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Antioxidative enzymes and increased oxidative stress in depressive women

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Abstract

Objectives: To investigate the activities of the main antioxidative enzymes and oxidative stress in women with depressive disorder (DD).

Methods: In 35 drug-naive women with DD and 35 age matched healthy women enzymes superoxide dismutase (CuZnSOD), catalase (CAT), glutathione peroxidase (GPX1), glutathione reductase (GR) and paraoxonase (PON1), concentrations of conjugated dienes (CD), reduced glutathione (GSH) and anthropometric and clinical data were investigated.

Results: Women with DD were found to have decreased activities of GPX1 ($p<0.05$), decreased concentrations of GSH ($p<0.05$), and increased activities of GR ($p<0.05$), CuZnSOD ($p<0.001$), and concentrations of CD ($p<0.05$). Activity of GPX1 was positively correlated with concentration of GSH ($p<0.05$). Concentrations of CD were positively correlated with TG ($p<0.01$).

Conclusion: Our set of depressive women was characterized by changes indicating an increased oxidative stress, as well as by certain features of metabolic syndrome.

Keywords: Depressive disorder; Oxidative stress; Antioxidative enzymes; Conjugated dienes

Introduction

Depressive disorder (DD) belongs to diseases, incidence of which is now increasing all around the world. In the USA, it was established, that about 16% of the population fall ill with major depressive disorder during the lifetime [1]. In Finland, 5% prevalence of the depression was described [2]. In 2006, 168 new cases of affective disorders per 100,000 inhabitants were noticed in the Czech Republic, the incidence being 2 times higher in women than in men [3]. The dysfunction of serotoninergic, noradrenergic and dopaminergic neurotransmission [4,5], abnormal regulation in the hypothalamic–pituitary–adrenal axis (HPA) [6], disturbance of cellular plasticity including reduced neurogenesis [7], or chronic inflammation, connected with higher oxidative stress [8] could play a role in the pathogenesis of DD.

Large consumption of oxygen (up to 20% of the total requirement of organism), high amount of polyunsaturated fatty acids, which are prone to oxidation, high amount of iron and low activities of antioxidant enzymes contribute to higher sensitivity of brain to oxidative stress [9]. Oxidative stress is defined as the imbalance between production of reactive oxygen and nitrogen species (RONS) and their insufficient decomposition by the antioxidative system [10]. This defence system involves enzymatic antioxidants — superoxide dismutase (EC 1.15.1.1.; SOD), glutathione peroxidase (EC 1.11.1.9; GPX), glutathione reductase (EC 1.6.4.2; GR), catalase (EC 1.11.1.6; CAT) and paraoxonase (EC 3.1.8.1; PON) as well as nonenzymatic antioxidants — reduced glutathione (GSH), provitamin A, vitamin C and E, coenzyme Q10, carotenoids and trace elements like copper, zinc or selenium. Increased production of RONS has been observed in patients with neurodegenerative and psychiatric diseases such as Alzheimer's and Parkinson's

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disease or schizophrenia [11–13]. Neurodegenerative changes, which are augmented by inflammation and oxidative stress, play an important role also in the pathogenesis of the DD [14,15]. The raised level of oxidative stress is supposed to be one of the factors, standing behind higher incidence of type 2 diabetes mellitus (DM2) and cardiovascular diseases (CVD), which were observed in patients with depression [16,17]. However, only few studies have studied an oxidative stress in DD and the results have been inconsistent. The aim of this study was to determine the activities of main antioxidative enzymes, concentrations of reduced glutathione and conjugated dienes (CD) as marker of lipoperoxidation, and their relations to anthropometric and selected metabolic parameters in women with DD in comparison with healthy controls.

Methods

Subjects

Thirty five women with DD, recruited from the consecutive outpatients of the Psychiatric Department of 1st Faculty of Medicine of Charles University in Prague from May 2006 to May 2008, and 35 age-matched healthy controls were included in the study. Depressive disorder was diagnosed according to Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition, DSM-IV (American Psychiatric Association, 1994) [18]. All patients were evaluated using Hamilton Depression Rating Scale (HAM-D).

Exclusion criteria of the study were: history of cardiovascular and cerebrovascular disease, DM, hepatic and/or renal diseases, hypothyroidism, malignancies, macroalbuminuria (proteinuria higher than 300 mg/day), excessive alcohol consumption (>30 g/day), treatment with hypolipidemic medications, supplementation by vitamins, polyunsaturated fatty acids and/or antioxidants. Patients have completed the 7 days dietary questionnaire. Food intake was processed by the software NutriMaster. We have evaluated intake of total energy, protein, fat, carbohydrate, vitamins and minerals. The study protocol was approved by the Joint Ethical Committee of the General Teaching Hospital and the 1st Faculty of Medicine of Charles University in Prague. Written informed consent was obtained from all participants.

Blood samples

Blood samples were obtained after overnight fasting. Activities of CAT, GR, GPX1 and CuZnSOD were measured in haemolysed erythrocytes. The blood samples were collected into the tubes with K_2 EDTA, erythrocytes were washed three times with a NaCl isotonic solution (9 g/L). Serum was used for the determination of all other parameters. The samples were stored at −80 °C until assay. The haemotological parameters were measured by routine laboratory techniques using an autoanalyzer (Coulter LH750 — haematological analyzer, Beckman Coulter).

Measurement of enzyme activities

Glutathione peroxidase 1

The activity was measured by the modified method of Paglia and Valentine using tert-butyl hydroperoxide as a substrate [19]. Briefly, 580 μL of 172.4 mM tris–HCl buffer containing 0.86 mM EDTA, pH=8.0; 100 μL of 20 mM GSH, 100 μL of 10 U/mL GR, 100 μL of 2 mM NADPH and 100 μL of diluted sample were pipetted into the cuvettes. The reaction was started after 10 min of incubation at 37 °C by the addition of 20 μL of 9.99 mM tert-butyl hydroperoxide. The rate of NADPH degradation was monitored spectrophotometrically at 340 nm. Blank was run for each sample. Activity of GPX1 was calculated using the molar extinction coefficient of NADPH 6220 M^{-1} cm^{-1} and expressed as U/g haemoglobin. One unit of GPX1 (U) is defined as 1 μmol of NADPH oxidized to NADP per minute.

Glutathione reductase

The activity was measured according to the method of Goldberg et al. [20]. Briefly, 700 μL of 0.127 M potassium phosphate buffer containing 0.633 mM $Na_2EDTA{\cdot}2H_2O$, pH=7.2 was added to cuvettes followed by 100 μL of 22 mM oxidized glutathione (GSSG) and 100 μL of diluted sample. The reaction was started after 10 min of incubation at 37 °C by addition of 100 μL of 1.7 mM NADPH. The rate of NADPH degradation was monitored spectrophotometrically at 340 nm. Blank was run for each sample. Activity of GR was calculated using the molar extinction coefficient of NADPH 6220 M^{-1} cm^{-1} and expressed as U/g haemoglobin. One unit of GR (U) is defined as the amount of enzyme catalyzing the reduction of 1 μmol of GSSG per minute.

Catalase

The activity was determined by the modified method of Aebi [21]. The reaction mixture in cuvettes contained 876 μL of 50 mM potassium phosphate buffer, pH=7.2 and 25 μL of diluted sample. The reaction was started after 10 min of incubation at 30 °C by addition of 99 μL of 10 mM H_2O_2. The rate of H_2O_2 degradation was monitored spectrophotometrically at 240 nm. Blank was run for each sample. Catalase activity was calculated using the molar extinction coefficient of H_2O_2 43.6 M^{-1} cm^{-1} and expressed as kU/g haemoglobin. One unit of CAT (U) is defined as 1 μmol of H_2O_2 decomposition per minute.

CuZn-Superoxide dismutase

The activity was determined according to the modified method of Štípek et al. [22]. The reaction mixture in cuvettes contained 700 μL of 50 mM potassium phosphate buffer, pH=7.2; 50 μL of xanthine oxidase; 100μL of NBT and 50 μL of diluted sample. The reaction was started after 10 min of incubation at 25 °C by addition of 100 μL of 1 mM xanthine. The rate of NBT-formazan generation was monitored spectrophotometrically at 540 nm. Blank was run for each sample. Superoxide dismutase activity was calculated by means of calibration curve and expressed as U/g haemoglobin. One unit

of SOD (U) is defined as the amount of enzyme needed to exhibit 50% dismutation of the superoxide radical. Superoxide dismutase standard (Cat. No. S9636-1kU) was purchased from Sigma Aldrich (St. Louis, MO USA).

Paraoxonase 1

The arylesterase activity of PON1 was measured according to the method of Eckerson et al. using phenylacetate as a substrate [23]. Briefly, 900 μL of 20 mM Tris–HCl buffer containing 1 mM $CaCl_2$, pH=8.0 was added to cuvettes followed by 50 μL of diluted serum sample. The reaction was started by addition of 50μL of 100 mM phenylacetate. The rate of phenol generation was monitored spectrophotometrically at 270 nm. Blank was run for each sample. Arylesterase activity of PON1 was calculated using the molar extinction coefficient of the produced phenol, 1310 M^{-1} cm^{-1} and expressed as U/mL serum. One unit of PON1 (U) is defined as 1 μmol of phenylacetate degradation to phenol per minute.

Measurement of concentration of reduced glutathione

Reduced glutathione was measured by the modified spectrophotometric method according to Griffith [24]. Suspension of washed erythrocytes (500 μL) was mixed with 100 μL of diluted acetic acid in water (6%, v/v), haemolysate was vortexed and 400 μL of 5-sulphosalicylic acid 10% (w/v) was immediately added. After centrifugation at 10 000 g for 2 min, supernatant solution was collected for analysis. This method is based on the determination of relatively stable product of reduction of 5.5′ dithiobis-2-nitrobenzoic acid (DTNB) reduction by sulfhydryl compounds to yellow product. Briefly, 50 μL of 0.125 M potassium phosphate buffer containing 6.3 mmol/L $Na_2EDTA{\cdot}2H_2O$, pH=7.5 was added to micro-cuvettes followed by 37.5 μL of the sample and 12.5 μL of 6 mmol/L DTNB. The absorbance of the yellow product (reduced chromogen) was measured at 412 nm. Concentration was calculated by means of calibration curve and was expressed as μg/g haemoglobin.

Measurement of concentration of conjugated dienes

Serum low density lipoproteins were isolated by precipitation method of Ahotupa et al. [25]. Concentrations of CD in precipitated LDL were measured by the modified method of Wieland et al. [26]. Serum samples were stabilized with EDTA (10:1 v/v) and analyzed within 2 weeks. The precipitation buffer consisted of 0.064 M trisodium citrate adjusted to pH 5.05 with 5 M HCl, and contained 50,000 IU/L heparin. Sample (110 μL) of serum with EDTA (10:1 v/v) was added to 1 mL of the heparin-citrate buffer. After mixing, the suspension was incubated for 10 min at room temperature. The precipitated lipoproteins were then separated by centrifugation at 2800 rpm for 10 min. Supernatant was removed and the pellet was resuspended in 100 μL of NaCl isotonic solution (9 g/L); this process, individual for each sample, did not exceed 3 s to prevent LDL oxidation. Lipids were extracted by chloroform–methanol (2:1), the mixture was incubated for 10 min with intermittent mixing, 250 μL redistilled water was used for phase separation. The mixture was centrifuged at 3000 rpm for 5 min. The 800 μL of lower layer (infranatant) was dried under nitrogen, redissolved in 300 μL of cyclohexane, and analyzed spectrophotometrically at 234 nm. The concentration of CD was calculated using the molar extinction coefficient 2.95×10^4 M^{-1} cm^{-1} and expressed as mmol/L serum.

Statistical analysis

All data were expressed as median (25th–75th percentiles). Normality of distribution of data was tested with Shapiro–Wilks *W* test. Differences between compared groups were tested with one-way ANOVA. Mann–Whitney *U* test was used for non-parametric comparison of groups. The Spearman correlation coefficients were used for correlation analysis. All statistical analyses were performed using version 8.0 of StatSoft software Statistica (2007, CZ).

Results

The basic characteristics and essential biochemical parameters observed in the studied groups are shown in Table 1, parameters of oxidative stress are presented in Table 2. Patients with DD had significantly raised values of waist circumference, TG, glucose and index of insulin resistance (HOMA-IR) in comparison with control group. The mean systolic and diastolic blood pressure (SBP and DBP) did not differ significantly. There were also no significant differences in concentrations of HDL-C, LDL-C, CRP, apo A-I and apo B, as well as those of calcium, zinc and copper. We have found no statistical

Table 1
Subject characteristics.

	Depression	Controls
N (female)	35	35
Age (years)	64.5 (50.0–75.1)	65.0 (53.2–77.0)
BMI (kg/m^2)	26.1 (24.1–29.4)$^{+}$	24.7 (22.7–25.9)
Waist (cm)	87.0 (77.0–96.0)$^{+}$	80.5 (77.0–85.5)
Systolic BP (mm Hg)	120.0 (120.0–135.0)	127.5 (120.0–130.0)
Diastolic BP (mm Hg)	80.0 (70.0–80.0)	80.0 (75.0–80.0)
TC (mmol/L)	5.42 (4.55–6.57)	5.92 (4.99–6.48)
TG (mmol/L)	1.32 (0.95–1.8)$^{+}$	1.06 (0.87–1.46)
HDL-C (mmol/L)	1.42 (1.24–1.71)	1.68 (1.49–1.94)
LDL-C (mmol/L)	3.14 (2.54–4.05)	3.56 (2.73–4.27)
Apo A-I (g/L)	1.41 (1.26–1.56)	1.45 (1.33–1.61)
Apo B (g/L)	1.02 (0.86–1.34)	1.04 (0.90–1.25)
Glucose (mmol/L)	5.0 (4.6–5.9)$^{++}$	4.70 (4.6–4.9)
HOMA-IR	2.32 (1.19–4.35)$^{++}$	1.65 (1.19–1.95)
CRP (mmol/L)	3.3 (2.0–7.9)	2.2 (2.0–5.5)
Ca (mmol/L)	2.35 (2.29–2.47)	2.35 (2.28–2.42)
Cu (mmol/L)	21.3 (17.8–23.5)	19.5 (18.3–21.8)
Zn (mmol/L)	15.2 (13.8–16.7)	14.8 (13.7–16.8)
Cu/Zn	1.33 (1.15–1.64)	1.26 (1.11–1.53)

Abbreviations used: BMI: body mass index, BP: blood pressure, TC: total cholesterol, TG: triglycerides, HDL-C: high density lipoprotein, LDL-C: low density lipoprotein, CRP: C-reactive protein; Data were expressed as median (25th–75th percentiles). Statistical analysis: $^{+}$ $p<0.05$; $^{++}$ $p<0.01$.

Table 2
Parameters of oxidative stress.

	Depression	Controls
GPX1 (U/g Hb)	53.7 (42.7–65.7)$^{+}$	64.0 (52.9–70.7)
GR (U/g Hb)	7.95 (6.84–8.62)$^{+}$	7.00 (6.19–8.30)
GSH (μg/g Hb)	568.75 (387.93–3484.01)$^{+}$	2374.93 (515.16–5668.35)
CuZnSOD (U/g Hb)	2356.2 (2080.75–2586.5)$^{+++}$	1930.5 (1309.2–2249.7)
CAT (kU/g Hb)	174.0 (155.2–217.9)	189.0 (166.6–215.4)
PON1 (kU/L)	161.3 (140.8–196.2)	175.9 (146.2–207.3)
CD (mmol/L)	55.7 (47.7–80.8)$^{+}$	53.3 (43.8–62.1)

Abbreviations used: GPX1: glutathione peroxidase1, GR: glutathione reductase, GSH: reduced glutathione, CuZnSOD: CuZn-superoxide dismutase, CAT: catalase, PON1: paraoxonase1, CD: conjugated dienes, Hb: haemoglobin; Data were expressed as median (25th–75th percentiles). Statistical analysis: $^{+}$ $p<0.05$; $^{++}$ $p<0.01$; $^{+++}$ $p<0.001$.

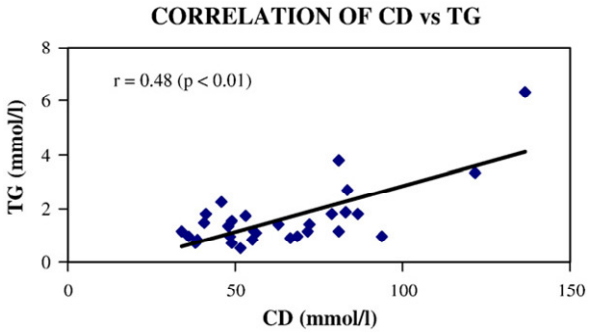

Fig. 2. Correlation of concentrations of conjugated dienes and concentration s of triglycerides. Abbreviation used: CD: conjugated dienes, TG: triglycerides; Statistical analysis: Spearman's rank correlation coefficient.

significant differences in nutritional habits between women with DD and control group (data not shown).

Erythrocyte activities of GR and CuZnSOD and concentrations of CD in precipitated LDL were increased in depressive women; however, activities of GPX1 were decreased. Reduced glutathione was significantly lower in depressive women than in the control group. Activities of CAT and PON1 were not altered in patients with DD.

In women with DD, activities of PON1 were positively correlated with concentrations of HDL-C ($r=0.457$, $p<0.01$), apo A-I ($r=0.379$, $p<0.05$) and calcium ($r=0.371$, $p<0.05$), but in control group we have found only positive correlation with apoA-I ($r=0{,}492$; $p<0.05$). Furthermore, activities of CuZnSOD were positively correlated with concentrations of zinc in DD (Fig. 1) and also in control group ($r=0.393$, $p<0.05$; $r=0.477$, $p<0.05$, respectively). There was no significant correlation of CuZnSOD with copper in both groups.

Activities of GPX1 were positively correlated with concentrations of GSH ($r=0.284$, $p<0.05$) in DD, but not in control group. There were no correlations observed between activities of individual antioxidant enzymes.

Concentrations of serum TG were positively correlated with concentrations of CD in precipitated LDL in the DD group (Fig. 2) and in the control one ($r=0.480$, $p<0.01$; $r=0.391$; $p<0.05$, respectively). We did not find any correlation between HAM-D score and any of observed parameters.

Discussion

The most important findings of this study were significantly increased concentrations of CD in precipitated LDL, indicating increased lipid peroxidation, accompanied by the decrease in activity of GPX1 and increase in activities of both CuZnSOD and GR in women with DD. The presence of IR and certain features of metabolic syndrome (MetS) in our set of women with DD were further important findings.

Oxidative stress was accepted to participate in the pathophysiology of neurodegenerative conditions such as Alzheimer's disease [27,28], HIV-associated dementia [29], Parkinson's disease [30]. Neurodegenerative changes of brain have been demonstrated in patients with DD, in which also markers of oxidative stress were previously described, such as altered activities of antioxidative enzymes and increased lipid peroxidation products [31–34].

Glutathione peroxidase is ubiquitous enzyme responsible for the degradation of lipid hydroperoxides and of H_2O_2 to hydroxyderivates and water. Decreased activities of GPX1 in erythrocytes were found in our depressive patients, similarly as in the study of Ozcan et al. [35], who described lower activities of GPX1 in patients with affective disorders in comparison with healthy controls. However, Bilici et al. [33] found increased

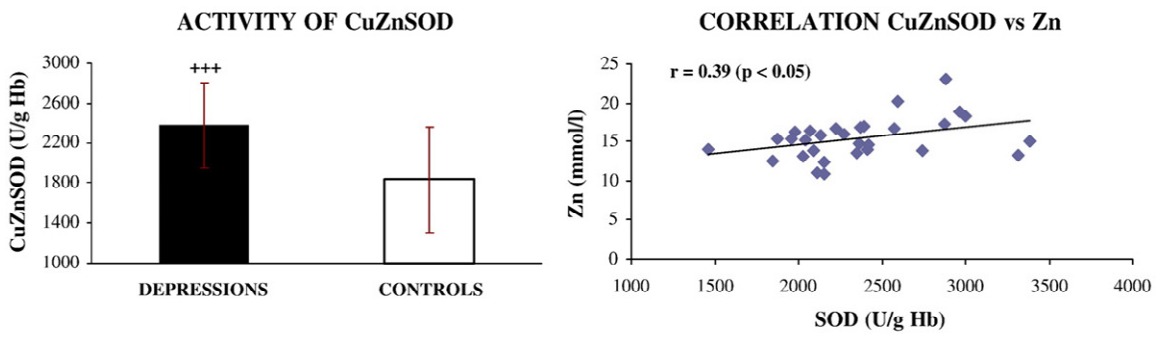

Fig. 1. Activity of CuZnSOD and its correlation with zinc in patients with depression. Abbreviation used: SOD: superoxide dismutase, Hb: haemoglobin; Statistical analysis: Spearman's rank correlation coefficient; $^{+++}p<0.001$ (Mann–Whitney U test).

activities of GPX1 in erythrocytes of patients with major depression, whereas Andrezza et al. [36] did not find any significant changes in patients with bipolar disorder. Activity of GPX1 could be decreased due to lower concentration of its substrate — GSH that we have found in women with DD. Reduced glutathione is one of the most important intracellular antioxidants in the cell and is enzymatically oxidized to GSSG in a number of biochemical pathways. In the present study we have observed significantly decreased concentrations of GSH in depressive women compared to control. To our knowledge, there has been no clinical study regarding data on GSH concentrations in patients with depressive disorders. The observed decrease of GSH were also described in patients with autism [37,38], schizophrenia [39] and Down syndrome [40] have reduced levels of total GSH. Reduced glutathione reacts also nonenzymatically with RONS leading to the glutathiol radical that reacts with further GSH to GSSG radical anion formation. Oxidized glutathione radical anion is involved in the conversion of oxygen to superoxide. The conversion of GSSG back to GSH is catalyzed by GR. In our study, we have found increased activities of GR in erythrocytes. Bilici et al. [33] described raised activities of GR in plasma, but no significant differences in erythrocytes in patients with major depression.

Studies have described a variety of intracellular sources of superoxide that include nitric oxide synthase, xanthine oxidase, cyclooxygenase and NADPH oxidase [41–44]. The most important source of superoxide in vascular cells is NADPH oxidase [45]. Decomposition of superoxide into H_2O_2 is catalyzed by SOD. We have found increased CuZnSOD activities in erythrocytes of depressive patients compared with healthy persons, similarly to Sarandol et al. [32]. They suggested that CuZnSOD activity is increased in response to increased ROS production. Bilici et al. [33] have also observed increased CuZnSOD activity in erythrocytes of depressive patients. Inconsistent results were published for serum CuZn-SOD activities. Herken et al. [34] have found decreased, whereas Khanzode et al. [46] elevated CuZnSOD activities in patients with major depression. We have found positive correlation between CuZnSOD activity and concentration of zinc, which is responsible for the stability of CuZnSOD structure as its cofactor [47].

Activities of CAT in erythrocytes were not altered in our set of women with DD, in accordance with Bilici et al. [33]. However, Szuster-Ciesielska et al. [48] found raised activities of CAT in serum of patients with major depression and Ozcan et al. [35] described decreased CAT activities in erythrocytes of patients with affective disorders. Induction of CAT or SOD does not necessarily lead to the induction of the other one [49]. The increased activity of SOD leads to increased amounts of hydrogen peroxide that is then degraded by GPX in its low concentrations and by CAT in its high concentrations [50]. It could be supposed that the concentration of hydrogen peroxide wasn't enough high to increase activity of CAT, and that the task of H_2O_2 degradation remains on GPX. But GPX activity is dependent on GSH, as its substrate. This antioxidant is rapidly consumed in oxidative stress. It is problematic whether GPX could function appropriately in low GSH concentrations.

The activities of PON1 were not altered in women with DD, as well as levels of apo A-I, HDL-C and calcium. Apolipoprotein A-I plays a key role for PON1 because of the connection of PON1 to HDL is through apo A-I. We have found positive correlation between PON1 activity and both apo A-I and HDL-C concentrations. Paraoxonase is calcium dependent enzyme; calcium is located in the active site of enzyme. It is consistent with our finding of a positive correlation between the PON1 activity and calcium concentrations in patients with DD.

Increased concentrations of CD in LDL indicate an elevation of minimally modified (oxidized) LDL in vivo. Raised concentrations of CD in LDL were found in insulin-resistant states such as MetS and DM2 [51–53], however, different results were published by Gavella et al. [54].

Observed hypertriglyceridemia (HTG) and higher glycaemia, the accumulation of visceral fat and IR could play a role in changes of oxidant/antioxidant balance in our set of depressive women. In nondiabetic human subjects, both BMI and waist circumference were closely correlated with the markers of systemic oxidative stress (plasma TBARS, urinary 8-epi-PGF2α) [55]. Hypertriglyceridemia was associated with an increased oxidative stress in experimental rats [56] and also in humans [57]. Inconsistent results were obtained with regard to the activities of antioxidant enzymes in insulin-resistant states. In one study, increased activity of CAT, decreased of GPX and non-changed of SOD was found in type 2 diabetic patients [58] while in another study [59] the activities of GPX, SOD and CAT in red blood cells were significantly decreased in diabetic subjects when compared with healthy controls. Some authors suggest decreased GPX1 activity as cardiovascular risk factor that was in the prospective study associated with increased extent of atherosclerotic lesions [60].

In summary, we have found significant increase in CuZnSOD and GR activity and simultaneous decrease of GPX1 activity as well as elevated concentrations of CD in precipitated LDL, which positively correlated with TG in our set of depressive women. These findings are in accordance with hypothesis that oxidative stress may play an important role in the pathogenesis of depression. Metabolic changes and markers of IR in women with DD suggest the relationships between MetS and DD. Increased oxidative stress could be a possible connection between depression, IR and increased incidence of both DM2 and CVD.

Acknowledgments

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Original Article

Altered Activities of Antioxidant Enzymes in Patients with Metabolic Syndrome

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Key Words
Metabolic syndrome · Antioxidant enzymes · Reduced glutathione · Conjugated dienes

Abstract
Objective: In the pathogenesis of the metabolic syndrome (MetS), an increase of oxidative stress could play an important role which is closely linked with insulin resistance, endothelial dysfunction, and chronic inflammation. The aim of our study was to assess several parameters of the antioxidant status in MetS. ***Methods:*** 40 subjects with MetS and 40 age- and sex-matched volunteers without MetS were examined for activities of superoxide dismutase (CuZnSOD), catalase (CAT), glutathione peroxidase 1 (GPX1), glutathione reductase (GR), paraoxonase1 (PON1), concentrations of reduced glutathione (GSH), and conjugated dienes in low-density lipoprotein (CD-LDL). ***Results:*** Subjects with MetS had higher activities of CuZnSOD ($p < 0.05$) and GR ($p < 0.001$), higher concentrations of CD-LDL ($p < 0.001$), lower activities of CAT ($p < 0.05$) and PON1 ($p < 0.05$), and lower concentrations of GSH ($p < 0.05$), as compared with controls. Activity of GPX1 was not significantly changed. ***Conclusions:*** Our results implicated an increased oxidative stress in MetS and a decreased antioxidative defense that correlated with some laboratory (triglycerides, high-density lipoprotein cholesterol (HDL-C)) and clinical (waist circumference, blood pressure) components of MetS.

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Introduction

Currently, the prevailing notion of the metabolic syndrome (MetS) is that it is characterized by a cluster of risk factors for atherosclerosis and type 2 diabetes mellitus and can be regarded as a physiological and clinical entity [1]. The main components of MetS are accumulation of intra-abdominal fat, impaired metabolism of glucose, atherogenic dyslipidemia (low high-density lipoprotein cholesterol (HDL-C), hypertriglyceridemia), and arterial hypertension. In pathogenesis, several mechanisms were shown to take part, namely insulin resistance, chronic low-grade inflammation, endothelial dysfunction, and oxidative stress; their interactions have not been fully elucidated at present. Elevated levels of oxidative stress in subjects with MetS were demonstrated in many experimental and clinical studies [2].

Oxidative stress is defined as an imbalance between the production of reactive oxygen and nitrogen species (RONS) and their insufficient decomposition by the antioxidant system which results in macromolecular damage and disruption of redox signaling and control [3]. Free radicals and non-radical oxidants belong to RONS. Free radicals could induce DNA mutations, structural disorders in proteins, and peroxidative damage of cell membrane and plasma lipids [4]. RONS play an important role in the pathogenesis of many cardiovascular and neurodegenerative diseases as well as in type 2 diabetes mellitus and its complications [5].

The defense mechanisms of the human body against oxidative stress are complex and involve cellular and extracellular antioxidant systems which are regulated at multiple levels [6]. Various enzymes, e.g. superoxide dismutase (CuZnSOD), glutathione peroxidase 1 (GPX1), catalase (CAT), paraoxonase 1 (PON1), glutathione reductase (GR), as well as nonenzymatic antioxidant compounds (e.g. metal chelators, low-molecular-weight antioxidants) take part in the antioxidant defense.

