Effects of Diabetes, Insulin and Antioxidants on NO Synthase Abundance and NO Interaction with Reactive Oxygen Species

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ABSTRACT

Background : Earlier studies have provided evidence for increased production of reactive oxygen species (ROS) and altered nitric oxide (NO) metabolism in diabetes. This study was intended to explore the effect of type I diabetes and its treatment with insulin alone or insulin plus antioxidant-fortified diet on expression of NOS isoforms and ROS interactions with lipids, glucose and NO.

Methods : Rats with streptozotocin-induced diabetes were divided into once-daily insulin (ultralente)-treated, insulin plus antioxidant (vitamin E and vitamin C)-treated and untreated groups. After four weeks, plasma malondialdehyde (MDA) and tissue endothelial (eNOS), neuronal (nNOS) NO synthases, carboxymethyllysine (CML) and nitrotyrosine were determined.

Results : The untreated diabetic animals exhibited severe hyperglycemia, elevated blood pressure, increased plasma MDA, high tissue CML and reduced tissue nitrotyrosine denoting enhanced lipid, glucose and protein oxidation but reduced NO oxidation by ROS. This was coupled with significant reduction of eNOS and nNOS expression in renal cortex and eNOS in the left ventricle. Insulin therapy partially lowered blood pressure, tissue CML, plasma glucose and MDA but significantly raised eNOS expression and nitrotyrosine abundance to supranormal levels. Combined insulin and antioxidant therapies resulted in normalization of blood pressure, plasma MDA, tissue CML and nitrotyrosine without affecting glucose level or NOS expression.

Conclusion : Oxidative stress in untreated diabetes, is associated with downregulation of NOS isoforms and increased ROS-mediated oxidation of lipid and glucose but not NO. Amelioration of hyperglycemia with once-daily insulin administration alone results in upregulation of NOS isoforms, reduction of lipid and glucose oxidation and increased NO oxidation. However, insulin plus antioxidant supplementation can normalize all three parameters.

INTRODUCTION

Production of the high energy compounds that fuel the biochemical, biophysical and mechanical functions of the body is coupled with ongoing generation of potentially cytotoxic reactive oxygen species (ROS). ROS can attack, denature or modify structural and functional molecules and thereby cause cytotoxicity, tissue injury and dysfunction. Oxidative stress has been implicated in the pathogenesis of tissue injury and dysfunction

in a wide range of human diseases including atherosclerosis, infection, inflammation, neoplasm, degenerative disorders, metabolic diseases, radiation injury, ischemia-reperfusion and hypertension to mention but a few[1, 2].

Numerous studies have provided convincing evidence for the presence of oxidative stress and its role in the pathogenesis of the complications of diabetes[3-7].

ROS react with and modify lipids, carbohydrates, proteins and DNA resulting in cytotoxicity and dysfunction[8]. In addition ROS avidly react with nitric oxide (NO) which is a major signaling molecule with diverse biological functions[9]. This can lead to functional NO deficiency and formation of highly reactive nitrogen species such as peroxynitrite[10] or peroxynitrous acid[11]. The latter agents can, in turn, attack, denature or modify various structural and functional molecules[12, 13]. For instance peroxynitrite can react with tyrosine or cystein residues of the proteins producing nitrotyrosine or nitrocystein which are considered as footprint of ROS interaction with NO[3].

Several in vivo and in vitro studies have demonstrated marked downregulation of NO production and NO synthase abundance in diabetic animals as well as cultured endothelial and mesangial cells subjected to simulated hyperglycemia [14-18]. In addition oxidative stress which is a common feature of diabetes can potentially result in ROS-mediated NO inactivation, thus, compounding the effect of downregulation of NOS. NO is the most potent endogenous vasodilator and as such plays an important role in regulation of renal and systemic vascular resistance, renal blood flow, glomerular filtration rate (GFR) and tissue perfusion[19, 20]. Uncontrolled hyperglycemia is invariably accompanied by increased renal blood flow and glomerular filtration rate. Earlier studies using NOS inhibitors have suggested the role of NO in the genesis of elevated renal blood flow and GFR in uncontrolled diabetes[21, 22].

