Effect of Equol on Vasocontractions in Rat Carotid Arteries Treated with High Insulin

Takayuki Matsumoto,* Keisuke Takeyaniagi, Shota Kobayashi, Mihoka Kojima, Kumiko Taguchi, and Tsuneo Kobayashi*

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

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Previous research has indicated that high insulin affects vascular function. Equol is an active metabolite of daidzein, an isoflavone produced from soy by intestinal microbial flora, with beneficial effects on the vascular system. This study investigated whether equol was beneficial for vascular function under high insulin conditions. Using organ culture techniques, rat carotid arteries were treated for 23 ± 1 h with a vehicle, high insulin (100 nM), or equol (100 µM) plus high insulin (100 nM). Vascular isometric forces were measured by the organ bath technique. In each endothelium-intact ring, the contractions induced by high-K⁺, noradrenaline, or by serotonin (5-HT) were similar for the vehicle, insulin, and equol + insulin treatments. Contractions induced by a selective 5-HT₁A receptor agonist (TCB2) increased with insulin treatment (vs. vehicle), but less so with equol + insulin. Under basal conditions, a selective 5-HT₂B receptor agonist (BW723C86) did not induce contraction; following precontraction by a thromboxane analog, it induced contraction but not relaxation. These responses were similar across the three treatments. Acetylcholine-induced relaxations were also similar for the three treatments. In the endothelium-denuded preparations, 5-HT-induced contraction was augmented with insulin treatment (vs. vehicle) but less so by equol + insulin treatment. These differences in 5-HT-induced contractions were eliminated by iberiotoxin, a large-conductance calcium-activated K⁺ channel (BKCa) inhibitor. These results suggest that equol exerts a preventive effect on the enhancement of 5-HT-induced contraction by high insulin (possibly mediated by the 5-HT₂A receptor), and that these effects may be attributed to the activation of BKCa channels in vascular smooth muscle.

Key words carotid artery; equol; insulin; serotonin; vasocontraction

INTRODUCTION

Equol represents the main active product of daidzein metabolism and is produced by specific microflora in the gut.¹⁻³ Previous studies have proposed that equol is responsible for the metabolic and cardiovascular benefits of soy.⁴⁻⁶ There is extensive evidence suggesting that equol has beneficial effects in patients with diabetes, animal models of diabetes, and cells under diabetic conditions.⁷⁻¹⁰ Several studies have also suggested that equol exerts beneficial effects on the vascular system, including antioxidative properties,¹¹⁻¹⁴ anti-atherosclerotic properties,¹⁵ and arterial stiffness suppression.⁶ Further, equol can cause vasorelaxations in the carotid arteries, cerebral arteries,¹¹ aorta,¹⁶,¹⁷ and basilar arteries⁸ of animal and human tissues. However, little is known about the relationship between equol and vascular function under diabetic conditions.

Hyperinsulinemia is one of the important phenomena in type 2 diabetes. Prolonged circulation of high insulin causes various systematic dysfunctions, including cardiovascular diseases.¹⁹,²⁰ Insulin also affects functions in the vascular system. For example, in healthy volunteers, exogenous insulin augments cardiovascular reactivity to high insulin, but not high glucose, led to an increase in serotonin (5-HT)-induced contractions in rat carotid arteries.²⁶ Therefore, insulin’s role in vascular function may be dependent on vessel types and/or (patho)physiological conditions. However, little attention has been given to the relationships among vascular function, high insulin, and equol.

The aims of our study were to assess the effects of equol on the vascular responsiveness to various stimuli including receptor-independent (high-K⁺) and -dependent (noradrenaline and 5-HT) stimulation in rat carotid arteries treated with high insulin as well as to identify some of the molecular mechanisms involved in the beneficial effects of equol under high insulin conditions. These effects were evaluated with organ culture techniques. In organ culture, conditions are easily manipulated and vascular function resulting from treatment incubation may be assessed purely without complicated interactions among other factors, such as neurohumoral substances and other cells.²⁶⁻³¹

MATERIALS AND METHODS

Animals Male Wistar rats (aged 13 ± 5 weeks old; specific-ic-pathogen-free, n = 44) were used. The animal experiments were approved by the Hoshi University Animal Care and Use Committee (Tokyo, Japan) (permission code: 29-086; permission date: 21 June 2018). All experiments were performed according to 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).