In the first step of the defense mechanism against superoxide anions (O_2^-), the enzyme CuZnSOD catalyzes their dismutation into oxygen and H_2O_2. In the second step, CAT and GPX1 independently convert H_2O_2 to water. Any increase in the CuZnSOD catalytic activity produces an excess of H_2O_2 that must be efficiently neutralized by either CAT or GPX1; otherwise, H_2O_2 reacts with O_2^- producing in a two-step reaction (the Haber-Weiss reaction) hydroxyl radicals OH which are even more dangerous [5]. Cytosolic GPX1 detoxifies H_2O_2 in the presence of reduced glutathione (GSH), which is oxidized to oxidized glutathione (GSSG) and subsequently recycled by GR. GPX1 with the aid of GSH protects lipids against peroxidation. The pool of GSH has to be replenished by de novo synthesis that is catalyzed by the enzyme glutamate-cystein ligase. The PON1 enzyme as HDL-associated enzyme is implicated in the anti-inflammatory and antioxidant activities of HDL and impedes oxidative modification of low-density lipoprotein (LDL) thus protecting cell membranes from the damage caused by products of lipoperoxidation [7].

This study is focused on the state of the antioxidant defense system in patients with MetS. We intend to investigate the wide variety of known antioxidants in association with MetS. The activities of several antioxidant enzymes as well as the concentration of GSH were determined in the erythrocytes. It has been noted that these cells maintain fairly constant concentrations of enzymes throughout the life span which had been synthesized during the maturation of erythroid precursors [8]. Furthermore, levels of albumin, bilirubin, and calculated total peroxyl radical trapping (cTRAP) were assessed in serum. As a global marker of systemic oxidative stress, conjugated dienes in precipitated low-density lipoproteins (CD-LDL) were determined.

Obes Facts 2013;6:39–47
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Participants and Methods

Participants

40 Caucasian subjects with MetS (20 male / 20 female) were recruited from outpatients who had been subsequently examined (from January 2008 until August 2010) at the Lipid Clinic of the 4th Department of Medicine, First Faculty of Medicine, Charles University in Prague. This study group was compared with a control group constituted from 40 volunteers without MetS matched for sex and age (20 male / 20 female), all Caucasian.

MetS was diagnosed according to the International Diabetes Federation criteria [9]. To be included, patients had to have central obesity (waist circumference ≥ 94 cm for men and ≥ 80 cm for women) and fulfill any two of the following four criteria: i) raised TG level (≥1.7 mmol/l), ii) reduced HDL-C (<1.03 mmol/l in males and <1.29 mmol/l in females) or specific treatment for these abnormalities, iii) raised blood pressure (BP) with systolic BP ≥ 130 or diastolic BP ≥ 85 mm Hg or treatment of previously diagnosed hypertension, and iv) raised fasting plasma glucose (≥5.6 mmol/l) or previously diagnosed type 2 diabetes mellitus. All samples were marked with unique anonymized identification numbers, and the data was merged only after the assays had been completed.

In the MetS group, 21 patients (52.5%) had three, 13 patients (32.5%) four, and 6 patients (15.0%) had all five of the above mentioned components of MetS. In the control group, only three subjects (7.5%) met two components of MetS, 15 (37.5%) controls met one, and the 22 (55.0%) volunteers showed no components of MetS. In the MetS group, 35 patients suffered from hypertension, and of these patients, 21 were under antihypertensive treatment. Among them, 12 were treated with an angiotensin converting enzyme (ACE) inhibitor or angiotensin receptor type 1 blockers, and the 9 remaining subjects were on a combination of ACE inhibitor with calcium channel blockers.

Exclusion criteria for both groups were the following: current antioxidant therapy, excessive alcohol consumption (>30 g/day), hormonal replacement therapy, supplementation with polyunsaturated fatty acids, manifestation of cardiovascular and/or cerebrovascular diseases, type 1 diabetes mellitus, liver (with exception of nonalcoholic fatty liver disease) and kidney diseases (creatinine >130 μmol/l), microalbuminuria (urinary albumine 30–300 mg/day), hypothyroidism as well as recent infections and malignancies.

Informed consent was obtained from all participants. The study protocol was approved by the Ethical Committee of the First Faculty of Medicine, Charles University in Prague.

Blood Samples

Blood samples were collected after a 12-hour overnight fast. Activities of antioxidant enzymes (with exception of PON1) and concentrations of GSH were measured in hemolysed erythrocytes which had been separated from the EDTA plasma and washed three times with saline. Serum was used for all other parameters. Samples were stored at –80 °C until the assay.

Methods

Activities of antioxidant enzymes were measured spectrophotometrically using kinetic methods previously described [10]. Briefly, the activity of GPX1 was measured using tert-butyl hydroperoxide as a substrate, and the rate of NADPH degradation was monitored. The molar extinction coefficient of NADPH (6,220 mol/l/cm) was used for calculation of activity which was then expressed as U/g hemoglobin. The activity of GR was measured by monitoring the rate of NADPH degradation. Activity was calculated using the molar extinction coefficient of NADPH and expressed as U/g hemoglobin. The CAT activity was calculated using the molar extinction coefficient of H_2O_2 (43.6 mol/l/cm), whose degradation rate was monitored at 240 nm. Activity is expressed as kU/g hemoglobin. The method of CuZnSOD activity assessment is based on the monitoring of the rate of NBT-formazan generation. Superoxide dismutase activity was calculated by means of a calibrating curve; superoxide dismutase standard (Cat. No. S9636-1kU) was purchased from Sigma Aldrich (St. Louis, MO, USA). Activity was expressed as U/g hemoglobin. The arylesterase activity of PON1 was measured using phenylacetate as a substrate. Arylesterase activity of PON1 was calculated using the molar extinction coefficient of the produced phenol (1,310 mol/l/cm) and expressed as U/ml serum.

GSH was assessed by the modified spectrophotometric method according to Griffith [11]; this method is based on the determination of the relatively stable product of the reduction of 5,5′ dithiobis-2-nitrobenzoic acid (DTNB). The concentration of CD-LDL was assessed by the modified method of Wieland and Seidel at 234 nm [12]; both methods have been fully described in the previously mentioned paper [10].

Obes Facts 2013;6:39–47
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All routine clinical tests were performed at the Institute for Clinical Biochemistry and Laboratory Diagnostics of General University Hospital in Prague: C-reactive protein (CRP) was determined by an immunoturbidimetric method using a K-ASSAY CRP kit (Kamiya Biomedical Company, Seattle, WA, USA; cv = max. 7.6%) on a Hitachi Modular analyzer (Tokyo, Japan). Copper and zinc were measured using atomic absorption spectrometry, uric acid by an enzymatic colorimetric method with the uricase-peroxidase system, and bilirubin by the 2,5-dichlorophenyldiazonium method with a Hitachi Modular analyzer. Plasma albumin was assessed by a colorimetric method using bromocresol green. Plasma concentrations of total cholesterol (TC) and triglycerides (TG) were measured by enzymatic-colorimetric methods (Boehringer, Mannheim, Germany). HDL-C was determined in the supernatant after precipitation of lipoproteins B by PTA/Mg^{2+}, using the kit from the same manufacturer; LDL-C was calculated according to Friedewald's formula. Concentrations of apolipoproteins apo B and apo A1 were measured by the Laurell rocket electroimmunoassay using standard and specific antibodies (Behringwerke, Marburg, Germany). The concentrations of insulin and C-peptide were determined with an electrochemiluminescence immunoassay (Roche, Basel, Switzerland). The homeostasis model assessment of insulin resistance (HOMA-IR) index was calculated as HOMA-IR = (fasting serum glucose (mmol/l) × fasting serum insulin (μU/ml)) / 22.5 [13]. The TRAP was calculated according to the formula: (0.63 (albumin) + 1.02 (uric acid) + 1.50 (bilirubin)) [14].

Statistical Analysis

Data was expressed as mean and standard deviation or median (25th–75th percentile) for data different from normal distribution. Normality of the distribution was tested by the Shapiro-Wilks W test. Comparisons between the groups were carried out by the independent t-test. Mann-Whitney U test was used for nonparametric comparisons and Spearman correlation coefficients for correlation analyses. All analyses were performed using version 8.0 of StatSoft Statistica software (2007, Czech version). The p value < 0.05 was considered statistically significant.

Results

Clinical and biochemical characteristics of the group of subjects with MetS and that of healthy controls are shown in table 1. The groups did not differ in age. In both groups there were no subjects with either type 1 or type 2 diabetes mellitus . The subjects included in the MetS group suffer from insulin resistance when the metabolism of glucose was impaired.

As expected, subjects with MetS had significantly higher values of body mass index and waist circumference. They also had higher values of systolic BP and diastolic BP, glucose, TC, TG, apolipoprotein B (apo B), and uric acid as well as a higher level of insulin and insulin resistance, as assessed by the homeostatic model HOMA-IR. Decreased values were observed for plasma concentrations of HDL-C and apo A1. The difference in CRP did not reach statistical significance. As expected, men have decreased levels of HDL-C and Cu and increased values of waist circumference compared to women.

Activities of antioxidant enzymes and concentrations of GSH and CD-LDL together with levels of cTRAP are presented in table 2. In the group of subjects with MetS, activities of CuZnSOD and GR as well as concentrations of CD-LDL and levels of cTRAP were significantly elevated. On the other hand, activities of CAT and PON1 as well as concentrations of GSH were found to be decreased.

Spearmen correlations (after Bonferroni adjustment) between selected variables are shown in table 3. All risk factors of MetS correlated significantly with the number of components of MetS, namely abnormal levels of glucose, waist circumference, TG, HDL-C, and SBP. Concentrations of CD-LDL significantly correlated with concentrations of TG and HDL-C.

Activities of CuZnSOD correlated positively with those of GR ($r = 0.341$, $p < 0.01$) and GPX1 ($r = 0.260$, $p < 0.05$), and with concentrations of Zn ($r = 0.363$, $p < 0.01$) as well as negatively with the ratio Cu/Zn ($r = -0.278$, $p < 0.05$). Activities of PON1 correlated positively with

Obes Facts 2013;6:39–47
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Table 1. Clinical and biochemical characteristics of subjects with the metabolic syndrome and of healthy controls[a]

	Metabolic syndrome			Controls		
	all	M	F	all	M	F
N	40	20	20	40	20	20
Age, years	58.4 (53.4–62.2)	57.0 (50.1–63.1)	58.7 (55.9–61.6)	58.5 (52.6–64.5)	57.8 (50.1–64.0)	59.5 (54.0–65.0)
Waist, cm	101.4 ± 9.1***	104.4 ± 6.4***+	98.3 ± 10.5***	82.5 ± 11.0	86.9 ± 13.2+	78.3 ± 6.6
BMI, kg/m^2	29.4 (27.4–31.7)***	29.0 (27.7–30.3)**	30.6 (25.9–32.4)***	23.9 (21.9–25.5)	25.0 (21.8–26.3)	23.8 (21.9–25.4)
Smoking, N (%)	10 (25.0)	6 (30.0)	4 (20.0)	4 (10.0)	0 (0.0)	4 (20.0)
Hypertension, N (%)	35 (87.5)	18 (90.0)	17 (85.0)	8 (20.0)	5 (25.0)	3 (15.0)
Systolic BP, mm Hg	140 (130–143)**	140 (130–140)***	140 (130–145)	130 (120–130)	128 (120–130)	130 (120–140)
Diastolic BP, mm Hg	90 (88–95)***	90 (90–95)***	90 (83–95)***	80 (80–80)	80 (80–85)	80 (80–85)
Glucose, mmol/l	5.4 (4.8–6.1)***	5.0 (4.7–5.8)	5.6 (5.1–6.3)**	4.7 (4.5–5.1)	4.9 (4.5–5.4)	4.7 (4.3–5.0)
Insulin, mU/l	11.4 (8.7–14.8)***	11.4 (8.6–15.1)*	11.3 (9.4–14.3)*	7.8 (4.6–9.5)	8.5 (4.8–10.1)	7.6 (4.6–9.1)
C-peptid, nmol/l	0.97 (0.84–1.19)***	0.99 (0.86–1.14)***	0.93 (0.81–1.28)***	0.64 (0.51–0.75)	0.59 (0.46–0.75)	0.68 (0.55–0.73)
HOMA-IR	3.0 (1.9–3.8)***	3.0 (1.8–3.7)*	3.0 (2.1–4.5)**	1.6 (1.0–2.1)	1.8 (1.0–2.2)	1.6 (1.0–1.9)
TC, mmol/l	6.3 (5.2–7.3)*	6.2 (5.2–7.1)*	6.5 (5.2–7.4)	5.7 (5.0–6.2)	5.7 (4.8–6.1)	5.8 (5.0–6.6)
TG, mmol/l	2.6 (1.9–3.7)***	2.3 (1.9–3.6)**	2.7 (1.7–3.9)***	1.1 (0.9–1.4)	1.1 (0.8–1.4)	1.0 (0.9–1.3)
HDL-C, mmol/l	1.2 (1.1–1.3)***	1.1 (1.0–1.2)***+	1.2 (1.1–1.3)***	1.6 (1.3–1.8)	1.5 (1.3–1.8)	1.6 (1.5–1.9)
LDL-C, mmol/l	3.6 (3.1–4.3)	3.5 (3.2–4.2)	3.6 (3.0–4.3)	3.5 (2.8–4.3)	3.4 (2.9–3.8)	3.6 (2.8–4.3)
Apo A1, g/l	1.26 ± 0.25**	1.24 ± 0.25	1.28 ± 0.26**	1.43 ± 0.21	1.36 ± 0.20+	1.50 ± 0.20
Apo B, g/l	1.34 ± 0.32***	1.39 ± 0.26***	1.28 ± 0.37	1.09 ± 0.25	1.06 ± 0.22	1.13 ± 0.28
NEFA, mmol/l	0.50 (0.39–0.72)	0.43 (0.35–0.68)	0.51 (0.43–0.75)	0.55 (0.43–0.71)	0.59 (0.435–0.83)	0.55 (0.40–0.61)
CRP, mg/l	2.7 (2.0–6.3)	2.8 (2.0–4.3)	2.7 (2.0–7.4)	2.3 (2.0–6.5)	2.0 (2.0–4.3)	4.6 (2.1–7.3)
Cu, µmol/l	17.7 (16.0–20.5)	17.0 (15.5–18.4)	19.6 (16.3–21.7)	18.5 (16.3–21.5)	16.3 (14.3–18.6)++	19.9 (18.5–23.4)
Zn, µmol/l	16.0 (13.4–17.7)	15.8 (13.4–17.8)	16.3 (13.8–17.1)	15.4 (14.6–19.9)	16.0 (14.5–18.3)	15.2 (14.7–20.8)
Bilirubin, µmol/l	9.1 (6.8–12.9)	10.9 (7.9–13.8)+	7.2 (6.1–9.6)	10.6 (8.0–15.2)	13.9 (9.8–18.2)++	9.0 (7.5–12.3)
Uric acid, µmol/l	346 (290–390)**	355 (312–420)*	329 (275–352)**	293 (236–346)	320 (291–370)+++	251 (195–293)

BP = Blood pressure; TC = total cholesterol; TG = triglycerides; HDL-C = high density lipoprotein; LDL-C = low density lipoprotein; Apo = apolipoprotein; HOMA-IR = homeostasis model assessment of insulin resistance; QUICKI = quantitative insulin sensitivity check index; NEFA = non-esterified fatty acids; CRP = C-reactive protein; Met = metabolic syndrome.

[a]Data presented as mean ± standard deviation (SD) for parametric and median (IQR) for non-parametric variables; MetS versus controls: *p < 0.05, ** p < 0.01, ***p < 0.001. Female versus male: +p < 0.05, ++p < 0.01, +++p < 0.001.

apo A1 (r = 0.479, p < 0.001). Concentrations of CD-LDL correlated positively with TC (r = 0.565, p < 0.001), apo B (r = 0.597, p < 0.001), and LDL-C (r = 0.384, p < 0.001), and negatively with CAT (r = –0.233, p < 0.05).

Discussion

In this study, comparing MetS patients with an age- and sex-matched control group, increased activities of CuZnSOD (+15%, p < 0.05) and GR (+19%; p < 0.001) and increased levels of CD-LDL (+14.4%; p < 0.001) and cTRAP (+6.5%; p < 0.01) were found in MetS

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Table 2. Parameters of oxidative stress of studied groups

	Metabolic syndrome			Controls		
	all	M	F	all	M	F
GPX1, U/g Hb	59.4 ± 15.8	57.6 ± 18.1	61.1 ± 13.4	59.1 ± 17.7	55.4 ± 19.2	62.8 ± 15.7
GR, U/g Hb	8.19 ± 1.54***	8.74 ± 1.21***+	7.63 ± 1.67	6.88 ± 1.66	6.92 ± 1.76	6.83 ± 1.60
GSH, mg/g Hb	0.57 (0.38–2.73)*	0.56 (0.40–0.70)	1.51 (0.38–5.01)	1.46 (0.41–5.22)	1.22 (0.43–5.40)	1.70 (0.40–5.05)
CAT, kU/g Hb	189.6 ± 31.8*	192.5 ± 27.8	186.7 ± 35.8	204.6 ± 33.0	206.1 ± 32.5	203.1 ± 34.4
CuZnSOD, kU/g Hb	2.3 (1.9–2.5)**	2.3 (2.2–2.5)*	2.0 (1.7–2.5)+	2.0 (1.2–2.5)	2.2 (1.1–2.6)	2.0 (1.3–2.4)
PON1, kU/l	158.9 ± 41.9*	152.0 ± 47.4	165.7 ± 35.4	179.9 ± 42.3	170.2 ± 36.1	189.5 ± 46.5
CD, mmol/l	61.9 (54.1–84.3)***	57.3 (53.4–68.7)*	63.8 (55.3–94.2)*	54.1 (41.3–63.6)	53.3 (33.8–63.6)	57.5 (42.9–68.1)
cTRAP, µmol/l	823 (766–877)**	875 (816–909)*++	785 (732–835)**	773 (691–820)	809 (768–865)+++	701 (655–776)

GPX1 = glutathione peroxidase 1; GR = glutathione reductase; GSH = reduced glutathione; CAT = catalase; CuZnSOD = CuZn-superoxide dismutase; PON1 = paraoxonase1 – arylesterase activity; CD = conjugated dienes in precipitated LDL; cTRAP = calculated total peroxyl radical trapping – calculation: [0.63 (albumin) + 1.02 (uric acid) + 1.50 (bilirubin)]; Met = metabolic syndrome; Data presented as mean ± standard deviation (S.D.) for parametric and median (IQR) for non-parametric variables.

MetS versus controls: *p < 0.05, ** p < 0.01, *** p < 0.001. Female versus male: $^{+}$p < 0.05, $^{++}$ p < 0.01, $^{+++}$p < 0.001.

Table 3. Spearman correlation coefficients for components of the metabolic syndrome and parameters of oxidative stress in the combined group (metabolic syndrome plus controls) (N = 80)

	SBP	TG	HDL-C	Glucose	HOMA-IR	MetSC	CD	PON1	GR	GPX1	CAT	CuZnSOD
Waist	0.313	0.533^{+++}	–0.602^{+++}	0.402^{++}	0.570^{+++}	0.717^{+++}	0.336	–0.103	0.377^{+}	–0.160	–0.115	0.049
SBP	–	0.270	–0.147	0.141	0.103	0.405^{++}	0.338	–0.039	0.129	0.076	–0.108	–0.097
TG	–	–	–0.631^{+++}	0.396^{+}	0.453^{++}	0.736^{+++}	0.571^{+++}	–0.170	0.219	–0.067	–0.182	0.017
HDL-C	–	–	–	–0.357^{+}	–0.405^{+}	–0.681^{+++}	–0.374^{+}	0.321	–0.148	0.086	0.133	–0.015
Glucose	–	–	–	–	0.555^{+++}	0.540^{+++}	0.019	–0.103	0.127	–0.286	–0.081	–0.118
HOMA-IR	–	–	–	–	–	0.493^{+++}	0.099	–0.088	0.216	0.025	–0.066	–0.073
MetSC	–	–	–	–	–	–	0.442^{++}	–0.193	0.261	–0.097	–0.249	–0.115

SBP = Systolic blood pressure; TG = triglycerides; HDL-C = high density lipoprotein; HOMA-IR = homeostasis model assessment of insulin resistance; Met = metabolic syndrome; MetSC = number of components of the MetS (N = 1–5; waist circumference, glucose, triglycerides, HDL-C, SBP); GPX1 = glutathione peroxidase 1; GR = glutathione reductase; CAT = catalase; CuZnSOD = CuZn-superoxide dismutase; PON1 = paraoxonase-1-arylesterase activity; CD = conjugated dienes in precipitated LDL. $^{+}$p < 0.05; $^{++}$p < 0.01; $^{+++}$p < 0.001; after Bonferroni adjustment.

patients. In contrast, activities of CAT (–7.3%; p < 0.05) and PON1 (–11.7%; p < 0.05) as well as serum concentration of GSH (–61%; p < 0.05) were significantly decreased. The HOMA-IR demonstrated evidence of a significantly increased insulin resistance in subjects with MetS.

Under resting physiological conditions, biologic systems generate only small amounts of the superoxide anion. Its overproduction can result from mitochondrial electron leakage in hyperglycemia [15]. Other causes of superoxide overproduction are increased activities of NAD(P)H oxidases [16], xanthine oxidase, lipoxygenase, and cyclooxygenase as well as an imbalance in the thioredoxin system [17]. Large amounts of superoxide and other RONS arise in the accumulated fat, mainly due to increased activities of NAD(P)H oxidases and a decreased expression of antioxidant enzymes [18]. Adipose tissue is an important generator of oxidative stress and inflammation, contributing to the production of pro-inflammatory cytokines

Obes Facts 2013;6:39–47
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(TNFα, IL-1, IL-6 etc.). Oxidative stress is supposed to worsen the inflammatory state in MetS via activation of redox-sensitive transcription factors (particularly NFκB) by RONS, inducing the expression of TNFα and IL-6. These cytokines increased CRP synthesis. However, in our study, we did not find a statistically significant difference in CRP levels between MetS patients and controls. This could be caused by the method used for CRP measurement. The method used in our study lacks the sensitivity to differ between low-grade inflammation in MetS (CRP between 1.0 and 3 mg/l) and subjects without MetS (CRP < 1.0 mg/l).

The raised CuZnSOD activities in the erythrocytes of patients with MetS found in our study may be compared with the results of Mitrijevic-Sreckovic et al. [19], who described slightly increased CuZnSOD activities in children with MetS in comparison with obese children without MetS. Studies on serum CuZnSOD activities did not show consistent results [20, 21]. Increased CuZnSOD activity results in raised amounts of H_2O_2 which becomes toxic when activity of CAT is normal or decreased. Induction of one enzyme (CAT or CuZnSOD) does not necessarily lead to the induction of the other one [22]. Another source of H_2O_2 is its passage through the erythrocyte membrane [23]. The elevated production of ROS in the endothelium could thus lead to increased levels of ROS also in erythrocytes.

In our study, we have found a significantly decreased activity of CAT. Because of the increased activity of CuZnSOD in our study, elevated levels of H_2O_2 have to be expected. According to study of Kirkman et al. [24], during lengthy exposure of CAT to H_2O_2, the CAT-bound NADPH became oxidized to $NADP^+$ and activity of CAT fell to about one third of the initial activity. Consequently, the cause of the decrease of CAT activity could be the damage of erythrocyte CAT by H_2O_2. Contrary to our study, Cardona et al. [20, 21] found increased activities of CAT in patients with hypertriglyceridemia (concentration of TG > 1.7 mmol/l) apart from the presence of MetS, and these activities were further increased after fat overload. Decreased activities of CAT were described in patients bearing only individual components of MetS – obesity [25], hypertension [26], or insulin resistance [27]. Decreased activity of CAT implies stressed condition of erythrocytes when complete removal of H_2O_2 is not possible [28]. Low activities of CAT were associated with an increased risk of diabetes mellitus and its complications [5, 29].

The GPX1 activity in our study was not altered in MetS patients. This result is in accordance with the study of Mitrijevic-Sreckovic [19]. On the contrary, Cardona et al. [20, 21] found lower activities of GPX1 in a group of subjects with hypertriglyceridemia, a part of MetS presence, and the drop of its activity was almost to 75% of that of the control group. Bougoulia et al. [30] showed decreased activity of GPX1 in obese subjects as well as an increase after weight reduction.

As expected, concentrations of GSH were significantly decreased and activities of GR increased in our group of subjects with MetS. Decreased concentrations of GSH with opposite changes in GSSG levels were also found in MetS subjects in the study of Cardona et al. [20]. On the other hand, Cardona et al. [21] registered a significant drop in GR activity in MetS subjects. Increased activity of GR could be attributed to a compensatory protective mechanism of the cells against ROS. Furthermore, our expected increase in the GSSG/GSH ratio due to lower levels of GSH may stimulate compensatory increase in GR activity in blood to reduce higher levels of GSSG into GSH [31].

The finding of decreased arylesterase activities of PON1 in our subjects with MetS is in accordance with other studies [32, 33]. Because it was shown [34] that there is a strong positive correlation between arylesterase and paraoxonase activity of PON1, we could therefore discuss arylesterase and paraoxonase activity of PON1 together. Low activities of PON1 have been shown to be associated with oxidative stress, hypercholesterolemia, diabetes mellitus, cardiovascular diseases, and sepsis [34, 35].

In the present study, we found significantly higher concentrations of CD-LDL in subjects with MetS. This test was shown to be the most sensitive indicator of lipid peroxidation and can be regarded as a global marker of systemic oxidative stress [36]. In this study, several

anthropometric and biochemical characteristics of MetS correlated significantly with increased concentrations of CD-LDL, which reflect oxidation of the lipid component of LDL. This finding is in agreement with the results of our studies concerning the severity of MetS, oxidative stress, hypertriglyceridemia, and fatty acid metabolism [37, 38]. The important role of lipid peroxidation in the pathogenesis of MetS has been proven in many experimental and clinical studies [39].

Conclusion

In the present study, we estimated a wide variety of antioxidant enzymes, and activities of several enzymes were changed in subjects with MetS. Enzyme activities were assessed in the erythrocytes where the concentration of enzymes remain stable throughout the life span and reflect adaptive changes in their expression in erythroid precursors. According to our results, alterations of antioxidant enzymes related to MetS are not uniform. While activities of CuZnSOD and GR were higher in the MetS group than in healthy subjects, a decrease in CAT and PON1 as well as the absence of the expected increase in GPX1 indicate a disorder in antioxidant defense mechanisms. Our results could be interpreted that the erythrocytes and their GSH levels and activities of GR and GPX1 protect against oxidative stress in MetS. The severity of MetS, as assessed by the number of its components, significantly correlated with the concentrations of CD-LDL.

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Disclosure Statement

We hereby state that there is no conflict of interest.

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Increased inflammatory cytokines together with impaired antioxidant status persist long after clinical recovery from severe sepsis: correlation with HDL-cholesterol and albumin

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ABSTRACT

Objective: To observe markers of oxidative stress and antioxidant status in relation to inflammatory mediators in septic patients at onset of systemic inflammatory response syndrome (SIRS), one week later and one week after the clinical recovery from sepsis.

Design: The prospective study.

Setting: Multidisciplinary adult intensive care unit (11 beds).

Patients: 30 adult patients in severe sepsis and septic shock (SP); 19 SP completed 3 samplings (S1: enrolled within 24 hours after the onset of sepsis, S7: 7 days after S1, R7: 7 days after the recovery).

Interventions: None

Measurements: C-reactive protein, procalcitonin, interleukins (IL-1β, IL-6, IL-10), tumor necrosis factor α, oxidized-LDL (ox-LDL, conjugated dienes (CD), nitrites, nitrotyrosine, paraoxonase 1 activity, HDL cholesterol, apoprotein A1, serum amyloid, cofactors of antioxidant enzymes, non-enzymatic antioxidants and antioxidant enzyme activities (CuZn-superoxide dismutase, catalase, glutathione peroxidase 1, glutathione reductase).

Main Results: Comparing SP with healthy controls (HC), the enhanced concentrations of C-reactive protein, procalcitonin and bilirubin in serum as well CuZnSOD activity in erythrocytes was found in S1 only. The serum levels of ox-LDL, CD, nitrites and nitrotyrosine were increased in S1, culminated in S7 and reverted nearly to the HC level in R7. The reduction in CAT activity and increased concentration of SAA observed in S1 endured till S7. The increase in IL-6, IL-10 and TNFα accompanied by the decrease in the PON1, GPX1, apo-A1, HDL-C, Se, Zn and albumin appeared in S1 and persisted until R7. The increased TNFα in R7 was in the close negative correlation with HDL-C and albumin concentrations.

Conclusions: Increased level of cytokines, lasting after cessation of clinical signs of severe sepsis, was accompanied by significant depletion of antioxidant capacity and persistence of inflammatory activity. At this critical period of recovery, the patients should be dealt as high risk population thus carefully followed up and considered for special antioxidant, nutritional and physiotherapeutic interventions.

Key words: sepsis, oxidative stress, antioxidant enzymes, cytokines, reactants of acute phase, paraoxonase 1

INTRODUCTION

Sepsis is defined as a systemic inflammatory response syndrome (SIRS) in the presence of infection progressing with different degree of severity (1;2). Patients with severe sepsis and septic shock show deregulation of inflammatory process that corresponds to extensive exhaustion of individual functional reserves and development of organ dysfunction. These patients require intensive care in order to improve survival (3). Nevertheless, many of them who survive beyond intensive care and are clinically recovered still possess subclinical impairments and thus remain susceptible to secondary complications with negative impact for their long-term prognosis. With this respect, using appropriate markers for the identification of these patients at risk would enable the follow up care to concentrate the effort and resources on sufficient functional recovery.

