Original Article

A Role of Oxidative Stress-Generated Eicosanoid in the Progression of Arteriosclerosis in Type 2 Diabetes Mellitus Model Rats

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Diabetes mellitus (DM) is a well-established risk factor of cardiovascular diseases. We investigated the mechanism of the progression of arteriosclerosis in DM, focusing on the role of oxidative stress and insulin resistance *in vivo*. Male Otsuka Long-Evans Tokushima Fatty (OLETF) rats, an experimental model of type 2 DM, were assigned to 3 groups, based on supplementation with vitamin E (VE) or troglitazone (TR), a VE-derived agent which improves insulin-resistance. At 36 weeks, plasma and aortic tissue 8-iso-PGF₂ contents, a vascular proliferating eicosanoid produced *in vivo* by oxidative stress, were measured by EIA. TGF- α and TGF- β receptor II were immunohistochemically analyzed. Histopathologically, medial area and the nuclear number of smooth muscle cells of the aorta were measured. The tissue 8-iso-PGF₂ content (pg/g tissue) was significantly decreased by either VE or TR in the aorta (untreated-OLETF, 15,332 $\pm$ 3,254 vs. TR-treated-OLETF, 7,092 $\pm$ 1,992 or VE-treated-OLETF, 5,394 $\pm$ 836, both $p < 0.01$), but that in plasma decreased by only VE. VE and TR improved the increased level of the actual medial area and the number of smooth muscle cells. The expression of TGF- α was reduced, but TGF- β receptor II was not. 8-iso-PGF₂ may play an important role in the progression of arteriosclerosis. Antioxidant treatment may promise significant clinical benefits in the early diabetic stage. (*Hypertens Res* 2002; 25: 91–98)

Key Words: diabetes mellitus, arteriosclerosis, oxidative stress, isoprostanes, insulin-resistance

Introduction

Some clinical studies have suggested that the macro vascular complications of diabetes mellitus (DM) are more likely related to either the degree of prevailing hyperglycemia or its associated cardiovascular risk factors rather than due to the administration of exogenous insulin (1–4). There is considerable controversy about the precise mechanism through which hyperglycemia could contribute to the development of arteriosclerosis in DM. Although a number of equally tenable hypotheses, including the advanced glycation end product hypothesis (5, 6) or the oxidative stress hypothesis (7–9), have been suggested, none of these are definitive because of

the complexity of the pathophysiology of DM. Additionally, in the process of arteriosclerosis, many factors are reported to be involved (10), including the presence of vasoactive substances such as angiotensin II (11), mechanical stretches (12), growth factors, and cytokines (13). Particularly, transforming growth factor- β (TGF- β) is an important factor (14, 15), and its action has been reported to involve oxidative stress (13, 16).

Recently, a series of bioactive prostaglandin (PG) F₂-like compounds, known as isoprostanes, has been discovered (17). These eicosanoids are produced from arachidonic acid through a nonenzymatic process of lipid peroxidation, catalyzed by oxygen free radicals either on cell membranes or on LDL particles (18, 19). Particularly, 8-iso-PGF_{2 α} is of im-

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portance in inducing vasoconstriction (20) and stimulating vascular smooth muscle cell proliferation (21, 22). Enhanced *in vivo* production of F₂-isoprostanes has been reported in several clinical conditions related to oxidant stress: aging, smoking (23, 24), coronary ischemia-reperfusion (25, 26), and DM. Although DM is a clearly established risk factor of cardiovascular diseases, the detailed mechanism responsible for accelerated arteriosclerosis remains to be elucidated. Numerous epidemiological studies have shown that dietary intake of vitamin E (α -tocopherol) is inversely correlated to the risk of cardiovascular disease (27). However, randomized intervention trials have failed to prove the cardiovascular benefit from the treatment using this anti-oxidative vitamin (28). On the other hand, troglitazone, a thiazolidinedione possessing the molecular structure of α -tocopherol, was developed as an antidiabetic drug to improve peripheral insulin sensitivity and hyperinsulinemia both in diabetic animal models and in patients with type 2 DM. In the present study, for the purpose of elucidating the mechanism of the progression of arteriosclerosis in DM, and focusing on the role of oxidant stress and insulin resistance, we investigate the role of TGF- β -mediating vascular complications and the effects of vitamin E and troglitazone in Otsuka Long-Evans Tokushima Fatty (OLETF) rats as an experimental model of type 2 DM (29).

