VDAC: old protein with new roles in diabetes

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Sasaki K, Donthamsetty R, Heldak M, Cho YE, Scott BT, Makino A. VDAC: old protein with new roles in diabetes. Am J Physiol Cell Physiol 303: C1055–C1060, 2012. First published September 12, 2012; doi:10.1152/ajpcell.00087.2012.—A decrease in capillary density due to an increase in endothelial cell apoptosis in the heart is implicated in cardiac ischemia in diabetes. The voltage-dependent anion channel (VDAC) plays a crucial role in the regulation of mitochondrial metabolic function and mitochondria-mediated apoptosis. This study is designed to examine the role of VDAC in coronary endothelial dysfunction in diabetes. Endothelial cells (ECs) were more apoptotic in diabetic left ventricle of diabetic mice and mouse coronary ECs (MCECs) isolated from diabetic mice exhibited significantly higher mitochondrial Ca2+ concentration and VDAC protein levels than control MCECs. The expression of VDAC-short hairpin RNA (shRNA) not only decreased the resting mitochondrial Ca2+ concentration but also attenuated mitochondrial Ca2+ uptake in diabetic MCECs. Furthermore, the downregulation of VDAC in diabetic MCECs significantly decreased mitochondrial superoxide anion (O2−) production and the activity of the mitochondrial permeability transition pore (mPTP) opening (an indirect indicator of cell apoptosis) toward control levels. These data suggest that the increased VDAC level in diabetic MCECs is responsible for increased mitochondrial Ca2+ concentration and VDAC protein levels. Normalizing VDAC protein level may help to decrease endothelial cell apoptosis, increase capillary density in the heart, and subsequently decrease the incidence of cardiac ischemia in diabetes.

Ca2+ overload in mitochondria; apoptosis; vascular complications; vascular rarefaction

ENDOTHELIAL DYSFUNCTION is a common feature of diabetic vascular complications (8). Insufficient formation and rarefaction of capillaries, as a result of endothelial dysfunction, may represent one of the most critical mechanisms involved in cardiac ischemia. Along with other investigators, we have reported that capillary density in the heart is progressively decreased in diabetes (15, 25, 37, 39). Increased endothelial cell (EC) apoptosis (39) and attenuated regeneration of new capillaries by circulating endothelial progenitor cells (23, 39) both contribute to microvascular rarefaction in diabetes. Preventing coronary EC apoptosis would restore the decrease in capillary density, improve oxygen transport to cardiac tissues, and decrease the incidence of cardiac ischemia in diabetes.

Voltage-dependent anion channel (VDAC) was identified in 1976, and three VDAC isoforms have been characterized (VDAC1, VDAC2, and VDAC3). Among these three subtypes, VDAC1 is highly expressed in most cell types and is considered a key player in mitochondria-mediated apoptosis (36, 40, 41). Although other subtypes are also related with cell apoptosis, the molecular mechanisms proposed to explain how they regulate cell apoptosis are still controversial (4, 5). VDAC is located in the outer mitochondrial membrane and is a bidirectional transporter. Under physiological conditions VDAC serves as a shuttle of ATP and other small molecules (35), whereas under pathophysiological conditions VDAC contributes to cell apoptosis at the early stages [e.g., Ca2+ overload into mitochondria (12)] as well as during later stages [such as apoptotic protein release from the mitochondria by opening the mitochondrial permeability transition pore (mPTP) or rupture of the mitochondrial outer membrane (34, 38)]. The present study was designed to investigate the pathological role of VDAC in coronary endothelial dysfunction in diabetes.

MATERIALS AND METHODS

Materials. Medium 199 (M199) was obtained from Mediatech (Manassas, VA). Antibiotic reagents, dispase II, MitoSOX Red, Rhod-2 AM, and a LICE Mitochondrial Transition POre Assay Kit were purchased from Invitrogen (Carlsbad, CA). Anti-VDAC obtained from BioVision (Mountain view, CA), anti-hexokinase II from Cell Signaling Technology (Danvers, MA), and anti-actin from Santa Cruz Biotechnology (Santa Cruz, CA) were used for Western blot. Collagenase II was purchased from Worthington Biochemical (Lakewood, NJ). All other chemicals were from Sigma-Aldrich (St. Louis, MO).

Animal preparation. All investigations conformed to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH Publication No. 85-23, Revised 1985). This study was approved and conducted in accordance with the guidelines established by the Institutional Animal Care and Use Committee in the University of Illinois at Chicago. Six-week-old male C57BL/6 mice were purchased from Harlan Laboratories (Madison, WI), and mice in the diabetic group received a single injection of streptozotocin (133 mg/kg, dissolved in citrate buffer, i.v.). Data were obtained from mice 4–6 wk after diabetic induction with the exception of data for apoptotic EC determination in the heart (10 wk). Plasma glucose levels were 138.5 ± 4.3 mg/dl in control mice and 570.4 ± 18.1 mg/dl in diabetic mice.