Sepsis arises through the activation of an innate immune response, with changes in the expression and activity of many endogenous mediators of pro- and anti-inflammatory processes (4-6) interplaying in order to eliminate the insult and establish new homeostasis (7;8). SIRS, typically present in early sepsis and lasting 3-5days, is characterized by tachycardia, tachypnoe and abnormal body temperature or white blood count. This predominately pro-inflammatory period is usually followed by the development of so called Compensatory Anti-inflammatory Response Syndrome (CARS), a complex but incompletely defined pattern of immunologic responses to attenuate pro-inflammatory reaction of host that when unbalanced under severe infection can result in energy and immunosuppression with increased susceptibility to the development of a new infection (9-11). From this point of view, pro- and anti-inflammatory cytokines facilitating and modulating the response to the inflammatory stimulus seem to serve as an important prognostic marker of the subsequent patient outcome (12;13). Moreover recent clinical studies have shown that the increased levels of IL-6, TNF-α and IL-10 persisting after clinical recovery from sepsis, rather than their initial

peak, are more characteristic of those patients who ultimately have further complications or die (14;15) .

The activation of leukocytes and release of mediators in sepsis is indispensably accompanied by an increased production of reactive oxygen and nitrogen species (RONS) (16). RONS are well recognised for playing a dual role as both deleterious and/or beneficial species. Beneficial effects occur at low/moderate concentrations of RONS and involve physiological role in cellular responses as for example in defence against infectious agents and in the function of a number of cellular signalling pathways. Under physiological conditions, the balance is established between RONS production and antioxidant defence capacity. This balance can be disturbed through variable extent of increased RONS production and/or impaired antioxidant defence. The pro-anti-oxidant imbalance, in favour of the former, is known as oxidative stress (17). Overproduction of RONS is a deleterious process that can be an important mediator of damage to cell structures under pathological conditions (18). The oxidative modification of molecules occurring in adult and paediatric sepsis is probably an important promoter of sepsis progression toward shock and organ dysfunction (16;19).

The idea of the study was to describe inflammatory process of severe sepsis/septic shock in SIRS, CARS and 7 days after the clinical recovery in carefully selected group of ICU patients. The analysis of inflammatory mediators together with oxidative stress markers and antioxidant status would help to confirm clinical stages of sepsis emphasizing the persistence of risk after the recovery (usually after discharge from ICU or hospital) that should be addressed in standard follow up measures to determine the patient status and prognosis as well the choice of appropriate interventions. To our knowledge, studies of this completeness have not been published so far.

PATIENTS AND METHODS

This prospective study was carried out in medical adult intensive care unit (ICU) of the University Teaching Hospital. The study protocol was approved by the institutional review board and the Ethics Committee of the General Teaching Hospital in Prague. Written informed consent was obtained from all participants.

Patients: The population under study consisted of two groups: 30 septic patients (SP) and 30 age and sex matched healthy controls (HC). The sepsis was defined according to the Society of Critical Care Medicine/American College of Chest Physicians (SCCM/ACCP) definitions (2). SP had to fulfil the following inclusion criteria: APACHE II score > 10 and C-reactive protein in serum > 20 mg/l. Exclusion criteria for SP were: antioxidant therapy, chronic dialysis, history of diabetes, generalized tumours, immunosupressive therapy and chemotheraphy. Sepsis was treated according to guidelines (5). HC were defined as individuals without known major disease.

Data collection: Samples from SP were collected three times: during the first 24 hours after ICU admission (S1), 7 days after S1 (S7) and recovery (R7), e. g. 7 days after the cessation of septic clinical sings, CRP < 20 mg/l and temperature < 37 °C. Samples from HC group were obtained once. From the group of 30 SP 8 patients died because of sepsis and 3 SP were lost from follow up because they never fully recovered from sepsis thus all three samplings were available from 19 patients. These SP were compared with group of 19 sex and age matched HC. The main source of sepsis was lung, in 13 cases. In all study participants the medical history and the intake of any medicaments were documented at the study entry. The first seven days after ICU admission, the SOFA score (20;21) was calculated from laboratory and clinical parameters in SP. Blood was taken after overnight fasting from an arterial line (SP) or by puncturing a peripheral vein (HC).

The concentration of C-reactive protein (CRP), procalcitonin (PCT), interleukin 6 (IL-6), interleukin 10 (IL-10), tumor necrosis factor α (TNFα), serum amyloid A (SAA), oxidized LDL (ox-LDL), albumin, bilirubin, uric acid, Cu, Zn, Fe, Se, vitamins A and E and lipid parameters, as well as PON1 activity were measured in serum. Serum was prepared (after coagulation in vacutainer tubes) by centrifugation at 3500 rpm at 4 °C for 10 min. Conjugated dienes (CD) were measured in precipitated LDL. Activities of antioxidant enzymes were measured in haemolysed erythrocytes. The samples were stored at -80 °C until assay. All samples were marked with unique identification numbers, merging data only after assays had been completed.

Laboratory measurements: The routine biochemical tests were measured in Central Biochemical Laboratory of General Teaching Hospital in Prague.

Concentration of CRP was measured with immunoturbidimetric method using K-ASSAY CRP kit (Kamiya Biomedical Company, USA) on analyzer Hitachi Modular (Japan). Concentration of PCT was measured with immunoluminometric assay (ILMA) using BRAHMS PCT LIA-Kit (Brahms Diagnostica GmbH; catalogue number 54.1, Berlin, Deutschland). Cytokines: IL6, IL10 and TNFα were analyzed using Fluorokine MAP kits (R&D Systems, USA) and Luminex®100 analyzer. Fluorokine MAP kits are composed of a Base kit and a panel of Analyte kits. Each kit contains antibody-coated microparticles and biotinylated detection antibodies. SAA concentration was analysed by a solid phase sandwich ELISA kit (Invitrogen Corporation, USA). The arylesterase activity of PON1 was measured according to the method as previously described by Eckerson et al. using phenylacetate as a substrate (22). The rate of phenol generation was monitored spectrophotometrically at 270 nm. Arylesterase activity of PON1 was calculated using the molar extinction coefficient of the produced phenol (1310 $M^{-1}cm^{-1}$) and expressed as U/ml of serum. Oxidized-LDL measurement was performed by Oxidized LDL ELISA kit (Mercodia, Sweden). Activities of

antioxidant enzymes were determined by spectrofotometric kinetic methods and concentration of CD/LDL was measured as previously described by Kodydková et al. (23). Concentration of nitrotyrosine was measured by a solid phase sandwich ELISA kit (Biovendor, Czech Republic). The concentration of nitrites and nitrates in serum was assessed by the Griess reaction according to method of Guevara et al. (24). The total peroxyl radical trapping was calculated according to the formula: [0.63 (albumin) + 1.02 (uric acid) + 1.50 (bilirubin)](25).

Statistical analysis: Data are expressed as mean ± S.D. for parametric and median as median (25^{th}-75^{th} percentiles) for nonparametric variables. Normality of data distribution was tested with Shapiro-Wilks W test. Differences between SP and HC were tested with one-way ANOVA with Dunnettpost test. For nonparametric analysis Kruskal-Wallis ANOVA was used. Friedman ANOVA was used for dependent analysis. All statistical analyses were performed using version 8.0 of StatSoft software Statistica (2007, CZ). $P < 0.05$ was considered to be statistically significant.

RESULTS

Basic characteristics: Table 1 summarizes the demographic and clinical characteristics of 19 SP in all three samplings and 19 sex and age matched HC.

Acute-phase response markers: The serum PCT and CRP concentrations increased in S1 but no significant difference was observed, in S7compared to HC. The increased concentrations of interleukins (IL-6, IL-10, TNF-α) persisted from S1 till R7 and SOFA gradually decreased from S1 till S7 (Figure 1).

Serum markers of oxidative stress: The levels of ox-LDL, CD and nitrotyrosine increased in S1, culminated in S7 and returned to the HC values in R7. Enhanced serum concentration of nitrites/nitrates was observed only in S7 (Figure 2).

Antioxidant capacity: CuZnSOD activity was increased in S1 and returned to the HC value already in S7. The decline in CAT activity found in S1 and S7, returned to the HC level in R7 while the decrease in GPX1 activity persisted in all three samplings. No significant difference in GR activity between HC and individual SP samplings was found (Figure 3).

Table 2 presents non-enzymatic antioxidants and cofactors of antioxidant enzymes. The decrease in concentrations of vitamin A, vitamin E and bilirubin was found in S1 only, however, the decrease in Zn was observed in both S1 and S7. The significant decline of uric acid and the rise in Cu was observed only in S7 compared to HC. Nevertheless all these changes returned nearly to the HC values in R7. On the other hand, the substantial decrease in transferrin, Fe, Se and albumin as well the increase in the ferritin concentrations and Calculated TRAP observed already in S1, persisted still 7 days after recovery (R7) and never reached the HC levels. Marked fall in PON1 activity appeared at the onset (S1) and persisted until recovery (R7). The decline in the PON1 activity was closely followed by decreased HDL-C and ApoA1 concentrations. SAA concentration was significantly increased in S1 and in S7 reaching nearly HC level in R7 (Figure 4). We also measured TC (mmol/l): S1 = 3.3 (2.5-3.5), S7 = 3.7 (2.8-4.3), R7 = 4.4 (4.0-5.2), HC = 5.7 (4.8-6.7); LDL-C (mmol/l): S1 = 1.8 (1.2-2.2), S7 = 2.2 (1.1-2.4), R7 = 2.9 (2.2-3.2), HC = 3.7 (3.0-4.3) and TAG (mmol/l): S1 = 1.3 (0.8-1.9), S7 = 1.8 (1.1-2.4), R7 = 1.5 (1.1-2.1), HC = 1.5 (1.0-1.7).

DISCUSSION

The design of this study emerged from the recent clinical trials monitoring the basic pro-inflammatory (IL-6, TNF-α) and anti-inflammatory (IL-10) cytokines as innate immunity markers on greater population of patients with severe sepsis together with their clinical outcomes (14). These studies have concluded that despite clinical recovery, the patients leaving hospital with increased level of cytokines are exposed to increased risk of death

during next year (15). Our relatively small but carefully selected group of patients in early severe sepsis/septic shock diagnostic category, allowed us to analyse broader set of parameters characteristic for different stages and aspects of inflammatory process in the similar clinical setting and corresponding (similar) mortality rate (14). Nineteen patients were available for three samplings. The first sampling was done within 24 hours after onset of sepsis, the time for second sampling was chosen 7 days later when the signs of SIRS are usually over and organ function is restored. In accordance, we present that the SOFA score was improved by day 7 in this study. The third sampling, 7 days after cessation of all clinical sings of inflammation, reflected the time difference of illness progress in individual subjects. This timing enabled us to catch patients in the similar stage of recovery regardless of the sepsis duration and subsequent inflammatory complications occurrence. The hospital discharge as the time for last sampling, used in the study cited above (26), we considered as inappropriate due to bias caused by organisational aspects of health care system such as accessibility of follow up care. Despite of this difference in timing, we confirmed the persistence of increased levels of cytokines after the cessation of sepsis in R7. Many studies have evidenced a significant correlation between the level of individual cytokines and other markers of SIRS/sepsis together with its severity and patient outcome. TNF-α and IL-6 are known to mediate mainly pro-inflammatory SIRS while IL-10 is the most important in CARS response.

In our group of patients a significant decrease in IL-10/TNF- α ratio was caused mainly by the decrease of serum IL-10 level, whereas TNF- α level declined between S1 and S7 and remained practically unchanged after. As for the main acute-phase response markers (CRP, PCT), the enhanced concentrations were observed only in S1 that corresponds with other studies showing particularly PCT as a typical marker of early sepsis (27-29).

We hypothesized that increased levels of cytokines in monitored times would be reflected by the concomitant rearrangement of red-ox status that inspired us to analyse the markers of oxidative damage together with the levels of enzymatic and non-enzymatic antioxidants. As for lipid peroxidation markers, ox-LDL and CD were elevated in S1, persisted till S7 and both returned nearly to the values of HC range in R7. In line, the study of Behnes et al. also presented the increased concentration of ox-LDL in patients with severe sepsis during the first week of illness (30). Similarly, the endotoxin administration caused a sharp rise in plasma levels of CD in the porcine model of burn and sepsis (31). Another study showed increased TBARS and protein carbonyls as markers of lipid peroxidation and protein oxidation, respectively. While TBARS normalized during 7 days of sepsis, increased protein carbonyls persisted still three months after the onset of sepsis, probably due to the slow protein turnover (32). In accordance with other studies (33-35), we present decreased serum concentrations of vitamins E and A in S1. These vitamins are lipid phase antioxidants, crucial for prevention of lipid peroxidation(36). The increased level of the nitrotyrosine appeared already in S1, persisted till S7 while the nitrites/nitrates were increased just in S7 however both parameters were normalized after recovery in R7. The rise in these nitrogen compounds is in accordance with previous studies on septic shock patients indicating enhanced NO and RNS formation during the generalized inflammatory response(32). The observed shift between starting of growth in nitrotyrosine and nitrites/nitrates is in line with results of Strand et al. who showed that peak of nitrotyrosine need not coincide with the peak of nitrites/nitrates concentration in septic shock (37).

The important findings of our study reveal that whereas increased concentrations of peroxidation products are accompanied by diminished antioxidant capacity in the course of sepsis (SIRS and CARS), lowered antioxidant capacity is still persisting after the recovery (R7) while peroxidation products are nearly normalized with the close negative correlation of

ox-LDL to GPX1 and albumin (R = -0.528 and -0.519 respectively). Normal levels of lipid markers of peroxidation in R7 are accompanied with low level of antioxidant capacity

We confirmed reduced antioxidant defence capacity in septic critically ill patients (16) and we have found that reduction of some its components even lasted in R7. In our study, increased CuZnSOD and decreased CAT and GPX1 activities in erythrocytes were found in S1. While CuZnSOD normalization was observed already in S7, the decrease in GPX1 and the trend to the decline in CAT activities persisted still in R7. In line, Warner et al.(38) also found the increased activity of CuZnSOD in erythrocytes at the onset of sepsis. Similarly in pediatric sepsis there was also observed apparent trend towards the increase of CuZnSOD activity in erythrocytes (39). CuZnSOD is one of the most important antioxidant enzymes responsible for the decomposition of superoxide radical while producing H_2O_2 that is further transformed to H_2O by the CAT and GPX1 action. It is necessary to note that the increase in CuZnSOD activity observed in the early stage of sepsis cannot be, by principle, the result of the rise in protein amount because mature erythrocytes do not possess any transcriptional apparatus but it is the result of the activity stimulation (40). We propose that the increase of CuZnSOD in combination with simultaneous decrease in CAT and GPX1 activities may intensify the H_2O_2 accumulation with subsequent spontaneous formation of highly reactive hydroxyl radicals causing escalation of oxidative damage. Therefore, the increased CuZnSOD activity in S1 may act predominantly as a pro-oxidant (41) . Published results on erythrocyte CAT in sepsis are rather controversial to our study. Warner et al.(38) and Leff et al.(42) published increased activity of CAT in both erythrocytes and plasma of SP. The decrease in CAT activity observed in our group of SP could possibly be explained by the results of the *in vitro* study published by Kirkman et al.(43) where human erythrocyte CAT was exposed (for 12-24hr) to H_2O_2. The catalase-bound NADPH, important for its activity, became oxidized to $NADP^+$ causing subsequent CAT activity fell down to about one-third of the initial value (43).

We have found decreased activity of GPX1 during the sepsis and after recovery. The main reason could be low level of GSH as well decline in Se concentration observed in sepsis (35;44;45). Reduced glutathione (GSH) acts as a reducing substrate of GPX. and Se, bound in the active site of the enzyme in the form of one selenocysteine residue, is essential for its activity (46). In accordance suppressed activity of GPX1was accompanied by the decrease in the Se concentration till R7. Supplementation with Se has been shown to improve antioxidant capacity as demonstrated by increased GPX activity (47). As for the decrease of GPX1 activity in R7, we have also to consider relatively long regeneration of the enzyme due to the slow turnover of mature erythrocytes. The enzyme was shown to protect red blood cells against haemoglobin oxidation and haemolysis (48) that is why the diminished antioxidant capacity of erythrocytes could impact on the patient outcome in the case of secondary insult.

Serum PON1 is considered as further antioxidant enzyme playing important role in defence against oxidative stress (49;50). We confirmed our pilot study presenting the decline of PON1 activity in sepsis (51) and on larger set of patients we have shown that this decrease persisted till R7. Simultaneously another authors published the decrease of PON1activity in patients at the onset of sepsis compared to HC (52;53). It was found that antioxidative effect of HDL on LDL oxidative modifications is mediated by HDL-bound PON1. The inactivation of PON1 by ox-LDL involves the interaction of oxidized lipids with its free sulfhydryl group. Thus the ability of PON1 to protect LDL against oxidation is together accompanied by inactivation of the enzyme (54).

In this study, the decrease in PON1, HDL-C and apo-A1concentrations was closely followed by a marked increase of SAA persisting until R7. It is known that during inflammation SAA replaces Apo-A1 and displaces PON1 from the association with HDL, accompanied by the decrease in its activity (55). Our finding of decreased PON1 activity in SP is consistent with the aforementioned parallels and therefore this enzyme activity should

be classified among the negative acute phase parameters. Together with the PON1 decrease and in accordance with others, we observed the fall down of total cholesterol (TC) which just as PON1 and HDL-C did not normalized in R7. Similar decrease of HDL-C, in the course of severe sepsis, was also observed in the study of van Leeuwen et al.(56). The fall in HDL-C negatively correlated with persisting increase in TNF-α.

We have measured decreased values of TC, LDL-C and HDL-C in SP in all three samplings. Similarly to serum lipids, the decrease in serum albumin, Apo-A1, transferrin and Fe in all three samplings was also observed. In line with our results Gordon *et al*. showed that in critically ill patients, the mean high-density lipoprotein cholesterol (HDL-C) concentration was significantly lower in patients with an infection compared to patients without infection (57).

We have seen a good positive correlation of HDL-C with albumin and to a lesser extent with CRP (58) and the correlation with HDL-C found in this study points towards HDL-C as an acute phase reactant. Changes in acute-phase protein synthesis are mediated by cytokines produced in response to a variety of stimuli in multiple cell types that include macrophages, monocytes, T lymphocytes, endothelial and parenchymal cells (59). Several clinical and experimental studies suggest that high circulating levels of different cytokines may be responsible for the cholesterol decrease in acute illness (60).

We have seen a good correlation of HDL-C with albumin and, to a lesser extent, with CRP. Albumin and CRP are well known as acute phase proteins (49) and the correlation with HDL-C found in this study points towards HDL-C as an acute phase reactant. Changes in acute-phase protein synthesis are mediated by cytokines produced in response to a variety of stimuli in multiple cell types that include macrophages, monocytes, T lymphocytes, endothelial and parenchymal cells (49). Several clinical and experimental studies suggest that high circulating levels of different cytokines may be responsible for the cholesterol decrease

in acute illness (60-62). The correlation of HDL-C and IL-6 found in this study strengthens the association of HDL-C with the acute phase response. We observed also a correlation between HDL-C and procalcitonin. Clinical and laboratory parallels with low grade inflammatory process in atherosclerosis – the higher markers of inflammation the higher probability of complications (ischemia, infections etc.). Moreover in the well-functioning elderly subjects, preinfection systemic levels of TNF- α. and IL-6 were associated with higher risk of subsequent infection (15).

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Table 1. Clinical characteristics of studied groups

	SP			HC
	S1	**S7**	**R7**	
N (M/F)	10/9			10/9
AGE (years)	74 (56-79)			71 (56-78)
APACHE II	16.0 (13.0-23.0)	-	-	-
Diagnosis (medical/surgical)	11/8			-
Source of sepsis (lungs/others)	12/7			-
Day of sampling	1	7	22.0 (14.0-34)	-
ICU hospitalization (days)	20.0 (9-53)			-
Hospitalization (days)	24.0 (16.0-61)			-
Duration of sepsis (days)	14.0 (6.0-26)			-
SOFA	7.0 (2.5-10.0)	3.0 (1.5-9.0)	-	-
APV (number/percent)	7 (36.8 %)	7 (36.8 %)	1 (5.3 %)	-
CRRT (number/percent)	0	3 (15.8 %)	-	-

SP: septic patients; S1: SP enrolled within 24 hours after the onset of sepsis, S7: septic patients 7 days after S1 and R7: septic patient one week after the recovery from sepsis, HC: healthy controls; SOFA: Sequential Organ Failure Assessment, APV: Artificial Pulmonary Respiration, CRRT: Continuous Renal Replacement Therapy; data presented as median and interquartile range (25^{th}-75^{th} percentile).

Table 2. Non-enzymatic antioxidants, cofactors of antioxidant enzymes and other parameters of antioxidant capacity

	SP			HC
	S1 (n = 19)	**S7 (n = 19)**	**R7 (n = 19)**	**(n = 19)**
Vitamin E (mg/l)	12.2 ± 4.6[a,c]	14.5 ± 4.55	16.4 ± 5.0	18.2 ± 8.6
Vitamin A (mg/l)	0.52 ± 0.20[a,b,c]	0.81 ± 0.28	0.96 ± 0.44	0.97 ± 0.27
Fe (µmol/l)	2.8 (2.0-3.3)[a,b,c]	7.1 (4.8-10.0)[a,c]	11.6 (7.5-13.6)[a]	20.0 (15.6-27.3)
Ferritin (µg/l)	452 (240-1436)[a]	356 (222-1347)[a]	278 (194-646)[a]	84 (67-161.3)
Transferin (g/l)	1.58 (1.13-1.91)[a,b,c]	1.86 (1.55-2.18)[a,c]	2.19 (2.05-2.35)[a]	2.65 (2.45-3.09)
Ceruloplasmin (g/l)	0.43 ± 0.08	0.47 ± 0.12	0.45 ± 0.10	0.40 ± 0.07
Cu (µmol/l)	20.3 ± 3.7	22.5 ± 5.1[a]	21.6 ± 4.7	18.5 ± 3.2
Zn (µmol/l)	8.9 ± 2.9[a,b,c]	11.8 ± 2.6[a,c]	14.1 ± 3.6	15.1 ± 1.7
Se (µg/l)	33.3 ± 13.3[a,c]	46.5 ± 28.4[a]	53.7 ± 24.3[a]	72.5 ± 13.8
Albumin (µmol /l)	437 ± 95[a,c]	438 ± 118[a,c]	548 ± 944[a]	707 ± 63
Bilirubin (µmol/l)	14.8 (9.4-25.9)[c]	12.5 (6.1-21.4)	7.7 (6.7-17.0)	10.3 (7.3-14.5)
Uric acid (µmol/l)	270 ± 103	224 ± 106[a,c]	293 ± 122	331 ± 90
cTRAP (µmol/l)	585 ± 143[a,c]	535 ± 157[a,c]	669 ± 143[a]	781 ± 132

S1: patients enrolled within 24 hours after the onset of sepsis, S7: patients 7 days after S1 and R7: one week after the recovery, HC: healthy controls; cTRAP: calculated total peroxyl radical trapping - calculation: [0.63 (albumin) + 1.02 (uric acid) + 1.50 (bilirubin)]; data presented as mean ± S.D. for parametric and median (25^{th}-75^{th} percentile) for nonparametric variables; [a] septic patients (all samplings) vs. healthy controls, [b] S1 vs. S7, [c] S1 or S7 vs. R7; $p < 0.05$.

Table 3. Correlations of inflammatory markers, albumin, HDL-C and ox-LDL

		Albumin	HDL-C	Ox-LDL/LDL-C
CRP	**S1**	-0,556**	-0,384	0,398
	S7	-0,743***	-0,546***	0,464*
	R7	-0.306	-0.018	-0.070
	HC	-0.512*	-0,242	0,117
PCT	**S1**	-0,324	-0,472*	0,405
	S7	-0,563*	-0,680**	0,548*
	R7	-0.341	-0.140	0.511*
	HC	-0,173	0,051	0,075
IL-6	**S1**	-0,448	-0,123	-0,116
	S7	-0,641**	-0,351	0,465*
	R7	-0.120	-0.169	0.049
	HC	-0,637**	-0,247	-0,009
IL-10	**S1**	0,172	-0,052	-0,093
	S7	-0,456*	-0,387	0,349
	R7	-0.712***	-0.302	0.144
	HC	-0,380	0,026	-0,043
TNF	**S1**	0,060	-0,299	0,116
	S7	-0,503*	-0,523*	0,552*
	R7	-0.775***	-0.456*	0.464*
	HC	0,234	0,669**	0,526*

S1: patients enrolled within 24 hours after the onset of sepsis, S7: patients 7 days after S1 and R7: one week after the recovery, HC: healthy controls; *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$

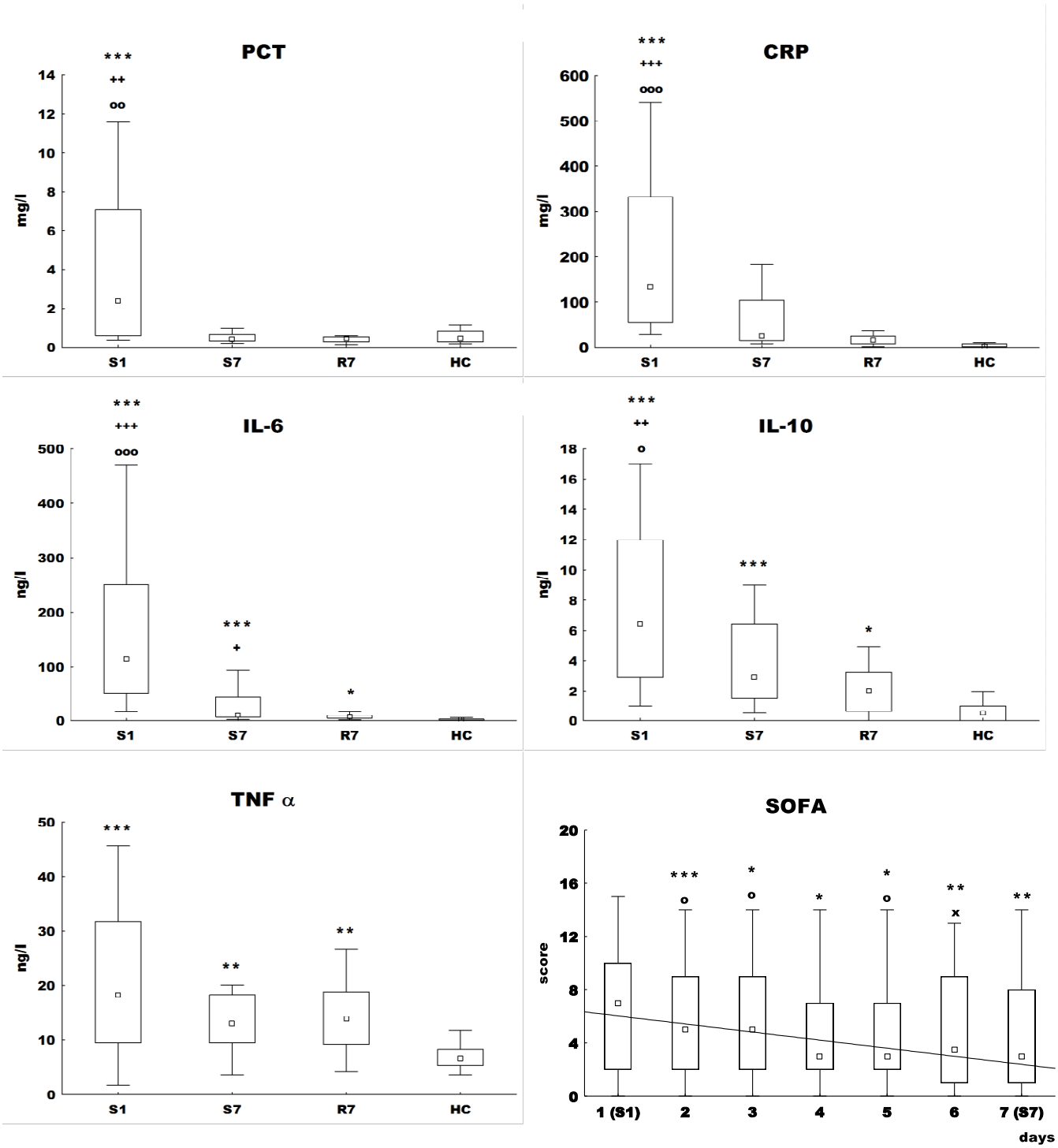

Figure 1. Changes in inflammation markers and SOFA in the course of sepsis.

