Glutathione Prevents Free Fatty Acids-Induced Oxidative Stress and Apoptosis in Human Brain Vascular Endothelial Cells Through Akt Pathway

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Keywords

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SUMMARY

Aims: The damage of human brain vascular endothelial cells (HBVECs) is the key pathogenesis of diabetes-associated cerebral vascular complications. The aim of this study was to elucidate the effects of glutathione (GSH) on free fatty acids (FFAs)-induced HBVECs apoptosis, oxidative stress, and the involved possible signaling pathway. **Methods:** After culturing HBVECs for 72 h with GSH and FFAs, we determined cell proliferation by CCK8, detected apoptosis by caspase-3 and Annexin V-FITC/PI staining, and judged oxygen stress by determining the reactive oxygen species (ROS) and the mitochondrial membrane potential (MMP). We investigated whether the Akt pathway was involved in FFAs-induced signaling pathway alteration and whether GSH influenced the above effects. **Results:** After being cultured in 200 μ M FFAs for 72 h, the HBVECs proliferation significantly decreased; HBVECs apoptosis increased; the ROS levels increased; and the HBVECs MMP subsequently decreased. FFAs induced a significant decrease in phosphorylated active Akt. These alterations were obviously prevented when 1 mM GSH was added to culture medium containing FFAs, and the above effects of GSH were blocked by Akt inhibitor. **Conclusion:** GSH may prevent FFAs-induced HBVECs damage, oxidative stress, and apoptosis through activating the Akt pathway.

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The first two authors contributed equally to this work.

Introduction

Evidence indicates that diabetes-associated atherosclerosis and vascular diseases are the principal causes of morbidity and mortality in patients with diabetes [1]. One study indicates that the endothelial cells (EC) play an important role in regulating the delicate balances between vasoconstriction and vasodilatation,

coagulation and fibrinolysis, and proliferation and apoptosis [2]. EC injury could be one of the earliest features of vascular damage leading to the development of atherosclerosis and vascular complications [3–5]. Environmental factors, such as the metabolic alterations observed in type 2 diabetes, may contribute to the loss of endothelial integrity and lead to vascular complications [6].

One of the main metabolic abnormalities observed in type 2 diabetes is the elevation of free fatty acids (FFAs) in plasma, leading to both cell apoptosis and insulin resistance [7–9]. Clinical studies have shown that increasing FFAs in plasma could induce endothelial damage [10]. Our research results demonstrated that high levels of FFAs-induced oxidative stress, which damaged human brain vascular endothelial cells (HBVECs) and resulted in apoptosis [11]. Moreover, FFAs-induced oxidative stress can be involved in diseases other than diabetes, including neurodegenerative disorders such as Alzheimer's disease (AD) and Parkinson's disease (PD) [12–14].

Glutathione (GSH, L- γ -glutamyl-L-cysteinyl-glycine) is one of the low-molecular-weight thiol-containing molecules and the most abundant intracellular antioxidant, playing an important role in protection against reactive oxygen species (ROS) toxicity [15–17]. Akt mediates both metabolic and mitogenic actions and therefore regulates a variety of cell functions, including cell proliferation and survival [18]. Because the Akt pathway has been shown to be involved in the suppression of apoptosis [19], we wondered about the role of the protein kinase Akt in FFAs-induced apoptosis and GSH cell protection.

As one of the most important diabetes-associated vascular complications, diabetes-associated cerebral vascular disease (DCVD) is inadequately studied in its pathogenesis mechanism. The values of damage to and protection of HBVECs in DCVD pathogenesis also require further research. Very few studies have been published concerning the oxidative stress, apoptosis, and possible signaling pathway involved in HBVECs cultured with FFAs. Thus, we wondered whether the physiological level of FFA pertinent to clinical diabetes could induce HBVECs apoptosis and oxidative stress and whether GSH could influence and protect the above FFAs-induced damages and apoptosis in HBVECs, which could induce DCVD, and the possible signaling pathway involved. Therefore, the aim of this study was to elucidate on the hypothesis whether or not GSH affects FFAs-induced HBVECs apoptosis and oxidative stress, and the possible signaling pathway involved.

Materials and Methods

Human brain vascular endothelial cells and trypsin were supplied by Sciencell Research Laboratories (San Diego, CA, USA). The rabbit Phospho-Akt (Ser473) antibody was from Cell Signaling Technology (Danvers, MA, USA). The fluorescent probe 20,70-dichlorofluorescein diacetate (DCFH-DA), fluorescent cationic dye Rhodamine 123 (Rh 123), and NO-sensitive fluorogenic probe DAF-AM were from Sigma Chemical Co. (St. Louis, MO, USA). LY294002 was purchased from Calbiochem (La Jolla, CA, USA). The Cell Counting Kit-8 (CCK-8) was from Dojindo Molecular Technologies, Inc. (Kumamoto, Japan). The Annexin V-FITC Apoptosis Detection Kit was purchased from Biovision (Linda Vista, CA, USA). The Caspase-3 Fluorometric Assay Kit was from R&D Systems, Inc. (Minneapolis, MN, USA). Rhodamine 123 and the fluorescent probe DCFH-DA were from Molecular Probes (Leiden, The Netherlands). Glutathione (GSH) and oleate were from Sigma-Aldrich (St. Louis, MO, USA). All other reagents were from Sigma Chemical Co.

