Journal of Pharmacological Sciences 144 (2020) 197–203

Contents lists available at ScienceDirect

Journal of Pharmacological Sciences

journal homepage: www.elsevier.com/locate/jphs

Full Paper

Cytoskeleton-disrupting agent cytochalasin B reduces oxidative stress caused by high glucose in the human arterial smooth muscle

Kazumi Takaishi [a], Hiroyuki Kinoshita [b, c, *], Guo-Gang Feng [c], Toshiharu Azma [d], Shinji Kawahito [b], Hiroshi Kitahata [a]

[a] Department of Dental Anesthesiology, Tokushima University Hospital, Tokushima, Japan
[b] Department of Anesthesiology, Tokushima University Hospital, Tokushima, Japan
[c] Department of Anesthesiology, Aichi Medical University School of Medicine, Aichi, Japan
[d] Department of Anesthesiology & Pain Medicine, Kohnodai Hospital, National Center for Global Health and Medicine, Ichikawa, Japan

ARTICLE INFO

Article history:
Received 14 May 2020
Received in revised form
1 August 2020
Accepted 19 August 2020
Available online 23 August 2020

Keywords:
Cytoskeleton
Glucose
Human
Oxidative stress
Vascular smooth muscle

ABSTRACT

The role of cytoskeleton dynamics in the oxidative stress toward human vasculature has been unclear. The current study examined whether the cytoskeleton-disrupting agent cytochalasin B reduces oxidative stress caused by high glucose in the human arterial smooth muscle. All experiments in the human omental arteries without endothelium or the cultured human coronary artery smooth muscle cells were performed in D-glucose (5.5 mmol/L). The exposure toward D-glucose (20 mmol/L) for 60 min reduced the relaxation or hyperpolarization to an ATP sensitive K^+ channel (KATP) opener levcromakalim (10^{-8} to 3×10^{-6} mol/L and 3×10^{-6} mol/L, respectively). Cytochalasin B and a superoxide inhibitor Tiron, restored them similarly. Cytochalasin B reduced the NADPH oxidase activity, leading to a decrease in superoxide levels of the arteries treated with high D-glucose. Also, cytochalasin B impaired the F-actin constitution and the membrane translocation of an NADPH oxidase subunit p47phox in artery smooth muscle cells treated with high D-glucose. A clinical concentration of cytochalasin B prevented human vascular smooth muscle malfunction via the oxidative stress caused by high glucose. Regulation of the cytoskeleton may be essential to keep the normal vascular function in patients with hyperglycemia.

© 2020 The Authors. Production and hosting by Elsevier B.V. on behalf of Japanese Pharmacological Society. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

1. Introduction

All cardiovascular cells express NADPH oxidases, which regulate diverse cellular functions, including differentiation, proliferation, apoptosis, senescence, inflammation, and oxygen sensing.[1] The enzymes are implicated in many cardiovascular pathologies in humans, such as diabetic cardiovascular complications, resulting from the oxidative stress caused by the activation.[1–3] Among several cytosolic NADPH oxidase subunits, the p47phox homologs play a critical role in the subunits' assembly, and thus, regulate the enzyme activation exclusively.[4,5] Our previous studies in the intact human arteries or cultured arterial smooth muscle cells found that p47phox presents significant relevance in the human vascular smooth muscle malfunction induced by the acute exposure to high concentrations of D-glucose.[2,3]

The actin polymerization resulting in the F-actin stress fiber formation has been shown to function in the migration process of human vascular smooth muscle cells.[6] Reactive oxygen species derived from NADPH oxidases are critical mediators acting on promigratory signaling pathways in the vascular smooth muscle.[7] The actin polymerization in the human neutrophil enhances the NADPH oxidase activity leading to increased superoxide production.[8,9] These results suggest the close interrelation between the actin polymerization and NADPH oxidase activation in the human vasculature. Indeed, the inhibition of actin polymerization in the cultured human vascular smooth muscle cells reduces the superoxide production mediated by the NADPH oxidase activation in terms of the p47phox subunit action via the angiotensin II signaling.[10] Whether the high glucose exposure to the human artery or arterial cells augments the F-actin constitution resulting in

* Corresponding author. Department of Anesthesiology, Institute of Biomedical Sciences, Tokushima University Graduate School, 3-18-15 Kuramoto, Tokushima, Tokushima, 770-8503, Japan. Fax: +81 88 633 7182.
E-mail address: hkinoshi@krc.biglobe.ne.jp (H. Kinoshita).
Peer review under responsibility of Japanese Pharmacological Society.

https://doi.org/10.1016/j.jphs.2020.08.004
1347-8613/© 2020 The Authors. Production and hosting by Elsevier B.V. on behalf of Japanese Pharmacological Society. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

the augmentation of NADPH oxidase activity simultaneously has never been determined.

The regulation targeted to the cytoskeleton of vascular smooth muscle cells, resulting in vascular tissue growth reduction as a means of suppressing the intimal hyperplastic response, is a potential pharmacological tool to treat patients with cardiovascular disorders.[11,12] Of these, cytochalasin B is a cytoskeleton-disrupting agent, which dose of just below 10^{-5} mol/L is clinically useful to prevent coronary stenosis after the intervention.[12] Whether the clinical concentration of cytochalasin B prevents oxidative stress of human vascular smooth muscle is increasingly critical since the superoxide produced by NADPH oxidase aggravates the proliferative response in human blood vessels.[13]

Therefore, the current study was conducted to determine if the clinical concentration of a cytoskeleton-disrupting agent impairs oxidative stress caused by the high glucose condition in the human vascular smooth muscle. We employed our high D-glucose-induced oxidative stress model to examine the human vascular smooth muscle function using the human omental artery without endothelium, and cultured human coronary artery smooth muscle cells (HCASMCs).[2,3]

2. Material and methods

The human research committees at Aichi Medical University (Aichi, Japan) approved the present study of the human arteries (No. 12-010), and the study was conducted following the Declaration of Helsinki. The written informed consent was obtained from all of the patients involved in the current study.