Organ Culture Procedure and Vascular Function Study

The organ culture procedures and measurements of vascular functions were as performed according to our previous studies. Briefly, common carotid artery was isolated from a rat anesthetized with isoflurane and euthanized by exsanguination. The carotid artery was cleaned of adhering fat and connective tissue, cut into rings under sterile conditions, and placed in the medium consisted of low-glucose (5.5 mmol/L) Dulbecco’s modified Eagle’s medium (DMEM) (Gibco BRL, Grand Island, NY, U.S.A.) supplemented with 1% fetal bovine serum (Biological Industries, Kibbutz Beit Haemek, Israel) and 1% penicillin/streptomycin (Gibco BRL). Tissues were then assigned to vehicle, insulin, and equol + insulin groups. For the vehicle group, the carotid arterial ring was pretreated with the medium containing 0.5% dimethyl sulfoxide (DMSO) for 30 min and then replaced into the medium containing 0.5% DMSO and 0.00001 n M HCl. For the insulin group, the carotid arterial ring was pretreated with the medium containing 0.5% DMSO for 30 min and then replaced into the medium containing 0.5% DMSO and 100 nM insulin (Sigma Chemical Co., St. Louis, MO, U.S.A.). For the equol + insulin group, the carotid arterial ring was pretreated for 30 min with the medium containing 100 µM S-equol (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan); this concentration was selected based on the findings of previous studies. It was then replaced into the medium containing 100 µM S-equol and 100 nM insulin. Following incubation under these three conditions at 37°C for 23 ± 1 h, vascular function was assessed by our previously-established organ bath technique.

Subsequently, the rings were mounted into the organ bath system. After stabilization, the rings were exposed to an artificial solution and washed with modified Krebs–Henseleit solution [KHS; comprising (in mM) 118.0 NaCl, 4.7 KCl, 25.0 NaHCO₃, 1.8 CaCl₂, 1.2 NaH₂PO₄, 1.2 MgSO₄, and 11.0 glucose]. Next, concentration–response curves of 5-HT (Sigma Chemical Co.) (10⁻⁹–10⁻⁴ M), high-K⁺ (10–80 mM), TCB2 (Tocris Bioscience, Ellisville, MO, U.S.A.) (10⁻⁹–10⁻⁴ M), and BW723C86 (Cayman Chemical, Ann Arbor, MI, U.S.A.) (10⁻⁹–10⁻⁴ M) were performed. BW723C86 (10⁻⁹–10⁻⁴ M) or acetylcholine (ACh) (10⁻⁹–10⁻⁵ M) was also applied cumulatively in carotid arterial rings precontracted with U46619 (a thromboxane analog, 10–30 nM). Following incubation, in some rings after organ culture, the endothelium was removed by gently rubbing the inner surface before mounting. We also performed 5-HT-induced contraction in the presence of the BKᵩ channel inhibitor iberiotoxin (100 nM) (Peptide Institute, Inc., Osaka, Japan) in endothelium-denuded rings to investigate the role of BKᵩ channels in the contraction. Following concentration–response curves, the rings were washed, stabilized, and precontracted with phenylephrine (1 µM). ACh (1 µM for endothelium-intact rings or 100 µM for endothelium-denuded rings) was applied to check endothelium integrity.

Data Analysis

Data were expressed as mean ± standard error, with n representing the number of animals used in the experiments. Contractile responses were presented as a percentage of 80 mM high-K⁺-induced contraction. Relaxation responses were presented as a percentage of U46619-induced contraction. Concentration–response curves were fitted using a nonlinear regression fitting program with a variable slope for high-K⁺-induced contraction and a standard slope for other drugs (Graph Pad Prism 7.0; GraphPad Software Inc., San Diego, CA, U.S.A.). Maximum response values (Eₘₐₓ) and area under the curve (AUC) were determined using the same software. Statistical evaluations were performed using one-way ANOVA followed by a Tukey’s post hoc test. p-Values <0.05 were considered significant.

RESULTS AND DISCUSSION

The present study used organ culture techniques to investigate the direct associations among equol, insulin, and vascular function without the influence of complicated factors from other organs. After prolonged treatment with equol and high insulin, each carotid arterial ring was mounted in an organ bath system where equol and insulin were completely removed. Thus, alterations of vascular function are resulted from prolonged but not acute effects.