Cell Culture

We first added oleate to a 22% fatty acid-free BSA solution in endothelial cell medium (ECM) to obtain a 10 mM FFAs stock solution. HBVECs were propagated in a fibronectin-coated 75-cm² flask in ECM at 37°C under 5% CO₂ and 95% air. The FFAs were then added to some of the cells (FFAs groups were, respectively, incubated with various FFAs concentrations for different periods of time according to our previous study [11]); and 2% BSA (FFAs free) was added to the other cells (control groups). Some HBVECs were also treated with FFA (200 μ M) $\pm$ GSH (1 mM) for 72 h without serum prestarvation. The intervention of 200 μ M FFAs for 72 h was based on the past experiment results [11], and the FFAs level was physiologically relevant to clinical diabetes patients. GSH intervention concentration was based on its clinical application method. In clinical treatment, GSH is usually used for one-time intravenous drip at 1.2–1.8 g/day (the average is approximate 1.5 g/day). The molar mass of GSH is 307 g/mol. Normal human blood volume is approximately equal to 7–8% of body weight. Average body weight of one diabetes adult is about 65 kg (60–70 kg). Average blood volume of one diabetes adult is approximate 4.9 L (4.2–5.6 L). After GSH intravenous drip, the average molar concentration in the diabetes adult body is about 1 mM (1.5 g/4.9 L/307 g/mol = 0.997 mM $\approx$ 1 mM). In this study, the HBVECs were treated with FFA (200 μ M) $\pm$ different concentrations GSH (61.4 mg/L [0.2 mM], 307 mg/L [1 mM] and 1535 mg/L [5 mM], respectively) for 72 h. It was discovered that the influences of 1 mM GSH and 5 mM GSH on HBVECs proliferation, apoptosis, and oxidative stress were similar, but both of them were obviously stronger than 0.2 mM GSH in decreasing apoptosis and ROS and increasing MMP. Therefore, we chose 1 mM (307 mg/L) GSH in this study, which was also physiologically relevant to clinical application in diabetes patients (data not shown).

Cell Viability Assay

Cell viability was assessed by CCK-8. Briefly, HBVECs (5×10^4 cells/100 μ L/well) were seeded in 96-well plates and used for the study on the second day *in vitro*; at this time, they were incubated with FFAs (50, 100, 200 μ M) and 2% BSA (FFAs-free), respectively, for different periods of time (24, 48, and 72 h), and FFA (200 μ M) $\pm$ GSH (1 mM) for 72 h. Then, 10 μ L of CCK8 solution was added to each well, and the culture was incubated for another 1 h at 37°C. The results were expressed as the percentage of viable treated cells relative to the control cells (untreated), using the absorbance at 450 nm, with a reference wavelength at 630 nm measured by a microplate reader (Spectra Max M7; Molecular Devices, Sunnyvale, CA, USA). This experiment and all the following experiments were performed in triplicate on three separate occasions, with each experiment containing eight readings for each experimental condition.

Evaluation of Apoptosis and Necrosis Using the Annexin V/propidium iodide Double-Staining Assay

At the end of the HBVECs' culture period, the cells were gently trypsinized and washed once with serum-containing media before

incubation with Annexin V-FITC (A.3–5). About $1-5 \times 10^5$ cells were collected by centrifugation, resuspended in 500 μ L of 1X binding buffer with 5 μ L of Annexin V-FITC and 5 μ L of propidium iodide (PI 50 μ g/mL, optional), and incubated at room temperature for 5 min in the dark. Annexin V-FITC binding was analyzed by flow cytometry (Ex = 488 nm; Em = 530 nm) using an FITC signal detector (usually FL1), and PI staining was detected by the phycoerythrin emission signal detector (usually FL2).

Assay of Caspase-3 Activity

Human brain vascular endothelial cells were scraped off in PBS after treatments and collected by centrifugation in a conical tube at $1000 \times g$ for 5 min. The supernatant was gently removed and discarded, while the cell pellet was lysed by the addition of the lysis buffer provided by the kit. The cell lysate was incubated on ice for 10 min and then clarified by centrifugation at $13,000 \times g$ for 10 min. The supernatant was quantitated in the BCA kit (Pierce Manufacturing Inc., Appleton, WI, USA). Each reaction mixture, containing an equal amount (100 μ g) of clear supernatant of the cell extract and 50 μ M DEVD-AFC (Sequence: Ac-Asp-Glu-Val-Asp-7-amino-4-trifluoromethyl coumarin, a caspase-3 fluorescent substrate, issued a blue to green fluorescence displacement) provided by the kit, was incubated at 37°C in the dark for 2 h. The absorbance was read using the microplate reader with excitation at 400 nm and emission at 505 nm.

Measurement of ROS

Intracellular ROS were detected using the fluorescent probe 2',7'-dichlorofluorescein diacetate (DCFH-DA) [20]. HBVECs grown on 96-well plates or glass-bottom dishes were subjected to various treatments. Following treatments, cells were incubated with DCFH-DA (final concentration in the mixture, 5 μ M) at 37°C in the dark for 10 min and then washed three times with PBS (0.1 M, pH 7.8). Fluorescence photomicrographs of HBVECs on glass-bottom dishes were taken using a Nikon (Mississauga, Canada) Diaphot inverted microscope equipped with a 100W Xenon lamp. The fluorescence intensity was quantified using the fluorescence microplate reader at an excitation of 485 nm and an emission of 538 nm.

Measurement of Mitochondrial Membrane Potential

The changes in mitochondrial membrane potential (MMP) were estimated using Rh 123, as previously described [21] with minor modifications. Briefly, after various treatments, HBVECs grown on glass-bottom dishes or 96-well plates were loaded with 10 μ M Rh123 at 37°C for 30 min and then washed three times with PBS. The cellular fluorescence intensity of Rh 123 was quantified using a fluorescence microplate reader with excitation at 485 nm and emission at 530 nm with an imaging system attached to a Zeiss Axioscope (Nikon).

