Pflugers Arch - Eur J Physiol (2016) 468:667–677
DOI 10.1007/s00424-015-1759-4

Insulin augments serotonin-induced contraction via activation of the IR/PI3K/PDK1 pathway in the rat carotid artery

Shun Watanabe[1] · Takayuki Matsumoto[1] · Mirai Oda[1] · Kosuke Yamada[1] · Junya Takagi[1] · Kumiko Taguchi[1] · Tsuneo Kobayashi[1]

Received: 23 September 2015 / Revised: 9 November 2015 / Accepted: 11 November 2015 / Published online: 17 November 2015

Abstract Hyperinsulinemia associated with type 2 diabetes may contribute to the development of vascular diseases. Although we recently reported that enhanced contractile responses to serotonin (5-hydroxytryptamine, 5-HT) are observed in the arteries of type 2 diabetes models, the causative factors and detailed signaling pathways involved remain unclear. The purpose of this study was to investigate whether high insulin would be an amplifier of 5-HT-induced contraction in rat carotid arteries and whether the contraction involves phosphoinositide 3-kinase (PI3K)/3-phosphoinositide-dependent protein kinase 1 (PDK1) signaling, an insulin-mediated signaling pathway. In rat carotid arteries organ-cultured with insulin (for 24 h), (1) the contractile responses to 5-HT were significantly greater (vs. vehicle), (2) the insulin-induced enhancement of 5-HT-induced contractions was largely suppressed by inhibitors of the insulin receptor (IR) (GSK1838705A), PI3K (LY294002), and PDK1 (GSK2334470), and (3) the levels of phosphorylated forms of both PDK1 and myosin phosphatase target subunit 1 (MYPT1) were greater upon 5-HT stimulation. In addition, in rat carotid arteries organ-cultured with an activator of PDK1 (PS48), the 5-HT-induced contraction was greater, and this was suppressed by PDK1 inhibition but not PI3K inhibition. In addition, MYPT1 and PDK1 phosphorylation upon 5-HT stimulation was enhanced (vs. vehicle). These results suggest that high insulin levels amplify 5-HT-induced contraction. Moreover, the present results indicated the direct linkage between IR/PI3K/PDK1 activation and 5-HT-induced contraction in rat carotid arteries for the first time.

Keywords Insulin receptor · Myosin phosphatase target subunit 1 · Phosphoinositide 3-kinase · Phosphoinositide-dependent kinase 1 · Vasoconstriction

Introduction

The prevalence of diabetes, particularly type 2 diabetes, has emerged as a significant problem worldwide in recent years. Although type 2 diabetes is remarkably associated with an increased incidence of vascular complications [5, 11, 13, 18, 31, 52, 53], the exact relationship between type 2 diabetes and vascular disease is not completely understood because multiple factors are involved in the development of these phenomena. Therefore, it is important to investigate and understand causal factors for altered vascular functions to prevent development of vascular complications in type 2 diabetes.

Serotonin (5-hydroxytryptamine, 5-HT) is a potent vasoactive amine that is considered to play pivotal roles in the physiological control of vascular tone and blood pressure as well as in the genesis and development of cardiovascular diseases such as atherosclerosis and hypertension [14, 22, 23, 55, 66, 67]. Several reports suggest a role of 5-HT in the pathogenesis of diabetic vascular complications [16, 21, 44, 48]. For instance, we recently found that the 5-HT-induced contractions were increased in superior mesenteric arteries of type 2 diabetic *ob/ob* mice [36] and in the carotid arteries of type 2 diabetic Goto-Kakizaki (GK) rats [40]. Although

Shun Watanabe and Takayuki Matsumoto contributed equally to this work.

✉ Tsuneo Kobayashi
tkoba@hoshi.ac.jp

Takayuki Matsumoto
t-matsu@hoshi.ac.jp

[1] Department of Physiology and Morphology, Institute of Medicinal Chemistry, Hoshi University, 2-4-41 Ebara, Shinagawa-ku, Tokyo 142-8501, Japan

responsiveness to 5-HT is altered in chronic type 2 diabetes, the causal factors of alterations in 5-HT signaling remain unclear.

It is a well-established theory that one of the important causative factors related to diabetic vascular dysfunction may be associated with hyperinsulinemia and insulin resistance [4, 5, 24, 61]. In vascular cells, including endothelial cells (ECs) and smooth muscle cells (VSMCs), various reports suggest that intracellular insulin signaling is altered under hyperinsulinemia and insulin resistance [4, 5, 24, 61, 62]. Indeed, several reports suggest that insulin resistance plays a key role in the development of hypertension and impairment of endothelium-dependent relaxation observed in insulin-receptor substrate (IRS)-1- or IRS-2-deficient mice [1, 30]. A major feature of insulin resistance in vascular cells is the specific impairment of insulin-induced IRS/phosphoinositide 3-kinase (PI3K) signaling with alteration of insulin signaling through mitogen-activated protein kinase (MAPK) and other growth pathways [5, 24, 61]. Previously, we reported that the coexistence of a high insulin level and established diabetes led to (1) excessive peroxynitrite generation and resulted in impaired endothelium-dependent relaxation in aortae [29] and (2) increased endothelin-1-induced aortic contraction via enhanced ET_A receptor/extracellular-signal-regulated kinase (ERK) signaling [27]. Therefore, insulin signaling could modulate arterial contractile responses to endogenous ligands; however, the mechanism by which signaling between insulin and 5-HT is integrated remains elusive.

