Repeated Glucose Spikes and Insulin Resistance Synergistically Increase Endothelial Function Vulnerability to High Glucose Levels through Redox Imbalance, and Bardoxolone Methyl (CDDO-Me) Ameliorates Endothelial Dysfunction

Kazuma Ogiso

Kagoshima University Graduate School of Medicine and Dental Sciences: Kagoshima Daigaku Daigakuin Ishigaku Sogo Kenkyuka <https://orcid.org/0000-0002-9626-0127>

Sigfrid Casmir Shayo

Kagoshima University Graduate School of Medicine and Dental Sciences: Kagoshima Daigaku Daigakuin Ishigaku Sogo Kenkyuka

Shigeru Kawade

Kagoshima University Graduate School of Medicine and Dental Sciences: Kagoshima Daigaku Daigakuin Ishigaku Sogo Kenkyuka

Hiroshi Hashiguchi

Kagoshima University Graduate School of Medicine and Dental Sciences: Kagoshima Daigaku Daigakuin Ishigaku Sogo Kenkyuka

Takahisa Deguchi

Kagoshima University Graduate School of Medicine and Dental Sciences: Kagoshima Daigaku Daigakuin Ishigaku Sogo Kenkyuka

Yoshihiko Nishio (✉ ynishio@m3.kufm.kagoshima-u.ac.jp)

Kagoshima University Graduate School of Medicine and Dental Sciences: Kagoshima Daigaku Daigakuin Ishigaku Sogo Kenkyuka

Original investigation

Keywords: Glucose spikes (GSs), CDDO, dihydroethidium (DHE), GS-induced endothelial dysfunction

Posted Date: July 26th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-731463/v1>

License: © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License. [Read Full License](#)

Abstract

Background

Glucose spikes (GSs) observed after a meal in metabolic syndrome have been reported to cause endothelial dysfunction. However, other insulin resistance-related factors can affect GS-induced endothelial dysfunction. To eliminate these confounding factors, we investigated the separate and combined effects of GSs and insulin resistance due to diet-induced obesity on endothelial function and clarified whether bardoxolone methyl (CDDO-Me), a novel nuclear factor erythroid 2-related factor 2 (Nrf2) activator, protects against GS-induced endothelial dysfunction.

Methods

In the first cohort, eight-week-old male Wistar rats were assigned to one of four groups: 1) control diet (CD)-GS (-); 2) CD-GS (+); 3) Western-type diet (WTD)-GS (-); and 4) WTD-GS (+). Rats were fed a CD or WTD for 13 weeks and intraperitoneally injected with saline or glucose for 1 week twice daily at 20 weeks of age. In the second cohort, four groups from the first cohort were additionally divided into vehicle and CDDO-Me (3 mg/kg) groups. At 21 weeks of age, endothelial function was evaluated using isolated thoracic aortas under normal (5.5 mM) and high-glucose (20 mM) conditions. Gene expression was analyzed, and endogenous superoxide anion was evaluated by dihydroethidium (DHE) staining of aortas.

Results

In the first cohort, endothelium-dependent relaxation (EDR) in the CD-GS (+) or WTD-GS (-) group was comparable to that in the CD-GS (-) group, but it deteriorated in the WTD-GS (+) group only under high-glucose conditions. Antioxidant agents, such as superoxide dismutase, catalase, apocynin (a NOX inhibitor) and Mito-TEMPO (a mitochondrial-targeted superoxide scavenger), improved endothelial function. In this group, upregulation of NOX2 expression and downregulation of SOD2 and catalase expression were observed in the aortas, and DHE intensity was enhanced. In the second cohort, pretreatment of the WTD-GS (+) group with CDDO-Me attenuated this endothelial dysfunction accompanied by a correction of redox imbalance in gene expression and an attenuation of DHE intensity.

Conclusion

We demonstrated that GSs and insulin resistance synergistically increased endothelial function vulnerability to high-glucose levels through redox imbalance, although each factor alone had little effect on endothelial function. Furthermore, we showed that pretreatment with CDDO-Me ameliorated endothelial dysfunction caused by GSs in metabolic syndrome model rats.

Introduction

Cardiovascular disease (CVD) is the leading cause of mortality and morbidity worldwide [1]. Metabolic syndrome is an important risk factor for atherosclerosis and CVD and is caused by insulin resistance due to the accumulation of excessive visceral fat [2]. Insulin resistance causes postprandial hyperglycemia, known as a glucose spike (GS), after a meal. GS causes endothelial dysfunction, which occurs early in the process of atherogenesis and contributes to the progression of atherosclerosis [3]. In fact, GSs rapidly suppress flow-mediated endothelium-dependent vasodilation (FMD) through free radical production [4,5] and enhance monocyte adhesion to arterial endothelial cells [6]. Repeated GSs also accelerate the formation of atherosclerotic lesions in mice fed an atherogenic diet [7]. Epidemiological studies have also suggested that GSs may be a more robust determinant of CVD risk than average glucose levels [8-12]. Previous studies have shown that glucose levels after an oral glucose tolerance test (OGTT) are more strongly associated with the carotid intima media thickness (cIMT), a marker of atherosclerosis, than the levels of fasting plasma glucose (FPG) or hemoglobin A1c (HbA1c) [13]. Glucose levels after an OGTT are also more strongly associated with cardiovascular mortality than HbA1c levels [14]. Moreover, pharmacological interventions with glinides reduced cIMT as well as postprandial hyperglycemia [15]. Even in people without diabetes, postprandial blood glucose (PBG) levels independently predict CVD in the nondiabetic glucose range [16]. Therefore, diabetic and nondiabetic individuals predisposed to GSs may be at increased risk of developing CVD.

However, it is not clear how GSs clinically impact CVD events because most intervention trials have not shown postprandial glucose to be a cardiovascular risk factor independent of HbA1c. Both prandial (rapid-acting) insulin and nateglinide, which are agents that target postprandial hyperglycemia, did not reduce CVD events (the HEART2D and NAVIGATOR trials) [17,18]. Even acarbose, an α -glucosidase inhibitor, did not reduce the risk of major adverse cardiovascular events (ACE trial) [19]. Although the reason for this finding has not been fully understood, other risk factors may contribute to endothelial dysfunction. In particular, some factors related to insulin resistance accompanied by postprandial hyperglycemia, such as dyslipidemia (including postprandial hypertriglyceridemia [20,21]) or hyperinsulinemia, may affect endothelial dysfunction in addition to GSs. Therefore, these confounding factors associated with insulin resistance should be eliminated to assess the effect of GSs themselves on endothelial function.

Furthermore, according to a review of previous reports related to GSs, oxidative stress is considered a major cause of endothelial dysfunction [22]. If oxidative stress can be attenuated, it may be useful as a new treatment for GS-associated endothelial dysfunction, even if GSs and insulin resistance affect endothelial function. Recently, the nuclear factor erythroid 2-related factor 2 (Nrf2) activator bardoxolone methyl (CDDO-Me) has been shown to have a renal protective effect on diabetic nephropathy, mainly through antioxidant and anti-inflammatory effects (TSUBAKI study) [23]. In fact, this Nrf2 activator was reported to protect endothelial function against chronic kidney disease (CKD) and diabetes in animal experiments [24,25]. These results suggest that CDDO-Me may have the potential to treat endothelial dysfunction due to GSs by attenuating oxidative stress.

In the present study, to explore the conditions in which endothelial function is most vulnerable to GSs, we investigated two factors separately, GSs and insulin resistance, and the combined effects of these two factors on vascular endothelial function. Specifically, we evaluated endothelial function in nonobese rats

and diet-induced obese rats that received glucose intraperitoneally twice a day for one week to reproduce GSs without postprandial hypertriglyceridemia. We also investigated whether CDDO-Me prevents GS-induced endothelial dysfunction.

Methods

Animal Studies

Seven-week-old male Wistar rats were obtained from KBT Oriental Co., Ltd. (Saga, Japan) and housed at $23 \pm 1^\circ\text{C}$ under a 12-h light/12-h dark cycle, with ad libitum access to food and water. After a 1 week acclimation period, the rats were fed a control diet (CD) or a Western-type diet (WTD) for 13 weeks (8 - 21 weeks old) according to their group. The CD contained 4.7% calories from fat, 23.3% calories from protein, and 55.6% calories from carbohydrates (3.6 kcal/g; MF, Oriental Yeast Co., Ltd., Tokyo, Japan), and the WTD contained 39.9% calories from fat, 15% calories from protein, and 44.3% calories from carbohydrates (4.5 kcal/g; F2WTD, Oriental Yeast Co., Ltd., Tokyo, Japan). The WTD group of rats were allowed access to feed ad libitum, and the CD group of rats were pair-fed (limited to the amount of food consumed by the rats in the WTD group) to prevent obesity due to excessive intake.