Methods

Animals

Forty male OLETF rats were used as the experimental subjects. Forty male Long-Evans Tokushima Otsuka (LETO) rats, which originated from the same colony as OLETF rats by selective mating but did not develop DM, were used as control animals (Tokushima Research Inst. Otsuka Pharmaceutical, Tokushima, Japan). The starting age was 5 weeks. All animals were maintained in a specific pathogen-free facility under controlled temperature (23°C) and humidity (5%) with a 12-h artificial light and dark cycle, in the Animal Experiment Center of Kagawa Medical University. Animals were given free access to standard laboratory rat chow (MF, Oriental Yeast Co., Tokyo, Japan) and tap water. All procedures were in accordance with institutional guidelines for animal research.

Experimental Protocol

At 20 weeks of age, animals were randomly assigned to 3 experimental groups: those given a standard diet, those given a diet supplemented with troglitazone (0.2% w/w MF) (Sankyo, Tokyo, Japan), or those given a diet supplemented with vitamin E (α -tocopherol acetate, 1,000 IU/kg MF) as follows:

OLETF rats fed with standard chow
(untreated-OLETF, $n = 16$)

OLETF rats fed with chow containing troglitazone
(TR-treated-OLETF, $n = 12$)

OLETF rats fed with chow containing vitamin E
(VE-treated-OLETF, $n = 12$)

LETO rats fed with standard chow
(untreated-LETO, $n = 16$)

LETO rats fed with chow containing troglitazone
(TR-treated-LETO, $n = 12$)

LETO rats fed with chow containing vitamin E
(VE-treated-LETO, $n = 12$)

At 24 and 36 weeks of age, body weight, heart rate, and blood pressure (tail-cuff method: sphygmomanometer PS-100 Rick, Riken Kaihatsu, Tokyo, Japan) were measured. At the age of 24 weeks, half of each of the OLETF or LETO rat groups was anesthetized with sodium pentobarbital, and blood was sampled by aortic puncture in order to measure the plasma concentration of glucose (blood glucose test meter, MIWA chemical laboratory, Nagoya, Japan), total cholesterol (COD-POD method, HITACHI automatic analyzer 7050, HITACHI Corp., Tokyo, Japan), and triglyceride (GK-GPO method, HITACHI automatic analyzer 7050). At 36 weeks of age, the other half of the OLETF and LETO rat groups was anesthetized with sodium pentobarbital. Blood was sampled through thoracic aorta for measurement of 8-iso-PGF_{2 α} and biochemical parameters including plasma glucose and lipids. The thoracic aorta was excised, weighed, and separated into 3 pieces. One was snap frozen in liquid nitrogen, and stored at -80°C for measurement of the extraction of tissue 8-iso-PGF_{2 α} . The other pieces were used for histopathological examination and immunohistochemical analyses of TGF- β ₁ and TGF- β ₁ receptor II.

Plasma and Tissue 8-iso-PGF₂ Determination

Plasma (0.5 ml) was thawed and hydrolyzed with 1.5 ml of 15% KOH at 40°C for 1 h. Its pH was adjusted to 3.0 with hydrochloric acid. After a Sep-Pak C18 column (Waters Associates, Milford, USA) was preconditioned with 5 ml of methanol followed by 10 ml of pH 3.0 hydrochloric acid, acidified plasma was loaded into the column followed by consecutive washing with 10 ml of hydrochloric acid (pH 3.0) and 10 ml of heptanes. The sample was eluted onto 1 g of sodium sulfate in a glass test tube with a 10 ml mixture of heptane and ethyl acetate (1:1). The sample was then loaded into a Sep-Pak Silica column (Waters Associates) preconditioned with 5 ml of methanol followed by 10 ml of ethyl acetate eluted by a 10 ml mixture of methanol and ethyl acetate (1:1) after the column had been washed with 10 ml of ethyl acetate. The eluted sample was dried under nitrogen gas and reconstituted with assay buffer supplied in an 8-isoprostane enzyme immunoassay (Cayman Chemical Co., Ann Arbor, USA), in which the concentration of free 8-iso-PGF_{2 α} was evaluated (30).