Among kinases known to be involved in insulin signaling, there is an emerging body of evidence suggesting that 3-phosphoinositide-dependent protein kinase 1 (PDK1), which is cytoplasmic membrane-associated enzyme activated by PI3K [2, 10, 54, 64, 69], plays a pivotal regulatory role in many cellular processes and signaling pathways including cell survival, growth, proliferation, and metabolism [15, 20, 59, 69, 70]. PDK1 activates a group of protein kinases belonging to the protein kinase A (PKA)/PKG/PKC kinase family that plays important roles in mediating diverse biological processes including vascular function [3, 6, 42, 69]. Indeed, Chen et al. [9] recently reported that PDK1 regulates platelet activation and arterial thrombosis. Tawaramoto et al. [63] found that EC-specific deletion of PDK1 enhances insulin sensitivity via reducing visceral fat and suppressing angiogenesis. In VSMCs, Weber et al. [68] found that platelet-derived growth factor (PDGF)-induced migration is reactive oxygen species (ROS)-dependent and the Src/PDK1/p21-activated protein kinase1 pathway contributes to ROS-sensitive migration. However, uncertainty surrounds the mechanisms by which PDK1 might contribute to vascular contractile response.

In the present study, we hypothesized that exposure to high insulin levels would amplify 5-HT-induced contraction in the carotid arteries of rats. Furthermore, we postulated that PDK1 might be involved in such alterations. To investigate our hypothesis, we used organ culture of the entire vascular wall [25, 26, 29, 51] because in this way, it is possible to incubate the vessel with a constant concentration of insulin over a prolonged period.

Methods

Reagents

The reagent sources were as follows: insulin, 5-HT, and monoclonal β-actin antibody (Sigma Chemical Co, St. Louis, MO, USA); (3S,6R)-1-[6-(3-amino-1H-indazol-6-yl)-2-(methylamino)-4-pyrimidinyl]-*N*-cyclohexyl-6-methyl-3-piperidinecarboxamide (GSK2334470) and (2Z)-5-(4-chlorophenyl)-3-phenyl-2-pentenoic acid (PS48) (Tocris Bioscience, Bristol, UK); U46619 and 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002) (Cayman Chemical, Ann Arbor, MI, USA); 2-(2-(1-(2-(dimethylamino)acetyl)-5-methoxyindolin-6-ylamino)-7H pyrrolo[2,3-d] pyrimidin-4-ylamino)-6-fluoro-*N*-methylbenzamide (GSK1838705A) (AdooQ Bioscience, Irvine, CA, USA); and dimethyl sulfoxide (DMSO) (Wako Pure Chemical Industries, Osaka, Japan). The antibody sources were as follows: phospho-PDK1 (Ser^{241}), PDK1, and insulin receptor β subunit (IR β) (Cell Signaling, Beverly, CA, USA); p-MYPT1 (Thr^{853}) (Santa Cruz Biotechnology, Santa Cruz, CA, USA); $5\text{-}HT_{2A}$ receptor (ImmunoStar, Hudson, WI, USA); and MYPT1, ROCK1, and ROCK2 (BD Biosciences, San Jose, CA, USA).

Animals and experimental design

Male Wistar rats were obtained at the age of 4–8 weeks (JLA, INC., Tokyo, Japan). All animals were allowed a standard laboratory diet (MF; Oriental Yeast Industry, Tokyo, Japan) and water ad libitum in a controlled environment (room temperature 21–22 °C, humidity 50±5 %) until the rats were 9–17 weeks old (body weight 0.3–0.6 kg). This study was approved by the Hoshi University Animal Care and Use Committee, and all studies were conducted in accordance with "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).

Arterial isolation and organ culture procedure

In all experiments, non-fasted rats were anesthetized with isoflurane (initially at 5 % and then maintained at 2.5 %) via a nose cone for surgical procedures and euthanized by thoracotomy and exsanguination via cardiac puncture. After euthanasia, common carotid arteries (diameter approx. 1 mm) were isolated under sterile conditions and placed in an ice-cold,

oxygenated, modified Krebs-Henseleit solution (KHS). Each artery was carefully cleaned and cut into rings. Some segments were placed in 300 μl of low-glucose (5.5 mM) or high-glucose (25 mM) Dulbecco's modified Eagle medium (DMEM: Gibco BRL, Grand Island, NY, USA) supplemented with 1 % penicillin streptomycin (Gibco BRL) and 1 % fetal bovine serum (FBS: Biological Industries, Kibbutz Beit Kaemek, Israel) in the absence or presence of insulin or PS48 (a PDK1 activator [19]). To investigate the effects of various signaling pathways on prolonged insulin treatment in carotid arteries, a given ring was incubated for 30 min in the appropriate drug-containing DMEM (viz., 1×10^{-6} M GSK1838705A [an IR inhibitor [56]], 1×10^{-5} M LY294002 [a PI3K inhibitor [40]], or 1×10^{-5} M GSK2334470 [PDK1 inhibitor [42]]), before insulin treatment, remaining present thereafter. Also, some rings treated these inhibitors alone (without insulin treatment). They were maintained at 37 °C in an atmosphere of 95 % air and 5 % CO_2 for approximately 24 h.

Measurement of isometric force

Vascular isometric force was recorded as described in our previous papers [39, 40]. After organ culture, the arterial rings were placed in oxygenated, modified KHS. This solution consisted of (in mM) 118.0 NaCl, 4.7 KCl, 25.0 $NaHCO_3$, 1.8 $CaCl_2$, 1.2 NaH_2PO_4, 1.2 $MgSO_4$, and 11.0 glucose. Isotonic high K^+ solution was prepared by replacing NaCl with KCl. Ring segments (2 mm in length) were suspended via a pair of stainless steel pins in a well-oxygenated (95 % O_2–5 % CO_2) bath containing 5 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 45 min. After stabilization, the contractile response to 80 mM KCl was measured. Force generation was monitored using an isometric transducer (model TB-611T; Nihon Kohden, Tokyo, Japan). For the contraction studies, 5-HT (1×10^{-9}–3×10^{-5} M) or U46619 (10^{-10}–$10^{-7.5}$ M) was added cumulatively to the bath until a maximal response was achieved.