S1: septic patients enrolled within 24 hours after the onset of sepsis, S7: septic patients 7 days after S1 and R7: septic patients one week after the recovery, HC: healthy controls; PCT: procalcitonin, TNF-α: tumor necrosis factor α, IL-6: interleukin-6, IL-10: interleukin-10; Data

presented as median (quartile, range), * S1 or S7 or SR7 vs. HC; $^{+}$ S1 or S7 vs. R7; ^{b} S1 vs. S7; *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$

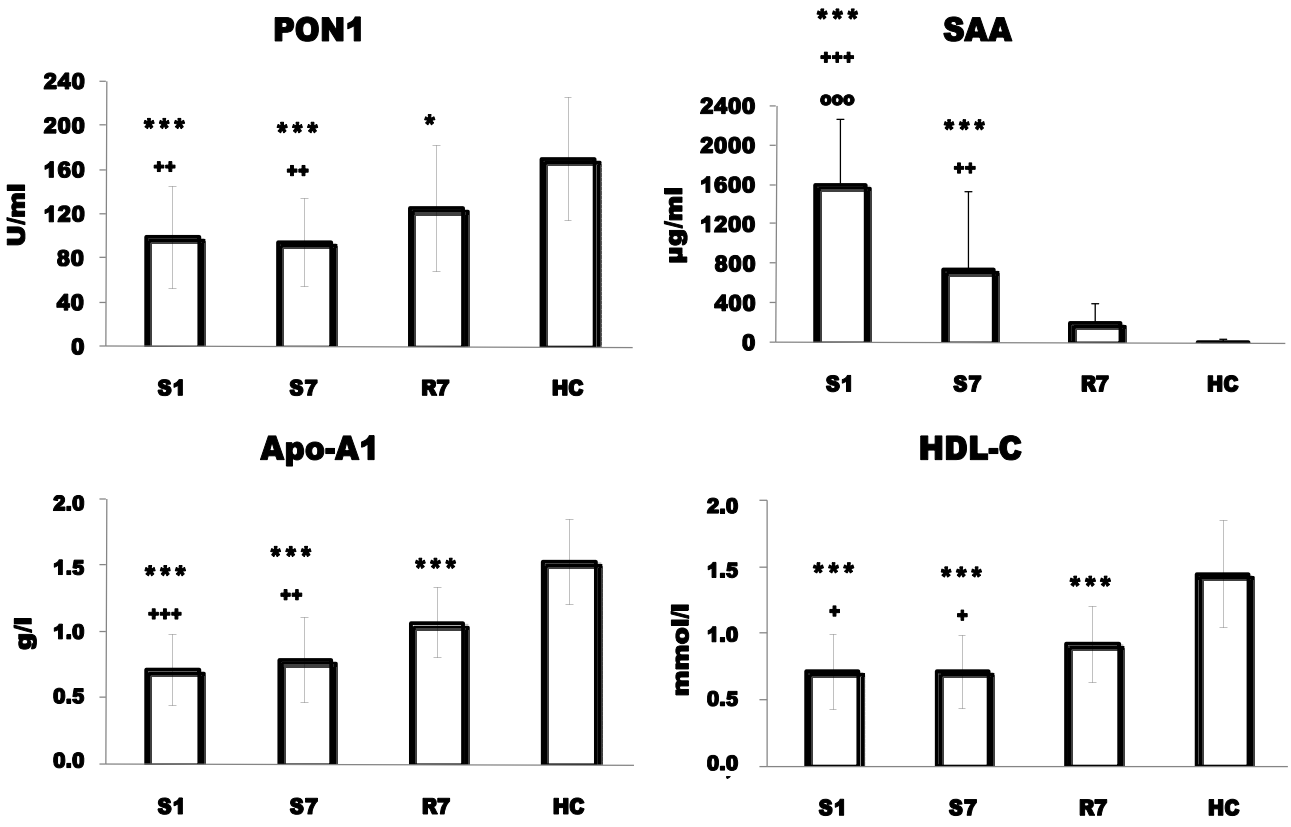

Figure 2. Changes in PON1 activity and associated parameters in the course of sepsis

S1: septic patients enrolled within 24 hours after the onset of sepsis (n = 19), S7: septic patients 7 days after S1 (n = 19) and R7: septic patients one week after the recovery (n = 19), HC: healthy controls (n = 19); PON1: enzyme paraoxonase1 – arylesterase activity, SAA: serum amyloid A, Apo-A1: apolipoprotein A1, HDL-C: high density lipoprotein cholesterol, data presented as mean ± S.D., * S1or S7 or R7 vs. HC; $^{+}$ S1 or S7 vs. R7; ^{b} S1 vs. S7; *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$

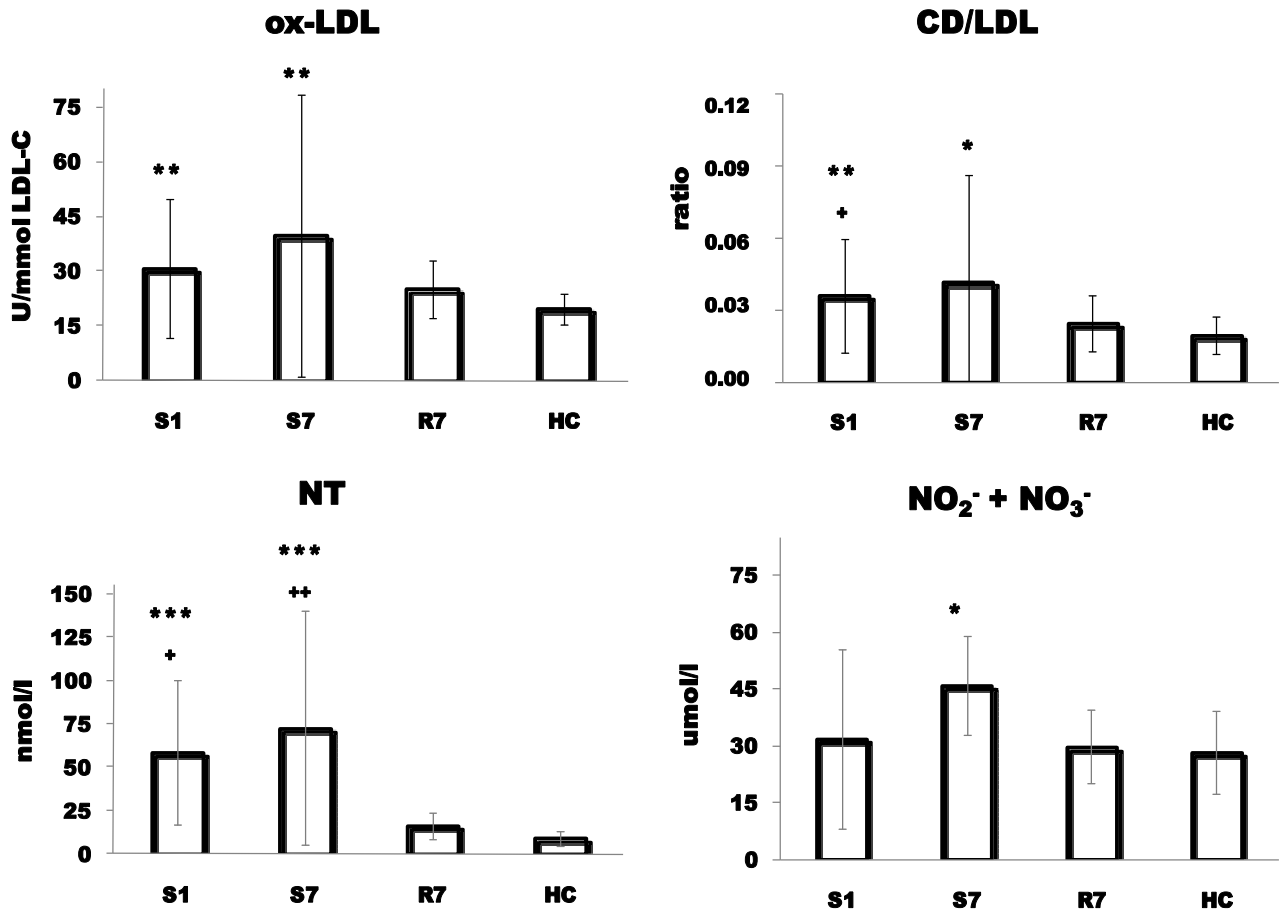

Figure 3. Changes in activities of oxidative stress parameters in the course of sepsis

S1: septic patients enrolled within 24 hours after the onset of sepsis (n = 19), S7: septic patients 7 days after S1 (n = 19) and R7: septic patients one week after the recovery (n = 19), HC: healthy controls (n = 19); Ox-LDL: oxidized low density lipoproteins, CD: conjugated dienes in precipitated LDL, LDL-C: low density lipoprotein cholesterol, NT: 3-nitrotyrosine; data presented as mean ± S.D., * S1or S7 or R7 vs. HC; $^{+}$ S1 or S7 vs. R7; ^{b} S1 vs. S7; *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$

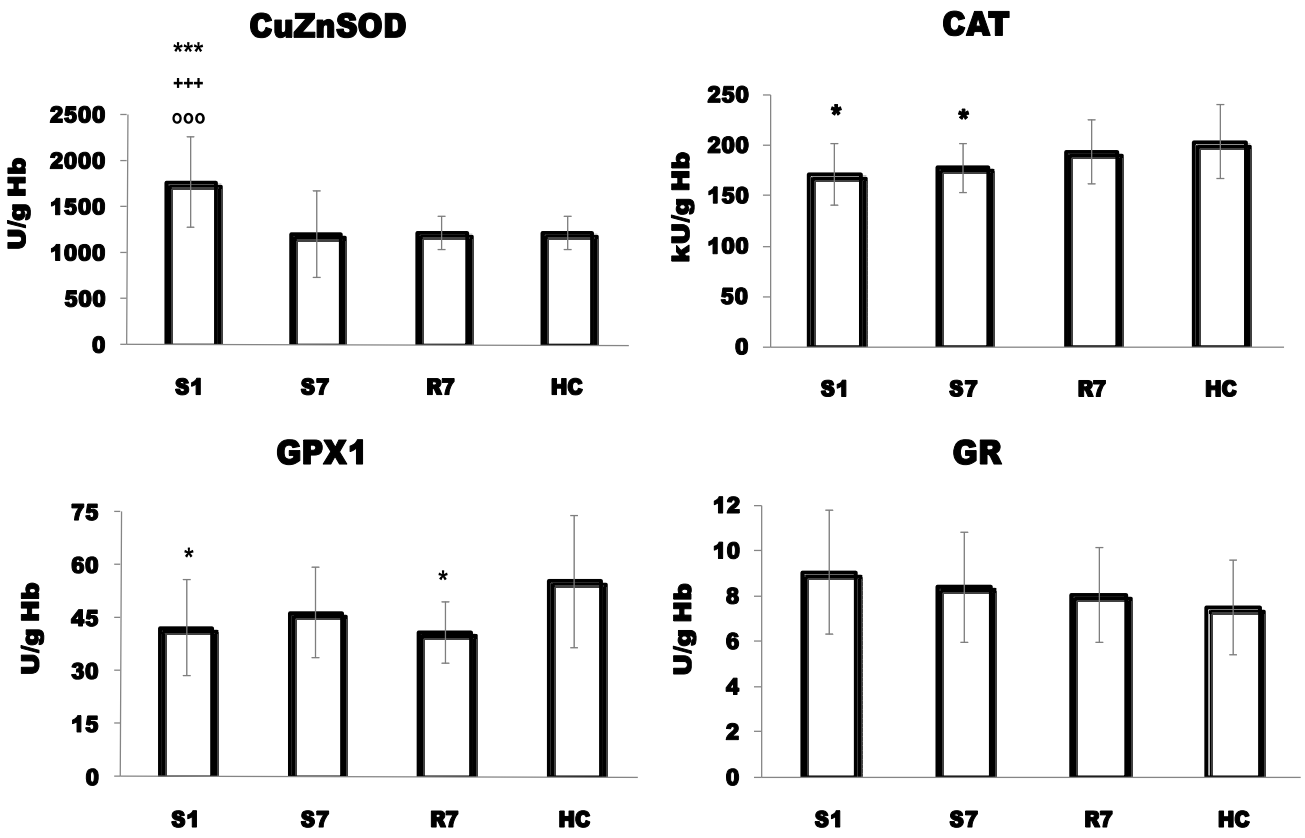

Figure 4. Changes in activities of antioxidant enzymes in the course of sepsis

S1: patients enrolled within 24 hours after the onset of sepsis (n = 19), S7: patients 7 days after S1 (n = 19) and R7: one week after the recovery (n = 19), HC: healthy controls (n = 19); CuZnSOD: superoxide dismutase, CAT: catalase, GPX1: glutathione peroxidase1, GR: glutathione reductase; data presented as mean ± S.D., * S1or S7 or R7 vs. HC; $^{+}$ S1 or S7 vs. R7; ^{b} S1 vs. S7; *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$

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Oxidační stres v průběhu akutní pankreatitidy

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SOUHRN
Cíl studie: stanovení parametrů oxidačního stresu a statutu antioxidačního systému v průběhu akutní pankreatitidy
Typ studie: observační, strukturálně vyvážená studie případů a kontrol
Materiál a metody: Do studie bylo zařazeno 13 pacientů s akutní pankreatitidou (AP) a dále na základě věku a pohlaví spárované dvě kontrolní skupiny, a to skupina zdravých osob (KON) a osob, které prodělaly v minulých 2-3 letech akutní pankreatitidu (PAP). Pacientům s AP byly odebírány vzorky celkem 4, nejprve během prvních 24 hodin od objevení příznaků, poté po 72 hodinách, třetí odběr byl prováděn 5. den a poslední odběr 10. den onemocnění. U všech pacientů byly stanovovány kromě základních klinických a biochemických parametrů aktivity antioxidačních enzymů, koncentrace některých antioxidantů (redukovaný glutation (GSH), vitamin A a E) a parametry oxidačního stresu (konjugované dieny v precipitovaných LDL (CD/LDL) a oxidované LDL(ox-LDL)). Ke statistickému zpracování výsledků byl použit program STATISTICA (Stat Soft, CZ).
Výsledky: Výsledky naší studie potvrzují zvýšený oxidační stres u pacientů s AP, a to zvýšenými hladinami CD/LDL u všech odběrů AP ve srovnání s CON ($p < 0,05$) a vzrůstajícími hladinami ox-LDL v průběhu AP s maximem 5. den AP. Pozorovali jsme rovněž změny v antioxidačním systému u AP pacientů; u těchto pacientů jsme zjistili snížené aktivity glutationperoxidázy a arylesterázové i laktonázové paraoxonázy během všech odběrů a dále pak snížené hladiny sérových antioxidantů – albuminu, vitaminu A a vitaminu E při porovnání s kontrolní skupinou.
Závěr: Ve studii byl pozorován zvýšený oxidační stres a porušený antioxidační systém v časné fázi AP s gradací mezi třetím a pátým dnem AP.
Klíčová slova: akutní pankreatitida, oxidační stres, antioxidační enzymy

SUMMARY
Vávrová L., Kodydková J., Macášek J., Ulrych J., Žák A.: Oxidative stress in the course of acute pancreatitis
Objective: to assess oxidative stress and antioxidant status in acute pancreatitis and their natural course over the 10-day period.
Design: observation, matched case-control study
Material and methods: Into our study 13 patients with acute pancreatitis (AP) were included together with 13 sex- and age-healthy controls (CON) and 13 sex- and age- matched controls enrolled from persons that suffered from AP 2 – 3 years ago (PAP). We observed the antioxidant status of AP patients during the disease and the samplings were taken four times – on the first 24 hours of disease (AP1), after 72 hours from disease onset (AP3), on the 5th (AP5) and on the 10th day (AP10). In all studied groups markers of oxidative stress (level of conjugated dienes in precipitated LDL, oxidized LDL) and levels of antioxidants were assessed. We measured activities of superoxide dismutase (CuZnSOD), catalase (CAT), glutathione peroxidase 1 (GPX1) and glutathione reductase (GR) in erythrocytes and arylesterase (PON1-A) and lactonase (PON1-L) activities of paraoxonase in serum and concentrations of reduced glutathione (GSH) in erythrocytes and concentrations of vitamins E and A in serum.
Results: In our study we confirmed increased oxidative stress in AP, with higher levels of CD/LDL in all AP samplings compared to CON ($p < 0.05$) and with increasing levels of ox-LDL during the AP with the maximum on the 5th day. We have shown altered status of antioxidant system; the activities of both PON1 activities as well as activity of GPX1 were depressed in all AP samplings in comparison to CON. We have also observed decreased levels of serum antioxidants – albumin, vitamin A and vitamin E in AP
Conclusion: High oxidative stress and impaired antioxidant status was observed during early phase of AP with the gradation between 3rd and 5th day of AP.
Key words: acute pancreatitis, oxidative stress, antioxidant enzymes

Úvod

V patogenezi všech akutních zánětlivých procesů hrají důležitou roli reaktivní formy kyslíku (ROS), které se uplatňují v časné fázi zánětu, jako vysoce aktivní metabolity vedoucí k poruše buněčné homeostázy, k poškození DNA a k peroxidaci membránových lipidů s následným zvýšením permeability a k buněčné smrti [1]. Udržení oxidační rovnováhy organismů zajišťuje antioxidační systém, tvořený antioxidačními enzymy – superoxiddismutáza (SOD), kataláza (CAT), glutationperoxidáza (GPX), glutationreduktáza GR) a paraoxonáza (PON) – a neenzymovými antioxidanty, kde nejdůležitějším je redukovaný glutation (GSH) [2].

Cílem naší práce bylo sledovat změny antioxidačního systému v průběhu akutní pankreatitidy (AP), která představuje rychle se rozvíjející zánětlivý proces spojený s významnými metabolickými změnami a významnou klinickou odezvou. Klíčovými patogenetickými pochody, které probíhají během rozvoje AP, jsou autodiges-

ce, patologická stimulace zánětlivých buněk, ischemie, reperfuze a hemoragie. Významným faktorem, který se uplatňuje v patogenezi AP je oxidační stres (OS), [1]. Mezi nejčastější etiologické faktory vedoucí k rozvoji AP se řadí alkohol a cholelitiáza [3].

Materiál a metody

Do pilotní observační studie bylo celkem zařazeno 13 pacientů s AP a dále pak na základě věku a pohlaví spárované dvě kontrolní skupiny – skupina 13 zdravých osob (CON) a skupina 13 osob, jež během 2-3 let před odběrem prodělaly akutní pankreatitidu a v době studie byly bez obtíží (PAP). U pacientů s AP byly prováděny celkem 4 náběry krevních vzorků: první náběr byl proveden během prvních 24 hodin od objevení prvních příznaků (AP1), druhý odběr po 72 hodinách (AP3), třetí náběr byl uskutečněn 5. den (AP5) a poslední náběr pak 10. den onemocnění (AP10). Pacienti s AP byli vybíráni na JIP IV. Interní kliniky a JIP I. chirurgické kliniky hrudní, břišní a úrazové chirurgie 1. LF UK a VFN v Praze. U těchto pacientů probíhala diagnostika a zařazení do studie na základě následujících kritérií: aktivita AMS, APACHE II skóre, Ransonova kriteria, koncentrace C-reaktivního proteinu (CRP), CTSI skóre, kontrastního CT vyšetření. Na základě nové klasifikace závažnosti AP dle Petrova et al. (2010) [4] se v jednom případě jednalo o kritickou AP (pacient v průběhu studie zemřel), ve 4 případech o středně těžkou a v 8 případech o lehkou formu AP. U 8 pacientů byla AP biliárního původu, u 2 pacientů se jednalo o etylickou AP a u 2 o idiopatickou AP, v jednom případě byla AP vyvolána endoskopickou retrográdní cholangio-pankreatografií (ERCP).

Do kontrolní skupiny CON byli zařazeni zdraví dobrovolníci, do druhé kontrolní skupiny PAP byli zařazeni dobrovolníci vybíraní z pacientů, kteří byli před 2-3 roky hospitalizováni na IV. Interní klinice s diagnózou akutní pankreatitidy a v době studie netrpěli žádným chronickým onemocněním pankreatu. Z těchto 13 osob, 6 prodělalo v minulosti těžkou formu AP a 7 lehkou formu AP, v 5 případech se jednalo o biliární, ve 4 případech o etylickou a ve 3 případech o idiopatickou pankreatitidu, v jednom případě byla AP vyvolána vyšetřením ERCP. Pro všechny osoby platila stejná vylučovací kritéria: zavedená terapie antioxidanty (farmakologické dávky vitaminu C a E, allopurinol, N-acetylcystein), chronická dialýza, imunosuprese, manifestní diabetes mellitus, generalizace tumoru a chemoterapie. Studie byla schválena Etickou komisí VFN Praha. Všechny osoby zařazené do studie podepsaly informovaný souhlas.

U všech osob zařazených do studie byly prováděny odběry krevních vzorků po celonočním lačnění (min. 10 hodin). Odebrané krevní vzorky byly zpracovány do 1 hodiny od náběru a materiál pro další analýzy byl uchováván při -80°C. U pacientů byly sledovány základní klinické, antropometrické a biochemické parametry, dále pak byly stanovovány aktivity antioxidačních enzymů CAT, GPX1, GR, CuZnSOD v erythrocytech a arylesterázové a laktonázové aktivity PON1 v séru, koncentrace antioxidantů jako je redukovaný glutation (GSH) v erythrocytech, či vitaminy E a A, albumin a bilirubin v séru. Jako parametr oxidačního stresu byla měřena koncentrace konjugovaných dienů v precipitovaných LDL (CD/LDL) a hladina oxidovaných LDL (ox-LDL) v séru. Speciální vyšetření (hladiny antioxidantů, markery oxidačního stresu) byla prováděna v laboratořích IV. Interní kliniky, rutinní biochemické parametry a stanovení hladin vitaminů bylo provedeno v Ústavu lékařské biochemie a laboratorní diagnostiky VFN Praha. Metody ke stanovení aktivity antioxidačních enzymů a koncentrací GSH a CD/LDL byly podrobně popsány v publikaci Kodydkové et al. (2009) [5], ke stanovení ox-LDL byl využit komerčně dodávaný ELISA kit od firmy Mercodia. Ke stanovení hladin selenu byla využita atomová absorpční spektrometrie s elektrotermickou atomizací (ETAAS) na Varian Spectra A220 FS. Koncentrace vitaminů A a E byla stanovena pomocí diagnostických kitů (Radanal s. r. o., ČR) a metody vysokoúčinné kapalinové chromatografie (HPLC) s UV detektorem (Ecom).

Výsledky jsou vyjádřeny jako průměr ± S. D. pro parametrické veličiny a jako medián (0,25-0,75 percentil) pro neparametrické veličiny. Normalita byla testována prostřednictvím Shapiro-Wilkova W testu. Rozdíly mezi jednotlivými skupinami AP vs. kontrolní soubory byly zkoumány pomocí jedno-faktorové ANOVY s Neuman-Keulsovým post-testem. Pro neparametrickou analýzu byla použita Kruskal-Wallisova ANOVA. Při testování rozdílů mezi jednotlivými odběry pacientů s AP byla použita ANOVA pro závislé vzorky. Pro všechny statistické analýzy byl používán program STATISTICA 10.0 (Stat Soft, CZ). Za statisticky signifikantní byly považovány výsledky s $p < 0,05$.

Výsledky

Do studie bylo zařazeno celkem 13 pacientů s diagnostikovanou AP s průměrným APACHE II skóre (APACHE II = 5,7 ± 3,8) při vstupu do studie. Základní biochemické charakteristiky jednotlivých skupin jsou shrnuty v Tabulce 1.

Hlavními sledovanými parametry byly antioxidanty a markery OS. Jako markery OS byly měřeny hladiny CD/LDL a ox-LDL. V koncentraci CD/LDL nebyly zjištěny žádné signifikantní rozdíly mezi jednotlivými odběry AP, ale vyšší hladiny CD/LDL byly pozorovány u pacientů s AP během všech odběrů ve srovnání s CON ($p < 0,05$). Hladina ox-LDL se v průběhu AP zvyšovala a svého maxima dosáhla 5. den onemocnění (obr. 1).

Ze sledovaných antioxidačních enzymů docházelo k největším změnám aktivit v průběhu AP u obou sledovaných aktivit PON1. Obě PON1 aktivity byly ve všech odběrech AP signifikantně snížené při srovnání s CON. Nejnižší aktivita u PON1-A byla pozorována 5. den AP (obr. 2). V aktivitách GPX1, GR a CuZnSOD nebyly pozorovány žádné rozdíly mezi jednotlivými odběry u AP. Aktivita CAT byla signifikantně zvýšená v AP1 oproti AP10 (231,7 ± 21,2 vs. 219,8 ± 26,0; $p < 0,05$).

Při srovnání aktivit těchto enzymů u AP s kontrolními skupinami, byla pozorována snížená aktivita

Table 1: Basic biochemical characteristics of the studied groups

	AP1	PAP	CON
N (M/F)	13 (9/4)	13 (9/4)	13 (9/4)
Age (years)	56.1 ± 21.5	54.8 ± 20.8	55.8 ± 19.4
Glucose (mmol/l)	6.6 ± 2.9**	6.1 ± 1.4**	5.2 ± 0.4
TC (mM)	4.9 ± 3.3	4.9 ± 1.3	5.2 ± 1.2
α-AMS (μkat/l)	10.5 (7.0 – 19.4)***++	0.4 (0.3 - 0.4)	0.5 (0.3 - 0.6)
ALT (μkat/l)	1.7 (0.7 – 4.6)***	0.4 (0.3 - 0.6)	0.5 (0.4 - 0.6)
AST (μkat/l)	1.8 (0.7 – 3.9)***	0.5 (0.4 - 0.6)	0.4 (0.4 - 0.5)
GGT (μkat/l)	4.3 (1.9 – 8.5)***++	0.6 (0.4 - 0.7)	0.4 (0.3 - 0.5)
WBC (*10⁹/l)	13,2 ± 5,5***+++	6.6 ± 1.0	6.6 ± 1.5
PCT (μg/l)	0.16 (0.13 – 0.84)***+	0.05 (0.05 - 0.05)*	0.03 (0.02 - 0.03)
Albumin (g/l)	36.5 ± 7.8***+++	48.4 ± 4.1	47.1 ± 3.1

AP1: acute pankreatitis- first sampling, CON: healthy controls, PAP: controls 2-3 years after AP; M: male, F: female, TC: total cholesterol, TG: triacylglycerols, α-AMS: panceatic α-amylase, ALT: alanin-amino-transferase, AST: Aspartat-amino-transferase, GGT: γ-glutamyl-transferase, PCT: procalcitonin, WBC: white blood cells; * AP or PAP vs. CON, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; + AP vs. PAP, + $p < 0.05$; ++ $p < 0.01$, +++ $p < 0.001$

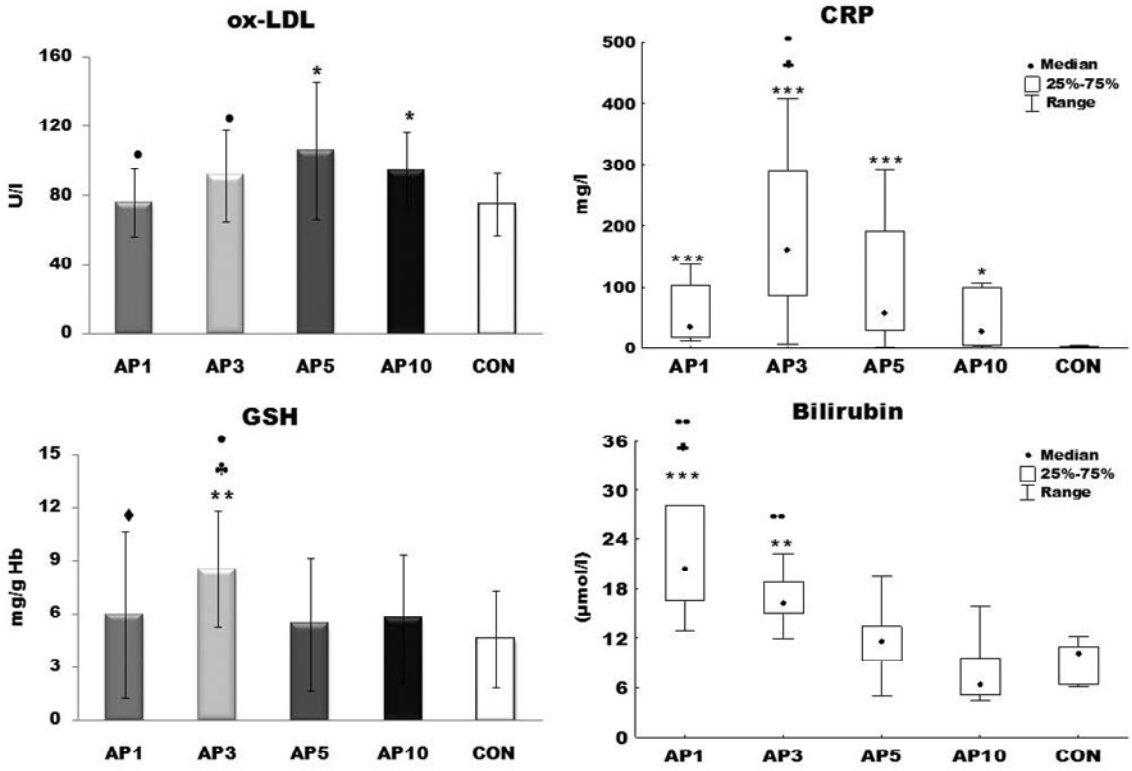

Fig. 1. Parameters of oxidative stress and antioxidant status in course of acute pancreatitis

ox-LDL: oxidized LDL, CRP: C-reactive protein, GSH: reduced glutathione; AP: patients with acute pancreatitis (1, 3, 5, 10: days of sampling), CON: healthy controls; * AP group vs. CON, * $p < 0.05$, ** $p < 0.01$; ♣ AP1 or AP3 or AP5 vs. AP10, ♣ $p < 0.05$; ♣♣ $p < 0.01$ • AP1 or AP3 vs. AP5, •$p < 0.05$, ••$p < 0.01$; ♦ AP1 vs. AP3, ♦ $p < 0.05$, ♦♦ $p < 0.01$;

GPX1 během všech AP odběrů v porovnání s CON, a dále pak snížená hodnota GPX1 u PAP ku CON (obr. 3). U CAT byla pozorována zvýšená aktivita u pacientů s AP během 1., 3. a 5. dne při srovnání s PAP ($p < 0,05$). Aktivita CAT při AP10 se již signifikantně nelišila od PAP, ale zato byl pozorován trend ke sníženým hodnotám vůči CON ($p = 0,06$). Při ostatních odběrech byla CAT u AP srovnatelná s hodnotami CON. Pro aktivity GR a CuZnSOD nebyly zjištěny žádné rozdíly mezi kontrolními skupinami a AP.

