Effect of Combined Vitamin E and Insulin Administration on Renal Damage in Diabetic Rats Fed a High Cholesterol Diet

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In the present study, we investigated the effects of a long-term treatment with vitamin E, an antioxidant vitamin, insulin, or their combination on renal damage in streptozotocin (STZ)-induced diabetic rats fed a high cholesterol diet. Increases in urinary albumin and lipid peroxide (LPO) excretions were observed in these diabetic rats, when both urinary parameters were measured at 8 and 15 weeks after STZ administration. Daily treatment with vitamin E, insulin, or their combination markedly suppressed the increase in the 24 h urinary albumin and lipid peroxide excretions. Furthermore, glycogen degeneration of distal tubules, fatty degeneration of glomerular endothelium and hypertrophy of glomeruli and mesangium were observed in the kidneys of the diabetic animals when histopathological evaluation was performed at 4, 8, and 15 weeks (glomerular and mesangial hypertrophy were observed only at 15 weeks). Combined vitamin E and insulin treatment was the most effective at suppressing these renal histopathological changes. These results indicate that combined vitamin E and insulin treatment additively prevents the development and progression of renal damage in diabetic rats. Possible mechanisms for the preventive effect of this combined treatment are discussed.

Key words diabetes; renal injury; hyperglycemia; hyperlipidemia; lipid peroxide

Diabetic nephropathy is characterized by persistent proteinuria, hypertension and declining renal function. Microalbuminuria is detected at the early stage of diabetic nephropathy. Approximately 30—40% of type 1 diabetic patients have renal complications, which are a major cause of mortality.1—3) Diabetes mellitus is a disease characterized by hyperglycemia. On the other hand, hyperlipidemia in addition to hyperglycemia has been frequently observed in insulin-dependent4,5) and non-insulin-dependent diabetic patients.6) Likewise, hyperlipidemia was also observed in experimental insulin-dependent7,8) and non-insulin-dependent diabetic animal models.9) Hyperlipidemia in addition to hyperglycemia has been thought to be a major risk factor for the development and progression of diabetic complications such as nephropathy.10,11)

Recently, oxidative stress has been indicated to play an important role in the development and progression of diabetic nephropathy.12) Under hyperglycemic conditions, oxygen-derived free radicals are produced mainly through a glycation reaction.13,14) Scheuer et al.15) have shown that hyperlipidemia can aggravate glomerulosclerosis and chronic tubulointerstitial damage in kidneys and oxidative stress contributes to the deleterious effects of hyperlipidemia on renal damage. Chen et al.16) have demonstrated that oxidized low density lipoprotein (LDL) enhances superoxide production by diabetic rat glomeruli. Their findings strongly suggest that oxidized LDL may play important roles in the pathogenesis of diabetic nephropathy through increased generation of oxygen-derived free radicals.

It is well known that vitamin E, a lipid-soluble and antioxidant vitamin, protects unsaturated fatty acid, a main component of cell membranes, from attack by oxygen-derived free radicals. There have been a few reports concerning the effect of vitamin E on experimental diabetes. Je et al.17) reported that vitamin E inhibited the oxidation of proteins in organs such as liver and kidney in streptozotocin (STZ)-induced diabetic rats. Baydas et al.18) have shown that vitamin E as well as melatonin protects organisms from oxidative damages. On the other hand, insulin has been widely used for the treatment of type 1 (insulin-dependent) diabetes mellitus as hormone supplement therapy. These findings suggest that insulin in combination with vitamin E may exert a more beneficial effect than insulin alone on diabetic nephropathy.

Recently, we reported that a long-term high cholesterol diet for STZ-induced diabetic rats not only precipitated the onset of cataracts but also increased the incidence of this complication with an increased death rate.19) Furthermore, in this report, we demonstrated that long-term combined treatment with vitamin E and insulin synergistically prevents the development and progression of cataracts of STZ-induced diabetic rats fed a high cholesterol diet.

In a pilot experiment, we observed that a long-term high cholesterol diet aggravated STZ-induced renal damage in rats with increased urinary lipid peroxide (LPO) excretion, an index of lipid peroxidation in kidneys. Therefore, in the present study, we investigated the effects of long-term treatment with vitamin E, an antioxidant vitamin, insulin, or a combination of the two, on renal damage in STZ-induced diabetic rats fed a high cholesterol diet.