In the first cohort, the rats were assigned to one of four groups according to the factor of diet or glucose spike (GS): 1) CD-GS (-), control diet and glucose spike (-); 2) CD-GS (+), control diet and glucose spike (+); 3) WTD-GS (-), Western-type diet and glucose spike (-); and 4) WTD-GS (+), Western-type diet and glucose spike (+). The CD group represented nonobese model rats without insulin resistance, and the WTD group represented obese model rats with insulin resistance. At 20 weeks of age, rats in the GS (-) and GS (+) groups were intraperitoneally injected with saline (5 mL/kg) and 20% glucose (1 g/5 mL/kg body weight [BW]), respectively, for one week twice daily at approximately 08:00 a.m. and 04:00 p.m. (Supplementary Fig. S1A for details). In the second cohort, four groups from the first cohort were additionally divided into vehicle and CDDO-Me groups. At 19 weeks of age, rats in the vehicle and CDDO-Me groups were orally administered vehicle (sesame oil, S3547, Sigma-Aldrich, St. Louis, MO, USA) or CDDO-Me (3 mg/kg BW; SMB00376, Sigma-Aldrich, St. Louis, MO, USA), which was solubilized in sesame oil, for two weeks once daily (Supplementary Fig. S1B for details). At 20 weeks of age, rats in the GS (-) and GS (+) groups were intraperitoneally injected with saline and 20% glucose for one week twice daily as described for the first cohort. For the insulin tolerance test (ITT), rats were deprived of food for 12 hours and then injected intraperitoneally with insulin (0.5 U/kg BW), and insulin resistance was evaluated as the decreasing blood glucose area under the curve (AUC). To collect additional samples, rats were deprived of food overnight (14 hours) in cages with fresh bedding. Visceral fat mass was evaluated as the adiposity index, which was defined as the ratio of epididymal, retroperitoneal and mesenteric fat grams to body weight [26]. Serum or plasma parameters were measured with a Rat Insulin ELISA (Morinaga Institute of Biological Science, Inc., Yokohama, Japan), LabAssay Triglyceride assay (TG; FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan), LabAssay NEFA assay (FFA, free fatty acid; FUJIFILM Wako), LabAssay Cholesterol assay (TC, total cholesterol; FUJIFILM Wako), Rat TNF α Quantikine ELISA (R&D Systems, Inc., McKinley Place, MN, USA) and Glucose Assay Kit II (Funakoshi Co., Ltd., Tokyo, Japan). Homeostasis model assessment of insulin resistance (HOMA-IR) was determined with the formula $HOMA\ IR = \text{serum insulin (mmol/L)} \times \text{blood glucose (mmol/L)} / 22.5$ [27]. All animal protocols were reviewed and approved by the Laboratory Animal Committees of Kagoshima University Graduate School and were performed in accordance with the guidelines for the care and use of laboratory animals (approval number: MD20086).

Continuous Interstitial Glucose Monitoring

When starting saline or glucose at 20 weeks of age, a FreeStyle Libre Pro[®] sensor (Abbott Diabetes Care, IL, USA), which continuously records interstitial glucose levels every fifteen minutes, was attached to the backs of the rats to record interstitial glucose levels for two days. After removing the sensor, the data were extracted using FreeStyle Libre Pro software (Abbott Diabetes Care). A GS was defined as an increased interstitial glucose level above 5 mM, which was the difference between baseline and peak interstitial glucose levels.

Vascular Reactivity

After animals were sacrificed at 21 weeks of age, each thoracic aorta was rapidly isolated, carefully cleared of perivascular fat and adventitia and placed in oxygenated physiological saline solution (PSS; 130 mmol/L NaCl, 14.9 mmol/L NaHCO₃, 4.7 mmol/L KCl, 1.18 mmol/L KH₂PO₄, 1.17 mmol/L MgSO₄·7H₂O, 1.6 mmol/L CaCl₂·2H₂O, 0.026 mmol/L EDTA, 5.5 mmol/L glucose [pH 7.4]). Two rings (2 mm long) were cut from each thoracic aorta per rat, mounted onto a Multiwire Myograph System 620M (Danish Myo Technology, Aarhus, Denmark) and perfused through the chambers with 5% CO₂ and 95% O₂ at 37°C. Changes in isometric tension were measured by using a LabChart Pro data acquisition system (ADInstruments Pty Ltd., Castle Hill, Australia) as previously described [28]. After 30 minutes of equilibration, the aortic rings were exposed to a high potassium physiological saline solution (KPSS) (74.7 mmol/L NaCl, 14.9 mmol/L NaHCO₃, 60 mmol/L KCl, 1.18 mmol/L KH₂PO₄, 1.17 mmol/L MgSO₄·7H₂O, 1.6 mmol/L CaCl₂·2H₂O, 0.026 mmol/L EDTA, 5.5 mmol/L glucose) to assess the maximal tension. After washout, one of the two samples collected per rat was equilibrated in a normal glucose PSS chamber (5.5 mmol/L glucose PSS [Glu 5.5 mM]), and the other was equilibrated in a high-glucose PSS chamber (20 mmol/L glucose PSS [Glu 20 mM]) for 2 hours. After an equilibration period, endothelium-dependent relaxation (EDR) was determined by the cumulative addition of acetylcholine (ACh; 3.2 nmol/L-100 μ mol/L) (NACALAI TESQUE, INC., Kyoto, Japan) to phenylephrine (PE; ~1 μ mol/L) (Sigma-Aldrich Japan Co., LLC, Tokyo, Japan)-precontracted segments. Sodium nitroprusside (SNP; 100 μ mol/L-100 μ mol/L) (Sigma-Aldrich Japan Co., LLC), an exogenous nitric oxide (NO) donor, was used to test endothelium-independent relaxation. Maximal relaxation was induced by papaverine (PPV; 100 μ mol/L) (Nichi-Iko Pharmaceutical Co., Ltd., Toyama, Japan). Endothelial integrity was tested by ACh-induced relaxation (100 μ mol/L) in segments previously contracted with a concentration of PE (~1 μ mol/L) that induced 50-70% of the contraction induced by 60 mmol/L KPSS. Relaxation in response to acetylcholine greater than 50% was considered demonstrative of endothelial functional integrity.

In some experiments, the role of free radicals in EDR was evaluated by incubating aortic rings with the following agents for 30 minutes before the EDR measurement: indomethacin (10 μ mol/L), a prostaglandin synthetase inhibitor (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) [29], allopurinol (100 μ mol/L), a xanthine oxidase inhibitor (Sigma-Aldrich Japan Co., LLC) [30]; insulin (10 nmol/L), Humulin R (Eli Lilly Japan K.K., Kobe, Japan) [31];

apocynin (100 $\mu\text{mol/L}$), an NADPH oxidase inhibitor (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan) [30]; superoxide dismutase (SOD) (150 U/mL), a superoxide scavenger (Sigma-Aldrich Japan Co., LLC) [32]; catalase (6,250 U/mL), a hydrogen peroxide scavenger (Sigma-Aldrich Japan Co., LLC) [33]; Mito-TEMPO (100 nmol/L), a mitochondria-targeted superoxide scavenger (Funakoshi Co., Ltd., Tokyo, Japan) [34]; and Mn(III) tetra(4-benzoic acid) porphyrin chloride (MnTABP) (10 $\mu\text{mol/L}$), a SOD mimetic and peroxynitrite scavenger (Funakoshi Co., Ltd.) [35]. L-N^G-nitro-arginine methyl ester (L-NAME) (100 $\mu\text{mol/L}$) and a nitric oxide synthase (NOS) inhibitor (FUJIFILM Wako Pure Chemical Corporation) [36] were also used as pretreatments to eliminate EDR.

Relaxation induced by ACh and SNP is expressed as the percentage of the maximum relaxation obtained with PPV. For each concentration-response curve, pD₂ (log of the half-maximal effective concentration [EC₅₀]) was calculated by using nonlinear regression analysis with statistical software R version 3.6.1 (The R Foundation for Statistical Computing, Vienna, Austria).

Quantitative Real-Time PCR

The excised thoracic aortas were frozen in liquid nitrogen, pulverized in a mill, and then homogenized with POLYTRON PT 2500 E (Kinematica AG, Luzern, Schweiz). Homogenized tissues were lysed with 900 μL QIAzol Lysis Reagent (QIAGEN K.K., Tokyo, Japan). Genomic DNA contamination of the aqueous phase was reduced with gDNA Eliminator Solution (QIAGEN K.K.). After separating the phases with the addition of 180 μL chloroform, RNA was isolated by using an RNeasy Mini Kit (QIAGEN K.K.). An Applied Biosystems High-Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (Thermo Fisher Scientific K.K., Tokyo, Japan) was used to synthesize cDNA from 1 μg total RNA. Quantitative PCR was performed on an Applied Biosystems StepOnePlus Real-Time PCR System with TaqMan™ Fast Universal PCR Master Mix (Thermo Fisher Scientific K.K.). Relative gene expression was calculated by using the $\Delta\Delta\text{Ct}$ method. Gene expression was normalized relative to GAPDH. The primers and probes used are shown in Supplementary Table S1 (Thermo Fisher Scientific K.K.).

Oxidative Stress Assessment

To evaluate local and systemic oxidative stress, we measured the dihydroethidium (DHE) fluorescence intensity in aortas and urinary 8-hydroxy-2'-deoxyguanosine (8-OHdG) levels. Reactive oxygen species (ROS) production in the en face endothelium of rat aortas was measured with DHE (Sigma-Aldrich Japan Co., LLC) under a fluorescence microscope [37]. In brief, the aortic rings were embedded vertically into OCT compound (Funakoshi Co., Ltd.) and immediately placed on crushed dry ice for freezing. Once frozen, 8 μm -thick sections were cut using a cryostat at -20°C . After rinsing the slides in pure H₂O for 30 seconds to wash out OCT compound, slides were immediately placed in a 5 μM DHE staining solution and incubated for 20 minutes at room temperature in the dark. Some sections were preincubated for 30 minutes with 250 U/mL polyethylene glycol-superoxide dismutase (PEG-SOD) (Sigma-Aldrich Japan Co., LLC) before incubation with DHE. DHE fluorescence intensity was measured with a fluorescence microscope (EVOS FL Auto 2 Imaging System; Thermo Fisher Scientific K.K.) at an excitation wavelength of 542 nm and an emission wavelength of 593 nm (EVOS Light Cube, RFP; Thermo Fisher Scientific K.K.). The background autofluorescence of elastin was measured at an excitation wavelength of 482 nm and an emission wavelength of 524 nm (EVOS Light Cube, RFP; Thermo Fisher Scientific K.K.) and subtracted from the fluorescence intensity of DHE. DHE fluorescence intensity was evaluated with ImageJ (version 1.51) (Rasband, W.S., U.S. National Institutes of Health, Bethesda, MD, USA) [38] and is represented as fold changes in fluorescence intensity relative to that of the control.