Uncontrolled diabetes is marked by hyperglycemia and elevation of circulating free fatty acids. ROS react with glucose to produce highly reactive carbonyl compounds which, in turn, can react with the free amino group of lysine residues leading to the formation of glycosylated proteins [4, 7]. In addition ROS avidly react with the fatty acids to produce lipoperoxides that can, in turn, attack various molecules, particularly proteins to generate lipoxidation products[23, 24].

The present study was intended to explore the effects of insulin therapy alone or insulin plus antioxidant therapy on ROS- mediated oxidation of glucose, lipids and NO, as well as, NOS expression in rats with streptozotocin-induced diabetes. The study revealed evidence for increased ROS-mediated glucose and lipid oxidation, reduced NOS expression and NO oxidation in untreated diabetes. Insulin therapy alone lessened lipid and glucose oxidation but increased NO production and NO oxidation. Antioxidant therapy augmented the effect of insulin administration by limiting ROS-mediated oxidation of, not only, lipids and glucose but NO as well.

METHODS

1. Animals

Nine week-old male Sprague-Dawley rats weighing 300-350 g were randomly assigned to the normal control rats fed regular diet (n=6), untreated diabetic rats (n=6), diabetic rats treated with insulin alone (n=5) and diabetic rats treated with the combination of insulin and antioxidant-fortified diet (n=5). Animals assigned to the diabetic group received streptozotocin (Sigma Chemical Co., St. Louis, MO) 65 mg/kg via tail vein. The control group received placebo injection. The insulin-treated subgroups received ultralente insulin (Eli Lilly Inc., Indianapolis, IN) subcutaneously at an initial dosage of 3 units/100 g once daily. The dosage was adjusted as needed using

twice weekly plasma glucose determinations. The antioxidant fortified diet consisted of a rat chow containing α-tocopherol 5,000 U/kg of food and ascorbic acid fortified deionized water (1,000 mg/liter). The control diet consisted of regular rat chow containing α-tocopherol 40 U/kg food and deionized water. The diets employed in the study were purchased from Purina Mills Inc. (St. Louis, MO). Animals were observed for four weeks. Body weight was determined weekly. Tail arterial pressure was measured by tail plethysmography as described in our earlier study[25]. Timed urine collections were obtained using metabolic cages. At the conclusion of the 4-week study period, animals were anesthetized by intraperitoneal injection of pentobarbital 50 mg/kg and killed by exsanguination using cardiac puncture. All of the tissues required for further analyses were immediately excised, cleaned with PBS, frozen in liquid nitrogen, and stored at -70℃. Plasma was separated and stored at - 70℃ until being processed.

2. Measurement of Plasma Glucose and Glycosylated Hemoglobin

Blood glucose level was monitored with a portable glucose-measuring device (Glucometer Elite, Bayer, Elkhart, IN) using blood obtained by tail prick of conscious rat. Blood glycosylated hemoglobin was determined by the affinity column binding method, using the reagents supplied by Sigma Chemical Co.

3. Measurement of Plasma Malondialdehyde (MDA)

Plasma MDA was measured by high pressure liquid chromatography as described in our earlier study[26].

4. Tissue Preparation

The frozen heart (left ventricle), aorta, and renal cortex of the animals studied were homogenized (25% wt/vol) in a solution containing 10 mM HEPES buffer, pH 7.4, containing 320 mM sucrose, 1 mM EDTA, 1 mM dithiothreitol, 10 μg/mL leupeptin, and 2 μg/mL aprotinin at 0℃ to 4℃ by means of a polytron homogenizer. Homogenates were centrifuged at 12,000 g for 5 minutes at 4℃, and the supernatant was used for determination of tissue carboxymethyllysine (CML) content, nitrotyrosine abundance and NOS expression. Protein concentration was determined by means of a bicinchoninic protein assay kit (Pierce, Rockford, IL).

