Glycoxidative and nitrosative stress in kidney of experimental diabetic rats: Effects of the prydoindole antioxidant stobadine

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Abstract

OBJECTIVES: Recent basic research and clinical data have provided new insights into the role of glycoxidative and nitrosative stresses (both oxidative stress) in diabetic complications, such as diabetic nephropathy, suggesting a different and innovative approach to antioxidant therapy. In streptozotocin-induced diabetic rat kidney, the present study investigated the effects of the sythetic pyridoindole antioxidant stobadine (STB) on renal total antioxidant potential (AOP) and protein oxidation parameters such as protein carbonyl content (PCC), advanced oxidation protein products (AOPPs) and nitrotyrosine (NT), a marker specific for protein modification by peroxynitrite.
MATERIALS AND METHODS: Wistar Albino rats were divided into two groups: normal and streptozotocin-induced diabetic rats. Each group of the animals was further divided into two groups: untreated and treated with stobadine (24.7 mg/kg) during 16 weeks daily by oral gavage.
RESULTS: The renal tissue AOP and the levels of AOPPs, PCC and NT were increased in diabetic rats compared with the untreated control animals. Furthermore, stobadine treatment significantly decreased protein carbonylation and AOPPs but not NT.
CONCLUSIONS: These findings indicate that STB is an antioxidant factor which can improve glycoxidative stress markers in kidney, while it has no effect on protein nitrosylation.

INTRODUCTION

Increased production of reactive oxygen and nitrogen metabolites, such as superoxide anion, hydrogen peroxide, nitric oxide, and peroxynitrite has been documented to occur in diabetes in association with hyperglycemia. Hyperglycemia can induce the formation of free radicals and activation of oxidative/nitrosative stress and it is also a key event in activating the other pathways involved in the pathogenesis of diabetic complications, such as nonenzymatic glycation of proteins (Sakurai & Tsuchiya 1998); increased polyol pathway (Ghahary *et al.* 1989); activation of protein kinase C

ORIGINAL ARTICLE

Abbreviations:

AOP	- Antioxidant Potential
AOPPs	- Advanced Oxidation Protein Products
PCC	- Protein Carbonyl Content
STB	- Stobadine
NT	- Nitrotyrosine

(Derubertis & Craven, 1994) and increased hexosamine pathway (Goldberg *et al.* 2002) Therefore excessive generation of reactive radicals has been suggested as one of the mechanisms involved in the development of clinical and experimental complications of diabetes, including nephropathy.

Under oxidative and nitrosative stress, a number of enzymes and structural proteins can be modified by reactive free radicals with direct formation of oxidized amino acids or indirectly by interaction with reactive carbonyls formed by auto-oxidation of carbohydrates and lipids. Oxidized amino acids can lead to the formation of protein carbonyl derivatives. Accumulation of modified derivatives can impair the cellular functions and may cause loss of catalytic and structural integrity (Suzuki & Miyata 1999, Strosova *et al.* 2008). The best known markers of oxidative and nitrosative protein damage are metal-catalyzed protein oxidations characterized by protein carbonyl content (PCC), advanced oxidation protein products (AOPPs) and nitrotyrosine (NT) formation (de Zwart *et al.* 1989; Ischiropoulos & al-Mehdi 1995)

AOPPs were described by Witko-Sarsat *et al.* (1996) for the first time in uremia. They are formed during oxidative stress via the action of chlorinated oxidants generated by myeloperoxidase in activated neutrophils. They are structurally similar to advanced gycation end products and exert similar biological functions, i.e. induction of proinflammatory cytokines and adhesive molecules. They are elevated in diabetic patients and are well-known indicators of oxidative protein damage (Witko-Sarsat *et al.* 1998; Kalousová *et al.* 2002).

Peroxynitrite is a highly cytotoxic oxidant formed by the reaction between superoxide and nitric oxide. Toxicity of peroxynitrite or its reactive intermediates can be mediated via multiple mechanisms, leading to cellular dysfunction. Peroxtnitrite can oxidize unsaturated fatty acids, low molecular mass antioxidants and nitrate tyrosine residues of cellular proteins. NT is a useful marker of peroxynitrite-mediated protein modification and plays a significant role in the pathogenesis of diabetic complications (Ischiropoulos & al-Mehdi 1995, Pacher *et al.* 2005).

