Na\textsuperscript{+}/H\textsuperscript{+} exchanger is required for hyperglycaemia-induced endothelial dysfunction via calcium-dependent calpain

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Aims Recent studies have reported that the calcium-dependent protease calpain is involved in hyperglycaemia-induced endothelial dysfunction and that the Na\textsuperscript{+}/H\textsuperscript{+} exchanger (NHE) is responsible for an increase in the intracellular calcium (Ca\textsuperscript{2+}) concentration in diabetes. We hypothesized that activation of NHE mediates hyperglycaemia-induced endothelial dysfunction via calcium-dependent calpain.

Methods and results Exposure of human umbilical vein endothelial cells (HUVECs) to high glucose (HG, 30 mM D-glucose) time dependently increased both the Ca\textsuperscript{2+} concentration and calpain activity. Chelation of free Ca\textsuperscript{2+} with 1,2-bis (2-aminophenoxy) ethane-N,N,N',N'-tetraacetic acid abolished the HG-increased calpain activity. In addition, HG activated NHE in a time-dependent manner, but cariporide, an NHE inhibitor, blocked the HG-induced increase in NHE activity. Furthermore, cariporide or NHE siRNA (small interfering ribonucleic acid) attenuated the HG-induced increases of both Ca\textsuperscript{2+} concentration and calpain activity. All of these HG-induced effects in HUVECs, including decreased endothelial nitric oxide synthase (eNOS) activity and NO (nitric oxide) production and increased dissociation of heat shock protein (hsp90) from eNOS, were NHE or calpain reversible.

In vivo experiments showed that cariporide treatment via inhibition of NHE activity significantly attenuated the hyperglycaemia-induced impairment of acetylcholine-induced endothelium-dependent relaxation in streptozotocin-injected diabetic rats.

Conclusion Activation of NHE via calcium-dependent calpain contributes to hyperglycaemia-induced endothelial dysfunction through dissociation of hsp90 from eNOS.

1. Introduction

Diabetes is associated with a significantly increased risk of cardiovascular disease, including atherosclerosis,\textsuperscript{1} coronary artery disease,\textsuperscript{2} and microvascular complications.\textsuperscript{3} Hyperglycaemia is thought to play a key pathogenic role in the development of diabetic cardiovascular disease. High blood glucose concentration results in endothelial dysfunction that is associated with a loss of endothelium-derived nitric oxide (NO), increased vascular permeability, increased endothelial adhesiveness, and thickening of the basement membrane of blood vessels.\textsuperscript{4} However, the exact mechanism responsible for endothelial dysfunction in diabetes remains largely unknown, limiting effective therapeutic interventions.

In endothelial cells, NO is produced from L-arginine in the catalysis of endothelial nitric oxide synthase (eNOS). Previous studies\textsuperscript{5,6} showed that the association of heat shock protein 90 (hsp90) with eNOS plays an important role in the generation of NO in endothelial cells. This process is controlled by calpain because hsp90 is a natural substrate for calpain.\textsuperscript{7,8} For example, exposure of pulmonary artery endothelial cells to hypoxia triggers calpain-mediated loss of hsp90 from the eNOS complex, resulting in decreased eNOS activity and NO release.\textsuperscript{9} Recent study by Stalker et al.\textsuperscript{10,11} has also reported that acute experimental hyperglycaemia up-regulated the endothelial-expressed \(\mu\)-calpain isoform in the microcirculation and induced endothelial dysfunction, however, the mechanism of calpain activation in hyperglycaemia is not fully understood.

The Na\textsuperscript{+}/H\textsuperscript{+} exchanger (NHE) is expressed ubiquitously in the plasma membrane of mammalian cells and exchanges...
intracellular H\(^+\) for extracellular Na\(^+\) to regulate intracellular pH (pH\(_i\)) value and the concentration of intracellular Na\(^+\) (Na\(^+_i\)).\(^{12}\) The activation of NHE increases Na\(^+_i\) that leads to Ca\(^{2+}\) overload through the Na\(^+\)/Ca\(^{2+}\) exchanger, which is assumed to be the crucial factor in diabetic injuries.\(^{13}\) Inhibition of NHE has been shown to have protective effects against diabetic nephropathy.\(^{14}\) Our previous study has demonstrated that cariporide, an NHE inhibitor, inhibited mediated by NHE and Ca\(^{2+}\). Accordingly, the aim of the current study is to investigate if hyperglycaemia-induced endothelial dysfunction is mediated by NHE and Ca\(^{2+}\)/calpain. Our results suggest that hyperglycaemia-induced endothelial dysfunction is due to the activation of NHE via dissociation of hsp90 from eNOS by Ca\(^{2+}\)-dependent calpain.

