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Diazoxide Suppresses Mitochondria-dependent Apoptotic Signaling in Endothelial Cells Exposed to High Glucose Media

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In the present study, we examined the effect of mitochondrial K^+ channel opener diazoxide on the mitochondria-dependent apoptotic signaling in endothelial cells exposed to high glucose (HG) media. Endothelial cells derived from human umbilical veins were exposed to HG media containing 30 mM glucose, and the degree of apoptotic cell death associated with activation of the mitochondria-dependent apoptotic signaling pathway was determined. Exposure to HG media was seen to enhance apoptotic cell death in a time-dependent manner. In these cells, activation of caspases 3, 8, and 9 was observed, and while caspase-3 and -9 inhibitors suppressed the HG-induced apoptotic cell death, a caspase-8 inhibitor did not. The HG-treated cells exhibited disruption of mitochondrial membrane potential, formation of permeability transition pores, and cytosolic release of cytochrome c. Subsequently, diazoxide was seen to attenuate the HG-induced apoptotic cell death; caspase-9 activation was suppressed but caspase 8 was not. Diazoxide also suppressed the depolarization of mitochondrial membrane potential, the formation of mitochondrial permeability transition pores, and the release of cytochrome c. These effects were significantly inhibited by 5-hydroxydecanoate, a selective blocker of ATP-sensitive K^+ channels (K_{ATP}). The present results demonstrate that diazoxide exhibits a beneficial effect to ameliorate HG-induced endothelial cell apoptosis. Opening the K_{ATP} could help preserve the functional integrity of mitochondria and provide an underlying mechanism to suppress HG-triggered apoptotic signaling.

Key words : Apoptosis, diazoxide, endothelial cell, high glucose, mitochondrial K^+ channel

Introduction

Diabetes mellitus can cause various vascular complications and close correlations have been observed between blood glucose level and vascular dysfunction [2, 26]. Vascular endothelium has been suggested to be primarily involved in the vascular lesions of experimental and human diabetes. Endothelial cells exposed to high glucose (HG) environment have been shown to exhibit delayed cell proliferation [5, 19], disturbed cell cycle [19, 21], enhanced DNA damage [20], and increased cell death [19]. In diabetes *in vivo*, high glucose concentration has been found to produce endothelial pathologies [6, 15, 18] such as loss of capillary endothelium and weakening of endothelial cell junctions. It was reported that HG also triggered apoptotic cell death in cultured human endothelial cells [3] and microvascular cells from retina [22]. Therefore, prevention of endothelial cell apoptosis in HG environment may have important implications for investigating pharmacological tools to alleviate diabetes-associated vascular complications.

A variety of potassium channel openers (KCOs) act on ATP-sensitive K^+ channels (K_{ATP}). Their activities are regulated in connection with cellular metabolism and are suppressed by a rise in the intracellular ATP [7, 8]. Noma [24] has suggested that these channels are endogenous mediators of myocardial protection in the ischemic heart. Mitochondrial membranes also have been shown to present ATP-sensitive K^+ conductance which has been recognized as the mitochontrial K_{ATP} [13]. Diazoxide a relatively selective opener of the mitochondrial K_{ATP} has been found to reduce infarct size in the whole heart, and survival of cardiomyocytes [14, 27]. With respect to diabetes, diazoxide has been shown to delay the development of diabetes [10, 11] and prevent pancreatic β-cell death induced by high glucose or tolbutamide [9]. An *in vivo* study with an established animal model of human type 2 diabetes demonstrated that it could prevent diabetes through suppression of pancreatic β-cell apoptosis [12].

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In this study, we postulated that diazoxide could provide a beneficial effect to ameliorate HG-induced vascular complications. To test this hypothesis, human umbilical vein endothelial cells (HUVECs) were exposed to HG media in the absence and presence of diazoxide and the degree of apoptotic cell death and changes in the mitochondria- dependent apoptotic signaling were examined.