There are both enzymatic and non-enzymatic mechanisms by which cells protect themselves against the destructive activity of reactive radicals on biomolecules. The enzyme protection mechanisms involve superoxide dismutase, catalase and glutathione peroxidase. On the other hand, low molecular weight antioxidants also play a significant role in maintaining the redox balance. Glutathione, ascorbate, tocopherols and a number of other compounds, such as cysteine, uric acid and bilirubin, are also known antioxidants. The antioxidant capacity comprises both antioxidant enzymes and some of the antioxidant molecules listed above (Grune 2002; Aydin *et al.* 2001; Rysz *et al.* 2007).

Stobadine, a pyridoindole derivative, is known as an efficient antioxidant and scavenger of free radicals, such as hydroxyl, peroxyl and alkoxyl radicals, and as an effective quencher of singlet oxygen. On the other hand, it only vaguely scavenges superoxide radical. Stobadine was shown to be able to repair oxidized amino acids, to prevent protein carbonylation and to protect against oxidation of protein and non-protein thiols. Stobadine has been shown to delay the development of pathogenesis of the late complications of diabetes (Horáková & Stolc 1998, Cumaoglu *et al.* 2007). In the present study, the effects of stobadine treatment on AOP, on formation of PCC, AOPPs and NT in the kidney tissue of streptozotocin diabetic rats were determined.

MATERIALS AND METHODS

Chemicals and instruments

Streptozotocin, 2,4-dinitrophenylhydrazine, folin ciocalteu reagent and other chemicals were purchased from Sigma chemicals Co (USA). Complete EDTA-free protease inhibitor cocktail tablets was purchased from Roche Applied Science (Indianapolis, USA). Stobadine dipalmitate was obtained from the Slovak Academy of Sciences, Institute of Experimental Pharmacology and Toxicology. Plasma glucose concentration was determined with a glucometer (Accutrend® GCT system, Roche Diagnostics, Basel, Switzerland). Nitrotyrosine levels were determined using a commercial kit Oxiss-Research® (Oxis International Inc., Portland, OR). Total antioxidant status was measured with OxissResearch® AOP-490 assay kit. For ELİSA and other microplate assays, a Bio-Tek ELX800 absorbance microplate reader (BioTek Instruments Inc., Vermont, USA) was used.

Animals and treatments

Male Wistar-Albino rats weighing 250–300 g and supplied by Ankara University Research Laboratory, Ankara, Turkey, were used in this experiment. Animals were designated randomly to be made diabetic or to remain as healty controls. Experimental diabetes was induced by a single intraperitoneal injection of STZ (55 mg/kg) dissolved in citrate buffer (pH 4.5). Two days after STZ administration, all animals with plasma glucose level >250 mg/dL were considered diabetic and were included in the study. Stobadine was administered both to diabetic rats (Diabet+STB) and non-diabetic (Control+STB) rats by gavage at the dose of 24.7 mg/kg body weight daily for 16 weeks. During the experiment, the animals were housed in conventional wire-mesh cages, each group per cage with bedding composed of wood shaving (exchanged daily). All rats were allowed free access to a standard laboratory diet and water by

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drinking bottle during the course of the experiment. The animal room was air-conditioned and the environment was continuously monitored for the temperature of 23±1 °C and relative humidity of 40–70% and light/dark cycles (12 h). Interventions concerning animals were performed according to "*Principles of Laboratory Animal Care*" (NIH publication No. 85-23, revised 1985).

Determination of protein carbonyl levels

Homogenates were prepared on ice in the ratio of 100 mg tissue per 900 µl of 50 mM phosphate buffer, pH 6.7, containing 1 mM Na_2EDTA. Protein carbonyl levels were measured spectrophotometrically by using the method of Reznick & Packer (1994). Protein carbonyl groups react with 2,4-dinitrophenylhydrazine (DNPH) to generate chromophoric dinitrophenylhydrazones. DNPH was dissolved in 2.5 M HCl and after the DNPH reaction, proteins were precipitated with an equal volume of 20% (w/v) trichloroacetic acid and washed three times with 1 ml of an ethyl acetate/ethanol mixture (1:1). Washings were achieved by mechanical disruption of pellets in the washing solution and re-pelleting by centrifugation at 6 000 g for 10 min. Finally, the precipitates were dissolved in 6 M guanidine-HCl solution and the absorbances were measured at 370 nm, using the molar extinction coefficient of DNPH, $e = 2.2 \times 10^4\ M^{-1}cm^{-1}$. The coefficients of intra- and inter-assay variations for carbonyl assay were 4.8% n=8 and 9.0% n=10, respectively.