Western blotting

Each carotid arterial ring was cultured with insulin (1×10^{-6} M) or vehicle (0.0001 N HCl) and PS48 (3×10^{-4} M) or vehicle (DMSO). After organ culture, the arterial rings were suspended via a pair of stainless steel pins in a well-oxygenated (95 % O_2–5 % CO_2) bath containing 5 ml of KHS at 37 °C, and then 3×10^{-5} M 5-HT was applied for 5 min. Next, carotid arterial rings were washed with ice-cold Ca^{2+}-free solution containing sodium orthovanadate (1×10^{-3} M) and EDTA (5×10^{-3} M) and rapidly removed, after which they were freeze-clamped in liquid nitrogen and stored at −80 °C for Western blotting. For measurements of IRβ and 5-HT_{2A} receptors, protein samples were obtained from freshly isolated carotid arteries and cultured with insulin (1×10^{-6} M) or vehicle (0.01 N HCl) for 24 h. After organ culture, carotid arterial rings were rapidly removed, and then, they were freeze-clamped in liquid nitrogen and stored at −80 °C for Western blotting. The protein levels of PDK1, phosphorylated PDK1, MYPT1, phosphorylated MYPT1, ROCK1, ROCK2, IRβ, and 5-HT_{2A} receptor were quantified using immunoblotting procedures, essentially as described previously [34, 38–40]. Carotid arterial protein extracts (20 μg/lane) were applied to 7.5 or 10 % SDS-PAGE and transferred to polyvinylidene difluoride membranes. Blots were incubated with anti-PDK1 (58–68 kDa; 1:1000), anti-phospho-PDK1 (Ser^{241}) (58–68 kDa; 1:1000), anti-MYPT1 (130 kDa; 1:1000), anti-phospho-MYPT1 (Thr^{853}) (130 kDa; 1:200), anti-ROCK1 (~160 kDa; 1:500), anti-ROCK2 (~160 kDa; 1:500), anti-IRβ (95 kDa; 1:500), anti-5-HT_{2A} receptor (53 kDa; 1:1000), and anti-β-actin (42 kDa; 1:5000) antibodies, with detection being achieved using a horseradish peroxidase-conjugated IgG followed by enhanced chemiluminescence. The resulting bands were analyzed using CS Analyzer 3.0 software (ATTO, Tokyo, Japan). The phosphorylation levels of PDK1 and MYPT1 were normalized by total PDK1 and total MYPT1, respectively, and then expressed as fold increase (relative to vehicle). The protein expressions of ROCK1, ROCK2, IRβ, and 5-HT_{2A} receptors were normalized by β-actin and then expressed as fold increase (relative to vehicle).

Statistical analysis

The contractile force exerted by carotid arterial rings is expressed as a percentage of the 80-mM KCl-induced contraction. Concentration-response curves with agonists were fitted using a nonlinear interactive fitting program (GraphPad Prism 5.0; GraphPad Software Inc., San Diego, CA, USA). Data are expressed as the mean±SE. Statistical evaluations were performed using Student's t test for comparisons between two groups. Statistical analysis of the values of E_{max} (the maximal effect generated by 5-HT) was performed using one-way ANOVA with Bonferroni's post hoc test. Statistical evaluations of concentration-response curves were performed using two-way ANOVA with repeated measures followed by Bonferroni's post hoc test. Values of $P<0.05$ were considered significant.

Results

Effects of prolonged treatment with insulin on 5-HT-induced contractile responses

We firstly examined the effects of high concentration of insulin or glucose on 5-HT (1×10^{-7}–3×10^{-5} M)-induced contraction. Exposure of organ-cultured carotid artery rings to 5-HT

led to a concentration-dependent rise in tension in both insulin- (1×10^{-7} or 1×10^{-6} M) and vehicle-treated arteries, although the contractile force of 5-HT was greater in insulin-treated arteries than in the vehicle-treated arteries in both normal (Fig. 1a) and high glucose (Fig. 1b) conditions. The E_{max} values of 5-HT-induced contractile response were significantly increased in insulin (1×10^{-6} M)-treated arteries in both normal and high glucose conditions (Fig. 1c). The reference contractions induced by 80 mM KCl were similar among all groups (Fig. 1d). On the other hand, the contractile force of another constrictor, U46619, was similar between insulin (1×10^{-7} M)-treated and vehicle-treated arteries under conditions of normal glucose (data not shown). These results suggested that high insulin but not high glucose could enhance 5-HT-induced contraction in rat carotid arteries. As glucose did not influence 5-HT-induced contraction in organ-cultured carotid arteries, all subsequent experiments were conducted in normal glucose DMEM for organ culture.

Effects of a selective IR antagonist and PI3K inhibitor on the insulin-induced enhancement of 5-HT-induced contraction

Because insulin signals were reported to involve the IR/IRS/PI3K pathway [43, 45, 50], in the second series of experiments, we examined whether 5-HT-induced contraction augmented by insulin could be suppressed by IR and PI3K inhibitors (Fig. 2). Cotreatment with the IR antagonist GSK1838705A (1×10^{-6} M) (Fig. 2a) or PI3K inhibitor LY294002 (1×10^{-5} M) (Fig. 2b) together with insulin (1×10^{-7} M) suppressed 5-HT-induced contraction in organ-cultured carotid arteries.

Effects of a selective PDK1 inhibitor on 5-HT-induced contraction enhanced by insulin

PDK1 was reported to be an immediate downstream effector of PI3K and a master kinase in various responses [2, 10, 54, 64, 69]. Next, to continue our investigation of the role of PDK1 on 5-HT-induced contraction augmented by insulin, we assessed the effect of PDK1 inhibition on the 5-HT-induced contraction of cultured carotid arteries in the presence of insulin (Fig. 2c). Treatment with the PDK1 inhibitor GSK2334470 (1×10^{-5} M) suppressed 5-HT-induced contraction in organ-cultured carotid artery in the presence of insulin (1×10^{-7} M).

When carotid rings were treated with each inhibitor alone (without insulin treatment) for 24 h, GSK1838705A (1×10^{-6} M), LY294002 (1×10^{-5} M), or GSK2334470 (1×10^{-5} M) did not influence 5-HT-induced contractions (Fig. 2d).