In the present experiment, we used doses of insulin and vitamin E that were insufficient for controlling blood glucose or scavenging oxygen-derived free radicals, because it would be difficult to evaluate the synergistic effect of combined vitamin E and insulin administration if their doses were both sufficient.

MATERIALS AND METHODS

Animals Seven-week-old male Sprague–Dawley rats (Charles River, Hino) were housed in an isolator caging system in an air-conditioned animal room at 23 ± 1°C. All experimental procedures were approved by the Committee for Ethics and Animal Experimentation of Nihon Bioresearch Inc.

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Drugs  The drugs used were vitamin E (α-tocopherol, Wako Pure Chemical Industries, Ltd., Osaka) and insulin (Isophan Insulin (Aqueous Suspension), NP Hiszilin®, Takeda Pharmaceutical Co., Ltd., Osaka).

Induction of Diabetes and Experimental Procedure

Rats were fasted for 20 h before the experiment and then divided into 5 groups. These rats were fed a standard diet before the experiment and a standard diet, high cholesterol diet, or vitamin E-supplemented high cholesterol diet after starting the experiment. The standard diet (pellets) consisted of 20% casein, 63.2% sucrose, 10% corn oil, 2% agar, a 0.8% vitamin mixture, and a 4% salt mixture.

The high cholesterol diet (pellets) consisted of the standard diet with 1% cholesterol (wt/wt) and 0.5% cholic acid (wt/wt) in place of an equal amount of sucrose. The vitamin E-supplemented diet contained 0.12% vitamin E (wt/wt) in the high cholesterol diet. These diets were produced by Oriental Yeast Co. Ltd. (Tokyo). To induce insulin-deficient diabetes, the fasted rats in four groups were injected intravenously with STZ (Sigma Chemical Co., Ltd., St. Louis, MO, U.S.A.) dissolved in a citrate buffer (0.1 M with pH 4.5) at a dose of 40 mg/kg. The fasted rats in one group were injected with the equivalent volume of citrate buffer as the non-diabetic group. The experimental groups were as follows (Table 1): (1) Standard diet-fed normal (SD-fed Nor) group, (2) high cholesterol diet-fed diabetic (HCD-fed Diab) group, (3) vitamin E-supplemented high cholesterol diet-fed diabetic (VE-suppl HCD-fed Diab) group, (4) high cholesterol diet-fed diabetic+insulin (HCD-fed Diab+Ins) group, and (5) vitamin E-supplemented high cholesterol diet-fed diabetic+insulin (VE-suppl HCD-fed Diab+Ins) group. The rats in the HCD-fed Diab+Ins and VE-suppl HCD-fed Diab+Ins groups received a daily s.c. injection (2 U/rat) of insulin at 10:00 a.m. from the day after the start of the experiment. Blood samples for the determination of serum glucose, total cholesterol, and triglyceride contents were withdrawn from the cavernous sinus with a capillary under light ether anaesthesia at 9:00 a.m. 0 (before the injection of STZ or citrate buffer), and 1, 2, 4, 8, 12, and 15 weeks after the start of the experiment. These serum biochemical parameters were determined using an Automated Chemistry Analyzer (AU 400, Olympus Optical Co., Ltd., Tokyo, Japan). At 0, 8 and 15 weeks after the start of the experiment, these animals were kept in individual metabolic cages and 24 h urine samples were collected for the determination of urinary albumin and LPO contents. The urinary albumin was determined by an immunoturbidity method using a kit for the determination of microalbumin (Bayer Diagnostics, Germany). Urinary LPO was determined as the concentration of malondialdehyde, a secondary product of lipid peroxidation, by adding thioobarbituric acid according to the method of Yagi. At 4, 8 and 15 weeks, animals from each group were sacrificed by decapitation under light ether anaesthesia and their kidneys removed for histopathological evaluation.