Materials and Methods

Cell culture

Human umbilical vein endothelial cells (HUVECs) were grown in Dulbeccos modified Eagles medium: Nitrient Mixture F-12 (Ham) (1:1) with 5% heat inactivated fetal bovine serum at 37℃ in humidified 95% air, 5% CO_2 incubator. When cells grew to reach confluence, they were detached using a 0.02% EDTA-0.05% trypsin solution and subculture was prepared.

Exposure to HG media

Cells were seeded and grown on 24-well culture plates or collagen-coated cover slips according to the purpose of the experiments. Experimental procedures were carried out 3 days after subculture. Cells were exposed to HG environment by replacing the control media containing 5.5 mM D-glucose with media containing 30 mM D-glucose. For osmotic controls, L-glucose or mannitol (each 25 mM) was added to the control media.

Analysis of apoptotic cell death

HG-induced apoptosis was analyzed by nuclear staining with Hoechst 33,258. After exposure to experimental procedures, cells were fixed with 4% paraformaldehyde and stained with 10 μM Hoechst 33,258. Cells presenting fragmented or condensed nuclei were counted as apoptotic cells.

Measurement of mitochondrial membrane potential

Mitochondrial membrane potential was analyzed with DiOC6(3). This fluorochrome accumulates into the micothondria depending upon the mitochondrial membrane potential, and thus, reduction in DiOC6(3) fluorescence indicates loss of the mitochondrial membrane potential. Cells were loaded for 30 min in the dark with DiOC6(3) at a final concentration of 50 nM at 37℃. Cells were harvested, washed and resuspended in PBS. The fluorescence intensity was analyzed with a FACsort Becton Dickinson Flow Cytometer.

Analysis of mitochondrial permeability transition pore formation

To examine the formation of mitochondrial permeability transition (MPT) pores, cells were double-stained with fluorescent dyes, calcein acetoxymethyl ester (calcein-AM) and tetramethylhodamine methyl ester (TMRM). Cells with mitochondrial permeability transition pores were analyzed under a confocal microscope as described by Lemasters et al [17].

Detection of cytochrom c release

Cells were washed three times with ice-cold phosphate-buffered saline and suspened in 1 ml of extraction buffer composed of (in mM) PIPES 50, KCl 50, $MgCl_2$ 5, EGTA 5, phenylmethylsulfonyl fluoride 1, leupeptin 10 mg/ml and pepstatin A 10 mg/ml, pH 7.0. Cells were lysed by five cycles of freezing in liquid nitrogen and thawing at 37℃. The cell lysates were then centrifuged for 1 hr at 100,000 g. The supernatant was then separated from the pellet containing the cellular debris. Western blot study was carried out using polyclonal anti-cytochrom c antibody.

Assay of caspase activity

A quantitative enzymatic assay of caspase activity was carried out using colorimetric assay kit (The R&D, Minneapolis, Mn) according to the manufacturer-provided instructions.

Chemicals

Reagents and medium for cell culture were purchased from GIBCO-BRL (Grand Island, NY). Ac-DEVD-CHO, Z-IETD-FMC, Z-LEHD-FMK, and Z-VAD FMK, were purchased from Calbiochem (California, USA). TMRM, Calcein/AM and DiOC6(3) was obtained from Molecular Probes (Eugene, OR, USA). Antibody of cytochrome-c was obtained from Cell Signaling Technology Inc. (Beverly, MA, USA). Other chemicals and reagents were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA).

Statistical analysis

Data were presented as means ± S.E. The significance of the difference between two groups was evaluated by student's t-test. When necessary, multiple group comparison was performed using one way analysis of variance followed by the post hoc Tukey test. A probability level less than 0.05 was used to establish significant difference.