2.1. Tissue and cells preparation

Human omental arteries (0.5–1.0 mm in diameter) were obtained from 25 patients (15 males and 10 females of 40–66 years), who were scheduled for elective gastric cancer surgery. The patients were without heart disease, diabetes mellitus, hypertension, hypercholesterolemia, smoking habit, and therapy with anti-cancer agents. The omental arteries without endothelium were examined in the Krebs–Ringer bicarbonate solution (the control Krebs–Ringer solution, pH 7.4) bubbled with a 95% O_2 − 5% CO_2 gas mixture; the solution had the following composition (mmol/L): NaCl 119, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.17, KH_2PO_4 1.18, $NaHCO_3$ 25, and glucose 5.5. Endothelial cells of the arterial rings were removed by rubbing the lumen with a needle insertion to avoid the modification mediated by endothelium-derived vasodilator substances.[2]

HCASMCs purchased from KURABO INDUSTRIES LTD., Osaka, Japan, were cultured and propagated in the specialized growth medium (medium A) supplemented with 5% fetal bovine serum, human epidermal growth factor (0.5 ng/mL), human fibroblast growth factor-B (2 ng/mL), insulin (5 μg/mL), gentamicin (50 μg/mL), and amphotericin B (50 ng/mL). Cells were grown in the humidified incubator insufflated with a 95% air − 5% CO_2 gas mixture at 37 °C.

All experimental protocols were performed in the existence of D-glucose (5.5 mmol/L) as the control condition.[2,14,15] Also, L-glucose (20 mmol/L) was employed to adjust the osmolarity, although our previous studies demonstrated a negligible role of the high osmolarity caused by D-glucose (20 mmol/L) in the human vascular smooth muscle function.[2,14,15]

2.2. Organ chamber experiments

Each omental artery (3-mm ring) without endothelium was suspended in an organ chamber filled with the control Krebs–Ringer solution (10 mL, 37 °C) bubbled with a 95% O_2 − 5% CO_2 gas mixture. The ring was connected to an isometric force transducer and stretched gradually to the optimal resting tension approximately at 1.0 g as determined by the contraction to a prostaglandin H_2/thromboxane receptor agonist U46619 (3×10^{-8} mol/L). The absence of relaxation in response to bradykinin (10^{-6} mol/L) verified the removal of endothelial cells in each ring.[2,14,15] Some rings were incubated for 60 min with L-glucose (20 mmol/L, $n = 15$), L-glucose (20 mmol/L) in combination with an ATP sensitive K^+ channel antagonist glibenclamide (10^{-6} mol/L, $n = 5$) or the cytoskeleton-disrupting agent cytochalasin B (3×10^{-6} mol/L, $n = 5$), D-glucose (20 mmol/L, $n = 15$), or D-glucose in combination with a superoxide inhibitor Tiron (10 mM, $n = 5$) or cytochalasin B (3×10^{-6} mol/L, $n = 5$). Control rings, however, did not receive any additional compound ($n = 5$). The concentration–response curves to an ATP-sensitive K^+ channel opener levcromakalim (10^{-8} to 3×10^{-6} mol/L) or a Ca^{2+} channel antagonist diltiazem (10^{-7} to 10^{-4} mol/L) were, after that, obtained during submaximal contraction to U46619 (3×10^{-8} mol/L).[2,14,15] The relaxation was expressed as a percentage of the maximal relaxation in response to papaverine (3×10^{-4} mol/L).[2,14,15]

2.3. Electrophysiological experiments

Each omental arterial ring was longitudinally cut and fixed on the bottom of an experimental chamber and perfused with the control Krebs–Ringer solution (10 mL, 37 °C) bubbled with a 95% O_2 − 5% CO_2 gas mixture. Some arterial segments were incubated for 60 min with L-glucose (20 mmol/L, $n = 5$), L-glucose (20 mmol/L) in combination with glibenclamide (10^{-6} mol/L, $n = 5$), D-glucose (20 mmol/L, $n = 5$), or D-glucose in combination with Tiron (10 mmol/L, $n = 5$), a superoxide inhibitor apocynin (1 mmol/L)[16] or cytochalasin B (3×10^{-6} mol/L, n = 5). A glass microelectrode (the tip resistance 40–80 MΩ) filled with KCl (3 mol/L) was, after that, inserted into a smooth muscle cell from the intimal side of the arterial segment.[2,14,15] The electrical signal was amplified (Electro 705™, World Precision Instruments Inc., FL), and the obtained membrane potential was monitored and recorded continuously (SS-250F-1™, SENKONIC Inc., Tokyo, Japan). Successful impalement was defined as a sudden negative shift, followed by a stable negative voltage for more than 2 min.[2,14,15] Levcromakalim (3×10^{-6} mol/L) was added subsequently to the experimental chamber, and the changes in membrane potentials were recorded continuously.

2.4. Measurements of in situ superoxide production and NADPH oxidase activity

Each omental arterial ring (5 mm in length) without endothelium was incubated in an experimental chamber and perfused with the control Krebs–Ringer solution (10 mL, 37 °C) bubbled with a 95% O_2 − 5% CO_2 gas mixture. The arterial rings were incubated for 60 min with L-glucose (20 mmol/L, n = 12), D-glucose (20 mmol/L, n = 13), or D-glucose in combination with Tiron (10 mmol/L, n = 6) or cytochalasin B (3×10^{-6} mol/L, n = 13) and were frozen at −80 °C immediately after the incubation. Twenty-micrometer unfixed sections of arteries were cut on a cryostat and mounted onto microscope slides. The slides in a light-protected chamber at 37 °C were further incubated with an oxidative fluorescent dye hydroethidine (2×10^{-6} mol/L) in PBS (1×, pH 7.4) for 20 min to obtain the semi-quantitative levels of superoxide in situ.[15,17] Hoechst 33258 (1 μg/mL) was simultaneously applied to stain nuclei of vascular cells. Images were acquired using a microscope fitted with BZ-II analyzer software (Model BZ-9000 Generation II, Keyence, Osaka, Japan). The settings were identical for the acquisition of images from all slides. The total hydroethidine

fluorescence determined in each specimen was standardized by making a ratio with the control fluorescence in the presence of Tiron (10 mmol/L) for arterial slices.[2,15]