Histopathological Evaluation of Kidneys

Kidneys removed for histopathological evaluation were fixed in 10% neutral buffered formalin. Paraffin sections (2 μm) were stained with hematoxylin and eosin (HE) or periodic acid-Schiff reaction (PAS) for light microscopy. Glycogen degeneration of distal tubules (HE stain), fatty degeneration of glomerular endothelium (HE stain), and glomerular and mesangial hypertrophy (PAS stain) were observed in 10 distal tubules or glomeruli per section for histopathological evaluation of the kidneys. The degree of degeneration of the tubules and glomerular endothelium was semiquantitatively scored from 0 to 3 as follows. Normal: score 0, mild: score 1, moderate: score 2 and severe: score 3. Representative micrographs of the degrees of degeneration are shown in Fig. 1. The average score for each group was calculated for the histopathological evaluation. Furthermore, glomerular and mesangial areas were measured using image analysis software (Win ROOF) (Mitani Corporation, Fukuji). The PAS-positive stained portion of each glomerulus was measured as the mesangial area.

Statistical Analysis

The data are expressed as the mean±S.E. The data were analyzed for statistical significance by Duncan’s test for multiple comparison or Student’s t-test for comparison between two groups.

RESULTS

Nonfasting Serum Glucose, Total Cholesterol, and Triglyceride Levels

In our previous study, the nonfasting serum glucose levels in the SD-fed Nor group were 127—144 mg/dl throughout the observation period of 1—15 weeks (Table 1A). The serum glucose levels in the HCD-fed Diab group increased markedly from 1 week after STZ administration (1 week: 546±17 mg/dl) and almost the same high levels were maintained up to 15 weeks (15 weeks: 601±28 mg/dl). The serum glucose levels in the VE suppl HCD-fed Diab group were not different from those in the HCD-fed Diab (control) group for the experimental period. These levels in the HCD-fed Diab+Ins and VE-suppl HCD-fed Diab+Ins groups were significantly lower than those in the HCD-fed Diab group, but higher than those in the SD-fed Nor group for 2—15 weeks. Thus, blood glucose control by the daily treatment with insulin (2 U/rat, s.c.) was insufficient.

The number of rats in all groups was 10 (n=10) at 0—4 weeks (Table 1). However, the number of rats in the HCD-fed diab group at 8 and 15 weeks was 8 and 4, respectively. The decreases in the number of animals were due to increases in the number of dead animals.

The nonfasting serum total cholesterol levels in the SD-fed Nor group were 67—74 mg/dl throughout the 15 week observation period (Table 1B). The total cholesterol levels in the HCD-fed Diab group were 21.1—75.7 times higher than those in the SD-fed Nor group throughout the observation period. The total cholesterol levels in the VE-suppl HCD-fed Diab, HCD-fed Diab+Ins, and VE-suppl HCD-fed Diab+Ins groups were significantly lower than those in the HCD-fed (control) Diab group for 4—12 weeks, 2—15 weeks, and 2—15 weeks, respectively. Thus, daily treatment with vitamin E, insulin, or the combination of both agents markedly suppressed hypercholesterolemia in the HCD-fed Diab rats.

The nonfasting serum triglyceride levels in the SD-fed Nor group were 122—169 mg/dl throughout the 15-week observation period (Table 1C). The triglyceride levels in the HCD-fed Diab group were 3.9—18.8 times higher than those in the SD-fed Nor group throughout the observation period. The triglyceride levels in the VE-suppl HCD-fed Diab group...
were significantly lower than those in the HCD-fed Diab group (control) at 1—12 weeks and these levels in the HCD-fed Diab + Ins and VE-suppl HCD-fed Diab + Ins groups were also significantly lower than those in the control group throughout 1—15 weeks.

**Urinary Albumin and LPO Excretions** Urinary albumin excretion in the SD-fed Nor group was 0.16 ± 0.02 mg/d at 8 and 15 weeks (Fig. 2A). The 24 h urinary albumin excretion in the HCD-fed Diab group was 12.6 and 16.2 times respectively, higher than that in the SD-fed Nor group at 8 and 15 weeks. The 24 h albumin excretion in the VE-suppl HCD-fed Diab, HCD-fed Diab + Ins, and VE-suppl HCD-fed Diab + Ins groups was markedly decreased compared with that in the respective HCD-fed Diab group (control) at 8 (% decrease: 75%, 85%, and 87%, respectively) and 15 weeks (% decrease: 78%, 89%, and 89%, respectively). There was no apparent difference in the effectiveness on the albumin excretion among these three treatment groups. Urinary LPO excretion in the SD-fed Nor group was 86.5 ± 2.2 nmol/d and 90.7 ± 2.3 nmol/d, respectively, at 8 and 15 weeks after the start of the experiment (Fig. 2B). LPO excretion in the HCD-fed Diab group was 4.3 and 6.9 times, respectively, higher than that in the SD-fed Nor group at 8 and 15 weeks. The 24 h LPO excretion in the VE-suppl HCD-fed Diab, HCD-fed Diab + Ins, and VE-suppl HCD-fed Diab + Ins groups was significantly decreased compared with that in the respective HCD-fed Diab group (control) at 8 (% decrease: 24%, 59%, and 67%, respectively) and 15 weeks (% decreases of 45%, 54%, and 67%, respectively). Combined treatment with vitamin E and insulin was significantly more effective at decreasing urinary LPO excretion than treatment with vitamin E alone at 8 weeks and treatment with each agent alone at 15 weeks. In addition, the dose of vitamin E (a high cholesterol diet supplemented with 0.12% w/w...
vitamin E) used as an antioxidant was not high enough to decrease LPO excretion to normal levels.