For tissue 8-iso-PGF_{2 α} content, an excised piece of the thoracic aorta weighing about 0.1 g was put in 1 ml of ice-

Table 1. Body Weight, Hemodynamics and Serologic Findings during and after Treatment with Troglitazone or Vitamin E in OLETF and LETO Rats

Group	(n)	Body weight (g)	Mean BP (mmHg)	Heart rate (bpm)	T-cholesterol (mg/dl)	Triglyceride (mg/dl)	Blood glucose (mg/dl)
24 weeks							
Untreated-LETO	(8)	491±37	144±20	410±22	80±9	21±3	152±12
Untreated-OLETF	(8)	627±25 [†]	148±24	375±26	117±9 [†]	115±29 [†]	483±28 [†]
TR-treated-OLETF	(6)	625±31 [†]	139±16	380±32	113±13 [†]	72±24 ^{††}	310±60 [‡]
VE-treated-OLETF	(6)	639±17 [†]	134±26	358±37	99±19 ^{††}	113±16 ^{††}	277±56 [‡]
36 weeks							
Untreated-LETO	(8)	543±36	109±9	446±36	79±11	15±2	156±22
Untreated-OLETF	(8)	683±22 [†]	132±7 [†]	387±37	127±23 [†]	195±37 [†]	418±82 [†]
TR-treated-OLETF	(6)	686±37 [†]	138±6 [†]	405±37	86±25 [‡]	142±27 ^{††}	262±30 [‡]
VE-treated-OLETF	(6)	688±19 [†]	134±16 [†]	399±21	100±16 ^{††}	178±34 [†]	240±19 [‡]

Data are expressed as mean ± SD. BP, blood pressure; TR, troglitazone; VE, vitamin E. [†], $p < 0.01$ vs. age-matched untreated-LETO; [‡], $p < 0.01$ vs. age-matched untreated-OLETF; ^{††}, $p < 0.05$ vs. age-matched untreated-OLETF; ^{††}, $p < 0.05$ vs. age-matched TR-treated-OLETF.

cold phosphate-buffered saline containing 0.005% butylated hydroxytoluene as an antioxidant in order to further inhibit lipid oxidation during the extracting procedure. The tissue was homogenized for 30 s after the addition of 2 ml of ethanol. The homogenate was vortexed vigorously, left on ice for 15 min, and was centrifuged at $800 \times g$ for 10 min at 4°C. The upper aqueous layer was used for total 8-iso-PGF_{2α} in the same manner as described above. Total tissue 8-iso-PGF_{2α} content was normalized by dividing the value with the wet tissue weight of the excised piece of the thoracic aorta.

Histopathological Examinations

The excised aorta was immediately fixed in 10% phosphate-buffered formalin solution and embedded in paraffin. Four 4-μm-thick sections were cut in each sample and stained with hematoxylin and eosin staining. Hematoxylin- and eosin-stained light-microscopic images were recorded directly through a 3-CCD video camera (Victor, Tokyo, Japan). Cross-sectional vessel and lumen areas were measured by tracing the adventitia-media border and intima surface, respectively, on the images at $\times 20$ magnification. Medial area was calculated as (vessel area) - (lumen area), and the ratio of medial to vessel area was calculated as (medial area) $\times$ 100/(vessel area). The nuclear number of smooth muscle cells per visual field was counted on the image at $\times 400$ magnifications. The nuclear number of smooth muscle cells per cross-sectional area was calculated as (nuclear number per visual field area) $\times$ (medial area)/(visual field area) (31).

Immunohistochemical Analysis of TGF- β_1 and TGF- β_1 Receptor II

Immunohistochemical analyses of TGF- β_1 and TGF- β_1 receptor II were carried out by using anti TGF- β_1 antibody and anti-TGF- β_1 receptor II antibody (Santa Cruz Biotechnology

Inc., Santa Cruz, USA), respectively, as the first antibodies. The Aorta was fixed in methyl Carnoy's solution (60% methanol, 30% chloroform, and 10% acetic acid), embedded in paraffin, and cut into 4-μm-thick sections (4 sections for each staining in each rat). The sections were deparaffinized in xylene followed by a graded series of ethanol. Immunostaining was carried out by means of the streptavidin-biotin immunoperoxidase method (Histofine Simple-Stain POX kit, Nichirei Corp., Tokyo, Japan) and counterstained with hematoxylin. The expressions of TGF- β_1 and TGF- β_1 receptor II in aortic wall were semiquantitatively analyzed according to the scoring method (24) by two independent pathologists who were unaware of the experimental conditions. Scores were as follows: 0, absent or very weak staining; 1+, staining involving 1% to 25% of the area of the smooth muscle cells and the endothelial cells; 2+, staining involving 25% to 50%; 3+, staining involving 50% to 75%; 4+, staining involving > 75%.