We substituted the NADPH-dependent superoxide production detected spectrophotometrically using the cytochrome-c reduction rate for the NADPH oxidase activity.[18,19] Human omental arteries without endothelium stocked at −80 °C were defrosted at 25 °C and rinsed three times with the Krebs–Ringer HEPES. Each vessel was incubated with L-glucose (20 mmol/L, n = 6), D-glucose (20 mmol/L, n = 6), or D-glucose in combination with cytochalasin B (3×10^{-6} mol/L, n = 6) for 60 min at 37 °C. After the incubation, the vessel was minced and immersed in a 200-μl portion of protease inhibitor cocktail consisting of cOmplete, EDTA-free (Roche CustomBiotech, Tokyo, Japan) dissolved in sucrose (150 mmol/L) and buffer-A (sodium phosphate [50 mmol/L] and EGTA (1 mmol/L, pH 7.0)). The tissue from each artery on the ice was pestled by a homogenizer with an electric mixer (BioMasher II, Nippi, Tokyo, Japan) and further had two 15-s cycles of auto power control sonication (10–50 W) for the disruption. The nuclear fraction and debris were removed from the disrupted tissue by the centrifugation at 800×*g* for 5 min. The protein concentration of the arterial cell membrane fraction (i.e., the supernatant) was determined using a Coomassie Plus Protein Assay Reagent Kit (Pierce Biotechnology, Rockford, IL). The superoxide production was determined as a part of the cytochrome-c reduction that is abolished by adding superoxide dismutase (3000 U/mL, Sigma Aldrich Inc., St. Louis, MO, USA).[20] A 20-μg portion of the arterial smooth muscle cell membrane fraction (the supernatant) in the prefinal mixture (total 320 μl) containing the oxidized form cytochrome-c (10^{-4} mol/L), NaN3 (2 mmol/L), FAD (10^{-5} mol/L), GTPγS (10^{-5} mol/L), and L-NAME (1 mmol/L) dissolved in the buffer-A was set on the spectrophotometer (Genesys 10S Bio, Thermo Scientific, Waltham, MA) and warmed at 25 °C. The basal absorbance at a 550-nm wavelength of the prefinal mixture was monitored spectrophotometrically for 5 min. An 80-μl aliquot of NADPH (0.5 mmol/L) in NaOH (4 mmol/L, 0.02 N) was added to the prefinal mixture to complete the final reaction mixture (total 400 μl), and immediately after, the absorbance of the mixture at 550 nm was determined for 10 min continuously. The reduction rate of cytochrome-c was calculated using the extinction coefficient 21.

2.5. Immunohistochemical analysis

The omental arterial ring (5 mm in length) without endothelium was incubated in an experimental chamber and perfused with the control Krebs–Ringer solution (10 mL) bubbled with a 95% O_2 − 5% CO_2 gas mixture at 37 °C. The rings were incubated for 60 min with L-glucose (20 mmol/L, n = 5), D-glucose (20 mmol/L, n = 5), or D-glucose in combination with cytochalasin B (3×10^{-6} mol/L, n = 5) and were subsequently immersed in 4% paraformaldehyde overnight at 4 °C.[21,22] Twenty-μm-thick coronal sections of the fixed artery were cut on a cryostat, mounted onto microscope slides and dried at 37 °C for 3 h. The sections were exposed to PBS with 3% bovine serum albumin in combination with 0.05% Triton X-100 at 24 °C for 60 min and further incubated for 24 h in the dark with the Alexa Fluor 488 Phalloidin (5 U/mL; Thermo Fisher Scientific Corp., Carlsbad, CA, USA). Images of cellular fluorescence were acquired using a microscope fitted with BZ-II analyzer software (Model BZ-9000 Generation II, Keyence, Osaka, Japan). Settings were adjusted based on the fluorescence intensity in tissues from the L-glucose group and were identical for the acquisition of images from all of the arterial tissues. The negative control did not show any nonspecific staining. The total F-actin green fluorescence was determined by subtracting that of the background in each specimen. Five fields of view were analyzed using five sections from different arteries.

2.6. Western immunoblotting analysis

Confluent HCASMCs in passages three to seven were set at the 60-mm tissue culture dishes to make quiescent for 2 h in low-serum medium containing 0.5% FBS (Neves 2018). HCASMCs were incubated for 60 min with the addition of L-glucose (20 mmol/L), D-glucose (20 mmol/L), or D-glucose (20 mmol/L) in combination with cytochalasin B (3×10^{-6} mol/L) to the medium containing D-glucose (5.5 mmol/L) insufflated with a 95% air − 5% CO_2 gas mixture at 37 °C. After the incubation, HCASMCs were washed with cold phosphate-buffered saline (−) solution (the control PBS solution, 1×, pH 7.4) and dissociated with trypsin/ethylenediamine tetraacetic acid solution. After that, the vascular smooth muscle cells were quickly frozen at −80 °C. Membranous fractions were prepared and used for western immunoblotting analysis.[23] The specimens, which were stored at −80 °C until use, were powdered under liquid nitrogen and dissolved in 300 μL cell permeabilization buffer containing 1% protease inhibitor. A portion of the supernatant fluid was centrifuged at 100,000×*g* for 60 min at 4 °C, and the pellet was used as the membrane fraction. Membranous fractions were prepared according to the protocol of Cell Fraction Kit (Cell Signaling Technology, Inc., Danvers, MA, USA). The protein concentrations in the membrane fraction were estimated by the BCA protein assay (Thermo Fisher Scientific Inc., Rockford, IL, USA). The same amount of protein was separated by SDS–PAGE and transferred to nitrocellulose membranes (Cell Signaling Technology, Inc., Danvers, MA, USA). These membranes were assessed with antibodies against p47phox (1:250 dilution; Santa Cruz Biotechnology, Dallas, TX, USA) and Na^+/K^+ ATPase (1:1000 dilution; Cell Signaling Technology, Inc., Danvers, MA, USA) for the membrane fraction. The results were quantified according to the expression level of Na^+/K^+ ATPase using the Image Analyzer System (Amersham Imager 600, GE Healthcare UK Ltd., Pollards Wood, UK).

2.7. Drugs

The following pharmacological agents were used: apocynin, bradykinin, diltiazem, dimethyl sulfoxide, glibenclamide, D-glucose, L-glucose, levcromakalim, papaverine and Tiron U46619 (Sigma Aldrich Inc., St. Louis, MO, USA), cytochalasin B (FUJIFILM Wako Pure Chemical Co., Osaka, Japan), and hydroethidine (Polysciences Inc., Warrington, PA, USA). Drugs were dissolved in distilled water such that volumes of <60 mL are added to the perfusion system. The stock solution of Tiron was prepared in dimethyl sulfoxide, and the highest concentration of dimethyl sulfoxide was 1.74×10^{-6} mol/L. Our preliminary confirmed that this vehicle does not affect the vasomotor function in our experimental condition.[24] The concentrations of drugs are expressed as the final molar concentration.

