrotizing pancreatitis and multiple organ failure (Adler and Kern, 1984). In experimental pancreatitis induced by supramaximal doses of the secretagogue cerulein, inhibition of exocytosis (Saluja et al., 1985) is followed by lysosomal degradation of intracellular organelles within autophagic vacuoles in acinar cells and marked interstitial edema (Gorelick et al., 1993). These features of cerulein-induced pancreatitis resemble the early phase of acute edematous pancreatitis in humans (Adler and Kern, 1984).

The role of oxidative stress in the pathogenesis of acute pancreatitis and the potential benefits of antioxidants have been the subject of numerous studies (see Sweiry and Mann, 1996). Intracellular levels of glutathione are depleted, whereas lipid peroxidation increases in pancreatic tissue during the development of acute pancreatitis (Schoenberg et al., 1992; Sweiry and Mann, 1996). Moreover, because restoration of intracellular glutathione levels ameliorates cerulein-induced pancreatitis in mice (Neuschwander-Tetri et al., 1992), it seems likely that generation of reactive oxygen radicals and the consequent depletion of glutathione play a pivotal role in the initiation of acute pancreatitis (Schoenberg et al., 1992). However, there is a lack of consensus whether lipid peroxidation and glutathione depletion are causes or consequences of acute pancreatitis (Dabrowski et al., 1988; Wisner et al., 1988; Schoenberg et al., 1992; Sweiry and Mann, 1996). In this context, Fu et al. (1997) have found that oxidative stress may be insufficient to initiate acute pancreatitis; however, the secretory block in this disease can be mimicked by oxidative stress induced by t-butylhydroperoxide (Sweiry et al., 1999). Beneficial effects of enzymic antioxidants, vitamin C analogs, and glutathione precursors are regarded as an indirect proof for the role of oxidative stress in this disease (Guice et al., 1986; Sandilands et al., 1990; Schoenberg et al., 1992; Sweiry and Mann, 1996; Sweiry et al., 1999).

Nitric oxide (NO) has also been implicated in the development of acute pancreatitis (Sweiry and Mann, 1996). As a reactive free radical, NO mediates the cytotoxicity caused by activated neutrophils and macrophages in the inflammatory response. Moreover, accumulating evidence suggests that NO contributes to oxidative stress in acute experimental pancreatitis (Dabrowski and Gabrylewicz, 1994). Pentoxifylline is a methylxanthine derivative that exhibits marked anti-inflammatory properties (Ward and Clissold, 1987) through its inhibition of cytokine production. It inhib-

ABBREVIATIONS: NO, nitric oxide; TNF-α, tumor necrosis factor-α; GSH, reduced glutathione; GSSG, oxidized glutathione.
its lipopolysaccharide-induced production of tumor necrosis factor-α (TNF-α) by monocytes and T cells as well as of interleukin-2-induced adherence of leukocytes (Edwards et al., 1991; Schandén et al., 1992). Thus, in this study we have examined whether treatment with pentoxifylline or antioxidants ameliorates pancreatic interstitial edema, inflammation, and depletion of intracellular glutathione associated with cerulein-induced pancreatitis in rats. A preliminary account of part of this work has appeared in abstract form (Gómez-Cambronero et al., 1997).

Materials and Methods

Animals. Male Wistar rats (400–450 g) were used. Animals were fed ad libitum on a standard diet (Panlab, Barcelona, Spain) and had free access to water. They were maintained on a 12-h light/12-h dark cycle at 21°C.

In dose-response studies, rats were treated with different doses of cerulein (Sigma, Madrid, Spain): 8 μg/kg, 20 μg/kg, 40 μg/kg, or 80 μg/kg b.wt. Cerulein was administered as four s.c. injections at hourly intervals, each injection containing 25% of the dose. A control group received four s.c. injections of 0.9% saline at hourly intervals. All these rats were sacrificed 2 h after the last injection.

To evaluate the effects of pentoxifylline or antioxidants, rats were divided into two groups: one group treated with cerulein (80 μg/kg b.wt.) and pentoxifylline (12 mg/kg b.wt.) and a second group treated with cerulein (80 μg/kg b.wt.) and an antioxidant mixture composed of retinol (1430 I.U./kg b.wt.), dl-α-tocopherol acetate (1.43 mg/kg b.wt.), ascorbic acid (14.3 mg/kg b.wt.), and N-acetyl cysteine (181 mg/kg b.wt.). The doses of these therapeutic agents were based on the corresponding therapeutic doses used in clinical trials. Therapeutic agents were administered i.p. as a single dose at the same time of the first injection of cerulein. These rats were sacrificed 2 h after the last injection of cerulein.

