Toll-Like Receptor 4 Inhibitor TAK-242 Augments Acetylcholine-Induced Relaxation in Superior Mesenteric Arteries of the Streptozotocin-Induced Diabetic Rat

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Received April 7, 2020; accepted May 15, 2020

Although vascular dysfunction is a key event in the development of diabetic complications, and abnormal toll-like receptor 4 (TLR4) may contribute to the pathophysiology of vascular diseases, the direct relationships between TLR4 and vascular function in diabetic arteries are still poorly understood. Thus, to investigate whether pharmacological blockade of TLR4 affects vascular function in the superior mesenteric artery (SMA) of streptozotocin (STZ)-induced diabetic rats, the SMA was isolated from male Wistar rat injected once with STZ (65 mg/kg, 27–34 weeks) which was treated with TAK-242 (10⁻⁵M), a TLR4 inhibitor, for approximately 1 d using organ culture techniques. After incubation, functional and biochemical studies were performed. In the functional study, treatment with TAK-242 increased acetylcholine (Ach)-induced relaxation of the diabetic SMA in the intact condition. Sodium nitroprusside (SNP)-induced relaxation was also increased in the TAK-242-treated group compared with the vehicle-treated group. Under cyclooxygenase (COX) blockade by indomethacin (10⁻⁵M), Ach-induced relaxation was similar in the vehicle- and TAK-242-treated groups. In addition, Ach-induced relaxation in the combined presence of the nitric oxide (NO) synthase inhibitor, N°C-nitro-L-arginine (L-NNA) (10⁻⁴M), and indomethacin (10⁻⁵M) was similar in the vehicle- and TAK-242-treated groups. The productions of thromboxane (TX) B₂ in cultured medium in the presence of ACh (10⁻⁵M) were lower in the TAK-242-treated group than in the vehicle-treated group. These data suggested that TAK-242 could augment endothelium-dependent relaxation by partly suppressing vasoconstrictor TXA₂, or increasing NO signaling. TLR4 inhibition may be a novel therapeutic strategy to assist in the management of diabetes-associated vascular complications.

Key words relaxation; streptozotocin; superior mesenteric artery; toll-like receptor 4; thromboxane

INTRODUCTION

Long-term diabetes leads to vascular dysfunction, including endothelial and smooth muscle dysfunction. The mechanisms underlying diabetes-associated vascular complications are multifactorial and complex. Therefore, understandings on the molecular basis of the initiation and development of vascular dysfunction in diabetes is an urgent issue for the management of diabetes-associated systematic complications.

Activation of vascular toll-like receptors (TLRs) is related to the development of vascular complications observed in hypertension and diabetes. Among TLRs, for example, TLR4 activation causes vascular dysfunction in hypertension, and blockade of TLR4 can decrease inflammation and oxidative stress in the cardiovascular systems, thereby blunting vascular abnormalities and reducing organ damage. In addition, high glucose leads to inflammation, production of reactive oxygen species (ROS), and nuclear factor-kappa B (NF-kB) activation via TLR4 signaling in endothelial and smooth muscle cells. Inhibition of TLR4 signaling suppressed mesenteric contraction in the diabetic rat. Although the regulation of TLR4 signaling may play a key role in controlling diabetes-associated vascular abnormalities, the relationship between TLR4 and the relaxation response remains unclear.

Several animal models have been used to investigate the pathophysiology of diabetes. The streptozotocin (STZ)-induced diabetic model is one of the classical major models. Although there are many reports suggesting alterations of vascular functions in STZ-diabetic models, responsiveness to endogenous substances [e.g., acetylcholine (ACh)] in vessels varies depending on the species, vessel types, duration of disease, and sex. For instance, ACh-mediated relaxation was impaired in the STZ-diabetic rat aorta, and mesenteric artery, and mouse aorta. ACh-induced endothelium-derived hyperpolarizing factor (EDHF)-type relaxation was reduced in the superior mesenteric artery (SMA) of STZ-diabetic rats and mice. However, no study has assessed the direct effects of inhibition of TLR4 on vasorelaxation in STZ-diabetic rats with long-term disease.

