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RAPID COMMUNICATION

Assessment of oxidative stress in chronic pancreatitis patients

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Abstract

AIM: To assess the levels of antioxidant capacity and oxidative damage in blood of chronic pancreatitis (CP) patients in comparison with those in healthy control subjects, by using several different analytical techniques.

METHODS: Thirty-five CP patients and 35 healthy control subjects were investigated prospectively with respect to plasma levels of thiols, ferric reducing ability of plasma (FRAP, i.e. antioxidant capacity), levels of protein carbonyls and thiobarbituric acid reactive substances (TBARS). Additionally, we evaluated the production of reactive oxygen species (ROS) in whole blood.

RESULTS: The antioxidative thiols including cysteine, cysteinylglycine and glutathione were significantly lower in CP patients. In addition, the non-enzymatic antioxidant capacity was significantly lower in CP patients, which correlated with the amount of oxidative protein (protein carbonyls) and the extent of lipid damage (TBARS), both were significantly higher in CP patients. The ROS production in whole blood after stimulation with phorbol 12-myritate 13-acetaat, demonstrated a strong tendency to produce more ROS in CP patients.

CONCLUSION: Oxidative stress may contribute to the pathogenesis of chronic pancreatitis by decreasing antioxidant capacity and increasing oxidative damage in CP patients may be a rationale for intervention with antioxidant therapy.

Key words: Chronic pancreatitis; Oxidative stress; Thiols; Ferric reducing ability of plasma; Protein carbonyls; Thiobarbituric acid reactive substances; Reactive oxygen species

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INTRODUCTION

Chronic pancreatitis (CP) is a progressive irreversible inflammatory disease that eventually leads to an impaired exocrine and/or endocrine function of the pancreas[1-4]. Although most cases have been attributed to alcohol abuse, the underlying causes of CP appear to be multi-faceted, including environmental as well as genetic factors. Chronic pancreatitis shares risk factors with pancreatic cancer such as smoking and alcohol abuse, but itself is also a risk factor for pancreatic adenocarcinoma[5]. A genetic predisposition to pancreatitis is supported by the identification of sequence alterations in the genes encoding cationic trypsinogen (PRSS1), the cystic fibrosis transmembrane conductance regulator (CFTR), and the serine protease inhibitor, Kazal type 1 (SPINK1) in patients with hereditary or idiopathic chronic pancreatitis[1,6-8]. Additionally, an increased frequency of SPINK1 mutations been reported in patients with alcohol-related chronic pancreatitis[3,9]. So far we have not completely understood the pathogenesis of CP[10]. Different hypotheses have been proposed, including the contribution of oxidative stress of endogenous origin or chemical stress by environmental or lifestyle-related xenobiotics[11-15]. There is growing recognition that an imbalance between reactive oxygen species (ROS) producing and ROS scavenging processes leads to the damage of pancreatic acinar cells, initiating auto-digestion of the entire pancreas. This insight is suggested by data from experimental and clinical studies[16-19]. Oxidative stress may be important in the pathogenesis of ethanol-induced pancreatic injury, although radiation, exposure to cigarette smoke, medication or trauma may stimulate the generation of free radicals, which subsequently may result in damage of lipids, proteins or nucleic acids. Activation of (enzymatic) antioxidative defence has been described in pancreatic disease independent of its origin[20]. Glutathione and cysteine are important mediators in the defence against oxidative stress and both molecules play a key role in the maintenance of cellular thiol redox status. Therefore, in the present study the concentrations of glutathione, cysteine and other thiols were measured in blood plasma of patients with CP and healthy control subjects. In addition, we also measured the non-enzymatic antioxidant capacity by applying the ferric reducing ability of plasma (FRAP) assay in patients with CP and healthy controls. Further assessment of the level of oxidative stress was performed by measuring the concentrations of protein carbonyls in plasma in order to determine the amount of oxidative protein damage. As an indicator of lipid peroxidation we established the concentrations of thiobarbituric acid-reactive substances (TBARS). Finally, we investigated the generation of ROS by chemiluminescence in whole blood of both patients and controls.