Statistical Analysis

Data are expressed as means ± SD. Statistical analysis was performed using Scheffe's multiple comparison procedure for multiple comparison analysis and Student's unpaired *t*-test for non-multiple comparison analysis, respectively, following analysis of variance. $P < 0.05$ was judged as significant.

Results

Blood Pressure, Heart Rate, and Body and Heart Weight

The difference in systolic blood pressure or heart rate was significant but small at all ages. The body weights of OLETF rats was significantly larger than those of LETO rats. Be-

Fig. 1. The plasma and aorta concentration of 8-iso-PGF_{2α}, as determined by EIA in LETO, untreated-OLETF, and TR or VE-treated-OLETF rats. Data are expressed as mean $\pm$ SD.

Fig. 2. The medial area, ratio of medial area to vessel area, and nuclear number of smooth muscle cells/cross-sectional area at 36 weeks of age. Data are expressed as mean $\pm$ SD.

tween any 2 of these 3 groups (standard diet, supplemented with troglitazone or vitamin E), no significant differences in body weight were observed (Table 1).

Glucose and Lipid Metabolism

At 24 and 36 weeks of age, the blood glucose, plasma cholesterol, and triglyceride in the untreated-OLETF group were higher than those in the untreated-LETO group. Blood glucose and plasma triglyceride levels in the TR-treated-OLETF group were significantly lower than the levels in the untreated-OLETF group. In the VE-treated-OLETF group, blood glucose and plasma cholesterol levels were significantly lower than in the untreated-OLETF group. Plasma triglyceride showed no differences between VE-treated-OLETF and untreated-OLETF groups at 24 and 36 weeks of ages. Blood glucose at 24 weeks of age was not significantly different

from that at 36 weeks of age in each group (Table 1).

8-iso-PGF₂ Measurements

Plasma 8-iso-PGF_{2α} was not different between untreated-OLETF and untreated-LETO groups at 36 weeks (135 ± 74 pg/ml vs. 127 ± 24 pg/ml). Following the treatment with vitamin E, plasma 8-iso-PGF_{2α} was markedly reduced (VE-treated-LETO, 69 ± 14 pg/ml and VE-treated-OLETF, 80 ± 15 pg/ml, both $p < 0.01$), while there was no change following troglitazone treatment (TR-treated-LETO, 104 ± 20 pg/ml and TR-treated-OLETF, 144 ± 42 pg/ml). Treatment with either troglitazone or vitamin E markedly reduced tissue 8-iso-PGF_{2α} of the aorta (untreated-OLETF, $15,332 \pm 3,254$ pg/g tissue vs. TR-treated-OLETF, $7,092 \pm 1,992$ pg/g tissue or VE-treated-OLETF, $5,394 \pm 836$ pg/g tissue; $p < 0.01$) (Fig. 1).

Fig. 3. Light micrographs of immunohistochemical staining of TGF- β_1 expression of aortic wall from 36-week-old LETO and OLETF rats. Panel (a) shows untreated-LETO rat (score 2). Panel (b) shows untreated-OLETF rat (score 3). Panels (c) and (d) show TR and VE-treated-OLETF rats (both, score 1), respectively.

Fig. 4. Light micrographs of immunohistochemical staining of TGF- β_1 receptor II expression of aortic wall from 36-week-old LETO and OLETF rats. Panel (a) shows untreated-LETO rat (score 0). Panel (b) shows untreated-OLETF rat (score 3). Panels (c) and (d) show TR (score 2) and VE-treated-OLETF rats (score 1), respectively.