The procedures were performed in accordance with the Helsinki Declaration of 1975 as revised in 1983. This study was approved by the Research Committee of the Facultad de Medicina de Valencia.

Histological Studies by Light and Electron Microscopy. For light microscopy, a piece from the central body of the pancreas was rapidly removed and fixed in 10% buffered formalin. Subsequently, it was embedded in paraffin, cut, and stained with hematoxylin and eosin. Assessment of tissue alterations was conducted by an experienced pathologist who was unaware of the treatments.

For electron microscopy, small pieces from the body of the pancreas were removed and cut into pieces not larger than 2 mm. They were fixed in phosphate-buffered (0.1 M, pH 7.2) 2.5% glutaraldehyde for 2 h and then postfixed in phosphate-buffered (0.1 M, pH 7.2) 2% osmium tetroxide solution. After embedding tissue blocks in Epon (Polyisciences, Inc., Eppelheim, Germany), ultrathin sections were cut using an ultramicrotome ULTRACUT-E (Reichert Jung, Wien, Austria), then contrasted with uranyl acetate and lead citrate for transmission electron microscopy. Electron microphotographs were taken with a JEOL (Tokyo, Japan) JEM-1010. Assessment of cellular alterations was conducted by an experienced pathologist who was unaware of the treatments.

Assays. Reduced glutathione (GSH) and oxidized glutathione (GSSG) levels in pancreatic tissue were determined as described by Fariss and Reed (1987), whereas GSSG levels in blood were determined as described by Asensi et al. (1994). Total glutathione levels were determined in serum using Ellman’s reagent (Tietze, 1969). Nitrate levels were measured as described by Gilliam et al. (1993). TFN-α levels were measured using the Cytoscreen ultrasensitive immunoassay kit for rat TNF-α from Biosource International (Camarillo, CA). Protein concentrations and lipase activity were determined by standard methods.

After the rats were sacrificed, a piece from the body of the pancreas was rapidly removed, weighed, and then blotted dry on filter paper. The ratio of weight with edema/weight without edema was expressed as a percentage and used as an index of the percentage of edema. The percentage of edema also was measured using the pancreatic dry weight with similar results (data not shown).

Statistical Analysis. Results are expressed as means ± S.D., with the number of experiments given in parentheses. Statistical analysis was performed in two steps. ANOVA was performed first, and then the sets of data in which F was significant were examined by using an unpaired Student’s t test.

Results

Effect of Pentoxifylline or Antioxidant Treatment on Cerulein-Induced Pancreatic Edema. The percentage of pancreatic edema was measured 2 h after treatment with the last dose of cerulein (8–80 μg/kg b.wt.). Figure 1 shows that treatment with 8 μg/kg of cerulein did not cause any edema, 20 μg/kg caused moderate pancreatic edema, and 40 or 80 μg/kg caused intense edema.

The percentage of pancreatic edema also was measured in rats treated with cerulein and pentoxifylline or with cerulein and antioxidants. Figure 2 shows that pentoxifylline treatment at a single dose of 12 mg/kg b.wt. significantly reduced the degree of edema, whereas antioxidant treatment was less effective. The effect of pentoxifylline at a dose of 48 mg/kg b.wt. on the degree of edema was similar to that obtained with a dose of 12 mg/kg b.wt. (data not shown).

Histologic Studies of Pancreas in Cerulein-Induced Pancreatitis: Effect of Pentoxifylline Treatment. Histologic studies of the pancreas using light microscopy showed that cerulein-induced pancreatitis leads to interstitial edema with fibrin accumulation, inflammatory infiltration of neutrophils and mononuclear cells into the pancreatic tissue, and cytoplasmatic vacuolation (Fig. 3A–C; Table 1). Treatment with pentoxifylline markedly reduced these histologic alterations in pancreas (see Fig. 3, D and E; Table 1).

As shown in the electron micrograph in Fig. 4A, cerulein treatment caused mitochondrial damage as well as a notable increase in intracellular zymogen granules, some of which appear to fuse. Treatment with pentoxifylline generally prevented pancreatic edema with similar results (data not shown). Values are mean ± S.D., for n = 3 to 7 animals, *P < .05; **P < .01 versus the control group.