In the second cohort, urinary 8-OHdG was also assessed using a commercially available ELISA kit (Cayman Chemical, Ann Arbor, MI, USA) to evaluate systemic oxidative radical levels. Measurements are expressed relative to urinary creatinine.

Statistical Analysis

Values are presented as the mean $\pm$ SEM. Statistical significance was determined by using one-way ANOVA to compare differences between groups. When diet and GS interaction effects were evaluated as dependent variables, two-way between-groups ANOVA was used; a significant interaction was interpreted by a subsequent simple-effects analysis with Bonferroni correction. Differences between vehicle and CDDO-Me were evaluated by using Bonferroni correction. Concentration response curves and body weight curves were analyzed by one-way or two-way repeated-measures ANOVA followed by the Bonferroni post hoc test. Univariate regression analysis using Pearson's correlation coefficient was performed to assess statistical associations between metabolic parameters and pD₂. The differences between groups were considered significant when $P < 0.05$. All data were analyzed with R version 3.6.1 statistical software (The R Foundation for Statistical Computing, Vienna, Austria).

Results

The GS Model Was Confirmed with Continuous Interstitial Glucose Monitoring

To confirm the glucose profile in the model rats, interstitial glucose levels were continuously recorded with Libre® for 48 hours from the 2nd day of administration of saline or glucose. All incremental interstitial glucose levels were above 5 mM in both the CD-GS (+) and WTD-GS (+) groups after glucose administration and were below 5 mM in both the CD-GS (-) and WTD-GS (-) groups after saline administration (Supplementary Fig. S2A and B). A significant main effect of GS was observed in both peak and incremental interstitial glucose levels ($F [1, 12] = 1619.1, P < 0.001$; $F [1, 12] = 513.7, P < 0.001$, respectively) without diet/GS interaction, although the main effect of diet on those was not significant.

Short-term Repeated GSs Deteriorated EDR in Diet-induced Obese Rats

To investigate the difference in EDR between the CD group (nonobese rats) and WTD group (obese rats) with or without GSs, we compared endothelial function among four groups: CD-GS (-), CD-GS (+), WTD-GS (-) and WTD-GS (+). The EDR of the thoracic aortas from the CD-GS (-), CD-GS (+) and WTD-GS (-) groups did not change under normal (5.5 mM) or high-glucose (20 mM) conditions, but the EDR of those from the WTD-GS (+) group deteriorated under high-

glucose conditions (Fig. 2A). This deterioration of EDR was not reproduced in the presence of 20 mM raffinose, an osmotic control for 20 mM glucose (Fig. 1A). Among the four groups under 20 mM glucose conditions, a significant interaction on pD2 (-log ACh EC 50) was detected between diet and GSs ($F [1, 24] = 17.4, P < 0.001$). Simple-effects analysis revealed that the WTD or GS (+) alone did not cause endothelial dysfunction; however, the combination of the WTD and GS (+) deteriorated endothelial function (Fig. 2B). There were no significant differences in the dose-response curves to SNP, an endothelium-independent vasodilator, or ACh in the presence of L-NAME, a NOS inhibitor, even under the 20 mM glucose condition among the four groups (Fig. 1C and D), suggesting that the impaired endothelial function in the WTD-GS (+) group under the 20 mM glucose condition was caused by a deterioration of NO-dependent relaxation. These results indicate that short-term repeated GSs deteriorated EDR only in diet-induced obese rats under high-glucose conditions but not in nonobese rats.

Endothelial Dysfunction Caused by Repeated GSs in Diet-Induced Obese Rats is Independent of Fat Mass and Blood Lipid Profiles

To understand the mechanism of endothelial dysfunction in the WTD-GS (+) group, metabolic parameters were evaluated. A significant main effect for diet was observed in some dependent variables as below without diet/GS interaction, although the main effect of GSs was not significant; body weight gain and visceral fat (adiposity index) were significantly higher, the ITT (decreasing AUC) was lower (Fig. 1E-G), and plasma insulin, serum TG, FFA, TNF α , and FPG levels and HOMA-IR in the WTD group were higher than those in the CD group (Table 1). Total cholesterol levels were comparable between the two groups (Table 1). Systolic and diastolic blood pressure in the WTD group tended to be higher than those in the CD group (the main effect of diet; $F [1, 24] = 3.93, P = 0.059$; $F [1, 24] = 3.14, P = 0.089$, respectively), but the differences were not significant (Table 1). These results indicate that changes in these parameters in the WTD group are compatible with metabolic syndrome. However, GSs did not affect these metabolic parameters in either the CD or WTD group.

Furthermore, to clarify whether the endothelial dysfunction observed in the WTD-GS (+) group under the 20 mM glucose condition was associated with these metabolic parameters, the correlation between each metabolic parameter and pD2 (-log ACh EC 50) was assessed. Among these parameters, HOMA-IR and serum FFA and TNF α levels were negatively correlated, and ITT (decreasing AUC) was positively correlated with the pD2 of the WGS (+) group under the 20 mM glucose condition (Supplementary Fig. S3D, G, H, E). Other metabolic parameters, such as body weight, adiposity index, and FPG and serum TG levels, were not significantly correlated with pD2 (Supplementary Fig. S3A, B, C, F).

A NOX Inhibitor, SOD and Catalase Ameliorate Endothelial Dysfunction Caused by High-Glucose Conditions in Diet-Induced Obese Rats with Repeated GSs

To clarify the mechanism of endothelial dysfunction in the WTD-GS (+) group, we used several pharmacological agents to evaluate endothelial function under the 20 mM glucose condition. Apocynin, a NOX inhibitor, improved EDR, represented as pD2, in the WTD-GS (+) group (Fig. 2) ($P < 0.001$). Similarly, extrinsic SOD and catalase, which are superoxide scavengers and hydrogen peroxide scavengers, also improved EDR ($P < 0.001$; $P = 0.005$, respectively) (Fig. 2). To investigate this finding in more detail, we used Mito-TEMPO and MnTAPB, a mitochondria-targeted superoxide scavenger and a peroxynitrite selective scavenger, respectively. Both of these agents also improved EDR in the WTD-GS (+) group ($P = 0.017$; $P = 0.013$, respectively) (Fig. 2). Indomethacin and allopurinol, a cyclooxygenase inhibitor and a xanthine oxidase inhibitor, respectively, however, did not improve EDR in the WTD-GS (+) group (Fig. 2), suggesting that prostaglandins and xanthine oxidase-derived radicals are unlikely to be responsible for endothelial dysfunction. We also evaluated the effect of insulin on endothelial function because repeated GSs were accompanied by transient hyperinsulinemia, and we found that even a high dose of insulin (10 nM) did not affect EDR, suggesting that direct exposure of the endothelium to insulin is neither harmful nor protective.

mRNA Expression of NOX2 Was Upregulated and that of SOD2 and Catalase Was Downregulated in the Thoracic Aortas from Diet-Induced Obese Rats with Repeated GSs

Then, we analyzed the gene expression of oxidoreductase in the thoracic aortas. Among the four groups, the interactive effect of diet and GS on the mRNA expression of NOX2 ($F [1, 20] = 4.69, P = 0.043$), p47phox ($F [1, 20] = 14.07, P = 0.039$) and SOD2 ($F [1, 20] = 5.20, P = 0.034$) was confirmed by two-way ANOVA (Fig. 3). Simple-effects analysis revealed that the combination of a WTD and GS (+) upregulated the mRNA expression of NOX2 and p47phox and downregulated that of SOD2; a WTD or GS (+) alone did not affect the gene expression of NOX2 or p47phox, but that of SOD2 was downregulated by a WTD ($P = 0.044$) but not by GS (+) ($P = 0.272$). The main effect of diet and GS on the mRNA expression of catalase was confirmed ($F [1, 20] = 13.25, P = 0.002$; $F [1, 20] = 4.80, P = 0.040$, respectively) without diet/GS interaction ($F [1, 20] = 1.13, P = 0.300$) (Fig. 3). Notably, there were no significant differences in other redox-related enzyme genes, such as NOX1, NOX4, SOD1 and GPX1, among all four groups (Fig. 3). These results suggest the following: 1) diet-induced obesity by a WTD downregulates that of SOD2 and catalase; 2) short-term repeated GSs downregulate the mRNA expression of catalase; and 3) the combination of repeated GSs and diet-induced obesity synergistically upregulates that of NOX2 and p47phox and downregulates that of SOD2. With regard to inflammatory genes, the mRNA expression of TNF α was significantly different between diet or GS factors ($F [1, 20] = 5.26, P = 0.033$; $F [1, 20] = 17.99, P < 0.001$, respectively) and that of IL1 β also different between GS factors ($F [1, 20] = 9.17, P = 0.007$) without diet/GS interaction (Fig. 3). An interactive effect of diet and GS on the mRNA expression of VCAM1 was detected ($F [1, 20] = 12.0, P = 0.002$), and simple-effects analysis revealed that the combination of a WTD and GS (+) upregulated that of VCAM1 (Fig. 3).