Results

HG-induced apoptosis

When cells were assessed by Hoechst staining after 72 hr exposure to HG, 38.7±7.4% of the cells were counted as apoptotic, which were characterized by fragmented or condensed nuclei as indicated by arrows (Fig. 1A). The time-dependent effect of HG to induce apoptosis was summarized in Fig. 1B. When cells were exposed to HG media the number of apoptotic cells increased in a time-dependent manner. Although, the concentration-effect relationship varied greatly according to experimental groups, the number of apoptotic cells tended to increase when the concentration of glucose in media was raised (Fig. 1C). Addition of L-glucose or mannitol instead of D-glucose did not affect the rate of apoptosis suggesting that the effect of HG was not ascribable to increased osmolality (Fig. 1C).

Role of caspases in HG-induced apoptosis

To examine the role of different caspases in the execution of HG-induced apoptosis, we determined caspase 3, 8 and 9 activities in the HG-treated cells. In cells exposed to HG media there was a time-dependent and sustained activation of caspase 9. The activity reached its peak (3.6 fold) in 36 hr and retained the peak level up to 48 hr. In contrast, HG-induced caspase 8 activation exhibited a transient pattern showing its peak activity (2.3 fold) in 24 hr then followed by a return to the basal level in 48 hr. Activation pattern of caspase 3 was comparable to that of caspase 9 (Fig. 2).

To delineate further the role of these caspases in the HG-induced apoptosis, effects of different caspase inhibitors were examined. Z-LEHD-FMK [25] and Ac-DEVD-CHO [1], the specific inhibitors of the caspases 9 and 3 respectively, as well as Z-VAD-FMK a broad range caspase inhibitor [29], significantly prevented the HG-induced apoptosis. In contrast, the caspase 8 inhibitor Z-IETD-FMK [28] did not show a significant protection (Fig. 3). These results suggested that activation of caspase 9 through a mitochondria-dependent

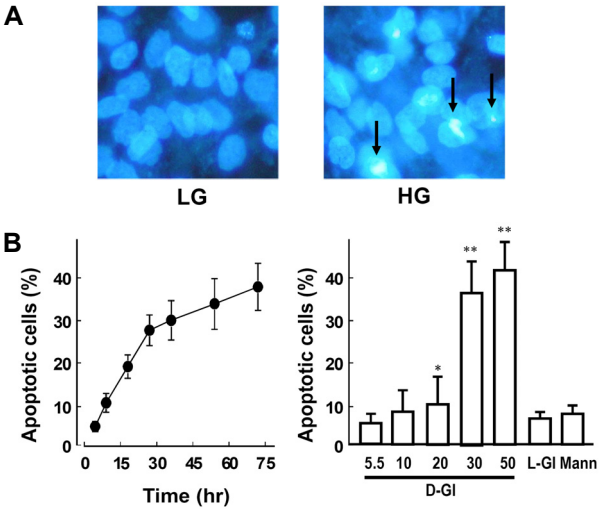

Fig. 1. Apoptosis of HUVECs in high glucose media. A. Representative micrographs of Hoechst 33,258-stained cell preparations after 72 hr exposure to low (LG, 5.5 mM) and high glucose (HG, 30 mM) media. Cells showing fragmented or condensed nuclei were counted as apoptotic (indicated by arrows) B. Time-dependent increase of apoptotic cell counts in HG media C. Concentration-dependent effect of D-glucose (D-Gl). The effects of L-glucose (L-Gl, 25 mM) and mannitol (Mann, 25 mM) as osmotic controls were presented together. Data are means ± S.E. of 4 experiments. $^{*}p<0.05$, $^{**}p<0.01$ vs. 5.5 mM D-Gl.