5. Measurement of tissue CML content

Noncompetitive ELISA was performed as described previously[27] with slight modification. Briefly, each well of 96-well immunoplate was coated with 100 μL of the above tissue preparation diluted at final concentration of 0.8-3 μg protein/mL in phosphated-buffered saline (PBS) containing 0.05% NaN_3. The coated plate was kept at 4℃ overnight, followed by washing three times with PBS containing 0.1% Tween 20 (buffer A). In preliminary experiments we had found that the given protein amounts were within the log-linear range of detection for our ELISA technique. Each well was then blocked with 200 μL of PBS containing 1% bovine serum albumin and 0.05% Tween 20 (blocking buffer) for 2 hours, then washed three times with buffer A. After washing, 100 μL of 1 : 2,000 diluted mouse anti-CML monoclonal antibody (ICN Biomedicals, Aurora, OH) in blocking buffer were added to the wells. Plates were incubated for 2 hours at room temperature and overnight at 4℃. Wells were then washed three times with buffer A and incubated with 100 μL of 1 : 2000 horseradish peroxidase-conjugated anti-mouse IgG antibody (Transduction Laboratories, Lexington, KY) in blocking buffer for 2 hours. After washing three times with buffer A, the wells were reacted with 100 μL of tetramethyl benzidine (Pierce, Rockford, IL) for 20 minutes. The reaction was terminated by the addition of 100 μL of 1.5 M sul-

furic acid, and the absorbance at 450 nm was read on a micro-ELISA plate reader (Molecular Device, Soft Max, Sunnyvale, CA). The results were expressed as optical densities per μg protein.

6. Western Blot Analysis

These measurements were carried out to determine the endothelial and neuronal NOS (eNOS and nNOS, respectively) expression and nitrotyrosine abundance, as previously described[28, 29]. Anti-eNOS and anti-nNOS monoclonal antibody and peroxidase-conjugated goat anti-mouse IgG antibody were supplied by Transduction Laboratories (Lexington, KY). Anti-nitrotyrosine antibody was purchased from Upstate Biotechnology (Charlottesville, VA). Briefly, the tissue extracts (70 μg of protein for aorta and heart and 125 μg of protein for renal cortex) were size fractionated on 4-12% Tris-glycine gel (Novex, San Diego, CA) at 120 V for 2-3 h. After electrophoresis, proteins were transferred onto Hybond-ECL enhanced chemiluminescence membrane (Amersham Life Sciences, Arlington Heights, IL) at 400 mA for 120 min, using the Novex transfer system. The membrane was prehybridized in 10 mL of blocking buffer containing 10% milk for 1 h and then hybridized for an additional 16 h period at 4℃ in the same buffer containing 10 μL of the given primary antibody (1 : 1,000). The membrane was then washed for 30 min in a shaking bath, with the wash buffer (Tris-buffered saline containing 0.1% Tween 20) changed every 5 min prior to 2 h of incubation in blocking buffer plus goat anti-mouse IgG-horseradish peroxidase at the final titer of 1 : 1,000. The washes were repeated before the membrane was developed with a light emitting nonradioactive method using ECL Western blotting detection reagents (Amersham Life Sciences, Arlington Heights, IL). The membrane was then subjected to autoluminography from 30 s to 5 min. The autoluminographs were scanned with a laser densitometer (model PD1211; Molecular Dynamics, Sunnyvale, CA) to determine the relative optical densities of the bands. In all instances, the membranes were stained with Ponceau stain, which verified the uniformity of protein load and transfer efficiency across the test samples.

7. Data Analysis

Data are presented as mean±SEM. Analysis of variance (ANOVA) with post hoc multiple comparison test and Wilcoxon signed-rank test were used in statistical analysis of the data. p values less than 0.05 were considered significant.

RESULTS

1. General data

Data are summarized in Table 1. As expected, the untreated diabetic subgroup exhibited marked hyperglycemia and elevated glycosylated hemoglobin. Daily administration of ultralente insulin ameliorated hyperglycemia and lowered glycosylated hemoglobin concentration but did not reduce plasma glucose values to the level seen in the control animals. Concomitant use of antioxidant with insulin did not significantly alter either plasma glucose level or glycosylated hemoglobin concentration in the study animals. The mean daily insulin dosage in diabetic animals treated with insulin alone (15.3±0.06 unit) was comparable with that used in the combined insulin and antioxidant-treated group (15.5±0.05 unit).