Here, we hypothesized that inhibition of TLR4 affects endothelium-dependent vasorelaxation in STZ-diabetic rats. We aimed to investigate the effects of long-term treatment with TAK-242, a cell-permeable small-molecule inhibitor of TLR4 by binding to the intracellular Cys747 residue of TLR4 and interfering with interactions between TLR4 and its adaptor molecules including Toll/interleukin-1 receptor domain-containing adaptor protein (TRAP) or Toll/interleukin-1 receptor domain-containing adaptor protein inducing interferon-β-related adaptor molecule (TRAM), on ACh-induced relaxation and related components in the SMA of STZ-diabetic rats by using the organ culture method, which is a useful tool for directly evaluating the vascular effects of target substances.

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on vascular functions without complicated interactions among other humoral and neuronal factors and other cells, including immune cells.\textsuperscript{15)}

MATERIALS AND METHODS

Animals, Organ Culture, and Vascular Function Male Wistar rats 7 weeks of age received a single injection through the tail vein of 65 mg/kg STZ (Sigma-Aldrich, St. Louis, MO, U.S.A.) dissolved in a citrate buffer, as in our previous studies.\textsuperscript{11)} Water and food were given ad libitum until the rats were sacrificed at 34–40 weeks of age (i.e., 27–34 weeks after STZ injection). Experiments were performed in accordance with the Guiding Principles for the Care and Use of Laboratory Animals from the Committee for the Care and Use of Laboratory Animals of Hoshi University (Tokyo, Japan).

All experiments, including organ culture, vascular functional study,\textsuperscript{11,15)} and release of thromboxane (TX) B\textsubscript{2},\textsuperscript{10)} were based on our previous studies. Body weight and blood glucose were measured in nonfasted STZ-induced rats at sacrifice (body weight, 350.2 ± 10.5 g; glucose, 470.8 ± 14.7 mg/dL; n = 24). The rats were anesthetized with isoflurane and euthanized by thoracotomy and exsanguination. After euthanasia, the SMA was rapidly isolated, cleaned, cut into arterial rings in an oxygenated modified Krebs–Henseleit solution (KHS; composition in mmol/L: NaCl 118.0, KCl 4.7, NaHCO\textsubscript{3} 25.0, CaCl\textsubscript{2} 1.8, Na\textsubscript{2}HPO\textsubscript{4} 1.2, MgSO\textsubscript{4} 1.2, and glucose 11.0). Subsequently, the arterial rings were placed in Dulbecco’s modified Eagle’s medium (low-glucose, Gibco BRL, Grand Island, NY, U.S.A.) containing antibiotics and 1.0% fetal bovine serum (Biological Industries, Kibbutz Beit Kaemek, Israel), in the presence of vehicle (1.0% dimethyl sulfoxide) or TAK-242 (10\textsuperscript{−6} M, Calbiochem, San Diego, CA, U.S.A.) and incubated in a humidified atmosphere of 95% air and 5% CO\textsubscript{2} at 37°C for approximately 1 d. After incubation, the rings were suspended in organ bath as reported previously.\textsuperscript{11,15)}

Measurement of TXB\textsubscript{2} Release After organ-cultured SMA was treated with TAK-242 (10\textsuperscript{−6} M) or vehicle (1.0% dimethyl sulfoxide) for approximately 1 d, the SMA was stimulated with ACh (10\textsuperscript{−6} M) for 20 min. Subsequently, the arterial rings were placed in Dulbecco’s modified Eagle’s medium (low-glucose, Gibco BRL, Grand Island, NY, U.S.A.) containing antibiotics and 1.0% fetal bovine serum (Biological Industries, Kibbutz Beit Kaemek, Israel), in the presence of vehicle (1.0% dimethyl sulfoxide) or TAK-242 (10\textsuperscript{−6} M, Calbiochem, San Diego, CA, U.S.A.) and incubated in a humidified atmosphere of 95% air and 5% CO\textsubscript{2} at 37°C for approximately 1 d. After incubation, the rings were suspended in organ bath as reported previously.\textsuperscript{11,15)}

Relaxations are presented as the percentage in the contraction induced by U46619. There were no differences in U46619-induced contraction between vehicle and TAK-242 groups in each condition (data not shown).