Radical Formation Is Enhanced by Repeated GSs in the Thoracic Aortas of Diet-induced Obese Rats

Repeated GSs in diet-induced obese rats showed an imbalance in redox enzyme gene expression. To investigate radical formation in the aorta, superoxide anion was evaluated by DHE staining of thoracic aortas isolated 2 hours after the administration of glucose (1 g/kg BW) or saline. The fluorescence intensity of DHE was higher in the WTD-GS (+) group administered glucose than in the other three groups (Fig. 4A). Among the four groups, the interactive effect of diet and GS on the fluorescence intensity of DHE ($F [1, 12] = 37.42, P < 0.001$) was confirmed by two-way ANOVA (Fig. 4A). Simple-effects analysis revealed that the combination of a WTD and GS (+) increased the fluorescence intensity of DHE, although a WTD or GS (+) alone did not. This enhanced intensity of DHE in the WTD-GS (+) group was diminished by preincubation with 250 U/mL PEG-SOD (Fig. 4B) ($P < 0.001$). In addition, the WTD-GS (+) group administered saline showed a significantly lower fluorescence intensity of DHE than the WTD-GS (+) group administered glucose (Fig. 4B) ($P < 0.001$). These results were

consistent with those of the vascular reactivity experiment in which endothelial function deteriorated only under high-glucose conditions in the WTD-GS (+) group.

CDDO-Me Protects the Endothelial Function of Diet-induced Obese Rats against Repeated GSs

Because repeated GSs induced oxidative stress in the thoracic aortas of diet-induced obese rats, we evaluated whether the administration of CDDO-Me, an activator of the Nrf2 system, could prevent endothelial dysfunction. To determine the optimal dose of CDDO-Me, five doses of CDDO-Me (0 [vehicle], 0.3, 1, 3 and 15 mg/kg BW) were orally administered to rats in the WTD-GS (+) group. As shown in Supplementary Fig. S4, endothelial function was most strongly preserved against repeated GSs at 3 mg/kg BW CDDO-Me, although this protective effect was diminished at 15 mg/kg BW.

We orally administered vehicle (sesame oil) or CDDO-Me (3 mg/kg BW) to four groups of 19-week-old rats for two weeks and intraperitoneally administered saline or glucose for one week beginning at 20 weeks old, similar to the first cohort. Thoracic aortas were isolated from these groups, and EDR was evaluated under 5.5 mM or 20 mM glucose conditions. Under the 20 mM glucose condition, the administration of CDDO-Me to rats in the WTD-GS (+) group showed a significant amelioration of EDR and pD2 compared with vehicle ($P < 0.001$), although no significant differences were observed under the 5.5 mM glucose condition (Fig. 5A and B). Among the CD-GS (-), CD-GS (+), and WTD-GS (-) groups, there were no differences in EDR or pD2 among the vehicle and CDDO-Me groups under either the 5.5 mM or 20 mM glucose conditions (Fig. 5A and B).

CDDO-Me Does Not Affect Metabolic Parameters

The effects of CDDO-Me on metabolic parameters were assessed. In the comparison of treatment with vehicle or CDDO-Me for each group, no significant differences in body weight, adiposity index (visceral fat mass), ITT (insulin resistance), or levels of plasma insulin, serum TG, FFA, FPG, or serum TC were observed (Supplementary Fig. S5A-H). There were also no differences in HOMA-IR, serum TNF α levels or blood pressure (Supplementary Fig. S5I-L).

CDDO-Me Suppresses the mRNA Expression of NOX2 and Enhances that of SOD2 and Catalase in the Thoracic Aortas of Diet-induced Obese Rats with Repeated GSs

To clarify the mechanism of the amelioration of endothelial dysfunction by treatment with CDDO-Me, we evaluated the changes in gene expression in the thoracic aortas between vehicle and CDDO-Me in each group. The mRNA expression of NQO1, a target gene of the Nrf2 system, was markedly enhanced by CDDO-Me in the four groups (Fig. 6A). CDDO-Me in the WTD-GS (+) group significantly reduced the mRNA expression of NOX2 and p47phox ($P = 0.004$; $P = 0.004$, respectively) and significantly increased that of SOD2 and catalase (Fig. 6B-E) ($P = 0.007$; $P = 0.033$, respectively). With regard to inflammatory genes, the mRNA expression levels of VCAM1 in the WTD-GS (+) group treated with CDDO-Me were significantly lower than those in the WTD-GS (+) group treated with vehicle ($P = 0.039$) (Fig. 6H). CDDO-Me also reduced those of TNF α and IL1 β , but there were no significant differences between vehicle and CDDO-Me (Fig. 6F and G).

CDDO-Me Suppresses Local and Systemic Oxidative Stress Caused by Repeated GSs in Diet-induced Obese Rats

To evaluate the effect of CDDO-Me on local and systemic oxidative stress, we measured DHE fluorescence intensity in the thoracic aortas and urinary 8-OHdG levels between the vehicle and CDDO-Me groups. These samples were collected 2 hours after the intraperitoneal administration of glucose (1 g/kg BW). Treatment with CDDO-Me in the WTD-GS (+) group significantly decreased DHE fluorescence intensity and urinary 8-OHdG levels ($P = 0.020$ and $P = 0.048$, respectively) (Fig. 6I and J). In the other three groups, no significant differences in DHE fluorescence intensity or urinary 8-OHdG levels were observed between the vehicle and CDDO-Me groups (Fig. 6I and J).

Discussion

In the present study, we clearly demonstrated that short-term repeated GSs cause high-glucose-dependent endothelial dysfunction in the aortas of obese rats with insulin resistance induced by a Western-type diet but not in the aortas of nonobese rats without insulin resistance fed a CD. Endothelial dysfunction was associated with the increased detection of superoxide anion in the wall of the aorta, the decreased expression of genes encoding enzymes that eliminate ROS, such as SODs and catalase, and the elevated expression of genes encoding enzymes that produce ROS, such as NOXs. In addition, this endothelial dysfunction induced by short-term repeated GSs was prevented by the administration of CDDO-Me, a Nrf2 system activator.

A decrease in the bioavailability of NO resulting in endothelial dysfunction is generally caused by an increase in radical levels or ROS production [39–42], and high-glucose conditions have been reported to increase superoxide anion levels in endothelial cells [43, 44]. We also found that apocynin, SOD, catalase or MnTAP, a selective peroxynitrite remover [45], improved endothelial dysfunction under high-glucose conditions, suggesting an increase in oxidative stress. Interestingly, however, the high-glucose condition induced endothelial dysfunction only in the aortas from the diet-induced obese rats with repeated GSs but not in the aortas from nonobese rats with repeated GSs or diet-induced obese rats without repeated GSs. These results indicate that high-glucose conditions, repeated GSs and insulin resistance and/or hyperinsulinemia are additive factors for the impairment of endothelial function.

In previous reports, each factor of GSs or diet-induced obesity was reported to cause endothelial dysfunction, but each factor was insufficient to impair endothelial function in the present study. Considering that GSs require 3 months to accelerate arteriosclerosis [7], the period of GSs may have been too short to induce endothelial dysfunction in nonobese rats with GSs. In addition, endothelial dysfunction by diet-induced obesity has been reported to be partly caused by eNOS uncoupling in perivascular adipose tissue (PAVT), and endothelial dysfunction was not observed in PAVT-free aortas isolated from obese mice [46]. We removed PAVT from the thoracic aorta, resulting in endothelial dysfunction that could not be observed in diet-induced obese rats without GSs in our experiment.

The mRNA expression of SOD2 and catalase in the aortas of the diet-induced obese rats without repeated GSs decreased and tended to decrease, respectively, while repeated GSs tended to increase the mRNA expression of NOX2 in the aortas of the nonobese rats fed a CD. These results are consistent with previous reports that a high-fat diet reduces the expression of SOD2 without any changes in the expression of NOX2 [47], that obesity decreases the expression of catalase in the aorta [48], and that intermittent high glucose levels stimulate ROS production through the protein kinase C-dependent activation of NOX [49]. In addition, the combination of diet-induced obesity and repeated GSs synergistically enhanced these effects on the genes in our experiment. Although the mechanism of this synergistic effect of obesity and GSs on gene expression in the aorta is unclear, serum free fatty acids might be a key factor because free fatty acids have been reported to increase the gene expression of NOX [50, 51] and decrease the gene expression of antioxidant genes such as NQO1, SOD and catalase via Nrf2 system suppression in endothelial cells, resulting in enhanced ROS production [52]. In our experiment, serum free fatty acid levels correlated negatively with pD2 under high-glucose conditions (Supplementary Fig. S3G), suggesting that repeated GSs and an elevation in serum free fatty acid levels due to diet-induced obesity might synergistically stimulate changes in the gene expression of NOX2, SOD2 and catalase. Accordingly, these changes in gene expression in the aorta may increase the vulnerability of endothelial function to ROS.