Determination of advanced oxidation protein products levels

AOPP levels were measured as previously described method by Witko-Sarsat *et al.* (1998). Plasma (200 µl) diluted 1/5 in PBS, 20 µl of acetic acid and 10 µl of potassium iodide was placed in wells of a 96-well microtiter plate. The absorbance of the reaction mixture was immediately read at 340 nm on the microplate reader. Concentrations of AOPP were calculated by using the extinction coefficent 261 $mM^{-1}cm^{-1}$ The coefficients of intra- and inter-assay variations for AOPP assay were 4.0% n=10 and 8.2% n=10, respectively.

Determination of nitrotyrosine levels

NT in kidney homogenates was measured according to the method of Beckman (2002). In brief, kidney homogenates were prepared in phosphate buffered saline (PBS) containing protease inhibitor cocktail. The homogenates were centrifuged at 10 000 × g for 10 min. The supernatants were assayed for NT content using NT ELISA Kit according to the manufacturer's instructions.

Determination of total antioxidant potential

The antioxidant potential of tissue homogenate was determined using the Bioxytech AOP-490 assay kit (Oxis Research, Portland, OR), which measures the ability of all available antioxidants to reduce copper. Samples were diluted in an assay buffer containing bathocuproine to a final volume of 100 µL and they were added to a 96-well microplate in duplicate. A reference measurement was taken at 490 nm using the microplate reader followed by the addition of 25 µL of assay buffer containing Cu^{2+} for 5 min of incubation at room temperature. Finally, 25 µL of stop solution was added, mixed, and the plate was read again at 490 nm. Following subtraction of the reference measurement from the final measurement, the standard uric acid (2.0 to 0.03125 mM) curve was used to calculate the copper reducing equivalents.

Determination of total protein levels

Total protein concentration in the kidney homogenates were determined by the Lowry method using bovine serum albumin as a standard (Lowry *et al.* 1951).

Statistical analysis

The statistical program SPSS 11.5 was used for data analysis. The reported data are the means of measurements and their S.E.M. values. Kruskall Wallis ANOVA, Mann Whitney U tests were used for comparisons of the groups as appropriate. A value of $p<0.05$ was set as the limit of statistical significance.

RESULTS

Blood glucose and body weight

The blood glucose levels were found to be significantly ($p<0.001$) higher in the group of STZ-diabetic rats as compared with those of controls. Stobadine treatment produced a significant ($p<0.05$) decrease in final blood glucose levels in Diabet+STB group. There was a significant ($p<0.05$) weight reduction in the STZ-Diabetic group when compared with controls. STB treatment caused significant ($p<0.05$) increase of the final average body weights compared with untreated diabetic group, as shown in Table 1.

Total antioxidant potential

Total antioxidant potential was found to be significantly ($p<0.05$) higher in diabetic rat kidney tissue than in nondiabetic control cases. There was no significant effect of STB treatment on antioxidant potential of diabetic kidney as shown in Table 2.

Markers of oxidative and nitrosative protein damage

The main characteristics of all oxidative and nitrosative protein damage markers are presented in Table 2. Statistically significant ($p<0.05$) elevated protein carbonyl contents were detected in kidney homogenates of diabetic group as compared with those of control cases. Protein carbonyl levels were decreased in stobadine treated non-diabetic controls (Control+STB) but the difference did not reach significance. Stobadine treatment significantly ($p<0.01$) decreased the levels of protein carbonyl in the Diabet+STB group compared with nontreated diabetic rats.

Tab. 1. The effects of stobadine (STB) supplementation on blood glucose levels and body weights of rats in all experimental groups.