MATERIALS AND METHODS

Subjects

The study was approved by the local medical ethical review committee and all subjects gave their written informed consent. This study was conducted at the Department of Gastroenterology of the University Medical Centre Nijmegen, the Netherlands and all subjects studied were Caucasians of Dutch extraction. A total of 35 consecutive CP patients were recruited between January 2004 and June 2004 at the out-patient clinic of the department. In 29 patients an alcohol-related etiology was indicated (ACP), the remaining 6 CP patients had a family history of CP (HCP). The clinical diagnosis of CP was based on one or more of the following criteria: presence of typical complaints (recurrent upper abdominal pain, radiating to the back, relieved by leaning forward or sitting upright and increased after eating), suggestive radiological findings such as pancreatic calcifications or pseudocysts, and pathological findings (pancreatic ductal irregularities and dilatations) revealed by endoscopic retrograde pancreaticography or magnetic resonance imaging of the pancreas before and after stimulation with secretin. ACP was diagnosed in patients who consumed more than 60 g (females) or 80 g (males) of ethanol per day for more than two years before they were diagnosed, during their treatment they all gave up drinking alcohol. HCP was diagnosed based on the presence of two first-degree relatives or three or more second-degree relatives in two or more generations, suffering from recurrent acute pancreatitis or chronic pancreatitis for which there was no precipitating factor. For comparison, we collected a control group consisting of 35 healthy subjects. We recruited our healthy controls by advertisement in a local paper and did not apply any monetary incentive for the controls to participate.

Analysis of thiols

Samples of blood were taken by venapuncture into EDTA tubes. Whole blood was centrifuged at 1500 × *g* for 10 min within 1 h after collection and plasma was stored at -30℃ until analysis. Concentrations of the thiols including cysteine, homocysteine, cysteinylglycine and glutathione (the sum of reduced-, oxidised- and protein-bound thiols) in plasma were quantified using high performance liquid chromatography (HPLC) with fluorescent detection, essentially as described by Fortin *et al*[21] and modified by Raijmakers *et al*[22]. Thiol levels were calculated using four-point calibration curves for each thiol, which were run in parallel with the samples, and values were expressed in μmol/L.

Analysis of FRAP

The antioxidant capacity in blood plasma was measured using the ferric reducing ability of plasma (FRAP) assay, according to the method of Benzie and Strain[23]. The reduction of ferric to ferrous ion at low pH formed a coloured ferrous-tripyridyltriazine complex. Absorbance changes were linear over a wide concentration range with antioxidant mixtures, including plasma. FRAP values were obtained using a seven-point calibration curve of known amounts of Fe^{2+} and expressed in mmol Fe^{2+}/L.

Analysis of protein carbonyls

The amount of oxidative protein damage, as a marker for oxidative stress, was determined using an enzyme linked immunosorbent assay (ELISA) for estimation of protein carbonyls in body fluids, as essentially described by Buss *et al*[24] and adapted by Zusterzeel *et al*[25]. Samples were incubated with dinitrophenylhydrazine and then adsorbed to wells of an ELISA plate before probing with a commercial antibody raised against protein-conjugated dinitrophenylhydrazine. The binding of biotin-conjugated primary antibody was then quantified after incubation with streptavidin-biotinylated horseradish peroxidase and staining with o-phenylenediamine. Calibration took place using oxidised and fully reduced albumin, and carbonyl levels were expressed in μmol/g protein.

Analysis of TBARS

Thiobarbituric acid-reactive substances (TBARS), mainly malondialdehyde (MDA) in plasma were evaluated by recording the fluorescence spectrum between 500 and 600 nm on a Shimadzu RFF-5000 spectrofluorometer, of the thiobarbituric acid-malonaldehyde complex, as described by Conti *et al*[26]. Levels of TBARS were expressed in μmol MDA/L.

