

SYMPOSIUM

Time dependent changes in oxidative metabolism during chronic diabetes in rats[†]

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ABSTRACT The stability and capacity of antioxidant status during chronic diabetes seriously influence the outcome of the long-term complications caused by oxidative stress. In the present study we investigated the effects of chronic streptozotocin-induced diabetes on the parameters of antioxidant status: activity of scavenging enzymes, glutathione-related and total antioxidant capacity, and degree of lipid peroxidation. Changes in the activities of superoxide-dismutase (SOD), glutathione peroxidase (GSH-Px), glutathione reductase (GSH-R), and catalase, and in the content of reduced glutathione (GSH), and oxidised glutathione (GSSG), and in the ratio of GSH/GSSG in blood samples were determined by means of biochemical methods. The degree of lipid peroxidation was measured via thiobarbituric acid assay (TBARS). Hyperglycaemia, ketosis and the accumulation of glycated proteins were estimated by measuring blood glucose, 3-OH-butirate, fructosamine and haemoglobin A_{1c}. In the course of chronic insulin-dependent diabetes, *i.e.* at 2 and 7 days, 10 weeks, and 6 and 8.5 months after streptozotocin injection, hyperglycaemia slightly while ketosis markedly attenuated. Lipid peroxidation was also attenuated. SOD activity decrease in the acute phase only. The activity of GSH-Px increased in the early phase while that of GSH-R mostly decreased in the chronic phase. GSH and GSSG concentrations moved into opposite direction in a time dependent manner. In conclusion, in chronic diabetes an attenuation of severity of diabetes was present throughout the post-injection period, which was well reflected in the improved antioxidant status and capacity.
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KEY WORDS

antioxidant status,
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Diabetes mellitus is accompanied by hyperglycaemia and a disturbed intracellular carbohydrate metabolism. Hyperglycaemia generates glucose auto-oxidation and auto-oxidative glycosylation of proteins, which may be considered as a combined source of generation of an additional amount of reactive oxygen species (ROS, Baynes 1991; Van Dam et al. 1995, 1996). The formation of oxygen free radicals in diabetes results in an extra charge on the antioxidant capacity of the organism and leads to complications in different tissues including vascular bed (Giugliano et al. 1996).

One of the most prominent sources of oxygen free radicals like superoxide and hydroxyl radicals ($\cdot\text{O}_2^-$ and $\cdot\text{OH}$, respectively) is the process of autooxidation of glucose into enediols in the presence of transition metals (Wolff et al. 1991). The enhanced generation of ROS in diabetes accelerates protein glycation, while glycated proteins become more susceptible to oxidation leading to the accumulation of advanced glycation endproducts (Bonfont-Rousselot 2002). The ROS-scavenging capacity through the antioxidant systems becomes insufficient in diabetes and a constant

oxidative stress develops. Oxidation of lipids, proteins and other macromolecules like DNA become overt with time during the development of diabetes. Lipid peroxides like malonyldialdehyde are the most frequently used compounds for the assessment of the degree of oxidative stress by measuring thiobarbituric acid reactive substances (TBARS, Ohkawa et al. 1979).

In the present study we investigated how the antioxidant capacity in the blood changes during the course of chronic diabetes in rats. Markers of the altered glucose metabolism, *i.e.* blood glucose, 3-OH-butirate, fructosamine and haemoglobin A_{1c} were assayed for estimation of the severity of diabetes. The activities of several scavenging enzymes were also followed. Among them superoxide-dismutase (SOD), glutathione peroxidase (GSH-Px), glutathione reductase (GSH-R), and catalase were selected. Regarding the endogenous antioxidant defence mechanisms the function of glutathione system was selected and the concentrations of reduced and oxidised glutathione (GSH and GSSG) were measured in the red blood cells (RBC). Changes in concentration of lipid peroxidation products in both RBC and plasma were also estimated as TBARS levels. By this approach a relation between the severity of diabetes and the

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[†]In memory of Professor Béla Matkovic

