EXPERIMENTAL STUDY

Thyroid hormones-mediated effects of insulin on antioxidant enzymes from diabetic rat hearts

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Abstract: Free radicals, oxidative stress, and antioxidants have become commonly used terms in modern discussion of disease mechanisms. Accumulation of evidence suggests that toxic oxygen-derived reactive free radicals (superoxide, peroxide, and hydroxyl radicals) play a crucial role in etiology of diabetes and its complication. Thus, it was aimed to determine the role of thyroid hormones in reversal of antioxidatant enzyme activities and lipid peroxidation alterations observed in experimentally induced diabetic rat hearts. The present study investigates the antioxidant enzyme activities such as SOD, CAT, GSH-Px and lipid peroxidation products in cardiac tissues of streptozotosin (STZ)-induced diabetic rats before and after thyroidectomy. Our results showed that CAT, GPx enzyme activities and FOX, MDA levels were increased (p<0.05) and SOD, Mn-SOD, Cu-SOD activities were decreased (p<0.05) during diabetes, hypothyroidism and hypothyroidism with diabetes, which can be regulated in different percentages with treatment of insulin and various doses of thyroid hormone (p<0.05). In conclusion, in this study, the possible contribution of thyroid hormones to the insulin effect of normalizing the induced diabetic changes in cardiac tissue and serum of rat has been seen (Tab. 5, Ref. 32). Full Text in PDF www.elis.sk.

Key words: diabetes mellitus, thyroid hormone, superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (CAT), malondialdehyde (MDA), ferrous oxidation in xylenol orange (FOX).
Materials and methods

The study protocol was reviewed and approved by the Animal Care Committee and Surgical Research Center of Gazi University Faculty of Medicine (GUDAM). Guiding principles for experimental procedures found in the Declaration of Helsinki of the World Medical Association regarding animal experimentation were followed in the study. We focused on the possible effect of insulin treatment on antioxidant enzyme activities in hypothyroid and hypothyroid diabetic heart tissues in male albino rats (150–200 g of body weight). The experimental group was injected intraperitoneally with streptozotocin (STZ) (freshly dissolved in saline, 55 mg/kg) whereas the control group was injected with saline only. Three days after the administration, the rats were fasted overnight and blood glucose levels were determined. The animals which had basal glycemia levels of 400 mg/dl were used in the experiments. Animals had free access to food and water after STZ injection.

Rats were assigned to eight groups: Group 1: control, Group 2: diabetes, Group 3: diabetes + insulin (five weeks after diabetes induction, some of the rats were given insulin, 7–10 U/kg day, Insulatard- HM® Penfill®, Novo Nordisk, 100 IU/ml NPH, subcutaneously), Group 4: surgical thyroidectomized control rats, Group 5: thyroidectomized rats + diabetes (diabetes was induced 3 weeks after the surgical operation at hypothyroid state), Group 6: thyroidectomized rats + diabetes + insulin (after diabetes inductions, rats were treated with insulin for 5 weeks, 7–10 U/kg day, subcutaneously), Group 7: thyroidectomized rats + diabetes + insulin + thyroid hormone (after diabetes induction, rats were treated both with insulin (7–10 U/kg day, subcutaneously) and thyroid hormone, levothyroxin sodium, 2.5 μg/kg, Tefor® (Organon) for five weeks), Group 8: thyroidectomized rats + diabetes + insulin + thyroid hormone (after diabetes induction, rats were treated both with insulin (7–10 U/kg day, subcutaneously) and thyroid hormone, levothyroxin sodium, 5 μg/kg, Tefor® (Organon) for five weeks).

Body weights were obtained before treatment and prior to sacrifice. Blood samples were collected from the tail vein and with cardiac puncture at the time of sacrifice. Blood glucose levels were determined by glucose oxidase enzymatic assay (Glucometer, Ames, Miles Laboratories Inc, Elkhart, IN, USA). Free T_{4} (FT_{4}), free T_{3} (FT_{3}), total T_{3} (T_{3}) and total T_{4} (T_{4}) levels were measured in the serum by TOSOH automated analyzer (TOSOH AIA-21, TOSOH Bioscience, N.V., Tessenderlo, Belgium). HbA1c levels in the serum by TOSOH automated analyzer (TOSOH AIA-21, TOSOH Bioscience, N.V., Tessenderlo, Belgium). HbA1c levels were analyzed in the day of experiments by Olympus autoanalyser (Olympus AU 400 analyzer, Olympus Diagnostica GmbH, Clarecastle, Ireland) depending on the turbidimetric inhibition of hemolyzed blood samples.

The rats were anesthetized using ketamine hydrochloride (ke-talar®, Eczacibasi, Turkey). The tissues were briefly washed in ice-cold 0.9 % saline (w/v), frozen in liquid nitrogen and stored at -70 °C until the subsequent protein and enzyme assays.