The untreated diabetic animals exhibited a significant weight loss during the 4-week study period. Insulin therapy prevented diabetes-induced weight loss and facilitated the growth of animals, albeit, at a moderately slower rate than seen in the control animals. Antioxidant supplementation did not significantly affect body weight in the study animals. The untreated diabetic rats showed a significant rise in creatinine clearance, sig-

Table 1. Body Weight, Plasma Concentrations of Glucose and Glycosylated Hemoglobin (HB-A1) and Creatinine (Pcr) and Creatinine Clearance (Ccr) in the Normal Control Rats Fed Regular Diet (CTL), Untreated Diabetic Rats (DM), Diabetic Rats Treated with Once-daily Ultralente Insulin Alone (DM+I) or the Combination of Insulin and Vitamin E and C-fortified Diet (DM+I+EC). Data are Mean±SEM

	Control	DM	DM+I	DM+I+EC
Body weight (g)				
Week 0	302±14	326±7	303±5	313±5
Week 4	404±34	296±14†	345±7	364±10
Plasma glucose* (mg/dL)	106±5	525±21†	180±6†	167±15†
HB-A1 (%)	6.59±0.76	11.95±0.44†	8.04±0.53	7.54±0.43
Pcr (mg/dL)	0.48±0.03	0.44±0.07	0.44±0.05	0.42±0.03
Ccr (mL/min/kg)	6.90±0.58	10.15±0.86†	8.62±1.42	9.07±0.73
N	6	6	5	5

**Average of plasma glucose levels measured twice weekly at 10 : 00, †p<0.05 versus control group, ‡p<0.005 versus all other groups*

nifying glomerular hyperfiltration. Insulin therapy attenuated the diabetes-induced hyperfiltration but did not restore creatinine clearance values to the level seen in the control animals. Antioxidant supplementation did not significantly affect creatinine clearance in the study groups.

2. Blood Pressure Data

Data are shown in Fig. 1. The untreated diabetic group exhibited a steady rise in arterial pressure during the observation period. Once-daily administration of ultralente insulin significantly ameliorated the diabetes-induced elevation of blood pressure. Antioxidant supplementation augmented the blood pressure lowering effect of insulin therapy leading to complete normalization of blood pressure in the insulin-treated diabetic animals.

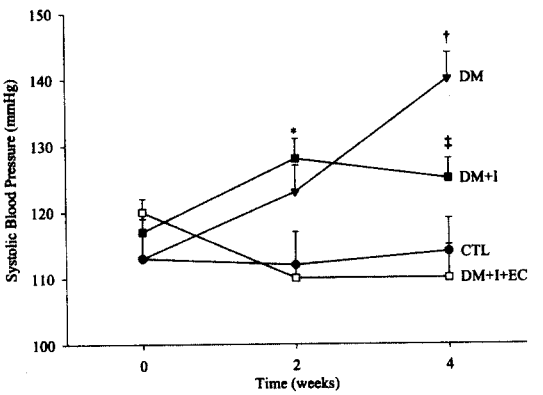

Fig. 1. *Systolic blood pressure in the normal control rats fed regular diet (CTL, n=6), untreated diabetic rats (DM, n=6), diabetic rats treated with once-daily ultralente insulin alone (DM+I, n=5) or the combination of insulin and vitamin E and C-fortified diet (DM+I+EC, n=5). *p<0.05 versus CTL and DM+I+EC groups; †p<0.05 versus all other groups and week 0 values; ‡p<0.05 versus DM+I+EC.*

3. Plasma MDA Results

Data are illustrated in Fig. 2. The untreated diabetic animals exhibited a marked increase in plasma MDA concentration. This observation points to increased ROS-mediated lipid peroxidation in the diabetic animals. Insulin therapy resulted in a significant but incomplete reduction of plasma MDA concentration in diabetic animals. Concomitant administration of antioxidants, vitamins E and C with insulin resulted in complete normalization of plasma MDA concentration.

4. CML data

Data are shown in Fig. 3. Untreated diabetes resulted in a significant rise in CML contents in all tested tissues. This phenomenon is indicative

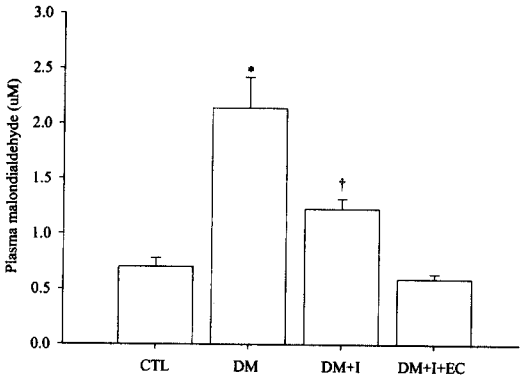

*Fig. 2. Plasma MDA in the normal control rats fed regular diet (CTL, n=6), untreated diabetic rats (DM, n=6), diabetic rats treated with once-daily ultralente insulin alone (DM+I, n=5) or the combination of insulin and vitamin E and C-fortified diet (DM+I+EC, n=5). *p<0.005 versus other groups; †p<0.05 versus CTL and DM+I+EC groups.*

of enhanced ROS mediated protein carbonylation and formation of advanced glycation end product in diabetes[7]. Insulin therapy led to a partial reduction of CML content in all tested tissues. Concomitant administration of vitamins E and C with insulin therapy resulted in further reduction of tissue CML content in all tested tissues to values which were comparable with those found in the control group.