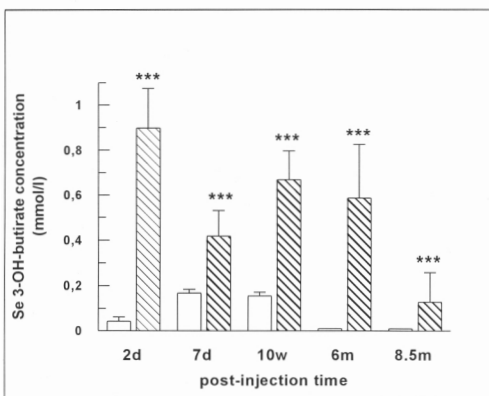
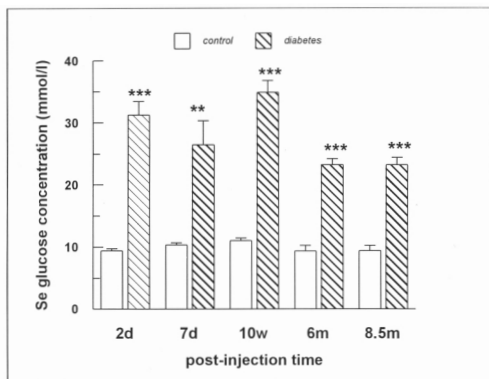


Figure 1. Time course of hyperglycaemia (upper panel) and ketosis (lower panel) in streptozotocin-induced chronic diabetes in rats. ** $p < 0.01$, *** $p < 0.001$ compared to controls (Student's t-test).

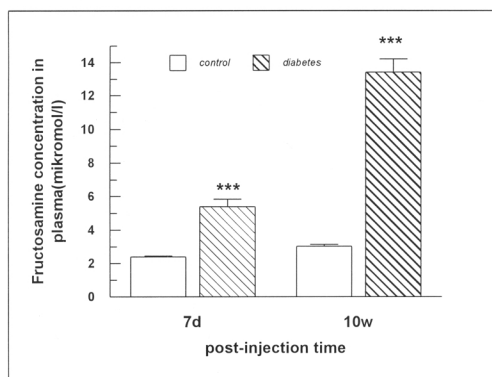
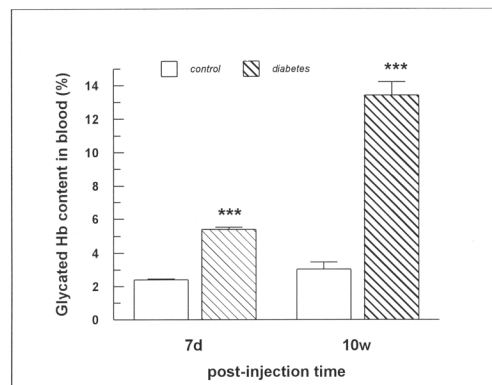


Figure 2. Increment in glycated haemoglobin content (upper panel) and in plasma fructosamine level (lower panel) in rats exposed to streptozotocin injection, during the time of chronic diabetes. *** $p < 0.001$ compared to controls (Student's t-test).

antioxidant capacity of the diabetic organism could be compared.

Materials and Methods

Animals and treatment

Sixty young adult male Wistar rats (250-300g) were divided into two groups: chronic diabetes and control. The rats were kept under standard laboratory conditions (12/12 h dark/light cycle, lights on at 7.00, room temperature set on $21 \pm 1^\circ\text{C}$, controlled humidity). All experiments on rats were performed in strict compliance with National Institutes of Health Guide for Care and Use of Laboratory Animals (1985).

Diabetes was induced with injection of streptozotocin (Sigma, St. Louis, MO, USA) into the tail vein in a dose of 60 mg/kg body weight. The control rats were injected with saline. Twelve animals were sacrificed after different survival periods at each time, at 2 and 7 days, 10 weeks, 6 and 8.5 months. From the 12 animals 6 were streptozotocin-treated and 6 controls. At the conclusion of the experiment the rats were deeply anaesthetised with chloral hydrate (375 mg/kg body weight; Sigma) and blood was taken from the aorta

during exsanguination. Disodium-ethylenediamine-tetraacetate (EDTA, 1 mg/ml, Sigma) was added to blood samples to prevent coagulation. In each case another blood sample was taken without anticoagulation to obtain serum as well. After a short storage at 4°C (maximum 1/2 hour) the anticoagulated blood samples were processed for centrifugation at 3,000 G for 10 min at 4°C . The erythrocytes and plasma fractions were processed separately afterwards. After 3 times washing with ice-cold physiological saline the erythrocyte fraction were hemolysed with distilled water reaching 10X or 2X dilutions and the aliquots were stored at -20°C until biochemical assays.