High-glucose conditions have been reported to induce mitochondria-derived superoxide production [43, 44]. We also observed that Mito-TEMPO, a mitochondria-targeted superoxide scavenger, improved high-glucose-dependent endothelial dysfunction. Since endothelial cells take up glucose in an insulin-independent manner via glucose transporter 1 (GLUT1) [53] and no downregulation of GLUT1 expression in response to high extracellular glucose levels has been reported, endothelial function is thought to be susceptible to hyperglycemia [54]. Increased glucose uptake and glycolytic conversion to pyruvate can increase the production of mitochondrial ROS [55, 56]. Based on these reports, an increase in mitochondria-derived ROS production could be observed during temporal hyperglycemia. Although mitochondria-derived ROS are easily removed by antioxidant enzymes, ROS may not be eliminated completely under the suppression of antioxidant enzymes, such as SOD2 and catalase, in the aortas of diet-induced obese rats with repeated GSs. In addition, the gene expression of NOX2 in the aorta was increased, and apocynin improved endothelial dysfunction in these aortas, suggesting that NOX2-derived superoxide might also contribute to endothelial dysfunction. Considering that endothelial function was impaired only under high-glucose conditions, NOX2-derived superoxide might enhance mitochondrial superoxide production under high-glucose conditions [57].

Based on the hypothesis that the synergistic effects of repeated GSs and insulin resistance induce oxidative stress in endothelial cells under high-glucose conditions, resulting in endothelial dysfunction, we evaluated the effect of CDDO-Me (a Nrf2 activator) administration on endothelial dysfunction. The administration of CDDO-Me to the WTD-GS (+) group improved endothelial dysfunction, reduced ROS production, and normalized redox gene expression in the aorta. It has been reported that CDDO-Me systematically induces the expression of antioxidant enzymes, including SODs and catalase, in addition to NQO1 via Nrf2 activation [58–61] and has an anti-inflammatory effect by inhibiting nuclear factor κ B (NF- κ B) through the direct inhibition of I κ B kinase β (IKK β) [62]. In our experiment, CDDO-Me administration decreased the gene expression of NOX2, probably due to the suppression of TNF α [63], and increased that of SOD2, catalase and proinflammatory cytokines such as TNF α and IL1 β in the aortas of diet-induced obese rats with repeated GSs. Because oxidative stress has been proposed as a cause of CVDs, the efficacy of antioxidants such as vitamins C and E, beta-carotene and selenium for the prevention of CVDs has been assessed in interventional studies; however, there has been no evidence to support the benefit of antioxidants for the prevention of CVDs [64]. One possible reason for this finding is that these antioxidants target only specific ROS and may not be adequate for suppressing oxidative stress in vivo, since many kinds of ROS and other radicals are involved in oxidative stress. On the other hand, CDDO-Me induces many antioxidant enzymes and suppresses the expression of proinflammatory genes by activating the transcription factor Nrf2. It has been reported that CDDO-Me improves endothelial dysfunction in diabetes model mice with insulin secretion deficiency and aging model mice [65, 66]. In our GS model with insulin resistance, CDDO-Me improved endothelial dysfunction and reduced ROS production and proinflammatory gene expression in the aorta at the same time. In addition, CDDO-Me was reported to reduce plaques in streptozotocin- (STZ)-induced diabetic ApoE $^{-/-}$ mice through the suppression of ROS production and proinflammatory mediators [67], suggesting that CDDO-Me can suppress not only endothelial dysfunction in the early stage of atherosclerosis but also the progression of atherosclerosis, such as plaque formation. These results indicate the possibility of CDDO-Me as a treatment for GS-induced endothelial dysfunction leading to the development of CVDs. However, the dose of CDDO-Me may be critical for the treatment of endothelial dysfunction, since it was reported that a higher concentration of CDDO-Me caused excessive mitochondrial uncoupling [68]. In addition, S.M. Tan *et al.* reported that a higher concentration of CDDO-Me upregulated the gene expression of TNF α and monocyte chemoattractant protein-1 (MCP-1), resulting in the deterioration of antiatherogenic effects [67]. We also observed that an optimal concentration of CDDO-Me was needed to protect endothelial function from GSs.

Conclusion

We demonstrated that repeated GSs in diet-induced obese rats with insulin resistance made endothelial function vulnerable to high-glucose conditions. Mitochondria-derived superoxide may be increased and induce endothelial dysfunction under high-glucose conditions against the background of changes in gene expression related to redox balance and inflammation in the aortas from rats with repeated GSs and insulin resistance. Repeated GSs and free fatty acids, an insulin resistance-related factor, may synergistically cause an imbalance in redox gene expression in the aorta. The administration of CDDO-Me corrected these changes in gene expression and protected against endothelial dysfunction caused by repeated GSs and insulin resistance.

Abbreviations

CVD: Cardiovascular disease; Nrf2: Nuclear factor erythroid 2-related factor 2; CDDO-Me: Bardoxolone methyl; CD: Control diet; WTD: Western-type diet; GS: Glucose spike; EDR: Endothelium-dependent relaxation; Ach: Acetylcholine; PE: Phenylephrin; SNP: Sodium nitroprusside; NO: Nitric oxide; PPV: Papaverine; NADPH: Nicotinamide adenine dinucleotide phosphate; SOD: Superoxide dismutase; MnTABP: Mn(III) tetra(4-benzoic acid) porphyrin chloride; L-NAME: L-N^G-nitro-arginine methyl ester; PCR: Polymerase chain reaction; DHE: Dihydroethidium; 8-OHdG: 8-Hydroxy-2-deoxyguanosine; ROS: Reactive oxygen species; BW: Body weight; ITT: Insulin tolerance test; AUC: Area under the curve; FPG: Fasting plasma glucose; TG: Triglycerides; FFA: Free fatty acid; TC: Total cholesterol;

TNFi: Tumor necrosis factor α ; HOMA-IR: Homeostasis model assessment of insulin resistance; BP: Blood pressure; NOX: NADPH oxidase; CAT: Catalase; GPX1: Glutathione peroxidase 1; IL1 β : Interleukin 1 β ; VCAM1: Vascular cell adhesion molecule 1

Declarations

Ethics approval and consent to participate

This study was approved by the Laboratory Animal Committees of Kagoshima University Graduate School and was performed in accordance with the guidelines for the care and use of laboratory animals (approval number: MD20086).

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

Funding

This research was supported by JSPS KAKENHI grant number 20K08890.

Authors' contributions

K.O. and Y.N. conceived and designed the research; K.O., S.S., and S.K. performed the experiments; K.O., S.S., S.K., and Y.N. analyzed the data; K.O., S.S., S.K., H.H., T.D., and Y.N. interpreted the results of the experiments; K.O. and S.S. prepared the figures; K.O. drafted the manuscript; K.O. and Y.N. edited and revised the manuscript; K.O., S.S., S.K., H.H., T.D., and Y.N. approved the final version of the manuscript.

Acknowledgments

We wish to thank the Joint Research Laboratory, Kagoshima University Graduate School of Medical and Dental Sciences, for the use of their facilities.

References

1. Weber C, Noels H. Atherosclerosis. Current pathogenesis and therapeutic options. *Nat Med*. 2011;17:1410–22.
2. Varghese JF, Patel R, Yadav UCS. Novel Insights in the Metabolic Syndrome-induced Oxidative Stress and Inflammation-mediated Atherosclerosis. *Curr Cardiol Rev*. 2018;14:4–14.
3. Iciar MT, Cristina SC, Amparo SG, Francisco JC. Type 2 diabetes and cardiovascular disease: Have all risk factors the same strength? *World J Diabetes*. 2014;5:444–70.
4. Kawano H, Motoyama T, Hirashima O, Hirai N, Miyao Y, Sakamoto T, Kugiyama K, Ogawa H, Yasue H. Hyperglycemia rapidly suppresses flow-mediated endothelium-dependent vasodilation of brachial artery. *J Am Coll Cardiol*. 1999;34:146–54.
5. Antonio C, Katherine E, Ludovica P, Michael AI, Jessica ET, Roberto T, Massimo B, Dario G. Oscillating glucose is more deleterious to endothelial function and oxidative stress than mean glucose in normal and type 2 diabetic patients. *Diabetes*. 2008;57:1349–54.
6. Otsuka A, Azuma K, Iesaki T, Sato F, Hirose T, Shimizu T, Tanaka Y, Daida H, Kawamori R, Watada H. Temporary hyperglycaemia provokes monocyte adhesion to endothelial cells in rat thoracic aorta. *Diabetologia*. 2005;48:2667–74.
7. Shuto Y, Asai A, Nagao M, Sugihara H, Oikawa S. Repetitive Glucose Spikes Accelerate Atherosclerotic Lesion Formation in C57BL/6 Mice. *PLoS One*. 2015. doi:10.1371/journal.pone.0136840.
8. Bonora E, Muggeo M. Postprandial blood glucose as a risk factor for cardiovascular disease in Type II diabetes: the epidemiological evidence. *Diabetologia*. 2001;44:2107–14.
9. Ceriello A, Hanefeld M, Leiter L, Monnier L, Moses A, Owens D, Tajima N, Tuomilehto J. Postprandial glucose regulation and diabetic complications. *Arch Intern Med*. 2004;164:2090–95.
10. Kataoka Y, Yasuda S, Morii I, Otsuka Y, Kawamura A, Miyazaki S. Quantitative coronary angiographic studies of patients with angina pectoris and impaired glucose tolerance. *Diabetes Care*. 2005;28:2217–22.
11. Saely CH, Drexel H, Sourij H, Aczel S, Jahnel H, Zweiker R, Langer P, Marte T, Hoefle G, Benzer W, Wascher TC. Key role of postchallenge hyperglycemia for the presence and extent of coronary atherosclerosis: an angiographic study. *Atherosclerosis*. 2008;199:317–22.
12. Jiang J, Zhao L, Lin L, Gui M, Aleteng Q, Wu B, Wang S, Pan B, Ling Y, Gao X. Postprandial blood glucose outweighs fasting blood glucose and HbA1c in screening coronary heart disease. *Sci Rep*. 2017. doi:10.1038/s41598-017-14152-y.
13. Temelkova-Kurktschiev TS, Koehler C, Henkel E, Leonhardt W, Fuecker K, Hanefeld M. Postchallenge plasma glucose and glycemic spikes are more strongly associated with atherosclerosis than fasting glucose or HbA1c level. *Diabetes Care*. 2000;23:1830–34.