5. Nitrotyrosine data

Data are depicted in Fig. 4. The untreated diabetic animals showed a significant reduction in nitrotyrosine abundance in the left ventricle and renal cortex compared with the control group. However nitrotyrosine abundance in the aorta of the untreated diabetic animals was similar to that of the control group. Insulin therapy alone increased nitrotyrosine abundance in all tested tissues to values which were significantly higher than those found in the control group. Concurrent antioxidant therapy attenuated the insulin-induced rise in tissue nitrotyrosine abundance.

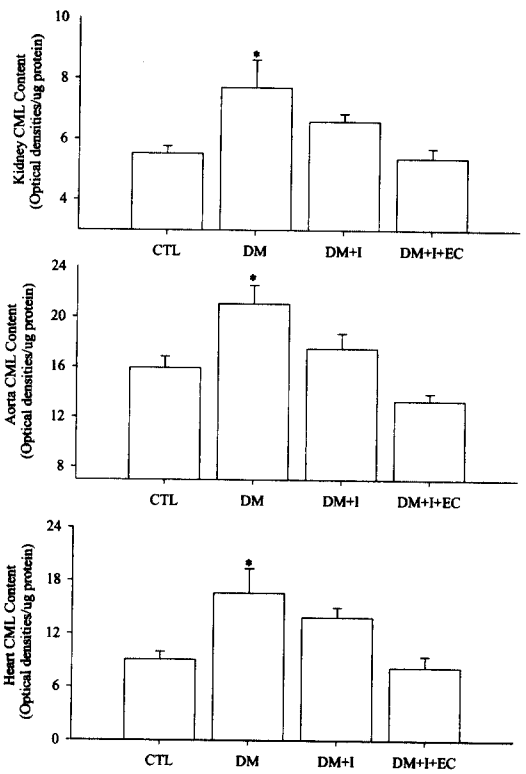

*Fig. 3. CML content of kidney, aorta and heart in the normal control rats fed regular diet (CTL, n=6), untreated diabetic rats (DM, n=6), diabetic rats treated with once-daily ultralente insulin alone (DM+I, n=5) or the combination of insulin and vitamin E and C-fortified diet (DM+I+EC, n=5). *p<0.05 versus CTL and DM+I+EC groups.*

6. NOS data

Data are given in Fig. 5. The untreated diabetic animals exhibited a significant reduction in eNOS and nNOS abundance in the renal cortex and eNOS abundance in the left ventricular tissues. However eNOS abundance in the aorta was unchanged in the untreated diabetic group. Insulin therapy alone raised the eNOS abundance in the renal cortex, aorta and left ventricular tissues to values which were significantly higher than those found in the control group. In addition insulin therapy alone restored renal cortex nNOS to a near normal level. Concomitant antioxidant sup-