2.8. Statistical analysis

Statistical analysis was performed using PASW Statistics 18 (IBM Japan Inc., Tokyo, Japan). The continuous variables are expressed as mean ± SD, and the data were analyzed by nonparametric statistical tests, including the Mann–Whitney and Kruskal–Wallis tests. Differences were considered to be statistically significant when P was <0.05. The current study protocol employed previous studies to estimate sample sizes,[2,21] that is, not formal a priori power analysis, and sample size projection was performed. A 21% difference in the human omental arterial relaxation to levcromakalim (10^{-6} mol/L) gave 83% power to detect at a significance level of 0.05

with the sample size of 5 (SD = 10). The power calculation was done using Sample Power 3.0™ (IBM Japan Inc., Tokyo, Japan).

3. Results

3.1. Organ chamber experiments

D-glucose (20 mmol/L), but not L-glucose (20 mmol/L), attenuated the vasorelaxation in response to levcromakalim (10^{-8} – 3×10^{-6} mol/L) compared with the condition without L-glucose (Fig. 1A). The incubation with glibenclamide (10^{-6} mol/L) abolished the relaxation (Fig. 1A). The addition of cytochalasin B (3×10^{-6} mol/L) or Tiron (10 mmol/L) to D-glucose (20 mmol/L) restored the levcromakalim-induced relaxation similarly. However, cytochalasin B did not alter the relaxation to levcromakalim with L-glucose (20 mmol/L, Fig. 1B). The contraction in response to U46619 (3×10^{-8} mol/L) did not differ among L-glucose (20 mmol/L), D-glucose (20 mmol/L), L-glucose (20 mmol/L) plus cytochalasin B (3×10^{-6} mol/L), and D-glucose (20 mmol/L) plus cytochalasin B (3×10^{-6} mol/L) groups (2.4 ± 0.4, 2.1 ± 0.5, 1.8 ± 0.5, and 2.0 ± 0.5 g, respectively, $P = 0.294$). Also, D-glucose (20 mmol/L), compared with L-glucose (20 mmol/L) did not alter the vasodilation to diltiazem (Fig. 1C).

3.2. Electrophysiological experiments

The incubation with D-glucose (20 mmol/L) reduced the vascular smooth muscle hyperpolarization in response to levcromakalim (3×10^{-6} mol/L), and glibenclamide (10^{-6} mol/L) abolished it. However, cytochalasin B (3×10^{-6} mol/L), apocynin (1 mmol/L), and Tiron (10 mmol/L) restored it in the arterial segment treated with D-glucose (20 mmol/L) similarly (Fig. 2).

3.3. Measurements of in situ superoxide production and NADPH oxidase activity

The exposure of D-glucose (20 mmol/L) to the arterial segment enhanced ethidium bromide fluorescence, indicating the levels of in situ superoxide, compared with the incubation with L-glucose (20 mmol/L; Fig. 3B). Both cytochalasin B (3×10^{-6} mol/L) and Tiron (10 mmol/L) similarly abolished the increased levels seen in the artery incubated with D-glucose (20 mmol/L) (Fig. 3A and B).

The incubation of the arterial segment with D-glucose (20 mmol/L) enhanced the NADPH oxidase activity, whereas the addition of cytochalasin B (3×10^{-6} mol/L) to D-glucose (20 mmol/L) reduced the increment (Fig. 3C).

3.4. Immunohistochemical analysis

The exposure of D-glucose (20 mmol/L) to the arterial segment increased the F-actin constitution (Fig. 4). The addition of cytochalasin B (3×10^{-6} mol/L) to D-glucose (20 mmol/L) inhibited the F-actin constitution in the arterial segment (Fig. 4).

3.5. Western immunoblotting analysis

The incubation with D-glucose (20 mmol/L), but not L-glucose (20 mmol/L), augmented the membrane levels of p47phox protein in the HCASMCs whereas the addition of cytochalasin B