Biochemical determinations

A) Diabetes markers

To monitor the development of diabetes several variables were measured: serum glucose and fructosamine, plasma 3-hydroxi-butyrate concentration and glycosylated haemoglobin content in the whole blood. Blood glucose concentration from serum was determined spectrophotometrically by a

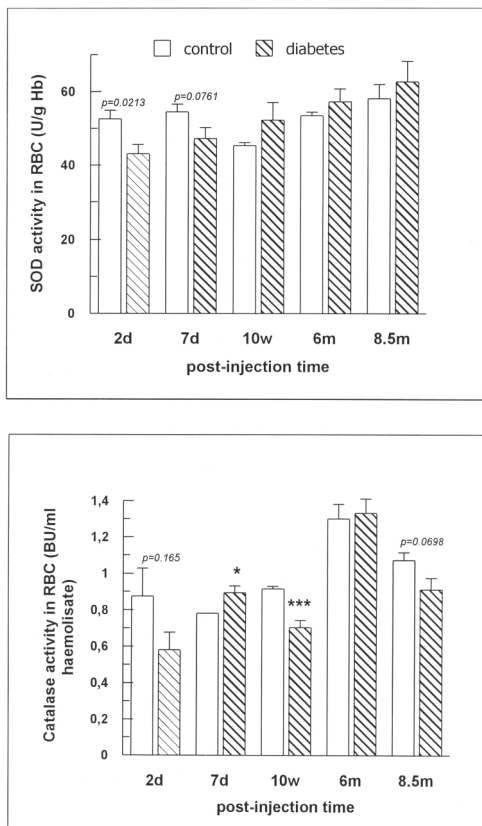


Figure 3. Time dependent changes in red blood cell (RBC) SOD activity (upper panel) and in catalase activity (lower panel) following streptozotocin injection. * $p < 0.05$, *** $p < 0.001$ compared to controls (Student's t-test).

GOD/POD/PAP kit, an enzymatic colorimetric method assaying the reaction product at 505 nm (Trinder et al. 1969). The plasma 3-OH-butyrate concentration was determined by the method of McMurray et al. (1984) by a Randox-kit (United Kingdom). The fuctosamine concentration of the serum was measured by a commercially available kit (Roche). The amount of glycated haemoglobin in whole blood samples was measured after a chromatographic separation on resin with a colorimetric method at 415 nm.

B) Antioxidant parameters

Assaying markers of antioxidant systems the haemoglobin (Hb) content of hemolysates was determined by using MINOS SPE Automate system (Roche, France). Among the antioxidant enzymes the Cu/Zn-SOD activity was determined by the method of Mishra et al. (1972) and Matkovic et al. (1977) based on the inhibition of superoxide ($\cdot\text{O}_2^-$)-dependent transformation of epinephrine to adrenochrome. The adrenochrome was measured at 480 nm with Hitachi U-2001 spectrophotometer on standard temperature of 32°C using the 2X-diluted haemolysate. Listing analytical steps

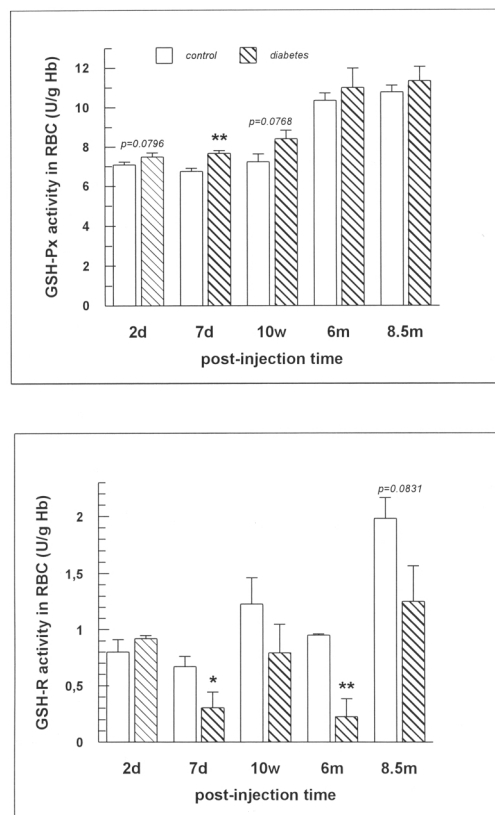


Figure 4. Changes in red blood cell GSH-Px and GSH-R enzyme activities (upper and lower panels, respectively) in the course of chronic diabetes after streptozotocin injection. * $p < 0.05$, ** $p < 0.01$ compared to controls (Student's t-test).

briefly, the haemolysate was extracted with chloroform:ethanol = 1:2, the water phase was obtained after centrifugation with 15,000 rpm for 10 min. The activity of SOD was expressed in U/gHb. One unit of SOD activity corresponds to a 50% inhibition of epinephrine-adrenochrome conversion.

