Resveratrol Protects Vascular Endothelial Cells from High Glucose–Induced Apoptosis through Inhibition of NADPH Oxidase Activation–Driven Oxidative Stress

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SUMMARY
Introduction: Hyperglycemia-induced oxidative stress has been implicated in diabetic vascular complications in which NADPH oxidase is a major source of reactive oxygen species (ROS) generation. Resveratrol is a naturally occurring polyphenol, which has vasoprotective effects in diabetic animal models and inhibits high glucose (HG)–induced oxidative stress in endothelial cells. Aims: We aimed to examine whether HG-induced NADPH oxidase activation and ROS production contribute to glucotoxicity to endothelial cells and the effect of resveratrol on glucotoxicity. Results: Using a murine brain microvascular endothelial cell line bEnd3, we found that NADPH oxidase inhibitor (apocynin) and resveratrol both inhibited HG-induced endothelial cell apoptosis. HG-induced elevation of NADPH oxidase activity and production of ROS were inhibited by apocynin, suggesting that HG induces endothelial cell apoptosis through NADPH oxidase–mediated ROS production. Mechanistic studies revealed that HG upregulated NADPH oxidase subunit Nox1 but not Nox2, Nox4, and p22phox expression through NF-κB activation, which resulted in elevation of NADPH oxidase activity and consequent ROS production. Resveratrol prevented HG-induced endothelial cell apoptosis through inhibiting HG-induced NF-κB activation, NADPH oxidase activity elevation, and ROS production. Conclusions: HG induces endothelial cell apoptosis through NF-κB/NADPH oxidase/ROS pathway, which was inhibited by resveratrol. Our findings provide new potential therapeutic targets against brain vascular complications of diabetes.

Introduction
A substantial body of evidence implicates oxidative stress as an important pathogenic factor in diabetic vascular complications. The drivers of the oxidative stress include hyperglycemia, hyperinsulinemia, and the elevated free fatty acids and lipids that are usually associated with diabetes. Reactive oxygen species (ROS) production emanates from several different sources, including NADPH oxidase, mitochondrial electron transport system, xanthine oxidase, cytochrome p450, uncoupled nitric oxide synthase, and myeloperoxidase [1]. NADPH oxidase has been implicated as the major source of ROS generation in the vasculature in response to high glucose (HG) [2,3]. Therefore, inhibition of ROS production through the inhibitors against NADPH oxidase is proposed as an alternative approach to conventional antioxidant therapies against diabetes and vascular complications [4].

Resveratrol (3, 4′, 5-trihydroxystilbene) is a natural polyphenol found in grapes, red wine, berries, knotweed, peanuts, and other plants. Collected evidence suggests that resveratrol has cardioprotective, anticancer, antiinflammatory, and antioxidative properties [5]. Resveratrol exerts significant vasoprotective effects in animal models of type 2 diabetes [6]. In cultured coronary arterial endothelial cells, resveratrol attenuates HG–induced oxidative stress through induction of mitochondrial content [7], upregulation of manganese superoxide dismutase expression, and increase in cellular GSH content through activation of SIRT1
[8], as well as activation of nuclear factor-E2-related factor-2 (Nrf2), a transcription factor that regulates the expression of numerous ROS detoxifying and antioxidant genes [9]. However, the effect of resveratrol on HG–induced NADPH oxidase activation and ROS production in vascular endothelial cells is not clear. In this study, we investigated the effect of resveratrol on NADPH oxidase–mediated oxidative stress and apoptosis of brain microvascular endothelial cells in response to HG and explored the underlying mechanisms.

Materials and Methods

Cell Culture and Treatment

Murine brain microvascular endothelial cell line bEnd3 was cultured in Dulbecco’s Modified Eagle Medium (DMEM) containing 1000 mg/L (5.6 mM) glucose, 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin in a humidified atmosphere at 37°C with 5% CO₂. High-glucose treatment was performed by incubating cells in DMEM containing 4500 mg/L (25 mM) glucose for the indicated times. For ROS measurements and Western blot assays, cells were cultured overnight in DMEM containing 5.6 mM glucose and 0.5% bovine serum albumin before experiments.

Cell Viability Assay

Cell viability was measured using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; Sigma, St. Louis, MO, USA) according to the manufacturer’s protocol. Briefly, endothelial cells were cultured in 96-well plates with medium containing 5.6 mM glucose or 25 mM glucose with without different concentrations of resveratrol or apocynin for 24 h. MTT (0.5 μg/μL per well) was added and incubated at 37°C for 4 h. The intracellular purple formazan product was dissolved with hydrogen chloride/isopropanol (0.04 mM). The absorbance at 570 nm (reference at 630 nm) was determined by a microplate reader Multiskan JX (Thermo LabSystems, Franklin, MA, USA). Each experiment was repeated three times in triplicate samples. The cell viability was expressed as a percentage of the cells cultured in medium containing 5.6 mM glucose.