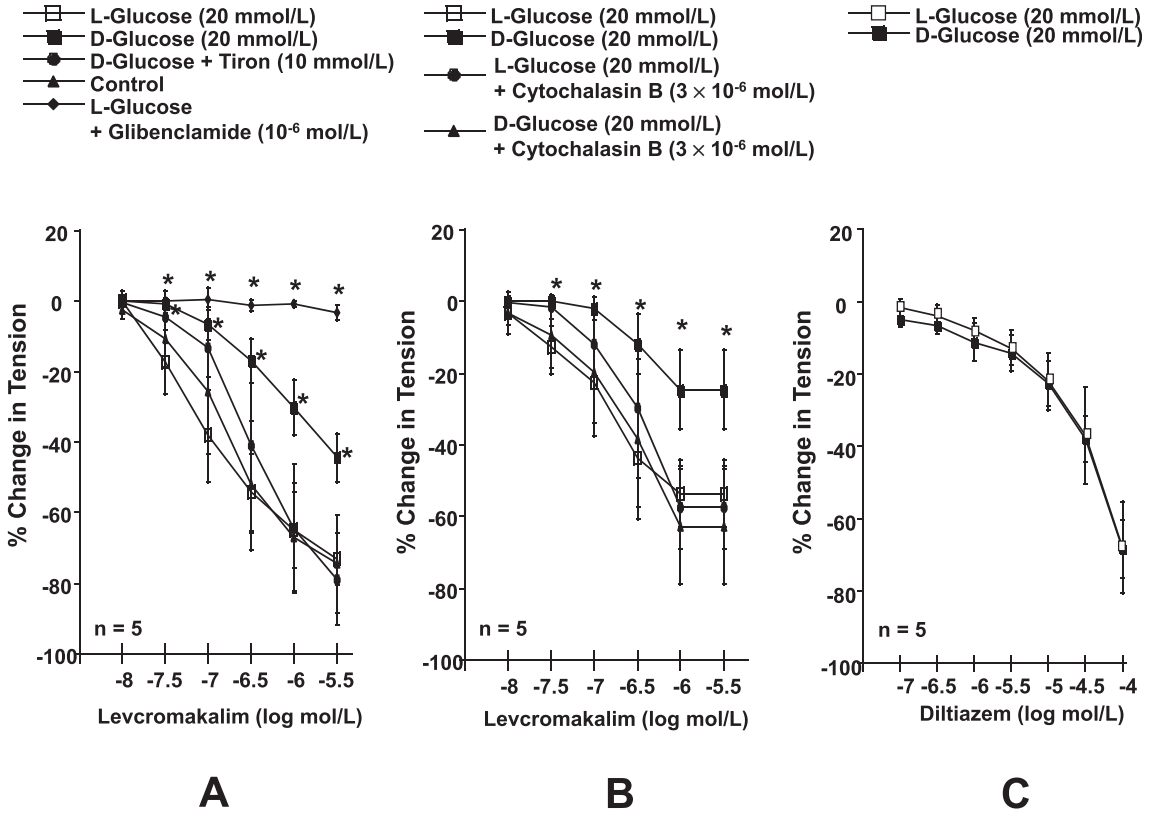

Fig. 1. **(A)** Levcromakalim-induced relaxation of the human omental arterial rings, in the presence or absence of L-glucose (20 mmol/L), D-glucose (20 mmol/L), Tiron (10 mmol/L), glibenclamide (10^{-6} mol/L), or the combination. **(B)** Levcromakalim-induced relaxation of the human omental arterial rings, in the presence of L-glucose (20 mmol/L), D-glucose (20 mmol/L), cytochalasin B (3×10^{-6} mol/L) or the combination. **(C)** Diltiazem-induced relaxation of the human omental arterial rings, in the presence of L-glucose (20 mmol/L) or D-glucose (20 mmol/L). Differences between the control rings and rings treated with D-glucose and glibenclamide in the graph A and differences between rings treated with L-glucose and those treated with D-glucose in the graphs A and B are statistically significant (*: $P < 0.05$).

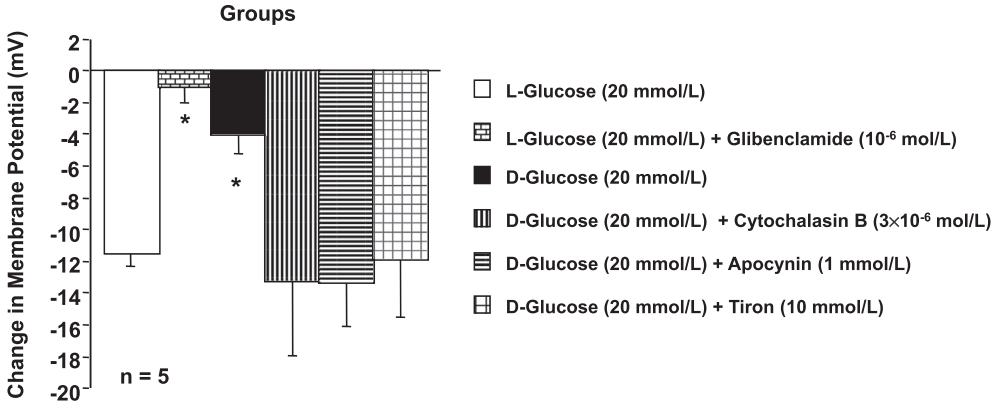

Fig. 2. Changes in the membrane potential caused by levcromakalim (3×10^{-6} mol/L) in the human omental arterial segment without endothelium incubated with L-glucose (20 mmol/L), L-glucose in combination with glibenclamide (10^{-6} mol/L), D-glucose (20 mmol/L), or D-glucose combined with cytochalasin B (3×10^{-6} mol/L), apocynin (1 mmol/L), or Tiron (10 mmol/L) for 60 min *Difference between arterial segments treated with L-glucose and those treated with D-glucose or L-glucose in combination with glibenclamide is statistically significant (*: $P < 0.05$).

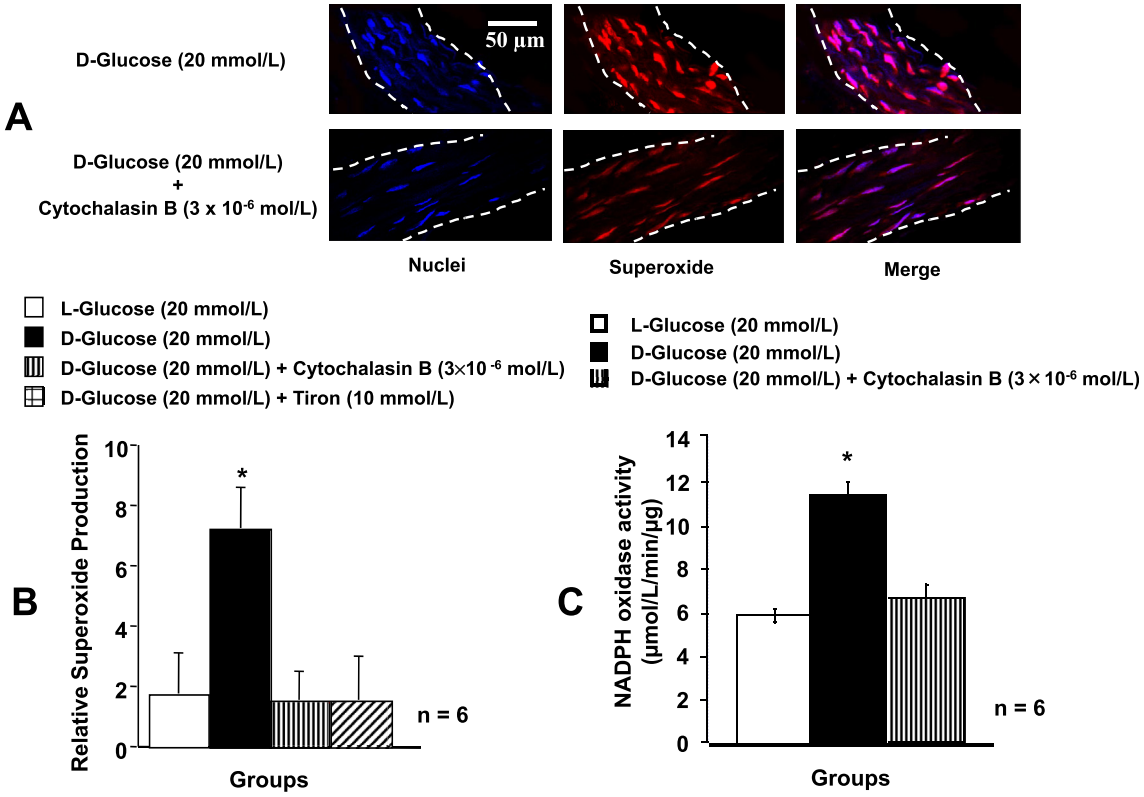

Fig. 3. **(A)** Representative images of in situ superoxide (the red fluorescence) and nuclei (the blue fluorescence) in the omental arterial segment incubated with D-glucose (20 mmol/L) or D-glucose in combination with cytochalasin B (3×10^{-6} mol/L) for 60 min. White dots indicate the outer margins of the artery without endothelium. **(B)** Relative superoxide levels in the omental arterial segment incubated with L-glucose (20 mmol/L), D-glucose (20 mmol/L), or D-glucose in combination with cytochalasin B (3×10^{-6} mol/L) or Tiron (10 mmol/L) for 60 min. The total hydroethidine fluorescence ratio was used for the semiquantitative superoxide. *Difference between the arterial segments treated with L-glucose and those treated with D-glucose is statistically significant (*: $P < 0.05$). **(C)** Relative NADPH oxidase activity in the omental arterial smooth muscle tissues incubated with L-glucose (20 mmol/L), D-glucose (20 mmol/L), or D-glucose in combination with cytochalasin B (3×10^{-6} mol/L) for 60 min *Difference between the arterial smooth muscle tissues treated with L-glucose and those treated with D-glucose is statistically significant (*: $P < 0.05$). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

