Oxidative stress and nitric oxide in rats with alcohol-induced acute pancreatitis

Gülner Andican, Remisa Gelisgen, Ethem Unal, Osman Baran Tortum, Sergülen Dervisoglu, Tayfun Karahasanoğlu, Gülden Burçak

Gülner Andican, Remisa Gelisgen, Gülden Burçak, Department of Biochemistry, Cerrahpaşa Medical Faculty, Istanbul University, Istanbul, Turkey
Ethem Unal, Osman Baran Tortum, Tayfun Karahasanoğlu, Department of Surgery, Cerrahpaşa Medical Faculty, Istanbul University, Istanbul, Turkey
Sergülen Dervisoglu, Department of Pathology, Cerrahpaşa Medical Faculty, Istanbul University, Istanbul, Turkey
Correspondence to: Dr. Gülner Andican, Ataköy 7-8, Kışım L13 Blok, E kapısı Da: 45, İstanbul, Turkey. andican@e-kolay.net
Fax:+90-212 559 55 91
Received: 2004-05-25   Accepted: 2004-05-30

Abstract

AIM: Oxygen free radical mediated tissue damage is well established in pathogenesis of acute pancreatitis (AP). Whether nitric oxide (NO) plays a deleterious or a protective role is unknown. In alcohol-induced AP, we studied NO, lipooxidative damage and glutathione in pancreas, lung and circulation.

METHODS: AP was induced in rats (n = 25) by injection of ethyl alcohol into the common biliary duct. A sham laparatomy was performed in controls (n = 15). After 24 h the animals were killed, blood and tissue sampling were done.

RESULTS: Histopathologic evidence confirmed the development of AP. Marked changes were observed in the pulmonary tissue. Compared with controls, the AP group displayed higher values for NO metabolites in pancreas and lungs, and thiobarbituric acid reactive substances in circulation. Glutathione was lower in pancreas and in circulation. Glutathione and NO were positively correlated in pancreas and lungs of controls but negatively correlated in circulation of experimental group. In the experimental group, plasma thiobarbituric acid reactive substances were negatively correlated with pancreas thiobarbituric acid reactive substances but positively correlated with pancreas NO.

CONCLUSION: NO increases in both pancreas and lungs in AP and NO contributes to the pathogenesis of AP under oxidative stress.

© 2005 The WJG Press and Elsevier Inc. All rights reserved.

Key words: Thiobarbituric acid reactive substances; Glutathione; Nitric oxide; Acute pancreatitis

INTRODUCTION

The etiological basis of acute pancreatitis (AP) is multifactorial. However, as in other inflammatory diseases, a final common pathway mediated by reactive oxygen species (ROS) appears to play a role in the associated tissue destruction both in the initiation and progression of AP. Augmented production of ROS in a self-perpetuating manner, in excess of antioxidant defenses, occurs predominantly in activated neutrophils. Once produced, ROS could trigger various inflammatory processes. They can directly attack the lipoid matrix of biological membranes, stimulate arachidonic acid metabolism with increased production of prostaglandins, thromboxane, and leukotrienes, thereby enhancing the accumulation and adherence of neutrophils and platelets to the capillary wall. Thus ROS could impair the microcirculation and disturb the microvascular integrity, resulting in decreased perfusion, increased capillary permeability and fluid transudation. Neutrophils infiltrating the pancreas have also been very recently demonstrated to contribute, via ROS, to the pathologic activation of digestive enzymes in acinar cells.

Nitric oxide (NO) has also been implicated in the pathogenesis of AP. NO, being a reactive free radical, contributes to the cytotoxicity of neutrophils and macrophages in the inflammatory response. Moreover, under circumstances of oxidative stress, the interaction of NO with superoxide radicals gives rise to peroxynitrite which can cause platelet aggregation, disseminated intravascular coagulation, lipid peroxidation and ubiquitous cell damage. On the other hand, NO induces vasodilatation and prevents endothelial damage by inhibiting platelet aggregation and leukocyte adherence. Due to improvement in both pancreatic microcirculation and capillary organ perfusion throughout the body, NO has been suggested to have a protective role in AP. Thus the role of NO in the pathogenesis of AP remains controversial. Accordingly both inhibitors of NOS and NO donors are suggested to be protective in AP.

