Pyrrolidine Dithiocarbamate Reduces Vascular Prostanoid-Induced Responses in Aged Type 2 Diabetic Rat Model

Takayuki Matsumoto¹, Keiko Ishida¹, Tsuneo Kobayashi¹, and Katsuo Kamata¹,*

¹Department of Physiology and Morphology, Institute of Medicinal Chemistry, Hoshi University, Shinagawa-ku, Tokyo 142-8501, Japan

Received April 9, 2009; Accepted May 13, 2009

Abstract. It has been shown that enhancement of vasoconstrictor prostanoids plays an important role in the development of cardiovascular diseases. The aim of the present study was to examine the effects of pyrrolidine dithiocarbamate (PDTC), a low-molecular-weight thiol antioxidant and a potent inhibitor of nuclear factor-κB (NF-κB), on both the response to and production of prostanoids in arterial vessels isolated from rats at the chronic stage of type 2 diabetes. Using aortas from type 2 diabetic Otsuka Long-Evans Tokushima Fatty (OLETF) rats, control Long-Evans Tokushima Otsuka (LETO) rats, and LETO and OLETF rats treated with PDTC (30 mg/kg, s.c., daily, for 1 week), we measured the production of prostanoids and NF-κB activity. The arachidonic acid–induced contraction and the acetylcholine-induced endothelium-derived contracting factor (EDCF)-mediated contraction in mesenteric arteries were also compared among these groups. OLETF rats exhibited (vs. age-matched LETO rats) the following: increased responses to both arachidonic acid and EDCF and greater productions of PGE₂ and TXA₂. Treatment with PDTC resulted in the following: 1) reduced arachidonic acid– and EDCF-mediated contractions, 2) suppressed the production of prostanoids, and 3) normalized NF-κB activity. These results suggest that PDTC has beneficial effects against the abnormal vasoconstrictor prostanoid signaling present in rats at the chronic stage of type 2 diabetes.

Keywords: diabetes, endothelium-derived contracting factor (EDCF), nuclear factor-κB (NF-κB), pyrrolidine dithiocarbamate (PDTC), prostanoid

Introduction

Vascular complications are responsible for the excess mortality associated with diabetes (1 – 3), and endothelial dysfunction has been described both in human diabetics and in animal models of the disease (3 – 10). Among the variety of proposed mechanisms, an abnormality of prostanoid signaling has been implicated in the pathogenesis of diabetic complications (11 – 13). Vasoconstrictor prostanoids include endothelium-derived contracting factor (EDCF) (14); and to judge from animal experiments, EDCF-mediated responses are exacerbated by aging, hypertension, and diabetes (14, 15).

Pyrrolidine dithiocarbamate (PDTC) is a low-molecular-weight thiol compound (16) with a variety of biochemical activities such as redox state alternation (17), heavy metal chelation (18), and enzyme inhibition (19). PDTC was initially regarded as a potent inhibitor of nuclear factor-κB (NF-κB) (17), and it has been used as an antioxidant compound both to counteract the toxic effects of free radicals (20) and to interfere with the generation of pro-inflammatory cytokines (21). Although the protective effects of PDTC have been attributed to its ability to inhibit NF-κB (22), it also has the potential to increase the expressions of genes for endogenous antioxidants, such as superoxide dismutase (23), independently of any effects on NF-κB. Although there is an accumulating body of evidence to show that PDTC has beneficial effects on several vascular diseases (20, 24), no study has yet investigated its effects on vascular function in a type 2 diabetic model.

There are several reports of abnormalities of vascular function in type 2 diabetic Otsuka Long-Evans Tokushima Fatty (OLETF) rats (25 – 29). We recently
demonstrated (30) the following: 1) that endothelial dysfunction is present in the mesenteric arteries of aged OLETF rats, 2) that this may result from an imbalance between endothelium-derived factors [reduced relaxing factor (EDRF) signaling and increased EDCF signaling], and 3) that the mechanisms underlying this abnormality may involve increments in the activities of cyclooxygenases (COXs). Moreover, we very recently reported that eicosapentaenoic acid improves endothelial dysfunction in OLETF rats by correcting the imbalance between endothelium-derived factors (i.e., reduced EDCF signaling and increased EDRF signaling), at least partly, by decreasing NF-κB activation (31). We therefore proposed important roles for vasoconstrictor prostanoids in the altered regulation of mesenteric arterial responsiveness seen in OLETF rats at the established chronic stage of diabetes (29 – 32).