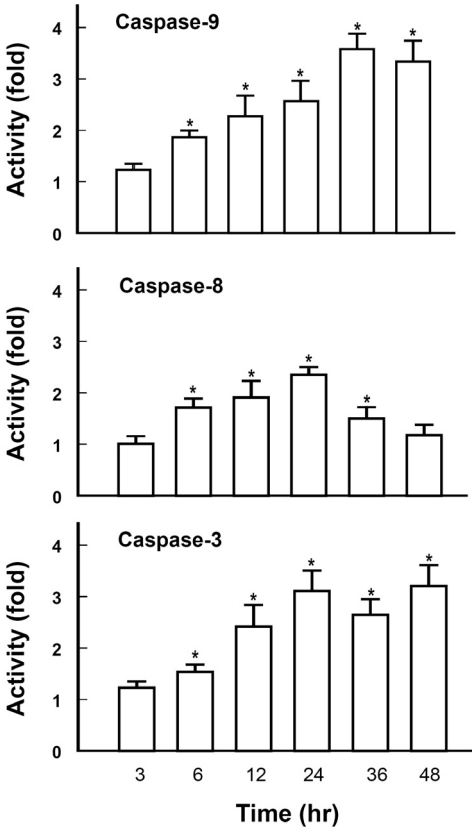

Fig. 2. Effect of high glucose on caspase activities. Cells were exposed to media containing 30 mM glucose for indicated time periods and assayed for caspase 3, 8, and 9 activities. Each bar represents means ± S.E. of 4 experiments. $^{*}p<0.01$ vs. the respective control determined in cells in low (5.5 mM) glucose media.

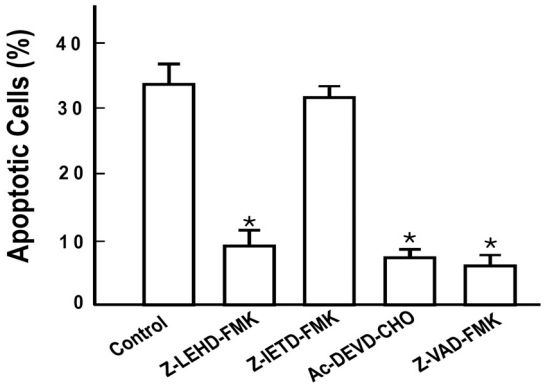

Fig. 3. Effects of caspase inhibitors on high glucose-induced apoptosis. Cells were exposed for 48 hr to media containing 30 mM glucose in the presence of different caspase inhibitors (each 20 μM), and apoptotic cells were detected by Hoechst 33,258-staining. The number of apoptotic cells was presented as percentile of total cell counts. Each bar represents means ± S.E. of 4 experiments. *p<0.01 vs. control.

pathway played a major role in the HG-induced apoptosis of HUVECs.

Effect of diazoxide on HG-induced apoptosis and caspase activation

Apoptotic cells were counted in different cell preparations exposed to HG in the presence of diazoxide and 5-hydroxydecanoate, a well-known prototype of mitochondrial K_{ATP} opener and blocker [27]. Diazoxide remarkably decreased the apoptotic cell counts. However, in cells pretreated with 5-hydroxydecanoate, the protective effect of diazoxide was significanly blocked. Diazoxide also showed a significant inhibition of caspase 9 which was comparable to the effect of Z-LEHD-FMK, a specific inhibitor of caspase-9. In contrast, caspase-8 activity was not affected by diazoxide (Fig. 4). These results strongly suggested that diazoxide exerted its anti-apoptotic effect via opening of K_{ATP} and suppression of the mitochondria-dependent apoptotic signaling.

HG-indiuced mitochondrial depolarization and permeability transition

To elucidate further the role of mitochondria in the HG-induced apoptosis, changes in mitochondrial integrity was determined. As shown in the representative graph (Fig. 5A) and summarized data (Fig. 5B) of flow cytometry analysis of DiOC6(3)-stained cells, HG significantly depolarized the mitochondrial membrane potential in a time-dependent manner, suggesting disruption of the mitochondrial membrane potential preceded the HG-induced apoptosis.