Apoptosis Determination

The morphological change in the nuclei in apoptotic cells was examined under fluorescence microscope as described previously with modification [10]. Briefly, cells cultured on glass slides were washed with PBS and fixed with 4% (wt/vol) of neutral buffered formalin for 30 min, then washed with PBS, and stained with 0.6 mg/mL Hoechst 33258 (Sigma). The images were taken at 400× magnification under a fluorescence microscope (Olympus BX61, Tokyo, Japan). The percentage of apoptotic bodies (fragmented and condensed nuclei) were examined with 500 nuclei from control and experimental cells, respectively.

RNA Extraction and RT-PCR

Total RNA was extracted from bEnd3 cells using the TRIZOL reagent (Invitrogen, Carlsbad, CA, USA) and depleted contaminating DNA with RNase-free Dnase. cDNA was synthesized from 2 μg RNA with M-MuLV reverse transcriptase and random hexamer according to the manufacturer’s instructions (Fermentas, Burlington, ON, Canada). The cDNA fragment was amplified by PCR using the following specific primers: Nox1: 5’-ATCCCTGAT-TCCGTGTTGTCG-3’ (sense), 5’-GGGTCCTCCGTAGGTCG-3’ (antisense); Nox2: 5’-CTCAGGGGTCTTACGTGCGT-3’ (sense), 5’-CCATTTCCAGTCAATAGGGG-3’ (antisense); p22phox: 5’-GGGGAAAGGGAAAAAGG-3’ (sense), 5’-CACACAGAAATGGAGAGCA-3’ (antisense); β-actin: 5’-TGGTATGTTGGAATGGGCAG-3’ (sense), 5’-TTGATGTCAGCGACGTTC-3’ (antisense). PCR products were visualized by ethidium bromide staining in 1.5% agarose gel and quantified using the Dolphin-Doc image analyzer (WEALTEC Corp., Sparks, NV, USA). Amplification of the target cDNA was normalized to β-actin expression.

Western Blot

The cells were lysed with cold lysis buffer and, the proteins were electrophoresed on 10% SDS-PAGE gel and transferred onto polyvinylidene difluoride membrane [11]. The membranes were blocked with 5% nonfat milk and then were incubated with primary antibodies overnight at 4°C. After incubation with a horseradish peroxidase–conjugated secondary antibody, the protein bands were detected with a Supersignal West Pico chemiluminescent substrate (Pierce, Rockford, IL, USA) and X-Omat BT film (Eastman Kodak Co., Rochester, NY, USA). Polyclonal antibody against Nox1 was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibody against phosphorylated IκBα was from Cell Signaling Technology (New England Biolabs, Beverly, MA, USA). Monoclonal antibody against β-actin was bought from Abcam (Abcam, Cambridge, UK).

NADPH Oxidase Assay

NADPH oxidase activity was measured by the lucigenin chemiluminescence method [12]. The cells were washed three times with ice-cold PBS and scraped from the plate followed by centrifugation at 1000 × g at 4°C for 10 min. The cell pellets were resuspended in lysis buffer containing 20 mM KH₂PO₄, pH 7.0, 1 mM ethylene glycol tetraacetic acid (EGTA), 1 mM phenylmethylsulfonyl fluoride, 10 μg/mL aprotinin, and 0.5 μg/mL leupeptin. Cell suspensions were homogenized with 100 strokes in a Dounce homogenizer on ice. Hundred microliters of homogenate was added into 900 μL of phosphate buffer (50 mM, pH 7.0), containing 1 mM EGTA, 150 mM sucrose, 5 μM lucigenin (TCI, Tokyo, Japan), and 100 μM NADPH. Photon emission was measured every 15 second for 5 min in a luminometer (Berthold, Bad Wildbad, Germany). A buffer blank was subtracted from each reading before calculation of the data. NAPD oxidase activity was defined as relative chemiluminescence (light) units per second per milligram of protein.