Measurement of TXB\textsubscript{2} Release After organ-cultured SMA was treated with TAK-242 (10\textsuperscript{−6} M) or vehicle (1.0% dimethyl sulfoxide) for approximately 1 d, the SMA was stimulated with ACh (10\textsuperscript{−6} M) for 20 min. Subsequently, the SMA was weighed and the cultured medium was rapidly frozen in liquid N\textsubscript{2} and stored at −80°C for later analysis. The levels of TXB\textsubscript{2}, a metabolite of TXA\textsubscript{2}, in the medium were measured by an enzyme immunoassay kit (Item No. 501020, Cayman Chemical, Ann Arbor, MI, U.S.A.) containing antibodies and 1.0% fetal bovine serum (Biological Industries, Kibbutz Beit Kaemek, Israel), in the presence of vehicle (1.0% dimethyl sulfoxide) or TAK-242 (10\textsuperscript{−6} M, Calbiochem, San Diego, CA, U.S.A.) and incubated in a humidified atmosphere of 95% air and 5% CO\textsubscript{2} at 37°C for approximately 1 d. After incubation, the rings were suspended in organ bath as reported previously.\textsuperscript{11,15)}

The concentration–response curves for ACh were obtained in the SMAs. Inhibitors were treated for 30 min before applying U46619 and were present thereafter. Relaxations are presented as the percentage in the contraction induced by U46619. There were no differences in U46619-induced contraction between vehicle and TAK-242 groups in each condition (data not shown).
Statistical Analysis The data were expressed as means ± standard error of the mean (S.E.M.), with n representing the number of animals used in the experiments. The concentration–response curves were fitted using a nonlinear regression-fitting program with a standard slope for each curve (GraphPad Prism ver. 8.0; GraphPad Software Inc., San Diego, CA, U.S.A.). The significance of differences between the two groups was determined by Student’s t-test. Statistical comparisons between concentration–response curves were made using a two-way repeated ANOVA with Bonferroni’s multiple comparisons test. The results were considered significant when the p value was less than 0.05.

RESULTS

After treatment of SMAs of STZ-diabetic rats with vehicle or TAK-242 for 1 d, exposure of SMA rings to ACh (10⁻⁹–10⁻⁵ M) led to a concentration-dependent relaxation in both the vehicle- and the TAK-242-treated groups (Fig. 1A). The ACh-induced relaxation was greater in the TAK-242 group than in the vehicle group, at intermediate concentrations of ACh (i.e., 10⁻⁷ M), the ACh-induced relaxation was significantly stronger in rings from the TAK-242 group than in those from the vehicle group (Fig. 1A). The maximal ACh-induced relaxation was significantly greater in the TAK-242 group than in the vehicle group (Fig. 1B). The pD₂ values for the ACh-induced relaxations were 7.41 ± 0.11 (n = 11) and 7.56 ± 0.09 (n = 12) in vehicle and TAK-242 group, respectively (p > 0.05). SNP-induced relaxation was slightly greater in SMA rings from the TAK-242 group than in SMA rings from the vehicle group (Fig. 1C). The maximal SNP-induced relaxation was significantly greater in the TAK-242 group than in the vehicle group (Fig. 1D). The pD₂ values for the SNP-induced relaxations were 7.94 ± 0.15 (n = 11) and 7.99 ± 0.10 (n = 12) in vehicle and TAK-242 group, respectively (p > 0.05).

COX-derived prostanoids play a key role in the regulation of vascular tone.¹ It is known that prostacyclin is one of the endothelium-derived relaxing factors (EDRFs), and that vasoconstrictor prostanoids exert an endothelium-derived contracting factor (EDCF) in various conditions, including aging, diabetes, and hypertension.¹ To investigate the component of COX-derived prostanoids that is mediated by ACh-induced relaxation, concentration–response curves for ACh were assessed in SMA rings precontracted by U46619 in the presence of indomethacin (Figs. 2A, B). The concentration–response curve (Fig. 2A) and the maximal response (Fig. 2B) for ACh in the presence of indomethacin (10⁻⁵ M) (A, B) and indomethacin (10⁻⁵ M) plus L-NNA (10⁻⁴ M) (C, D) in STZ-diabetic SMAs treated with vehicle (1.0% dimethyl sulfoxide) or TAK-242 (10⁻⁶ M) for 1 d. n = 8 or 12. ACh, acetylcholine; L-NNA, N⁵-nitro-L-arginine; ns, not significant; SMA, superior mesenteric artery; SNP, sodium nitroprusside; STZ, streptozocin.