Z neenzymatických antioxidantů byla sledována koncentrace GSH, která byla signifikantně nejvyšší 3. den AP (obr. 1) a hladiny sérového albuminu (Tabulka 1) a bilirubinu. Hladiny albuminu byly u všech AP odběrů signifikantně snížené oproti oběma kontrolním skupinám a mezi sebou se nelišily. Koncentrace bilirubinu byly nejvyšší při záchytu AP a postupně docházelo k jejich poklesu (obr. 1). Dále pak byla stanovována koncentrace vitaminů E a A při AP1 a srovnávána s oběma kontrolními skupinami (obr. 3), koncentrace obou vitaminů byla snížená u AP1 ve srovnání s CON.

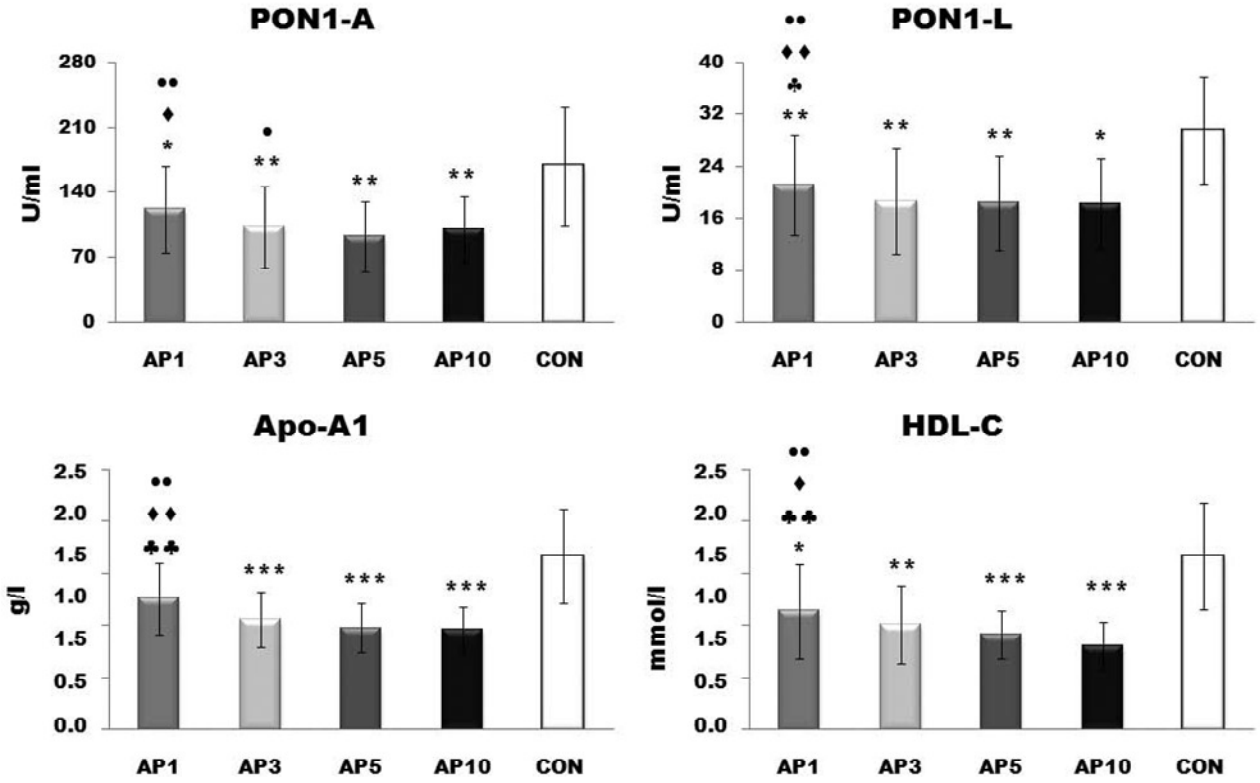

Fig. 2. Paraoxonase and its associate parameters in course of acute pancreatitis

PON1-A: arylesterase activity of paraoxonase 1, PON1-L: lactonase activity of paraoxonase 1, HDL-C: high density lipoprotein, Apo-A1: apolipoprotein A1; AP: patients with acute pancreatitis (1, 3, 5, 10: days of sampling), CON: healthy controls; * AP group vs. CON, * $p < 0.05$, ** $p < 0.01$; ♣ AP1 or AP3 or AP5 vs. AP10, ♣ $p < 0.05$; ♣♣ $p < 0.01$ • AP1 or AP3 vs. AP5, •$p < 0.05$, ••$p < 0.01$; ♦ AP1 vs. AP3, ♦ $p < 0.05$, ♦♦ $p < 0.01$;

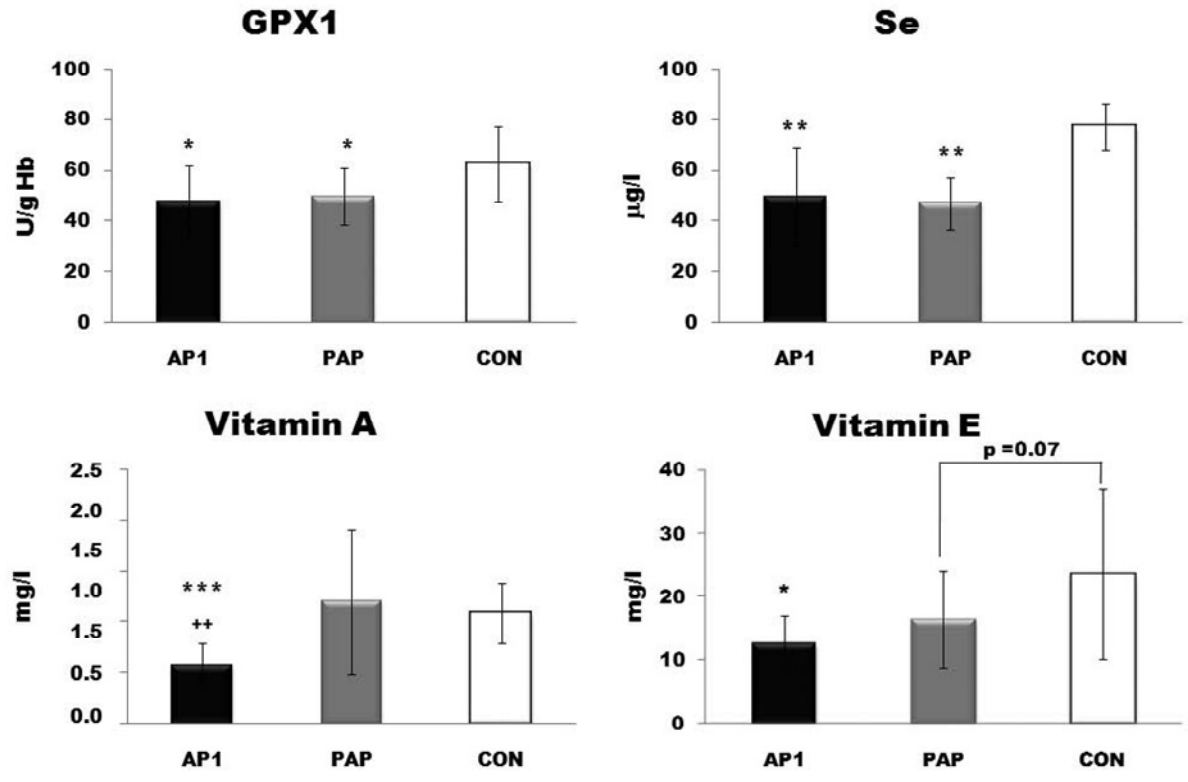

Fig. 3. Antioxidants in acute pancreatitis

GPX1: glutathione peroxidase 1, AP1: patients with acute pancreatitis, CON: healthy controls, PAP controls 2-3 years after AP; * AP or PAP vs. CON, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; $^{+}$ AP or R vs. CON, $^{++}$ $p < 0.01$

Diskuse

V naší studii jsme se zaměřili na sledování jednotlivých komponent antioxidačního systému a měření markerů peroxidace v průběhu AP. Naše výsledky ukazují na zvýšený oxidační stres u tohoto onemocnění, který je doprovázen změnou ve fungování některých složek antioxidačního systému. Největší změny je možno pozorovat v arylesterázové a laktonázové aktivitě PON1 a v aktivitě GPX1, dále pak v koncentraci vitaminů A a E.

U některých antioxidačních enzymů jsme však nepozorovali žádné změny spojené s onemocněním AP. K těmto enzymům se řadí CuZnSOD, u které byly hodnoty aktivit téměř konstantní v průběhu AP. Nepozorovali jsme ani rozdíl mezi aktivitou CuZnSOD u AP a u kontrolních skupin. Dosud publikované výsledky aktivit CuZnSOD v erytrocytech u pacientů s AP jsou nejednotné. Byly publikovány jak snížené [6, 7], tak zvýšené [8] aktivity CuZnSOD u pacientů s těžkou i lehkou formou AP. Zvýšená aktivita extracelulární SOD (EC-SOD) byla pozorována v průběhu AP (1., 3., 7.den) v porovnání s kontrolami [9], kdy 1. den byla signifikantně vyšší než 3. a 7. den. Zvýšenou EC-SOD u AP při srovnání s kontrolami pozorovali ve svých studiích i Góth (1982, 1989) [10, 11] a Szuster-Czielska (2001a) [12]. CuZnSOD má v organismu za úkol odbourávat superoxidový radikál, ze kterého při této reakci vzniká peroxid vodíku, za jehož degradaci jsou zodpovědné CAT, GPX1 a peroxiredoxiny. Při nízkých – fyziologických – koncentracích je H_2O_2 odbouráván GPX1 a peroxiredoxiny, naopak při zvýšeném oxidačním stresu a vyšších koncentracích je za odbourávání odpovědná CAT [13].

V naší studii byly aktivity CAT 1., 3. a 5. den srovnatelné s hodnotami zdravých kontrol, ale signifikantně se lišily od hodnot získaných u skupiny osob, které AP prodělaly před 2-3 lety. Při odběru prováděném desátý den (AP10) byl pozorován signifikantní pokles v aktivitách CAT ve srovnání s AP1 a i se zdravými kontrolami, i když zde je možno mluvit pouze o trendu. Tyto výsledky ukazují, že při dlouhodobém vystavení CAT působení oxidačního stresu, může dojít k poklesu její aktivity. Kirkman a Gaetani (1987) ve své studii ukázali, že dlouhodobé vystavení CAT působení H_2O_2 může vést k oxidaci NADPH na $NADP^+$ a následnému snížení aktivity CAT až na 1/3 její původní aktivity [14]. Ve studii, která se zabývala erythrocytární aktivitou CAT nebyly pozorovány žádné významné rozdíly mezi pacienty s AP a kontrolami [8]. Doposud získané výsledky aktivity CAT v séru ukazují zvýšené aktivity u pacientů s AP ve srovnání s kontrolní skupinou [10 – 12, 15].

Degradace H_2O_2 není jedinou funkcí GPX1, dále je také zodpovědná za odbourávání lipidových peroxidů. Glutationperoxidáza 1 je selenoprotein, jehož aktivita je závislá nejen na dostatku selenu, ale ke své funkci potřebuje GSH jako druhý substrát. U pacientů s AP jsme pozorovali snížené koncentrace selenu a snížené aktivity GPX1 ve srovnání s CON. Aktivita GPX1 byla snížená u všech odběrů AP a také u skupiny PAP. Ve studii, kde se zabývali aktivitou GPX1 v erythrocytech v průběhu AP, pozorovali sníženou hladinu GPX1 u AP až při odběru 9. den AP [16]. V séru byly pozorovány snížené hladiny GPX1 u pacientů s AP vzhledem ke kontrolám již v několika dřívějších studiích [17 – 19], i když existuje i studie, kde nenašli žádný rozdíl mezi pacienty a kontrolami [12]. U koncentrací GPX1 v séru nebyl nalezen rozdíl mezi AP a ambulantními kontrolami [20]. Také snížené koncentrace Se v séru u pacientů s AP byly již dříve publikovány [16, 19], i když opět ne ve všech pracech [21].

Koncentrace GSH byla u našich pacientů s AP srovnatelná s koncentracemi u CON, pouze při odběru 3. den nemoci (AP3) bylo pozorováno zvýšení koncetrace GSH oproti ostatním odběrům AP i oproti CON. Na rozdíl od naší studie Rahman et al. (2004, 2009) [22, 23] ve svých studiích pozoroval snížené hladniny GSH v erytrocytech u lehké i těžké formy AP ve srovnání s kontrolní skupinou, stejně tak pro GSH v séru byly pozorovány signifikantně snížené koncentrace u AP v porovnání s CON [17]. Možným vysvětlením zvýšených hladin GSH u AP3 je obranná reakce organismu na aktuálně vzniklý oxidační stres, ale i možná desynchronizace aktivit GPX1 a GR v období 2. odběru (AP3).

S GPX1 spolupracuje v organismu GR, která udržuje hladinu GSH zpětnou redukcí oxidovaného glutationu vzniklého působením GPX1. V naší studii jsme nepozorovali žádné signifikantní změny v aktivitě GR v průběhu akutní pankreatitidy a nezjistili jsme ani žádný rozdíl při srovnání pacientů s AP s kontrolními skupinami, tento výsledek je ve shodě s již dříve publikovanou studií [17].

Dalšími sledovanými antioxidanty byly vitaminy A a E. Koncentrace obou vitaminů byla signifikantně snížená u pacientů s AP ve srovnání s CON. Snížené hladiny vitaminu A byly pozorovány též ve studii Musil et al. (2005) [12], zatímco u koncentrace vitaminu E nebyl nalezen žádný rozdíl [12, 21].

Posledním sledovaným antioxidačním enzymem byla s HDL asociovaná paraoxonáza, u níž byly měřeny dvě její různé aktivity, a to arylesterázová a laktonázová. Obě tyto aktivity byly v celém průběhu AP signifikantně snížené oproti zdravým kontrolám. U obou aktivit též došlo k dalšímu snížení v rámci odběrů AP3 a AP5, kdy arylesterázová aktivita dosáhla svého minima u odběru 5. den AP. V tento den byly naměřeny též nejvyšší koncentrace oxidovaných-LDL, jako markeru lipidové peroxidace. Kinetika změn aktivit PON v průběhu AP odpovídá změnám aktivit PON1, které byly pozorovány v průběhu sepse a během jejího zotavování, a které mají zřejmě obecnější zákonitosti [24].

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Antioxidant Status and Oxidative Stress Markers in Pancreatic Cancer and Chronic Pancreatitis

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Objectives: Oxidative stress has been implicated in the pathogenesis of chronic pancreatitis (CP) and pancreatic cancer (PC). The study aim was to assess the oxidative stress markers and antioxidant defense system in patients with CP and those with PC.

Methods: Activities of superoxide dismutase 1 (SOD1), catalase (CAT), glutathione peroxidase 1 (GPX1), glutathione reductase (GR), arylesterase (PON1-A) and lactonase (PON1-L) activities of paraoxonase 1 (PON1) and concentrations of reduced glutathione, conjugated dienes in low-density lipoprotein (CD/LDL) and oxidized LDL (ox-LDL/LDL) were assessed in 50 PC and 50 CP patients and 50 age- and sex-matched controls.

Results: Comparison of PC and CP groups to controls found the following changes: glutathione peroxidase 1 (GPX1) (−20.2%, −25.5%; $P < 0.001$), glutathione reductase (GR) (−9.5%, −11.9%; $P < 0.05$), SOD1 (+22.9%; $P < 0.01$), CAT (−10.6%; $P < 0.05$), PON1-A (−34.3%, −16.0%; $P < 0.001$) PON1-L (−44.2%; −17.6%; $P < 0.01$), conjugated dienes in LDL (CD/LDL) (+20%, +33.3%; $P < 0.05$) and ox-LDL/LDL (+42.2%, +14.4%; $P < 0.05$). The patients with PC had changed activities and levels of SOD1 (+24.2%), CAT (−10.4); $P < 0.01$), PON1-A (−21.7%), PON1-L (−32.9%), and ox-LDL/LDL (+24.3%); (all $P < 0.01$) compared with the patients with CP.

Conclusions: Reduced antioxidant defense system capacity and increased markers of oxidative stress were found in PC and CP. PON1-L and CAT activities, along with ox-LDL/LDL levels, were the independent factors differentiating the patients with PC from the patients with CP.

Key Words: oxidative stress, oxidative stress markers, antioxidant enzymes, chronic pancreatitis, pancreatic cancer, discriminant analysis

Abbreviations: CAT – catalase, CD – conjugated dienes, CP – chronic pancreatitis, CT – computed tomography, EUS – endoscopic ultrasonography, GPX1 – glutathione peroxidase 1, GR – glutathione reductase, GSH – reduced glutathione, HDL – high-density lipoprotein, HOMA-IR – homeostasis model assessment of insulin resistance, LDL – low-density lipoprotein, MDA – multivariate discriminant analysis, MRCP – magnetic resonance cholangiopancreatography, MRI – Nutritional Risk Index, NT – nitrotyrosine, ox-LDL – oxidized LDL, PC – pancreatic carcinoma, PON – paraoxonase 1, PON1-A – PON1 arylesterase, PON1-L – PON1 lactonase, RONS – reactive oxygen and nitrogen species, ROS – reactive oxygen species, SAA – serum amyloid A, SOD1 – Cu-Zn superoxide dismutase

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Overproduction of reactive oxygen and nitrogen species (RONS) and oxidative stress have been implicated in the pathogenesis of pancreatitis, both in its acute and chronic form, as well as in the pathogenesis of pancreatic cancer (PC).[1] Chronic pancreatitis (CP) shares risks with PC such as smoking and alcohol abuse as well as being a risk factor per se for PC.[1–3] Among them, cigarette smoking, alcohol abuse, diabetes mellitus, and other insulin resistance (IR) states are connected with increased RONS formation and oxidative stress.[1] Chronic pancreatitis is a progressive inflammatory disease with irreversible damage to the pancreas and the destruction of exocrine and endocrine tissue.[4] The underlying causes of CP seem to be multifaceted, including environmental as well as genetic factors, but its pathogenesis to date has not been completely understood. Although most cases of CP have been attributed to alcohol abuse and/or genetic predisposition, other etiologic risk factors such as enhanced oxidative stress could play an important role.[5,6]

Reactive oxygen and nitrogen species are generated during endogenous oxidative stress that is linked to the pancreatic renin-angiotensin system[7] or exogenous oxidative stress caused by environmental or lifestyle-related xenobiotics, which is connected with the detoxification system.[3] It has been proposed that local oxidative stress and reactive oxygen species (ROS) generation, caused by overexpression of membrane nonmitochondrial nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase, is connected with pancreatic (patho)biology such as cell growth regulation and apoptosis, acinar cell inflammation, fibrosis, and disturbed islet microcirculation.[7,8] The inflammatory process is associated with increased production of RONS resulting in local or systemic oxidative stress.[9,10] A number of experimental and clinical studies have demonstrated impaired antioxidant status that may be a contributing factor for increasing oxidative stress in CP. The involvement of oxidative stress in CP has been described both in experimental and clinical studies.[1–3]

Oxidative stress could not only be the cause of CP (and PC) but also a consequence of the underlying disease (CP or PC, respectively). Moreover, increased RONS production and oxidative stress seem to be independent from the etiology of CP.[1,11]

In patients with CP, decreased levels of antioxidant thiols (cysteine, glutathione, and cysteinylglycine), decreased total antioxidant capacity, along with increased carbonylated proteins, thiobarbituric acid-reactive substances, malondialdehyde and 4-hydroxynonenal levels were found.[10,12–14] Similarly, in patients with CP (both alcoholic and tropical), decreased concentrations of glutathione, vitamin C, and zinc in erythrocytes were connected with elevated thiobarbituric acid–reactive substances.[15]

Levels of conjugated dienes (CD) are the most sensitive indicator of lipid peroxidation and can be regarded as a global marker of systemic oxidative stress[16] and also are a marker of minimally oxidatively modified low-density lipoprotein (LDL). On the contrary, oxidized LDL (ox-LDL) reflects concentration of malondialdehyde and 4-hydroxynonenal, the highly reactive

end products of lipid peroxidation that are bound, as adducts, to ε-amino group of lysine in molecule of apolipoprotein B.[17] Increased susceptibility of LDL to oxidation (LDL oxidability) was observed in CP.[18] Concentration of ox-LDL has not yet been studied in human PC.

Among enzymes that regulate RONS, glutathione peroxidase (GPX) and catalase (CAT) play an important role by the reduction of hydrogen peroxide, which is generated by superoxide dismutase (SOD) in the dismutation of superoxide. The main ROS scavenger in the pancreas is supposed to be reduced glutathione (GSH), which is recycled back to its reduced form by glutathione reductase (GR).[1,19] The enzyme paraoxonase 1 (PON1) as high-density lipoprotein (HDL)-associated enzyme is implicated in the anti-inflammatory and antioxidant activities of HDL and impedes oxidative modification of LDL, protects cell membranes from the damage caused by products of lipoperoxidation, and eliminates carcinogenic lipid-soluble radicals.[20–22]

The activities of some antioxidant enzymes in CP were already studied. SOD activity in the studies by Girish et al,[15] Quillot et al,[18] and Szuster-Ciesielska et al[23] and GPX1 activity in the studies by Quillot et al[18] and Szuster-Ciesielska et al.[18,23] These studies show lowered antioxidant capacity in CP.

The aim of the study was to ascertain the importance of lipoperoxidation markers (CD and ox-LDL) in relation to the group of main antioxidant enzymes, such as SOD, CAT, GPX1, GR, and PON1 in patients with CP and PC. Because there are intercorrelations between oxidative stress markers and antioxidant enzymes activities, both in CP and PC groups, we used the multivariate discriminant analysis to differentiate PC from CP as well as to evaluate the discriminative power of different oxidative stress markers and antioxidant enzymes.

MATERIALS AND METHODS

This prospective study was carried out at the Fourth Department of Internal Medicine of General University Hospital from January 2009 to September 2011. The study protocol was approved by the institutional review board and the Ethics Committee of the General University Hospital in Prague. Written informed consent was obtained from all participants.

Patients

The study population consisted of 3 groups: 50 patients with PC, 50 patients with CP, and 50 healthy controls. All groups are age and sex matched.

Diagnosis of PC was confirmed in all of the patients (based on histological examination of pancreatic resection or endoscopic ultrasonography-guided aspiration cytology). The tumor staging was evaluated by the combination of criteria issued by the Union Internationale Contre le Cancer and the American Joint Committee on Cancer (UICC/AJCC 2002).[24] The clinical diagnosis of CP was based on clinical features (abdominal pain, nausea and/or vomiting, anorexia and/or malnutrition, and steatorrhea) confirmed by 2 or more imaging methods (abdominal ultrasonography [USG], contrast-enhanced computed tomography [CT]), endoscopic retrograde cholangiopancreatography, magnetic resonance cholangiopancreatography (MRCP), and endoscopic ultrasonography (EUS). Only patients with definite CP were included. The grade of CP (mild, moderate, or severe) was assessed according to the M-ANNHEIM pancreatic imaging criteria[25] (M-ANNHEIM stands for M, multiple risk factor classification; A, alcohol consumption; N, nicotine consumption; N, nutritional factors; H, hereditary factors; E, efferent pancreatic duct factors; I, immunological factors; M, miscellaneous and metabolic factors). All the patients were assessed by the combination of EUS and other imaging methods (CT, or USG, or MRCP) because EUS does not differentiate between the moderate and severe grades, and other methods (CT, or USG, or MRCP) cannot differentiate between mild and moderate changes.[25]

Exclusion criteria for all the 3 groups were the following: current antioxidant therapy (eg, vitamin C, vitamin E, allopurinol, *N*-acetylcysteine, supplementation with n-3 polyunsaturated fatty acids), kidney disease (creatinine >150 μmol/L), clinically manifest proteinuria (urinary protein >500 mg/L), and liver cirrhosis, decompensate diabetes mellitus, concomitant malignancies, chronic, immunosuppressive, and anti-inflammatory therapy, as well as chemotherapy. Further criteria for exclusion were the following: endocrine disease, acute pancreatitis, or acute relapse of CP; unstable angina pectoris, stage within 1 year after acute myocardial infarction; coronary aortic bypass grafting, or percutaneous coronary intervention, and stroke. Persons who were operated on in the upper gastrointestinal tract (in the previous year) and subjects after systemic inflammation in the previous 6 months were also excluded. Patients with CP enrolled into the study were reexamined after 2 years to exclude the development of PC and thus to avoid enrollment of patients with initial stages of PC into the study.

Among our 50 patients with PC, there were 22 patients with diabetes. In this group were the following stage distributions: 9 patients with stage II (2 patients with stage IIA and 7 patients with stage IIB) disease, 24 patients with stage III disease, and 17 patients with stage IV disease. Alcoholic CP was diagnosed in 38 patients, obstructive CP in 5 subjects, and idiopathic CP in 7 patients. Among the 50 patients with CP, there were 30 patients with severe grade, 17 patients with moderate, and 3 patients with mild grade changes in pancreatic morphology. Severe exocrine dysfunction (concentration of pancreatic stool elastase 1 <200 ng/g) was found in 29 patients with CP. Complications (ascites, bleeding, obstruction or stricture ductus choledochus, pancreatic fistula, duodenal stenosis, splenic and/or portal vein thrombosis, and segmental portal hypertension) were found in 28 patients with CP.

Data Collection

Samples from all participants were obtained after overnight fast (at least 10 hours). All study participants' medical history and intake of any medications were documented at study entry. Blood was taken by puncturing a peripheral vein. Concentrations of C-reactive protein (CRP), conjugated dienes (CD/LDL) in precipitated LDL, serum amyloid A (SAA), 3-nitrotyrosine (NT), tumor markers (CA 19-9, CA 72-4, and CEA), albumin, bilirubin, uric acid, calcium, copper, zinc, iron, selenium, vitamins A and E, and lipid parameters, as well as activity of routine biochemical tests (pancreatic amylase, alanine transaminase, aspertate aminotransferase, γ-glutamyltransferase, cholinesterase alkaline phosphatase [data not shown]), PON1 arylesterase (PON1-A), PON1 lactonase (PON1-L), and oxidized-LDL (ox-LDL) were measured in serum. Serum was prepared after coagulation in vacutainer tubes by centrifugation at 3500 rpm at 4°C for 10 minutes. Activities of antioxidant enzymes CAT, GPX1, GR, and Cu-Zn superoxide dismutase (SOD1), as well as the concentration of GSH were measured in hemolyzed erythrocytes. The samples were stored at −80°C until assay. All samples were marked with unique identification numbers made anonymous, and the data were merged only after the assays had been completed.

Laboratory Measurements

Activities of antioxidant enzymes were determined by spectrophotometric kinetic methods, and the concentration of

GSH were assessed spectrophotometrically as previously described by Kodydková et al.[26] The lactonase activity of PON1 was measured according to the modified method described earlier[27] using dihydrocoumarin (final concentration, 1 mmol/L) as a substrate. The increase in absorbance at 270 nm was monitored for 2 minutes. The enzyme activity was calculated from the molar extinct coefficient of the reaction product [3-(2-hydroxyphenyl)-propionate ($\varepsilon = 1295$ dm^3 mol^{-1} cm^{-1})] estimated during the linear phase of reaction.[28] The concentration of CD in precipitated LDL was determined by the Wieland modified spectrophotometric method at 234 nm.[29,30]

The levels of SAA, 3-NT, and ox-LDL were established using sandwich enzyme-linked immunosorbent assay kits (Invitrogen, Camarillo, Calif; Biovendor, Brno, Czech Republic, Czech Republic; and Mercodia, Uppsala, Sweden; respectively).

