

Gene expressions of Mn-SOD and GPx-1 in streptozotocin-induced diabetes: effect of antioxidants

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Abstract Increased oxidative stress and impaired antioxidant defense mechanisms are believed to be the important factors contributing to the pathogenesis and progression of diabetes mellitus. In this study, we have reported the effects of the streptozotocin-induced diabetes on the gene expression and the activities of two antioxidant enzymes, manganese superoxide dismutase (MnSOD) and glutathione peroxidase (GPx). We also studied the effects of two antioxidants, vitamin C and DL- α -lipoic acid (LA), on the system. Our results showed no significant change in both enzymes activities in diabetic animals compared to controls. Similarly, mRNA and protein profiles of MnSOD showed no change. Though the mRNA expression of GPx did not show any change, Western-blot analysis results demonstrated that protein expression is increased. LA, which is a water- and lipid-soluble antioxidant, decreased the protein expression of MnSOD, though mRNA levels and activities remained unchanged. LA treatment increased the GPx activities in diabetic tissues, significantly, and RT-PCR and Western-blot analysis results demonstrated that this increase in activity is not regulated at the gene level, as both mRNA and protein levels did not change. Supplementing the animals with vitamin C, a powerful water-soluble antioxidant, increased the mRNA expression of MnSOD, though the protein expression and the activity did not change statistically. On the other hand GPx activity increased significantly through post-translational modifications, as both mRNA and protein expressions did not change. These results together with our previous findings

about the gene expressions of catalase and Cu–Zn SOD indicate the presence of very intricate control mechanisms regulating the activities of antioxidant enzymes in order to prevent the damaging effects of oxidative stress.

Keywords Diabetes · MnSOD · GPx · Gene expression · Lipoic acid · Vitamin C

Introduction

Diabetes mellitus is a common disease characterized by the disordered metabolism and abnormally high blood glucose levels (hyperglycemia) resulting from insufficient levels of insulin or its action on cells. Elevated blood glucose levels cause free radicals to be produced via glucose autoxidation [1]; non-enzymatic protein glycation [2] increased influx toward polyol pathway [3], activation of protein kinase C, and increased flux through hexosamine pathway [4]. Under normal circumstances, there are antioxidant enzymes and macromolecules that remove and protect the cells from the damaging effects of free radicals [5]. Cytosolic superoxide dismutase (SOD-1 or CuZnSOD) and mitochondrial superoxide dismutase (SOD-2 or MnSOD) convert intracellular superoxide radicals into hydrogen peroxide which are cleared by glutathione peroxidase (GPx) and catalase (CAT) further by converting it into water. In the mammals, there are five isoenzymes of GPx and the levels of each isoform vary depending on the tissue type. GPx1 is a cytosolic and mitochondrial enzyme reducing fatty acid hydroperoxides and H₂O₂ by using of glutathione. GPx1 and the phospholipid hydroperoxide glutathione peroxidase (GPx4) are found in most tissues. Latter is located in both the cytosol and the membrane and directly reduce the phospholipid hydroperoxides, fatty acid

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hydroperoxides, and cholesterol hydroperoxides that are produced in peroxidized membranes and oxidized lipoproteins [6]. GPx1 is found in erythrocytes, kidney, and liver, and GPx4 is highly expressed in renal epithelial cells and testes. Cytosolic GPx2 and extracellular GPx3 are present in the gastrointestinal tract and kidney, respectively. The final isoenzyme which is GPx5 is expressed especially in mouse epididymis, and it is selenium-independent [7]. Furthermore, free radicals are also quenched non-enzymatically with antioxidant macromolecules such as vitamin C, α -lipoic acid (LA), carotenoids, and tocopherols.