Measurement of Intracellular ROS Production

The membrane permeable indicator 2′,7′-dichlorodihydrofluorescein diacetate (H₂DCF-DA) (Invitrogen) was used to detect intracellular ROS production by bEnd3 cells. The cells were cultured in...
medium containing 5.6 mM or 25 mM glucose with or without different concentrations of apocynin or resveratrol for the indicated length of time, then were loaded with 10 μM H$_2$DCF-DA in serum-free DMEM containing 5.6 mM or 25 mM glucose at 37°C for 30 min, and washed twice with PBS. Intracellular ROS production was detected by the FlexStation II384 fluorometric imaging plate reader (Molecular Devices, Sunnyvale, CA, USA) at an excitation wavelength of 488 nm and an emission wavelength of 525 nm.

**Cell Transfection**

bEnd3 cells were transfected with IκBα-2N, a dominant-negative IκBα expressing plasmid, or control vector flag-zeo [13] (a kind gift from Dr. R. Lin (McGill University, Montreal, QC, Canada) using SuperFect Transfection Reagent (Qiagen, Valencia, CA, USA). Thirty-six hours after transfection, the cells were stimulated with 25 mM glucose for 36 h, and Nox1 protein expression was detected by Western blot.

**Inhibition of Nox1 Expression by RNA Interference**

siRNA against mouse Nox1 and the control siRNA were synthesized by GenePharma (Shanghai, China). The sequences of Nox1 siRNA are 5′-CCUUACUGAGUGAUUGCCACUGUA-3′ (sense) and 5′-UACAGUGGCAACUCACUAAGG-3′ (antisense) [14]. The sequences for control siRNA are 5′-UUCUCCGAACGUGUCACGUTT-3′ (sense) and 5′-ACGUGACACGUUGAATT-3′ (antisense). bEnd3 cells were transfected with siRNA at final concentration of 100 nM using SuperFect Transfection Reagent (Qiagen, Hilden, Germany). After transfection for 36 h, the cells were cultured in medium containing 5.6 mM or 25 mM glucose for additional 24 h and then Nox1 protein expression and NADPH oxidase activity were examined.

**Statistical Analysis**

Data are presented as means ± SD. Statistical differences between groups were analyzed by unpaired Student’s t-test.

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**Results**

**Resveratrol Inhibits High Glucose–Induced Endothelial Cell Apoptosis**

We cultured bEnd3 cells in medium containing 5.6 mM or 25 mM glucose and found that 25 mM glucose significantly reduced endothelial cell viability detected by MTT assay (Figure 1A) and increased cell apoptosis measured by nuclear staining with Hoechst 33258 (Figure 1B). These results are consistent with previous reports [15,16]. Apocynin, an NADPH oxidase inhibitor, and resveratrol both protected bEnd3 cells from the toxicity of 25 mM glucose, while these two compounds had no effect on viability of bEnd3 cells cultured in medium containing 5.6 mM glucose (Figure 1A,B). These results indicate that NADPH oxidase activation–driven oxidative stress mediates HG–induced endothelial apoptosis. As resveratrol has been reported to inhibit the basal expression of NADPH oxidase subunit Nox4 in human umbilical vein endothelial cells [17], the protective effect of resveratrol from HG–induced apoptotic cell death may be mediated thorough inhibition of NADPH oxidase activation by HG.

**Resveratrol Inhibits High Glucose–Induced NADPH Oxidase Activation and ROS Production in Endothelial Cells**

We then examined the effect of resveratrol on NADPH oxidase activation and ROS production by HG. High-glucose (25 mM) treatment resulted in significant increase in NADPH oxidase activity in bEnd3 cells (Figure 2A), which could be markedly inhibited by 25 μM resveratrol or 200 nM apocynin (Figure 2B). Further studies showed that HG triggered ROS production in a time-dependent manner (Figure 3A), which was also reversed by apocynin (Figure 3B). Similarly, resveratrol dose dependently inhibited ROS production induced by HG (Figure 3C). These results indicate that resveratrol inhibits HG–induced ROS production through inhibition of NADPH oxidase activation by HG.
High Glucose Enhances NADPH Oxidase Activity in Endothelial Cells Through Upregulation of Nox1 Expression

We examined the effect of glucose on the expression of NADPH oxidase subunits Nox1, Nox2, NOx4, and p22phox and found that HG (25 mM) induced Nox1 expression at mRNA and protein levels in bEnd3 cells (Figure 4A,B). Transfection of bEnd3 cells with Nox1 siRNA significantly inhibited HG–induced Nox1 protein expression (Figure 4C) and abolished HG–induced NADPH oxidase activation (Figure 4D). These results demonstrate that the augmentation of NADPH oxidase activity by HG is mediated by upregulation of Nox1.