All routine clinical tests were measured at the Institute for Clinical Biochemistry and Laboratory Diagnostics of General University Hospital in Prague. Concentration of CRP was measured by the immunoturbidimetric method using a K-ASSAY CRP kit (Kamiya Biomedical Company, Seattle, Wash) on a Hitach. Modular analyzer (Tokyo, Japan). Tumor markers (CEA, CA 19–9, and CA 72–4) were measured by chemiluminescence assay on ADVIA Centaur analyzer, Siemens (Tarrytown, NY). Selenium, copper, and zinc were measured using atomic absorption spectrometry. Concentrations of total cholesterol and triglycerides were measured by enzymatic-colorimetric methods. High-density lipoprotein cholesterol was determined in the supernatant after precipitation of lipoproteins B by phosphotungstic acid/Mg^{2+} (Boehringer Mannheim, Germany); LDL cholesterol was calculated according to the Friedewald formula. Apolipoprotein B and apolipoprotein A1 were measured by the Laurell rocket electroimmunoassay using standards and specific antibodies (Beluingwerke Marburg, Germany). The homeostasis model assessment of insulin resistance (HOMA-IR) index was calculated as HOMA-IR = [fasting serum glucose (mmol/L) × fasting serum insulin (μU/mL)] / 22.5.[31] Malnutrition was categorized into the mild, moderate, and severe forms according to the Nutritional Risk Index (NRI).[32] The NRI was calculated according to the following formula: NRI = (1.519 × albumin + 41.7 × current body weight/usual body weight), and the classification was as follows: normal nutrition, NRI > 100; mild malnutrition, NRI = 97.5–100; moderate malnutrition, NRI = 83.0–97.4; severe malnutrition, NRI < 83.0.

Statistical Analysis

Data are expressed as mean ± SD for parametric and as median and interquartile range (25th–75th percentiles) for nonparametric data. Normality of data distribution was tested with the Shapiro-Wilks *W* test. Differences between the compared groups (PC, CP, and controls) were tested with the one-way analysis of variance with Scheffé and Newman-Keuls post-tests. For nonparametric analysis, the Kruskal-Wallis analysis of variance was used. For correlation analysis the Spearmen coefficient was used. All previously described statistical analyses were performed using StatSoft Statistica version 9.0 software (2007, Czech Republic). $P < 0.05$ was considered statistically significant.

The multivariate discriminant analysis (MDA) was carried out by using Statistical Analysis System (SAS, Cary, NC), JMP version 9 software. The process of MDA is carried out in a stepwise manner using the minimum Wilks λ (within-groups sum of squares–total sum of squares ratio) as a measure of group discrimination. At each step in the process, the variable, which contains the most discriminating power, is identified and its coefficient is determined. The relative importance of each variable is indicating by so-called approximate F statistic. This is a transformation of Wilks λ, which can be compared with F distribution. The process is stopped when the remaining variables are determined to lack significant discriminating power ($P > 0.05$). We used discriminant models for classification into the groups of PC and CP. Only variables with an appropriate final F statistic ($P < 0.05$) were included in our discriminant functions. The oxidative stress and inflammatory markers (CD/LDL, ox-LDL/LDL, and SAA), the antioxidant enzymes (SOD1, CAT, GPX1, GR, PON1-A, PON1-L), and their combinations as variables were subjected into the discriminant functions.

RESULTS

This study was focused on the antioxidant status in patients with PC and CP. Fifty patients with PC and 50 patients with CP were enrolled in the study. The basic clinical and biochemical data of the studied groups are summarized in Table 1.

As markers of oxidative stress, the levels of CD/LDL, ox-LDL/LDL, and NT were determined. The levels of CD/LDL and ox-LDL/LDL were significantly increased in both the patients with PC and those with CP compared with the controls (Figs. 1A, B). Furthermore, the patients with PC had higher levels of ox-LDL/LDL than the patients with CP ($P < 0.001$). There was an increase in the concentration of 3-NT only in the patients with CP ($P < 0.01$) in comparison with the controls (Fig. 1C).

Erythrocyte activity of SOD1 was increased and CAT activity was decreased in the patients with PC compared with the patients with CP and the controls (Figs. 2A, B). In addition, the serum concentration of SOD1 cofactor Cu was observed as elevated; and the serum concentration of Fe, the CAT cofactor, was decreased in the patients with PC in comparison with the controls (Table 2). The concentration of Zn was increased in the patients with CP compared with the controls. Conversely, decreased erythrocyte activities of GPX1 and GR were found in patients with PC and those with CP as compared with the controls (Figs. 2C, D). The decreased GPX1 activities in both the PC and CP groups were accompanied with lower serum selenium levels compared with the controls (Table 2). The concentration of GSH in erythrocytes in the patients with PC differed from that of the subjects with CP and controls, respectively (Fig. 1D). We have found that serum concentrations of Zn negatively correlate with activity of SOD1 in the entire group ($r = -0.312$; $P < 0.001$), in the patients with PC ($r = -0.357$; $P < 0.05$) and also in the patients with CP ($r = -0.458$; $P < 0.001$). There was a significant correlation between Se and GPX1 in the entire group ($r = 0.319$; $P < 0.01$) and also in the patients with CP ($r = 0.470$; $P < 0.01$).

The PON1-A and PON1-L activities in serum were decreased in the patients with PC and those with CP in comparison with the controls (Figs. 2E, F). Furthermore, decreased activities of these enzymes in the patients with PC compared with the patients with CP (both $P < 0.001$) were observed. Both PON1 activities were significantly correlated in the PC ($r = 0.711$; $P < 0.001$) and CP ($r = 0.811$; $P < 0.001$) groups and in the controls ($r = 0.687$; $P < 0.001$) as well as in all the studied groups ($r = 0.806$; $P < 0.001$). The levels of both PON1 activities correlated negatively with ox-LDL/LDL ($r = -0.309$; $P < 0.001$; and $r = -0.358$; $P < 0.001$; respectively) in the entire group.

Serum amyloid A concentrations were higher in the patients with PC than in the patients with CP and in the controls (both $P < 0.001$; Table 1). The studied groups did not differ in concentrations of PON1 cofactor—calcium. Additionally, we

TABLE 1. Basal Clinical and Biochemical Characteristics

	PC	CP	CON
No. patients (M/F)	50 (40/10)	50 (40/10)	50 (40/10)
Age (range), yrs	63 (56–68)	59 (55–65)	60 (55–65)
Smokers, n (%)	34 (68)	15 (30)	13 (26)
DM, no. patients (M/F)	28 (24/4)	30 (22/7)	–
NRI, kg/m^2	96.4 ± 12.3*	109.1 ± 7.3	–
CRP, mg/L	10.9 (5.8–54.8)***; +++	4.5 (2.3–10.3)*	2.1 (1.0–4.9)
SAA, μg/mL	49.0 (21.7–134.2)***; +++	14.5 (7.2–49.8)	12.7 (4.6–25.6)
CEA, μg/L	2.75 (1.65–6.45) ***	2.37 (1.44–3.42)***	0.71 (0.5–1.45)
CA 19–9, kU/L	105.2 (24–2301.3)***; +++	14.6 (8.5–26.5)*	8.7 (6.1–10.7)
CA 72–4, kU/L	2.32 (1.31–9.70)*; +*	1.45 (0.97–2.23)	1.44 (1.02–3.86)
Glucose, mmol/L	6.5 (5.2–8.9) ***	6.6 (5.3–7.6)***	5.1 (4.9–5.3)
Glycated hemoglobin, mmol/mol	5.46 ± 1.85***	5.11 ± 1.49***	3.91 ± 0.34
TC, mmol/L	4.7 (3.7–6.6)*	4.9 (4.4–6.0)	5.4 (4.6–5.9)
TG, mmol/L	1.67 (1.26–2.12) ***	1.48 (1.10–2.01)***	1.06 (0.79–1.34)
HDL-C, mmol/L	0.92 (0.73–1.05)***; +++	1.42 (1.21–1.59)	1.5 (1.27–1.73)
LDL-C, mmol/L	2.76 (2.12–3.24)*	2.75 (2.34–3.17)*	3.33 (2.72–3.73)
Apo A1, g/L	0.88 ± 0.32***; +++	1.61 ± 0.44	1.53 ± 0.30
NEFA, mmol/L	0.71 ± 0.35***	0.62 ± 0.40*	0.48 ± 0.24

Data are expressed as mean ± SD for parametric variables, and as median and interquartile range (IQR, 25th–75th percentile) for nonparametric variables;

PC or CP versus CON: ***$P < 0.001$, **$P < 0.01$, and *$P < 0.05$; PC versus CP: +++$P < 0.001$, +$P < 0.05$ (one-way analysis of variance (ANOVA) with Newman-Keuls posttest), and ++$P < 0.01$.

Apo-A1, apolipoprotein A1; CA, carbohydrate antigen; CEA, carcinoembryonic antigen; CON, control subjects; NEFA, nonesterified fatty acids; TC, total cholesterol; TG, triglycerides.

have observed statistically decreased concentrations of vitamin A, albumin, and uric acid and higher levels of bilirubin in the patients with PC compared with the patients with CP and the controls (Table 2).

Discriminant models for classification into the groups of PC and CP are shown in Table 3. The concentrations of ox-LDL and CD/LDL were the best discriminators (model A) when only oxidative stress and inflammatory markers were entered into the

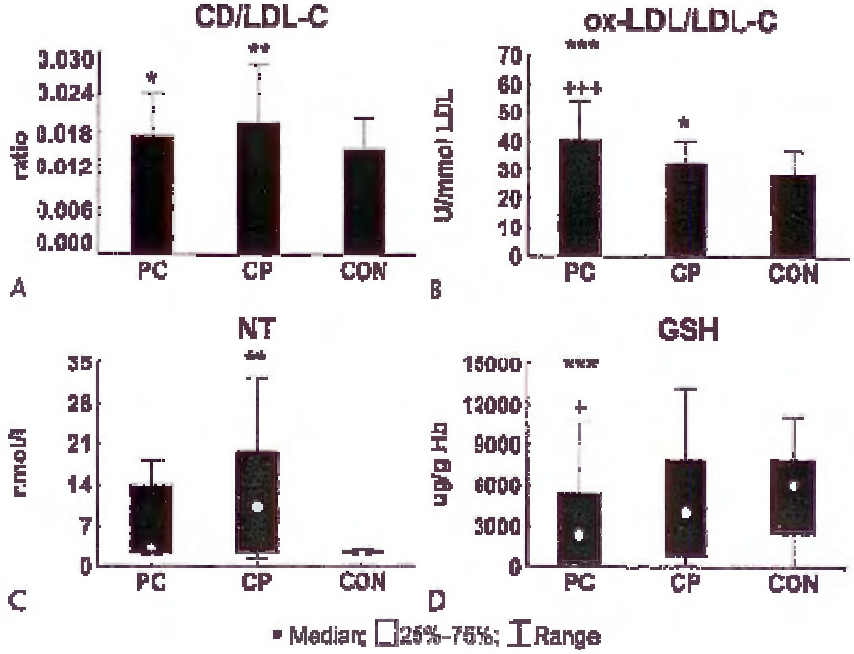

FIGURE 1. Serum concentration of oxidative stress markers and reduced glutathione. Data are expressed as mean ± SD for parametric variables and as median and IQR (25th–75th percentile) for nonparametric variables. PC or CP versus CON: ***$P < 0.001$, **$P < 0.01$, and *$P < 0.05$; PC versus CP: ***$P < 0.001$ and +$P < 0.05$ (one-way ANOVA with Newman-Keuls posttest or Kruskal-Wallis ANOVA).

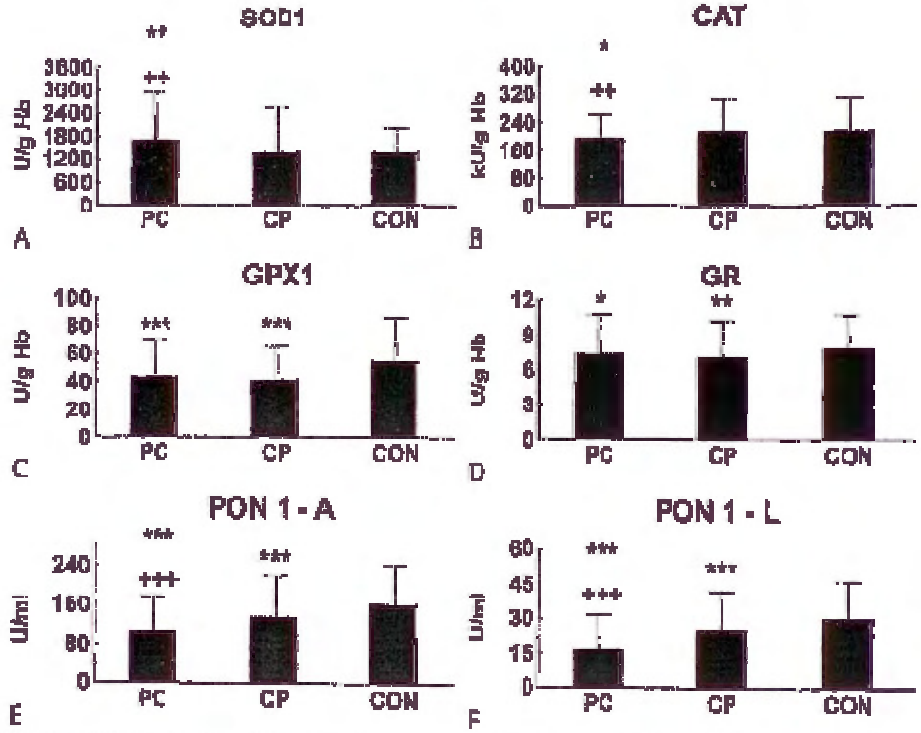

FIGURE 2. Activities of antioxidant enzymes. Data are expressed as mean ± SD; PC or CP versus CON: $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$; PC versus CP: $^{++}P < 0.01$ (one way ANOVA, Newman-Keuls posttest) and $^{+++}$ and $^{+++}P < 0.001$ $P < 0.001$.

MDA. Using only antioxidant enzymes as discriminating variables resulted in 73% of the final correct classification. Variables subjected into the analysis were PON1-L, SOD1, and CAT (model B). A combination of both models resulted in 83% of the final correct classification. The activity of PON1-L, the levels of ox-LDL/LDL, and the activity of CAT, in that order, were found to be the best set of independent factors discriminating PC and CP, with higher values for PC (model C).

Table 2 presents data of nonenzymatic antioxidants and of cofactors of antioxidant enzymes.

DISCUSSION

The present study demonstrates contemporary changes in the levels of selected inflammatory and oxidative stress markers as well as a set of the antioxidant defense system (both enzymatic and nonenzymatic) in the patients with PC and those with CP in comparison with age- and sex-matched controls. The reduced capacity of the antioxidant defense system and an increased oxidative stress in patients with PC and those with CP was confirmed in our study. The antioxidant system seems to be more affected in the patients with PC compared with the patients with CP.

The groups of CP and PC differed statistically significantly in many oxidative stress and antioxidant variables using univariate analysis. Moreover, there were intercorrelations between oxidative stress markers and activities of antioxidant enzymes. Therefore, multivariate discriminant analysis was performed to determine the set of independent oxidative stress

TABLE 2. Nonenzymatic Antioxidants, Cofactors of Antioxidant Enzymes, and Other Parameters of Antioxidant Capacity

	PC	CP	CON
Vitamin A, mg/L	0.51 ± 0.24$^{**; +}$	0.80 ± 0.41	0.83 ± 0.35
Vitamin E, mg/L	16.0 ± 7.7	12.3 ± 5.0	14.4 ± 6.1
Fe, μmol/L	13.5 ± 8.3**	16.4 ± 7.2	19.2 ± 7.9
Ca, mmol/L	2.26 ± 0.16	2.24 ± 0.13	2.27 ± 0.13
Cu, μmol/L	21.9 ± 6.3$^{***; +++}$	18.8 ± 4.5	15.6 ± 3.1
Zn, μmol/L	19.2 ± 4.7	19.9 ± 3.9*	17.8 ± 2.6
Zn/Cu	0.86 ± 0.34$^{***; +++}$	1.13 ± 0.38	1.15 ± 0.40
Se, μg/L	31.3 ± 10.9**	42.1 ± 21.3*	58.9 ± 26.0
Albumin, g/L	41.4 (37.7–45.5)$^{***; +++}$	45.7 (42.3–46.9)	47.2 (44.9–48.6)
Bilirubin, μmol/L	18.7 (11.0–64.1)$^{***; +++}$	10.2 (7.2–14.2)	11.0 (8.0–15.3)
Uric acid, μmol/L	257 ± 108$^{**; ++}$	324 ± 90	310 ± 76

Data are expressed as mean ± SD for parametric variables and as median and IQR (25th–75th percentile) for nonparametric variables;
PC or CP versus CON: $^{***}P < 0.001$, $^{**}P < 0.01$, and $^{*}P < 0.05$; PC versus CP: $^{+++}P < 0.001$, $^{++}P < 0.01$, and $^{+}P < 0.05$ (one-way ANOVA with Newman-Keuls posttest).

TABLE 3. Discriminant Models for Classification of CP and PC

	F-Statistic (df)	Final Correct Classification	Percent
Model A			
ox-LDL/LDL	13.80 (1, 98)	CP	74.0
ox-LDL/LDL + CD/LDL	11.32 (2, 97)	PC	72.0
		Total	73.0
Model B			
PON1-L	31.77 (1, 98)	CP	68.0
PON1-L + SOD1	19.33 (2, 97)	PC	74.0
PON1-L + SOD1 + CAT	14.83 (3, 96)	Total	71.0
Model C			
PON1-L	31.77 (1, 98)	CP	80.0
PON1-L + ox-LDL/LDL	27.72 (2, 97)	PC	86.0
PON1-L + ox-LDL/LDL + CAT	21.32 (3, 96)	Total	83.0

Model A: Only oxidative stress and inflammatory markers included in the analysis (CD/LDL, Ox-LDL/LDL, and SAA). Model B: Only activities of antioxidant enzymes included in the analysis (SOD1, CAT, GPX1, GR, PON1-A, and PON1-L). Model C: combination of the model A and the model B.

df, degrees of freedom;

F statistic (so-called an approximate F statistic), transformation of Wilks λ.

and antioxidant variables giving the most discrimination power to separate CP and PC.

Multivariate discriminant analysis indicated that the activities of PON1-L and CAT, along with ox-LDL/LDL levels (in the order of PON1-L, ox-LDL/LDL, and CAT), are the independent factors discriminating the patients with PC and those with CP.

Antioxidant enzymes play an important role in the defense of cells against ROMS and thus may protect the pancreas against development of CP, which is a risk factor of PC. The first scavenger of ROS is SOD, which converts superoxide radical to H_2O_2, which should be later removed by GPX1 and CAT. Our study found increased SOD1 activity in erythrocytes of the patients with PC in comparison with the patients with CP and the controls. Some experimental studies have described relationships between the expression of SOD, its activity, and PC cell growth in vitro, on the one hand, and tumor growth and survival in nude mice, on the other.[8] The insignificant differences in the erythrocyte activities of SOD1 in the patients with CP and controls found in our study were consistent with the study of Quillot et al.[18] On the other hand, decreased SOD1 activity in the patients with CP was found in the study of Girish et al.[15] Inconsistent results concerning serum SOD activities in hereditary and alcohol-related pancreatitis have been published. Some reports have described increased[14,33] serum SOD activity, and in some studies, no differences in serum SOD activities were found.[18,34] It could be supposed that discordance in elevated activity of SOD1 without an appropriate change in the GPX1 and/or CAT activities in the patients with PC resulted in the increased production of H_2O_2, which cannot be detoxified by the action of GPX1 and CAT. Accumulation of H_2O_2 can thus participate in a Haber-Weiss reaction and generate hydroxyl radicals.[35,36] Catalase and glutathione peroxidase are both able to detoxify H_2O_2. Under physiologic conditions, H_2O_2 is mainly removed by GPX1. The activity of CAT is involved in the degradation of H_2O_2 in severe oxidative stress connected with higher H_2O_2 concentrations.[37] It was previously shown that long-term exposure of CAT to H_2O_2 leads to the oxidation of the catalase-bound NADPH to $NADP^+$ and to a decrease in the activity of CAT to approximately 30% of the initial activity.[38] Because our patients with PC had the highest level of oxidative stress markers associated with decreased erythrocyte CAT activity, our results implicated that under stressed conditions, erythrocyte CAT is unable to detoxify H_2O_2. In contrast, no changes in serum CAT activity in the patients with PC were found.[39] The insignificant differences in CAT activities in erythrocytes of the patients with CP observed in our study were consistent with the results of Fukui et al,[39] dealing with serum CAT activities in patients with CP. In the contrast, other authors described increased serum CAT[13,40] or decreased serum and erythrocyte CAT[18] activities in patients with CP.

Glutathione peroxidases use GSH to metabolize H_2O_2 and lipid hydroperoxides to water-related alcohols.[1] We have found decreased activity of erythrocyte GPX1 in the patients with PC and those with CP compared with the controls. The decreased erythrocyte GPX1 in CP was also found in the study of Girish[15] but also no differences in erythrocyte GPX1 activity in patients with CP and the controls were observed.[16,33] Published results in serum and plasma GPX activities are inconsistent.[18,23,33,34,40] Decreased GPX1 activity may be explained by the lowered serum levels of selenium in both groups and/or decreased concentrations of reduced glutathione in erythrocytes found in the patients with PC. Selenium is bound as the selenocysteine at the active site of GPX1, and it is essential for its activity. Moreover, selenium deficiency leads to decreased GPX1 activities.[35]

Glutathione reductase is a NADPH-dependent enzyme that catalyzes the regeneration of GSH from oxidized glutathione (GSSG) and thus maintains a constant supply of GSH for GPX.[41] In the present study, a decrease in GR erythrocyte activity was observed in the patients with PC and those with CP and decreased erythrocyte levels of GSH in PC but not in the patients with CP in comparison with the controls. It is supposed that depletion in GSH concentration may be caused by accumulation of GSSG as a result of impaired GR (pentose-phosphate pathway may limit NADPH supply). Formed GSSG could react with the sulfhydryl group, via mixed disulphide reactions, or could be secreted out of the cell. It is supposed that the aforementioned processes may lead to GSH depletion.[42] In

contrast to our study, decreased levels of reduced glutathione were observed in the patients with CP.[43]

We found a decrease in serum activities of arylesterase as well as lactonase activity of PON1 in the patients with PC and those with CP. The lowest PON1 activities were observed in the patients with PC. At present, the decreased PON1 activity in patients with PC has been described only in one study,[44] and there is no study dealing with PON1 activities in CP. A number of studies have shown decreased serum arylesterase and/or paraoxonase activities of PON1 in different malignancies.[45]

Under conditions of systemic inflammation and/or oxidative stress, several mechanisms are implicated in a drop of PON1 activities. Among them, displacement of PON1 from its linkage to apolipoprotein A1 in HDL by SAA,[46] down-regulation of liver PON1 lipopolysaccharides and cytokines (tumor necrosis factor α and interleukin-1) via IL-6,[20] and inhibition of PON1 activity by oxidized phospholipids[47] are the most important. All the abovementioned mechanisms can be related to cancer-related decrease in PON1 activities. Using MDA, the PON1-L activity had the most discriminating power to differentiate PC from PC.

The finding of increased SAA levels in the patients with PC in our study is consistent with the results of other studies.[48,49] Serum amyloid A is implicated in carcinogenesis, and it was associated with tumor progression and its metastasizing.[50] Some authors considered SAA as a tumor marker for PC. However, SAA did not reach appropriate specificity and sensitivity as tumor marker for PC diagnostics.[48,49]

Human serum PON1 should contribute to the detoxification of organophosphorus compounds and carcinogenic lipid-soluble radicals from lipid peroxidation and, moreover, should impede oxidative modification of LDLs.[2,7] In this study, we found a negative correlation between PON1 activities and ox-LDL/LDL levels. The reduced PON1 activities in the patients with PC and those with CP could lead to the increase in ox-LDL/LDL levels. Oxidatively modified LDL represents heavily oxidized LDL characterized by oxidative altered both the lipid and the apolipoprotein B, moiety of particle. Low-density lipoprotein is supposed to be oxidize not only within the artery wall (by endothelial cells, smooth muscle cells, and monocyte/macrophages) but also at peripheral tissues altered by inflammation (by neutrophils and fibroblasts).[51]

In our study, increased concentrations of CD/LDL in the patients with PC and those with CP compared with the controls were found. Concentrations of CD/LDL are partly considered as a marker of systemic oxidative stress and partly reflect minimally modified LDL, in which only the lipid component is oxidatively modified.[29,52] Currently, serum concentration of CD in CP patients was described only in the study of Santini et al,[53] where the levels of CD and lipid hydroperoxides did not differ between patients with CP and controls. However, the patients with CP, in comparison with the controls, had increased levels of CD and lipid hydroperoxides in pancreatic juice after secretin stimulation. These results indicated local enhanced oxidative stress in pancreas without systemic oxidative stress response. An increase of lipid peroxidation connected with PC and CP were observed in many studies using products of lipid peroxidation (such as thiobarbituric-reactive substances, malondialdehyde, 4-hydroxynonenal, lipid hydroperoxides).[1,3] The importance of ox-LDL/LDL and CD/LDL was pointed out in discriminating PC from CP using MDA.

In conclusion, our study demonstrates the persisting oxidative stress in patients with CP and those with PC, which is associated with the reduced capacity of the antioxidant defense system. The oxidative stress defense system seems to be more affected in patients with PC compared with those with CP. Multivariate discriminant analysis indicates the importance of PON1-L and CAT activities, along with ox-LDL/LDL levels, as the independent factors discriminating patients with PC and those with CP. Further studies concerning antioxidant defense systems and oxidative stress are warranted, especially with respect to potential diagnostic and therapeutic implications.

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Přehledový článek

Reaktivní kyslíkové a dusíkové sloučeniny v klinické medicíně

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SOUHRN

V poslední době dochází k rychlému růstu poznatků o reaktivních kyslíkových a dusíkových sloučeninách (RONS, reactive oxygen and nitrogen species) v klinické medicíně. Jejich významná úloha byla popsána v patogenezi mnoha chorob včetně těch, které značně zatěžují zdravotnické systémy vyspělých států. Výzkumu reaktivních kyslíkových a dusíkových radikálů je proto věnováno velké úsilí. Jedná se o nestabilní částice ochotně reagující s biomolekulami v organismech. Tyto reakce se řetězově propagují a vedou k mnohočetnému poškození buněčných systémů, což se uplatňuje v patogenezi mnoha chorob. Chemickou podstatou těchto částic je přítomnost nespárovaného elektronu v zevním orbitalu. Patří sem také sloučeniny snadno oxidující jiné molekuly. Volné kyslíkové radikály vznikají během fyziologických procesů, jako jsou oxidativní fosforylace v mitochondriích, fagocytóza či při metabolismu purinů. Při nadměrné tvorbě ROS během těchto procesů může dojít k poškození tkáně. Dusíkaté radikály vznikají především při metabolismu oxidu dusnatého, který reguluje mnoho procesů v organismu, rozpřažením jeho syntézy působením např. asymetrického dimetylargininu. Při vzniku radikálů či oxidačně působících látek hrají roli mnohé enzymy jako peroxizomální oxidázy, NAD(P)H oxidáza, xanthinoxidáza, syntáza NO, myeloperoxidáza, lipooxygenáza a mnoho dalších. RONS svůj negativní účinek zprostředkovávají chemickou modifikací DNA, proteinů a lipidů, čímž zasahují do základních biochemických a molekulárně biologických dějů buněk. Proti působení RONS zasahují antioxidační systémy, které se dělí na enzymatické a neenzymatické. RONS se uplatňují v rozvoji mnoha chorobných stavů, z nichž jmenujme aterosklerózu a její kardiovaskulární komplikace, diabetes mellitus, hyperlipidémii, neurodegenerativní či psychiatrická onemocnění.

Klíčová slova: RONS, radikály, superoxidový anion, radikál oxidu dusnatého, antioxidanty, ateroskleróza, diabetes mellitus, neurodegenerativní a psychiatrická onemocnění.

SUMMARY

Macášek J, Zeman M, Vecka M, Vávrová L, Kodydková J, Tvrzická E, Žák A. Reactive oxygen and nitrogen species in the clinical medicine

Vast knowledge has accumulated recently on the role of reactive oxygen and nitrogen species (RONS) in clinical medicine. Strong evidence was disclosed on their important role in the pathogenesis of several diseases. Free radicals have unpaired electron and this is the reason for extreme reactivity causing propagation reactions that lead to the multiple damage to cells. Oxidizing agents belong to the family of reactive species. Reactive oxygen species are produced during biochemical processes such as oxidative phosphorylation, phagocytosis and metabolism of purins. Overproduction of reactive oxygen species can cause the tissue damage. Reactive nitrogen species are produced by inhibition of nitric oxide synthase by the action of asymmetric dimethylarginine. Peroxisomal oxidases, NAD(P) oxidase, xanthinoxidase, nitric oxide synthase, myeloperoxidase and lipooxygenase catalyze biochemical reactions producing reactive oxygen and nitrogen species. Biochemical and molecular processes in cells are negatively influenced by chemical modification of DNA, proteins and lipids caused by the action of reactive oxygen and nitrogen species. Antioxidant metabolites and enzymes work together to stop and to prevent oxidative modification of biomolecules. Reactive oxygen and nitrogen species play an important role in the pathogenesis of many diseases such as atherosclerosis, diabetes, hyperlipidaemia and neurodegenerative diseases.