Protein concentration was measured in tissue homogenate by the method used by Lowry (13), using bovine albumin standard.

For SOD assay, tissue samples were homogenized in ratio of 1/10 in phosphate buffer (pH: 7.4) and the supernatant was carefully separated, then 3/5 (v/v) chloroform and ethanol were added. This mixture was centrifuged at 5000 x g for 2 h. The supernatant was used for assay of superoxide dismutase. This assay for superoxide dismutase activity involves xanthine oxidase used as superoxide generator (14).

The results were expressed as unit per mg protein tissue. One unit of SOD is defined as an amount of protein inhibiting the rate of NBT reduction by 50 %.

To determine GSH-Px activity, tissue samples were homogenized in ratio of 1/10 in phosphate buffer (pH 7.0) and centrifuged at 1900 x g for 15 min. Glutathione peroxidase activity was measured by a coupled modification of the coupled assay procedure of Paglia and Valentine (15). The results were expressed as nmoles of NADPH oxidized per min per mg protein.

Tissue catalase activity was measured by the method used by Aebi (16). Tissue samples were homogenized in phosphate buffer (pH 7.0) and centrifuged at 2400 nm for 3 min. The results were expressed as Katal/mg protein.

The levels of malondialdehyde were determined in tissue samples homogenized in ratio of 1:10 in 1.5 % (w/v) cold KCl solution, using thiobarbituric acid method (17) and the results were obtained in nmol/g tissue wt.

To determine FOX activity, tissue samples were homogenized in ratio of 1/10 in cold methanol (pH 7.4) and then centrifuged at 1000 x g for 15 min. FOX activity was measured by a coupled modification of the coupled assay procedure of Hermes-Lima (18). This study shows that lipid hydroperoxides can be effectively quantified in animal tissue extracts using an assay based on the formation of a Fe (III) xylene orange complex. Addition of H_{2}O_{2}, cumene hydroperoxides, or metholic tissue extracts to an acidic mixture containing 0.25 mM Fe (II) and 0.1 mM xylene orange resulted in the formation of a broad Fe (III) xylene orange peak at 440 nm. Complex formation measured at 580 nm was saturable with both xylene orange and Fe (II) concentration and the results were expressed as nmol/g tissue wt.

Non-parametric methods were performed in the cross-sectional analysis of biomedical data (Mann-Whitney U test). Two-tailed probability (p) values were calculated throughout, and statistical significance was defined as p<0.05. All analyses were performed by statistical software SPSS 10.0.

Results

In the present study, rats demonstrated polyphagia, polydipsia and stable hyperglycemia after STZ treatment for 5 weeks as determined by measuring blood glucose levels every 3 days. Body weights were obtained before treatment and prior to sacrifice. Body weight measurements revealed a significant difference in all groups compared to group 2, and also groups 2 and 4 revealed a significant difference compared to groups 1 and 5 at the time of sacrifice (p<0.05). In group 6, body weight measurements were unchanged compared to groups 3, 7 and 8 (p>0.05). Furthermore, blood glucose determination showed a significant hyperglycemia.
in groups 2 and 5 compared to group 1 (p<0.05). In group 3, blood glucose levels were unchanged while the blood glucose levels in groups 7 and 8 were significantly decreased compared to group 6 (p<0.05). HbA1c levels were significantly increased in groups 2 and 5 compared to group 1 (p<0.05). However, HbA1c levels were significantly decreased in groups 3, 7 and 8 compared to group 6 (p<0.05). Table 1 summarizes the mean changes in bodyweight, blood glucose concentrations and HbA1c levels of all groups.

Serum Free T₃ (FT₃), free T₄ (FT₄), total T₃ (T₃) and total T₄ (T₄) levels were significantly decreased (p<0.05) in group 2 after 5 weeks of diabetes, and serum FT₃ levels were normalized in group 3 depending on insulin treatment compared to control group 1. In group 5, FT₄ and T₄ levels were significantly decreased after the induction of diabetes (p<0.05) compared to group 4. In groups 7 and 8, thyroid hormone levels significantly increased after the treatment (p<0.05). FT₃, FT₄, T₃ and T₄ levels were significantly increased in groups 3, 7 and 8 compared to group 6 (p<0.05). Table 2 summarizes the mean changes of serum FT₃, FT₄, T₃ and T₄ levels in all groups.

In the present study, group 2 (SOD, Cu-Zn SOD, MnSOD) levels signiﬁcantly increased compared to group 6, Thy + DM (p<0.05). However, SOD, Cu-Zn SOD, MnSOD were signiﬁcantly decreased compared to respective group 1 (control) (p<0.05).