14. De Vegt F, Dekker JM, Ruhe HG, Stehouwer CD, Nijpels G, Bouter LM, Heine RJ. Hyperglycaemia is associated with all-cause and cardiovascular mortality in the Hoorn population: the Hoorn Study. *Diabetologia*. 1999;42:926–31.
15. Esposito K, Giugliano D, Nappo F, Marfella R. Campanian Postprandial Hyperglycemia Study G. Regression of carotid atherosclerosis by control of postprandial hyperglycemia in type 2 diabetes mellitus. *Circulation*. 2004;110:214–19.
16. Levitan EB, Song Y, Ford ES, Liu S. Is nondiabetic hyperglycemia a risk factor for cardiovascular disease? A meta-analysis of prospective studies. *Arch Intern Med*. 2004;164:2147–55.
17. Raz I, Wilson PW, Strojek K, Kowalska I, Bozikov V, Gitt AK, Jermendy G, Campaigne BN, Kerr L, Milicevic Z, Jacober SJ. Effects of prandial versus fasting glycemia on cardiovascular outcomes in type 2 diabetes: the HEART2D trial. *Diabetes Care*. 2009;32:381–86.
18. NAVIGATOR Study Group. Holman RR, Haffner SM, McMurray JJ, Bethel MA, Holzhauer B, Hua TA, Belenkov Y, Boolell M, Buse JB, Buckley BM, Chacra AR, Chiang FT, Charbonnel B, Chow CC, Davies MJ, Deedwania P, Diem P, Einhorn D, Fonseca V, Fulcher GR, Gaciong Z, Gaztambide S, Giles T, Horton E, Ilkova H, Jenssen T, Kahn SE, Krum H, Laakso M, Leiter LA, Levitt NS, Mareev V, Martinez F, Masson C, Mazzone T, Meaney E, Nesto R, Pan C, Prager R, Raptis SA, Rutten GE, Sandstroem H, Schaper F, Scheen A, Schmitz O, Sinay I, Soska V, Stender S, Tamás G, Tognoni G, Tuomilehto J, Villamil AS, Vozár J, Califf RM. Effect of nateglinide on the incidence of diabetes and cardiovascular events. *N Engl J Med*. 2010;362:1463–76.
19. Holman RR, Coleman RL, Chan JCN, Chiasson JL, Feng H, Ge J, Gerstein HC, Gray R, Huo Y, Lang Z, McMurray JJ, Rydén L, Schröder S, Sun Y, Theodorakis MJ, Tendera M, Tucker L, Tuomilehto J, Wei Y, Yang W, Wang D, Hu D, Pan C, ACE Study Group. Effects of acarbose on cardiovascular and diabetes outcomes in patients with coronary heart disease and impaired glucose tolerance (ACE): a randomised, double-blind, placebo-controlled trial. *Lancet Diabetes Endocrinol*. 2017;5:877–86.
20. Aslam M, Aggarwal S, Sharma KK, Galav V, Madhu SV. Postprandial Hypertriglyceridemia Predicts Development of Insulin Resistance Glucose Intolerance and Type 2 Diabetes. *PLoS One*. 2016. doi:10.1371/journal.pone.0145730.
21. Bae JH, Bassenge E, Kim KB, Kim YN, Kim KS, Lee HJ, Moon KC, Lee MS, Park KY, Schwemmer M. Postprandial hypertriglyceridemia impairs endothelial function by enhanced oxidant stress. *Atherosclerosis*. 2001;155:517–23.
22. Ceriello A. Postprandial hyperglycemia and diabetes complications: is it time to treat? *Diabetes*. 2005;54:1–7.
23. Nangaku M, Kanda H, Takama H, Ichikawa T, Hase H, Akizawa T. Kidney. Randomized Clinical Trial on the Effect of Bardoxolone Methyl on GFR in Diabetic Kidney Disease Patients (TSUBAKI Study). *Int Rep*. 2020;5:879–90.
24. Aminzadeh MA, Reisman SA, Vaziri ND, Shelkovnikov S, Farzaneh SH, Khazaali M, Meyer CJ. The synthetic triterpenoid RTA dh404 (CDDO-dhTFEA) restores endothelial function impaired by reduced Nrf2 activity in chronic kidney disease. *Redox Biol*. 2013;1:527–31.
25. Sharma A, Rizky L, Stefanovic N, Tate M, Ritchie RH, Ward KW, de Haan JB. The nuclear factor (erythroid-derived 2)-like 2 (Nrf2) activator dh404 protects against diabetes-induced endothelial dysfunction. *Cardiovasc Diabetol*. 2017. doi:10.1186/s12933-017-0513-y.
26. Venu L, Padmavathi IJ, Kishore YD, Bhanu NV, Rao KR, Sainath PB, Ganeshan M, Raghunath M. Long-term effects of maternal magnesium restriction on adiposity and insulin resistance in rat pups. *Obesity (Silver Spring)*. 2008;16:1270–76.
27. Haffner SM, Miettinen H, Stern MP. The homeostasis model in the San Antonio Heart Study. *Diabetes Care*. 1997;20:1087–92.
28. Wong WT, Tian XY, Chen Y, Leung FP, Liu L, Lee HK, Ng CF, Xu A, Yao X, Vanhoutte PM, Tipoe GL, Huang Y. Bone morphogenic protein-4 impairs endothelial function through oxidative stress-dependent cyclooxygenase-2 up-regulation: implications on hypertension. *Circ Res*. 2010;107:984–991., 2010.
29. Matsumoto T, Miyamori K, Kobayashi T, Kamata K. Specific impairment of endothelium-derived hyperpolarizing factor-type relaxation in mesenteric arteries from streptozotocin-induced diabetic mice. *Vascul Pharmacol*. 2006;44:450–60.
30. White CR, Darley-Usmar V, Berrington WR, McAdams M, Gore JZ, Thompson JA, Parks DA, Tarpey MM, Freeman BA. Circulating plasma xanthine oxidase contributes to vascular dysfunction in hypercholesterolemic rabbits. *Proc Natl Acad Sci USA*. 1996;93:8745–49.
31. Cheang WS, Wong WT, Zhao L, Xu J, Wang L, Lau CW, Chen ZY, Ma RC, Xu A, Wang N, Tian XY, Huang Y. PPAR δ Is Required for Exercise to Attenuate Endoplasmic Reticulum Stress and Endothelial Dysfunction in Diabetic Mice. *Diabetes*. 2017;66:519–28.
32. Rubanyi GM, Vanhoutte PM. Superoxide anions and hyperoxia inactivate endothelium-derived relaxing factor. *Am J Physiol*. 1986;250:H822–7.
33. Morikawa K, Shimokawa H, Matoba T, Kubota H, Akaike T, Talukder MA, Hatanaka M, Fujiki T, Maeda H, Takahashi S, Takeshita A. Pivotal role of Cu,Zn-superoxide dismutase in endothelium-dependent hyperpolarization. *J Clin Invest*. 2003;112:1871–79.
34. Olgar Y, Degirmenci S, Durak A, Billur D, Can B, Kayki-Mutlu G, Arioglu-Inan EE, Turan B. Exp Gerontol. Aging related functional and structural changes in the heart and aorta: Mito-TEMPO improves aged-cardiovascular performance. *Exp Gerontol*. 2018;110:172–81.
35. Blackwell KA, Sorenson JP, Richardson DM, Smith LA, Suda O, Nath K, Katusic ZS. Mechanisms of aging-induced impairment of endothelium-dependent relaxation: role of tetrahydrobiopterin. *Am J Physiol Heart Circ Physiol*. 2004;287:H2448-53.
36. Matsumoto T, Noguchi E, Kobayashi T, Kamata K. Mechanisms underlying the chronic pioglitazone treatment-induced improvement in the impaired endothelium-dependent relaxation seen in aortas from diabetic rats. *Free Radic Biol Med*. 2007;42:993–1007.
37. Wang Q, Zou MH. Measurement of Reactive Oxygen Species (ROS) and Mitochondrial ROS in AMPK Knockout Mice Blood Vessels. *Methods Mol Biol*. 2018;1732:507–17.
38. Rasband WS. ImageJ US. National Institutes of Health, Bethesda, Maryland, USA, 1997–2012; <http://imagej.nih.gov/ij/>.
39. Landmesser U, Dikalov S, Price SR, McCann L, Fukai T, Holland SM, Mitch WE, Harrison DG. Oxidation of tetrahydrobiopterin leads to uncoupling of endothelial cell nitric oxide synthase in hypertension. *J Clin Invest*. 2003;111:1201–9.
40. Potenza MA, Gagliardi S, Nacci C, Carratu' MR, Montagnani M. Endothelial dysfunction in diabetes: from mechanisms to therapeutic targets. *Curr Med Chem*. 2009;16:94–112.