AP is known to be often complicated by multiple organ failure. In particular, acute lung injury occurs at an early stage of pancreatitis and significantly contributes to
morbidity and mortality of this disease\cite{12,13}. The etiology of this extrapancreatic disease has not yet been clearly elucidated. The injury in lung and the resulting adult respiratory distress syndrome have been attributed to the release of pancreas-derived proteolytic enzymes into the circulation\cite{14}. There is also evidence of neutrophil related, ROS-mediated microvascular injury. The release of ROS from activated alveolar macrophages (AMs) has also been shown to lead to the progression of lung injury\cite{15}. AM-derived NO was also reported to contribute to lung injury\cite{16}.

As alcoholism is the most common etiology in humans and the mechanism of injury remains unknown, we focused our attention on alcohol-induced AP. Only a few studies have been noted on alcohol-induced AP and in most of these, alcohol has not been used alone but in combination with another agent\cite{17-19}. With regard to alcohol usage, an emerging concept is that a single episode of binge drinking may be sufficient to induce an episode of AP\cite{20}. However, the previous dogma was that consistent ingestion of alcohol for a prolonged period was required to prime the pancreas before an episode of pancreatitis.

Thus in this experimental study we aimed to induce AP with a single high dose of alcohol and to assess NO production (stable metabolites of NO, nitrite plus nitrate; NO\textsubscript{3}\textsuperscript{–}\textsuperscript{–}) and oxidative stress as reflected by lipid peroxidation and glutathione depletion in the pancreas, lungs and systemic circulation as far as our literature survey was concerned. In an alcohol-induced AP model these assessments were simultaneously made for the first time.

**MATERIALS AND METHODS**

**Animal treatment**

The study was carried out on 40 young adult male (200-320 g) Wistar Albino rats. The rats, cared for in accordance with the Guide for the Care and Use of Laboratory Animals\cite{21}, were permitted ad libitum access to standard laboratory chow (20-30 g/rat/d) and tap water for 10 d prior to experimental procedures.

The rats were divided randomly into two groups. The experimental group consisted of 25 rats and control group of 15 rats. Animals were anesthetized with ether to undergo a midline laparotomy and the duodenal portion of the pancreas was exposed. AP was induced by injection of ethyl alcohol (48%, 1 mL) into the common biliary duct. A sham laparotomy was performed in the control group. After 24 h, heparinized blood samples were taken with intracardiac puncture and rats were killed by decapitation under ether anesthesia. Lung and pancreatic tissues were obtained peroperatively and immediately frozen at -80 °C for biochemical measurements and fixed in 10% buffered formalin, embedded in paraffin for standard histologic examinations.

Thiobarbituric acid-reactive substances (TBARS), glutathione (GSH) and NO metabolites were measured in the blood and tissue samples. Tissues prepared in cold potassium phosphate buffer 100 g/L were homogenized using a Potter homogenizer. The homogenates were centrifuged at 3 000 r/min for 15 min and the supernatant was used for the biochemical measurements.

**Measurement of lipid peroxidation**

Lipid peroxidation was determined by measuring the TBARS by the modified method of Buege et al\cite{22} in the plasma and by the method of Okhawa et al\cite{23} in tissue using 1. 56×10\textsuperscript{5} mol/L/cm as molar extinction coefficient. TBARS concentration in the supernatant of the tissue homogenates was expressed as µmol/g protein.

**Measurement of GSH**

GSH concentration in erythrocytes and supernatants of the tissue homogenates was determined according to the method of Buettler et al\cite{24} using metaphosphoric acid for protein precipitation and 5’5’-dithiobis-2-nitrobenzoic acid for color development. Erythrocyte GSH concentration was expressed as mg/g Hb using 1.36×10\textsuperscript{5} mol/L/cm as molar absorption coefficient. Hemoglobin concentration was determined by the cyanomethemoglobin method\cite{25}. GSH concentration in supernatants of the tissue homogenates was expressed as mg/g protein.

**Measurement of nitric oxide metabolites (NO\textsubscript{x})**

The stable metabolites of NO, nitrate (NO\textsubscript{3}\textsuperscript{–}) and nitrite (NO\textsubscript{2}\textsuperscript{–}) were measured as an index of NO production. NO colorimetric assay kit (Roche-Boehringer Mannheim) was used. Nitrate was first reduced to nitrite by NADPH in the presence of nitrate reductase. Nitrite was reacted with sulphanilamide and naphthyl-ethylenediamine dihydrochloride to give a red-diazo dye. The diazo dye was measured spectrophotometrically at 550 nm. The concentration of NO metabolites was determined by comparison with a standard curve, which was constructed using a set of serial dilutions of nitrate.