2. Methods

2.1 Animals

Male Sprague–Dawley rats, 180 ± 20 g, 6–8 weeks of age, were obtained from the Animal Department of Central South University (Changsha, China). Rats were housed in temperature-controlled cages with a 12 h light–dark cycle and given free access to water and normal feed. The animal protocol was reviewed and approved by the Central South University Institute Animal Care and Use Committee. The current investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

2.2 Materials

The NHE inhibitor, cariporide, was kindly provided by Hoechst (Frankfurt, Germany). Calpain inhibitor, ZLLaL (benzyloxy carbonyl-leucyl-lysine) was from Biomol Research Laboratories, PA, USA. Calcium chelator \([1,2\text{-bis (2-aminophenoxy) ethane-N,N,N',N'-tetraacetic acid (BAPTA)}]\) was obtained from Invitrogen Corporation, CA, USA. Fluorescent indicators, dianmiofluorescein (DAF) and 2-carboxyethyl-5(6)-carboxyfluorescein were purchased from Calbiochem (CA, USA). Both [\(^{14}\)H]-arginine and \([\(^{32}\)P]ATP were obtained from NEN (Boston, MA, USA). Streptozotocin (STZ), phenylephrine, acetylcholine (ACh), sodium nitroprusside (SNP), hydroxyethyl piperazine ethanesulphonic acid (HEPES), and other chemicals were purchased from Sigma Chemical Co, MO, USA. Primary antibodies (NHE, hsp90, eNOS, and \(\beta\)-actin), human NHE siRNA (small interfering ribonucleic acid), and control siRNA were obtained from Santa Cruz Biotechnology (CA, USA). All chemicals were of reagent grade.

2.3 Cell culture

Human umbilical vein endothelial cells (HUVECs) purchased from the American Type Culture Collection (ATCC) were grown in endothelial basal medium (EBM-2) (Clonetics Inc., Walkersville, MD, USA) supplemented with 2% fetal bovine serum, 12.5 mg/mL ECGF, 1 mg/mL hydrocortisone, 100 \(\mu\)g/mL penicillin and 100 mg/mL streptomycin. The cells were cultured at 37 °C in a humidified atmosphere of 5% CO\(_2\) and 95% air. Culture medium was replaced twice a week, and cells were subcultured when 80% confluent. Cells at passage 4 were used for all experiments.

2.4 High-glucose treatment of human umbilical vein endothelial cells

After reaching 80% confluence, HUVECs were exposed to normal glucose (NG; 5 mM d-glucose), HG (30 mM d-glucose), or hyperosmotic control (HO, 5 mM d-glucose plus 25 mM L-glucose) for different lengths of time with a daily change of culture media.

2.5 Transfection of small interfering ribonucleic acid into human umbilical vein endothelial cells

Transient infection of siRNA into cells was carried out according to Santa Cruz’s protocol.\(^{16}\) Briefly, 100 \(\mu\)L transfection medium containing 6 \(\mu\)L siRNA (10 \(\mu\)M) stock solution was added to 100 \(\mu\)L transfection medium containing 6 \(\mu\)L transfection reagent (Lipofectamine 2000, Invitrogen, CA, USA) and mixed gently. After 30 min incubation at room temperature, 200 \(\mu\)L siRNA-lipid complex solution was added to each well (6-well plate) in 1.0 mL transfection medium. After incubation for 6 h at 37 °C, the medium was replaced with normal medium and cultured for 24–48 h.

2.6 Western blot

After treatment, HUVECs were lysed in cell-lysis buffer (Cell Signaling Company, MA, USA). The protein content was assayed by bicinchoninic acid protein assay reagent (Pierce, IL, USA). Twenty microgram of protein was loaded to sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE) and then transferred to membrane. Membrane was incubated with a 1:1000 dilution of primary antibody, followed by a 1:2000 dilution of horseradish peroxidase-conjugated secondary antibody. Protein bands were visualized by enhanced chemiluminescence (GE Healthcare, Chicago, IL, USA). The intensity (area \(\times\) density) of the individual bands on western blots was measured by densitometry (model GS-700, Imaging Densitometer; Bio-Rad, CA, USA). The background was subtracted from the calculated area.