Histopathological Examination

At 36 weeks of age, the medial area of untreated-OLETF was larger than that of untreated-LETO ($1.22 \pm 0.04 \text{ mm}^2$ vs. $0.54 \pm 0.08 \text{ mm}^2$, $p < 0.01$). In OLETF rats, the medial area was significantly reduced following the treatment with either troglitazone or vitamin E, and more in TR-treated-OLETF

than in VE-treated-OLETF. The ratio of the medial area to vessel area showed a similar tendency to that seen in the results of the medial area. The nuclear numbers of smooth muscle cells per cross-sectional area in the treated groups (TR-treated-OLETF, $2,697 \pm 292$ and VE-treated-OLETF, $1,881 \pm 337$) were smaller than that in untreated group (untreated-OLETF, $3,728 \pm 456$, $p < 0.01$). In particular, the

Table 2. Aortic Wall Staining Score for TGF- β_1 and TGF- β_1 -receptor II at 36 Weeks

Group	(n)	TGF- β_1	TGF- β_1 -receptor II
Untreated-LETO	(8)	1.88 $\pm$ 0.99	1.13 $\pm$ 0.64
Untreated-OLETF	(8)	2.38 $\pm$ 1.06	2.25 $\pm$ 0.70 [†]
TR-treated-OLETF	(6)	1.50 $\pm$ 1.04	2.16 $\pm$ 0.75 [†]
VE-treated-OLETF	(6)	1.20 $\pm$ 0.83 [‡]	1.80 $\pm$ 0.83 [†]

Data are expressed as mean $\pm$ SD. BP, blood pressure; TR, troglitazone; VE, vitamin E. [†], $p < 0.05$ vs. age-matched untreated-LETO; [‡], $p < 0.05$ vs. age-matched untreated-OLETF.

number in VE-treated-OLETF was significantly smaller than that in TR-treated-OLETF (Fig. 2).

The immunohistochemical stainings of TGF- β_1 (Fig. 3) and TGF- β_1 receptor II (Fig. 4) were found much more abundantly in the endothelium and medial smooth muscle cells of the aorta in OLETF rats than in LETO rats. The immunohistochemical staining score of TGF- β_1 was decreased in the treated groups, but the expression of TGF- β_1 receptor II was not changed (Table 2).

Discussion

On the basis of oral glucose tolerance testing in our previous study (32), the OLETF rats were prediabetic, and insulin resistance occurred at 10 to 20 weeks of age. At 30 weeks of age, the OLETF rats showed minor abnormalities in fasting blood glucose level (112 $\pm$ 12 mg/dl), and elevation of 2-h blood glucose (294 $\pm$ 41 mg/dl) and insulin (7,967 $\pm$ 1,436 pg/ml) levels, suggesting that the OLETF rats were in the stage of impaired glucose tolerance with postprandial hyperglycemia. In the present study, the casual blood glucose levels at 24 and 36 weeks of age were similarly elevated. These data regarding glucose level suggested that the OLETF rats in the present study were also in the stage of impaired glucose tolerance at 36 weeks of age. In this stage, impairment of lipid metabolism was simultaneously apparent (32). With regard to lipid and glucose metabolism, vitamin E also improved these parameters to a degree similar to that observed in the troglitazone group. Kaneto H *et al.* (33) demonstrated in a type 2 diabetes mice model that apoptosis induced by oxidative stress causes reduction of β -cell mass, and that antioxidant treatment (*N*-acetyl-L-cysteine) suppressed apoptosis in β -cells. Although vitamins C and E were not effective in their experiment, the usefulness of antioxidants in the treatment of type 2 diabetes has been suggested. Consequently, the exact reason for the improvement of lipid and glucose metabolism in the present experiment remains unknown, but it could have been due to the preservation of β -cell function or the amelioration of insulin resistance through anti-oxidation by vitamin E.