Measurement of NO and eNOS Activity

Either treated or control HBVECs were incubated at 37°C for 30 min in ECM containing 5 μ M of the NO-sensitive fluorogenic

probe DAF-AM. After incubation, cells were washed twice with ECM. The cellular NO generation was expressed as a percentage of that of control cells. NOS activity was measured according to the instruction of an assay kit. Briefly, after various treatments, cells were loaded with 5 μ M DAF-AM in ECF either containing or lacking a relatively selective eNOS inhibitor, L-N(5)-(1-iminoethyl) ornithine(L-NIO; 240 μ M); the fluorescence of DAF-AM was measured by a fluorescence microplate reader at an excitation of 495 nm and emission of 519 nm.

Western Blot Analysis

After treatments, the HBVECs were washed three times with ice-cold PBS, harvested, and centrifuged at $1000 \times g$ for 10 min at 4°C. The pellets were lysed for 10 min in ice-cold lysis buffer (containing 1% Triton, 0.1% SDS, 0.5% deoxycholate, 1 mM EDTA, 20 mM Tris [pH 7.4], 150 mM NaCl, 10 mM NaF, 0.1 mM phenylmethylsulfonyl fluoride [PMSF] and a cocktail of protease inhibitors) and then clarified by centrifugation at $13,000 \times g$ for 20 min. The protein in the supernatant was quantified using the BCA kit. The samples were normalized for protein concentrations. The proteins were electrophoretically separated with 8–10% SDS-PAGE and transferred onto nitrocellulose membranes. The membranes were soaked in blocking buffer (1X Tris-buffered saline [TBS, pH 7.6] containing 5% nonfat dry milk) for 1 h at room temperature and incubated overnight at 4°C with a polyclonal antibody against phospho-Akt-ser 473 (1:500) and Akt (1:1000). After being washed three times with TBS-Tween (0.1% Tween 20), the membranes were subsequently probed with secondary HRP-conjugated anti-rabbit IgG antibodies (diluted 1:2000) for 1 h at room temperature. The reaction was developed with an enhanced chemiluminescence detection system. The specific signaling was revealed by autoradiography. Equal loading was confirmed by probing the membranes with anti-actin antibody (1:4000). In every experiment, we ensured that an equal amount of protein was present in each lane by measuring the actin protein content. Quantification of protein bands was achieved by densitometric analysis using ChemImage 5500 software (ChemImage Corp., Pittsburgh, PA, USA).

Statistics

All the experiments were performed in triplicate, and data are shown as mean $\pm$ SD based on three separate experiments. *T*-test was used to compare two groups mean values. One-way ANOVA test followed by Fisher's projected *post hoc* test was used to compare the difference among more than two groups. The probability values of $P < 0.05$ were considered significant.

Results

High FFAs Effects on HBVEC Cell Viability, Apoptosis, and ROS Production

Incubation with FFAs induced a dose- and time-dependent decrease in HBVEC viability, increase in HBVEC apoptosis, caspase-3 activity, and ROS production, and decrease in DCF fluores-

cence intensity, with maximum effects at 200 μ M for 72 h according to our previous report [11] (data not shown).

Effects of FFA on NO Production and eNOS Activity

Compared with cultures under normal conditions, micrographs of representative experiments showed that NO production was most obviously reduced after exposure to 200 μ M FFAs for 72 h (Figure 1A). The NO production of HBVECs cultured with 200 μ M FFAs for 72 h was significantly lower than in control cells (0.6 ± 0.067 vs. 1.0 in control cells, $P < 0.001$, $n = 8$; Figure 1B); similarly, eNOS activity was also lower (0.65 ± 0.07 vs. 1.0 in control cells, $P < 0.001$, $n = 8$; Figure 1C). Meanwhile, when incubating HBVECs with 200 μ M FFAs for different time periods, the NO production and eNOS activity after 72 h were obviously lower than those after 24 h (NO production, 0.6 ± 0.067 vs. 0.89 ± 0.1 , $P < 0.001$; eNOS activity, 0.65 ± 0.07 vs. 0.86 ± 0.088 , $P < 0.001$; Figure 1B,C). The above results demonstrated that FFAs induced different degrees of dose- and time-dependent decreases in the activity of eNOS and production of NO. To investigate the role of eNOS in antiapoptotic effect to FFA-induced apoptosis of GSH, we inhibited eNOS activity with L-NIO (240 μ M), a known inhibitor. However, inhibition of eNOS did not reduce the antiapoptotic effect of GSH on endothelial cells (data not shown).

Effects of FFAs on MMP

To investigate the effects of FFAs on the HBVEC MMP, the fluorescent cationic dye Rh123 was used to monitor changes in MMP. Continuous incubation with 200 μ M FFAs for 24, 48, and 72 h induced decreases in Rh123 fluorescence intensities to 90%, 52%, and 29% of that of the control cells, respectively (0.90 ± 0.04 vs. 0.52 ± 0.06 , $P < 0.01$; 0.52 ± 0.06 vs. 0.29 ± 0.04 , $P < 0.01$, $n = 8$) [11] (data not shown).

Mechanisms of FFAs-induced Injury and Apoptosis: The Role of the Akt Pathway

We mainly investigated whether Akt pathway affects HBVECs injury and apoptosis caused by FFAs [18,19,22]. HBVECs exposed to FFAs contained the same amount of total Akt as the control cells (Figure 2A,B). In contrast, an obvious inhibitory effect in phosphorylated active Akt was already present after a 48-h exposure in 50 μ M FFAs (134 ± 14 AU in 50 μ M FFAs vs. 158 ± 12 AU in control cells, $n = 4$, $P < 0.05$) and became more significant in 100 μ M FFAs and 200 μ M FFAs (95 ± 8 AU in 100 μ M FFAs vs. 158 ± 12 AU in control cells, 65 ± 7 AU in 200 μ M FFAs vs. 158 ± 12 AU in control cells, respectively, $n = 4$, all $P < 0.001$; 95 ± 8 AU in 100 μ M FFAs vs. 134 ± 14 AU in 50 μ M FFAs, 65 ± 7 AU in 200 μ M FFAs vs. 134 ± 14 AU in 50 μ M FFAs, respectively, $n = 4$, all $P < 0.05$; Figure 2A). Meanwhile, HBVECs exposed to 200 μ M FFAs for 24, 48, and 72 h showed different decreases in phosphorylated active Akt content in relation to every corresponding control ($n = 3$, all $P < 0.05$; Figure 2B).