41. Wolin MS, Gupte SA, Neo BH, Gao Q, Ahmad M. Oxidant-redox regulation of pulmonary vascular responses to hypoxia and nitric oxide-cGMP signaling. *Cardiol Rev.* 2010;18:89–93.
42. Kojda G, Harrison D. Interactions between NO and reactive oxygen species: pathophysiological importance in atherosclerosis, hypertension, diabetes and heart failure. *Cardiovasc Res.* 1999;43:562–71.
43. Ungvari Z, Bailey-Downs L, Gautam T, Jimenez R, Losonczy G, Zhang C, Ballabh P, Recchia FA, Wilkerson DC, Sonntag WE, Pearson K, de Cabo R, Csizsar A. daptive induction of NF-E2-related factor-2-driven antioxidant genes in endothelial cells in response to hyperglycemia. *Am J Physiol Heart Circ Physiol.* 2011;300:H1133-40.
44. Piconi L, Quagliaro L, Assaloni R, Da Ros R, Maier A, Zuodar G, Ceriello A. Constant and intermittent high glucose enhances endothelial cell apoptosis through mitochondrial superoxide overproduction. *Diabetes Metab Res Rev.* 2006;22:198–203.
45. Batinić-Haberle I, Cuzzocrea S, Rebouças JS, Ferrer-Sueta G, Mazzon E, Di Paola R, Radi R, Spasojević I, Benov L, Salvemini D. Pure MnTBAP selectively scavenges peroxynitrite over superoxide: comparison of pure and commercial MnTBAP samples to MnTE-2-PyP in two models of oxidative stress injury, a SOD-specific *Escherichia coli* model and carrageenan-induced pleurisy. *Free Radic Biol Med.* 2009;46:192–201.
46. Xia N, Horke S, Habermeyer A, Closs EI, Reifenberg G, Gericke A, Mikhed Y, Münzel T, Daiber A, Förstermann U, Li H. Uncoupling of Endothelial Nitric Oxide Synthase in Perivascular Adipose Tissue of Diet-Induced Obese Mice. *Arterioscler Thromb Vasc Biol.* 2016;36:78–85.
47. García-Prieto CF, Hernández-Nuño F, Rio DD, Ruiz-Hurtado G, Aránguez I, Ruiz-Gayo M, Somoza B, Fernández-Alfonso MS. High-fat diet induces endothelial dysfunction through a down-regulation of the endothelial AMPK-PI3K-Akt-eNOS pathway. *Mol Nutr Food Res.* 2015;59:520–32.
48. Chen JY, Tsai PJ, Tai HC, Tsai RL, Chang YT, Wang MC, Chiou YW, Yeh ML, Tang MJ, Lam CF, Shiesh SC, Li YH, Tsai WC, Chou CH, Lin LJ, Wu HL, Tsai YS. Increased aortic stiffness and attenuated lysyl oxidase activity in obesity. *Arterioscler Thromb Vasc Biol.* 2013;33:839–46.
49. Quagliaro L, Piconi L, Assaloni R, Martinelli L, Motz E, Ceriello A. Intermittent high glucose enhances apoptosis related to oxidative stress in human umbilical vein endothelial cells: the role of protein kinase C and NAD(P)H-oxidase activation. *Diabetes.* 2003;52:2795–804.
50. Inoguchi T, Li P, Umeda F, Yu HY, Kakimoto M, Imamura M, Aoki T, Etoh T, Hashimoto T, Naruse M, Sano H, Utsumi H, Nawata H. High glucose level and free fatty acid stimulate reactive oxygen species production through protein kinase C-dependent activation of NAD(P)H oxidase in cultured vascular cells. *Diabetes.* 2000;49:1939–45.
51. Chinen I, Shimabukuro M, Yamakawa K, Higa N, Matsuzaki T, Noguchi K, Ueda S, Sakanashi M, Takasu N. Vascular lipotoxicity: endothelial dysfunction via fatty-acid-induced reactive oxygen species overproduction in obese Zucker diabetic fatty rats. *Endocrinology.* 2007;148:160–5.
52. Mahmoud AM, Wilkinson FL, Jones AM, Wilkinson JA, Romero M, Duarte J, Alexander MY. A novel role for small molecule glycomimetics in the protection against lipid-induced endothelial dysfunction: Involvement of Akt/eNOS and Nrf2/ARE signaling. *Biochim Biophys Acta Gen Subj.* 2017;1861:3311–22.
53. Kaiser N, Sasson S, Feener EP, Boukobza-Vardi N, Higashi S, Moller DE, Davidheiser S, Przybylski RJ, King GL. Differential regulation of glucose transport and transporters by glucose in vascular endothelial and smooth muscle cells. *Diabetes.* 1993;42:80–9.
54. Eelen G, de Zeeuw P, Treps L, Harjes U, Wong BW, Carmeliet P. Endothelial Cell Metabolism. *Physiol Rev.* 2018;98:3–58.
55. Liemburg-Apers DC, Willems PH, Koopman WJ, Grefte S. Interactions between mitochondrial reactive oxygen species and cellular glucose metabolism. *Arch Toxicol.* 2015;89:1209–26.
56. Balaban RS, Nemoto S, Finkel T. Mitochondria, oxidants, and aging. *Cell.* 2005;120:483–95.
57. Dikalov SI, Nazarewicz RR, Bikineyeva A, Hilenski L, Lassègue B, Griendling KK, Harrison DG, Dikalova AE. Nox2-induced production of mitochondrial superoxide in angiotensin II-mediated endothelial oxidative stress and hypertension. *Antioxid Redox Signal.* 2014;20:281–94.
58. Chen XL, Dodd G, Thomas S, Zhang X, Wasserman MA, Rovin BH, Kunsch C. Activation of Nrf2/ARE pathway protects endothelial cells from oxidant injury and inhibits inflammatory gene expression. *Am J Physiol Heart Circ Physiol.* 2006;290:H1862-70.
59. Dreger H, Westphal K, Wilck N, Baumann G, Stangl V, Stangl K, Meiners S. Protection of vascular cells from oxidative stress by proteasome inhibition depends on Nrf2. *Cardiovasc Res.* 2010;85:395–403.
60. Itoh K, Tong KI, Yamamoto M. Molecular mechanism activating Nrf2-Keap1 pathway in regulation of adaptive response to electrophiles. *Free Radic Biol Med.* 2004;36:1208–13.
61. Jung KA, Kwak MK. The Nrf2 system as a potential target for the development of indirect antioxidants. *Molecules.* 2010;15:7266–91.
62. Lee Y, Shin DH, Kim JH, Hong S, Choi D, Kim YJ, Kwak MK, Jung Y. Caffeic acid phenethyl ester-mediated Nrf2 activation and IκappaB kinase inhibition are involved in NFκappaB inhibitory effect: structural analysis for NFκappaB inhibition. *Eur J Pharmacol.* 2010;643:21–8.
63. Yoshida LS, Tsunawaki S. Expression of NADPH oxidases and enhanced H₂O₂-generating activity in human coronary artery endothelial cells upon induction with tumor necrosis factor-α. *Int Immunopharmacol.* 2008;8:1377–85.
64. Myung SK, Ju W, Cho B, Oh SW, Park SM, Koo BK, Park BJ, Korean Meta-Analysis Study Group. Efficacy of vitamin and antioxidant supplements in prevention of cardiovascular disease: systematic review and meta-analysis of randomised controlled trials. *BMJ.* 2013. doi:10.1136/bmj.f10.
65. Sharma A, Rizky L, Stefanovic N, Tate M, Ritchie RH, Ward KW, de Haan JB. The nuclear factor (erythroid-derived 2)-like 2 (Nrf2) activator dh404 protects against diabetes-induced endothelial dysfunction. *Cardiovasc Diabetol.* 2017. doi:10.1186/s12933-017-0513-y.
66. Angulo J, El Assar M, Sevilleja-Ortiz A, Fernández A, Sánchez-Ferrer A, Romero-Otero J, Martínez-Salamanca JI, La Fuente JM, Rodríguez-Mañías L. Short-term pharmacological activation of Nrf2 ameliorates vascular dysfunction in aged rats and in pathological human vasculature. A potential target for therapeutic intervention. *Redox Biol.* 2019. doi:10.1016/j.redox.2019.101271.
67. Tan SM, Sharma A, Stefanovic N, Yuen DY, Karagiannis TC, Meyer C, Ward KW, Cooper ME, de Haan JB. Derivative of bardoxolone methyl, dh404, in an inverse dose-dependent manner lessens diabetes-associated atherosclerosis and improves diabetic kidney disease. *Diabetes.* 2014;63:3091–103.

68. Szczesny-Malysiak E, Stojak M, Campagna R, Grosicki M, Jamrozik M, Kaczara P, Chlopicki S. Bardoxolone Methyl Displays Detrimental Effects on Endothelial Bioenergetics, Suppresses Endothelial ET-1 Release, and Increases Endothelial Permeability in Human Microvascular Endothelium. *Oxid Med Cell Longev* Oct. 2020. doi:10.1155/2020/4678252.