Key words: RONS, radicals, superoxide anion, nitric oxide radical, antioxidants, atherosclerosis, diabetes mellitus, neurodegenerative and psychiatric diseases. *Ma.*

Čas Lék čes 2011; 150: 423–432

ÚVOD

V klinické medicíně je dnes věnována velká pozornost pochodům spojeným s oxidačním stresem a působením reaktivních sloučenin kyslíku a dusíku (RONS – reactive oxygen and nitrogen species). Mezi tyto látky patří nejen volné radikály, ale i sloučeniny, které nejsou v chemickém slova smyslu radikály, ale snadno oxidují jiné látky, nebo se na radikály mění. Většina chemických sloučenin obsahuje v zevních orbitalech spárované elektrony. Tzv. volné radikály obsahují v zevním orbitalu jeden nepárový elektron, což je pro ně energeticky nevýhodné, a tudíž se snaží spárovat elektron vazbou s jiným atomem či molekulou. Získáním elektronu od jiného atomu či molekuly (redukce) nebo jeho odevzdáním na jiný atom či molekulu (oxidace) přejde atom či molekula do energeticky stabilnějšího stavu s nižší reaktivitou. Mezi radikály v organismu patří například superoxidový anion $^{\cdot}O_2^-$, oxid dusnatý $^{\cdot}NO$, nebo hydroxylový radikál $^{\cdot}OH$. Mezi neradikálové reaktivní substance patří například peroxid vodíku (H_2O_2), ky-

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Tab. 1. Přehled reaktivní sloučenin kyslíku a dusíku

Reaktivní formy kyslíku	**volné radikály**	tripletový kyslík $^3O_2$[a)]
		superoxidový anion $O_2^{\cdot-}$
		hydroxylový radikál $HO^{\cdot}$
		alkoxyl $RO^{\cdot}$
		hydroperoxyl $HO_2^{\cdot}$
		peroxyl $ROO^{\cdot}$
	látky, které nejsou volnými radikály	peroxid vodíku H_2O_2
		kyselina chlorná HClO
		ozon O_3
		singletový kyslík $^1O_2$
Reaktivní formy dusíku	**volné radikály**	oxid dusnatý $NO^{\cdot}$
		oxid dusičitý $NO_2^{\cdot}$
		nitroxylový anion $NO^{\cdot-}$
	látky, které nejsou volnými radikály	nitrosonium NO^+
		dimer oxidu dusičitého N_2O_4
		kyselina dusitá HNO_2
		oxid dusitý N_2O_3
		nitronium NO_2^+
		peroxynitrit $ONOO^-$
		alkylperoxynitrit ROONO

[a] Kyslík v základním energetickém stavu ($^3S_g^-$) je vlastně biradikál; jeho reaktivita je díky tomu, že reakce tripletové molekuly se singletovou (většina molekul) je spinově zakázána, relativně nízká. Protože je ale molekula kyslíku hojně rozšířena, v přehledu ji uvádíme.

selina chlorná (HClO) a peroxynitrit (ONOO-). Příklady nejdůležitějších reaktivních látek jsou uvedeny v tabulce 1.

Reaktivní formy kyslíku i dusíku vznikají v průběhu metabolických pochodů u všech aerobních organismů. Na jejich vzniku se podílejí i vnější vlivy, jako je ionizační záření, xenobiotika, toxiny či léky. Buňky a tkáně živých organismů jsou před poškozením těmito látkami chráněny antioxidačními ochrannými systémy (enzymatickými i neenzymatickými). V organismu však RONS nepůsobí pouze jako patogeny, ale podílejí se také na obraně vůči infekčním agens a v přiměřených koncentracích ovlivňují signální transdukci a genovou transkripci, přičemž oxid dusnatý ($NO^{\cdot}$) je jednou z nejvýznamnějších signálních molekul v lidském organismu (1).

Nadměrná tvorba a/nebo nedostatečné odstraňování RONS, resp. zvýšený poměr prooxidační k antioxidační aktivitě je označována pojmem **oxidační stres** (OS) (2). V důsledku OS může dojít k: 1. adaptaci buňky nebo organismu se zvýšením aktivity obranných systémů, 2. poškození buňky s oxidativní modifikací lipidů, DNA, proteinů, sacharidů atd., 3. buněčné smrti (3). Oxidační stres podle současných názorů hraje roli zejména u onemocnění, v jejichž patogenezi se uplatňuje zánět, který je s OS spojen. Jde o řadu rozšířených chorob, jako je ateroskleróza a její komplikace (ischemická choroba srdeční – ICHS), ischemická kolitida, ischemické cévní mozkové příhody, arteriální hypertenze, diabetes mellitus, neurodegenerativní neurologická onemocnění (Alzheimerova nemoc, roztroušená skleróza, Parkinsonova nemoc), psychiatrická onemocnění (schizofrenie, deprese) i zhoubné nádory (4).

ZDROJE A VZNIK RONS V LIDSKÉM ORGANISMU

Reaktivní sloučeniny kyslíku (ROS)

K nejvýznamnějším ROS se počítají superoxidový anion $^{\cdot}O_2^-$, hydroxylový radikál $^{\cdot}OH$ a látka neradikálové povahy peroxid vodíku H_2O_2. K hlavním zdrojům $^{\cdot}O_2^-$ patří v lidském organismu reakce provázející oxidativní fosforylaci v mitochondriích, reakce katalyzované peroxizomálními oxidázami, NAD(P)H oxidázami, xantinoxidázou nebo jednoelektronová redukce kyslíku syntázami NO v případě deficitu argininu nebo tetrahydrobiopterinu (obr. 1). Při oxidativní fosforylaci probíhá v dýchacím řetězci v mitochondriích redukce molekuly atmosférického kyslíku na dvě molekuly vody, spojená s tvorbou ATP (4). Redukce molekulárního kyslíku na vodu vyžaduje celkem čtyři elektrony, pokud se uskuteční transfer pouze jednoho elektronu, vzniká superoxidový anion (4).

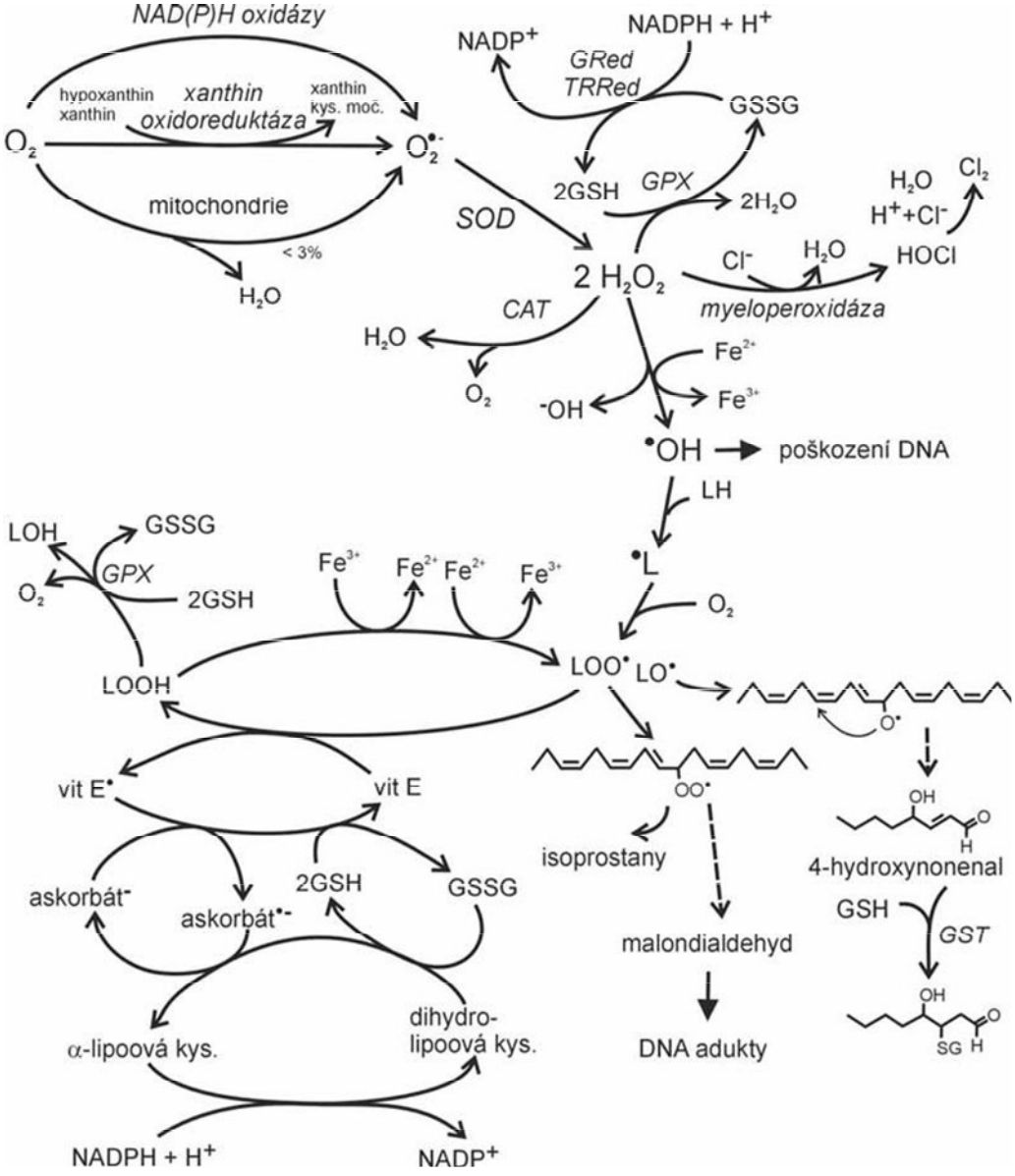
$$O_2 + e^- \rightarrow O_2^{\cdot-} \qquad [1]$$

Na superoxidový anion je převedeno až 3 % molekul mitochondriálního kyslíku (5). NAD(P)H oxidáza je enzym vázaný na buněčnou membránu, který používá elektrony pocházející z NADPH k redukci molekulárního kyslíku na $^{\cdot}O_2^-$:

$$NAD(P)H + 2\ O_2 \rightarrow NAD(P)^+ + H^+ + 2\ ^{\cdot}O_2^- \qquad [2]$$

Enzym se nachází v neutrofilních leukocytech, monocytech či makrofázích, kde je zdrojem velkého množství $^{\cdot}O_2^-$, který má baktericidní účinky. Strukturálně poněkud odlišná je NAD(P)H oxidáza obsažena v endotelu cév. Její produkce $^{\cdot}O_2^-$ je ve srovnání s formou obsaženou ve fagocytech o několik řádů nižší. Aktivita cévní NAD(P)H oxidázy a následná tvorba $^{\cdot}O_2^-$ je zvyšována působením řady faktorů účastnících se v patogenezi aterosklerózy, jako jsou angiotenzin II působící vazokonstrikci, PDGF (platelet derived growth factor) působící hyperplazii hladkých svalových buněk nebo trombin (6). Superoxid vzniká také působením enzymu xantinoxidoreduktázy. Tento flavoproteinový enzym obsahující molybden existuje ve dvou formách: xanthin oxidáza (XO) a xantin dehydrogenáza (XD). Enzym katalyzuje postupnou oxidativní hydroxylaci hypoxanthinu na xanthin a dále na kyselinu močovou (ve formě XD), ale může také redukovat kyslík na $^{\cdot}O_2^-$ (forma XO). Je zajímavé, že je lokalizován hlavně v endoteliálních a epiteliálních buňkách, což dobře nekoresponduje s jeho funkcí v metabolismu purinů, ale spíše naznačuje význam v systému antimikrobiální ochrany (7). Xantinoxidáza hraje významnou úlohu v patofyziologii reperfuzního syndromu. Při hypoxii způsobené nedostatečným přívodem kyslíku krví (nízká perfuze tkání např. při infarktu myokardu) dochází k vzestupu hladiny ADP, který je za fyziologických okolností přeměňován působením XO na hypoxantin, xantin a močovou kyselinu. Při hypoxii je enzym inhibován; poté, co dojde k reperfuzi a opětovnému obnovení dodávky kyslíku, zvýší XO svoji aktivitu s cílem odstranit nahromaděné ADP a jako vedlejší produkt jsou ve zvýšeném množství produkovány enzymem XO i ROS s následným paradoxním prohloubením poškození po obnově dodávky kyslíku.

Vznikající $^{\cdot}O_2^-$ je působením enzymu superoxid dismutázy (SOD) přeměňován na H_2O_2, ze kterého pak účinkem lyzozomální katalázy nebo mitochondriální glutathion peroxidázy (GPx) vzniká voda a kyslík (obr. 2). Glutathion, používaný GPx jako donor vodíku během eliminace H_2O_2, je regenerován glutathion reduktázou (GR). Součástí obranných mechanismů lidského organismu proti infekčním agens je také enzym myeloperoxidáza (MPO), nacházející se v azurofilních granulech



Obr. 1. Vznik reaktivních forem kyslíku v lidském organismu a jejich další osud (dle 6, 19)
vit – vitamin, GSH – glutathion, GSSG – glutathion disulfid, GPx – glutahion peroxidáza, GRed – glutathion reduktáza, GST – glutathion S-transferáza, TRRed – thioredoxin reduktáza, CAT – kataláza, LH – mastná kyselina, SOD – superoxid dismutáza

neutrofilů a lyzozomech monocytů. Enzym, který hraje roli ve fagocytóze, vytváří kyselinu chlornou (HClO) z peroxidu vodíku (H_2O_2) a chloridů. Reakce HClO se superoxidem může vést ke vzniku mimořádně reaktivního hydroxylového radikálu (8).

$$HOCl + {}^{\cdot}O_2^- \rightarrow {}^{\cdot}OH + Cl^- + O_2 \quad [3]$$
$$HOCl + Fe^{2+} \rightarrow {}^{\cdot}OH + Cl^- + Fe^{3+} \quad [4]$$

Chlornanové anionty reagují také s nízkomolekulárními aminy za vzniku chloraminů, které stejně jako chlornany mají silný baktericidní účinek (4). Hemo-peroxidázy, jako je MPO i eozinofilní peroxidázy, katalyzují také v přítomnosti H_2O_2 a nitritu NO_2^- nitraci tyrozinu v proteinech, a mohou tak nepříznivě modifikovat jejich funkci, jako např. v případě apolipoproteinu A-1 a apolipoproteinu B.

Reaktivní sloučeniny dusíku

Mezi nejvýznamnější reaktivní formy dusíku patří radikál oxidu dusnatého ·NO a peroxynitrit $ONOO^-$. Radikál oxidu dusnatého ·NO je tvořen oxidací L-argininu působením syntázy oxidu dusnatého (NOS) za vzniku citrulinu a NO. U člověka lze rozlišit tři hlavní izoformy NOS: endoteliální NOS (eNOS), inducibilní (iNOS) a neuronální (nNOS). nNOS a eNOS jsou konstitutivně exprimované enzymy, aktivované vzestupem hladiny intracelulárního kalcia (Ca^{2+}). Ca^{2+} se váže na kalmodulin a komplex Ca^{2+}/kalmodulin aktivuje nNOS nebo eNOS. iNOS obsahuje pevně vázaný kalmodulin s kalciem a její syntéza je indukována zánětlivými cytokiny, jako je interleukin-1 (IL-1), tumor nekrotizující faktor alfa (TNF-α), interferon gamma (IFN-γ), ale i antigeny bakterií a nádorových buněk.

Za fyziologických okolností se ·NO významně podílí na tzv. endotel-dependentní vazodilataci a regulaci cévního tonu, má protizánětlivé účinky, inhibuje agregaci krevních destiček a adhezi leukocytů i destiček na endotel a reguluje proliferaci a diferenciaci buněk cévní stěny. V buňce hladkého svalu cévy aktivuje ·NO enzym guanylát cyklázu. Aktivace guanylát cyklázy vede k syntéze cyklického GMP a k vazorelaxaci.

Ke správné funkci vyžadují NOS pět kofaktorů: flavinadenindinukleotid (FAD), flavinmononukleotid (FMN), hem, tetrahydrobiopterin (BH_4) a Ca^{2+}-kalmodulin. Jestliže chybí L-arginin – substrát pro NOS nebo jeden z jeho kofaktorů, může NOS produkovat $\cdot O_2^-$ místo ·NO, což je označováno jako roz-

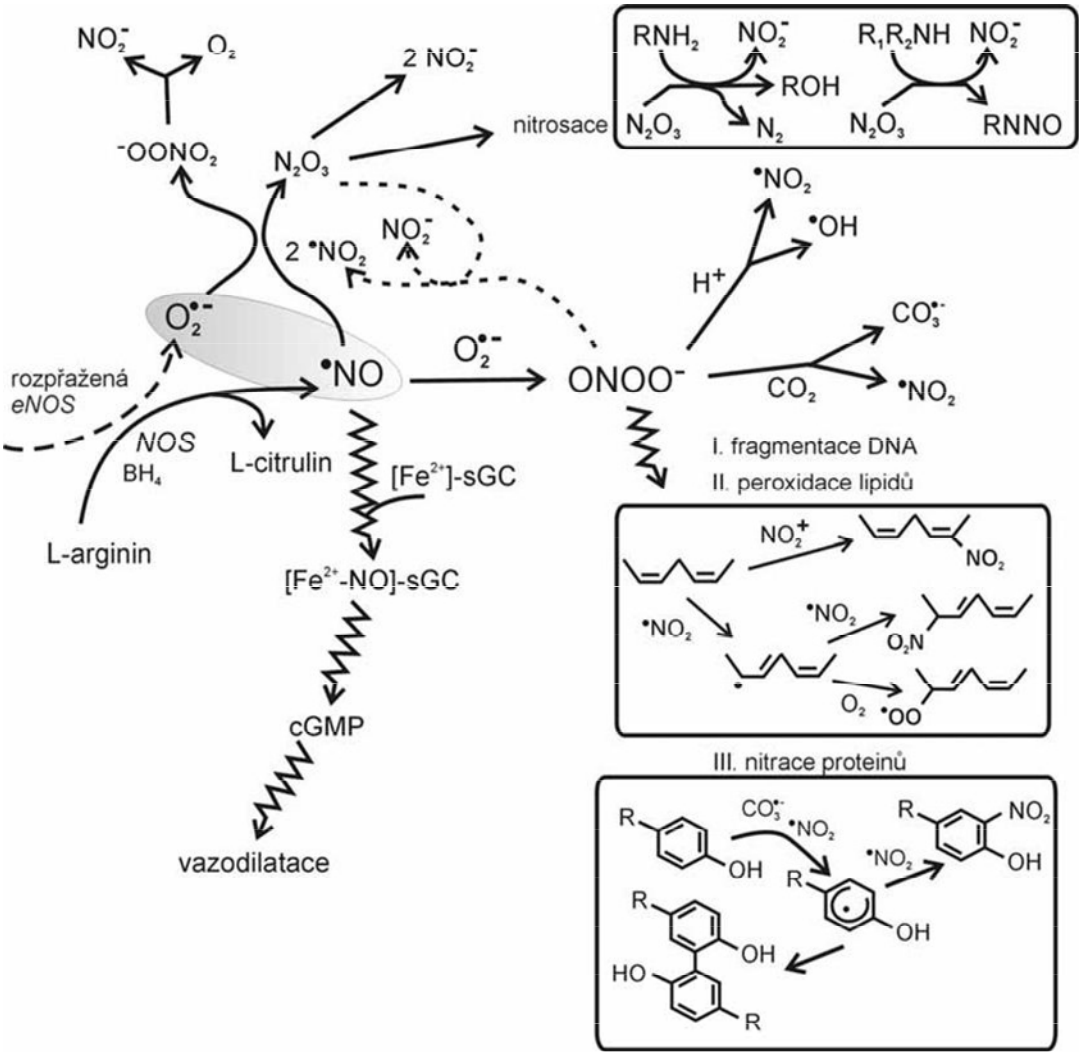
přažený (uncoupled) stav NOS (9). K rozpřažení reakce vede též zvýšení hladiny inhibitoru NOS, asymetrického dimetylargininu (ADMA), jehož zvýšené koncentrace jsou spojeny s endoteliální dysfunkcí u hypercholesterolémie, inzulínové rezistence či hyperhomocysteinémie. Rozpřažení eNOS v cévní stěně působí oxidační stres jednak poklesem tvorby $^{\cdot}NO$, jednak zvýšenou tvorbou $^{\cdot}O_2^-$. Pokud jsou tvořeny současně $^{\cdot}NO$ a $^{\cdot}O_2^-$, vzniká toxický peroxynitrit $ONOO^-$. Peroxynitrit reaguje s CO_2, který je v tělesných tekutinách obsažen ve vysokých koncentracích, a vytváří jednoelektronové oxidanty $^{\cdot}NO_2$ a $CO_3^{\cdot-}$, které oxidací aminokyseliny tyrozinu vedou ke vzniku tyrozinového radikálu $Tyr^{\cdot}$ a následně 3-nitrotyrozinu, 3-NO_2-Tyr . Z $ONOO^-$ může také vzniknout $^{\cdot}OH$, působící peroxidaci lipidů, mutace DNA, jejich fragmentaci nebo modifikace proteinů (obr. 2). Peroxynitrit vedle přímého toxického působení oxiduje BH_4, což rovněž přispívá k rozpřažení eNOS.

Lipoxygenáza

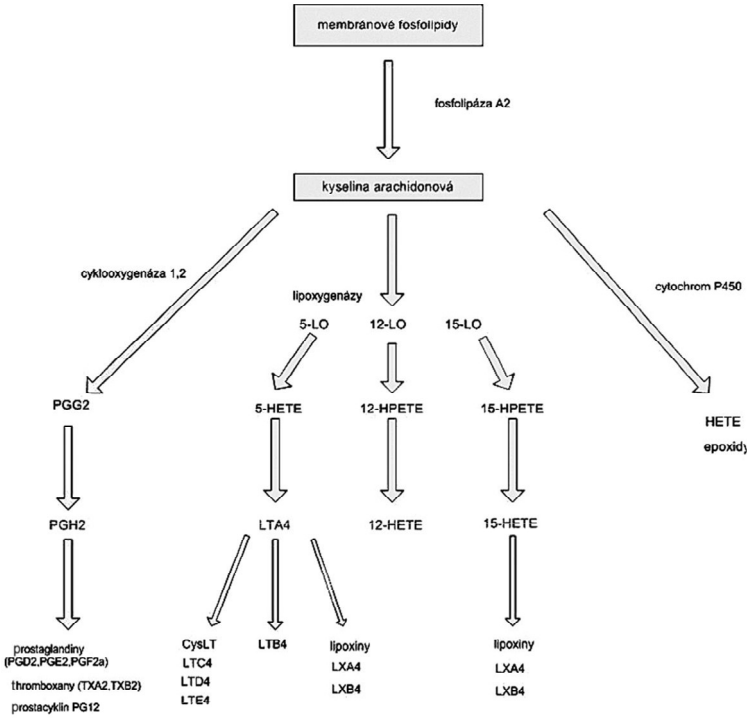
Lipoxygenázy jsou dioxygenázy obsahující železo, které katalyzují stereospecifickou inzerci molekulárního kyslíku do molekuly vícenenasycené mastné kyseliny. Aktivní forma enzymu obsahuje v katalytickém centru trojmocné železo, forma lipoxygenázy s dvojmocným železem není aktivní. Působení lipoxygenáz na kyselinu arachidonovou vede k tvorbě 5-, 11- a 15- hydroperoxyeikosatetraenových mastných kyselin (HPETE), které jsou v tkáních rychle redukovány na odpovídající hydroxyeikosatetraenové kyseliny (HETE), jako jsou 5*S*-hydroxy-6t,8c,11c,14c-, 12*S*-hydroxy-5c,8c,10t,14c- a 15*S*-hydroxy-5c,8c,11c,13t-eikosatetraenové mastné kyseliny (10). Z těchto derivátů jsou 5-hydroxy izomery předchůdci biologicky aktivních leukotrienů a lipoxinů (obr. 3), které hrají významnou roli v patofyziologii zánětu. 5-HETE má chemotaktický účinek na neutrofily, leukotrien B_4 (LTB_4) působí chemotakticky na fagocyty, LTC_4, LTD_4 a LTA_4 působí vazokonstrikci, bronchokonstrikci a zvyšují permeabilitu cév. Lipoxiny mají naopak účinky protizánětlivé.

Myeloperoxidáza

Myeloperoxidáza (MPO) je enzym, obsahující hem, který se nachází v azurofilních granulech neutrofilů a lyzozomech monocytů. Enzym vytváří kyselinu chlornou (HClO) z peroxidu vodíku (H_2O_2) a chloridů. MPO hraje roli při fagocytóze a produkty jejího působení se podílejí na destrukci bakterií, intracelulárních parazitů i nádorových buněk. Reakce kyseliny chlorné se superoxidem může vést ke vzniku mimořádně reaktivního hydroxylového radikálu (8). Chlornanové anionty reagují také s nízkomolekulárními aminy za vzniku chloraminů, které stejně jako chlornany mají silný baktericidní účinek (4). Hemo-peroxidázy, jako je MPO i eozinofilní peroxidázy, katalyzují také v přítomnosti H_2O_2 a nitritu NO_2^- nitraci tyrozinu v proteinech, a mohou tak nepříznivě modifikovat jejich funkci, jako např. v případě apolipoproteinu A-1 a apolipoproteinu B. Oxidované produkty působení MPO jsou ve vysokých



Obr. 2. Vznik a působení reaktivních forem dusíku (dle 6, 11, 16, 17)
BH_4 – tetrahydrobiopterin, eNOS – endoteliální syntháza NO, sGC – solubilní guanylát cykláza, cGMP – cyklický guanylmonofosfát



Obr. 3. Metabolizmus eikosanoidů
HETE – hydroxyeikosatetraenová kyselina, HPETE – hydroperoxyeikosatetraenová kyselina, LO-x – lipoxygenáza, COX – cyklooxygenáza, LX – lipoxiny, LT – leukotrieny, cysLT – cysteinylleukotrieny

koncentracích prokazovány v částicích LDL lokalizovaných v aterosklerotických plátech. Předpokládá se, že aktivita MPO souvisí s vulnerabilitou ateromových plátů (11). MPO může také modifikovat lipoprotein HDL, což vede k poruše reverzního transportu cholesterolu. Nejvyšší kvartily MPO v krvi a leukocytech významně korelovaly s přítomností koronární aterosklerózy. Ve studii u nemocných s AKS předpovídaly hladiny MPO rozvoj IM nezávisle na jiných rizikových faktorech, jako např. CRP (12). Zdá se, že MPO je významným činitelem, podílejícím se na destabilizaci ateromového plátu a stanovení MPO by mohlo sloužit jako nezávislý prognostický ukazatel u nemocných přijatých k observaci pro bolest na hrudi.

MECHANISMY PŮSOBENÍ REAKTIVNÍCH ČÁSTIC

Nukleové kyseliny, lipidy a proteiny mohou být poškozeny působením RONS, což může vést až k buněčné smrti (5). Jejich působení není však jen nepříznivé, aktivují také různé buněčné signální kaskády, které regulují proliferaci, detoxifikaci, reparaci DNA nebo apoptózu. V případě snížené tvorby RONS může dojít k poruše imunitní odpovědi na cizorodou noxu nebo k poruše proliferace. V závislosti na koncentraci a typu RONS mohou být aktivovány buď signální cesty protektivní (např. reparace DNA) anebo buněčná apoptóza.

Poškození lipidů

Působením radikálové látky, nejčastěji $^{\cdot}OH$, na lipidy, zejména na vícenenasycené mastné kyseliny (polyunsaturated fatty acids, PUFA), vede k lipoperoxidaci (viz obr. 2). Oxidující látka vytrhne elektron z metylenové skupiny uhlovodíkového řetězce PUFA ($-CH_2^{\cdot}$) za vzniku lipidového radikálu ($L^{\cdot}$). Po vytržení vodíku dojde ke změně elektronového uspořádání v uhlovodíkovém řetězci PUFA tak, že mezi dvěma dvojnými vazbami je jedna vazba jednoduchá (konjugovaný dien) (4). Konjugované dieny se snadno spojují s molekulárním kyslíkem za vzniku peroxylového radikálu $LOO^{\cdot}$. Peroxylový radikál může vytrhnout elektron ze sousední mastné kyseliny, která se stává radikálem, zatímco peroxyl se mění na hydroperoxid LOOH. Radikálová reakce se pak řetězovitě šíří dál (propagace), dokud není ukončena (terminace) setkáním radikálu mastné kyseliny s jiným radikálem nebo vitaminem E (4). Lipoperoxidačními pochody vznikají hydroperoxidy a cyklické peroxidy mastných kyselin, které v přítomnosti přechodných kovů (jako Ca^{2+}, Fe^{2+}) podléhají tzv. Fentonově reakci za vzniku alkoxylového radikálu $LO^{\cdot}$ a hydroxidového aniontu OH.

$$LOOH + Fe^{2+}\{Cu^{+}\} \rightarrow LO^{\cdot} + Fe^{3+}\{Cu^{2+}\} + OH^{-} \quad [5]$$

Lipoperoxidace nakonec vede ke vzniku stabilních látek, které lze laboratorně stanovit, jako je např. MDA (malondialdehyd) a nebo 4-hydroxynonenal (4-HNE). Malondialdehyd velmi ochotně reaguje s nukleofilními skupinami (aminoskupiny), a způsobuje tak modifikaci struktury a následně funkce proteinů (zesíťování kolagenu). Další produkt oxidace vícenenasycených mastných kyselin 4-hydroxynonenal, elektrofilní α,ß-nenasycený aldehyd, způsobuje kovalentní modifikaci DNA, což způsobuje vznik mutací, a proteinů signálních drah, a ovlivňuje tak genovou expresi zodpovědnou za produkci složek antioxidačního systému, heat shock proteinů a proteinů účastnících se reparace poškozené DNA. 4-hydroxynonenal je též užíván jako biomarker oxidativního poškození buněk. Jinými významnými produkty působení oxidačního stresu na lipidy jsou F_2-izoprostany (13). Izoprostany jsou látky podobné F_2-prostaglandinu vznikající neenzymatickou peroxidací kyseliny arachidonové působením radikálů. F_2-izoprostany však *in vivo* prodělávají další přeměnu v E_2-, D2-, A2-, J2-izoprostany, izotromboxany a vysoce reaktivní ketoaldehydy zvané izoketaly. Podobné sloučeniny vznikají též z kyseliny dokosahexaenové, která je hojná v neuronech, a proto se sloučeniny vzniklé její radikálovou neenzymatickou peroxidací nazývají neuroprostany či neuroketaly. F_2-izoprostany jsou nejenom markery lipoperoxidace, ale jako ligandy specifických receptorů způsobují i vazokonstrikci. U různých onemocnění (např. diabetu) dochází ke vzniku oxidativně modifikovaných LDL částic tzv. oxLDL, jejichž hlavními kompo-

nentami jsou 9-hydroxy-10,12-oktadekadienová (9-HODE) a 13-hydroxy-9,11-oktadekadienová (13-HODE) kyselina. Vznikají působením ROS na linolovou kyselinu. Bylo zjištěno, že 9-HODE i 13-HODE jsou endogenními aktivátory PPAR-γ (peroxisome proliferator-activated receptor gamma) a hrají významnou úlohu například při rozvoji diabetické nefropatie tím, že stimulují mezangiální proliferaci.