The similar inhibitory effect in phospho-Akt-thr308 was already present when incubating HBVECs with FFAs (data not

shown). Moreover, when culturing HBVECs in the presence of a PI3K/Akt inhibitor (Ly-294002, 10 mM for 72 h), we found an increased number of apoptotic cells ($30.3 \pm 2.4\%$ vs. $4.8 \pm 0.45\%$, $n = 3$, $P < 0.001$) that paralleled the inhibition of phosphorylated active Akt (83.2 ± 5.1 AU, $n = 3$, $P < 0.001$; data not shown).

Effects of GSH on FFAs-induced injury and apoptosis

Here, we investigated whether GSH was able to prevent the effect of FFAs on HBVECs. In HBVECs cultured in the presence of both 200 μ M FFAs and 1 mM GSH, we found a significant increase in the OD values of CCK8 compared with 200 μ M FFAs-cultured HBVECs (0.71 ± 0.06 vs. 0.48 ± 0.03 in FFAs, $n = 8$, $P < 0.05$; Figure 3A). In HBVECs cultured only with 1 mM GSH, apoptosis was similar to that of control cells ($4.45 \pm 0.52\%$ vs. $5.31 \pm 0.75\%$ in control cells, $n = 5$, $P > 0.05$), but in HBVECs cultured with both 200 μ M FFAs and 1 mM GSH, the number of apoptotic cells was significantly decreased compared with that in 200 μ M FFAs samples ($12.11 \pm 1.32\%$ vs. $20.62 \pm 2.21\%$ in FFA, $n = 5$, $P < 0.05$; Figure 3B). Assays of relative caspase-3 enzymatic activity showed that 1 mM GSH prevented increase of caspase-3 activity induced by 200 μ M FFAs (1.48 ± 0.11 vs. 2.2 ± 0.28 in FFAs, $n = 8$, $P < 0.05$; Figure 3C).

Compared with cultures under normal conditions, fluorescent micrographs of representative experiments showed the effect of 1 mM GSH on ROS content, NO production, and MMP induced by incubation with 200 μ M FFAs (Figure 4A). The fluorescence intensity quantified assay showed that 1 mM GSH could obviously prevent the increase of relative ROS content induced by 200 μ M FFAs (7.10 ± 0.44 vs. 14.40 ± 1.90 in FFAs, $n = 8$, $P < 0.05$; Figure 4B), restrain the decrease of eNOS/NO and relative NO content, identified by DAF-FM (the former 0.82 ± 0.06 vs. 0.65 ± 0.07 in FFAs, $n = 8$, $P < 0.05$; the latter 0.84 ± 0.06 vs. 0.60 ± 0.07 in FFAs, $n = 8$, $P < 0.05$; Figure 4C,4D), and elevate the reduced MMP induced by incubation with 200 μ M FFAs (0.77 ± 0.04 vs. 0.55 ± 0.06 in FFAs, $n = 8$, $P < 0.05$; Figure 4E).

The Western blots results indicated that FFAs could induce obvious decreases in phosphorylated Akt level. Under the same experimental conditions, 1 mM GSH was able to prevent partially the decrease in phosphorylated Akt level induced by FFAs (121.3 ± 9.2 vs. 68.3 ± 6.1 AU, $n = 4$, $P < 0.05$; Figure 5A,B). As expected, in cells cultured only with 1 mM GSH, western blot analysis showed an increase in the phosphorylated active form of Akt compared with HBVECs cultured without GSH, but the difference was not notable (172.1 ± 11.4 vs. 166.6 ± 13.2 AU, $n = 4$, $P > 0.05$; Figure 5A). However, the similar up-regulated effect of 1 mM GSH in phospho-Akt-thr308 was not present when incubating HBVECs with 200 μ M FFAs and 1 mM GSH (data not shown). In addition, some experiments were performed using the PI3K/Akt inhibitor Ly-294002. In HBVECs simultaneously exposed to FFAs, GSH, and Ly-294002 (the PI3K inhibitor) prevented the effect of 1 mM GSH on Akt phosphorylated protein level (53.3 ± 3.9 vs. 87.8 ± 5.3 AU, $n = 4$, $P < 0.05$) and on apoptosis ($22.6 \pm 2.4\%$ vs. $12.11 \pm 1.32\%$, $n = 4$, $P < 0.05$; Figure 5B, C).

Figure 1 Dose- and time-course of free fatty acids (FFA) effect on NO content in human brain vascular endothelial cells (HBVECs). **(A)** Fluorescent micrographs of representative experiments show the dose and time course of NO production induced by FFA incubation (dose for 50, 100, and 200 μ M, time for 24, 48, and 72 h, respectively); **(B)** Dose- and time-course of FFA effect on NO content in HBVECs, as identified by using a fluorescent probe (DAF-FM). Data are mean $\pm$ SD (following the same). * P < 0.05, ** P < 0.001 versus control cells; # P < 0.05 versus FFA-treated cells (200 μ M for 24 h); **(C)** Dose- and time-course of FFA effect on eNOS/NOS in HBVECs. * P < 0.05, ** P < 0.001 versus control cells; # P < 0.05 versus FFA-treated cells (200 μ M for 24 h).