2.7 Measurement of intracellular calcium concentration

The intracellular calcium (Ca\(^{2+}\)) concentration was measured by using a Fluo-4 NW kit from Invitrogen following kit protocol. Briefly, HUVECs were treated as indicated, the cell culture medium was aspirated, washed with HEPES buffer (pH 7.4) once, and 1 mL of HEPES buffer with fluorescent dye was added to cultured cells. After 30 min incubation, fluorescence strength was measured in wavelength of excitation/emission of 485/520 nm.

2.8 Calpain activity

The calpain activity was measured by using the fluorogenic peptide Suc-Leu-Leu-Val-Tyr-AMC as a substrate following the procedure described previously with slight modification.\(^{17}\) Shortly, cells were cultured in 24-well plates in EBM with different treatments. After being washed twice with phosphate buffered saline (PBS), fluorogenic peptide was added to a final concentration of 80 \(\mu\)M in PBS. Immediately after addition of fluorogenic peptide, fluorescence was recorded at 2 min intervals for 20 min at excitation 360 nm and emission 460 nm using a Synergy HT Multi-Detection Microplate Reader (BIO-TEK Instruments Inc., VT, USA). The initial rate of peptide-AMC hydrolysis was used as the velocity of enzyme activity.

2.9 Determination of Na\(^{+}/H^+\) exchanger activity

To evaluate NHE activity in HUVECs or rat aortas, the pH\(_r\), rate of recovery from an induced acidification, cellular buffer capacity of the HUVECs or rat aortas, and the calibration of the fluorescence to pH values were determined as described previously.\(^{14,15}\)

2.10 Endothelial nitric oxide synthase activity assay

eNOS activity was monitored by \(\text{L-[^{14}]}\text{H}\text{citrulline production from L-[^{14}]}\text{H}\text{arginine as described previously.}\(^{18}\) Briefly, protein samples were incubated in reaction buffer [1 mM L-arginine/100 mM NADPH/1 mM tetrahydrobiopterin/0.2 \(\mu\)M of L-[\(^{14}\)H] arginine control (HO, 5 mM d-glucose plus 25 mM L-glucose) for different lengths of time with a daily change of culture media.
High glucose (HG) increases intracellular calcium (Ca\textsuperscript{2+}) and calpain activity in human umbilical vein endothelial cells (HUVECs). Cultured HUVECs were incubated with HG (30 mM d-glucose) for 3, 6, 12, 24, and 48 h. (A) Ca\textsuperscript{2+} concentration was detected by Fluo-4 fluorescence. (B) Calpain activity was assayed by fluorogenic peptide in situ. (C) HUVECs were incubated with normal glucose (NG, 5 mM d-glucose), HG, or hyperosmotic control (HO, 5 mM d-glucose plus 25 mM L-glucose) for 24 h in presence or absence of BAPTA [1,2-bis-(2-aminophenoxy) ethane-N, N, N',N'-tetraacetic acid] (0.5 mM), a Ca\textsuperscript{2+} chelator, and then calpain activity was detected. Data are expressed by mean ± SEM (n = 5). *P < 0.05 vs. Control, #P < 0.05 vs. HG.
3. Results

3.1 High glucose increases intracellular calcium and calpain activity in human umbilical vein endothelial cells

Previous studies have demonstrated that calpain is activated by hyperglycaemia via calcium overload and causes endothelial dysfunction. To investigate whether HG alters the activity of calpain and Ca\(^{2+}\) concentration in HUVECs, both Ca\(^{2+}\) concentration and calpain activity were determined in HUVECs incubated with HG (30 mM) at different times. As shown in Figure 1A and B, HG significantly increased both Ca\(^{2+}\) concentration and calpain activity in a time-dependent manner.

3.2 High glucose-induced increase in calpain activity is calcium dependent

Calpains are a family of Ca\(^{2+}\)-dependent cysteine proteases found in mammals and many lower organisms. In the presence of an elevated Ca\(^{2+}\) concentration, calpain is activated in endothelial cells. We next detected whether the HG-increased calpain activity is dependent on Ca\(^{2+}\) in HUVECs. As shown in Figure 1C, in NG and HO groups, BAPTA, a Ca\(^{2+}\) remover, did not change basal calpain activity. However, BAPTA inhibited the enhanced calpain activity induced by HG.

