Molecular and Cellular Biochemistry **243:** 147–152, 2003.

Oxidative stress and gene expression of antioxidant enzymes in the renal cortex of streptozotocin-induced diabetic rats

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Received 29 May 2002; accepted 7 August 2002

Abstract

The present study was aimed at addressing the effect of hyperglycemia on antioxidant enzymes. The expression of catalase, superoxide dismutase and glutathione peroxidase, the three primary scavenger enzymes involved in detoxifying reactive oxygen species has been evaluated in the renal cortex of rats after 6 weeks of streptozotocin-induced diabetes. Lipid peroxidation and protein oxidation in the renal cortical homogenate were first performed to confirm a state of oxidative stress. The enzyme assays showed significant and varied alterations in catalase, superoxide dismutase and glutathione peroxidase activities. An opposing response of catalase and glutathione peroxidase activities to diabetes was observed. RT-PCR analysis was used to ascertain whether steady-state transcription levels were altered. While an increase in glutathione peroxidase and Cu-Zn superoxide dismutase mRNA parallels the increase in the activities of the enzymes, an increase in catalase gene expression in contrast to a decrease in enzyme activity suggests a role for post-translational modification in altering the activity of this enzyme. (Mol Cell Biochem **243**: 147–152, 2003)

Key words: oxidative stress, catalase, superoxide dismutase, glutathione peroxidase, diabetes mellitus, reverse transcriptase polymerase chain reaction

Introduction

Diabetes is a group of metabolic diseases characterized by hyperglycemia resulting from defects in insulin secretion, or action or both. In diabetes, significant damage occurs in tissues such as the kidney, since the entry of glucose in this tissue is not regulated by insulin. Associated changes in kidney function in terms of increased levels of blood urea nitrogen and distinct proteinuria have been reported in streptozotocin-induced diabetic rats [1] as well as histopathological changes such as increase in the glomerular mesangial matrix have been measured [2]. Hyperglycemia–induced increases in glucose autoxidation, protein glycation and the subsequent oxidative degradation of glycated protein leads to enhanced production of reactive oxygen species (ROS) [2]. The levels of ROS are regulated by a variety of cellular defense mechanisms consisting of enzymic and non-enzymic [glutathione, α-tocopherol and other ROS scavengers] systems. High levels and/or inadequate removal of ROS may cause severe metabolic imbalance and oxidative damage to biological macromolecules [3]. Oxidative stress may be both a cause and an effect of tissue damage, both a primary and a secondary source of diabetic pathology [4]. Oxidative stress *in vivo* is of importance, since it can not only damage receptors, enzymes, signal transduction pathways and transport proteins but also give rise to secondary damage to other biomolecules. The oxidatively modified proteins may be recognized as 'foreign' by the immune system, triggering antibody formation [5].

Oxidative stress is known to elicit varying effects on the activity of antioxidant enzymes. The three primary scavenger enzymes involved in detoxifying ROS in mammalian systems are catalase, superoxide dismutase and glutathione

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peroxidase [3]. There have been contradictory reports in the literature regarding the effect of diabetes-induced hyperglycemia on these antioxidant enzymes. They have been reported to decrease, increase or remain unaltered in diabetic animals with wide variations depending on age of the animal or duration of diabetes [6–8] or tissues examined, [9–10]. These discrepancies may arise due to variations in enzyme activity over time (e.g. compensatory increases in enzyme activity to overcome raised oxidative stress or direct inhibitory effects of ROS) as well as due to the type of tissue under examination. The present study was carried out using the renal cortical homogenates in order to reinvestigate the effects of diabetes-induced hyperglycemia.

Materials and methods

Materials

Bovine serum albumin, streptozotocin, reduced glutathione, pyrogallol, thiobarbituric acid, 2,4-dinitrophenylhydrazine and guanidine hydrochloride were from Sigma Chemical Co., MO, USA. The glucotest strips were supplied by Ames Diastix, Mumbai, India. The 100 bp DNA ladder was purchased from MBI Fermentas, USA. Agarose, bromophenol blue and ethidium bromide were from Sisco Research Laboratories Ltd., Mumbai, India. All other chemicals used were of the highest analytical grade available. RT-PCR kits were purchased from Qiagen Germany, Trizol reagent was from Gibco laboratories, USA.