Discussion

Diabetes-associated oxidative stress is an attractive hypothesis for the pathogenesis of diabetic cerebral vascular complications.

Our current study clearly demonstrates that the HBVECs cultured in FFA environments causes oxidative stress and induces apoptosis, which is in agreement with previous studies [11,23]. Excessive production of ROS may contribute to cell damage

Figure 2 Dose- and time-course of free fatty acids (FFA) effect on phosphorylated Akt content in human brain vascular endothelial cells (HBVECs). (A) Dose-course effect of FFA incubation for 48 h on phosphorylated active Akt content in HBVECs identified by western blot. * $P < 0.05$, ** $P < 0.001$ versus control cells; # $P < 0.05$ versus FFA-treated cells (50 μ M for 48 h); (B) Time-course effect of 200 μ M FFA on phosphorylated active Akt content in HBVECs identified by western blot. * $P < 0.05$, ** $P < 0.001$ versus control cells; # $P < 0.05$ versus FFA-treated cells (200 μ M for 24 h).

Figure 3 The effect of GSH on CCK8, apoptosis and caspase-3 induced by free fatty acids (FFA). (A) The effect of 1 mM GSH on CCK8 induced by 200 μ M FFA incubation for 72 h. * $P < 0.05$, ** $P < 0.001$ versus control cells; # $P < 0.05$ versus FFA 200 μ M; (B) The effect of 1 mM GSH on apoptosis identified by Annexin-V/PI FITC induced by 200 μ M FFA incubation for 72 h. * $P < 0.05$, ** $P < 0.001$ versus control cells; # $P < 0.05$ versus FFA 200 μ M; (C) The effect of 1 mM GSH on caspase-3 induced by 200 μ M FFA incubation for 72 h. * $P < 0.05$, ** $P < 0.001$ versus control cells; # $P < 0.05$ versus FFA 200 μ M.

either directly, by interacting with and destroying cellular structural molecules, or indirectly, by affecting normal cellular signaling pathways and gene regulation [24–26]. Inappropriate elevation of FFAs is a main abnormality observed in type 2 diabetes and conditions associated with a high risk of cardiovascular disease [1]. Our data show that HBVECs exposure to

high-level FFAs increases cell injury and apoptosis. Although apoptosis is essential to cell normal development [27], when cell death via apoptosis exceeds cell proliferation, apoptosis can manifest itself as a tissue damage [28].

In fact, the survival of ECs critically determines vessel growth and inflammatory processes in the vessel wall [29]. Previous stud-

Figure 4 The effect of GSH on reactive oxygen species (ROS), NO and MMP production induced by free fatty acids (FFA). **(A)** Fluorescent micrographs of representative experiments show the effect of 1 mM GSH on ROS, NO and MMP production induced by 200 μ M FFA incubation for 72 h; **(B)** The effect of 1 mM GSH on ROS content identified by DCFH-DA induced by 200 μ M FFA incubation for 72 h. * P < 0.05, ** P < 0.001 versus control cells; # P < 0.05 versus FFA 200 μ M; **(C)** The effect of 1 mM GSH on NO content identified by DAF-FM induced by 200 μ M FFA incubation for 72 h. * P < 0.05, ** P < 0.001 versus control cells; # P < 0.05 versus FFA 200 μ M; **(D)** The effect of 1 mM GSH on eNOS/NOS induced by 200 μ M FFA incubation for 72 h. * P < 0.05, ** P < 0.001 versus control cells; # P < 0.05 versus FFA 200 μ M; **(E)** The effect of 1 mM GSH on MMP content identified by Rh123 induced by 200 μ M FFA incubation for 72 h. * P < 0.05, ** P < 0.001 versus control cells; # P < 0.05 versus FFA 200 μ M.

ies demonstrated that many main risk factors for vascular diseases, such as ROS, hyperglycemia, and oxidized low-density lipoproteins, elicited ECs injury and apoptosis *in vitro* [30–32]. Although all vascular ECs share certain common functions, it has become clear that considerable structural and functional heterogeneity

exists along the vascular system. The use of HBVECs, as in this research, to represent a special and rare EC model has not been reported frequently in other studies *in vitro*.

Evidence obtained over the past two decades has shown that ROS are involved in several diseases [33]. Excessive production of

Figure 5 The effects of GSH on phosphorylated Akt and apoptosis induced by FFA. **(A)** The effects of 1 mM GSH on total and phosphorylated Akt levels induced by 200 μ M FFA incubation for 72 h. The graphs show representative blots. * P < 0.05, ** P < 0.001 versus control cells; # P < 0.05 versus FFA 200 μ M; **(B)** The effects of PI3K/Akt inhibitor (Ly-294002) on phosphorylated Akt induced by FFA and GSH. * P < 0.05, ** P < 0.001 versus control cells; # P < 0.05 versus FFA 200 μ M; & P < 0.05 versus FFA + GSH; **(C)** The effects on apoptosis exposed simultaneously to FFA, GSH and Ly-294002. * P < 0.05, ** P < 0.001 versus control cells; # P < 0.05 versus FFA 200 μ M; & P < 0.05 versus FFA + GSH.

ROS may lead to oxidative stress, loss of cell function, and ultimately apoptosis or necrosis [34]. ROS are also known to be the mediators of intracellular signaling cascades [35]. Mitochondria, which have a critical role in cell death or survival, are the primary intracellular sources of ROS through the electron transport system, and ROS induce a large number of oxidation–reduction reactions [36,37]. Treatment with vitamins C and E decreased maleic dialdehyde (MDA), and increased total antioxidant capacity (TAC) and glutathione peroxidase (GPx) activity significantly in stroke-prone spontaneously hypertensive rats (SHR-SP), while also decreasing the infarct area, which indicate that oxidative stress plays an important role in the pathogenesis of cerebral ischemia [38].