Protein concentration was determined according to the method of Lowry (Sigma-Aldrich, Germany).

**Histology**

For histologic examination, the samples were cut into 4-μm thick sections and stained with hematoxylin and eosin. All slides were evaluated “blindly” by two independent histopathologists for the presence of edema, hyperemia, infiltration by inflammatory cells, hemorrhage and necrosis.

**Statistical analysis**

All data were presented as mean±SD. Student’s t-test was performed to evaluate significant differences between the groups. Correlation between different variables was studied by Pearson’s correlation coefficient. P<0.05 was considered statistically significant.

**RESULTS**

Histopathologic study of the pancreas in the experimental group showed hyperemia, interstitial edema, hemorrhage, inflammatory infiltration of neutrophils and mononuclear cells and focal necrotic areas. Marked changes were also observed in pulmonary histology, with hyperemia and focal atelectasis areas, peribronchial mononuclear and neutrophilic infiltration, early neutrophilic exudation in some alveoli. Some of the cases revealed abscess formation in lung parenchyma. These findings were considered as the evidence of an
established AP. They were summarized as semiquantitatively analyzed results in control and AP group (Table 1).

TBARS concentration in the systemic circulation was observed to be higher in the experimental group in comparison to the control (8.992±3.52 vs 6.208±0.95, P<0.05). However no significant differences were noted between the groups in pancreas or in lung (Figure 1).

GSH concentration was observed to be significantly lower in the pancreas (2.978±1.52 vs 5.375±1.38, P<0.001) and erythrocytes (1.576±0.44 vs 1.947±0.27, P<0.05) in the experimental group compared with the control group. No significant difference was noted in GSH concentration in the lung (Figure 2).

NOx were observed to be higher in both the pancreas (4.079±0.78 vs 3.080±0.37, P<0.01) and in the lung (1.809±0.89 vs 1.023±0.55, P<0.05) in the experimental group compared with the control group. No significant difference was noted in the circulation (Figure 3).

The data evaluated by correlation analysis yielded the following results (Table 2). In the control group, GSH and NO were positively correlated both in the pancreas and in the lung (r = 0.757, P<0.001; r = 0.533, P<0.05, respectively).

In the experimental group, GSH and NOx were negatively correlated in the circulation (r = -0.426, P<0.05). GSH and TBARS were positively correlated in the pancreas (r = 0.462, P<0.05). Plasma TBARS was negatively correlated with pancreas TBARS (r = -0.402, P<0.05) but positively correlated with pancreas NO (r = 0.654, P<0.01). Pancreas TBARS was positively correlated with lung TBARS (r = 0.650, P<0.01).

**DISCUSSION**

The pathophysiology of alcohol-related AP is not well understood. The loss in pancreatic duct integrity and/or changes in duct permeability are responsible for the initiation. Elevated intraductal pressure due to increased secretion and/or the sphincter of Oddi spasm and the presence of alcohol are considered necessary for the production of permeable ducts[^17,27].

As in experimental animals not adapted to chronic alcohol ingestion ethanol given orally or intravenously had only a weak stimulatory effect on the pancreas[^27], we administered alcohol to the common bile duct. Our histopathologic examination showed interstitial edema, infiltration of neutrophils and focal necrotic and hemorrhagic areas in the pancreas 24 h after the administration of alcohol. We considered these findings as lines of evidence of mild-moderate, edematous pancreatitis. Marked changes were

---

**Table 1** Histopathologic lesions observed in pancreas and lung

|                  | Pancreas | Lung |
|------------------|----------|------|
| Edema            | +/-      | +/-  |
| Hemorrhage       | +        | ++   |
| Hyperemia        | -        | -    |
| Necrosis         | -        | +/–  |
| PMN infiltration | -        | -    |
| PMN and lymphocytic infiltration | -        | -    |
| Alveolar neutrophilic exudation | -        | -    |
| Hyperemia        | -        | -    |
| Focal atelectasia| -        | -    |

The semiquantitative evaluation scale was the mean of the lesions observed in each group evaluated by independent observers: -, no lesion; +/-, minimal lesion; +, mild lesion; ++, moderate lesion; ++++, intense lesion. AP: acute pancreatitis.
also observed in pulmonary tissue with hyperemia, focal atelectasia, peribronchial mononuclear and neutrophilic infiltration and alveolar neutrophilic exudation resulting in abscess formation in some of the rats with pancreatitis.