Pentoxifylline Ameliorates Acute Pancreatitis 671
The GSH/GSSG ratio was 9.1 ± 0.6 for and used as an estimate of the degree of edema. Values are mean ± S.D. for n = 3 to 7 animals. **P < .01 versus the control group; *P < .01 versus the cerulein-treated group.

vented these histologic changes (Fig. 4B) and mitochondrial swelling and damage to mitochondrial cristae.

**Serum Lipase Activity and Glutathione Status in Cerulein-Induced Pancreatitis: Effects of Pentoxifylline and Antioxidants.** Lipase activity was measured in the serum of control and cerulein-treated rats. Figure 5 shows that lipase activity increases nearly 12-fold after cerulein treatment, whereas it increases only ~4-fold when pentoxifylline was administered together with cerulein.

GSH levels were measured in the pancreas 2 h after the last treatment with different doses of cerulein. Figure 1 shows that treatment with 8 μg of cerulein/kg b.wt. had no effect on pancreatic GSH levels, whereas treatment with doses of 20 to 40 μg/kg caused a moderate depletion of GSH, and treatment with higher doses of cerulein led to severe GSH depletion. Indeed, pancreatic GSH levels were only 22% of control levels after treatment with the 80-μg/kg dose. A marked depletion of GSSG was also observed in the pancreas after treatment with high doses of cerulein (see Fig. 1). The pancreatic GSH/GSSG ratio did not change significantly in rats treated with low or moderate doses of cerulein (8–40 μg/kg), but it increased significantly (P < .01) 2 h after the last treatment with the highest dose of cerulein (80 μg/kg). The GSH/GSSG ratio was 9.1 ± 1.7 (n = 4) after cerulein treatment (80 μg/kg) versus 4.6 ± 1.9 (n = 4) in controls.

Some researchers have reported a pancreatic GSH/GSSG ratio in control rats of 20 to 40 (Altomare et al., 1996; Janjic et al., 1996). The method used to determine GSSG levels is critical for measuring the GSH/GSSG ratio. We have used the HPLC method described by Fariss and Reed (1987), which has the advantage of measuring simultaneously both GSH and GSSG in the same aliquot. Some researchers have shown a pancreatic GSH/GSSG ratio in control rats similar to our own. Thus, Anjaneyulu et al. (1982) reported a pancreatic ratio of 6.7, and Toyooka et al. (1989) reported a ratio of 5.6. Furthermore, Ammon et al. (1983) reported a ratio less than 1.5 in rat pancreatic islets.

Table 2 shows that blood GSH and GSSG levels were not altered 2 h after treatment with the highest dose of cerulein (80 μg/kg). Moreover, total glutathione levels in serum did not change significantly 2 h after treatment with the highest dose of cerulein (see Table 2). The glutathione redox status in the pancreas also was examined 2 h after treatment with cerulein (80 μg/kg) and antioxidants or pentoxifylline. Glutathione depletion was much less intense after treatment with pentoxifylline (Fig. 6A). Thus, pentoxifylline treatment maintained GSH levels at 71% of values in control rats. In rats treated with cerulein and antioxidants, GSH levels were maintained only at 34% of controls (Fig. 6A).

GSSG depletion in pancreas also was less marked after treatment with antioxidants or pentoxifylline (Fig. 6B). The pancreatic GSH/GSSG ratio in cerulein-treated rats did not change when antioxidants or pentoxifylline were administered. Indeed, it was 11.9 ± 1.6 (n = 3) for the antioxidant group and 12.6 ± 3.1 (n = 4) for the pentoxifylline group (versus 9.1 ± 1.7, n = 4, for the group treated with cerulein alone).

**NO and TNF-α in Cerulein-Induced Pancreatitis: Effect of Pentoxifylline.** Nitrate levels were measured in serum as an index of NO production in vivo. We found an increase in serum nitrate levels in cerulein-induced pancreatitis that was prevented by pentoxifylline treatment (Table 3). We also studied the effect of pentoxifylline on NO release from cultured macrophages, but pentoxifylline did not alter NO synthesis in cytokine-activated macrophages (data not shown). We found a small but significant increase in serum TNF-α levels in cerulein-induced pancreatitis that was prevented by pentoxifylline treatment (Table 3).

**Discussion**

This study has confirmed that experimental pancreatitis induced by hyperstimulation with cerulein is characterized by marked pancreatic inflammation, mitochondrial swelling and damage to mitochondrial cristae, and depletion of glutathione. Cotreatment of animals with pentoxifylline ameliorated these inflammatory responses and generally prevented the depletion of pancreatic glutathione induced by hyperstimulation with cerulein.