It has been reported that oxidative stress induced by sublethal concentrations of lipid peroxidation products and oxysterols sig-

nificantly increased the cellular GSH [39]. It is still not very clear whether GSH outside the cell could indeed enhance tolerance against oxidative stress and apoptosis, which is exactly our main purpose in this study.

In addition, in diabetic patients, the increase of FFAs is associated with hyperglycemia and hyperinsulinemia [7,40]. To study the mechanism by which FFAs induced HBVEC apoptosis in our study, we investigated the PI3K/Akt pathway, which has been known to be involved in apoptosis in a variety of cell types [19,41–43].

In this study, we demonstrated that FFAs affects Akt signaling pathway in HBVECs and that the decrease of the phosphorylated active form of Akt is associated with HBVEC apoptosis. Blocking the AKT pathway via the AKT inhibitor LY294002 suppressed the R1PostC-induced autophagy and resulted in the activation of cas-

pase-3 in R1PostC rats, suggesting a critical role for AKT/GSK3 beta-dependent autophagy in reducing cell death after cerebral ischemia [44].

Our study showed that 1 mM GSH could prevent Akt inhibition induced by FFAs and partially decreased the number of apoptotic cells. Furthermore, the antiapoptotic effect of GSH was prevented when HBVECs were cultured with Ly294002, also suggesting that Akt phosphorylation mediates the antiapoptotic action of GSH in ECs. In addition, in endothelial cells cultured with high levels of glucose for a long period of time, apoptosis was associated with impaired Akt activation by insulin [27]. A number of pro-apoptotic proteins, including caspase-3, caspase-9, and eNOS, have been identified as direct Akt substrates [18,45]. Caspase-3 activation is a distal event in the apoptotic cascade. We found an obvious increase in the level of caspase-3 active form when incubating HBVECs with 200 μ M FFAs for 72 h, while adding 1 mM GSH to the culture medium could significantly inhibit caspase-3 activity (Figure 3). Similarly, under low/no GSH conditions, the mechanism of arsenite-induced toxicity is to attenuate Akt and cause cell cycle dysfunction and apoptosis [46]. Applying GSH could protect cells from arsenite-induced ubiquitination [47].

These above data are similar to our study and those of Hermann *et al.* [30], further demonstrating that apoptotic death of ECs is mediated by AKT/caspase-3 signaling and could be reduced by the protection of GSH.

The production of NO by eNOS may have beneficial effects. An enhanced inactivation and/or reduced synthesis of NO is seen in conjunction with risk factors for cardiovascular disease [48], whereas NO generated from nNOS and iNOS might be involved in brain injury [49,50]. eNOS plays a key role in endothelial cell function and survival, and eNOS/NO is an important index reflecting the function of EC. Thus, in this study, we also assayed the generation of NO and activity of eNOS. eNOS could be activated by insulin through eNOS phosphorylation leading to NO production, and this effect is dependent on the activation of the PI3-kinase/Akt pathway [51,52]. eNOS activity is impaired in endothelial apoptosis induced by several pro-apoptotic factors [53]. Our results demonstrate that NO production and eNOS activity in HBVECs greatly decreased after exposure to FFA. These data indirectly indicate that FFAs can decrease the level of eNOS activity and NO production and cause HBVEC damage. GSH could resist FFAs-induced HBVEC damage via an eNOS-mediated mechanism that probably involves direct or indirect interference with AKT pathways.

Mitochondria are a sensitive target for oxygen radicals, which contribute to cell death by reducing ATP production, increasing ROS production, and releasing death regulatory and signaling molecules from the intermembrane space [31]. Here, we also demonstrated that exposure to FFAs resulted in a marked decrease in MMP in cultured HBVECs, and this decrease was partly pre-

vented by GSH. Moreover, GSH also attenuated the activation of caspase-3 induced by FFAs and increased the activity levels of phosphorylated Akt reduced by FFAs. These data reveal that phosphorylated Akt participates in FFAs-induced mitochondria dysfunction and activation of the apoptotic cascades.

In this study, we demonstrated that decreased Akt activity is associated with a reduction in NO and eNOS/NO and that co-incubation with GSH abrogated this effect. However, inhibition of eNOS by L-NIO did not reduce the antiapoptotic effect of GSH on HBVECs, suggesting that the Akt-mediated antiapoptotic effect of GSH is not dependent on eNOS activity and NO generation. These results are similar to previous evidence from Hermann *et al.* [18], who showed that L-NAME did not reduce the antiapoptotic effect of insulin mediated by Akt.

Conclusion

We have demonstrated the existence of FFA-induced oxidative stress in HBVECs, as evidenced by elevated ROS and decreased NO, eNOS/NO and MMP levels. Our data show that high levels of FFAs cause HBVEC apoptosis through Akt-P-dependent pathways, which could be prevented by GSH. The increased levels of apoptosis are accompanied by a reduction in CCK8 and an increase in caspase-3 activity induced by FFAs, which can be partially reversed by GSH. These results suggest that inappropriate FFAs elevation could be a key independent risk factor in the development of endothelial injury and may contribute to the development of vascular disease in diabetes. Inappropriate FFAs elevation may affect the vascular endothelium by directly impairing cells and inducing apoptosis, thus contributing to the development of vascular disease in type 2 diabetic patients. In conclusion, our study found that GSH could activate the Akt pathway and prevent FFAs-induced oxidative stress and apoptosis *in vitro*, which indicated that GSH perhaps possesses an endothelial cell protecting against oxidative stress-induced endothelial apoptosis in clinic.

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Disclosures

None.