(3×10^{-6} mol/L) to D-glucose (20 mmol/L) inhibited the protein expression of the NADPH oxidase subunit (Fig. 5A and B).

4. Discussion

The current study documented that the acute D-glucose, but not L-glucose, exposure for 60 min attenuated the relaxation and hyperpolarization in response to ATP-sensitive K^+ channel activation in the human omental artery smooth muscle cells and that the reduction resulted from the enhanced superoxide production via the NADPH oxidase activation, consistently with our previous studies.[2,3,14,15] We are unknown why only D-glucose is a source of oxidative stress in our experimental condition. Nevertheless, L-glucose is not available as a substrate for the higher animals, and

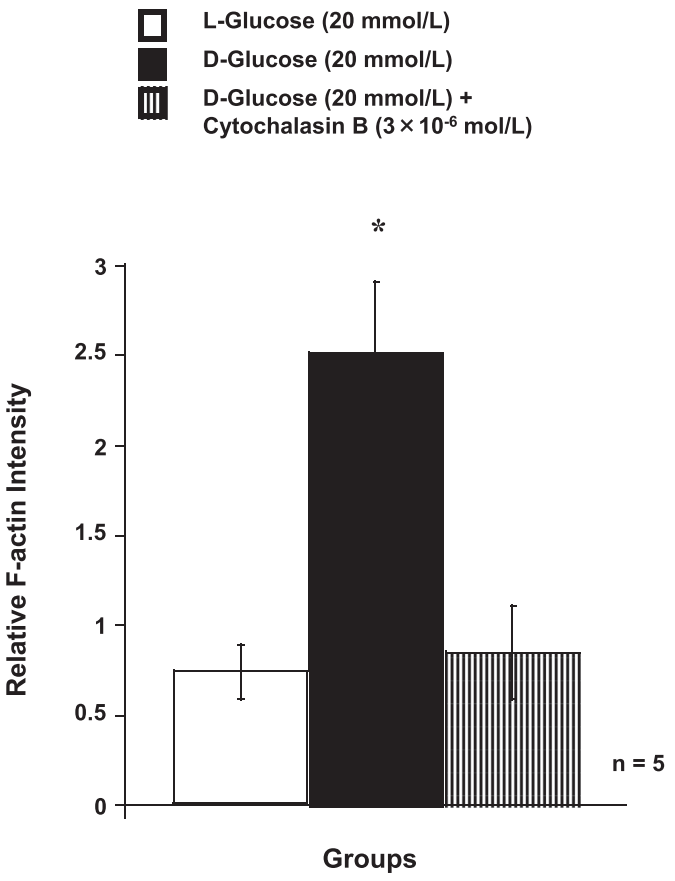

Fig. 4. Relative F-actin constitution in the omental arterial segment incubated with L-glucose (20 mmol/L), D-glucose (20 mmol/L), or D-glucose in combination with cytochalasin B (3×10^{-6} mol/L) for 60 min *Difference between the arterial segments treated with L-glucose and those treated with D-glucose is statistically significant (*: $P < 0.05$).

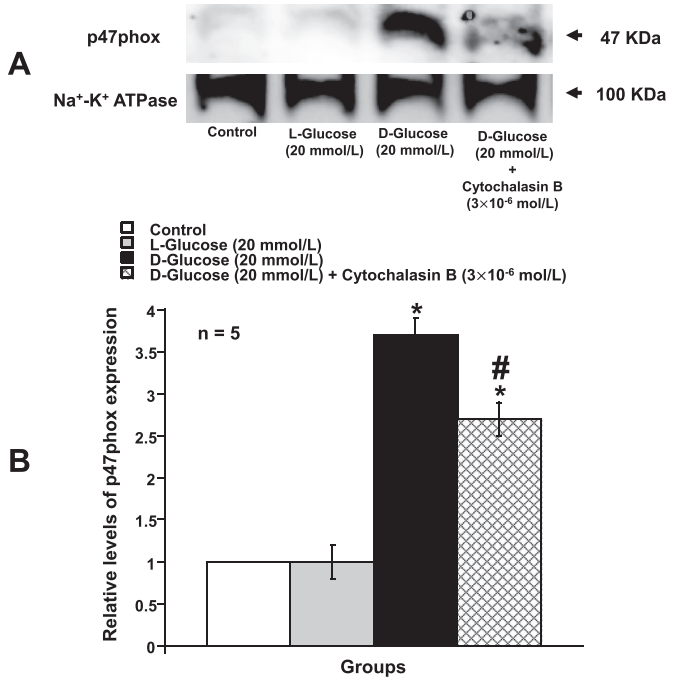

Fig. 5. Representative images **(A)** and the bar graph **(B)** of the protein expression of p47phox compared with Na^+/K^+ ATPase in the membrane fraction of the cultured HCASMCs. The results were quantified based on the expression level of Na^+/K^+ ATPase. The differences between the control HCASMCs and the cells treated with D-glucose (20 mmol/L) or D-glucose in combination with cytochalasin B (3×10^{-6} mol/L) are statistically significant (*: $P < 0.05$). Also, the difference between the cells treated with D-glucose and those with D-glucose combined with cytochalasin B is statistically significant (#: $P < 0.05$).