Poškození DNA

Podobně jako lipidy jsou nukleové kyseliny poškozovány především ·OH. Hydroxylový radikál reaguje se všemi složkami DNA a poškozuje jak purinové, tak pyrimidinové báze i strukturu deoxyribózy. Dochází k vyjmutí vodíkového atomu z deoxyribózy s následnou destrukcí sacharidu a přerušení řetězce. Hydroxylový radikál vytváří addukty s purinovými i pyrimidinovými bázemi a modifikované báze pak slouží jako marker poškození DNA, např. 8-hydroxydeoxyguanozin (8-OH-dG), 8-hydroxy-guanin a 8-hydroxy-guanozin (4). Modifikace nukleových kyselin pak vede k chybným párováním bázi při replikaci DNA a k následným změnám genetické informace.

Poškození bílkovin

Oxidativní modifikace poškozuje strukturu bílkovin. Oxidace aminokyselin v proteinech vede k nevratným změnám. Dochází k fragmentaci a agregaci bílkovin. V důsledku konformačních změn se zvyšuje citlivost k proteolytickému štěpení. Citlivost proteinů vůči oxidaci je ovlivňována také přítomností iontů kovů schopných katalyzovat reakci Fentonova typu (14). Modifikovány jsou zejména aminokyseliny postranních řetězců, zejména cystein a methionin, přičemž oxidací cysteinových zbytků vznikají smíšené disulfidy mezi thiolovými skupinami bílkovin (-SH) a nízkomolekulárními thioly, zejména GSH (15). Oxidace proteinů vede také k fragmentaci polypeptidových řetězců a k intra- i intermolekulárnímu síťování (cross-linking). Takto modifikované proteiny snáze podléhají degradaci. Je známo, že glykovaný kolagen zvyšuje tvorbu malondialdehydu a 4-hydroxynonenalu, produktů oxidace vícenenasycených mastných kyselin. Malondialdehyd je velice reaktivní sloučenina vytvářející vazby především s amino-skupinami aminokyselin, což zapříčiňuje zesítování proteinů a ztrátu jejich funkce. U diabetiků se zvyšuje glykace kolagenu, jenž se působením malondialdehydu zesíťuje a tento děj sekundárně urychluje rozvoj aterosklerózy.

ANTIOXIDAČNÍ SYSTÉM V LIDSKÉM ORGANISMU

Složité biochemické děje neustále probíhající v živých organismech vytvářejí RONS, které mohou mít jak nepříznivé, tak i příznivé účinky. Pro správné fungování metabolických procesů je tak nutné stále ustavovat rovnovážný stav mezi vznikem a odbouráváním RONS. K udržení homeostázy v situaci, kdy jsou neustále vytvářeny RONS, slouží systém antioxidantů (tab. 2). Dříve se pomýšlelo, že RONS mají pouze negativní účinky, ukázalo se však, že mají i příznivé účinky. V leukocytech slouží k likvidaci infekčních částic cestou respiračního vzplanutí za účasti NADPH oxidázy, hrají důležitou úlohu v signálních dráhách (ovlivňují nukleární faktor κB, mitogen- activated proteinkinázu atd.), proliferaci, přežívání, migraci a adhezi buněk.

Enzymatické antioxidanty

Mezi enzymatické antioxidanty patří např. superoxid dismutáza (SOD), kataláza (CAT), glutathion peroxidáza, glutathion reduktáza. Enzymy glutathion peroxidáza a glutathion reduktáza se nacházejí v cytoplazmě, mitochondriích i v jádře. Glutathion peroxidáza mění H_2O_2 na vodu za spoluúčasti glutathionu (GSH) jako dárce vodíku. Vznikající glutathion disulfid (GSSG) je přeměňován zpět na GSH působením GR, jejímž kofaktorem je NADPH. Působením SOD dochází k přeměně $^{\Sigma}O_2^{\cdot-}$ na H_2O_2, který je detoxikován buď katalázou, která v lyzozomech rozkládá H_2O_2 na vodu a kyslík, nebo účinkem GPx v mitochondriích (viz obr. 1). Glutathion reduktáza regeneruje GSH, který je používán jako donor vodíku glutathion peroxidázou během eliminace H_2O_2.

Tab. 2. Důležité komponenty antioxidačního systému (dle 30, 31, 36)

Lokalizace		
Intracelulární Antioxidanty	**buněčná membrána**	**extracelulární antioxidanty**
enzymové složky		
superoxiddismutáza kataláza glutathionperoxidáza peroxidáza DT-diaforáza proteolytické enzymy hem oxygenáza I	fosfolipázy	SOD-3 EC-CAT GPx-3 paraoxonáza selenoprotein P? peroxiredoxiny
neenzymové složky		
Intracelulární Antioxidanty	**buněčná membrána**	**extracelulární antioxidanty**
Glutathion kyselina askorbová kyselina lipoová vazebné proteiny kovů – feritin (Fe^{2+}ġFe^{3+}), metallothioneiny (Cu^{+}) opravné systémy DNA – excize basí glykosylázami, homologní rekombinace, spojování nehomologních konců	vitamín E ß-karoten	glutathion kyselina askorbová vitamin E * proteiny vázající přechodné kovy – transferin (Fe^{3+}), laktoferin (Fe^{3+}), ceruloplasmin (Cu^{2+}), haptoglobiny (hemoglobin) a hemopexin (hem) albumin, bilirubin, kyselina močová, thioredoxin

* Vitamin E je nejsilnějším antioxidantem v membránách, mimo ně vykazuje pouze slabé antioxidační schopnosti.

Neenzymatické antioxidanty

Mezi neenzymatické antioxidanty patří vitaminy A, C a E, glutathion, kyselina alfa-lipoová, dále karotenoidy, stopové prvky jako měď, zinek a selen, koenzym Q_{10} (Co Q_{10}) a kofaktory jako kyselina listová, vitaminy B_1, B_2, B_6 a B_{12}, dále též močovina, albumin či bilirubin. Hlavním intracelulárním antioxidantem je GSH, který působí jako přímý scavenger a současně jako kosubstrát pro GPx. Vitamin E je označení pro skupinu osmi příbuzných tokoferolů a tokotrienolů, které zabraňují peroxidaci lipidů. U lidí je nejaktivnější forma α-tokoferolu. Hydroxylový radikál reaguje s tokoferolem za vzniku stabilního fenolického radikálu, který je redukován zpět na fenol askorbátem a NAD(P)H dependentními reduktázami (16). Koenzym Q_{10} působí jako elektronový nosič v komplexu II mitochondriálního elektronového transportního řetězce. Je to v tucích rozpustný antioxidant, který ve vyšších koncentracích působí jako scavenger $^{\Sigma}O_2^-$ (17). Vitamin C (kyselina askorbová) stabilizuje kofaktor NOS, tetrahydrobiopterin (BH_4), což podporuje tvorbu NO (18). Kyselina α-lipoová je hydrofilní antioxidant, působící jak ve vodném, tak v lipidovém prostředí. Její redukovaná forma, dihydrolipoát, je schopna regenerovat jiné antioxidanty, jako jsou vitamin C nebo vitamin E (18). Bilirubin je v poslední době intenzivně studován jako neenzymatický antioxidant. Kromě toho působí antiaterogenně tím, že inhibuje oxidaci LDL částic a lipidů obecně a pohlcuje kyslíkové radikály. Mnohé studie prokázaly inverzní vztah hladin bilirubinu k výskytu kardiovaskulárních chorob. Lidé s Gilbertovým syndromem (nekonjugovaná hyperbilirubinémie) mají nižší incidenci koronární choroby (19).

UPLATNĚNÍ RONS V KLINICKÉ MEDICÍNĚ

Volné radikály i ostatní RONS plní v organismu významné funkce. Jsou součástmi **obranného systému** organismu proti bakteriální infekci, intracelulárním parazitům, cizorodým látkám i nádorovým buňkám. V případě bakteriální infekce se v neutrofilních leukocytech a makrofázích aktivuje enzym NAD(P)H oxidáza, vzniká superoxidový anion. Takto aktivované buňky zvýší spotřebu O_2 (tzv. oxidační nebo respirační vzplanutí, respiratory burst). Vznikající $^{\Sigma}O_2^-$ se přeměňuje na H_2O_2. Enzym myeloperoxidáza zase v polymorfonukleárních leukocytech katalyzuje tvorbu kyseliny chlorné z H_2O_2 a chloridového iontu. Významnou součástí obrany organismu proti různým mikrobům, intracelulárním parazitům i nádorovým buňkám, je aktivita iNOS. Exprese enzymu je indukována působením mikrobů a různých cytokinů a vede k produkci NO mnohem vyšší, než ke které dochází v důsledku aktivity eNOS. Současně vytvářený $^{\Sigma}O_2^-$ však působí zvýšení koncentrací peroxynitritu $ONOO^-$, který má baktericidní účinky (4). V nízkých koncentracích se RONS podílejí na nitrobuněčných signálních pochodech. V tzv. **signální transdukci** je zprostředkován přenos informace přicházející zvenčí prostřednictvím hormonů, cytokinů, růstových faktorů či neurotransmiterů až do buněčného jádra. Transkripční faktory po vazbě na specifické sekvence DNA regulují aktivitu RNA polymerázy II. Některé signální cesty v buňce jsou zprostředkovány RONS, které v tomto případě hrají roli „druhotných poslů" (second messengers). Pravděpodobně nejvýrazněji se RONS uplatňují při ovlivnění systému MAP kináz (mitogen associated protein kinase), který představuje kaskádu fosforylačních reakcí, ve kterých se postupně aktivují enzymy a další proteiny s výsledným ovlivněním jaderných transkripčních faktorů, regulujících buněčný růst, diferenciaci i apoptózu. Patří sem faktory NF-κB (nuclear factor kappa B), významný u zánětlivých procesů, AP-1 (activated protein-1), ovlivňující růst a diferenciaci buněk a p53, což je protein, který pomáhá udržovat stabilitu genomu (zásahem do reparačních mechanismů DNA a také do regulace proliferace a diferenciace buňky) (20).

VYBRANÁ ONEMOCNĚNÍ, V JEJICHŽ ETIOPATOGENEZI JSOU VÝZNAMNÉ VOLNÉ RADIKÁLY

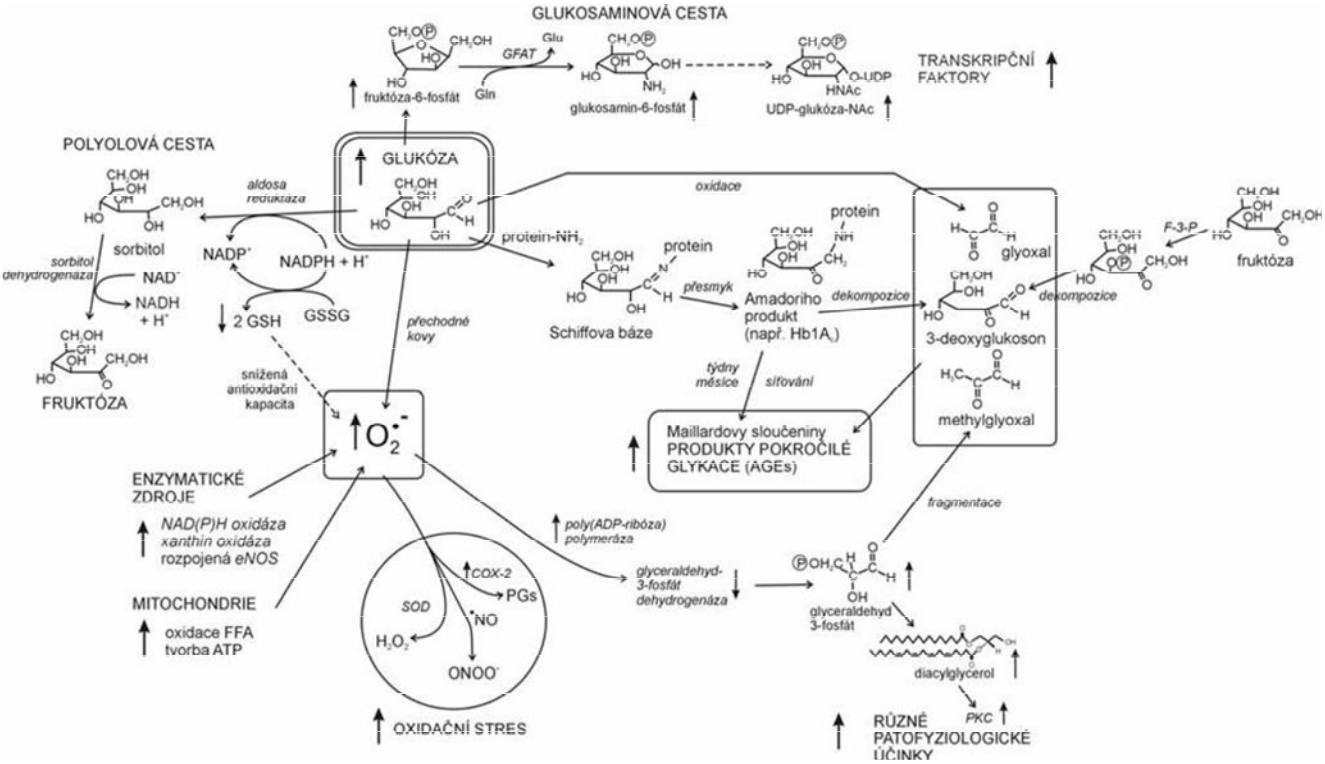
Ateroskleróza a její komplikace

Aterosklerózu je možno charakterizovat jako chronické zánětlivé fibroproliferativní onemocnění, ve kterém hraje podstatnou roli proliferace hladkých svalových buněk (SMC) a makrofágů, tvorba pojivové tkáně buňkami hladké svaloviny a hromadění lipidů, zejména volného (FC) a esterifikovaného cholesterolu (CE), v buňkách a mezibuněčné hmotě. V aterogenezi hraje oxidační stres významnou roli. Uplatňuje se zejména $^{\cdot}O_2^-$, H_2O_2 a $^{\cdot}NO$. Nadměrná tvorba RONS vede k aterogenním a trombogenním změnám ve smyslu zvýšené adheze monocytů, agregace krevních destiček a porušené vazodilatace. Působením RONS jsou ve stěně cévy modifikovány LDL částice a vznikají tzv. minimálně modifikované a oxidované LDL (mmLDL – minimally modified and oxLDL-oxidized), mmLDL mají zoxidovanou pouze lipidovou složku a u oxLDL dochází k oxidační modifikaci i proteinové složky. Tyto částice následně inhibují vazodilataci a působí proaterogenně tím, že aktivují zánětlivou odpověď, proliferaci buněk, ale i jejich apoptózu. Syntéza proaterogenních adhezních molekul je zvyšována cytokiny (interleukiny, tumor necrosis factor alfa, angiotenzin II, endoteliální růstový faktor VEGF) mechanismy zahrnujícími RONS. Inaktivace $^{\cdot}NO$ působením $^{\cdot}O_2^-$ a zvýšená tvorba H_2O_2 inhibuje vazodilataci. Oxidační stres působí také zvýšenou apoptózu endoteliálních buněk cestou aktivace signální cesty proteinkinázy C (21). H_2O_2 i $^{\cdot}O_2^-$ významně ovlivňují migraci hladkých svalových buněk do cévní stěny indukcí MCP-1 (monocyte chemotactic protein-1) i proliferaci hladkých svalových buněk. Zvýšením sekrece i aktivity metaloproteinázy 9 se RONS podílejí i na zvýšeném odbourávání extracelulární matrix (22), což má význam při vývoji nestabilního ateromového plátu a následné trombózy.

Diabetes mellitus

U diabetes mellitus zvýšený oxidační stres pochází z několika zdrojů: 1. neenzymatické zdroje, tj. zejména hyperglykémie, 2. zdroje enzymatické, kde vznikají RONS v důsledku aktivity enzymů, hlavně NAD(P)H oxidázy, xanthinoxidázy, cyklooxygenázy a 3. mitochondriální elektronový řetězec v průběhu oxidativní fosforylace (23). Hyperglykémie zvyšuje tvorbu volných radikálů několika způsoby (24). Během tzv. autooxidace glukózy, katalyzované přechodnými kovy, dochází ke vzniku redukovaných kyslíkových derivátů, jako jsou $^{\Sigma}O_2^-$, $^{\Sigma}OH$ a H_2O_2 ale i reaktivních ketoaldehydů. Glukóza je dále schopna vázat se neenzymaticky adicí k aminoskupině proteinu (glykace), tímto vznikají přes meziprodukt (Schiffovy báze) tzv. Amadoriho produkty. Důsledkem intramolekulárních přesmyků v Amadoriho produktech je vznik vysoce reaktivních dikarbonylových látek, glyoxalu, methylglyoxalu a 3-deoxyglukosonu. Řádově během týdnů jsou Amadoriho produkty po intra- a intermolekulárních přestavbách přeměňovány na novou třídu molekul, tzv. Maillardovy sloučeniny, neboli AGE (advanced glycation end products). U hyperglykémie se metabolismus glukózy ubírá i polyolovou cestou, která také vede ke zvýšené tvorbě $^{\Sigma}O_2^-$. Glukóza je nejprve redukována aldozoreduktázou za účasti NADPH na sorbitol, ten je oxidován NAD^+ s následným zvýšením poměru NADH/NAD^+ v cytosolu (hyperglykemická pseudohypoxie).

Zdrojem zvýšené tvorby superoxidu $^{\Sigma}O_2^-$ jsou u diabetu vedle hyperglykémie také enzymatické aktivity NAD(P)H oxidázy, xanthinoxidázy i cyklooxygenázy, jejichž působením vzniká $^{\Sigma}O_2^-$ jednoelektronovou redukcí kyslíku. Se vzestupem hladiny $^{\Sigma}O_2^-$, možná v důsledku poklesu BH_4 jsou spojeny stavy spojené s inzulinovou rezistencí jako obezita, arteriální hypertenze a diabetes mellitus (25). Superoxid, tvořený v mitochondriálním systému za hyperglykemických podmínek,



Obr. 4. Oxidační stres a hyperglykémie v patofyziologii diabetu (dle 26)
COX-2 – izoforma 2 cyklooxygenázy, SOD – superoxid dismutáza, PKC – proteinkináza C, F-3-P – fruktóa 3-fosfatáza, GFAT – glutamin: fruktóza 6-fosfát aminotransferáza, Gln – glutamin, Glu – glutamát, PGs – prostaglandiny

aktivuje enzym poly (ADP-ribóza) polymerázu (PARP). To působí inhibici glyceraldehydfosfát dehydrogenázy (GAPDH) s následným zvýšením koncentrace glyceraldehyd-3-fosfátu a aktivací 4 patologických mechanismů: 1. polyolové cesty, 2. glukozaminové cesty, 3. zvýšené tvorby methylglyoxalu a AGE, 4. vzniku diacylglycerolu (DAG), který aktivuje PKC (proteinkinázu C) (26). Aktivací PKC je možné vysvětlit některé cévní abnormality pozorované u diabetu (změny funkcí buněk endoteliálních, mezangiálních, buněk hladkého svalstva cév s výslednými změnami permeability, kontraktility a syntézy bazální membrány). PKC může také modulovat působení hormonů, růstových faktorů a iontových kanálů. Nástin působení oxidačního stresu v patofyziologii komplikací diabetu je podán na obrázku 4.

Hyperlipidémie

Hypercholesterolémie i hypertriglyceridémie (HTG) jsou zdrojem zvýšeného oxidačního stresu. Je u nich zjišťována zvýšená tvorba $^{\Sigma}O_2^{-}$, zřejmě v důsledku zvýšené aktivity xantin oxidázy (27) a NAD(P)H oxidázy (28). Léčiva užívaná k léčbě těchto dvou stavů, statiny a fibráty, nesnižují pouze hladinu lipidů, ale mají tzv. pleiotropní účinky, mezi které patří i příznivé účinky na oxidační stres. U nemocných s HTG léčených fibráty byl popsán pokles hladiny konjugovaných dienů, prodloužení lag fáze lipoproteinových částic VLDL a LDL i vzestup aktivity SOD a GPx (29).

Neurodegenerativní onemocnění

Mozek je vůči oxidačnímu stresu vysoce citlivý, protože využívá 20 % kyslíku spotřebovávaného organismem (30). Mozek také obsahuje velké množství vícenenasycených mastných kyselin a železa a nízkou koncentraci antioxidačních enzymů.

Parkinsonova nemoc. U Parkinsonovy nemoci dochází k degeneraci neuronů v substantia nigra, secernujících dopamin, které se podílí na kontrole a plánování pohybu. Předpokládá se, že v patofyziologii choroby se uplatňuje tvorba RONS a oxidace dopaminu (31). U pacientů s Parkinsonovou nemocí jsou silné důkazy o působení oxidačního stresu. V mozku pacientů s Parkinsonovou nemocí byla zjištěna zvýšená množství oxidovaných forem proteinů, lipidů a nukleových kyselin, jako jsou karbonyly proteinů, 4-hydroxy-2-nonenol a 8-hydroxy-2-deoxyguanozin (2, 3, 5, 9–11).

Alzheimerova nemoc. Podobně se oxidační stres podle současných názorů uplatňuje také v patogenezi Alzheimerovy nemoci. Alzheimerova nemoc (AD) je heterogenní onemocnění, za jejíž hlavní rys je považováno ukládání amyloidu beta (Aß) v mozku. Beta-amyloid je ukládán extracelulárně v tzv. senilních placích a je tvořen z těla vlastního amyloidového prekurzorového proteinu (APP). Dalším patologickým proteinem u AD je degenerovaný protein tau, uložený intracelulárně (32). Tvorba RONS, jako např. H_2O_2 provázející redukci kovových iontů, vedla k oxidačnímu poškození neuronů a vzniku Aß. Aß sám je zdrojem oxidačního stresu. Během progrese AD byla prokázána lipoperoxidace, oxidační poškození proteinů i DNA.

V mozkové tkáni jsou u pacientů s AD prokazovány markery oxidačního stresu, jako je zvýšená aktivita hem-oxygenázy 1 (HO-1) a koncentrace 8-hydroxyguaninu (8-OHG). Senilní plaky nesou známky oxidativního poškození jako modifikace Maillardovými sloučeninami (AGE), karbonylace, „síťování“ (cross-linking) proteinů. V mozkové tkáni nemocných s AD jsou také prokazovány zvýšené koncentrace železa a mědi. Přesné mechanismy spojení mezi oxidačním stresem a smrtí neuronů, vedoucí k poruchám poznávacích procesů, však zatím nebyly objasněny (33).

Psychiatrická onemocnění

V poslední době je úloha oxidačního stresu sledována i u některých psychiatrických onemocnění, zejména schizofrenie, ale i depresivních poruch, obsedantní kompulzivní poruchy (OKP) a autismu.

Schizofrenie. U pacientů se schizofrenií je většinou prokazována dysfunkce antioxidačního systému spojená s vystupňovanou lipoperoxidací. Mechanismy vzniku a působení oxidačního stresu u schizofrenie nejsou jasné, někteří autoři ukazují na význam zvýšeného obratu katecholaminů u nemocných se schizofrenií (34).

Depresivní poruchy. V etiopatogenezi deprese se předpokládá účast oxidačního stresu, a protože mozek obsahuje velké množství vícenenasycených mastných kyselin, železa a nízkou koncentraci antioxidačních enzymů je k jeho působení náchylný. Deprese je často spojena i se subklinickým zánětem provázeným zvýšenými hladinami zánětlivých cytokinů, zvyšujících tvorbu reaktivních částic (35). Byla popsána korelace závažnosti symptomů deprese s hladinou lipoperoxidů v séru.

ZÁVĚR

RONS i antioxidační systémy hrají v organismu důležitou úlohu. Jejich vývoj šel ruku v ruce s vývojem aerobního metabolismu a ochrany před toxicitou kyslíku. Role RONS není pouze negativní účastí v patofyziologických mechanismech různých chorob, ale RONS mají též řadu příznivých účinků a jsou součástí přirozených buněčných signálních drah. Jejich patologické působení závisí hlavně na nerovnováze prooxidačních a antioxidačních systémů. V současné době je jejich studiu věnována zvýšená pozornost a lze doufat v brzké uplatnění poznatků pro léčbu chorob, v jejichž rozvoji se RONS uplatňují.

Zkratky

13-HODE – a 13-hydroxy-9,11-oktadekadienová kyselina
4-HNE – 4-hydroxynonenal
8-OH-dG – 8-hydroxydeoxyguanozin
8-OHG – 8-hydroxyguanin
9-HODE – 9-hydroxy-10,12-oktadekadienová kyselina
AD – Alzheimerova nemoc
ADMA – asymetrický dimetylarginin
AGE – advanced glycation end products
AP-1 – activated protein-1
APP – amyloidový prekurzorový protein
Aß – amyloid beta
BH_4 – tetrahydrobiopterin
Ca^{2+} – intracelulární kalcium
CAT – kataláza
CE – esterifikovaný cholesterol
cGMP – cyklický guanylmonofosfát
Co Q_{10} – koenzym Q_{10}
COX – cyklooxygenáza
COX-2 – izoforma 2 cyklooxygenázy
CRP – C-reaktivní protein
cysLT – cysteinylleukotrieny
DAG – diacylglycerol
eNOS – endotheliální syntáza NO
F-3-P – fruktóza 3-fosfatáza
FAD – flavinadenindinukleotid
FC – volný cholesterol
FMN – flavinmononukleotid
GAPDH – glyceraldehydfosfát dehydrogenázy
GFAT – glutamin: fruktóza 6-fosfát aminotransferáza
Gln – glutamin
Glu – glutamát
GPx – glutahion peroxidáza
GR – glutathion reduktáza
GSH – glutathion
GSSG – glutathion disulfid
GST – glutathion S-transferáza
H_2O_2 – peroxid vodíku
HclO – kyselina chlorná
HETE – hydroxyeikosatetraenová kyselina
HO-1 – hem-oxygenáza
HPETE – hydroperoxyeikosatetraenová mastná kyselina
HTG – hypertriglyceridémie
IFN-γ – interferon gamma
ICHS – ischemická choroba srdeční
IL – interleukin
iNOS – inducibilní NOS
LDL – lipoproteiny o nízké hustotě
LH – mastná kyselina
LO-x – lipoxygenáza
LT – leukotrieny
LX – lipoxiny
MCP-1 – monocyte chemotactic protein-1
MDA – malondialdehyd
mmLDL – minimálně modifikované a oxidované LDL (minimally modified and oxLDL-oxidized)
MPO – myeloperoxidáza
NF-κB – nuclear factor kappa B
nNOS – neuronální NOS
NO – oxid dusnatý
NOS – syntáza oxidu dusnatého
OKP – obsedantní kompulzivní porucha
$ONOO^-$ – peroxynitrit
OS – oxidační stres
PDGF – platelet derived growth factor
PGs – prostaglandin
PKC – proteinkináza C
PPAR-γ – peroxisome proliferator-activated receptor gamma
PUFA – polyunsaturated fatty acids
RONS – reactive oxygen and nitrogen species
ROS – reaktivní sloučeniny kyslíku
sGC – solubilní guanylát cykláza
SMC – proliferace hladkých svalových buněk
SOD – superoxid dismutáza
TNF-α – tumor nekrotizující faktor alfa
TRRed – thioredoxin reduktáza
XD – xanthin dehydrogenáza
XO – xanthaxanthin oxidáza

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