Depletion of pancreatic glutathione may be attributable in part to inflammation and/or to the activation of proteases because it is known that activated proteases such as carboxypeptidase can cleave GSH (Meister, 1991). Glutathione plays a role in acinar stimulus-secretion coupling (Stenson et al., 1983), in the maintenance of the cytoskeleton (Jewell et al., 1982), and in appropriate protein folding in the endoplasmatic reticulum (Scheele and Jakoby, 1982). Thus, a depletion of intracellular glutathione may contribute to impairedzymogen granule transport (secretory block) and to the premature activation of pancreatic proteases (Lütken et al., 1995). To study whether depletion of glutathione in cerulein-induced pancreatitis is attributable to oxidation, we measured GSSG levels. Our results show that glutathione depletion, but not glutathione oxidation, occurs in the pancreas in cerulein-induced pancreatitis. Therefore, detoxification of reactive oxygen species does not appear to be the major cause for depletion of glutathione. Additional evidence that glutathione oxidation is not responsible for GSH depletion is the fact that antioxidants did not protect against GSH depletion caused by cerulein. The aim of this study was to demonstrate whether pentoxifylline exhibits beneficial effects on cerulein-induced pancreatitis. Pasquier et al. (1991) reported that
pentoxifylline may act as a hydroxyl radical scavenger. We used acute antioxidant administration to compare its putative protective effect on cerulein-induced pancreatitis with that of an acute administration of pentoxifylline. We found that in our experimental conditions, antioxidants did not protect against cerulein-induced pancreatitis. However, we cannot rule out that other kinds of treatment using antioxidants can ameliorate cerulein-induced pancreatitis. This issue already has been the subject of numerous studies (Sweiry and Mann, 1996).

An increased efflux of glutathione from pancreas across the basolateral membrane does not seem to account for the loss of glutathione related to acute pancreatitis because glutathione levels in serum or blood did not increase in our model of pancreatitis. It can be calculated that if 80% of pancreatic glutathione released from pancreas remained in plasma, its

Fig. 3. Pancreatic histology of rats treated with cerulein alone or with cerulein and pentoxifylline. A, severe pancreatic interstitial edema; B, a diffuse infiltrate of inflammatory cells into the pancreatic tissue; and C, intracellular vacuolization. D, only a mild edema without inflammatory infiltrate occurs in the pancreas of rats treated with cerulein and pentoxifylline. D and E, the minimal intracellular vacuolization present in the pancreas of rats treated with cerulein and pentoxifylline.
concentration would rise up to 20 times, i.e., from 7 to 140 nmol/ml. However, we have not found any increase in glutathione levels of plasma in cerulein-induced acute pancreatitis and, hence, a loss of pancreatic glutathione toward circulation does not appear to account for glutathione depletion.

The low half-life of glutathione in plasma (Wendel and Cikryt, 1980; Ammon et al., 1986) cannot account for its total disappearance. During the elimination phase, the half-life of plasma GSH may be greater than 50 min (Ammon et al., 1986). Hence, the increase of total glutathione in plasma should have been detected 1 h after the last dose of cerulein. As we show in Results, this is not the case.

Mitochondrial damage is closely associated with severe glutathione depletion (Meister, 1991). Mitochondria cannot synthesize glutathione because they lack γ-glutamylcysteine synthetase or glutathione synthetase activities (Meister, 1991) and thus obtain glutathione by transport from the cytosol. Marked depletion of mitochondrial glutathione causes mitochondrial damage that is completely prevented by administration of glutathione monoesters (Meister, 1991). Depletion of pancreatic glutathione may be responsible for the mitochondrial damage associated with acute pancreatitis. As evidenced in this study, partial restoration of intracellular glutathione levels after pentoxifylline treatment prevented damage to pancreatic mitochondria (see Fig. 4, A and B). TNF-α is known to cause mitochondrial damage, and it is worth noting that pentoxifylline also prevented increases in serum TNF-α levels induced by cerulein hyperstimulation. The noted damage to mitochondria in cerulein-induced pancreatitis may well account for the increased cellular damage associated with progression of pancreatitis in animal models of the disease and in humans.

Several studies have reported substantial improvements in acute pancreatitis on treatment with enzymic antioxidants (Guice et al., 1986; Wisner et al., 1988; Sandilands et al., 1990; Schoenberg et al., 1992). In our study, cotreatment of animals with an antioxidant mixture containing N-acetyl cysteine plus vitamins A, C, and E (administered at the beginning of the first cerulein injection) was of limited value in cerulein-induced pancreatitis.