Resveratrol Attenuates Nox1 Upregulation in Endothelial Cells by High Glucose through Inhibition of NF-κB Activation

We further investigated the effect of resveratrol on Nox1 upregulation by HG and explored the underlying mechanisms. Pretreatment of bEnd3 cells with 25 μM resveratrol significantly inhibited Nox1 mRNA and protein elevation by HG (25 mM) (Figure 5A,B). Sulfasalazine, an NF-κB inhibitor, suppressed the increase in Nox1 protein by HG (Figure 5B). Transfection of bEnd3 cells with dominant-negative IκBα plasmids also inhibited HG–induced Nox1 expression (Figure 5C). These results demonstrate that HG induces Nox1 expression through NF-κB.

We further found that sulfasalazine could inhibit HG–induced ROS production (Figure 5D) and reverse the inhibition of cell viability by HG (Figure 5E). These results combined with the previous results indicate that HG induces vascular endothelial cell death through NF-κB-mediated NADPH oxidase subunit Nox1 expression, NADPH oxidase activation, and subsequent ROS production.

To elucidate whether resveratrol directly inhibit NF-κB activation by HG, we performed Western blot to examine IκBα phosphorylation. As shown in Figure 5F, HG induced IκBα phosphorylation in a time-dependent manner, and resveratrol significantly inhibited HG–induced IκBα phosphorylation. These results together with our previous results demonstrate that resveratrol inhibits HG–induced Nox1 elevation in endothelial cells through suppression of NF-κB/Nox1/ROS pathway by HG.

Discussion

In this study, we demonstrated that resveratrol attenuated hyperglycemia-induced endothelial apoptosis through inhibition of oxi-
Dative stress. The protective effect of resveratrol is mediated by inhibiting the activation of NF-κB/Nox1/NADPH oxidase/ROS pathway by HG.

Oxidative stress mediated by hyperglycemia-induced generation of ROS contributes significantly to the development and progression of diabetes and related vascular complications [18]. NAD(P)H oxidase has been implicated as the major source of ROS generation in the vasculature in response to HG [19]. Vascular NADPH oxidase is a multicomponent enzyme composed of membrane-bound subunits (Nox protein and p22phox) and cytosolic subunits (p47phox, p67phox, and Rac). Nox protein acts as the catalytic subunit of NADPH oxidase. NADPH oxidases can be divided into different isoforms according to Nox proteins. Nox1, Nox2, and Nox4 are expressed significantly in the vascular system [20]. NADPH oxidase subunits have been reported to be elevated in different vascular beds of rodent diabetic models, including Nox2 in retinal vessels; Nox2, Nox4, p22phox, p47phox, p67phox, and Rac1 in kidney vessels; Nox1, Nox2, Nox4, p22phox, and Rac1 in aorta vessels [19]. Expression of p22phox is increased in mouse pancreatic islet endothelial cell line and rat coronary microvascular endothelial cells exposed to HG [21,22]. High glucose induces p22phox, p47phox, p67phox, and Rac1 expression in human umbilical endothelial cells and upregulates Nox4 level in human aortic endothelial cells [16,23,24]. As mouse cerebral vascular system expresses Nox1, Nox2, and Nox4, and different NADPH oxidase isoforms has a common p22phox subunit but different cytosolic subunits [20], we examined the effect of HG on the expression of Nox1, Nox2, Nox4, and p22phox and activation of NADPH oxidase. We found that HG induced ROS production in mouse brain microvascular endothelial cells through upregulation of Nox1 expression and enhanced NADPH oxidase activity. The difference in NADPH oxidase subunits upregulation by HG may be due to the differences in species and cell origin.

Accumulating evidences support that NF-κB activation is involved in the pathogenesis of diabetes and diabetic vascular complications. In patients with type 2 diabetes, NF-κB activation is associated with increased transcription of the p65 subunit of NF-κB [25]. The activation of NF-κB in human kidney biopsies has been shown to correlate with severity of proteinuria and degree of glycemic control in diabetic nephropathy [26]. NF-κB was activated in endothelial cells, retina, heart, and kidneys of diabetic rats [27], and administration of NF-κB inhibitor protects retinal capillary cells from apoptotic death [28,29]. In vitro studies