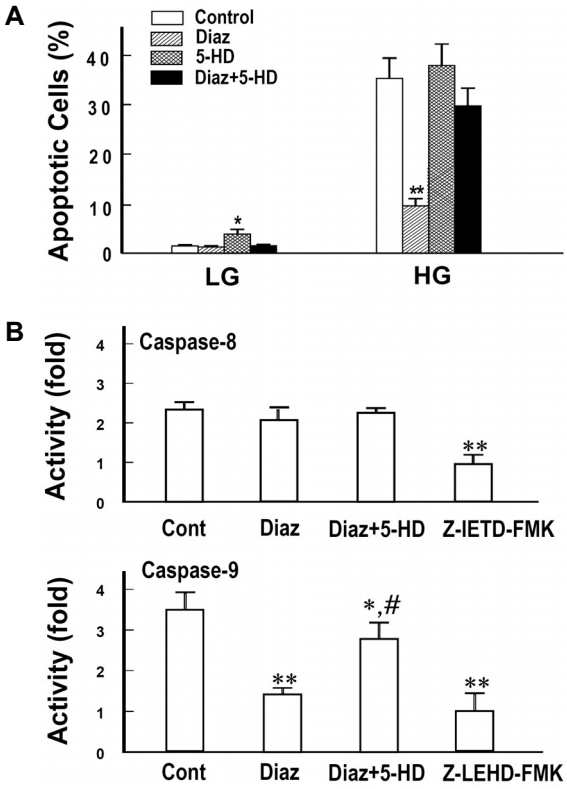

Fig. 4. Effect of diazoxide on high glucose-induced apoptosis and caspase activities. A. Cells were exposed for 72 hr to low (LG, 5.5 mM) and high (HG, 30 mM) glucose media in the presence of diazoxide (Diaz, 10 μM) with or without 5-hydroxydecanoate (5-HD, 100 μM) and apoptotic cells were detected by Hoechst 33,258-staining. B. Cells were exposed for 24 hr to high glucose media in the presence of Diaz with or without 5-HD and analyzed for caspase-8 and 9 activities. For a comparison, effects of each specific caspase inhibitors were presented together. Data are means ± S.E. of 4 experiments. $^{*}p$< 0.05, $^{**}p$<0.01 vs. the respective control and $^{\#}p$<0.01 vs. Diaz alone.

Formation of MPT pores and disruption of the permeability barrier in the inner mitochondrial membrane has been suggested to be a crucial event to initiate the mitochondria-dependent apoptotic pathway. The MPT pores have been known to mediate the release of cytochrome c [16]. In confocal microscopic analysis, cells with intact mitochondria were able to be discriminated by their mitochondrial contours visualized as bright red spots. On the other hand, in cells with MPT pores mitochondria tended to lose TMRM and become permeable to calcein. As a result, it became hard to distinguish mitochondrial contours from cytosol as indicated by arrows in a representative micrograph (Fig. 6A).