Alpha-lipoic acid (LA) is a molecule involved in the metabolism and energy production [8]. In its free form, LA is a powerful antioxidant, functioning as a reactive oxygen species scavenger. Antioxidant effects of LA are based on their interactions with peroxy radicals, which are essential for the initiation of lipid peroxidation and ascorbyl radicals of vitamin C. Reduced form of lipoic acid, dihydrolipoic acid (DHLA), can recycle ascorbyl radicals and reduce dehydroascorbate generated in the course of ascorbate oxidation by radicals. Alpha-lipoic acid also plays an important role in the synergism of antioxidants. Therefore, dihydrolipoic acid may act as a strong chain-breaking antioxidant and may enhance the antioxidant potency of other antioxidants like vitamin C in both the aqueous and in hydrophobic membrane phase [9].

In our previous study [10], we have reported the oxidative stress related changes in the mRNA and protein expressions of Cu–Zn SOD and catalase in streptozotocin-induced diabetic rat liver tissues. The purpose of this study is to investigate the effects of diabetes on the status of the other two important antioxidant enzymes, cytosolic glutathione peroxidase (GPx-1), and manganese superoxide dismutase (MnSOD). The effects of the administration of two powerful antioxidants α -lipoic acid and vitamin C will also be studied. The results will help us to better understand the molecular mechanism of the antioxidant defense system.

Materials and methods

Male Wistar rats were randomly divided into four groups. The induction of diabetes and the antioxidant treatments were carried out as previously described [10]. All experiments were carried out with the approval of local animal use ethical committee. The procedures involving animals and their care are conformed to the institutional guidelines [11]. At the end of the 4 week growing period, rats were decapitated and livers were removed and quickly frozen in liquid nitrogen and kept -85°C for subsequent biochemical analysis.

Liver tissues were homogenized in ice cold homogenization buffer containing 1.15% (w/v) KCl, 5 mM EDTA, 0.2 mM PMSF, 0.2 mM DTT, in 25 mM phosphate buffer, and pH 7.4 using teflon glass homogenizer. The homogenates were centrifuged at 1,500g and the supernatants were sampled for determination of MnSOD activity and remaining supernatants were recentrifuged at 16,000g for the determination of GPx activity. Protein concentrations of cytosolic fractions were determined according to Lowry method [12].

MnSOD activities were determined according to the method of Marklund and Marklund [13] where CuZnSOD activities were inhibited by 1.5 mM KCN and MnSOD activities were measured. One unit MnSOD activity was described as the amount of total protein that cause 50% inhibition of pyrogallol autoxidation.

GPx activities were determined according to the method of Paglia and Valentine [14] and one unit activity was described as the amount of NADPH consumed in 1 min by 1 mg protein containing cytosolic fraction.

Polyacrylamide gel electrophoresis and Western-blot analysis of GPx and MnSOD were performed as described previously [10]. Rat liver cytoplasmic fractions were diluted and 10 μg of total protein for MnSOD and 50 μg protein for GPx were applied to the gels. After electrophoresis, proteins were electroblotted onto PVDF membrane [15]. Incubation with primary antibodies for MnSOD (Anti-SOD-2 Rabbit IgG, Santa Cruz, USA) and with primary antibodies for GPx (Anti-GPx Rabbit IgG, Abcam: Cambridge, USA) for 2 h followed by the 1 h further incubation with alkaline phosphatase conjugated secondary antibodies (Goat anti rabbit IgG-AP conjugate, Abcam: Cambridge, USA). Then, the bands on the membrane were visualized by BCIP/NBT substrate solution. As an internal standard, GAPDH antibody (Anti-GAPDH Rabbit IgG, Santa Cruz, USA) was used for normalization procedures. Intensities of bands corresponding to antioxidant enzymes and reference protein were measured with Image J software [16]. Intensities were proportional to the amount of desired protein present.

Semiquantitative reverse transcriptase PCR (RT-PCR) was used in order to compare the mRNA expressions of GPx and MnSOD in control, diabetic, and antioxidant supplemented animals. Total RNAs were isolated from liver tissues by guanidine isothiocyanide method [17]. After isolation of total RNAs, cDNA synthesis from 1 μg total RNA was carried out as described previously [10] by using M-MuLV reverse transcriptase (MBI Fermentas, USA). After synthesizing cDNA, they were stored at ambient temperature until subsequent PCR reactions.