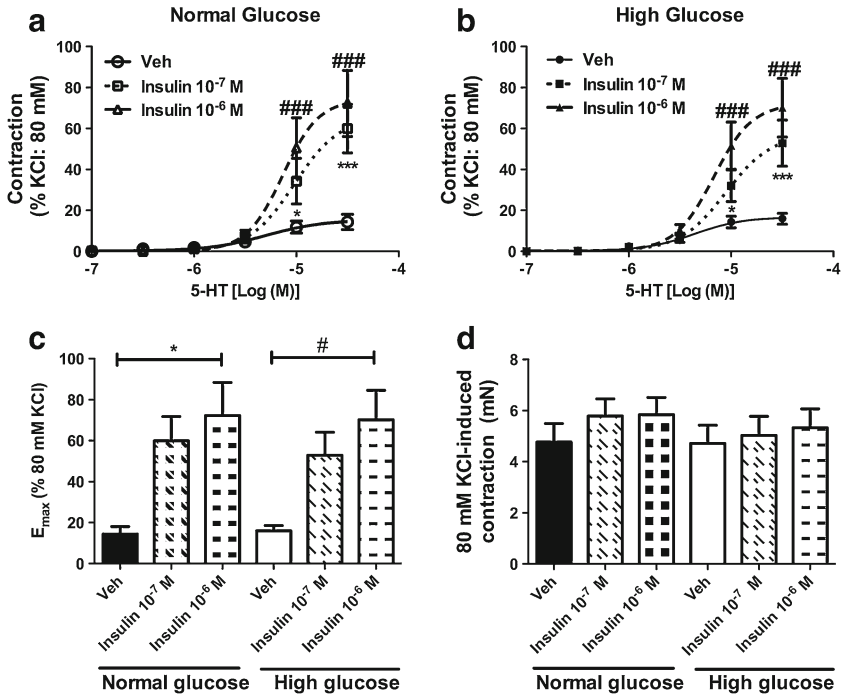

Fig. 1 Effects of insulin treatment on 5-hydroxytryptamine (5-HT)-induced contraction in organ-cultured rat carotid arteries. Carotid arteries were preincubated with vehicle (0.0001 N HCl) or insulin (1×10^{-7} or 1×10^{-6} M) in normal- (5.5 mM glucose) (**a**) or high-glucose (25 mM) Dulbecco's modified Eagle's medium (**b**). E_{max} of 5-HT-induced contraction (**c**). **d** The 80 mM KCl-induced contraction in carotid arteries cultured with vehicle or insulin in normal or high-glucose medium. Each data-point represents the mean±SE from five experiments. **a**, **b** $^{*}P<0.05$, $^{***}P<0.001$, vehicle vs. insulin 1×10^{-7} M, $^{\#\#\#}P<0.001$, vehicle vs. insulin 1×10^{-6} M. **c** $^{*}P<0.05$, vehicle vs. insulin 1×10^{-6} M in normal-glucose medium. $^{\#}P<0.05$, vehicle vs. insulin 1×10^{-6} M in high-glucose medium

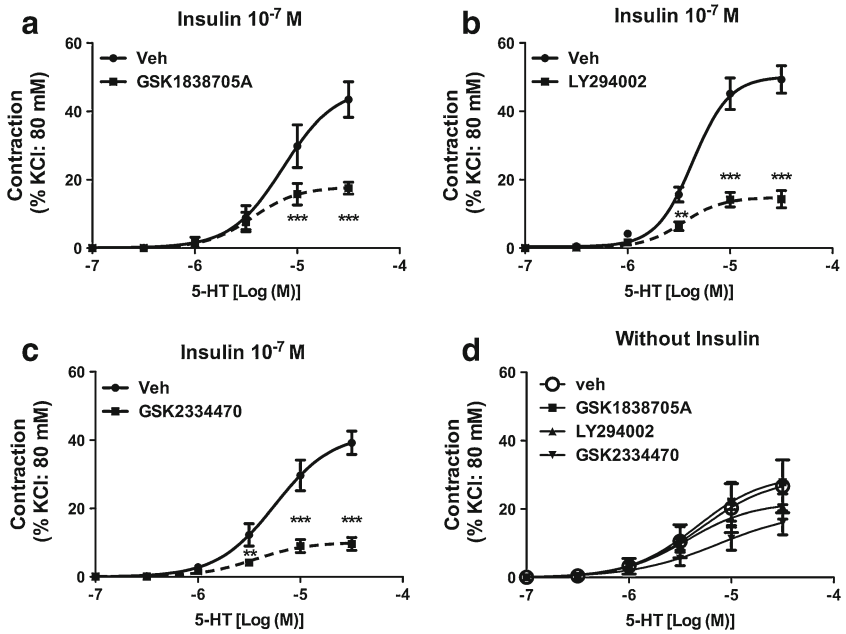

Fig. 2 Effect of an insulin receptor (IR) antagonist, phosphoinositide 3-kinase (PI3K) inhibitor, or 3-phosphoinositide-dependent protein kinase 1 (PDK1) inhibitor on the enhancement of 5-hydroxytryptamine (5-HT)-induced contraction in organ-cultured rat carotid arteries exposed to insulin. Concentration-response curves for 5-HT-induced contraction in insulin (1×10^{-7} M for 24 h)-treated carotid arteries in the absence or presence of GSK1838705A (IR inhibitor, 1×10^{-6} M) (**a**), LY294002 (PI3K inhibitor, 1×10^{-5} M) (**b**), or GSK2334470 (PDK1 inhibitor, 1×10^{-5} M). **d** Concentration-response curves for 5-HT-induced contraction in carotid arterial rings treated with vehicle (DMSO), GSK1838705A (1×10^{-6} M), LY294002 (1×10^{-5} M), or GSK2334470 (1×10^{-5} M) for 22–24 h. Data are the means±SE from seven (**a**), nine (**b**, **c**), or six (**d**) experiments: $^{**}P<0.01$, $^{***}P<0.001$ vs. vehicle