The role of NO in the pathogenesis of acute pancreatitis remains controversial (Sweiry and Mann, 1996), with some studies suggesting that NO potentiates pancreatic oxidative stress and damage (Tani et al., 1990; Dabrowski and Gablylewicz, 1994), whereas others report that NO ameliorates...
Pentoxifylline ameliorates acute pancreatitis

Pentoxifylline (1430 I.U./kg b.wt.) or an antioxidant mixture composed of ascorbic acid (14.3 mg/kg b.wt.), and N-acetyl cysteine (181 mg/kg b.wt.). Curcumin was injected s.c. at the dose of 80 g/kg b.wt. Cerulein-treated rats were injected i.p. with pentoxifylline (12 mg/kg b.wt.) or an antioxidant mixture composed of vitamin E (1.45 mg/kg b.wt.), vitamin C (14.3 mg/kg b.wt.), and N-acetyl cysteine (181 mg/kg b.wt.). The ratio of pancreatic weights with and without edema was expressed as a percentage and used as an estimate of the degree of edema. Values are mean ± S.D. for n = 4 to 5 animals; *P < .05, **P < .01 versus the control group, ***P < .01 versus the cerulein-treated group.

**Table 3** Effect of pentoxifylline on serum TNF-α and nitrates levels in acute pancreatitis

Values are mean ± S.D. for n = 4 to 10 different animals. Cerulein was injected s.c. at the dose of 80 μg/kg b.wt. Cerulein-treated rats were injected i.p. with pentoxifylline (12 mg/kg b.wt.).

<table>
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<th>Group</th>
<th>TNF-α (pg/ml)</th>
<th>Nitrates (nmol/ml)</th>
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<tr>
<td>Control</td>
<td>7.1 ± 2.3</td>
<td>9.7 ± 5.2</td>
</tr>
<tr>
<td>Cerulein</td>
<td>11.3 ± 3.4*</td>
<td>21.0 ± 4.5*</td>
</tr>
<tr>
<td>Cerulein + pentoxifylline</td>
<td>7.4 ± 1.7*</td>
<td>6.6 ± 3.9*</td>
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*P < .05 versus the control group.

Pentoxifylline exhibits marked anti-inflammatory properties that are mediated by inhibition of TNF-α production and interleukin-2-induced injury (Edwards et al., 1991; Schandén et al., 1992). Pentoxifylline also inhibits the inflammatory actions of interleukin-1 and TNF-α on neutrophil function (Sullivan et al., 1988). Sugita et al. (1997) have reported recently that propentoxifylline inhibited the rise in serum TNF-α levels in rats treated with cerulein and lipopolysaccharide. Our results establish that pentoxifylline significantly reduced pancreatic inflammation, edema, infiltration of inflammatory cells into pancreatic tissue, and the increase in serum lipase activity caused by cerulein pancreatitis. The anti-inflammatory effect of pentoxifylline also was associated with a decrease in both circulating TNF-α and nitrate levels. Pentoxifylline may have directly inhibited TNF-α formation and consequently NO production from activated leukocytes. On the other hand, pentoxifylline prevents leukocyte infiltration, and thus may prevent the release of interferon-γ by leukocytes that might be responsible, at least in part, for the suppression of NO production. It is worth noting that in vitro experiments, we could not directly inhibit cytokine-induced NO production in a murine macrophage cell line, J774. Nevertheless, we cannot rule out a beneficial effect of pentoxifylline in acute pancreatitis also attributable to its rheologic properties because this methylxanthine derivative is able to increase blood cell deformability, decrease platelet aggregation, lower blood viscosity, and reduce thrombus formation (Ward and Clissold, 1987). These characteristics result in improved microvascular flow and have prompted the use of pentoxifylline clinically in peripheral and cerebrovascular disease (Ward and Clissold, 1987).

Contrary to our results, Bassi et al. (1994) found no protective effect of pentoxifylline in severe acute pancreatitis induced in rats by cerulein plus glycodeoxycholate. This discrepancy may be explained if pentoxifylline ameliorates mild edematous pancreatitis but not severe pancreatitis as in the study of Bassi et al. (1994). The anti-inflammatory and rheologic properties of pentoxifylline may not be sufficient to overcome pancreatic damage involving acinar necrosis and hemorrhage. However, because we have shown that pentoxifylline ameliorates interstitial edema, inflammatory infiltration, glutathione depletion, and limits the increase in serum lipase, TNF-α, and nitrate levels associated with cerulein-induced pancreatitis, pentoxifylline could be considered a
potential therapy to prevent progression of mild edematous to severe pancreatitis.

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References