Multiplex PCR was performed in order to amplify the gene of interest (MnSOD and GPx) and the internal standard β -Actin simultaneously. Primer pairs for the

amplification of GPx, MnSOD, and internal standard β -actin cDNA are given in Table 1. cDNA mixture was amplified in a PCR reaction in which, initial denaturation at 94°C for 3 min, denaturation at 94°C for 30 s, annealing at 58°C, extension at 72°C for 45 s (28 cycle), and final extension at 72°C for 5 min was carried out by using Eppendorf gradient type mastercycler (Eppendorf Germany). After the reaction and agarose gel electrophoresis on 2% agarose gels, intensities of bands were measured with Image J software [16] and mRNA expressions were measured in control, diabetic, and antioxidant supplemented groups.

Statistical analysis

Data were expressed as mean \pm standard error of mean (SEM) and differences in measured parameters between control, diabetic, and antioxidant supplemented animals were assessed by two tailed student *t*-test with the help of MINITAB 12.1 statistics software. The relationships between oxidative parameters characterizing diabetic and control rat liver status were analyzed and a probability of 0.05 and 0.005 was set as the level of statistical significance.

Results

The effects of diabetes and α -lipoic acid (LA) and vitamin C treatments on GPx and MnSOD activities are given in Figs. 1c and 2c. According to our results, though the activities of MnSOD in diabetic animals seemed to decrease compared to control values, this decrease was not statistically significant and GPx activities also remained unchanged. Application of LA and vitamin C increased the diabetic GPx activities without changing the MnSOD activities. The increases in the GPx activities in both treatments were statistically significant.

We have also performed multiplex RT-PCR for the simultaneous amplification of internal standard β -actin gene and our gene of interests. Results of the multiplex amplification of MnSOD and β -actin mRNAs in whole groups are shown in Fig. 3. Figure 1a represents the ratios of the densities of MnSOD and β -actin bands measured by Image J software. As seen from both figures, diabetes

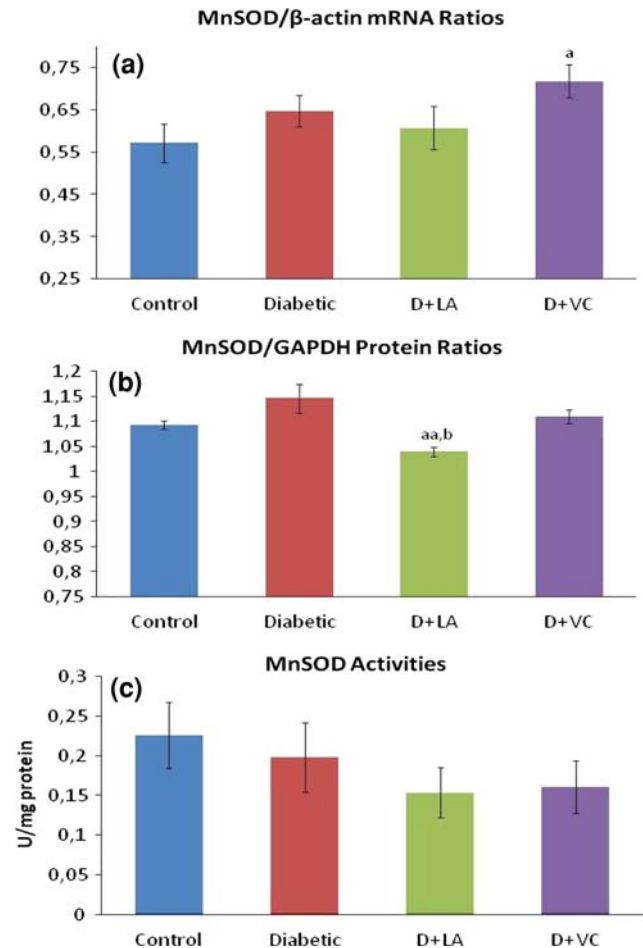


Fig. 1 MnSOD mRNA expressions (a), protein expressions (b) and activities (c) in control, diabetic, diabetic animals supplemented with α -lipoic acid (D + LA) and diabetic animals supplemented with vitamin C (D + VC). ^a Represents significance at $P < 0.05$ as compared with control groups. ^{aa} Represents significance at $P < 0.005$ as compared with control groups. ^b Represents significance at $P < 0.05$ as compared with untreated diabetic groups