The aims of the present study, on arterial vessels isolated from OLETF rats at the chronic stage of type 2 diabetes, were to assess the effects of a 1-week treatment of such rats with PDTC on i) prostanoid production and ii) the responses to prostanoids.

Materials and Methods

Reagents
Arachidonic acid, \( N^\alpha \)-nitro-L-arginine (L-NNA), PDTC, and tumor necrosis factor-\( \alpha \) (TNF-\( \alpha \)) were all purchased from Sigma Chemical Co. (St. Louis, MO, USA). Acetylcholine chloride was purchased from Daiichi-Sankyo Pharmaceuticals (Tokyo). Drugs were dissolved in saline.

Animals and experimental design
Five-week-old male rats [OLETF rats and Long-Evans Tokushima Otsuka (LETO) rats, a genetic control for OLETF] were supplied by the Tokushima Research Institute (Otsuka Pharmaceutical, Tokushima). Food and water were given ad libitum in a controlled environment (room temperature 21°C – 22°C, room humidity 50 ± 5%) until the rats were 65 – 70-week-old. Some OLETF and LETO rats were given PDTC (30 mg/kg, s.c., daily) for 1 week starting at 65 – 69 weeks of age. Thus, we studied four groups: PDTC-untreated LETO and OLETF groups and PDTC-treated LETO and OLETF groups. This study was approved by the Hoshi University Animal Care and Use Committee, and all studies were conducted in accordance with the “Guide for the Care and Use of Laboratory Animals” published by the US National Institutes of Health, and “Guide for the Care and Use of Laboratory Animals” adopted by the Committee on the Care and Use of Laboratory Animals of Hoshi University (which is accredited by the Ministry of Education, Culture, Sports, Science, and Technology, Japan).

Measurement of blood glucose and blood pressure
Plasma glucose and systemic blood pressure were measured, as described previously (10, 31), by the use of a commercially available enzyme kit (Wako Chemical Company, Osaka). After a given rat had been in a constant-temperature box at 37°C for a few minutes, its blood pressure was measured by the tail-cuff method using a blood pressure analyzer (BP-98A; Softron, Tokyo).

Measurement of isometric force
Vascular isometric force was recorded as in our previous papers (27, 29 – 33). At 65 – 70 weeks of age, rats were anesthetized with diethyl ether and then euthanized by decapitation. The superior mesenteric artery was rapidly removed and immersed in oxygenated, modified Krebs-Henseleit solution (KHS). This solution consisted of 118.0 mM NaCl, 4.7 mM KCl, 25.0 mM NaHCO\(_3\), 1.8 mM CaCl\(_2\), 1.2 mM Na\(_2\)PO\(_4\), 1.2 mM MgSO\(_4\), and 11.0 mM dextrose. The artery was carefully cleaned of all fat and connective tissue, and ring segments (2 mm in length) were suspended by a pair of stainless-steel pins in a well-oxygenated (95% \( O\_2 \) – 5% \( CO\_2 \)) bath containing 10 mL of KHS at 37°C. The rings were stretched until an optimal resting tension of 1.0 g was loaded and then allowed to equilibrate for at least 60 min. Force generation was monitored by means of an isometric transducer (model TB-611T; Nihon Kohden, Tokyo).

For the contraction studies, mesenteric rings were first contracted using 80 mM K\(_+\), these responses being taken as 100%. There was no significant difference in the response to 80 mM K\(_+\) among the LETO (n = 9), OLETF (n = 9), PDTC-treated LETO (n = 9), and PDTC-treated OLETF (n = 9) groups (1.60 ± 0.05, 1.63 ± 0.06, 1.76 ± 0.05, and 1.77 ± 0.04 g, respectively). To investigate the arachidonic acid–mediated response and EDCF-mediated response, mesenteric rings were treated with 100 \( \mu \)M L-NNA for 30 min. After this incubation period, arachidonic acid (100 nM – 10 \( \mu \)M), or acetylcholine (10 nM – 10 \( \mu \)M) was cumulatively applied. After the addition of sufficient aliquots of the agonist to produce the chosen concentration, a plateau response was allowed to develop before the addition of the next dose of the same agonist.