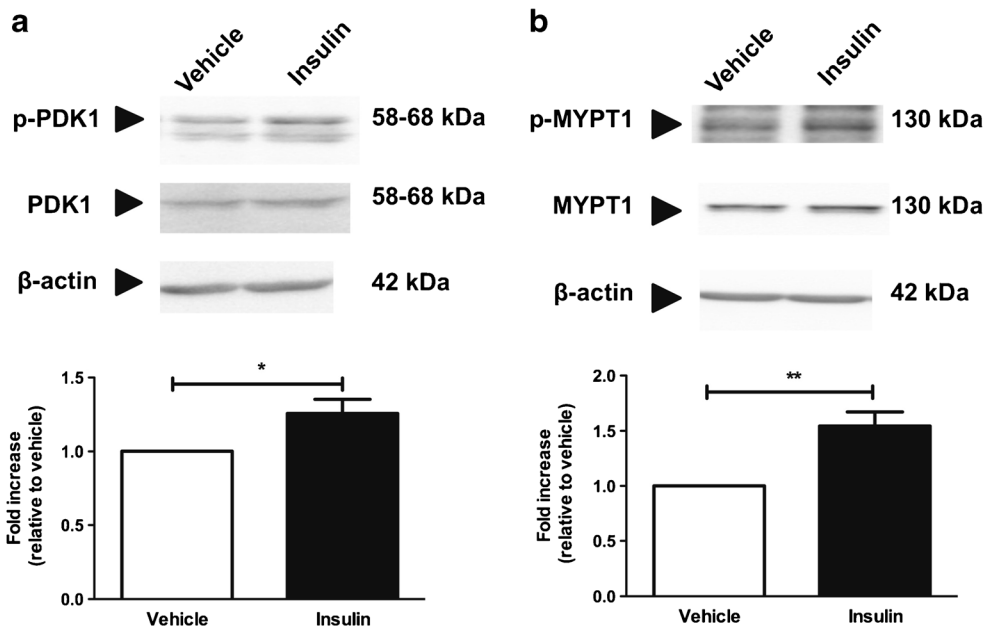

Fig. 3 Effects of prolonged treatment with insulin on the phosphorylation of 3-phosphoinositide-dependent protein kinase 1 (PDK1) and myosin phosphatase target subunit 1 (MYPT1) in organ-cultured rat carotid arteries. Western blots for p-PDK1/PDK1 (**a**) and p-MYPT1/MYPT1 (**b**) in organ-cultured carotid arteries exposed to insulin (1×10^{-6} M). After organ-cultured carotid arteries were stimulated with 5-hydroxytryptamine (3×10^{-5} M) for 5 min. Representative Western blots (*upper panels*). Bands were quantified as described in the "Methods" (*lower panels*). Results were shown as the fold increase relative to control (*vehicle*). Equal protein loading was confirmed using β-actin antibody. *Each column* represents the mean±SE from six (**a**) or eight (**b**) experiments. $^{*}P<0.05$, $^{**}P<0.01$ vs. vehicle

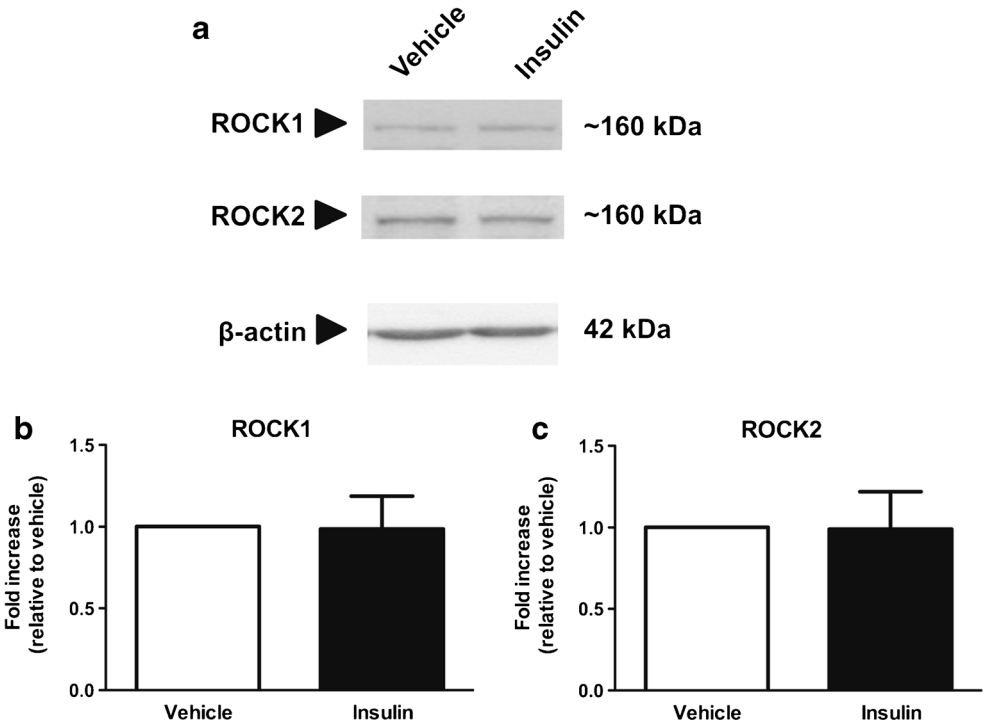

Fig. 4 Effects of prolonged treatment with insulin on the expression of ROCK in organ-cultured rat carotid arteries. Western blots for ROCKs in organ-cultured carotid arteries exposed to insulin (1×10^{-6} M for 24 h). After organ-cultured carotid arteries were stimulated with 5-hydroxytryptamine (3×10^{-5} M) for 5 min. **a** Representative Western blots. **b**, **c** Bands of ROCK1 (**b**) and ROCK2 (**c**) were quantified as described in the "Methods." Results are shown as the fold increase relative to control (*vehicle*). *Each column* represents the mean±SE from six experiments