Following the vitamin E and troglitazone treatments, the

nuclear number of smooth muscle cells decreased. Vascular smooth muscle cells have been found to have insulin receptors and to exhibit insulin-induced responses (34), and Law RE *et al.* (35) have demonstrated that the proliferation and migration of cultured smooth muscle cells are inhibited by troglitazone in an arterial balloon injury model in rats. In addition to insulin-induced responses, Rao GN and Berk BC (36), and Kyaw M *et al.* (37) have shown that vascular smooth muscle cell proliferation is strongly related to active oxygen species production. Our *in vivo* study also demonstrated that the potent anti-oxidant activity of vitamin E sufficiently prevented cell proliferation, suggesting that oxidative stress was relatively important as a mechanism of cell proliferation. Troglitazone is not only an insulin-sensitizing compound, but also is a potent anti-oxidant due to a structural similarity to vitamin E, and therefore, both the hyperinsulinemia and oxidative stress implicated in the development of type 2 DM are considered the cause of smooth muscle cell proliferation.

In the previous study, collagen was significantly accumulated in the aortic media of OLETF rats (31). Although we did not measure the concentration of hydroxyproline, an indicator of collagen, collagen accumulation may have also contributed to the increase in medial wall thickness. Gene expression of the TGF- β_1 or enhanced gene expression of collagen might participate in the onset of tissue fibrosis by stimulating extracellular matrix synthesis. In the present study, an expression of TGF- β_1 and an increase in aortic wall thickness were simultaneously observed. Additionally, a potent anti-oxidant activity decreased the expression of TGF- β_1 , which may have decreased the stimulation of extracellular collagen synthesis. On the other hand, 8-iso-PGF $_{2\alpha}$ is formed during the reactive oxygen species-dependent peroxidation of lipid-esterified arachidonic acid, and its increase accurately indicates oxidative stress. Montero A *et al.* (38) measured the plasma and urine concentrations of 8-iso-PGF $_{2\alpha}$ and TGF- β_1 expression in glomeruli in streptozotocine-induced diabetic rats, and their results clearly indicate the relationship between 8-iso-PGF $_{2\alpha}$ production and TGF- β_1 expression *in vivo* despite its expression in mesangial cells. Consequently, our data may demonstrate that the production of 8-iso-PGF $_{2\alpha}$ due to oxidative stress induced the expression of TGF- β_1 and medial fibrosis of the aorta in DM rats. Both TGF- β_1 and TGF- β_1 receptor expression were higher in diabetic rats than in our nondiabetic control animals. TGF- β_1 expression was suppressed by either troglitazone or vitamin E, while TGF- β_1 receptor expression was affected by neither of these two agents. This inhibition of TGF- β_1 expression paralleled the suppression of medial proliferation by these two agents, suggesting that the regulation of TGF- β_1 expression may contribute to smooth muscle cell proliferation.

Natarajan R *et al.* (39) have studied the formation of 8-iso-PGF $_{2\alpha}$ in porcine vascular smooth muscle cells cultured under hyperglycemic conditions, and suggested that 8-epi-PGF $_{2\alpha}$ may lead to increased vascular smooth muscle cell

growth. Indeed, our *in vivo* study demonstrates the increase in the nuclear number of vascular smooth muscle cells following an augmentation of 8-iso-PGF_{2α} production. The direct addition of 8-iso-PGF_{2α} to vascular smooth muscle cells for 24 h leads to an increased formation of the matrix protein fibronectin, which is known to play a causative role in vascular hypertrophy and neointima formation (hypertrophic and hyperplastic effects). In our *in vivo* study, the concentration of 8-iso-PGF_{2α} in aortic tissue was reduced following treatment with both vitamin E and troglitazone, but the reduction in plasma 8-iso-PGF_{2α} was significantly observed only after the administration of vitamin E. Although the actual reason for this discrepancy is unknown, a decrease in 8-iso-PGF_{2α} and a reduction of medial proliferation due to anti-oxidation were accurately demonstrated. Therefore, it is likely that the 8-iso-PGF_{2α} directly stimulated vascular smooth muscle cell proliferation and further participates in the progression of arterial wall remodeling and arteriosclerosis.

In summary, our results demonstrate that antioxidant treatment as well as correction of insulin sensitivity improved histopathological changes of the aorta in type 2 DM rats. This improvement was associated with a decrease in plasma and tissue levels of 8-iso-PGF_{2α}, a lipid peroxidation product of arachidonic acid metabolism, suggesting its contribution to arteriosclerosis by initiating the pathogenic process in the vascular system. Our findings provide a basis for understanding the mechanisms of the effects of antioxidants on arteriosclerosis, and may promise significant clinical benefits of antioxidant treatment in the early diabetic stage.

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