Release of prostanoids
Prostanoid release was measured as in our previous papers (29 – 32). For this purpose, we used aortas because in our preliminary experiment, the acetyl-
choline-induced EDCF–mediated contraction and the arachidonic acid–induced contraction were significantly increased in the aorta from chronic stage (i.e., 36-week-old) OLETF rats compared to those from age-matched LETO rats (data not shown); this was also true in the mesenteric arteries (31). To allow us to measure the release of prostanoids, aortas from all four groups were cut into transverse rings, 8 mm in length. These were placed for 30 min in siliconized tubes containing 0.5 ml KHS at 37°C, and then 10 μM acetylcholine was applied for 15 min. Next, after the aortic rings had been removed, the tubes were freeze-clamped in liquid nitrogen and stored at −80°C for later analysis. The prostaglandins (PGs) were measured using a commercially available EIA kit (Cayman Chemical, Ann Arbor, MI, USA). Two-fold diluted samples were used for measurements of PGE₂ and thromboxane B₂ (TXB₂) (a stable metabolite of TXA₃). The various assays were performed as described in the manufacturer’s procedure booklet. The amounts of prostaglandins released are expressed as pg/mg wet weight of aorta.

**Trans-AM NF-κB transactivation factor assay**

NF-κB activity was measured as described previously (31). Briefly, aortas from all four groups were cut into transverse rings, 8 mm in length. These were placed for 30 min in siliconized tubes containing 0.5 ml KHS at 37°C, and then 1 ng/ml TNF-α was applied for 30 min. Next, the aortic rings were removed, frozen in liquid nitrogen, and physically crushed to a fine powder in liquid nitrogen using a Cryo-Press (Microtech Nichion, Chiba). Nuclear protein extracts were then isolated from these aortic tissues, a Nuclear Extract Kit (Active Motif, Carlsbad, CA, USA) being used according to the manufacturer’s instructions. Aliquots of nuclear protein were stored at −80°C. Finally, NF-κB activity was assayed using Active Motif’s (Carlsbad, CA, USA) ELISA-based transactivation Trans-AM kit (according to the manufacturer’s protocol). The NF-κB Trans-AM kit contains a 96-well plate with immobilized oligonucleotides encoding an NF-κB consensus site (5'-GGGACTTTCC-3'). The active form of NF-κB contained in a nuclear extract of aortic tissue specifically binds to this oligonucleotide. The primary antibody used to detect NF-κB recognizes an epitope on p65 that is accessible only when NF-κB is activated and bound to its target DNA. An HRP-conjugated secondary antibody provides a sensitive colorimetric readout easily quantified by spectrophotometry.

**Measurement of nitrite (NO⁻) and nitrate (NO₃⁻)**

The concentrations of nitrite and nitrate in the plasma were measured by the method described previously (30, 31). For the determination of plasma NO metabolites, 0.3 mL of 100% methanol was added to 0.3 mL of each plasma sample, and the sample was then centrifuged at 5000 × g for 10 min at 4°C. The concentrations of nitrite and nitrate in plasma were measured using an automated NO detector/high-performance liquid chromatography system (ENO20; Eicom, Kyoto).

**Statistical analyses**

Data are expressed as means ± S.E.M. Each contractile response was expressed as a percentage of the response to 80 mM KCl. When appropriate, statistical differences were assessed by Dunnett’s test for multiple comparisons after a one-way analysis of variance (ANOVA), a probability level of P<0.05 being regarded as significant. Statistical comparisons between concentration–response curves were made using a two-way ANOVA, with Bonferroni’s correction for multiple comparisons being performed post hoc (P<0.05 again being considered significant).