	Untreated control	Stobadine treated control (Control+STB)	Untreated diabetic	Stobadine treated diabetic (Diabet+STB)
Final Blood Glucose level (mmol/L)	6.33±1.3 n=10	6.77±2.4 n=7	20.38±0.6$^{\omega}$ n=8	16.66±1.6$^{\varepsilon}$ n=9
Starting Body Weight (g)	240±2.8 n=10	232±3.1 n=7	252.3±3.8 n=8	248.4±4.2 n=9
Final Body Weight (g)	288±3.0 n=10	279±4.9 n=7	220.4±3.3$^{\gamma,*}$ n=8	269.5±2.8$^{\varepsilon}$ n=9

Values are mean ± S.E.M
$^{\gamma}$*p*<0.01, $^{\omega}$*p*<0.001 vs. untreated control. $^{\varepsilon}$*p*<0.05 vs. untreated diabetic, **p*<0.05 vs. starting body weight.

Tab. 2. The effects of stobadine (STB) supplementation on total antioxidant potential (AOP; μM Copper Reducing Equivalents), protein carbonyl content (PCC; nmol/mg protein), advanced oxidation protein products (AOPPs; nmol/mg protein) and nitrotyrosine (NT; nmol/mg protein) in the kidney homogenates of rats in all experimental groups.

	Untreated control	Stobadine treated control (Control+STB)	Untreated diabetic	Stobadine treated diabetic (Diabet+STB)
AOP	0.133±0.0006 n=7	0.133±0.0013 n=7	0.138±0.0014^{b,e} n=8	0.134±0.0008 n=7
PCC	5.77±0.33 n=7	4.75±0.40 n=5	8.01±0.66^{a,e} n=7	4.12±0.29^{b,c} n=7
AOPPs	6.23±0.43 n=10	5.37±0.47 n=7	9.31±0.17^{b,e} n=8	7.63±0.32^{a,e,c} n=9
NT	248.48±9.91 n=5	256.21±7.02 n=5	299.10±6.91^{a,f} n=6	263.32±4.81 n=8

Values are means ± S.E.M
^{a}*p*<0.05, ^{b}*p*<0.01 vs. untreated control, ^{c}*p*<0.05, ^{d}*p*<0.01 vs. untreated diabetic, ^{e}*p*<0.05, ^{f}*p*<0.01 vs. stobadine treated control

AOPPs are a well-used biomarker of oxidative protein damage. We found significant (*p*<0.01) elevation of AOPPs in rat kidney homogenats of diabetic rats in comparison with non-diabetic controls. Slight but not statistically significant differences between control and STB treated control groups were observed. In the STB treated diabetic group, AOPPs levels were significantly decreased (*p*<0.05) as compared with untreated diabetic rats.

The nitrated tyrosine residues are a useful marker for detecting peroxynitrite in biological samples. The concentration of NT was significantly (*p*<0.05) higher in diabetic groups compared with those of control cases. Stobadine treatment decreased NT levels in the diabetic group but the difference did not reach statistical significance in comparison with the untreated diabetic rats.

DISCUSSION

In the present study, protein carbonyl compounds and advanced oxidation protein products were found to be elevated in renal tissue homogenates of non-treated diabetic rats compared with healthy controls. This is in accordance with the findings of early studies of other investigators (Cederberg *et al.* 2001; Kyselova *et al.* 2005; Suryanarayana *et al.* 2005; Cumaoglu *et al.* 2007). In diabetes, autoxidation of reducing sugars yields dicarbonyls, which react with proteins and eventually lead to the formation of AOPPs and advanced glycation end-products (AGEs). AOPPs are supposed to be structurally similar to AGE proteins and to exhibit similar biological toxic effects as AGEs. Oxidative modifications of structural proteins and enzymes also play a significant role in the progression of diabetes and its complications (Miyata *et al.* 2008).