Methods

Induction of diabetes

Male Wistar rats weighing 200–250 g were used in the present study. The rats were weighed and divided into two groups. They were made diabetic by a single i.p injection of streptozotocin in 0.1 M citrate buffer, pH 4.5 at a dose of 75 mg/kg body weight. Diabetes was confirmed by glucosuria and blood glucose estimation after 24 h. Six rats showing blood glucose values above 300 mg/dl and persistent glucosuria were selected for the study. Six controls were injected with an equal volume of isotonic saline. The blood samples were immediately deproteinised and glucose values determined using the glucose estimation kit. Urinary glucose was monitored using glucotest strips. The rats were kept for 6 weeks after which they were weighed, bled, sacrificed and immediately surgically opened. Kidneys were removed, washed free of blood, blotted dry and processed immediately.

Preparation of homogenate

Kidneys were de-capsulated, dissected, cortices removed, weighed and minced. A 10% (w/v) homogenate was made in 10 mM sodium phosphate buffer, pH 7.4. This homogenate was centrifuged at 10,000 rpm for 10 min at 4°C refrigerated centrifuge in a Sorvall RC 5B using SS-34 rotor and the supernatant used for all the assays.

Enzyme assays

Activity of total superoxide dismutase (SOD) was measured by the inhibition of autoxidation of pyrogallol by the method of Eugene *et al.* [11]. Catalase (CAT) activity was assayed by the method of Aebi [12]. Glutathione peroxidase (GPx) was assayed by the method of Rotruck *et al.* [13]. Lipid peroxidation was measured using thiobarbituric acid [14]. The samples were treated with thiobarbituric acid and the absorbance was read at 532 nm. The concentration of thiobarbituric acid reactive substances was calculated using a molar absorbance coefficient value of 153,000 $(mol/L)^{-1}$ cm^{-1} and expressed as nmoles of thiobarbituric acid reactive substances per mg protein. Determination of protein oxidation was performed as described by Levine *et al.* [15]. The extent of protein oxidation was determined by using 2,4-dinitrophenylhydrazine and the absorbance of the resulting hydrazones was determined spectrophotometrically at 366 nm. The carbonyl content was calculated using a molar absorbance coefficient of 22,000 $(mol/L)^{-1}$ cm^{-1} and the results expressed as nmoles of carbonyls per mg protein. Determination of the protein content was carried out by the method of Lowry *et al.* using bovine serum albumin as the standard [16].

Total RNA isolation and RT-PCR

Total RNA was purified from freshly isolated rat renal cortices using 1 ml of the Trizol reagent as described by the suppliers (Gibco laboratories, USA). The purity and integrity of the total RNA was determined by spectrophotometry and agarose gel electrophoresis [17].

Reverse transcriptase polymerase chain reaction (RT-PCR) was performed with Qiagen one-step RT-PCR kit (Qiagen, Germany) as per the manufacturers instructions, using 1 μg each of the template RNA, 0.6 μM of each of the forward and reverse gene specific primers GPx/CAT/SOD/β-actin in a Techne (UK) thermal cycler.

The GPx and β-actin primers were taken from the literature [18] while CAT and SOD primers were designed manually (Table 1) using the rat sequences from the Genbank database. The primers were checked for their T_m values, hairpin loops, dimers, cross- dimers and number of repeats and runs using Net Primer (Premier Biosoft Corp., USA). The T_m values of the forward and reverse primers were designed to be within 5°C of each other. The length was chosen to be between 18–30 nucleotides and the G/C content between 40–60%. The primers were designed to flank a region that contains at least one intron so that the products amplified from

Table 1. Details giving primer sequences and expected product sizes for the genes amplified

cDNA	Genbank Accession No.	Forward primer	Reverse primer	RT-PCR product size
β-actin	V01217	5′-CCTGCTTGCTGATCCACA	5′-CTGACCGAGCGTGGCTAC	505bp
Cu-Zn SOD	X05634	5′-GCAGAAGGCAAGCGGTGAAC	5′-TAGCAGGACAGCAGATGAGT	387bp
GPx	M21210	5′-CTCTCCGCGGTGGCACAGT	5′-CCACCACCGGGTCGGACATAC	290bp
CAT	AH004967	5′-GCGAATGGAGAGGCAGTGTAC	5′-GAGTGACGTTGTCTTCATTAGCACTG	670bp

Cu-Zn SOD – copper zinc superoxide dismutase; GPx – glutathione peroxidase; CAT – catalase.

the cDNAs will be smaller than those from the contaminating genomic DNA, if any. β-Actin gene was used as an internal standard. The RT-PCR conditions were as follows: (1) reverse transcription, 30 min, 50°C, (2) initial PCR activation step, 15 min, 95°C, (3) 3-step cycling for 30 cycles, each cycle consisting of denaturation for 30 sec at 94°C followed by annealing for 30 sec at 58°C and extension for 1 min at 72°C.