Evaluations of PDK1, MYPT1, ROCK1, and ROCK2 protein expression in the absence or presence of insulin in cultured carotid arteries stimulated with 5-HT

Using pharmacological approaches, enhanced 5-HT-induced contraction was observed in insulin-treated arteries, and this was suppressed by inhibiting IR/PI3K/PDK1 signaling. To investigate the possible mechanisms underlying the alterations in 5-HT-induced contraction in organ-cultured carotid arteries in the presence of high insulin concentrations, we investigated whether the activity of PDK1 and MYPT1, which is the regulatory subunit of myosin light chain phosphatase and the phosphorylation of which at Thr^{853} stimulated by constrictors in a Rho kinase-dependent manner, affects contractile responses [17, 47, 60, 65]. Moreover, we assessed that the expression of ROCK1/2 was altered in carotid arteries subjected to prolonged insulin treatment with 5-HT stimulation. The 5-HT-stimulated carotid arterial expression of phosphorylated PDK1 (Fig. 3a) and MYPT1 (Fig. 3b) was significantly greater in the insulin-treated group (vs. vehicle). Conversely, the protein expression of ROCK1 (Fig. 4a) and ROCK2 (Fig. 4b) was not significantly different between insulin and vehicle treatment.

Effects of prolonged PDK1 activation on 5-HT-induced contraction and the activities of PDK1 and MYPT1 in organ-cultured carotid arteries

Next, to assess the direct relationship between PDK1 and contraction in response to 5-HT in carotid arteries, we investigated the effect of prolonged PDK1 activator treatment on 5-HT-induced contraction (Fig. 5) and the phosphorylation of MYPT1 and PDK1 (Fig. 6). Prolonged treatment with a PDK1 activator (PS48; 3×10^{-4} M for 24 h) [19, 58] enhanced 5-HT-induced contraction (Fig. 5a). This enhancement of 5-HT-induced contraction induced by PS48 was suppressed by pretreatment with GSK2334470 (1×10^{-5} M) but not by LY294002 (1×10^{-5} M) (Fig. 5b). Moreover, the 5-HT-induced contraction in carotid arteries cultured with (PS48 3×10^{-4} M) was not further modified by cotreatment with insulin (1×10^{-7} M) (Fig. 5c). The phosphorylation of MYPT1 upon 5-HT stimulation was significantly greater in PS48-treated arteries than in vehicle-treated arteries (Fig. 6a). Predictably, the expression of phosphorylated PDK1 was significantly greater in the PS48-treated group (vs. vehicle) (Fig. 6b).

Expression of IRβ and 5-HT_{2A} receptors

Finally, to investigate the possible mechanisms underlying the above amplifier effects of insulin on 5-HT-induced contractions in carotid arteries, we finally examined the protein expression of the IRβ and 5-HT_{2A} receptors (Fig. 7). Although the protein expressions of IRβ were not significantly altered between fresh and vehicle-cultured carotid arteries, the protein expressions of IRβ were significantly decreased in the insulin (1×10^{-6} M)-treated group compared to vehicle-treated group (Fig. 7b) in cultured carotid arteries. Conversely, the protein expressions of the 5-HT_{2A} receptor were similar for the three groups (Fig. 7c).

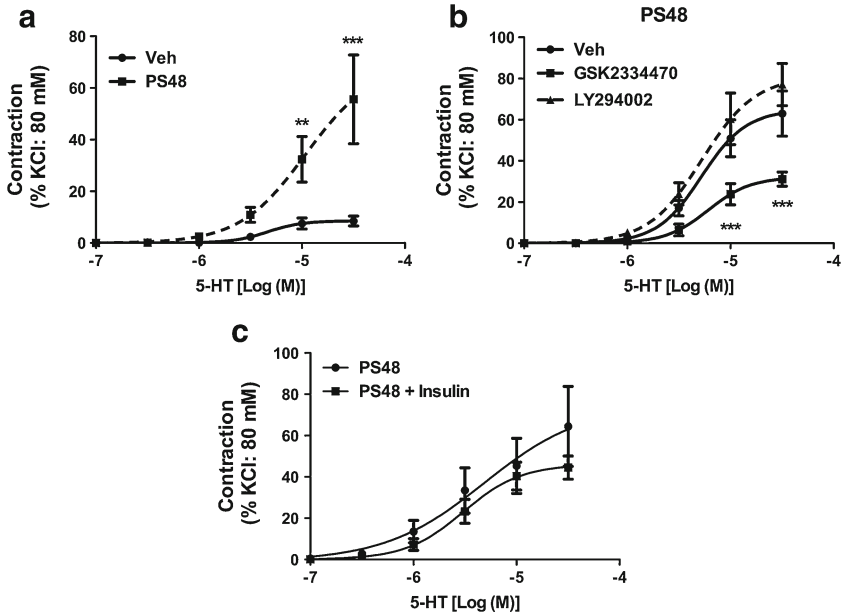

Fig. 5 Prolonged treatment with a 3-phosphoinositide-dependent protein kinase 1 (PDK1) activator augments 5-hydroxytryptamine (5-HT)-induced contraction in rat carotid arteries. Effects of a PDK1 activator (PS48: 3×10^{-4} M for 24 h) on 5-HT-induced contraction in organ-cultured rat carotid arteries (**a**). Effects of GSK2334470 (1×10^{-5} M) and LY294002 (1×10^{-5} M) on the concentration-response curves for 5-HT in the presence of PS48 (3×10^{-4} M) in organ-cultured rat carotid arteries (**b**). **c** Effects of coincubation with PS48 (3×10^{-4} M) and insulin (1×10^{-7} M) for 22–24 h on the 5-HT-induced contraction. The data represent the mean±SE from four to six experiments. **a** $^{**}P<0.01$, $^{***}P<0.001$ vs. vehicle. **b** $^{***}P<0.001$, vehicle vs. GSK2334470