The results of our study showed that STB treatment significant decreases renal PCCs and AOPPs in diabetic rats. These results support our early findings (Cumaoglu *et al.* 2007) and they are in accordance with the *in vitro* results of Stefek *et al.* (2005) who demonstrated that the aggregation and cross-linking of 2,2'-azobis (2-amidinopropane) hydrochloride (AAPH)-treated proteins along with the generation of free carbonyls was significantly inhibited by the chain-breaking antioxidant STB. Furthermore, in an experimental glycation model *in vitro*, the glycation-related fluorescence changes of bovine serum albumin were inhibited by the pyridoindole antioxidant STB, and so was the yield of

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2,4-dinitrophenylhydrazine-reactive carbonyls (Stefek *et al.* 1999).

On the other hand, peroxidation of polyunsaturated fatty acids yields reactive carbonyl coumponds such as malondialdehyde (MDA), 4-hydroxynonenal (HNE) and acrolein in biological systems. The latter reactive carbonyl compounds produce lipoxidation products via protein amino acid residues attack. These are MDA-lysine, HNE-lysine adducts and acrolein-protein adducts, also termed advanced lipoxidation end products. Non-enzymatic alterations in proteins by lipid aldehydes play an important role in the progression of diabetic nephropathy (Suzuki & Miyata 1999; Dalle-Donne *et al.* 2003). In several *in vivo* experiments, STB was shown to reduce oxidative damage of kidney tissue, as indicated by decreased conjugated diene and malondialdehyde levels in tissue homogenates of STZ-diabetic rats (Stefek *et al.* 2002; Kyselova *et al.* 2005; Yülek *et al.* 2007).

Peroxynitrite formation is a process of key importance in the development of diabetic complications. Peroxynitrite anion is cytotoxic because it oxidizes thiol groups in proteins, initiates lipid peroxidation, and nitrates amino acids such as tyrosine. On the other hand, nitration can cause antioxidant enzyme inhibition, signal transduction pathway disturbance and islet cell destruction (Suarez-Pinzon *et al.* 1997; Szabó *et al.* 2002; Pacher *et al.* 2005). In this study, protein nitration, measured as NT, was found to be elevated in renal tissues of non-treated diabetic rats compared with healthy controls. This is in accordance with the findings of others who have shown that nitrotyrosine formation is increased in diabetic rats (Kowluru & Odenbach 2004; Drel *et al.* 2006; Kavak *et al.* 2008). Our results showed that STB treatment decreased NT levels in the diabetic group but the difference was not statistically significant. This finding is supported by our previous experimental data (Cumaoglu *et al.* 2007).

It has been shown that hyperglycemia results in oxidative stress, which is defined as an imbalance between cellular oxidants and antioxidants. There is also evidence that elevation in glucose concentration may depress natural antioxidant defenses, including enzymatic antioxidants or low molecular weight non-enzymatic antioxidants. A number of experiments have been performed to evaluate changes in the antioxidative system of the kidney tissues of diabetic rats (Mak *et al.* 1996; Rauscher *et al.* 2001; Craven *et al.* 2001; Agardh *et al.* 2002, Zurova-Nedelcevova 2006). Gumieniczek *et al.* (2002) demonstreated increased activity of antioxidant enzymes in rats after 24 weeks of diabetes. Sechi *et al.* (1997) showed that renal Cu, Zn-SOD and catalase mRNA levels were increased in diabetic rats 17 days after administration of STZ. Transient changes in total antioxidant activity may also be observed after induction of diabetes. Acute toxicity of streptozotocin or acute hyperglycemia may decrease the tissue antioxidant defence, whereas in prolonged hyperglycemia, antioxidant defences can be activated (Crouch *et al.* 1978; Pieper *et al.* 1995).

The results of the present study showed that streptozotocin injection caused increase in total AOP in kidney homogenates of rats. In addition, STB treatment decreased total antioxidant potential but this difference was not found statistically significant. Thus elevated total antioxidant potential may be considered a compensatory response to oxidative stress due to an increase in endogenous reactive free radical production. Additionally, the tissue AOP may vary with the duration of diabetes. Other factors might be involved in dependence on experimental protocols.

In conclusion, elevated markers of oxidative stress, namely AOPP, PCC and NT, were recorded in the kidney tissue of STZ-diabetic rats. Treatment with the pyridoindole antioxidant STB significantly decreased protein carbonylation and advanced oxidation protein product accumulation in diabetic animals. These findings indicate that STB is a protective factor which reduces oxidative protein damage in renal tissues under conditions of experimental diabetes.

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