Catalase activity of the red blood cells was measured by the method of Beers et al. (1952) by means of monitoring the rate of H_2O_2 consumption at 240 nm on 25°C. The determination was carried out in the 100X-diluted hemolysates. The activity of catalase was expressed in g H_2O_2 hydrolysed/min/ml hemolysate at 25°C.

GSH-Px activity was measured spectrophotometrically by the method of Sedlak et al. (1968; 412 nm; Hitachi U-2001 spectrophotometer) using the 10X-diluted hemolysate by applying 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) in the presence of cumene hydroperoxide as a cosubstrate. The activity of GSH-Px was expressed in μmol GSH oxidised/min/gHb at 37°C.

GSH-R activity was determined by the method of Bergmeyer (1963) from the 10X-diluted hemolysate. In this

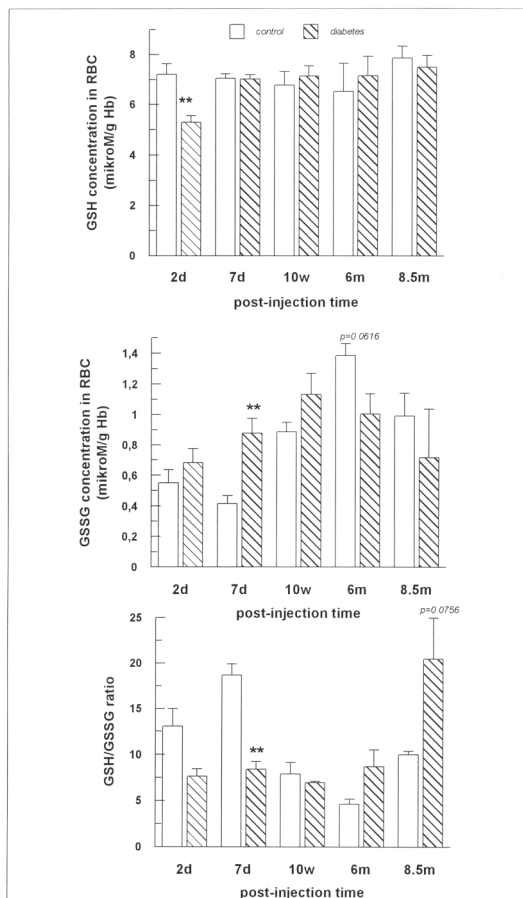


Figure 5. Concentrations and ratio of GSH and GSSG in the red blood cells in the course of chronic diabetes after streptozotocin injection. ** $p < 0.01$ compared to controls (Student's t-test).

assay the oxidised form of glutathione is reduced to GSH by GSH-R in the presence of reduced nicotinamide adenine dinucleotide phosphate (NADPH), which serves as a H^+ donor in the chemical reaction. The oxidation of NADPH was followed spectrophotometrically (340 nm, Hitachi U-2001 spectrophotometer) throughout a 3 min period. The activity of GSH-R was expressed in $\mu\text{mol NADPH oxidised/min/gHb}$ at 37°C .

The GSH content of the same hemolysate was measured spectrophotometrically (412 nm, Hitachi U-2001 spectrophotometer), with DTNB, by the method of Beutler et al. (1963) and Tietze (1969). The amount of GSH in the hemolysate was expressed in nmol/gHb .

The determination of oxidised glutathione in the hemolysate was carried out by the method of Tietze (1969) on 25°C , applying a spectrophotometric measurement with the help of DTNB (412 nm, Hitachi U-2001 spectrophotometer). The amount of GSSG was expressed in nmol/gHb . Finally the ratio of GSH/GSSG was also calculated.