Analysis of ROS

ROS production in whole blood was evaluated using luminol- enhanced chemiluminescence, as measured in an automated LB96V Microlumat Plus Luminometer (EG & G Berthold, Belgium). Briefly, the signal-amplifying molecule luminol reacts with oxygen species (mainly superoxide anion) generated by neutrophils in whole blood, to produce an excited state intermediate that emits light as it returns to its ground state. ROS production was determined in the absence of a cellular stimulator, as well as in the presence of either a receptor-dependent (serum-treated zymosan, STZ) or a receptor-independent stimulus (phorbol 12-myritate 13-acetaat, PMA). Freshly obtained heparinized blood was 1:100 diluted in HBSS containing 1 mmol/L calcium. Two hundred μL of this diluted blood was added to each well of a 96-well plate. In addition, reaction mixtures contained 0.45 g/L bovine serum albumin (BSA), 0.83 mmol/L luminol and either 1 g/L STZ, 0.4 mg/L PMA or no stimulating agents. As an internal positive control for the luminescence process, samples of 1 g/L ammonium persulphate (APS) in phosphate-buffered solution (PBS) were run simultaneously. Chemiluminescence was monitored every 60 s for 1 h. EDTA blood, taken together with the heparinized blood samples, was tested for leukocyte counts and differentiation, in order to adjust the chemiluminescence produced during one hour ('area under the curve') in relative light units (RLU) per cell for neutrophil counts. All measurements were performed in quadruplicate and corrected for background values (absence of a stimulus). Opsonized zymosan particles were prepared by incubation of STZ with pooled human serum for 30 min at 37℃, as previously described[27]. These particles were then washed twice in PBS and finally suspended at 12.5 g/L in PBS.

Statistical analysis

Data were analysed using SPSS version 12.0. Differences in the baseline characteristics of patients and controls were estimated with Fisher's exact test and Student's *t*-test. The Mann-Witney U-test was used to estimate differences in biochemical parameters between the patient and control population in a non-parametrical manner. Differences were considered significant if $P < 0.05$. Finally, we examined the correlation between the non-enzymatic antioxidant capacity with the amount of oxidative protein and lipid damage in CP patients, using Spearman rank correlation test.

RESULTS

The characteristics of patients with CP and healthy controls are denoted in Table 1. The mean age of the CP patients was 51 years (range 25 to 74 years) and was not significantly different from that of the healthy controls (45 years; range 27 to 68 years). There was no significant difference in the distribution of gender between CP patients and healthy control subjects. Smoking habits between CP patients and healthy controls were not different; 66% of the patients and 63% of the control subjects smoked or stopped smoking within the last 5 years.

The oxidative stress was measured in CP patients and healthy controls. The plasma concentrations of cysteine (Cys), homocysteine (Hcys), cysteinylglycine (CGS) and glutathione (GSH), the plasma antioxidant capacity (FRAP) as well as the plasma levels of protein carbonyls and TBARS and chemoluminescense in whole blood are depicted in Table 2.

The plasma concentrations of antioxidative thiols including cysteine, cysteinylglycine and glutathione were significantly lower in the CP patients than in the controls ($P = 0.021$, $P = 0.003$ and $P = 0.048$, respectively). The plasma levels of homocysteine were similar in both groups. The antioxidant capacity as measured by the FRAP assay was also significantly lower in the CP patients than in the healthy control subjects ($P < 0.001$). The levels of both carbonyls and TBARS were significantly higher in the CP patients than in the healthy controls ($P < 0.001$). The chemiluminescence of diluted whole blood of CP patients and controls was not different, although there was a strong trend towards an increased ROS production after stimulation with PMA ($P = 0.058$). As expected, spontaneous generation of ROS in the absence of a stimulus was less than 10% of the amount of ROS measured in response to the stimuli of PMA and STZ. The leukocyte counts and differentiation within the ranges were considered normal at our hospital. The values obtained with either assay were not different in patients with ACP and HCP.