**Results**

**General parameters**

At the time of the experiment, all OLETF rats (non-fasted) exhibited hyperglycemia, their blood glucose concentrations (477.7 ± 34.3 mg/dl, n = 8) being significantly higher than those of the age-matched nondiabetic control LETO rats (also non-fasted) (148.5 ± 4.5 mg/dl, n = 8; P<0.001). Plasma glucose was not altered in either the LETO (147.5 ± 3.5 mg/dl, n = 8) or OLETF (495.8 ± 60.4 mg/dl, n = 8) rats treated with PDTC. Body weight was not significantly different among the four groups: LETO (595.6 ± 8.5 g, n = 8), OLETF (570.3 ± 47.8 g, n = 8), PDTC-treated LETO (582.0 ± 16.6 g, n = 8), and PDTC-treated OLETF (488.9 ± 11.6 g, n = 8). The systolic blood pressure of OLETF rats (146.9 ± 2.8 mmHg, n = 8) was greater than that of LETO rats (117.5 ± 2.8 mmHg, n = 8; P<0.001), while heart rate was similar between the OLETF group (400.2 ± 10.3 beats/min, n = 8) and the LETO group (421.1 ± 17.1 beats/min, n = 8). The systolic blood pressure and the heart rate were not altered in either the LETO (121.0 ± 3.4 mmHg, 423.2 ± 9.7 beats/min, n = 8) or OLETF (150.9 ± 2.5 mmHg, 411.8 ± 9.8 beats/min, n = 8) rats treated with PDTC.

**Effects of PDTC on arachidonic acid–induced and endothelium-mediated contractions in OLETF rats**

We previously demonstrated (29–32) that EDCF signaling was increased in mesenteric arteries from OLETF rats at the chronic stage of diabetes. When we added arachidonic acid (100 nM – 10 μM) cumulatively
to rings in the presence of 1-NNA (100 μM), a contractile response was seen in each group at higher concentrations of arachidonic acid (Fig. 1). This contraction was significantly greater in rings from OLETF rats than in those from LETO rats (Fig. 1) and was completely blocked by indomethacin, an inhibitor of cyclooxygenase (data not shown). Surprisingly, mesenteric rings from OLETF rats treated for 1 week with PDTC showed a normalized response (i.e., it was not different from that in the LETO group) (Fig. 1). Next, to investigate the effect of PDTC on the EDCF-mediated response, we added acetylcholine (10 nM – 10 μM) cumulatively to rings in the presence of 1-NNA (100 μM). As shown in Fig. 2, under these conditions an acetylcholine-induced contraction was observed at higher acetylcholine concentrations (i.e., 0.3 – 10 μM) in rings from all four groups. This acetylcholine-induced contractile response was i) significantly greater in mesenteric arteries from OLETF rats than in those from LETO rats (Fig. 2) and ii) significantly weaker in the PDTC-treated OLETF group than in the untreated OLETF group (Fig. 2). As reported previously, these ACh-induced contractions were completely blocked by endothelium denudation (30) or by indomethacin treatment (30).

**Effects of PDTC on production of prostanoids in OLETF rats**

In view of the published evidence that overproduction of prostanoids contributes to vascular dysfunction in diabetic arteries (12, 30, 34), we examined the effects of one-week PDTC treatment on the endothelium-stimulated release of prostanoids in aortas from all four groups (Fig. 3). Acetylcholine (10 μM) evoked release of both TXB₂ (stable metabolite of TXA₂) (Fig. 3A) and PGE₂ (Fig. 3B) in aortic rings from all four groups. Both effects were significantly greater in rings from the OLETF group than in those from the LETO group. Compared to those in the OLETF group, the productions of TXB₂ and PGE₂ were significantly suppressed in the PDTC-treated OLETF group (Fig. 3).

**Effects of PDTC on aortic NF-κB activity in OLETF rats**

It is known that in diabetes, activation of NF-κB in the vasculature plays an important role in the development of endothelial dysfunction (35). Nuclear extracts prepared from aortic tissue were subjected to ELISA using a Trans-AM kit (Fig. 4). NF-κB activity tended to be greater in the OLETF group than in the LETO group (although statistical significance was not reached) (Fig. 4). Compared to that in the OLET group, this activity was significantly reduced in the PDTC-treated OLETF group (becoming similar to the levels seen in the LETO groups) (Fig. 4).
Determination of NO synthesis as plasma nitrite and nitrate

Since the plasma level of nitrite (an oxidation product of NO) correlates with the level of NO biosynthesis (36) and since the nitrate/nitrite ratio is often used as an indirect marker of oxidative stress (37), we measured the levels of nitrite and nitrate in rat plasma as a measure of NO synthesis. The plasma nitrite level tended to be lower in OLETF than in LETO (Fig. 5A), but the plasma nitrate level (Fig. 5B) and nitrate/nitrite ratio (Fig. 5C) was significantly greater in OLETF than in LETO. Compared with these levels in the OLETF group, the nitrite level (Fig. 5A) tended to be higher and the nitrate/nitrite ratio (Fig. 5C) was significantly lower in the PDTC-treated group.