To evaluate whether equol modulates vascular contractile activity under high insulin conditions, concentration–response curves for various stimuli in endothelium-intact carotid arteries of rats were performed (Fig. 1). The contractions induced by high-K⁺ were similar among the vehicle, insulin, and equol + insulin groups (Fig. 1A). The contractions induced by noradrenaline were also similar among the three groups (Fig. 1B). The similarity between noradrenaline-induced contractions observed with or without high insulin were consistent with a previous report of mesenteric arteries treated with high insulin (100 nM for 21–23h). Although there was no statistical difference observed, 5-HT-induced contraction was slightly increased in the insulin group (Eₘₐₓ 45.0 ± 6.5%, n = 8) compared with the vehicle group (Eₘₐₓ 31.2 ± 4.1%, n = 8) and equol + insulin (Eₘₐₓ 35.8 ± 6.0%, n = 8) (Fig. 1C). We further investigated the effects of two serotonin 5-HT₂ receptor agonists, because 5-HT₂ receptors play an important role in the control of vascular tone. Among the 5-HT receptor subtypes, 5-HT₂-induced vasoconstriction is mainly due to activation of 5-HT₂A receptors on vascular smooth muscle. On the other hand, the activation of 5-HT₂B receptors results in not only vasorelaxation but also vasoconstriction depending on vessel type or pathophysiological situations. Interestingly, the contraction induced by TCB2, an agonist of 5-HT₂A receptor, was more significantly increased in carotid arteries from the insulin group compared with those from the vehicle group in the present study. In the equol + insulin group, TCB2-induced contraction was significantly reduced compared with the insulin group (Fig. 1D). On the other hand, the selective 5-HT₂B receptor agonist BW723C86 did not cause vasoconstriction in any group (Fig. 1E). These results suggest that high insulin enhanced 5-HT stimulation, particularly in 5-HT₂A-mediated contraction, and equol can prevent such enhancement. Next, we investigated the equol’s potential to modulate relaxant activity.

Because 5-HT₂B receptor activation causes vasorelaxation in some vessels, concentration–response curves for BW723C86 in endothelium-intact carotid arteries precontracted with U46619 (10–30 nM) were confirmed. As shown in Fig. 2A, BW723C86 did not induce relaxation, whereas contractions were observed at higher concentrations and were similar among the three groups. To assess whether equol would modulate endothelial function, we performed concen-
tration–response curves for ACh, an endogenous endothelial stimulator. As shown in Fig. 2B, ACh-induced relaxations were similar among the three groups. These results suggest that 5-HT2B receptors do not contribute to the effect of equol on 5-HT-induced contraction.

Next, to evaluate whether equol modulates 5-HT-induced contraction in vascular smooth muscle, concentration–response curves for 5-HT in endothelium-denuded carotid arteries were performed. As shown in Fig. 3A, the 5-HT-induced contraction was significantly increased in endothelium-denuded carotid arteries from the insulin group compared with those from the vehicle group. Interestingly, the 5-HT-induced contractions were significantly reduced in the equol + insulin group than in the insulin group. These results suggest that the signaling in vascular smooth muscle, but not in the endothelium, is determinant of 5-HT-induced contraction enhancement under high insulin conditions and that equol exerts preventive effects on 5-HT-induced contraction in vascular smooth muscle.

To investigate the possible mechanisms underlying the suppressive effect of equol on 5-HT-induced contraction under high insulin conditions in vascular smooth muscle, we performed concentration–response curves for 5-HT in endothelium-denuded carotid arteries. Because some reports suggest
that equol modulates BK<sub>Ca</sub> channel activity, these curves were performed in the presence of a BK<sub>Ca</sub> channel inhibitor. Moreover, activation of BK<sub>Ca</sub> channels causes hyperpolarization and, subsequently, relaxation in vascular smooth muscle. Vascular reactivity to various substances is modulated by BK<sub>Ca</sub> channel activation. Interestingly, under inhibition of BK<sub>Ca</sub> channel by iberiotoxin (100 nM), the difference in 5-HT-induced contractions among the three groups was eliminated. These results suggest that the preventive effect of equol on 5-HT-induced contraction under high insulin conditions is due to an increase in BK<sub>Ca</sub> channel activation.

High insulin, which is an important pathophysiological condition of type 2 diabetes, results from insulin resistant states. The prolonged circulation of high insulin can result in various types of systematic dysfunctions, including cardiovascular diseases. The findings of this study suggest that equol exerts preventive effects on vascular dysfunction under high insulin conditions. Abnormalities of 5-HT-mediated vasoreactions have been observed in type 2 diabetic states. It is therefore possible that equol may be able to prevent the development of vascular dysfunction in type 2 diabetes. However, further investigations are required, not only to elucidate the underlying mechanisms, but also to determine equol’s beneficial effects for humans with and without type 2 diabetes.

In conclusion, our data suggest that equol prevents high insulin-induced enhancement of 5-HT-induced contraction in rat carotid arterial smooth muscle, and which may be due to increased BK<sub>Ca</sub> channel activity in carotid artery smooth muscle. Therefore, equol may be a promising and safe drug candidate for the prevention of vascular dysfunction development under high insulin conditions.

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**Conflict of Interest** The authors declare no conflict of interest.

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