In addition, the correlation between the non-enzymatic antioxidant capacity with the amount of oxidative protein and lipid damage in CP patients was examined and a negative correlation was found between the non-enzymatic antioxidant capacity and the amount of oxidative protein damage ($r_s = -0.44$, $P = 0.013$), as well as between the non-enzymatic antioxidant capacity and the amount of lipid damage ($r_s = -0.39$, $P = 0.004$). Finally, we found a positive correlation between oxidative protein and lipid damage ($r_s = 0.67$, $P = 0.001$).

Table 1 Main characteristics of patients with chronic pancreatitis and healthy controls

Characteristic	CP patients	Controls
n	35	35
Gender		
Male/Female	17/18	18/17
Mean age (yr) (range)	51(25-74)	45 (27- 68)
Smoking		
Yes/No	23/12	22/13

Table 2 Thiol plasma concentrations, FRAP antioxidant capacity, protein carbonyl plasma levels, TBARS plasma levels and ROS production in whole blood of patients with CP and controls mean (range)

Measure for oxidative stress	CP patients	Controls
Cys[1] (μmol/L)	225 (124-314)[a]	249 (212-328)
Hcys[2] (μmol/L)	13.6 (5.0-38.2)	12.7 (0.2-27.8)
CGS[3] (μmol/L)	34.8 (23.5-124)[a]	39.3 (25.2-56.7)
GSH[4] (μmol/L)	7.5 (2.4-18.5)[a]	8.9 (3.5-16.1)
FRAP[5] (mmol Fe^{2+}/L)	0.75 (0.31-1.73)[a]	0.99 (0.69-1.57)
Carbonyls (nmol/mg protein)	0.32 (0.02-1.47)[a]	0.04 (0.01-0.07)
TBARS[6] (μmol/L)	4.98 (0.23-27.79)[a]	0.35 (0.04-0.68)
ROS[7] production (RLU/10^4 cells)		
PMA[8]-stimulated	40 (1-83)	33 (1-87)
STZ[9]-stimulated	124 (11-266)	111 (9-2130)

[1]Cysteine; [2]Homocysteine; [3]Cysteinylglycine; [4]Glutathione; [5]Ferric reducing ability of plasma; [6]Thiobarbituric acid-reactive substances; [7]Reactive oxygen species; [8]Phorbol 12-myritate 13-acetate; [9]Serum treated zymosan. [a]$P < 0.05$ *vs* control.

DISCUSSION

Alcohol abuse is regarded as a major risk factor for the development of CP. However, the exact mechanism behind the effect of alcohol remains unknown. Some evidence obtained by animal studies, suggests that metabolism of ethanol catalysed by cytochrome P4502E1 (CYP2E1) may contribute to oxidative stress in the pancreas during chronic alcohol consumption[19]. Trauma, exposure to radiation, cigarette smoke, medication or other toxins generating free radicals also may increase the amount of oxidants. In the present study, we assessed the antioxidant capacity and levels of oxidative damage in CP patients as compared with healthy controls by means of several analytical techniques that are known to measure various components that together constitute oxidative stress. The major observations were that plasma concentrations of some thiols, having antioxidant properties, were significantly lower in CP patients. Likewise the non-enzymatic antioxidant capacity as measured by the FRAP assay, was significantly lower in CP patients than in healthy control subjects. This inferior antioxidant capacity in CP patients parallels significantly increased amounts of oxidative protein and lipid damage, whereas the generation of ROS in whole blood did not show a statistically significant difference between CP patients and healthy control subjects, with a similar age and gender distribution and smoking habits. Our results clearly indicate that oxidative stress is present in patients with CP and that this eventually might contribute to the initiation and maintenance of inflammation in CP patients, as has been previously suggested[17,19,28-31]. Thiols such as cysteine and glutathione play an essential role as antioxidants, and are involved in protein synthesis, redox sensitive transduction signalling, cell growth and proliferation, xenobiotic metabolism and immune regulation[32]. Glutathione is conjugated to many xenobiotics and essential for the optimal functioning of numerous enzymes and hence crucial for cell viability. Decreased levels of glutathione in plasma have been reported, but paradoxically also increased glutathione levels may be found as a result of outshoot after enhanced synthesis due to oxidative stress or conjugation of toxic compounds, as has been shown in different disorders[33-35]. We found significantly lower plasma concentrations of cysteine, cysteinylglycine and glutathione in CP patients as compared to healthy controls, whereas the control values measured here can be considered normal for the Dutch population[36]. We found no elevated concentrations of homocysteine in CP patients, however homocysteine does not always act as an antioxidant, moreover elevated plasma concentrations of homocysteine are positively associated with an increased risk of cerebral, coronary or peripheral vascular diseases[36]. In parallel with the lower plasma concentrations of the antioxidative thiols, the FRAP assay also demonstrated a significantly lower antioxidant capacity in CP patients. Since the FRAP assay does not measure sulfhydryl (SH)-containing antioxidants such as the thiols glutathione and cysteine, this indicates that other non-thiol related antioxidants are decreased in CP patients also. In patients with acute pancreatitis very low ascorbate concentrations in plasma have been described before [37,38].