Tables

Table 1. Metabolic parameters

		Control diet						Western-type diet						Two-way ANOVA	
		GS (-)			GS (+)			GS (-)			GS (+)			Diet	GS
		n = 7			n = 7			n = 7			n = 7				
Plasma insulin	pmol/L	107.1	±	12.5	88.4	±	12.5	314.8	±	33.8	343.7	±	38.4	< 0.001	0.758
Serum triglycerides	mmol/L	0.6	±	0.1	0.5	±	0.1	1.7	±	0.1	1.8	±	0.1	< 0.001	0.882
Serum free fatty acids	μEq/L	357.3	±	25.8	348.1	±	20.7	710.1	±	41.8	720.1	±	34.4	< 0.001	0.990
Serum TNFα	pg/mL	9.6	±	1.2	7.8	±	0.7	14.7	±	2.0	15.2	±	2.1	< 0.001	0.608
Fasting plasma glucose	mmol/L	5.0	±	0.3	4.6	±	0.3	5.7	±	0.3	5.6	±	0.2	0.003	0.501
Serum total cholesterol	mmol/L	1.5	±	0.1	1.4	±	0.1	1.4	±	0.1	1.4	±	0.1	0.535	0.507
HOMA-IR		3.6	±	0.5	2.8	±	0.4	13.1	±	1.8	12.9	±	1.5	< 0.001	0.645
Systolic blood pressure	mmHg	131.7	±	8.5	125.4	±	10.2	149.7	±	10.2	146.7	±	10.6	0.059	0.644
Diastolic blood pressure	mmHg	77.0	±	5.4	76.4	±	4.6	86.7	±	5.3	85.3	±	5.7	0.089	0.851

GS: Glucose spike; TNFα: tumor necrosis factor α; HOMA-IR: Homeostasis model assessment of insulin resistance

The data are presented as the mean ± SEM.

Figures

Figure 1

Glucose spikes deteriorate endothelium-dependent relaxation in diet-induced obese rats independent of weight gain. Rats were fed a control diet (CD) or Western-type diet (WTD) for 13 weeks and administered saline or glucose for 1 week twice daily (7 rats per group). **A:** Curves of endothelium-dependent relaxation (EDR) in the thoracic aorta in response to ACh under 5.5 mM glucose, 20 mM glucose and 20 mM raffinose conditions. * $P < 0.05$, † $P < 0.005$, § $P < 0.001$ vs. 5.5 mM glucose and 20 mM raffinose in the WTD-GS (+) group, one-way repeated-measures ANOVA with the Bonferroni post hoc test. **B:** Vascular sensitivity, plotted as pD2 (-log of the half-maximal effective concentration [EC50]) of ACh. A significant interaction was detected between diet and GS factors under 20 mM glucose conditions ($F[1, 24] = 17.4$, $P < 0.001$). § $P < 0.001$, two-way ANOVA followed by simple-effects analysis. **C:** Curves of vasorelaxation in response to SNP under 20 mM glucose conditions. **D:** Curves of EDR in response to ACh in the presence of L-NAME and 20 mM glucose. Two-way repeated-measures ANOVA. **E:** Body weight curve over the duration of feeding and administration. § $P < 0.001$ vs. between diet groups, two-way repeated-measures ANOVA. **F:** Adiposity index (the ratio of epididymal, retroperitoneal and mesenteric fat mass to body weight). **G:** Insulin tolerance test (ITT). Insulin resistance is presented as the decreasing glucose area under the curve (AUC). § $P < 0.001$ vs. between diet groups, two-way ANOVA. No interaction was detected between diet and GS factors. The data are presented as the mean $\pm$ SEM. Glu, glucose; Raffi, raffinose; SNP, nitroprusside. CD, control diet; WTD, Western-type diet; GS, glucose spike. The GS (-) group consisted of rats given saline, and the GS (+) group consisted of rats given glucose.

Figure 2

NOX inhibitors, SOD and catalase ameliorate endothelial dysfunction under high-glucose conditions in the WTD-GS (+) group. Rats were fed a control diet (CD) or Western-type diet (WTD) for 13 weeks and administered saline or glucose for 1 week. Vascular sensitivity under 20 mM glucose conditions was plotted as pD2 (- log of the half-maximal effective concentration [EC50]) of ACh. The control was administered to 7 rats, and other agents were administered to 4 rats each. * P < 0.05, † P < 0.01, § P < 0.001 vs. control in the WTD-GS (+) group, Dunnett's test. The data are presented as the mean ± SEM. Indomethacin, a cyclooxygenase inhibitor; allopurinol, a xanthine oxidase inhibitor; apocynin, a NADPH oxidase inhibitor; SOD, superoxide dismutase; Mito-TEMPO, a mitochondria-targeted superoxide scavenger; MnTAPB, a superoxide dismutase mimetic and peroxynitrite selective scavenger. CD, control diet; WTD, Western-type diet; GS, glucose spike. The GS (-) group consisted of rats given saline, and the GS (+) group consisted of rats given glucose.

Figure 3

Quantitative PCR analysis of gene expression in the thoracic aorta. mRNA expression of NOX2 and SOD2 was synergistically changed by the combination of a Western-type diet (WTD) and repeated glucose spikes (GSs). Rats were fed a control diet (CD) or Western-type diet (WTD) for 13 weeks and administered saline or glucose for 1 week twice daily (6 rats per group). The significant interactive effect of diet and GS on the mRNA expression of NOX2, p47phox, SOD2 and VCAM1 was confirmed by two-way ANOVA followed by simple-effects analysis (* P < 0.05, ‡ P < 0.005, § P < 0.001). The mRNA expression of catalase and TNFα was significantly different between diet or GS factors and that of IL1β was different between GS factors without diet/GS interaction. * P < 0.05, † P < 0.01, ‡ P < 0.005 vs. between diet or GS factors, two-way ANOVA. The data are presented as the mean ± SEM. CD, control diet; WTD, Western-type diet; GS, glucose spike. The GS (-) group consisted of rats given saline, and the GS (+) group consisted of rats given glucose.

A

B

Figure 4

Superoxide anion is enhanced by repeated glucose spikes in diet-induced obese rats. Rats were fed a control diet (CD) or Western-type diet (WTD) for 13 weeks and given saline or glucose for 1 week (4 rats per group). The thoracic aorta was removed 2 hours after the intraperitoneal administration of glucose (1 g/kg) to the 4 groups or saline to the WTD-GS (+) group. A: Representative images of DHE staining and DHE fluorescence intensity in the thoracic aorta. The significant interactive effect of diet and GS on DHE fluorescence intensity was confirmed by two-way ANOVA followed by simple-effects analysis ($P < 0.001$). B: DHE fluorescence intensity in the aortas incubated with PEG-SOD and in the aortas removed after the intraperitoneal administration of saline to the WTD-GS (+) group. The control group (Glu i.p.) was the same as the WTD-GS (+) group in Fig. 4A. $P < 0.001$, one-way ANOVA. The data are presented as the mean $\pm$ SEM. Glu, glucose; i.p., intraperitoneal; PEG-SOD, polyethylene glycol-superoxide dismutase; CD, control diet; WTD, Western-type diet; GS, glucose spike. The GS (-) group consisted of rats given saline, and the GS (+) group consisted of rats given glucose.

Figure 5

CDDO-Me protects endothelial function in diet-induced obese rats against repeated glucose spikes. Rats were fed a control diet (CD) or Western-type diet (WTD) for 13 weeks, treated with vehicle or CDDO-Me for 2 weeks and administered saline or glucose for 1 week (4 rats per group). A: Curves of endothelium-dependent relaxation in response to ACh under 5.5 mM glucose or 20 mM glucose conditions. † $P < 0.01$, ‡ $P < 0.005$ (to vehicle [Glu 5.5 mM] in the WTD-GS [+] group), one-way repeated-measures ANOVA with the Bonferroni post hoc test. B: Vascular sensitivity, plotted as pD2 (-log of the half-maximal effective concentration [EC50]) of ACh. § $P < 0.001$, one-way ANOVA with the Bonferroni post hoc test. The data are presented as the mean $\pm$ SEM. Glu, glucose; CD, control diet; WTD, Western-type diet; GS, glucose spike. The GS (-) group consisted of rats given saline, and the GS (+) group consisted of rats given glucose.

Figure 6

The effect of CDDO-Me on gene expression and oxidative stress. CDDO-Me suppresses NOX2 and enhances SOD2 and catalase gene expression in the thoracic aorta and suppresses local and systemic oxidative stress in the WTD-GS (+) group. Rats were fed a control diet (CD) or Western-type diet (WTD) for 13 weeks, treated with vehicle or CDDO-Me for 2 weeks and administered saline or glucose for 1 week (4 rats per group). A-H: Quantitative PCR analysis of the mRNA expression of NQO1 (A), NOX2 (B), p47phox (C), SOD2 (D), catalase (E), TNF α (F), IL1 β (G) and VCAM1 (H) in the thoracic aorta. I: DHE fluorescence intensity in the thoracic aorta. J: Urinary 8-OHdG levels. Thoracic aortas and urinary samples were collected 2 hours after the administration of glucose to evaluate oxidative stress. * $P < 0.05$, † $P < 0.01$, ‡ $P < 0.005$, Bonferroni correction. The data are presented as the mean $\pm$ SEM. CD, control diet; WTD, Western-type diet; GS, glucose spike. The GS (-) group consisted of rats given saline, and the GS (+) group consisted of rats given glucose.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [SupplementaryFigure.pptx](#)
- [SupplementaryTableS1.docx](#)