High dose of ethanol is known to affect the lipids of cellular membranes and to cause alterations in membranes, permeability and functioning of the integral membrane components. The loss in lysosomal membrane integrity within acinar cells and decompartmentalization of cathepsin B results in pathological activation of trypsinogen, chymotrypsinogen, proelastase and thus autodigestion of pancreatic acinar cells. Additionally, ethanol affects the antiprotease components and disturbs the protease-antiprotease balance in pancreatic juice, enabling more effective activation of proteolytic enzymes. Trypsin induced activation of the complement system could contribute to inflammatory interstitial infiltration, parenchymal necrosis, and vacuolization of acinar cells. Activation of proelastase could contribute to damage in microvasculature. There is also growing evidence that ethanol exerts direct toxic effects on pancreatic acinar cells. Certain toxic effects of ethanol have been attributed to both oxidative and nonoxidative metabolites of alcohol, acetaldehyde and fatty acid ethyl ester, respectively.

As to the pathogenesis of lung injury secondary to AP, it is complex and probably involves pancreatic proteases, phospholipase A2 activated complements, AMs and neutrophils. Ethanol could specifically amplify the effect of phospholipase A2 which damages pulmonary surfactants, induces AMs to release NO and promotes PAF or eicosanoid production.

ROS have been implicated as an important factor in both the initiation and progression of AP in all experimental models. Augmented ROS production could cause lipid peroxidation which induces either apoptosis or necrosis depending on its extent. It has been shown that a short-term, high peak radical attack is more injurious to pancreatic acinar cells than a long lasting, low-level ROS generating system. In our study, under the conditions of neutrophilic infiltration and necrosis in the pancreas, respiratory burst of polymorphonuclear leukocytes, derangement in oxidative phosphorylation, activations of NADPH oxidase, myeloperoxidase, xanthine oxidase and ethanol-catabolizing oxidases all contributed to augmented ROS production. The increased TBARS and the decreased GSH that we observed in the circulation of rats 24 h after establishment of alcohol-induced AP reflected oxidative stress on whole body basis. As sham-operated animals served as controls these findings did not reflect laparotomy or bowel-maneuver induced alterations.

Despite an increase of lipid peroxidation in the circulation, we could not demonstrate any increase of lipid peroxidation in tissue samples. The negative correlation of TBARS in pancreatic tissue, with TBARS in circulation suggested the release of lipooxidative damage end-products from the tissue to the circulation. This release might well be due to the autodigestion in the pancreatic tissue. On the other hand, it is also known that injury to the pancreatic tissue during AP was followed by a spontaneous reparative/regenerative process. Accordingly, in several experimental studies, lipid peroxidation, observed to be increased as early as 30 min after induction of AP was normalized within 12 h. Thus, in this respect, lipid peroxidation may perhaps be considered as the sequel of pancreatic inflammation and TBARS in circulation may likely reflect the severity of the systemic inflammatory response, rather than the pancreatic parenchymal damage. Accordingly various reactive aldehydes reflected by TBARS are rather long lived and therefore can diffuse from their site of origin and attack distant targets with some oxidative stress associated pathophysiologic effects. Interestingly we observed that the extent of lipooxidative damage in the lung was correlated with that in the pancreas.

Table 2 Correlation between studied parameters in control and experimental groups

| Parameter               | Control group (n = 15) | AP group (n = 25) |
|-------------------------|------------------------|-------------------|
| GSH<sub>plasm</sub>–NO<sub>plasm</sub> | r = 0.757<sup>a</sup> |                   |
| GSH<sub>plasm</sub>–NO<sub>panc</sub>  | r = 0.533<sup>b</sup> |                   |
| GSH<sub>panc</sub>–NO<sub>panc</sub> | r = –0.426<sup>c</sup> |                   |
| GSH<sub>panc</sub>–TBARS<sub>plasm</sub> | r = 0.462<sup>d</sup> |                   |
| TBARS<sub>plasm</sub>–TBARS<sub>panc</sub> | r = –0.402<sup>e</sup> |                   |
| TBARS<sub>panc</sub>–NO<sub>panc</sub> | r = –0.654<sup>f</sup> |                   |
| TBARS<sub>panc</sub>–TBARS<sub>plasm</sub> | r = 0.650<sup>g</sup> |                   |

AP: acute pancreatitis, NO: nitrite plus nitrate. <sup>a</sup>P<0.05 vs control, <sup>b</sup>P<0.01 vs control.
Ischemic injury associated with experimental acute pancreatitis. Br J Surg 1990; 77: 1256-1259.