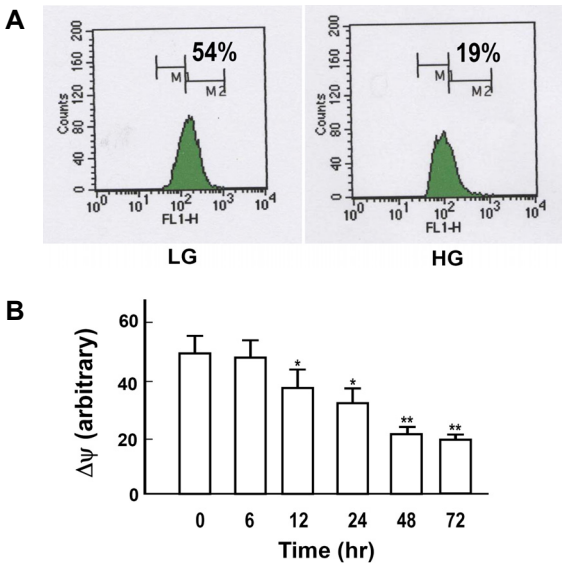

Fig. 5. Effect of high glucose on mitochondrial membrane potential. A. Cells were exposed to low (LG, 5.5 mM) and high (HG, 30 mM) glucose media for 72 hr. Cells were then loaded with DiOC6(3), and analyzed for mitochondrial membrane potential by flow cytometry analysis. B. Changes in mitochondrial membrane potential were determined as a function of exposure time periods to HG. Mitochondrial membrane potential was presented as arbitrary unit estimated by the degree of fluorescence quenching. Data are means ± S.E. of 4 experiments. $^{*}p<0.05$, $^{**}p<0.01$ vs. control.

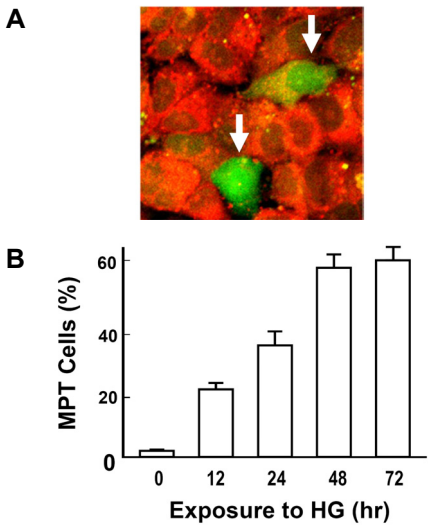

Fig. 6. Formation of mitochondrial permeability transition pores induced by exposure to high glucose. A. Cells were exposed to high glucose media for 72 hr and double-stained with fluorescence dyes TMRM and calcein-AM. Intact mitochondria accumulate TMRM (bright red), whereas injured mitochondria with mitochondrial permeability transition (MPT) pores lose TMRM and become permeable to and stained with calcein (green, indicated by arrows). B. The number of cells with MPT pores were presented as percentile of total cell counts. Data are means ± S.E. of 6 experiments.

These results showed clear evidence that formation of MPT pores took place in the HG-treated cells. The number of cells with MPT pores increased in a time-dependent manner in cells exposed to HG media (Fig. 6B).

Diazoxide-induced suppression of mitochondrial apoptotic signaling

As was shown in the above results, HG-induced apoptosis was likely to proceed mostly via a mitochondria-dependent pathway. The results also suggested that diazoxide exhibited its anti-apoptotic effect via suppression of the mitochondria-dependent apoptotic signaling. To elucidate further the effects of diazoxide on mitochondrial events associated with the HG-induced apoptosis, effects of diazoxide on mitochondrial membrane potential, formation of MPT pores and cytosolic cytochrome release were examined. As presented in the representative flow cytometric analysis of DiOC6(3)-stained cells in Fig. 7A and summarized data in Fig. 7B, diazoxide significantly reversed HG-induced depolarization of the mitochondrial membrane potential. In accordance with this result, diazoxide significantly reduced the number of cells with MPT pores (Fig. 8A) and cytosolic release of cytochrome c (Fig. 8B) in cells treated with HG.

Discussion

Diabetes impacts diverse organs and systems through different molecular mechanisms. HG-induced apoptosis has been suggested to be importantly implicated in diabetic pathologies and multiple mechanisms have been suggested to trigger and regulate the complicated signaling cascades involved in the HG-induced apoptosis [2].

Caspase enzymes play crucial roles in the process of apoptotic pathway. Cytochrome c released from mitochondria through the permeability transition pores is a main trigger to activate the caspase 9. Thus activation of the caspase 9 is a crucial event to initiate the mitochondria-dependent apoptotic signaling. On the other hand, activation of the caspase 8 is achieved largely through the mitochondria-independent mechanisms [17]. In the present study, exposure to HG resulted in activation of the caspase 8 as well as the caspase 9 and 3. However, an inhibitor of the caspase 8 did not show a significant effect to suppress the HG-induced