Discussion

In the present study, we examined whether high insulin concentrations cause increased vascular contractile responses to 5-HT. The major findings of the present study are that prolonged exposure to high insulin levels but not high glucose levels in rat carotid arteries enhances 5-HT-induced contraction and the increase in 5-HT-induced contraction induced by high insulin concentrations is due to the activation of IR, PI3K, and PDK1 pathways (Fig. 8). It was also found that PDK1 activation leads

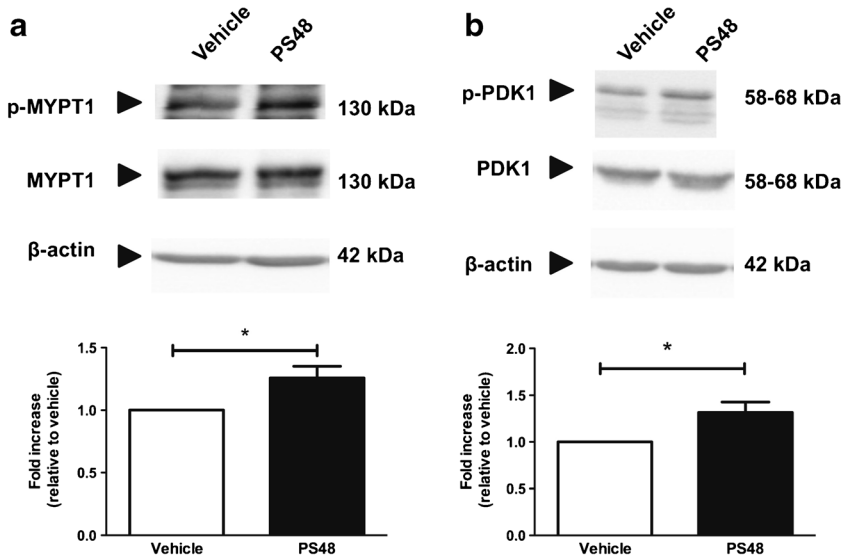

Fig. 6 Effects of prolonged treatment with PS48 (3×10^{-4} M) on the phosphorylation of myosin phosphatase target subunit 1 (MYPT1) and 3-phosphoinositide-dependent protein kinase 1 (PDK1) in organ-cultured rat carotid arteries stimulated with 5-hydroxytryptamine (3×10^{-5} M for 5 min). Representative Western blots for p-MYPT1/MYPT1 (**a**) and p-PDK1/PDK1 (**b**) (*upper panels*). Bands were quantified as described in the "Methods" (*lower panels*). Results are shown as the fold increase relative to control (*vehicle*). Equal protein loading was confirmed using β-actin antibody. *Each column* represents the mean±SE from eight experiments. $^{*}P<0.05$ vs. vehicle

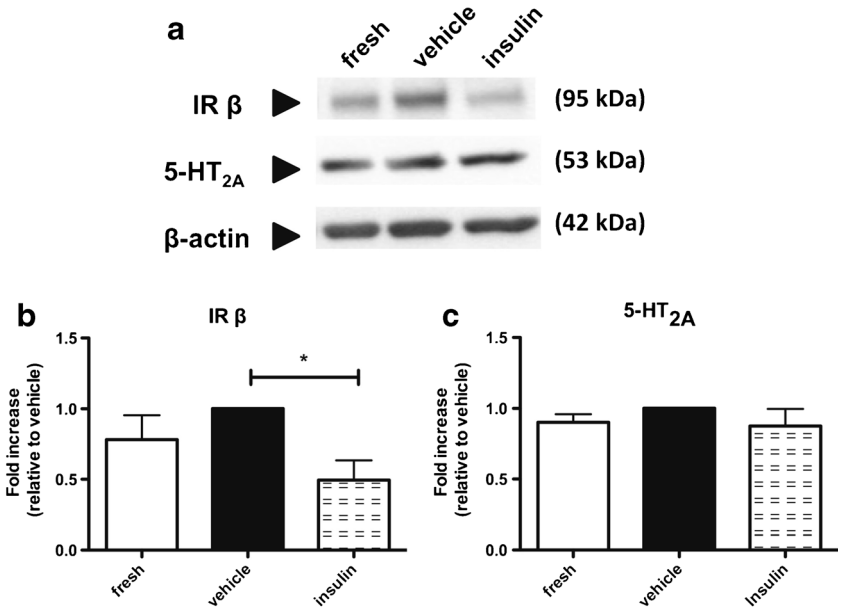

Fig. 7 Western blots for IRβ and 5-HT_{2A} receptor expressions in freshly isolated carotid arteries and vehicle- (0.01 N HCl) or insulin (1×10^{-6} M for 24 h)-cultured carotid arteries. **a** Representative Western blot is shown. **b**, **c** Corresponding densitometric analysis showing the expressions of IRβ (**b**) and 5-HT_{2A} receptor (**c**). Results are shown as the fold increase relative to control (*vehicle*). *Each column* represents the mean±SE from eight experiments. *$P<0.05$ vs. vehicle

to increased 5-HT-induced contraction similarly as exposure to high insulin levels in rat carotid arteries.