4. Cuzzocrea S, Mazzon E, Dugo L, Serraino I, Centorrino T, Ciccolo A, Van de Loo FA, Britti D, Caputi AP, Thiemermann C. Inducible nitric oxide synthase-deficient mice exhibit resistance to the acute pancreatitis induced by cerulein. Shock 2002; 17: 416-422.

5. Tsai K, Wang SS, Chen TS, Kong CW, Chang FY, Lee SD, Lu FJ. Oxidative stress: an important phenomenon with pathogenetic significance in the progression of acute pancreatitis. Gut 1998; 42: 850-855.

6. Sanfey H, Bulkley GB, Cameron JL. The role of oxygen-derived free radicals in the pathogenesis of acute pancreatitis. Ann Surg 1984; 200: 405-413.

7. Viola G, al-Mufi RA, Sohail M, Williamson RC, Mathie RT. Nitric oxide induction in a rat model of selective pancreatic ischemia and reperfusion. Hepatogastroenterology 2000; 47: 1250-1255.

8. Rodeberg DA, Chaet MS, Bass RC, Arkovitz MS, Garcia VF. Nitric oxide: an overview. Am J Surg 1995; 170: 292-303.

9. Liu X, Nakano I, Yamaguchi H, Ito T, Goto M, Koyanagi S, Kinjoh M, Nawata H. Protective effect of nitric oxide on development of acute pancreatitis in rats. Dig Dis Sci 1995; 40: 2162-2169.

10. Werner J, Fernandez-del Castillo C, Rivera JA, Kollias N, Lewandrowski KB, Rattner DW, Warshaw AL. On the protective mechanisms of nitric oxide in acute pancreatitis. Gut 1998; 43: 401-407.

11. Andrzejewska A, Jurkowska G. Nitric oxide protects the ultrastructure of pancreatic acinar cells in the course of caerulein-induced acute pancreatitis. Int J Exp Pathol 1999; 80: 317-324.

12. Schulz HU, Niederau C, Kronowiak-Stumpe H, Halangk W, Lüthen K, Lippert H. Oxidative stress in acute pancreatitis. Hepatogastroenterology 1999; 46: 2736-2750.

13. Sweiry JH, Mann GE. Role of oxidative stress in the pathogenesis of acute pancreatitis. Scand J Gastroenterol Suppl 1996; 219: 10-15.

14. Tsukahara Y, Horita Y, Anan K, Morisaki T, Tanaka M, Torisu M. Role of nitric oxide derived from alveolar macrophages in the early phase of acute pancreatitis. J Surg Res 1996; 66: 43-50.

15. Renner IG, Savage WT, Pantoya JL, Renner VJ. Death due to acute pancreatitis. A retrospective analysis of 405 autopsy cases. Dig Dis Sci 1985; 30: 1005-1018.

16. O’Donovan DA, Kelly CJ, Abdih H, Boucher-Hayes D, Watson RW, Redmond HP, Burke PE, Boucher-Hayes DA. Role of nitric oxide in lung injury associated with experimental acute pancreatitis. Br J Surg 1995; 82: 1122-1126.

17. Guice KS, Oldham KT, Caty MG, Johnson KJ, Ward PA. Neutrophil-dependent, oxygen-radical mediated lung injury associated with acute pancreatitis. Ann Surg 1989; 210: 740-747.

18. Closa D, Sabater L, Fernandez-Cruz L, Prats N, Gelpi E, Roselló-Catalá J, Activation of alveolar macrophages in lung injury associated with experimental acute pancreatitis is mediated by the liver. Ann Surg 1999; 229: 230-236.

19. Lüthen RE, Niederau C, Grondell JH. Glutathione and ATP levels, subcellular distribution of enzymes, and permeability of duct system in rabbit pancreas following intravenous administration of alcohol and cerulein. Dig Dis Sci 1994; 39: 871-879.

20. Rydzewska G, Jurkowska G, Dzieciol J, Faszczena A, Wroblewski E, Gabryelewicz M, Does chronic ethanol administration have influence on pancreatic regeneration in the course of caerulein induced acute pancreatitis in rats? J Physiol Pharmacol 2001; 52: 855-849.

21. Długosz JW, Wroblewski E, Poplawski C, Gabryelewicz A. Does antecedent ethanol intake affect course of taurourcholate pancreatitis in rats? Dig Dis Sci 1997; 42: 944-951.