Conflict of Interest

The authors have no conflict of interest.

References

1. Reusch JE, Wang CC. Cardiovascular disease in diabetes: Where does glucose fit in? *J Clin Endocrinol Metab* 2011;**96**:2367–2376.
2. Kassam SI, Chao L, Norman B, Robert MKWL. The mechanisms of propofol-induced vascular relaxation and modulation by perivascular adipose tissue and endothelium. *Anesth Analg* 2011;**112**:1339–1345.
3. Huerta-Garcia E, Ventura-Gallegos JL, Victoriano MEC, Montiel-Davalos A, Tinoco-Jaramillo G, Lopez-Marure R. Dehydroepiandrosterone inhibits the activation and dysfunction of endothelial cells induced by high glucose concentration. *Steroids* 2012;**77**:233–240.

4. Szmikto PE, Wang CH, Weisel RD, Jeffries GA, Anderson TJ, Verma S. Biomarkers of vascular disease linking inflammation to endothelial activation: Part II. *Circulation* 2003;**108**:2041–2048.
5. Verma S, Buchanan MR, Anderson TJ. Endothelial function testing as a biomarker of vascular disease. *Circulation* 2003;**108**:2054–2059.
6. Ahmad FK, He Z, King GL. Molecular targets of diabetic cardiovascular complications. *Curr Drug Targets* 2005;**6**:487–494.
7. Wyne KL. Free fatty acids and type 2 diabetes mellitus. *Am J Med* 2003;**115**(Suppl 8A):29S–36S.
8. Chavez JA, Summers SA. Characterizing the effects of saturated fatty acids on insulin signaling and ceramide and diacylglycerol accumulation in 3T3-L1 adipocytes and C2C12 myotubes. *Arch Biochem Biophys* 2003;**419**:101–109.
9. Unger RH. Lipotoxic diseases. *Annu Rev Med* 2002;**53**:319–336.
10. Steinberg HO, Baron AD. Vascular function, insulin resistance and fatty acids. *Diabetologia* 2002;**45**:623–634.
11. Zhou HG, Liu XY, Liu L, et al. Oxidative stress and apoptosis of human brain microvascular endothelial cells induced by free fatty acids. *J Int Med Res* 2009;**37**:1897–1903.
12. Murray IVJ, Proza JF, Sohrabji F, Lawler JM. Vascular and metabolic dysfunction in Alzheimer's disease: A review. *Exp Biol Med* 2011;**236**:772–782.
13. Reed TT. Lipid peroxidation and neurodegenerative disease. *Free Radic Bio Med* 2011;**51**:1302–1319.
14. Ruizperez V, Darios F, Davletov B. Alpha-synuclein, lipids and Parkinson's disease. *Prog Lipid Res* 2010;**49**:420–428.
15. Reliene R, Schiestl RH. Glutathione depletion by buthionine sulfoximine induces DNA deletions in mice. *Carcinogenesis* 2006;**27**:240–244.
16. Anderson ME. Glutathione: An overview of biosynthesis and modulation. *Chem Biol Interact* 1998;**111–112**:1–14.
17. Wu G, Fang YZ, Yang S, Lupton JR, Turner ND. Glutathione metabolism and its implications for health. *J Nutr* 2004;**134**:489–492.
18. Hermann C, Assmus B, Urbich C, Zeiher AM, Dimmeler S. Insulin-mediated stimulation of protein kinase Akt: A potent survival signaling cascade for endothelial cells. *Arterioscler Thromb Vasc Biol* 2000;**20**:402–409.
19. Asanuma H, Torigoe T, Kamiguchi K, et al. Survivin expression is regulated by coexpression of human epidermal growth factor receptor 2 and epidermal growth factor receptor via phosphatidylinositol 3-kinase/AKT signaling pathway in breast cancer cells. *Cancer Res* 2005;**65**:11018–11025.
20. Possel H, Noack H, Augustin W, Keilhoff G, Wolf G. 2',7'-Dihydrochlorofluorescein diacetate as a fluorescent marker for peroxynitrite formation. *FEBS Lett* 1997;**416**:175–178.
21. Zamzami N, Maisse C, Metivier D, Kroemer G. Measurement of membrane permeability and permeability transition of mitochondria. *Methods Cell Biol* 2001;**65**:147–158.
22. Varma S, Lal BK, Zheng R, Breslin JW, Saito S, Pappas PJ. Hyperglycemia alters PI3k and Akt signaling and leads to endothelial cell proliferative dysfunction. *Am J Physiol Heart Circ Physiol* 2005;**289**:H1744–H1751.
23. Salvatore P, Daniela S, Luisa S, Conchita EO, Francesco P, Agata MR. Direct apoptotic effects of free fatty acids on human endothelial cells. *Nutr Metab Cardiovasc Dis* 2008;**18**:96–104.
24. Lievre V, Becuwe P, Bianchi A, et al. Free radical production and changes in superoxide dismutases associated with hypoxia/reoxygenation-induced apoptosis of embryonic rat forebrain neurons in culture. *Free Radic Biol Med* 2000;**29**:1291–1301.
25. Chan PH. Reactive oxygen radicals in signaling and damage in the ischemic brain. *J Cereb Blood Flow Metab* 2001;**21**:2–14.
26. Sugawara T, Chan PH. Reactive oxygen radicals and pathogenesis of neuronal death after cerebral ischemia. *Antioxid Redox Signal* 2003;**5**:597–607.
27. Yonehara S. To reviews on physiological and pathological roles of cell death. *Cell Struct Funct* 2003;**28**:1–2.
28. Gorczyca W, Melamed MR, Darzynkiewicz Z. Programmed death of cells (apoptosis). *Patol Pol* 1993;**44**:113–119.