Alterations of contractile responses to endogenous ligands were often observed in the arteries of long-term models of type 2 diabetes [33–35, 37–39, 46, 57]. Other researchers [12, 19, 44, 48] and our group [36, 40] found that 5-HT-induced contraction was increased in arteries from type 2 diabetic animal models. In addition, we suggested that the enhancement of 5-HT-induced contraction was attributable to various signal transduction pathways including Src, Rho kinase, MAPKs, and PI3K pathways in type 2 diabetic arteries [36, 40]. The major hallmarks of type 2 diabetes are hyperglycemia and hyperinsulinemia. In the present study, we illustrated that prolonged exposure to high insulin levels could augment 5-HT-induced contraction in rat carotid arteries, whereas

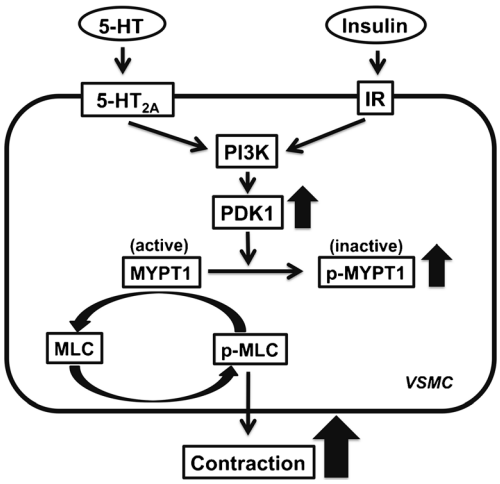

Fig. 8 Summary of the present results. Prolonged exposure of insulin increases 5-HT-induced contraction in rat carotid arteries via the activation of IR/PI3K/PDK1 pathway

high-glucose treatment did not influence this contraction. On the other hand, high insulin could not alter other constrictor U46619 (TP agonist)-induced contraction. Therefore, these results suggest that high insulin levels act as a specific amplifier of 5-HT-induced contraction and may speculate that insulin affects specific signal-transduction cascade to contract vascular smooth muscle upon stimulation of each G protein-coupled receptor.

Among the 5-HT receptor subtypes, it has been reported that 5-HT-induced arterial contractions were mainly mediated by 5-HT_{2A} receptors [67]. There was an increase in arterial contractions in response to some endogenous agonists in vascular diseases which may be associated with increase in their receptor expression. For example, Edvinsson's group clearly demonstrated that 5-HT-induced vasocontractions were associated with upregulation of 5-HT receptors (such as 5-HT_{2A}), in mesenteric arteries using an organ culture system [7, 32]. In the present study, we found that the protein expression of 5-HT_{2A} receptors did not change among freshly isolated and cultured (vehicle and insulin for 24 h) groups, suggesting that the extent of altered receptor expression (via upregulation and downregulation) did not influence the enhancement effects of insulin on 5-HT-induced contractions. Moreover, we and others previously observed that the increase in 5-HT-induced contractions in diabetic arteries was attributable to an increase in intracellular signaling rather than its receptor expression [36, 40, 48]. Therefore, we speculate that the site(s) of action resulted in enhancement of 5-HT-induced contraction by insulin that may be related to downstream molecules.

It was reported that insulin acts on various target organs including the vasculature through the activation of IR and subsequently activates multiple signaling pathways [41]. Hyperstimulation of the IR alters the balance of these signaling

pathways such as suppressing PI3K/Akt signaling and boosting MAPK signaling [24]. In the present study, the enhancement of 5-HT-induced contraction induced by prolonged insulin exposure was reduced by pretreatment with an IR antagonist and PI3K inhibitor. It was reported previously that PDK1 is one of the major downstream targets of PI3K upon insulin stimulation [2, 10, 28, 69]. We found that in carotid arteries subjected to prolonged exposure to insulin, (1) 5-HT-induced contraction was suppressed by pretreatment with a PDK1 inhibitor, and (2) the phosphorylation (activation) of PDK1 upon 5-HT stimulation was increased. Moreover, prolonged exposure to a PDK1 activator increased 5-HT-induced carotid arterial contraction similarly as high insulin levels. In addition, pretreatment with a PI3K inhibitor did not influence 5-HT-induced contraction in carotid arteries subjected to prolonged exposure to a PDK1 activator. Finally, no additive augmentation in 5-HT-induced contraction was seen in cotreatment with insulin and a PDK1 activator. These results strongly suggest that the IR/PI3K/PDK1 pathway contributes to increased 5-HT-induced contraction.

PDK1 has been implicated as a major hub of multiple signaling cascades in the regulation of various cellular processes [2, 6, 10, 15, 54, 64, 69]. In the present study, we focused on the relationship between PDK1 and MYPT1 phosphorylation because phosphorylation of MYPT1 at Thr^{853} by Rho kinase is a key process of vascular smooth muscle contraction [17, 47, 60, 65]. A novel, intriguing, and potentially important finding of the present study was that the linkage between PDK1 and MYPT1 phosphorylation is functionally present in rat carotid arteries. In the present study, we found that 5-HT-induced MYPT1 phosphorylation at Thr^{853} was increased by prolonged treatment with a PDK1 activator as well as high insulin exposure and treatment with insulin did not affect the expression of ROCK1 and ROCK2 upon 5-HT stimulation. These results suggest that the increased MYPT1 phosphorylation induced by insulin (and PDK1 activation) is independent of ROCK expression. There are few reports suggesting an interaction between PDK1 and Rho kinase. The colocalization of PDK1 and ROCK1 at the cell membrane and sustained RhoA/ROCK1 activation was observed in colorectal cancer cells [8]. Okada et al. [49] observed that chemotaxis induced by PDGF-D is mediated by the activation of the PDGF-ββ receptor/PI3K/PDK1/Akt/Rac1/ROCK pathway in malignant mesothelioma cells. Further investigations will be required on these points in the vasculature.

In conclusion, in the present study, we demonstrated that prolonged exposure of rat carotid arteries to high insulin levels enhanced 5-HT-induced contraction. Moreover, we for the first time demonstrated that PDK1 activation promotes 5-HT-induced vascular contraction. Further studies on PDK1 might contribute to the development of new pharmaceutical therapies for the prevention of type 2 diabetes-associated vascular diseases.

Acknowledgments We thank T. Adachi, K. Matsubara, Y. Noishiki, Y. Kimoto, H. Higa, M. Nagata, M. Ando, and M. Iguchi for technical assistance. This study was supported in part by JSPS KAKENHI Grant Numbers 26460107, 15K21419, and 15K07975.

Compliance with ethical standards

Conflict of interest No conflict of interest.

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