The template concentration and the cycle number were optimized to ensure linearity of response and to avoid saturation of the reaction.

The PCR products were then resolved on 2% agarose gels. The bands were identified based on the product size using a 100 bp DNA ladder, photographed using a Fisher Biotech Photo documentation camera, FB-PDC-34 (Polaroid instant) and the prints were scanned using a UMAX Astra 2000P scanner at a resolution of 400 dpi. The scanned images were quantified with the aid of the Gel Pro Analyzer version 3.1.00.00 for Windows 95/NT copyright: Media Cybernetics. Bio print Version 96.11 for Windows. The results were normalized to the levels obtained for the β-actin gene by taking a ratio of the value obtained for the gene of interest to that of β-actin. The values of the diabetic samples were expressed as percentages with respect to the control. All data are expressed as mean ± S.D. Statistical differences were analyzed using Student's *t*-test ($n = 6$) with $p < 0.05$ being regarded as significant.

Results and discussion

In most diseases characterized by tissue damage, oxidative stress may be either a cause or a consequence of this damage [5]. Cumulative or steady-state oxidative damage may increase in diabetes in response to an increase in oxidizable substrate (carbohydrate or lipid), an increase in the rate of autoxidation of substrate, a decline in the antioxidant defenses, or a combination of all these processes. The ROS generated by the autoxidation of carbohydrates and lipids may further auto-catalytically enhance the rate of autoxidation and propagate oxidative damage [4].

A precise understanding of oxidative stress in diabetes and the adaptive response to it requires knowledge of the time of onset of manifestations of oxidative stress, their characterization in terms of oxidative damage to lipids and proteins and the antioxidant enzymes affected and their regulation at the level of RNA or activity or both. Most of the studies in literature have addressed one or two of these questions under different experimental conditions, often leading to inconclusive or even contradictory conclusions as discussed later. Therefore the present study sought to address all the above aspects together in the rat renal cortex after 6 weeks of streptozotocin-induced diabetes, as it was already shown that it takes up to six weeks to clearly demonstrate histopathological changes and oxidative stress [2].

Figure 1 shows the relative percentage change in lipid peroxidation and protein oxidation. It is evident that both protein oxidation and lipid peroxidation increased significantly, by 78.45 and 53.75%, respectively, suggesting significant oxidative stress. Our lipid peroxidation data corroborate the findings of Kakkar *et al.* [2] who reported a 60% increase at six weeks of streptozotocin-induced diabetes. Our protein oxidation data provide additional evidence for carbonyl stress (Fig. 1), which can arise from oxidative and/or non-oxidative reactions and lead to increased chemical modification of proteins. The generalized increase in reactive carbonyl groups in diabetes may be due to alterations in the glyoxalase pathway, aldose reductase or aldehyde reductase, which are responsible for the detoxification of the reactive carbonyl species or from increased substrate stress [4, 5].

Hypoinsulinemia is known to increase the activity of the enzyme fatty acyl-CoA oxidase that initiates β-oxidation of fatty acids. This can result in increased production of H_2O_2 [19]. H_2O_2 is not only toxic to cells but can also be a precursor of other toxic species. It is permeable to cell membranes and therefore its site of action can be different from its site of production. In the extracellular environment, H_2O_2 can react with transition metals such as Fe^{2+} and Cu^{2+} generating highly reactive $OH^{\cdot}$ that can damage macromolecules in the vicinity. The lipid peroxidation induced by H_2O_2 has been shown to cause renal epithelial cell injury *in vitro*, under non-diabetic conditions. The data presented here on lipid peroxidation and antioxidant enzymes do not rule out the possibility of this mechanism operating in diabetes as well [20].

Figure 2 shows the alterations in the total SOD, GPx and CAT activity in the renal cortex of diabetic and control rats,

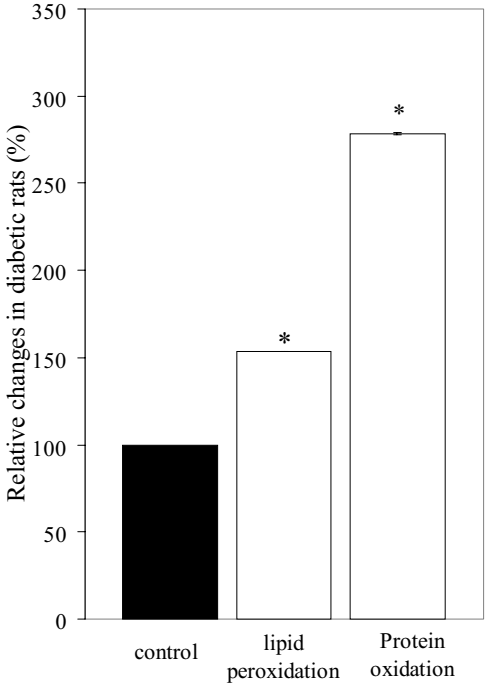

Fig. 1. Summary of the changes in lipid peroxidation and protein oxidation observed in diabetic rats expressed as percentages with respect to the controls. The results are expressed as mean ± S.D, n = 6 and the bars represent means of triplicate determinations, *p < 0.05.