increased the mRNA levels of MnSOD only slightly. Supplementing the animals with LA did not change the mRNA expression of MnSOD whereas, vitamin C treatment increased MnSOD mRNA expression significantly ($P < 0.05$).

The ratios of the mRNA expressions of the GPx and internal standard β -actin were also evaluated with the help of semiquantitative RT-PCR and the agarose gel electrophoresis of the multiplex RT-PCR amplification product of

Table 1 Primer sequences and expected product sizes for antioxidant enzymes and internal standard β -actin

cDNA	Genbank accession number	Forward primer sequence	Reverse primer sequence	RT-PCR product size
β -actin	NM_031144	5'-CCTGCTTGCTGATCCACA	5'-CTGACCGAGCGTGGCTAC	500 bp
MnSOD	NM_017051	5'-GCACATTAACGCGCAGATCA	5'-AGCCTCCAGCAACTCTCCTT	240 bp
GPx	NM_030826	5'-CTCTCCGCGGTGGCACAGT	5'-CCACCACGGGTCGGACATAC	290 bp

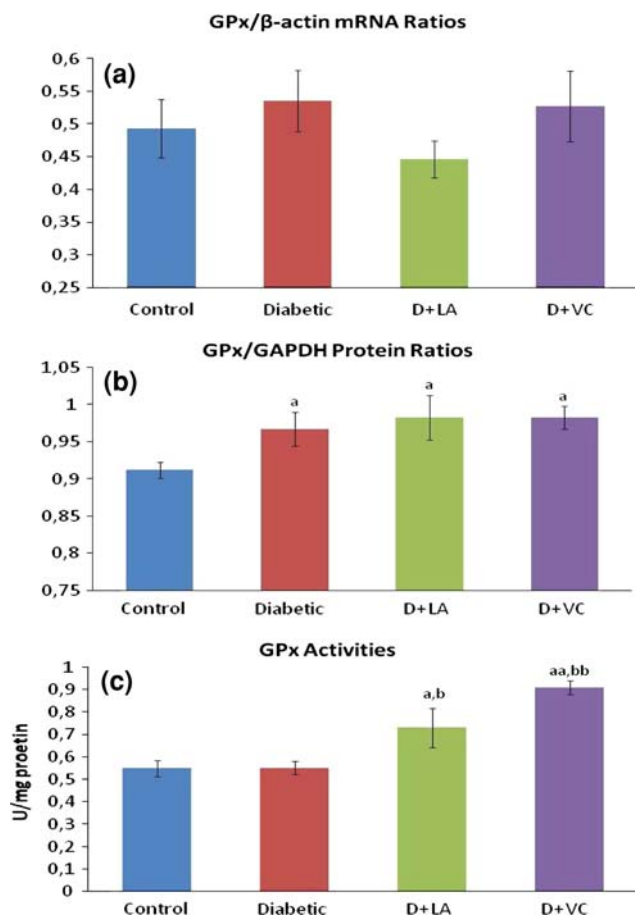
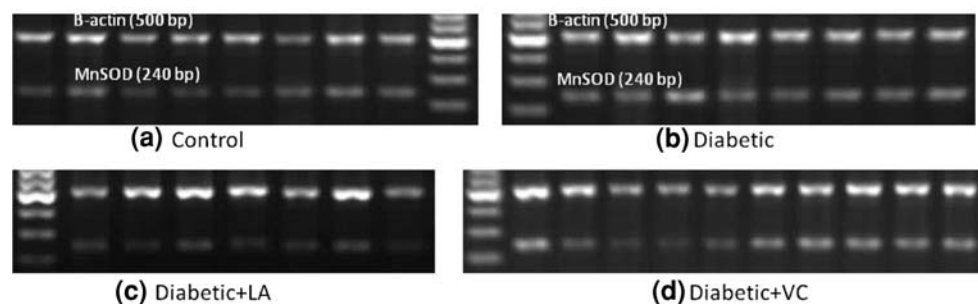