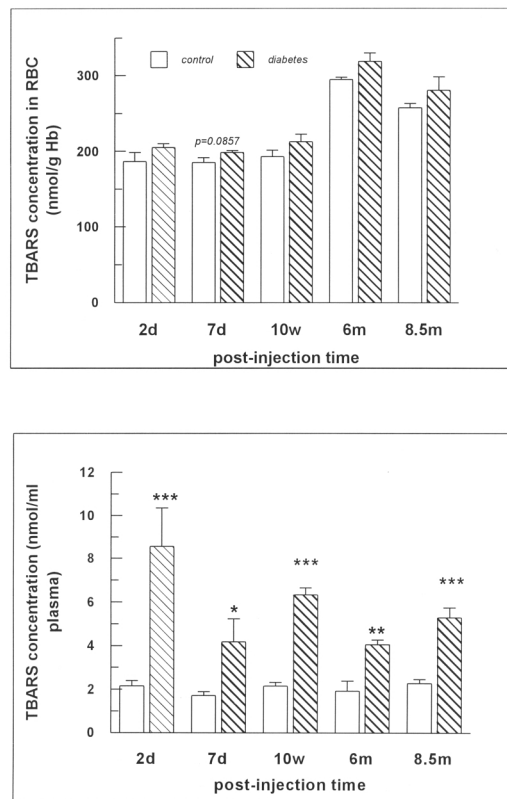


Figure 6. Lipid peroxidation expressed as TBARS concentration in red blood cells (upper panel) and in the plasma (lower panel) in rats exposed to streptozotocin injection. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to controls (Student's t-test).

The degree of lipid peroxidation was established by estimation of the thiobarbituric acid reactive substance (TBARS) concentrations in both erythrocytes and plasma. The TBARS content was determined as described by Ohkawa et al. (1979) and Yagi (1976) by using thiobarbituric acid (Merck, Darmstadt, Germany). The TBARS product was assayed by Hitachi U-2001 spectrophotometer at 532 nm. The RBC and plasma TBARS contents were expressed in nmol/gHb and nmol/ml plasma, respectively.

The ferric reducing ability of plasma (FRAP) as a measure of total antioxidant capacity was assayed by the method of Benzie and Strain (1996). The FRAP values was obtained with spectrophotometer measurements at 593 nm.

Results

Characteristics of chronic diabetes

Two days after streptozotocin treatment the blood glucose level increased to the maximum measured throughout this experiment (Fig. 1, upper panel). The serum concentration of 3-OH-butirate was also high on the second day and remained significantly higher during the entire course of the

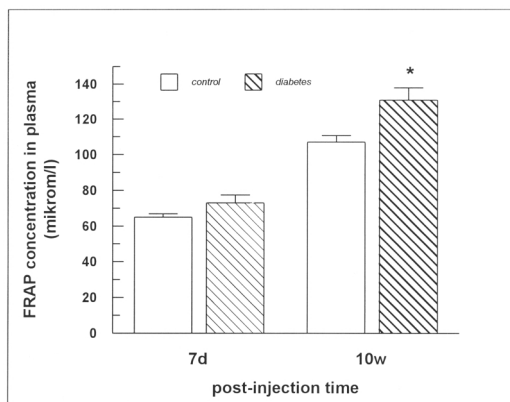


Figure 7. Total antioxidant capacity (FRAP) measured in the plasma of rats exposed to streptozotocin injection. * $p < 0.05$ compared to controls (Student's *t*-test).

study (Fig. 1, lower panel). By the end of observation period the glucose level decreased slightly and the 3-OH-butirate concentration showed a more marked diminution, especially at the 8.5 months time point. Those parameters, which reflect accumulation in the molecular effects of chronically high glucose level, *i.e.* glycated haemoglobin and fructosamine, are shown in Figure 2. It is clearly visible that with time the level of both parameters increased significantly.

Parameters of oxidative metabolic state

Activities of oxidative scavenging enzymes are summarised in Figure 3. SOD activity slightly decreased in the acute phase, up to 7 days of the experiment (upper panel). Catalase activity varied throughout the experiment into opposite directions, therefore, no clear change could be realised (lower panel). Enzymes related to the oxidation-reduction circuit of the GSH pathways, GSH-Px and GSH-R, showed also some activity changes (Fig. 4). GSH-Px activity increased significantly at day 7 as well as slightly at 10th week, while other time periods the increment did not reach statistical significance. GSH-R activity, contrary to GSH-Px, performed a decrement from the sub-acute phase (day 7) on.

The concentrations of reduced and oxidised molecular forms of glutathione, *i.e.* GSH and GSSG, as well as their ratio are shown in Figure 5. During the acute phase (day 2) GSH reduced significantly, but the level of this antioxidant marker has been restored from day 7 on (Fig. 5, upper panel). In case of GSSG at the beginning of the observation period the major effect was visible at day 7. This tendency could be seen up to the 10th week, but later the direction of change reversed (Fig. 5, middle panel). The ratio of these two parameters showed a decrease in the acute and sub-acute phases and in the chronic period it tended to be increased (Fig. 5, lower panel).

The degree of lipid peroxidation measured as TBARS level in the RBC and plasma is demonstrated in Figure 6. In the case of RBC a slight tendency of increase was visible throughout of all experimental time-points (upper panel), while in the plasma a marked increment was found in all time periods (lower panel). The highest reaction was visible at day 2 and from day 7 the increment in plasma levels became practically constant.