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Groups 3, 4, 5 and 6 (SOD, Cu-ZnSOD, Mn-SOD p<0.05) and groups 7 and 8 (SOD, Mn-SOD p<0.05) were significantly increased, while groups 7 and 8 (Cu-ZnSOD, p<0.05) were unchanged compared to respective group 2 (Diabetes). Groups 3, 4 and 7 (SOD, p<0.05) were significantly decreased; group 6 (SOD, p<0.05) was significantly increased, while group 8 (SOD, p<0.05), was unchanged, compared to respective group 5 (Thyroidectomy + Diabetes). Groups 3 and 8 (Cu-ZnSOD, p<0.05) were significantly decreased, while other groups (Cu-ZnSOD, p<0.05) were unchanged compared to respective group 5 (Thyroidectomy + Diabetes). Groups 4 and 7 (Mn-SOD p<0.05) were significantly decreased; group 6 (Mn-SOD p<0.05) was significantly increased, while other groups (Mn-SOD, p<0.05) were unchanged compared to respective group 5 (Thyroidectomy + Diabetes). Furthermore, groups 3, 7 and 8 (SOD, Cu-ZnSOD, Mn-SOD p<0.05) were significantly decreased compared to respective group 6 (Thy ± DM ± I) (Tab. 3).

Groups 2, 5 and 6 (GPx p<0.05) were significantly increased; group 7 (GPx p<0.05) was significantly decreased, while groups 3, 4 and 8 (GPx p<0.05) were unchanged compared to respective group 1 (control). Groups 3, 4, 6, 7 and 8 (GPx p<0.05) were significantly decreased, while group 5 (GPx p<0.05) was unchanged compared to respective group 2 (Diabetes). Groups 3, 4, 6, 7 and 8 (GPx p<0.05) were significantly decreased compared to respective group 5 (Thyroidectomy + Diabetes). Furthermore, groups 3, 7 and 8 (GPx p<0.05) were significantly decreased compared to respective group 6 (Thy ± DM ± I) (Tab. 4).

CAT activities in heart tissue in groups 2, 5 and 6 (CAT p<0.05) were significantly increased; in group 7 (CAT p<0.05), they were significantly decreased, while groups 3, 4 and 8 (CAT p<0.05) were unchanged compared to respective group 1 (control). Groups 5 and 6 (CAT p<0.05) were significantly increased; groups 3, 7 and 8 (CAT p<0.05) were significantly decreased while group 4 (CAT p<0.05) was unchanged, compared to respective group 2 (Diabetes). Groups 3, 4, 7 and 8 (CAT p<0.05) were significantly decreased while group 6 (CAT p<0.05) was unchanged compared to respective group 5 (Thyroidectomy + Diabetes). Furthermore, groups 3, 7 and 8 (CAT p<0.05) were significantly decreased compared to respective group 6 (Thy ± DM ± I) (Tab. 4).

FOX activities in heart tissue in groups 2, 5 and 7 (FOX p<0.05) were significantly increased while groups 3, 4, 6 and 8 (FOX p<0.05) were unchanged, compared to respective group 1 (control). Groups 3, 4, 6, 7 and 8 (FOX p<0.05) were significantly decreased while group 5 (FOX p<0.05) was unchanged compared to respective group 2 (Diabetes). Groups 3, 4, 6, 7 and 8 (FOX p<0.05) were significantly decreased compared to respective group 5 (Thyroidectomy + Diabetes). Furthermore, groups 3 and 7 (MDA p<0.05) were significantly decreased compared to respective group 6 (Thy ± DM ± I) (Tab. 5).
compared to respective group 2 (Diabetes). Groups 3, 4, 6, 7 and (FOX p<0.05) were significantly decreased compared to respective group 5 (Thyroidectomy + Diabetes). Furthermore, groups 3 and 7 (FOX p<0.05) were significantly increased while group 8 was unchanged compared to respective group 6 (Thy + DM ± I) (Tab. 5).

**Discussion**

Oxidative stress has been proposed as the major source of events causing late complications in patients with diabetes mellitus. Sensitive and reliable laboratory tools for detecting oxidative stress are a prerequisite for animal experimental models and studies in human diabetes (19).

Alterations in antioxidant enzyme activities have been reported in diabetes and thus the tissue antioxidant status seems to emerge as an important factor in the etiology of diabetic complications. Serum levels of thyroid hormones are also significantly reduced in streptozotocin-induced diabetic rats but they return to normal after administration of insulin (20).

Hyperglycemia is the primary clinical manifestation of diabetes, and is associated with the development of several clinical complications. Some blood components such as HbA1c and glucose levels are used as indicators of the metabolic status of diabetic individuals (21). In the present study, the levels of HbA1c and glucose were significantly increased in the diabetic group. Recent studies have shown that the activation of AMP-dependent kinase protein (AMPK) is responsible for the increase in glucose uptake in muscle (21).