3.3 Inhibition of Na\(^{+}/H^+\) exchanger abolishes high glucose-induced intracellular calcium and calpain activity

Activation of NHE by some pathologic factors, such as ischaemia and hypoxia, leads to an increased Ca\(^{2+}\) level and causes cellular damage in the vascular system. Next we determined if the HG-increased Ca\(^{2+}\)-dependent calpain activity is mediated by NHE. HUVECs were treated with HG in absence or presence of cariporide (10 \(\mu M\)) for 24 h. As shown in Figure 2A and B, in both NG and HG groups, inhibition of NHE by cariporide did not change basal calpain activity and Ca\(^{2+}\) level. However, cariporide blocked the increase in calpain activity and Ca\(^{2+}\) level induced by HG.

In order to further investigate whether the inhibitory effect of cariporide on HG-increased calpain activity is specific to NHE, we used siRNA to silence NHE protein expression. In Figure 2C, specific siRNA of NHE reduced NHE protein expression to 20%, but control siRNA did not change NHE protein expression. In Figure 2D, control siRNA did not block the increase in calpain activity induced by HG, however, NHE siRNA inhibited the HG-increased calpain activity.

3.4 High glucose induces Na\(^{+}/H^+\) exchanger activation

Since inhibition of NHE by pharmacologic inhibitor or siRNA abolished HG-induced increase in calpain activity, we next...
investigated the effects of HG on NHE activity in HUVECs. In Figure 3A, HG increased NHE activity in a time-dependent manner. However, cariporide, a new NHE inhibitor, dose-dependently inhibited NHE activity when HUVECs were treated with HG for 24 h (Figure 3B).

3.5 High glucose decreases endothelial nitric oxide synthase activity, association of endothelial nitric oxide synthase with heat shock protein 90 and nitric oxide production

One of the main functions of endothelium is to produce NO, which is catalysed by eNOS. We next studied whether HG affected NO production and eNOS activity in HUVECs. In Figure 4A, HG decreased NO release from endothelial cells in a time-dependent manner, associated with a decreased eNOS activity (Figure 4B).

There is evidence that hsp90 plays an important role in positively regulating eNOS activity.16,27 We further investigated whether HG decreased eNOS activity by decreasing its association with hsp90. The interaction of hsp90 and eNOS was assayed using immunoprecipitation of eNOS or hsp90 with the specific antibodies. In Figure 4C, exposure of HUVECs to HG up to 30 mM for 24 h did not alter the expression of hsp90 and eNOS. Compared with NG, however, decreased amounts of eNOS were detected when...
hsp90 was immunoprecipitated from HG-treated HUVECs. These results were further corroborated by decreased detection of hsp90 when eNOS was immunoprecipitated from HG-treated cells.

3.6 Inhibition of Na\(^{+}/\)H\(^{+}\) exchanger or calpain abolishes high glucose-induced reduction of endothelial nitric oxide synthase activity, association of endothelial nitric oxide synthase with heat shock protein 90, and nitric oxide production

Activation of calpain caused hsp90 degradation from eNOS–hsp90 complex, leading to inactivation of eNOS. Thus, we further investigated whether NHE or calpain was involved in HG-reduced eNOS association with hsp90. As shown in Figure 5A, inhibition of either NHE with cariporide or calpain with ZLLa1 reversed HG-decreased eNOS activity. Although inhibition of either NHE with cariporide or calpain with ZLLa1 alone did not alter NO production in basal condition, it significantly increased the NO release from HG-treated HUVECs (Figure 5B). In addition, inhibition of either NHE with cariporide or calpain with ZLLa1 restored the association of eNOS with hsp90 (Figure 5C).

3.7 Inhibition of Na\(^{+}/\)H\(^{+}\) exchanger with cariporide reverses streptozotocin-induced endothelial dysfunction via apoptosis-independent pathway

We next investigated whether cariporide reversed diabetes mellitus-induced endothelial dysfunction in vivo. As shown in Figure 6A, cariporide alone did not change Ach-induced endothelium-dependent relaxation in control rats, however, STZ-induced hyperglycaemia impaired Ach-induced endothelium-dependent relaxation. Furthermore, administration of cariporide in diabetic rats abolished hyperglycaemia-impaired endothelium-dependent relaxation but had no effects on NO donor-triggered endothelium-independent relaxation (Figure 6B).