The parameter of the overall antioxidant capacity reflected in the plasma, *i.e.* the FRAP concentration, slightly increased at day 7 and became significantly more pronounced at the chronic phase.

Discussion

The main findings of this experiment are the followings. (a) After streptozotocin injection in the acute phase of the diabetes (2nd day) blood glucose level showed already a maximum and was accompanied by a maximal production of 3-OH-butirate indicating that ketosis followed the acute diabetes. By the end of the observation period the remarkable decline in 3-OH-butirate indicated that the metabolic severity of diabetes attenuated. (b) FRAP concentration, *i.e.* the total antioxidant capacity showed an increment in the course of diabetes, which is in accordance with the decline in ketosis. (c) The glutathione (GSH) antioxidant system also has been compensated throughout the time-period of chronic diabetes: at the acute and subacute phase the ratio of GSH/GSSG decreased, but in the chronic phase this ratio turned into opposite direction and stayed elevated, indicating that the concentration of the reduced form of glutathione could meet the requirements of the increased oxidative stress. (d) The activities of the scavenging enzymes also changed. SOD declined temporarily but only in the acute phase. GSH-Px activity increased at the acute and subacute phases, while GSH-R activity declined in the advanced period of testing.

To combat ROS the organism has defence mechanisms of enzymatic and non-enzymatic types. The first line of enzymatic defence against the superoxide radical is formed by the superoxide dismutase enzymes (SOD). These enzymes catalyse the diffusion-limited dismutation of superoxide to hydrogen peroxide and oxygen. The cytosolic SOD enzyme contains the metal ions copper and zinc (Cu/Zn-SOD). Hydrogen peroxide in peroxisomes is metabolised by catalase (Oshino and Chance 1977). For further metabolism of cytosolic and mitochondrial hydrogen peroxide a selenium containing scavenger enzyme glutathione peroxidase (GSH-Px) plays an important function. GSH-Px catalyses a reaction in which hydrogen peroxide is reduced to water at the expense of reduced glutathione. The resulting oxidised glutathione molecule is a dimer (GSSG) and is recycled and reduced to GSH by the glutathione reductase enzyme (GSH-R).

In the present study an initial decrement in Cu/Zn-SOD activity was observed in the erythrocytes of diabetic rats (days 2 and 7). By the 10th week and longer periods, however, the activity tended to be higher. It is recognised that oxidative stress inactivates SOD by its enzymatic product H₂O₂ (Salo et al. 1990). At the same time superoxide radical is a signal for increased genetic expression of SOD (Matsuyama et al. 1993). The available amount and activity of SOD, therefore, is the subject of a balance between the production of enzyme molecule and its degradation. As it was shown here this balance shows a time dependent modulation in the course of diabetes.

Two enzymes, GSH-Px and catalase, cooperate in the degradation of H₂O₂. While catalase activity did not show consistent changes, GSH-Px displayed an increment, especially at the acute and subacute phases. It is probable that the increased activity of GSH-Px participated in the gradual but moderate recovery from the acute phase of oxidative stress caused by diabetes. In the very beginning of the diabetic oxidative stress the concentration of GSH decreased, while the oxidised form of glutathione (GSSG) increased. The ration of GSH/GSSG, therefore, decreased at days 2 and 7. In the more advanced periods of the present testing GSSG concentration decreased and the ration of GSH/GSSG tended to be increased. In conclusion, in the course of the observation period the glutathione defence system showed a gradual compensatory increment in its capacity.

The chronic decrement in the activity of GSH-R during prolonged diabetes found in the present study raises several questions. Like SOD the enzymes included in the glutathione redox cycle show susceptibility to oxidation and inactivation. In patients with insulin-dependent diabetes mellitus the GSH pathway enzymes proved to be susceptible to oxidation and this susceptibility increased in poorly controlled diabetics (Dincer et al. 2002). In our present study the activity of GSH-Px was not decreased but only that of GSH-R. It might mean that the GSH redox enzymes are differently sensitive to the destructing action of oxygen radicals.

Lipid peroxidation was studied by measuring TBARS concentration. In the erythrocytes TBARS level increased only moderately but in the plasma a marked elevation could be observed. TBARS concentration in the plasma decreased in the course of observation period with some fluctuation. This finding is in accordance with the idea that there is a functional recovery, at least partial, in the antioxidant defence systems in rats during long-term diabetes.

Acknowledgments

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