Protein carbonyl derivates are generated by direct oxidative attack of proteins or by indirect lipid peroxidation products and therefore represent a good biomarker for general oxidative stress[39,40]. The lower FRAP levels in CP patients are accompanied with high protein carbonyl concentrations, indicating that increased oxidative damage occurs as a result of the lower protective capacity as measured by FRAP. Former studies have demonstrated elevated plasma protein carbonyls in experimental animal models[41,42] and humans with acute pancreatitis[43]. Unless properly scavenged, ROS may lead to lipid peroxidation, which represents an important manifestation of oxidative stress. Lipid peroxidation is initiated when free radicals interact with polyunsaturated fatty acids. For instance, in cell membranes this may result in a chain reaction forming lipid hydroperoxides. Analysis of TBARS in plasma is a widely used method for the estimation of lipid peroxidation. In accordance with the elevated levels of protein carbonyls and lower antioxidant capacity, we found significantly increased plasma concentrations of TBARS in CP patients. The production of ROS, as measured in whole blood by chemiluminescence assay, was not significantly higher in CP patients than in healthy controls, although there was a strong trend to generate higher amounts of ROS after stimulation with PMA. We used luminol-enhanced chemiluminescence assay in 100-fold diluted whole blood to study ROS generation of peripheral blood cells in a non-disturbed system. As expected, ROS generation in the absence of a stimulus in CP patients was low, and not significantly different from the values in the healthy control group. Chemiluminescence measurements did not demonstrate a significantly increased ROS generation in CP patients, while the other analytical techniques applied here showed increased oxidative stress and damage in CP patients as compared to healthy control subjects, demonstrating that the oxidative damage in CP patients is caused by other reactive (oxygen) species rather than by leucocytes. Since most of the CP patients included in this study were alcoholics, the cause of oxidative damage might be mainly of exogenous origin. However, a contribution of oxidative stress of endogenous origin might also be possible, since we detected a strong tendency to produce ROS in CP patients after stimulation with PMA. This PMA-induced respiratory burst is receptor-independent and not absolutely dependent on priming[44], whereas priming does moderately influence the STZ-induced respiratory burst[45]. We measured the ROS production in whole blood and it is known that during isolation of neutrophils, these cells often become primed[46]. The clinical importance of oxidative stress in human pancreatic disease was first suggested by Braganza *et al*[47], and subsequently supported by data from other groups, showing increased levels of lipid hydroperoxides in pancreatic juice[14] and increased spontaneous production of ROS by neutrophils[27,48]. The assessment of oxidative stress in CP patients corroborates the hypothesis that oxidative stress leads to damage of pancreatic acinar cells, initiating auto-digestion of the entire pancreas as has been shown in the present study.

In summary, significantly higher levels of products of oxidative damage (protein carbonyls and TBARS), correlating with decreased levels of cysteine, cysteinylglycine, glutathione and non-enzymatic antioxidant capacity (FRAP) can be found in CP patients. Oxidative stress, defined as the imbalance between prooxidant and antioxidant capacity, is higher in CP patients, which may justify further studies on intervention with antioxidant therapies for this serious disease.

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