Fig. 2 GPx mRNA expressions (a), protein expressions (b) and activities (c) in control, diabetic, diabetic animals supplemented with α -lipoic acid (D + LA) and diabetic animals supplemented with vitamin C (D + VC). ^a Represents significance at $P < 0.05$ as compared with control groups. ^{aa} Represents significance at $P < 0.005$ as compared with control groups. ^b Represents significance at $P < 0.05$ as compared with untreated diabetic groups. ^{bb} Represents significance at $P < 0.005$ as compared with untreated diabetic groups

the GPx and β -actin mRNAs in whole groups is shown in Fig. 4.

According to our results, diabetes increased the relative expression of GPx mRNA compared to controls about 8% but this induction was not statistically significant. Both LA and vitamin C treatments did not show any effects on the

Fig. 3 Agarose gel electrophoresis of multiplex RT-PCR amplification of MnSOD and β -actin mRNA. In the figure, lower band corresponds to 240 bp MnSOD and upper band corresponds to 500 bp β -actin



expressions of GPx mRNA. The results of GPx RT-PCR analysis are summarized in Fig. 2a.

Protein amounts of GPx and MnSOD were also measured in whole groups with Western-blot analysis by using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as an internal control. Co-immunostaining of GPx and MnSOD and the internal standard allow us to compare relative expressions of GPx and MnSOD with respect to GAPDH. Figure 5 shows the results of Western-blot analysis of MnSOD and GAPDH with co-immunostaining. Intensities of bands were measured with Image J software and relative expressions of MnSOD with respect to housekeeping GAPDH protein are given in Fig. 1b.

The results showed that, diabetes did not change the levels of the protein synthesis of MnSOD in diabetic animals compared to controls significantly. Furthermore, vitamin C did not change the amount of MnSOD. On the other hand, LA treatment decreased the protein synthesis of MnSOD significantly ($P < 0.05$).

Figure 6 shows the results of Western-blot analysis of GPx and GAPDH with co-immunostaining. Intensities of bands were measured with the help of Image J software and relative expressions of GPx with respect to housekeeping GAPDH protein are given in Fig. 2b. The results of Western-blot analysis showed that diabetes increased the protein synthesis of GPx significantly ($P < 0.05$) compared to controls. Supplementing the animals with both antioxidants, LA and vitamin C did not cause any further increase in the protein synthesis of GPx.

Table 2 summarizes the overall changes in mRNA expressions, protein amounts and enzyme activities of MnSOD and GPx in control, diabetic, and antioxidant supplemented diabetic rat liver tissues.

Discussion

We found that, in diabetic animals both the mRNA expressions and the activities of two antioxidant enzymes, namely MnSOD and GPx, did not change, indicating that the control of activities were not at the level of genes. Interestingly, though the protein expression of GPx increased significantly in diabetic animals compared to controls, still the activities

Fig. 4 Agarose gel electrophoresis of multiplex RT-PCR amplification of GPx and β -actin mRNA. In this figure, upper band corresponds to 500 bp β -actin and lower band represents 290 bp GPx gene