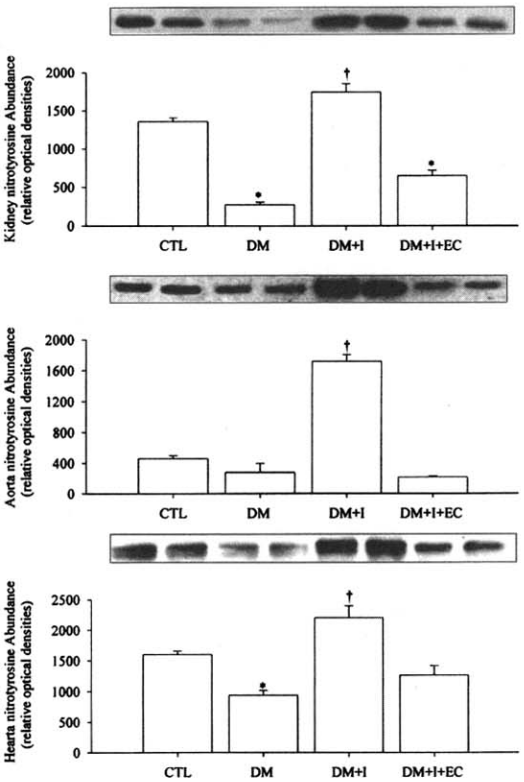

Fig. 4. *Representative Western blots and corresponding group data illustrating nitrotyrosine abundance of kidney cortex, aorta and heart in the normal control rats fed regular diet (CTL, n=6), untreated diabetic rats (DM, n=6), diabetic rats treated with once-daily ultralente insulin alone (DM+I, n=5) or the combination of insulin and vitamin E and C-fortified diet (DM+I+EC, n=5). *p<0.005 versus CTL and DM+I groups; †p<0.05 versus other groups.*

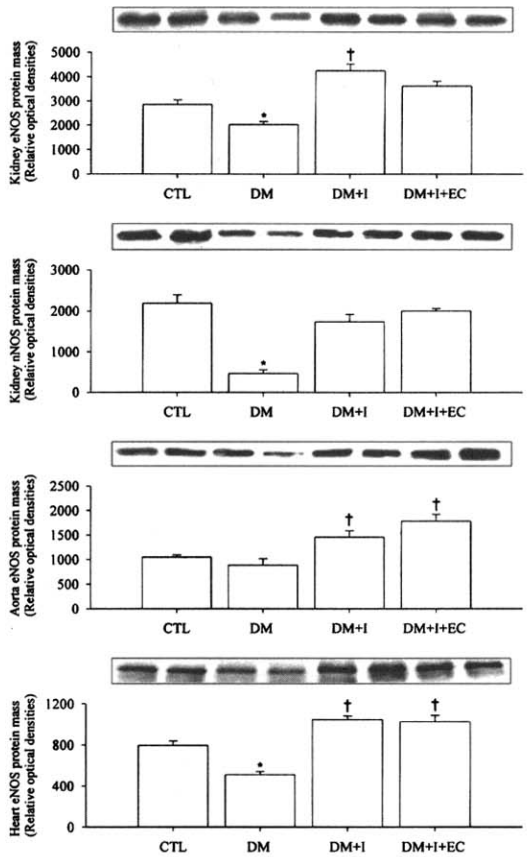

Fig. 5. *Representative Western blots and corresponding group data illustrating NOS protein mass of kidney cortex, aorta and heart in the normal control rats fed regular diet (CTL, n=6), untreated diabetic rats (DM, n=6), diabetic rats treated with once-daily ultralente insulin alone (DM+I, n=5) or the combination of insulin and vitamin E and C-fortified diet (DM+I+EC, n=5). *p<0.05 versus other groups; †p<0.05 versus CTL and DM groups.*

plementation had no additional impact on NOS abundance in the tested tissue.

DISCUSSION

The untreated diabetic animals showed significant increases in plasma MDA concentration and marked elevation of tissue CML content. These findings point to ROS-mediated modifications of lipid, carbohydrate and protein molecules denoting the presence of oxidative stress which is a known feature of diabetes[3, 5, 6].

Interestingly, tissue nitrotyrosine abundance was significantly reduced in the untreated diabetic animals despite presence of oxidative stress which could have raised nitrotyrosine abundance via ROS-NO interaction. This phenomenon can, in part, be explained by the downregulation of eNOS and nNOS abundance in the diabetic animals. In addition glucose and free fatty acids which are invariably elevated in uncontrolled diabetes may compete with NO as potential molecu-

lar targets of ROS.

Accordingly the byproducts of ROS interaction with glucose (CML) and fatty acids (MDA), were increased whereas that of ROS-NO interaction (nitrotyrosine) was reduced in diabetic animals. If true, increased competition for ROS interaction by glucose and fatty acids may have an NO-sparing effect. This can, in part, contribute to elevation of renal blood flow and glomerular filtration rate in uncontrolled diabetes despite downregulation of NOS expression. The authors wish to acknowledge the speculative nature of this statement and acknowledge the previously demonstrated role of enhanced proximal tubular sodium reabsorption leading to attenuation of tubuloglomerular feedback as the primary factor[30].