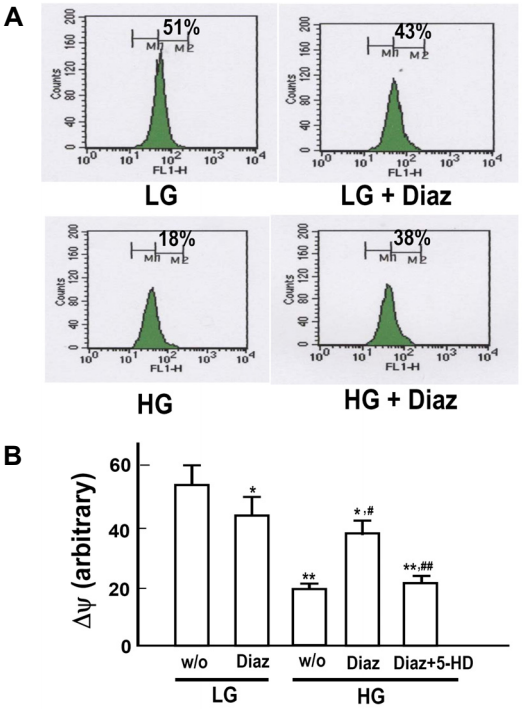

Fig. 7. Effect of diazoxide on high glucose-induced depolarization of mitochondrial membrane potential. A. Cells were exposed to low (LG, 5.5 mM) and high (HG, 30 mM) glucose media for 72 hr. Cells were then loaded with DiOC6(3) and analyzed for mitochondrial membrane potential by flow cytometry analysis. B. Mitochondrial membrane potential was presented as an arbitrary unit estimated by the degree of fluorescence quenching. Diaz, diazoxide (10 μM); 5-HD, 5-hydroxydecanoate, 100 μM. Data are means ± S.E. of 5 experiments. $^{*}p<0.05$, $^{**}p<0.01$ vs. LG without Diaz; $^{\#}p<0.01$ vs. HG without Diaz and 5-HD; $^{\#\#}p<0.01$ vs. HG with Diaz.

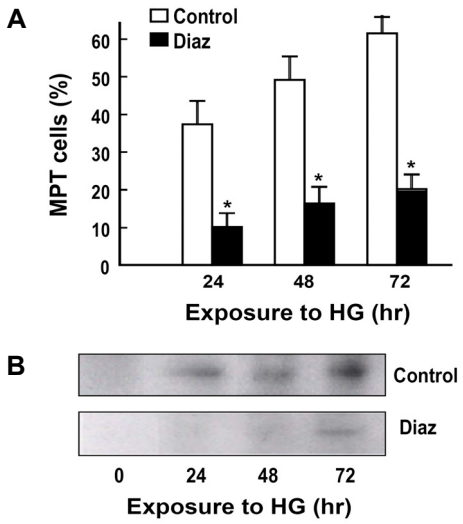

Fig. 8. Effect of diazoxide on high glucose-induced formation of mitochondrial permeability transition pores and cytochrome c release. A. Cells were exposed to high glucose (HG, 30 mM) media for indicated time periods in the presence and absence of diazoxide (Diaz, 10 μM). Cells with mitochondrial permeability transition (MPT) pores were analyzed by double fluorescence imaging with TMRM and calcein-AM. B. Western blot analysis of cytochrom C release was performed in the cytosolic fraction of cells exposed to HG for indicated time periods. Data are means ± S.E. of 4 experiments. $^{*}p<0.01$ vs. the respective controls.

apoptosis, suggesting that mitochondria-dependent activation of the caspase 9 be a major trigger which leads to the execution of the HG-induced apoptosis.

In this study, HG caused depolarization of mitochondrial membrane potential and cytochrome *c* release from the mitochondria to the cytosol. Confocal imaging studies with TMRM and calcein-AM demonstrated that the cytosolic release of cytochrome c is a result of formation of the MPT pores. These findings strongly suggested that deterioration of mitochondrial functional integrity is tightly associated with the initiation of the HG-induced apoptotic signaling.