Fig. 2. Effect of TAK-242 on Relaxations Induced by ACh in the SMA of STZ-Diabetic Rats under Cyclooxygenase Inhibition (A, B) or Cyclooxygenase/Nitric Oxide Synthase Inhibitions (C, D)
22) In addition, deletion of TLR4 abrogated molecular patterns, ROS generation by smooth muscle cells, but also in vascular smooth muscle cells. 6,19) Based on studies in TLR4-deficient mice, a growing body of evidence suggests that TLR4 plays an important role in the development of cardiovascular and metabolic diseases. 8,10) Previous reports also suggest that impaired endothelium-dependent relaxation in the SMA of STZ-diabetic rats. In general, stimulation of the endothelium (e.g., by ACh) can release EDRFs including NO, prostacyclin, and EDHF/EDH, 1) and their contributions to relaxation depend on the vessel type. For example, NO contributes greatly in large arteries, and the contribution of EDHF/EDH gradually increases as vessel diameter decreases. 9) Although prostacyclin is another EDRF, it may be unrelated to relaxation induced by ACh in the SMA of STZ-diabetic rats. This possibility is supported by previous reports that vaso-dilator prostanooids do not make a prominent contribution to endothelium-dependent relaxation in the SMA. 12,25) In chronic diseases including hypertension and diabetes, and aging, vasocostrctor prostanooids rather than vasodilator prostanooids play a role in alteration of the responses induced by ACh. 1,25) In the present study, indomethacin abolished the difference in ACh-induced relaxations between the vehicle and TAK-242 groups. Moreover, TAK-242 reduced the generation of TXA₂ in SMAs stimulated with ACh. Furthermore, TAK-242 slightly increased SNP-induced relaxation in the SMA of STZ-diabetic rats. Based on our data and published evidence, we suggest that the enhancement of ACh-induced relaxation by treatment with TAK-242 is due to increased NO signaling and/or suppression of TXA₂ generation, but not affecting EDHF/EDH signaling.

Although the molecular mechanisms underlying the enhancement of ACh-induced relaxation by TAK-242 treatment in the SMA of STZ-diabetic rats remain unclear, oxidative stress might be involved. Activation of TLR4 induced generation of ROS in endothelial cells 8,10) and vascular smooth muscle cells. 6) Increased bioavailability of NO 8) or increased generation of TXA₂ was observed in arteries from STZ-diabetic models, and these changes were partly due to increased oxidative stress. 8,10) Previous reports also suggest that impaired ACh-induced relaxation was increased by reducers of oxidative stress in STZ-diabetic vessels. 8,27) Although ROS induced by TLR4 activation during chronic diabetes may be a causal factor of decreased NO signaling and increased TXA₂ generation, further investigation will be required to determine the underlying mechanisms.

In summary, we have shown that TAK-242 enhances ACh-induced relaxation in the SMA of STZ-diabetic rats by increased NO signaling and suppressing TXA₂ production. Because TAK-242 enhances endothelium-dependent relaxation.
in STZ-diabetic rats, it is expected to be employed as a therapeu- 
tic agent for diabetes-associated vasculopathies.

Acknowledgments We would like to thank Yurika Ezaki, 
Yurina Mae, Hiyori Yokoyama, Marina Ito, Tamayo Hashimoto, Amane Kurakata, Yuzuki Sato, and Tomoki Katome for their excellent technical assistance. This work was supported in part by Grants JSPS KAKENHI Grant numbers JP18K06861 (to TM), JP17K08318 (to KT), and JP18K06974 (to TK), and The Promotion and Mutual Aid Corporation for Private Schools of Japan.

Author Contributions TM designed the study. TM, KT, 
MK, TK, and KT conducted the experiments and analyzed the 
data. TM and TK wrote the manuscript. All authors have 
read and approved the manuscript.

Conflict of Interest The authors declare no conflict of interest.

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