Discussion

The main conclusion to be drawn from the present study is that in aged OLETF rats, one-week treatment with PDTC suppresses signaling by vascular prostanoids.

OLETF rats manifest stable clinical and pathological features that resemble human type 2 diabetes (38). Briefly, OLETF rats are characterized by 1) increasing body weight just after weaning; 2) a late onset of hyperglycemia (after 18 weeks of age) and diagnosable diabetes after 24 weeks of age; 3) a hyperinsulinemia that is present at 24 weeks of age and declines after 55 weeks of age, and conversion to insulin-dependent diabetes after approximately 40 weeks of age; and 4) increasing plasma cholesterol and triglyceride concentrations after 21 weeks of age (38). Moreover, we and others have demonstrated that abnormalities of vascular function are present in several arteries in OLETF rats (25–28, 39), and there is recent evidence of an imbalance between endothelium-derived factors (viz. decreased EDRFs signaling and increased EDCFs signaling) in mesenteric arteries isolated from OLETF rats at the chronic stage of diabetes (29–32). In the present study, aged OLETF rats (i.e., 65–70 weeks of age), who are at the insulin-dependent stage of diabetes, were used to investigate signaling by vasoconstrictor prostanoids, which includes EDCF-mediated signaling. The reason for choosing such rats was as follows. Although various studies conducted on animal models of cardiovascular diseases have demonstrated prevention of disease when treatment is initiated early [viz. prior to the onset of complications (21, 40, 41)], treatment of human diabetes does not normally begin until after the symptoms have become evident and in many instances, when complications are already present. We therefore used aged OLETF rats (a) because long-term diabetic conditions entail severe diabetic complications associated with cardiovascular dysfunction and (b) because no previous study has investigated the therapeutic, not preventive, effect of PDTC on diabetic vasculopathy (in
particular, on vasoconstrictor prostanoid signaling). In the present study, we found that in such aged OLETF rats, one-week treatment with PDTC 1) reduced arachidonic acid- and EDCF-mediated contractions in mesenteric arteries, 2) suppressed the aortic productions of PGE$_2$ and TXA$_2$, and 3) normalized aortic NF-$\kappa$B activity.

Recently, the possibility of a key role for low-grade vascular inflammation in the development of diabetic vascular complications has received considerable attention (42). Among other factors, PGs are important mediators in several inflammatory mechanisms (43). However, it is also known that many PG derivatives have specific vasoactive properties, thereby contributing to the local regulation of arteriolar diameter (12, 44). Moreover, there are several reports suggesting that an interrelationship between oxidative stress and PG-mediated regulation of vascular tone may be present in diabetic states (13, 34, 45). NF-$\kappa$B activation plays an important role in diabetes-associated inflammation and is strongly stimulated by reactive oxygen species (22, 35, 46, 47). Therefore, maneuvers that reduce oxidative stress and/or prevent activation of NF-$\kappa$B might be capable of improving diabetic vasculopathy. Of interest in this regard are the dithiocarbamates, which are antioxidants and potent inhibitors of NF-$\kappa$B. Among them, the most effective NF-$\kappa$B inhibitor is the pyrrolidine derivative of dithiocarbamate (PDTC) as a result of its ability to traverse the cell membrane and its prolonged stability at a physiological pH (48). The possibility of modulating both cell activation and the effects of oxidants by the use of PDTC suggests that such an agent might offer therapeutic benefits in conditions in which activation of NF-$\kappa$B plays a major role. In the present study, we found that NF-$\kappa$B activity in aortas tended to be greater in the OLET group than in the LETO group, and this activity was significantly reduced in aortas from PDTC-treated OLETF rats (vs. those from untreated OLETF rats). NF-$\kappa$B controls, at least in part, the expression of COX-2 (21, 49). Importantly, COX-2 activity has been found to contribute to the augmented vasoconstriction seen in vessels from several diabetic models (12, 13). Moreover, in a very recent study we found that chronic treatment with eicosapentaenoic acid ameliorated endothelial dysfunction in OLETF rats at least partly by decreasing NF-$\kappa$B activation and reducing COX-2 expression, leading to a correction of the imbalance between endothelium-derived factors (viz. normalized endothelium-derived relaxing factor signaling and decreased EDCF signaling) (31). Therefore, we speculate that the observed suppressive effect of PDTC on vasoconstrictor prostanoids in OLETF rats might be at least in part attributable to an induced decrease in NF-$\kappa$B activity.