thus, it might explain the difference. The enzymatic activation coincided with the p47phox membrane translocation, confirming the cytosolic subunit's critical role in the oxidative stress caused by high D-glucose in the human artery smooth muscle cells.[2,3] Indeed, the p47phox homologs regulate the subunits' assembly among several cytosolic NADPH oxidase subunits upon the NOX1 or NOX2 subtype enzyme activation induced by many pathological stimuli including high D-glucose.[2,4,5,10,25]

tThe actin polymerization leading to the F-actin stress fiber formation has been shown to play roles in the vascular smooth muscle pathophysiological processes, including migration and remodeling.[6,26] Previous studies on the cultured animal vascular smooth muscle cells documented that the prolonged incubation with high concentrations of D-glucose (25–45 mmol/L for 24 h to six weeks) increases the F-actin constitution, suggesting that the actin polymerization mediates the vascular smooth muscle pathology in the glucose intolerance.[27,28] We have first demonstrated in the isolated human vascular smooth muscle that the short-term high glucose exposure (25.5 mmol/L for 60 min) enhances the actin polymerization, inhibited by a cytoskeleton-disrupting agent cytochalasin B.[11] In the current study, treatment with the disrupting agent prevented the vascular smooth muscle malfunction in which the acute exposure to high glucose reduced ATP-sensitive K^+ channel-mediated vasorelaxation. These results indicate that the modulation of F-actin dynamics contributes to the maintenance of the normal vascular smooth muscle function in humans.

Cytochalasin B reduced the NADPH oxidase activity coincided with the inhibition of the subunit p47phox membrane expression, leading to a decrease in superoxide levels of the human arteries treated with high glucose. These results suggest that the interrelation between the actin polymerization and NADPH oxidase activation plays a critical role in the oxidative stress caused by acute high glucose in humans. How the actin polymerization activates NADPH oxidase, has been unclear yet. A previous study on the cultured human vascular smooth muscle cells documented the association among p47phox, F-actin, and an actin-binding protein cortactin upon the exposure toward angiotensin II, and the results suggest a possible role of the actin-binding protein in the NADPH oxidase activation.[10] However, further studies will require to confirm the involvement of various actin-binding proteins, including cortactin, ADF, and cofilin in the p47phox activation caused by high glucose.[29]

The possible nonselective effect of cytochalasin B used as the cytoskeleton-disrupting agent has to be discussed. Cytochalasin B is also known as an inhibitor targeting glucose transporter (GLUT),[30] and thus, it may reduce the high glucose-induced oxidative stress by its intracellular glucose concentration lowering effect via the GLUTs inhibition. Indeed, GLUTs 1–4 have established roles as the glucose transporter in various tissues and cell types, including blood vessels.[31,32] However, the cytochalasin B effect as a GLUTs inhibitor is unlikely in the current study since the transporter inhibitor should only play a role in the condition with insulin, which is capable of activating the glucose transportation.[32] Recent studies have documented that the cytoskeletal remodeling resulting from actin polymerization associated with prolonged vasoconstriction, indicating a critical role of F-actin formation in the pathogenesis of hypertension.[33] Indeed, the concentrations of a cytoskeleton-

disrupting agent, cytochalasin B equal and higher than 10^{-5} mol/L are known to reduce vasoconstriction,[11,34] and the dose of 10^{-5} mol/L is the highest limit to administer locally for patients to prevent coronary stenosis after the intervention.[12] Therefore, we selected 3×10^{-6} mol/L cytochalasin B in the current study since it appears to be the highest clinical dose, which does not affect the vasoconstriction. As we expected, the concentration did not alter the human arterial contraction caused by the thromboxane receptor activation, indicating that the acute cytoskeleton modulation by the concentration of cytochalasin B probably does not affect the human vasoconstrictor response.

In conclusion, a clinical concentration of the cytoskeleton-disrupting agent cytochalasin B prevents the acute high glucose-induced human vascular smooth muscle malfunction caused by oxidative stress originated from superoxide production to be involved with the NADPH oxidase activation. Regulation of the cytoskeleton may be essential to keep the normal vascular smooth muscle function in patients with hyperglycemia.

Authors' contribution

Kazumi Takaishi, D.D.S., Ph.D. collected the data and prepared the manuscript; Hiroyuki Kinoshita, M.D., Ph.D. designed the study, collected the data, performed the analysis, and prepared the manuscript; Guo-Gang Feng, M.D., Ph.D., and Toshiharu Azma, M.D., Ph.D. collected the data; Shinji Kawahito, M.D., Ph.D., and Hiroshi Kitahata, M.D., Ph.D. performed the analysis.

Declaration of competing interest

None.

Acknowledgments

This work was presented in part at the annual meetings of the American Society of Anesthesiologists, San Francisco, CA, October 13-17. 2018. This work was supported in part by Grant-in-Aid, 17K11910 (Kazumi Takaishi), 18K08873 (Hiroyuki Kinoshita), 17K11082 (Shinji Kawahito), and 17K11909 (Hiroshi Kitahata) for Grant-in Aid, Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan, Tokyo, Japan.