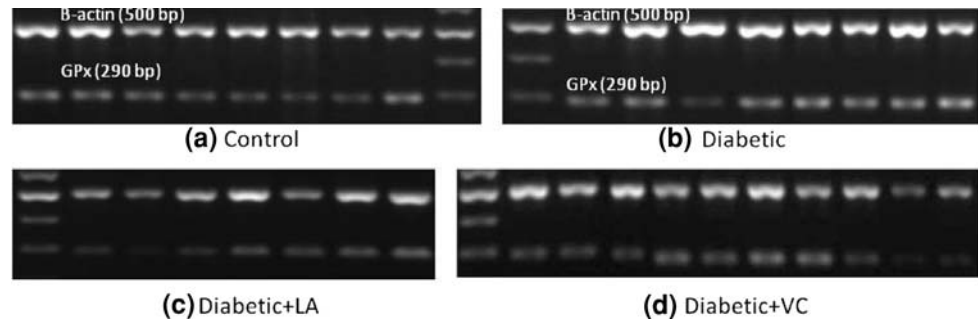


Fig. 5 Western-blot analysis of MnSOD and GAPDH proteins in control (a), diabetic (b), α -lipoic acid treated diabetic (c), and vitamin C treated diabetic (d) groups. Upper band represents 36kD GAPDH protein and lower band represents 22kD MnSOD

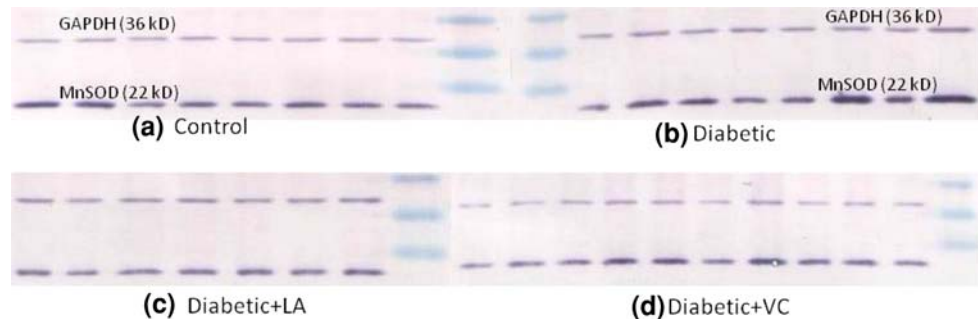


Fig. 6 Western-blot analysis of GPx and GAPDH proteins in control (a), diabetic (b), α -lipoic acid treated diabetic (c), and vitamin C treated diabetic (d) groups. Upper band represents 36kD GAPDH protein and lower band represents 23kD GPx

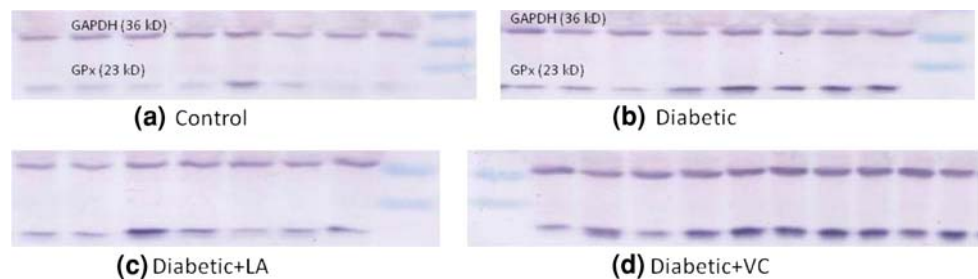


Table 2 Summary of overall changes in the mRNA expressions, protein amounts, and enzyme activities of antioxidant enzymes (GPx and MnSOD) in control, diabetic and antioxidant supplemented diabetic rat liver tissues