Histopathology of Kidneys

When histopathological changes in the kidneys were evaluated at 4, 8 and 15 weeks after STZ administration, glycogen degeneration in the distal tubules (Fig. 3A) and fatty degeneration in the glomerular endothelium (Fig. 3B) were observed at all weeks in the kidneys of animals evaluated in the HCD-fed Diab group. The distal tubule glycogen degeneration scores in the VE-suppl HCD-fed Diab, HCD-fed Diab + Ins, and VE-suppl HCD-fed Diab + Ins groups were significantly decreased compared with those in the HCD-fed Diab group (control) at 4 (% decrease: 46%, 69% and 77%, respectively), 8 (% decrease: 57%, 64% and 79%, respectively) and 15 weeks (% decrease: 53%, 87% and 93%, respectively). Thus, the combined vitamin E and insulin treatment tended to be the most effective at suppressing glycogen degeneration in the distal tubules. The glomerular endothelium fatty degeneration score in the VE-suppl HCD-fed Diab group was markedly decreased compared with that in the HCD-fed Diab group (control) at all weeks measured (% decrease: 21%, 19% and 36%, respectively). The combined vitamin E and insulin treatment was significantly more effective at suppressing the increase in glomerular area than either vitamin E or insulin alone.

The mesangial area in the VE-suppl HCD-fed Diab, HCD-fed Diab + Ins, and VE-suppl HCD-fed Diab + Ins groups was significantly decreased compared with that in the HCD-fed Diab group (control) (% decrease: 17%, 25% and 48%, respectively). The combined vitamin E and insulin treatment was significantly more effective at suppressing the increase in mesangial area than treatment with either agent alone. Thus, the combined treatment with both agents was the most effective at suppressing renal pathological changes.

DISCUSSION

The results of the present study demonstrate that long-
Term combined treatment with vitamin E, a lipid-soluble and antioxidant vitamin, and insulin additively prevents the development and progression of renal damage in STZ-induced diabetic rats fed a high cholesterol diet.

In the present experiment, an increase in urinary albumin excretion was observed in STZ-induced diabetic rats fed a high cholesterol diet, compared with normal rats fed a standard diet at 8 and 15 weeks after STZ administration. Furthermore, glycogen degeneration in the distal tubules, fatty degeneration in the glomerular endothelium, and glomerular and mesangial hypertrophy were observed in diabetic rats fed a high cholesterol diet at 4, 8 and 15 weeks (glomerular hypertrophy and mesangial hypertrophy were observed only at 15 weeks). The severity of the urinary albumin excretion and these histopathological changes in kidneys was greater in diabetic rats fed a high cholesterol diet than in diabetic rats fed a standard diet (data not shown). However, massive albuminuria and severe histopathological changes such as glomerulosclerosis were not observed in STZ-induced diabetic rats fed a high cholesterol diet even at 15 weeks after STZ administration. Thus, the degree of the changes in renal function was observed at 10 weeks after STZ injection. Therefore, it is conceivable that hypertension may be the major risk factor for the progression to glomerulosclerosis observed at the late stage of diabetic nephropathy.