**Figure 4** High glucose (HG) enhances NADPH oxidase activity in endothelial cells through upregulation of Nox1 expression. (A–B) bEnd3 cells were cultured in medium containing 5.6 mM glucose (NG) or 25 mM glucose (HG) for different periods of time, the mRNA levels of Nox1, Nox2, Nox4, and p22phox were examined by RT-PCR (A), and protein levels of Nox1 were examined by Western blot (B). (C–D) bEnd3 cells transfected with control siRNA or Nox1 siRNA for 36 h were incubated with 5.6 mM glucose (NG) or 25 mM glucose (HG) for additional 36 h and then examined for Nox1 expression by Western blot (C) and NADPH oxidase activity (D). All results represent the mean ± SD of three independent experiments. The representative images of RT-PCR and Western blot are shown in the upper part of A, B, and C, respectively. A–B: *P < 0.05, **P < 0.01 compared with cells cultured with NG. C–D: ***P < 0.01 compared with control siRNA transfected cells cultured with NG. # P < 0.05, ## P < 0.01 compared with control siRNA transfected cells cultured with HG.
showed that through activation of NF-κB in endothelial cell, HG induced the expression of adhesion molecules and chemokines and promoted apoptotic cell death [30–32]. Our present studies demonstrated that through activation of NF-κB in brain vascular endothelial cells, HG induced NADPH subunit Nox1 expression, which resulted in NADPH activation, ROS production, and apoptotic cell death. High glucose has been reported to activate NF-κB in human glomerular endothelial cells through IκBα phosphorylation and p65 nuclear translation [33]. Our results revealed that the activation of NF-κB by HG in murine endothelial cells was also mediated by phosphorylation of IκBα.

Resveratrol has been reported to improve vascular responses in streptozotocin-induced diabetic rats [34]. In a mouse model of diabetes, resveratrol restored endothelial function and vascular responses by inhibiting TNF-α-induced activation of NAD(P)H oxidase and preserving endothelial nitric oxide synthase phosphorylation [6]. In vitro studies showed that resveratrol attenuated HG-induced oxidative stress in endothelial cells through multiple pathways as described in the introduction section [7–9]. Our new finding is that resveratrol protects endothelial cells from HG-induced apoptosis through inhibition of NADPH oxidase–driven oxidative pathway.

**Figure 5** Resveratrol attenuates Nox1 upregulation in endothelial cells by high glucose (HG) through inhibition of NF-κB activation. (A) bEnd3 cells pretreated with or without 25 μM resveratrol (RV) for 2 h were cultured in medium containing 5.6 mM glucose (NG) or 25 mM glucose (HG) for 36 h, and then, Nox1 mRNA level was examined by RT-PCR. (B) bEnd3 cells pretreated with 25 μM resveratrol (RV) or 50 μM sulfasalazine (Sul) for 1 h were incubated with medium containing 5.6 mM glucose (NG) or 25 mM glucose (HG) for 36 h and then examined for Nox1 expression by Western blot. (C) bEnd3 cells transfected with dominant-negative IκB (DN-IκB) or control vector flag-zeo for 36 h were treated with 5.6 mM glucose (NG) or 25 mM glucose (HG) for another 36 h and then examined for Nox1 expression by Western blot. (D–E) bEnd3 cells were exposed to 5.6 mM glucose (NG) or 25 mM glucose (HG) in the presence or absence of 50 μM sulfasalazine, reactive oxygen species production was examined with H2DCF-DA after 12 h (D), and cell viability was examined with MTT assay after 24 h (E). (F) bEnd3 cells pretreated with or without 25 μM resveratrol for 1 h were incubated with medium containing 5.6 mM glucose (NG) or 25 mM glucose (HG) for the indicated times and then examined for IκBα phosphorylation by Western blot. All results represent the mean ± SD of three independent experiments. The representative images of RT-PCR and Western blot are shown in the upper part of A–C and F. A, B, D, E: **P < 0.01 compared with cells cultured with NG alone; *P < 0.05, ***P < 0.01 compared with cells cultured with HG alone. C: *P < 0.05 compared with control plasmid flag-zeo-transfected cells cultured with NG; **P < 0.01 compared with flag-zeo-transfected cells cultured with HG. F: *P < 0.05, **P < 0.01 compared with cells cultured with NG. *P < 0.05, **P < 0.01 compared with cells treated with HG alone for corresponding time.
In summary, using a murine brain microvascular endothelial cell line, we demonstrated that resveratrol protected endothelial cells from HG-induced apoptosis through inhibition of NF-κB/Nox1/NADPH oxidase/ROS pathway. Further studies with mouse primary brain microvascular endothelial cells and related animal models will be helpful to confirm our in vitro results and to identify signaling molecule(s) in this pathway as target to prevent vascular complications under hyperglycemic conditions.

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References

2. Christ M, Bauersachs J, Liebetrau C, Heck M, Günther A, Wehling M. Glucose increases endothelial-dependent superoxide formation in coronary arteries by NAD(P)H oxidase activation: Attenuation by the 3-hydroxy-3-

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Conflict of Interest

The authors declare no conflict of interest.