	N	MnSOD activity (U/mg protein)	MnSOD mRNA (MnSOD/ β -actin)	MnSOD protein (MnSOD/GAPDH)	GPx activity (U/mg protein)	GPx mRNA (GPx/ β -actin)	GPx protein (GPx/GAPDH)
Control	8	0.226 \pm 0.041	0.573 \pm 0.045	1.092 \pm 0.009	0.548 \pm 0.035	0.493 \pm 0.044	0.912 \pm 0.011
Diabetic	8	0.198 \pm 0.044	0.648 \pm 0.037	1.145 \pm 0.029	0.551 \pm 0.030	0.535 \pm 0.047	0.967 \pm 0.023 ^a
Diabetic + α -lipoic acid	7	0.154 \pm 0.032	0.608 \pm 0.051	1.039 \pm 0.010 ^{aa,b}	0.729 \pm 0.087 ^{aa,b}	0.446 \pm 0.028	0.983 \pm 0.030 ^a
Diabetic + vitamin C	10	0.161 \pm 0.033	0.719 \pm 0.039 ^a	1.110 \pm 0.013	0.908 \pm 0.030 ^{aa,bb}	0.527 \pm 0.054	0.983 \pm 0.016 ^a

^a Represents significance at $P < 0.05$ and ^{aa} represents significance at $P < 0.005$ as compared with control groups

^b Represents significance at $P < 0.05$ and ^{bb} represents significance at $P < 0.005$ as compared with untreated diabetic groups

remained constant. In our previous study [10], we demonstrated that, the excessive oxidative stress occurred in the liver of diabetic rats resulted in the suppression of the expressions of both catalase (CAT) and copper zinc containing superoxide dismutase (CuZnSOD) the other two important antioxidant enzymes. The decrease in the mRNA expressions of these antioxidant enzymes also effected both the expression of proteins and the activities of

those enzymes. Catalase is a major primary antioxidant defense component that works primarily to catalyze the decomposition of H_2O_2 to water, sharing this function with GPx. Therefore, both of these enzymes detoxify H_2O_2 derived from SOD activities. In the presence of low H_2O_2 levels, H_2O_2 is the preferred substrate for GPx since it has low K_m value against H_2O_2 . At high concentrations, organic peroxides are metabolized by GPx [18] and catalase is

responsible for H_2O_2 removal. In our previous study, we have shown that Cu–Zn SOD and CAT decreased significantly in diabetics compared to controls. Reduced Cu–Zn SOD activities reduce H_2O_2 production leading to catalase to be deficient of its substrate. Furthermore, decrease in SOD activity might lead to build up high concentration of superoxide radicals which might inhibit CAT activities which was demonstrated by Kono and Fridovich [19]. In this study, we have shown that, decreasing trend in CAT activity, mRNA, and protein seemed to be counterbalanced by the increasing trends in GPx activities, protein, and mRNA expressions in diabetics. In our study, the decrease in the activity of SOD was probably due to the decrease in gene expression of only CuZnSOD, as we found that MnSOD gene expression and activity did not change at all. In that respect, it seems that cytosolic CuZnSOD is more sensitive to oxidative stress caused by diabetes. It has been shown that oxidative stress or changes in the balance between oxidation and reduction in a cell can affect the translocation of redox-sensitive transcription factors into the nucleus [20]. Therefore, the changes in the mRNA expressions of antioxidant enzymes in diabetes could be due to oxidation of transcriptional factors responsible for the initiation machinery of antioxidant enzymes transcription process.

In literature, several articles showed that MnSOD can be induced to protect against oxidative stress such as cytokine treatment, ultraviolet light, irradiation, certain tumors, amyotrophic lateral sclerosis, and ischemia/reperfusion [21–24]. On the other hand, several others have also reported a decrease in MnSOD activity during cancer, aging, asthma, and transplant rejection [25, 26]. The reason for the unchanged activities of MnSOD in diabetes in our case might be the result of the overall mechanism of the antioxidant enzyme system. As the gene expressions of CuZnSOD decreased, the unchanged gene expressions of MnSOD prevent the further decrease in the total SOD activities.