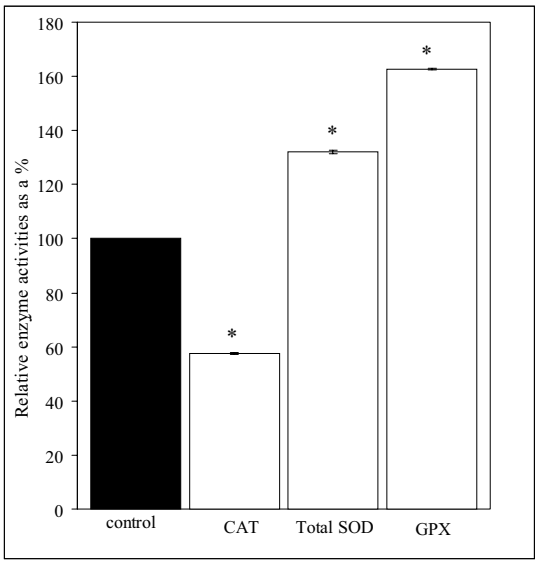

Fig. 2. Summary of the changes in catalase, total superoxide dismutase, glutathione peroxidase observed in diabetic rats expressed as percentages with respect to the controls. The results are expressed as mean ± S.D, n = 6 and the bars represent means of triplicate determinations, *p < 0.05. Total SOD – total superoxide dismutase; GPx – glutathione peroxidase; CAT – catalase.

and Fig. 3 shows the gene expression of the above enzymes measured by RT-PCR using gene-specific primers (Table 1). Six weeks of streptozotocin induced-diabetes resulted in a reduced CAT activity by an average of 42.53% as judged from 6 experiments each done in triplicate (Fig. 2). However, RT-PCR analysis did not show a corresponding decrease in the CAT transcript and has increased instead (Fig. 3) by an average of 33.28 % in 6 independent experiments. This indicates that the inhibition of CAT activity may operate at the post-translational level. Direct glycation of the enzyme protein could be one such means, as suggested from *in vitro* experiments [21]. Alternatively, CAT activity may be inhibited by ROS attack, as demonstrated *in vitro* under non-diabetic conditions [22]. In diabetic rats, however, the effect of oxidative stress on renal CAT activity reported in literature varied significantly from no change [7], decrease [6, 8] and increase [23, 24] depending on the experimental conditions such as age of the animal and duration of diabetes. Moreover, it is not known whether the effect is at the level of RNA or activity or both and attempts to address this question by Northern blot experiments reported opposite results [25, 26].

As far as our results are concerned, an increase in CAT gene expression seems to be a natural response of the cell to cope with oxidative stress, especially when CAT enzyme itself is inhibited by glycation, ROS or both. This also explains the inability of increased total SOD activity (see below) to protect CAT.

Unlike CAT, which is specifically responsible for the destruction of H_2O_2, GPx has a wider spectrum since it also reduces lipid peroxides. GPx plays a role in cellular defense against ROS. In the present study the GPx activity increased by 62.58% in diabetic rats and the mRNA levels also increased by 44.74%, following the same trend. The increase in GPx activity and a decrease in the CAT activity in the kidney cortex suggest that there could be compensatory mechanisms between the antioxidant enzymes in response to oxidative stress (Fig. 1). The need for enhanced production of CAT and GPx enzymes to cope with oxidative stress is also reflected in their gene expression as measured by RT-PCR in our study (Fig. 3). However, tissues in which CAT activity suffers damage may be critically dependent on GPx [2]. The GPx activity in kidney has been found to increase in most of the studies [7, 9, 10] except one report of a decrease [27]. However, none of these have carried out the gene expression analysis. An increase in GPx mRNA in the presence of higher concentrations of H_2O_2 has been reported [28], but under non-diabetic conditions. Our data on GPx gene expression as measured by RT-PCR are backed by activity data and are in agreement with Northern blotting studies, with [25] or without [26] enzyme activity data.