As described in the Introduction, in the present experiment, we used doses of insulin (2 U/rat/d, s.c.) and vitamin E (a high cholesterol diet supplemented with 0.12% w/w vitamin E) that were insufficient for controlling blood glucose or scavenging oxygen-derived free radicals because it would be difficult to evaluate any synergistic effect of combined vitamin E and insulin administration if their respective doses were both sufficient. As a result, daily treatment with vitamin E, insulin, or their combination markedly decreased urinary albumin excretion in diabetic rats fed a high cholesterol diet. However, there was no apparent difference in the effect on albumin excretion. In addition, daily treatment with vitamin E, insulin, or their combination was effective at suppressing all histopathological changes in the kidneys such as glycogen degeneration in the distal tubules, fatty degeneration in the glomerular endothelium, and glomerular and mesangial hypertension in diabetic rats fed a high cholesterol diet. Combined vitamin E and insulin treatment was the most effective at suppressing these renal pathological changes.
Recently, oxygen-derived free radicals and LPO, which are easily formed in the diabetic state, are thought to play important roles in the development of diabetic complications. It has been reported that under diabetic conditions, oxygen-derived free radicals are produced mainly through the glycation reaction.\(^{13,14}\) Furthermore, it has been shown that the generation of oxygen-derived free radicals is enhanced in the arteries of hypercholesterolemic rabbits\(^ {23}\) and in diabetic rat glomeruli incubated with native and oxidized LDL.\(^ {15}\) These findings suggest that the enhanced production of oxygen-derived free radicals in the diabetic state may be attributed to hyperlipidemia in addition to hyperglycemia. In the present experiment, we measured the 24-h urinary LPO content as an index of lipid peroxidation in vivo in kidneys, especially at 8 and 15 weeks after STZ administration. Marked increases in urinary LPO excretion were observed in diabetic rats fed a high cholesterol diet at both 8 and 15 weeks. Treatment with vitamin E, insulin, or a combination of both agents markedly suppressed the increase in urinary LPO excretion. Combined treatment with both agents was the most effective at decreasing LPO excretion. Insulin, unlike vitamin E, does not possess a direct antioxidative action. Therefore, the suppressive action of insulin on the increase in urinary LPO excretion in the diabetic rats may be a secondary action due to its hypoglycemic action. It has been reported that two endogenous antioxidants, alpha-lipoic acid and reduced glutathione, directly improve the impaired endothelium-dependent response of renal arterioles and aortic rings of alloxan-induced diabetic rabbits.\(^ {25}\) Vitamin E, an antioxidant vitamin, as well as the above endogenous antioxidants may prevent the progression of diabetic nephropathy by improving the impaired endothelium-dependent vascular response. It has been shown that the protein expression of hepatic superoxide dismutase, catalase, and glutathione peroxidase is decreased in STZ-induced diabetic rats compared with those in normal controls and the reductions in these antioxidant enzymes can be ameliorated by insulin and/or antioxidant (vitamins E and C) therapy.\(^ {24}\) Therefore, employing antioxidants such as vitamin E as a supplement to insulin therapy for diabetic nephropathy may be clinically useful. It is unclear whether combined vitamin E and insulin treatment is more effective than a sufficient dose of insulin alone. However, intensive insulin therapy for type 1 diabetes has been demonstrated to exert some undesirable vascular effects, such as increased blood pressure and worsening lipid profiles which counteract its benefit as a hypoglycemic drug.\(^ {25}\) In this connection, it has been reported that intensive insulin therapy may cause a transient worsening of retinopathy in patients with diabetes through increased retinal vascular endothelial growth-factor gene expression.\(^ {26}\) From the above reports, further experiments are needed to clarify whether treatment with insulin alone at a sufficient dose exhibits more beneficial effects on diabetic renal damage than combined vitamin E and insulin treatment, or if insulin alone produces any undesirable effects.

In summary, it is concluded from these results that combined vitamin E and insulin treatment additively prevents the development and progression of diabetic renal damage. Treatment with vitamin E, insulin, and their combination markedly improved hypercholesterolemia, hypertriglyceridemia, and increased urinary LPO excretion in diabetic rats fed a high cholesterol diet. In addition, treatment with insulin and the combination of vitamin E and insulin markedly improved hyperglycemia, although vitamin E alone had no effect. Therefore, the results also suggest that the preventive effect of the combined treatment with both agents may be mainly due to their hypoglycemic, hypolipidemic, and free radical scavenging actions.

REFERENCES

15. Scheuer H., Gwinner W., Hohbach J., Grone E. F., Brandes R. P., Malle