Sindhu and coworkers [27] found that, even though there was no significant change in the activities, the protein expression of GPx was decreased in diabetes. Contrary to these findings, we found that the activities of the GPx were remained unchanged though the protein expression of the enzyme was elevated. Considering an insignificant change in mRNA expression of GPx, it is probable that the mechanism affected only the protein expression of GPx. According to Bhor et al. [28] in the small intestine of streptozotocin-induced diabetic rats, though mRNA expression of GPx was not changed, activities increased. In another study, the GPx activity in kidney has been found to increase in diabetes and this increment was in parallel with the increase in mRNA expressions [29]. The effect of oxidative stress on antioxidant enzyme status reported in literature significantly varied [8, 29–31] depending on the experimental conditions, like the age of the animals and the

duration of the diabetes. All these studies indicate that oxidative stress effect the status of the antioxidant enzyme systems of different tissues in different ways in order to protect the tissues.

We further supplement the animals with α -lipoic acid (LA) and vitamin C in order to see their effects of these antioxidants on the gene and protein expressions of MnSOD and GPx. LA and its reduced form, dihydrolipoic acid (DHLA) can reduce oxidized antioxidants at the interphase between lipid and water because of their water and lipid soluble characteristics. In literature there are studies which showed that LA reversed the changes in diabetic antioxidant enzyme activities [10, 32–35]. According to our findings, LA decreased the protein expression of MnSOD with no significant change in the activity. That is, LA exerted its action on MnSOD at the level of translation and this effect could not be observed in the activities. On the other hand though LA showed no effect on both the mRNA and protein expressions of GPx, it increased the GPx activity significantly, indicating a post-translational modification.

There is limited number of articles showing the effect of LA on the MnSOD and GPx and these articles only focus on the effect of LA on enzyme activities. Maritim and coworkers [8] found that when LA was supplemented, GPx activity was decreased in livers, and increased in kidneys of diabetic rats.

Vitamin C, also known as ascorbic acid, is a water-soluble vitamin. Under physiological conditions, vitamin C predominantly exists in its reduced form. It is a powerful antioxidant, quenching ROS and reactive nitrogen species, and in that respect it can prevent the damaging effects of oxidative stress [36, 37]. The critical role of vitamin C in eliminating the adverse effects of ROS has been well studied. Its protective effect on antioxidant enzyme activities was presented by Garg and Bansal [38]. According to our results, though vitamin C did not change the activities of the MnSOD drastically, it affected the mRNA expressions of the enzyme. It enhanced the mRNA expression of MnSOD and seemed to induce the transcription without affecting the translation and activity. In the case of GPx, similar to the action of LA, vitamin C also increased the activity of GPx in diabetic groups. That is, supplementing the animals with either antioxidant increased the activities in diabetic groups without any change in both the mRNA and protein expressions. While vitamin C affected the transcription of the MnSOD enzyme only without a significant change in the enzyme activities, it has almost no effect on the mRNA expressions of the GPx. Knirsch and Clerch [39] previously showed that a cytoplasmic protein bound to the 3' untranslated region of MnSOD mRNA and this binding was regulated by the phosphorylation of MnSOD binding protein affecting the MnSOD gene

expression. On the other hand, the effect of vitamin C on the GPx can be at post-translational level probably through phosphorylation. According to Cao and coworkers [40] the mammalian c-Abl and Arg are the nonreceptor tyrosine kinases and they are activated by the cellular response to oxidative stress and both c-Abl and Arg form a heterodimer and become activated with the H₂O₂ application. This activated complex further mediate the phosphorylation on Tyr-96 of GPx1 and this phosphorylation stimulate the activity of GPx providing a protection to cells against oxidative stress.

As a result, it seems that, diabetes and the resulting oxidative stress coordinately regulate the activities of the antioxidant enzymes at different control levels. Though the total SOD and catalase activities decreased as a result of diabetes, no change in the GPx activity was observed. LA and vitamin C, two powerful antioxidants increased all antioxidant enzyme activities but their effects were at different levels of transcription and translation. As both the activity and the mRNA expressions of MnSOD did not change, the increase in total SOD activity with LA and vitamin C was totally related with the increase in the gene expression of CuZnSOD [10]. In order to understand the molecular mechanism better, future studies should emphasize on the identification of the transcriptional and translational factors required for the activation of the antioxidant enzymes.

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