29. Dimmeler S, Hermann C, Zeiher AM. Apoptosis of endothelial cells. Contribution to the pathophysiology of atherosclerosis? *Eur Cytokine Netw* 1998;**9**:697–698.
30. Hermann C, Zeiher AM, Dimmeler S. Shear stress inhibits H₂O₂-induced apoptosis of human endothelial cells by modulation of the glutathione redox cycle and nitric oxide synthase. *Arterioscler Thromb Vasc Biol* 1997;**17**:3588–3592.
31. Ido Y, Carling D, Ruderman N. Hyperglycemia-induced apoptosis in human umbilical vein endothelial cells: Inhibition by the AMP-activated protein kinase activation. *Diabetes* 2002;**51**:159–167.
32. Iwakiri Y. S-nitrosylation of proteins: A new insight into endothelial cell function regulated by eNOS-derived NO. *Nitric Oxide* 2011;**25**:S95–S101.
33. Christophe M, Nicolas S. Mitochondria: A target for neuroprotective interventions in cerebral ischemia–reperfusion. *Curr Pharm Des* 2006;**12**:739–757.
34. Verrax J, Beck R, Dejeans N, et al. Redox-active quinones and ascorbate: An innovative cancer therapy that exploits the vulnerability of cancer cells to oxidative stress. *Anticancer Agents Med Chem* 2011;**11**:213–221.
35. Sauer H, Wartenberg M. Reactive oxygen species as signaling molecules in cardiovascular differentiation of embryonic stem cells and tumor-induced angiogenesis. *Antioxid Redox Signal* 2005;**7**:1423–1434.
36. Kim J-S, He L, Lemasters JJ. Mitochondrial permeability transition: A common pathway to necrosis and apoptosis. *Biochem Biophys Res Commun* 2003;**304**:463–470.
37. Jezek P, Hlavata L. Mitochondria in homeostasis of reactive oxygen species in cell, tissues, and organism. *Int J Biochem Cell Biol* 2005;**37**:2478–2503.
38. Zhang XH, Lei H, Liu AJ, Zou YX, Shen FM, Su DF. Increased oxidative stress is responsible for severer cerebral infarction in stroke-prone spontaneously hypertensive rats. *CNS Neurosci Ther* 2011;**17**:590–598.
39. Chen ZH, Yoshida Y, Saito Y, Sekine A, Noguchi N, Niki E. Induction of adaptive response and enhancement of PC12 cell tolerance by 7-hydroxycholesterol and 15-deoxy-delta(12,14)-prostaglandin J2 through up-regulation of cellular glutathione via different mechanisms. *J Biol Chem* 2006;**281**:14440–14445.
40. Belfort R, Mandarino L, Kashyap S, et al. Dose-response effect of elevated plasma free fatty acid on insulin signaling. *Diabetes* 2005;**54**:1640–1648.
41. Rasul A, Ding C, Li XM, et al. Dracorhodin perchlorate inhibits PI3K/Akt and NF-kappa B activation, up-regulates the expression of p53, and enhances apoptosis. *Apoptosis* 2012;**17**:1104–1119.
42. Chen SF, Tsai HJ, Hung TH, et al. Salidroside improves behavioral and histological outcomes and reduces apoptosis via PI3K/Akt signaling after experimental traumatic brain injury. *PLoS ONE* 2012;**7**:e45763.
43. Bezler M, Hengstler JG, Ullrich A. Inhibition of doxorubicin-induced HER3-PI3K-AKT signalling enhances apoptosis of ovarian cancer cells. *Mol Oncol* 2012;**6**:516–529.
44. Qi ZF, Luo YM, Liu XR, et al. AKT/GSK3 ss-dependent autophagy contributes to the neuroprotection of limb remote ischemic preconditioning in the transient cerebral ischemic rat model. *CNS Neurosci Ther* 2012;**18**:965–973.
45. Fulton D, Gratton JP, McCabe TJ, Fontana J, Fujio Y, Walsh K. Regulation of endothelium-derived nitric oxide production by the protein kinase Akt. *Nature* 1999;**399**:597–601.
46. Habib GM. Arsenite causes down-regulation of Akt and c-Fos, cell cycle dysfunction and apoptosis in glutathione-deficient cells. *J Cell Biochem* 2010;**110**:363–371.
47. Habib GM, Shi ZZ, Lieberman MW. Glutathione protects cells against arsenite-induced toxicity. *Free Radic Bio Med* 2007;**42**:191–201.
48. Forstermann U. Nitric oxide and oxidative stress in vascular disease. *Pflugs Arch* 2010;**459**:923–939.
49. Iadecola C, Zhang F, Xu X. Inhibition of inducible nitric oxide synthase ameliorates cerebral ischemic damage. *Am J Physiol* 1995;**268**:R286–R292.
50. Iadecola C, Zhang F, Xu S, Casey R, Ross ME. Inducible nitric oxide synthase gene expression in brain following cerebral ischemia. *J Cereb Blood Flow Metab* 1995;**15**:378–384.
51. Zeng G, Nystrom FH, Ravichandran LV, et al. Roles for insulin receptor, PI3-kinase, and Akt in insulin-signaling pathways related to production of nitric oxide in human vascular endothelial cells. *Circulation* 2000;**101**:1539–1545.
52. Dimmeler S, Fleming I, Fisslthaler B, Hermann C, Busse R, Zeiher AM. Activation of nitric oxide synthase in endothelial cells by Akt-dependent phosphorylation. *Nature* 1999;**399**:601–605.
53. Hayashi T, Yano K, Matsui-Hirai H, Yokoo H, Hattori Y, Iguchi A. Nitric oxide and endothelial cellular senescence. *Pharmacol Ther* 2008;**120**:333–339.