In the present study, there was an increase in the total SOD activity by 32% (Fig. 2) in the kidney cortex with the Cu-Zn SOD mRNA levels increased by 35.68 % (Fig. 3). Thus, the

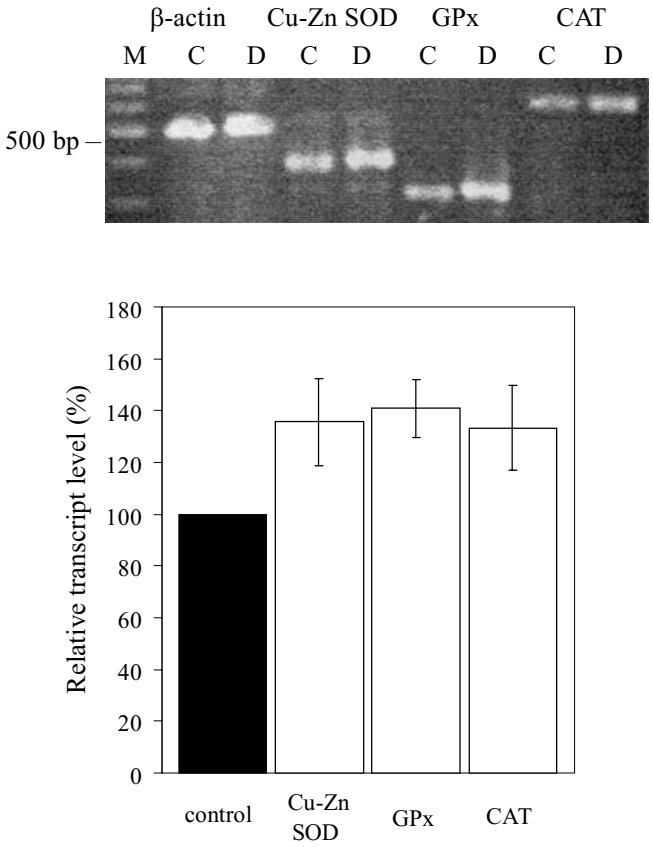

Fig. 3. Agarose gel electrophoresis of RT-PCR products from total RNA isolated using Trizol. The gels were stained with ethidium bromide and photographed using Polaroid camera. M – 100 bp DNA ladder; C – control; D – diabetic; Cu-Zn SOD – copper-zinc superoxide dismutase; GPx – glutathione peroxidase; CAT – catalase. The gel photographs were scanned and quantitated using Gel Pro Analyzer 3.1.00.00 for Windows 95/NT copyright: Media Cybernetics Bioprint Version 96.11 for Windows. The results depicted are normalized to levels of β-actin gene. Data are mean ± S.D. of ratios of intensity for each gene divided by that for β-actin (n = 6).

possibility of increased Mn-SOD activity and/or its gene expression accounting for the increase in total SOD activity cannot be excluded. There have been discrepancies in the reports in SOD activities in various tissues during diabetes. The Cu-Zn SOD activity has been reported to decrease in the heart, liver as well as the kidney [9], but the RNA levels have not been examined. An increased total SOD activity in the liver, heart, pancreas with unchanged activity in the kidney was observed during streptozotocin-induced diabetes [7]. Increased SOD activity has also been observed in the erythrocytes of type I and type II diabetic patients [9]. A decrease of 20.6% in the activity of renal total SOD of rats during six weeks of diabetes has been considered significant [2]. According to one report, diabetes did not alter total SOD in the renal cortex, but increased Mn-SOD and decreased Cu-Zn SOD activity [29]. This response was reversed by insulin treatment [29]. None of these studies have performed gene expression analysis. A decrease in mRNA levels of both Mn SOD and Cu-Zn SOD has been reported in the aorta of diabetic rats and attributed to be the cause of rapid destruction of nitric oxide by superoxide radical, but enzyme activities of SOD have not been assayed [30]. These issues can only be resolved by further work to examine the expression of SOD at both mRNA and activity levels for both the isoforms.

Elevated oxidative stress was found to be involved in the activation of the NF-kappa B and AP-1 transcription factors in cardiac tissues of diabetic rats, suggesting a possible mechanism for altered gene regulation [5]. While this may explain the elevated mRNA levels and correspondingly high enzyme activities of GPx and SOD in the present study, the decrease in CAT activity indicates post-translational effects of oxidative damage in diabetes.

Acknowledgements

We wish to thank the Universities Grants Commission for the award of fellowship to one of the authors Pallavi V. Limaye. We also wish to thank Ms. Kaushiki Kadam, of IRR Mumbai for her help with the densitometric analysis of the PCR products.

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