In the present study, the activity of SOD, Cu-ZnSOD and Mn-SOD decreased in diabetic heart, hypothyroidism and hypothyroidism with diabetes, which can be regulated in different percentages with the treatment of insulin and various doses of thyroid hormone. The decrease in SOD activity observed in the heart could be due to glycation of the enzyme and/or result from excessive accumulation of free radicals (6).

The increased CAT activity observed in the heart of diabetic rats agrees with the findings of Asayama et al (22), Godin et al (5) and Bukan et al (6). Insulin deficiency promotes β-oxidation of fatty acids with resulting H₂O₂ formation (6, 23). The elevation of CAT activity may be due to a compensatory increase in endogenous H₂O₂ production in the heart (6). In our study, we observed that CAT levels in insulin-treated groups (Tab. 4) (group 3) were not effective, and this alteration was increased in hypothyroid-diabetic groups (Tab. 4) (group 6), which indicates that the insulin treatment can regulate CAT levels related to thyroid hormone levels.

The results suggest that GPx is primarily responsible for the decomposition of H₂O₂. Jones et al (24) and Nicholls (25) reported that at low H₂O₂ generation rates, GPx plays a key role in H₂O₂ metabolism, while at higher H₂O₂ generation rates, the role of CAT becomes more important. In the present study, the GPx activity increased in diabetic heart, hypothyroidism and hypothyroidism with diabetes, which can be regulated at different percentages with the treatment of insulin and various doses of thyroid hormone.

Free radicals affect the cell components such as lipid, protein, DNA and carbohydrates, but of which lipid is the most sensitive part. The increase in free radicals in many diseases is suggested to be caused by increased lipid peroxidation and damaged antioxidant defense system (26).

The increase in MDA levels in spite of increased activity of antioxidant enzymes could be due to increased production of oxygen free radicals in amount exceeding the capacity of antioxidant enzymes to metabolize them (26).

H₂O₂ is postulated to be a biomarker of oxidative stress, a phenomenon of considerable clinical interest as oxidative stress is associated with a number of clinical conditions including diabetes mellitus (27). Hydrogen peroxide production was increased by insulin and reduced by thyroid hormones and glucocorticoids. The changes in antioxidant enzyme activities caused by these hormones in macrophages may indicate important mechanisms for establishing the impaired immune function in endocrine pathologies (28). Plasma hydroperoxide concentration was previously estimated by FOX-2 assay (29).

The main findings of our recent study are increased rat heart MDA and FOX levels during the diabetes, hypothyroidism and hypothyroidism with diabetes, which can be regulated at various percentages with insulin and various doses of thyroid hormone. Moreover, it was observed that the data were regulated by insulin treatment. The results obtained in this study are in general agreement with those of our earlier studies (6).

During diabetic condition, thyroid hormones (T3 and T4) are suppressed and it is suggested that serum insulin levels regulate the basal metabolic rate (28). These decreases reflected the significant reduction in T3/T4 ratio in the diabetic group. T3 and T4 depletions in serum might be due to the lack of thyroid binding proteins needed for their transport (28). Karasu et al (29) demonstrated that thyroid hormones were needed for insulin to reverse the diabetes-induced alterations in heart tissue. In another study with experimental diabetic rats, the decreased levels of thyroid hormones were maintained by insulin treatment (30). In our previous study, DNA damage could not be possibly controlled with insulin treatment but in surgically thyroidectomized rats with diabetes treated with both insulin and appropriate thyroid hormone, the doses were succeeded (31). In our other studies (32), the stz-induced diabetic group data support this knowledge while showing a decrease in all serum thyroid hormone parameters in diabetes groups and an increase after insulin treatment. In addition to those, also in this study, low leptin levels can be explained by inadequate thyroid hormones synthesis in surgically hypothyroid and hypothyroid with diabetes group. In these animals, serum leptin levels were significantly lower in both groups compared to diabetes group.

Thus, our results suggest that diabetes-induced hypothyroidism may contribute to antioxidant enzyme alterations in diabetic rats. However, the decrease in thyroid hormone levels due to diabetes seems to have no influence on lipid peroxidation changes in these rats. In the absence of thyroid hormones, insulin may not work as sufficiently as expected to control and balance the effects of oxidative stress on protein, lipids, and DNA. As a result, insulin and thyroid hormones must work together. In conclusion, during diabetic hypothyroidism, insulin is not sufficient to balance the metabolic pathways, thus the mediated effects of
insulin on free radicals and antioxidant regulation via thyroid hormones are an increased possibility. In diabetes it might be that the changes of radical enzyme and antioxidant activities come into existence through different mechanisms or the possibility of affecting both insulin and thyroid hormones through increasing the gene transcriptions.

References

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