Another possibility for the mechanism underlying the beneficial suppressive effect of PDTC on vasoconstrictor prostanoid production is related to its effect on oxidative stress. There are several reports suggesting 1) that the beneficial effects of PDTC may result from the ability of this agent to interfere with reactive oxygen species (50); 2) that in particular, inhibitions of reactive oxygen intermediates and superoxide anions are possible factors in the protective effect of PDTC (51); and 3) that PDTC has the potential to increase the expressions of genes for endogenous antioxidants such as superoxide dismutase (23). Indeed, our present study found that the increased plasma nitrate/nitrite ratio [often used as an indirect marker of oxidative stress (37)] in OLETF rats was significantly reduced by PDTC treatment. Therefore, we speculate that the suppressive effect of PDTC on vasoconstrictor prostanoid signaling may be partly attributable to modulation mediated by its antioxidant activity since oxidative stress can enhance vasoconstrictor prostanoid signaling (13, 15, 34, 45). However, it remains unclear to what extent individual PDTC-induced beneficial effects (viz. its NF-$\kappa$B activity–suppressing effect, antioxidant effect, and other as yet unknown effects) might contribute to the suppression of vasoconstrictor prostanoid signaling. Further research will therefore be required on this point.

Hypertension is one of the major risk factors for cardiovascular diseases, and it is closely related to type 2 diabetes (52). The literature contains several reports on the relationship between vasoconstrictor prostanoids and blood pressure (12, 14). On the other hand, there several reports have suggested that the treatment with PDTC led to decreased blood pressure in spontaneous hypertensive rats (40, 53). In the present study, we set out to investigate the therapeutic, rather than the preventive, effect of PDTC on the abnormal prostanoid signaling, and to this end, we administered PDTC for 1 week to established diabetic OLETF rats (which exhibited hypertension). Within the time scale studied here, PDTC did not affect blood pressure, even though it suppressed the enhanced vasoconstrictor prostanoids that are related to the regulation of blood pressure (14, 15). Possibly, if OLETF rats were treated with PDTC for a longer time, or if treatment were started at an earlier stage, this drug might reduce blood pressure. To establish if it has such a beneficial effect, future research will need to focus, for example, on time-course changes in blood pressure and in vasoconstrictor prostanoid responses in animals in a diabetic state.

We conclude that PDTC has beneficial effects against the enhancement of vascular prostanoid signalings that exists in the chronic stage of type 2 diabetes. The
mechanism of action of PDTC may involve a reduction in NF-κB activity. We speculate that PDTC, or compounds with similar mechanisms of action, may prove useful against the augmented vasoconstrictor prostanooid signaling seen in disease states such as hypertension and diabetes. Our finding that a significant attenuation of vasoconstrictor prostanooid signaling can be achieved by one-week administration of PDTC should stimulate further research aimed at (a) a better understanding of the specific mechanisms responsible for the normalization by PDTC of the endothelial dysfunction associated with diabetes and (b) an elucidation of the potential roles of this agent in clinical settings.

Acknowledgments

We thank Otsuka Pharmaceutical for providing LETO and OLETF rats. This study was supported in part by the Ministry of Education, Culture, Sports, Science, and Technology, Japan and by the Open Research Center Project.

References

27 Matsumoto T, Kobayashi T, Kamata K. Mechanisms underlying the impaired EDHF-type relaxation response in mesenteric