In order to confirm whether HG activates NHE in rat aorta endothelium, the activity of NHE was assayed in rat aortas. As shown in Figure 6C, STZ-induced hyperglycaemia activates NHE in rat aortas. Administration of cariporide attenuated the hyperglycaemia-increased NHE, but cariporide did not change the NHE activity in control rat aortas.

It has been reported that HG-induced apoptosis contributes to endothelial dysfunction in diabetes. In order to study whether apoptosis is involved in the NHE-mediated endothelial dysfunction in diabetes, we detected the apoptosis of endothelial cells treated with HG. We used tumour necrosis factor, TNF\(_{\alpha}\) (20 ng/mL), a well-known positive control of apoptosis inducer, and HG to treat HUVECs for 24 h. As shown in Figure 6D, we did not see the increased apoptosis in HG-treated HUVECs. However, TNF\(_{\alpha}\) caused HUVECs apoptosis after 24 h incubation without altering NHE activity and the association of eNOS with hsp90 (data not shown).

4. Discussion

The current study demonstrates that HG (or hyperglycaemia) via NHE induces vascular endothelial dysfunction by...
activating calpain-dependent dissociation of eNOS from hsp90. Not only did inhibition of NHE by a pharmacologic inhibitor, cariporide, reverse HG-induced increase in Ca\(^{2+}\) concentration and calpain activity, but its actions were also mimicked by silencing of NHE with siRNA. In addition, inhibition of either NHE by cariporide or calpain by ZLLa1 blocked the decrease in eNOS activity and eNOS association with hsp90 caused by HG. Treatment of diabetic mice with cariporide restored endothelial function in vivo. These results strongly suggest that NHE is required for hyperglycaemia-induced endothelial dysfunction via calcium-dependent protease calpain. Recent studies have reported that calpain inhibition exerts anti-inflammatory effects in diabetes. Ruetten et al.\(^2\) showed that calpain inhibitors significantly improve leukocyte-endothelium interactions cardiovascular outcome and ameliorate multiple organ dysfunction during endotoxic shock. Similarly, Scalia R et al.\(^2\) reported that hyperglycaemia is a major determinant of albumin permeability in diabetic microcirculation via calpain. These studies imply that calpain plays a role in diabetes-induced endothelial dysfunction. Our data clearly indicated that inhibition of NHE by cariporide or siRNA blocked the increase in either calpain activity or Ca\(^{2+}\) concentration caused by HG and improved endothelial function in diabetic rats. It strongly suggested to us that NHE is essential for hyperglycaemia-induced endothelial dysfunction. To our knowledge, this is the first study to report that inhibition of NHE prevents endothelial dysfunction in diabetes via Ca\(^{2+}\)/calpain-dependent pathway. Our data also clearly indicate that inhibition of NHE abolished HG-induced increase in calpain activity via Ca\(^{2+}\). The calpains are a family of calcium-dependent proteases that cleave a number of cellular substrates, including kinases, phosphatases, transcription factors, and cytoskeletal proteins.\(^3\) In this study, we found that HG increased calpain activity in vascular endothelial cells as well as increased Ca\(^{2+}\). In addition, chelation of intracellular free-calcium by BAPTA inhibited the activation of calpain induced by HG. So we speculated that HG-induced calpain activation is calcium dependent because it has been reported that calpain is activated in response to large calcium fluxes.\(^2\)

To determine whether or not the elevated glucose produced a cariporide-sensitive activation of NHE in endothelial cells, we measured NHE activity in vitro and in vivo. These results showed that HG produced a cariporide-sensitive activation of NHE in endothelial cells. A potential mechanism of glucose-induced increase in NHE activity is a phosphorylation-dependent increase in the activity of existing exchangers or the activation of dormant membrane-associated exchangers. Indeed, Sardet et al.\(^3\) had demonstrated that the NHE is rapidly phosphorylated in response to various mitogens and concluded that this phosphorylation of the NHE is temporally correlated with its activation. Additional experiments\(^3\) support the hypothesis that protein kinase C is one of the kinases responsible for this phosphorylation.
In summary, we have demonstrated that diabetic hyperglycaemia activates NHE and results in the increased Ca\(^{2+}\) and calpain activity, which degraded hsp90, a positive regulator of eNOS activity, and subsequently impaired endothelium-dependent vessel relaxation (Figure 6E). These results are particularly relevant to hyperglycaemic conditions and endothelial dysfunction, both of which are prevalent in type 1 diabetes mellitus.

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References