References

1. Lassègue B, Martin AS, Griendling KK. Biochemistry, physiology, and pathophysiology of NADPH oxidases in the cardiovascular system. *Circ Res*. 2012;110:1364–1390.
2. Kinoshita H, Matsuda N, Kaba H, et al. Roles of PI3K-Akt and NADPH oxidase in ATP-sensitive K^+ channel function impaired by high glucose in the human artery. *Hypertension*. 2008;52:507–513.
3. Kinoshita H, Watanabe K, Azma T, et al. Human serum albumin and oxidative stress in preeclamptic women and the mechanism of albumin for stress reduction. *Heliyon*. 2017;3, e00369. https://doi.org/10.1016/j.heliyon.2017.e00369.
4. Marcoux JM, Man P, Petit-Haertlein I, et al. $p47^{phox}$ molecular activation for assembly of the neutrophil NADPH oxidase complex. *J Biol Chem*. 2010;285:28980–28990.
5. Brandes RP, Weissmann N, Schröder K. Nox family NADPH oxidases: molecular mechanisms of activation. *Free Radic Biol Med*. 2014;76:208–226.
6. Kundumani-Sridharan V, Singh NK, Kumar S, Gadepalli R, Rao GN. Nuclear factor of activated T cells c1 mediates p21-activated kinase 1 activation in the modulation of chemokine-induced human aortic smooth muscle cell F-actin stress fiber formation, migration, and proliferation and injury-induced vascular wall remodeling. *J Biol Chem*. 2013;288:22150–22162.
7. San Martin A, Griendling KK. Redox control of vascular smooth muscle migration. *Antioxid Redox Signal*. 2010;12:625–640.
8. Morimatsu T, Kawagoshi A, Yoshida K, Tamura M. Actin enhances the activation of human neutrophil NADPH oxidase in a cell-free system. *Biochem Biophys Res Commun*. 1997;230:206–210.
9. Bengtsson T, Orselius K, Wetterö J. Role of the actin cytoskeleton during respiratory burst in chemoattractant-stimulated neutrophils. *Cell Biol Int*. 2006;30:154–163.
10. Touyz RM, Yao G, Quinn MT, Pagano PJ, Schiffrin EL. p47phox associates with the cytoskeleton through cortactin in human vascular smooth muscle cells: role in NAD(P)H oxidase regulation by angiotensin II. *Arterioscler Thromb Vasc Biol*. 2005;25:512–518.
11. Boer C, van Nieuw Amerongen GP, Groeneveld ABJ, et al. Smooth muscle F-actin disassembly and rhoA/rho-kinase signaling during endotoxin-induced alterations in pulmonary arterial compliance. *Am J Physiol*. 2004;287:L649–L655.
12. Lehmann KG, Popma JJ, Werner JA, Lansky AJ, Wilensky RL. Vascular remodeling and the local delivery of cytochalasin B after coronary angioplasty in humans. *J Am Coll Cardiol*. 2000;35:583–591.
13. West NEJ, Guzik TJ, Black E, Channon KM. Enhanced superoxide production in experimental venous bypass graft intimal hyperplasia: role of NAD(P)H oxidase. *Arterioscler Thromb Vasc Biol*. 2001;21:189–194.
14. Kinoshita H, Azma T, Nakahata K, et al. Inhibitory effect of high concentration of glucose on relaxations to activation of ATP-sensitive K^+ channels in human omental artery. *Arterioscler Thromb Vasc Biol*. 2004;24:2290–2295.
15. Kinoshita H, Azma T, Iranami H, et al. Synthetic peroxisome proliferator-activated receptor-γ agonists restore impaired vasorelaxation via ATP-sensitive K^+ channels by high glucose. *J Pharmacol Exp Ther*. 2006;318:312–318.
16. Heumüller S, Wind S, Barbosa-Sicard E, et al. Apocynin is not an inhibitor of vascular NADPH oxidases but an antioxidant. *Hypertension*. 2008;51:211–217.
17. Miller FJ, Gutterman DD, Rios CD, Heistad DD, Davidson BL. Superoxide production in vascular smooth muscle contributes to oxidative stress and impaired relaxation in atherosclerosis. *Circ Res*. 1998;82:1298–1305.
18. Moriguchi T, Seres T, Ravichandran V, Sasada M, Johnston Jr RB. Diamide primes neutrophils for enhanced release of superoxide anion: relationship to S-thiolation of cellular proteins. *J Leukoc Biol*. 1996;60:191–198.
19. O'Brien WJ, Heimann T, Rizvi F. NADPH oxidase expression and production of superoxide by human corneal stromal cells. *Mol Vis*. 2009;15:2535–2543.
20. Az-ma T, Saeki N, Yuge O. Cytosolic Ca^{2+} movements of endothelial cells exposed to reactive oxygen intermediates: role of hydroxyl radical-mediated redox alteration of cell-membrane Ca^{2+} channels. *Br J Pharmacol*. 1999;126:1462–1470.
21. Yasuda Y, Feng GG, Li J, et al. High oxygen modifies vasodilator effect of cysteine via enhanced oxidative stress and thromboxane production in the rat mesenteric artery. *Pflugers Arch*. 2016;468:1555–1564.
22. Nakamura E, Kinoshita H, Feng G-G, et al. Sevoflurane inhalation accelerates the long-term memory consolidation via small GTPase overexpression in the hippocampus of mice in adolescence. *PLoS One*. 2016;11, e0163151. https://doi.org/10.1371/journal.pone.0163151.
23. Yamamoto Y, Saito T, Feng G-G, et al. Intermittent local periodontal inflammation causes endothelial dysfunction of the systemic artery via increased levels of hydrogen peroxide concomitantly with overexpression of superoxide dismutase. *Int J Cardiol*. 2016;222:901–907.
24. Haba M, Kinoshita H, Matsuda N, et al. Beneficial effect of propofol on arterial adenosine triphosphate-sensitive K^+ channel function impaired by thromboxane. *Anesthesiology*. 2009;111:279–286.
25. Gimenez M, Veríssimo-Filho S, Wittig I, et al. Redox activation of Nox1 (NADPH oxidase 1) involves an intermolecular disulfide bond between protein disulfide isomerase and $p47^{phox}$ in vascular smooth muscle cells. *Arterioscler Thromb Vasc Biol*. 2019;39:224–236.
26. Staiculescu MC, Galiñanes EL, Zhao G, et al. Prolonged vasoconstriction of resistance arteries involves vascular smooth muscle actin polymerization leading to inward remodeling. *Cardiovasc Res*. 2013;98:428–436.
27. Hien TT, Turczynska KM, Dahan D, et al. Elevated glucose levels promote contractile and cytoskeletal gene expression in vascular smooth muscle via Rho/protein kinase C and actin polymerization. *J Biol Chem*. 2016;291:3552–3568.
28. Schaeffer G, Levak-Frank S, Spitaler MM, et al. Intercellular signalling within vascular cells under high D-glucose involves free radical-triggered tyrosine kinase activation. *Diabetologia*. 2003;46:773–783.
29. Bamburg JR. Proteins of the ADF/cofilin family: essential regulators of actin dynamics. *Annu Rev Cell Dev Biol*. 1999;15:185–230.
30. Kapoor K, Finer-Moore JS, Pedersen BP, et al. Mechanism of inhibition of human glucose transporter GLUT1 is conserved between cytochalasin B and phenylalanine amides. *Proc Natl Acad Sci USA*. 2016;113:4711–4716.
31. Mueckler M, Thorens B. The SLC2 (GULT) family of membrane transporters. *Mol Asp Med*. 2013;34:121–138.
32. Nazari H, Takahashi A, Harada N, et al. Angiotensin II inhibits insulin-induced actin stress fiber formation and glucose uptake via ERK1/2. *J Med Invest*. 2007;54:19–27.
33. Touyz RM, Alves-Lopes R, Rios FJ, et al. Vascular smooth muscle contraction in hypertension. *Cardiovasc Res*. 2018;114:529–539.
34. Dresel PE, Knickle L. Cytochalasin-B and phloretin depress contraction and relaxation of aortic smooth muscle. *Eur J Pharmacol*. 1987;144:153–157.