Molecular events which link the HG-induced changes in the mitochondrial functional integrity and apoptosis are still unclear. Recent studies have suggested that uncoupling proteins might be key mediators of HG-induced apoptotic signaling [4]. Uncoupling proteins are proton carriers which reside on inner mitochondrial membrane. They have been suggested to prevent ROS formation and maintain negative mitochondrial membrane potential. HG causes disruption of the mitochondrial membrane potential and loss of the uncoupling proteins. These events result in increased oxidative stress, cytosolic release of cytochrome c and activation of caspases [4, 30].

Opening of K_{ATP} by KCOs has been shown to provide cardioprotection which mimics the endogenous cardioprotective mechanism known as 'ischemic preconditioning' a paradoxical phenomenon whereby brief conditioning periods of ischemia render the heart resistant to subsequent lethal ischemia [23]. The ability to recruit such protection pharmacologically opened new prospects for limiting damage to the heart as a consequence of ischemic or oxidative insults. It is now evident that such a protection mechanism provided by KCOs is functioning not only in the heart but also in the various organs or tissues including brain, vessels and skeletal muscle [14].

The inner mitochondrial membrane as well as the plasma membrane harbors the K_{ATP} [13]. Diazoxide is a relatively

selective opener of this mitochondrial K_{ATP} [27]. In the present study, diazoxide protected HUVECs against HG-induced apoptosis and the protection was associated with inhibition of HG-induced activation of caspase 9 and 3. The effect of diazoxide to suppress caspase activation and apoptosis was significantly blocked when cells were pre-treated with the selective blocker of the mitochondrial K_{ATP}, 5-hydroxydecanoate [27], suggesting that the diazoxide-induced protection was associated with opening of the mitochondrial K_{ATP}.

The results in this study consistently suggested that the beneficial effect of diazoxide to preserve mitochondrial functional integrity in HG-exposed cells might be a key event responsible for its protective effect against apoptosis. It helped mitochondria to preserve the mitochondrial membrane potential, suppressed the formation of MPT pores, and its related event, cytochrome c release. In conclusion, it was suggested that diazoxide prevented deterioration of mitochondrial functional integrity through opening of the mitochondrial K_{ATP} and provided a protection mechanism against HG-induced apoptosis in HUVECs.

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초록 : 고농도 당에 노출된 혈관 내피세포에서 미토콘드리아 의존성 세포사멸 기작 활성화에 미치는 diazoxide의 억제 효과

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본 연구에서는 사람의 제대정맥 내피세포에서 고농도 당에 의해 유도되는 세포사멸과 연관된 미토콘드리아의 기능적 지표 변화에 미치는 diazoxide의 효과를 관찰하였다. 고농도 당에 노출된 내피세포에서 세포사멸이 시간에 따라 증가하였고, caspase 3와 8, 9의 활성 증가가 동반되었다. Caspase 3와 9의 억제제들이 세포사멸을 감소시킨 반면 caspase 8의 억제제는 효과가 없었다. 고농도 당에 노출된 세포에서 미토콘드리아 막전위의 탈분극과 막투과도의 증가, 치토크롬 C (cytochrome C)의 유리가 동반됨을 관찰할 수 있었다. Diazoxide는 고농도 당에 의한 미토콘드리아 의존성 세포사멸 신호의 활성화를 억제하였다. Diazoxide의 이러한 효과들은 미토콘드리아막의 ATP-억제성 칼륨통로 차단제인 5-hydroxydecanoate에 의해 차단되었다. 이상의 결과들을 종합하면 diazoxide가 미토콘드리아막의 ATP-억제성 칼륨통로 개방을 통해 미토콘드리아 의존성 세포사멸 신호기작의 활성화를 차단하여 고농도 당에 의해 유도되는 세포사멸을 억제하는 것으로 사료된다.