Insulin therapy alone resulted in a marked but incomplete reduction of plasma glucose concentration. This was accompanied by a significant but incomplete reduction of plasma MDA and tissue CML levels. The reduction in plasma MDA and tissue CML was coupled with a significant rise in tissue nitrotyrosine abundance. Elevation of tissue nitrotyrosine with insulin therapy could be, in part, explained by the upregulation of NOS isoforms in all tested tissues and the expected rise in NO production capacity. This coupled with the residual oxidative stress with incomplete glycemia control in once-daily-insulin-treated animals can account for the observed elevation of tissue nitrotyrosine abundance. In addition possible reduction in competition for ROS interaction by glucose and fatty acids can further contribute to elevation of tissue nitrotyrosine abundance.

Antioxidant therapy resulted in complete normalization of MDA, CML and significant reduction in tissue nitrotyrosine levels in the insulin-treated animals. These findings can be explained by the interactions of vitamins E and C with ROS which necessarily spared lipids, glucose and NO alike from attack by ROS.

The untreated diabetic animals exhibited a significant downregulation of eNOS and nNOS protein expressions in the renal cortex and of eNOS in the left ventricle. These findings are consistent with our earlier study demonstrating marked downregulation of eNOS expression in cultured endothelial cells subjected to simulated hyperglycemia and insulin deprivation[14]. Similar observations have been reported by other investigators in numerous in vivo and in vitro experiments[15-18].

In contrast a number of other studies have shown upregulation of NO production and NOS expression in diabetic animals and cultured endothelial cells exposed to high-glucose concentration [22, 31-33]. The reason for the variance in the results of the latter studies with those of the present study and previous studies from our group and other investigators is not clear. However differences in the model used, duration of diabetes, severity of hyperglycemia and other factors may be involved.

Insulin therapy resulted in a significant increase in eNOS and nNOS expression in the renal cortex and eNOS expression in the aorta and left ventricular tissue. This is consistent with the known effect of insulin on NO production and eNOS gene expression in endothelial cells via activation of phosphatidylinositol-3 kinase[34, 35]. Interestingly eNOS expression rose to values which were significantly greater in all tested tissues than the corresponding values found in the control group. The reason for the exaggerated response to insulin therapy is not clear. However it may be due to higher peak insulin levels achieved with once-daily insulin administration than that achieved by normal release of insulin from the pancreas in the normal condition[36]. Moreover the amount of exogenous insulin required to maintain reasonable glucose control averaged 44 U/kg/day which far exceeds the amount normally produced by the pancreas in intact animals. This insulin dosage required in the diabetic rats employed in

the present study was comparable with that reported by other investigator[22]. Given the upregulatory action of insulin on eNOS expression shown by us and others previously[14, 34, 35], the observed eNOS upregulation to supranormal value in the insulin-treated diabetic animals is not surprising.

Antioxidant therapy resulted in a significant reduction in arterial pressure in the insulin-treated diabetic animals. This phenomenon is consistent with the earlier studies which demonstrated improvement in hypertension by amelioration of oxidative stress in various forms of genetic and acquired hypertension[26, 37-40].

In the present study, diabetic rats were treated with once-daily injection of a long-acting insulin preparation resulting in significant but incomplete reduction of plasma glucose concentration. A higher concentration of insulin or more frequent injections per day may result in a more complete control of plasma glucose level and oxidative stress. In that case, antioxidants supplementation may be of no added benefit. However, complete normalization of blood glucose concentration requiring multiple insulin injections is not always practical for long-term clinical management of diabetic patients. Therefore, the use of dietary antioxidant supplementation can be an effective adjunct in the amelioration of oxidative stress associated with incomplete glycemia control with current method of insulin administration. In fact, oxidative stress has been shown to contribute to renal injury and antioxidant supplementation has been shown to mitigate renal disease in diabetic and nondiabetic rats[41].

In conclusion, uncontrolled diabetes of 4 weeks duration in rats is associated with enhanced lipid, glucose and protein oxidation, hypertension, down regulation of NOS expression and reduced nitrotyrosine abundance. Once-daily insulin administration results in partial glycemia control and incomplete correction of oxidative stress and hypertension as well as upregulation of eNOS expression and nitrotyrosine formation. Concomitant antioxidants therapy leads to complete correction of oxidative stress, blood pressure and nitrotyrosine abundance. These findings support the potential benefit of antioxidant therapy in diabetes.

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