Analysis of EC apoptosis in left ventricular myocardium. Transferrase-mediated dUTP nick end-labeling (TUNEL) assay was performed to detect apoptotic cells with an in situ cell detection kit (Roche). Sections of subepicardial regions of the left ventricular (LV) free wall were costained with BS-1-FITC to identify ECs. The images were photographed in sequence by a CCD camera connected to a fluorescence microscope with a ×20 objective lens. Apoptotic ECs were defined as TUNEL-positive cells colocalized with the EC marker and were counted with ImageJ software (National Institutes of Health, Bethesda, MD). Data were described as the percentage of apoptotic ECs (ratio of TUNEL-positive ECs/total ECs).

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Isolation of mouse coronary vascular endothelial cells. Mouse coronary vascular endothelial cells (MCECs) were isolated as described previously (26, 27). Briefly, dissected heart tissues were minced and incubated with M199 containing 1 mg/ml collagenase II and 0.6 U/ml dispase II for 1 h at 37°C. The digested material was filtered through sterile 40-μm nylon mesh and washed in 2% (vol/vol) FCS-M199. Subsequently, the cells were incubated with Dynabeads (Invitrogen), which were prepared as follows: beads coated with sheep anti-rat IgG were incubated with purified rat anti-mouse CD31 monoclonal antibody (1 μg/ml) at 4°C overnight and then washed with PBS containing 0.1% (wt/vol) BSA and 2 mM EDTA. The cell suspension was incubated with beads for 1 h at 4°C and then the beads attached to ECs were captured and isolated by the Dynal magnet (Invitrogen).

Western blot analysis. Freshly isolated MCECs were used for protein extraction. Cell lysates were centrifuged at 16,000 g for 10 min at 4°C. Supernatants were used as sample protein. Samples were separated on SDS-polyacrylamide gels and transferred to nitrocellulose membranes. Blots were then incubated with a primary antibody (anti-VDAC [1:2,000] or anti-actin [1:4,000]) followed by incubation with a horseradish peroxidase-conjugated secondary antibody. The immunoblots were detected with SuperSignal West Pico reagent (Thermo Fisher Scientific, Rockford, IL). Band intensity was normalized to actin controls and expressed in arbitrary units.

Mitochondrial Ca\(^{2+}\) concentration measurement. Mitochondrial Ca\(^{2+}\) concentration ([Ca\(^{2+}\)\(_{\text{mit}}\)]) in MCECs was measured using a modification of previously described methods (7). Isolated MCECs were cultured in M199 (containing 5 mmol/l glucose) supplemented with 10% (vol/vol) FBS, 100 U/ml endothelial cell growth supplement (ECGS), 100 μM penicillin, 100 μg/ml streptomycin, 50 mg/l d-valine, and 16 U/ml heparin. Cells were plated on glass chamber slides coated with 5% (wt/vol) gelatin. Three days after isolation, [Ca\(^{2+}\)\(_{\text{mit}}\)] was measured by a digital imaging fluorescence microscope. Cells on coverslips were loaded with the membrane-permeable acetoxyethyl ester form of Rhod-2 (Rhod-2-AM; 2 μmol/l for 20 min in the dark at 37°C. The Rhod-2-AM-loaded cells were then superfused with physiological salt solution (PSS) for 90 min at 32°C to wash away extracellular and extramitochondrial dye. Rhod-2 fluorescence (530 nm excitation; 580 nm emission) from the cells and background fluorescence were imaged using a Nikon Eclipse Ti-E inverted fluorescence microscope. The background intensity was normalized to actin controls and expressed in arbitrary units.

RESULTS

More ECs are apoptotic in diabetic left ventricle and [Ca\(^{2+}\)\(_{\text{mit}}\)] and VDAC protein expression are increased in MCECs isolated from diabetic mice. ECs in the LV were stained with BS-lectin (EC marker) (25), and apoptotic cells were detected by TUNEL assay. The percentage of apoptotic ECs (ratio of TUNEL-positive ECs/total ECs) is significantly higher in diabetic versus control mice (Fig. 1A). In addition, [Ca\(^{2+}\)\(_{\text{mit}}\)] and VDAC protein levels are significantly increased in MCECs from diabetic mice compared with control (Fig. 1, B and C).

VDAC-shRNA expression decreases VDAC protein levels and restores the resting [Ca\(^{2+}\)\(_{\text{mit}}\)] and rate of Ca\(^{2+}\) uptake into mitochondria in diabetic MCECs. To test whether the increased [Ca\(^{2+}\)\(_{\text{mit}}\)] in diabetic MCECs results from the increased levels of VDAC protein, we generated mouse VDAC1 (VDAC)-shRNA Adv to decrease VDAC protein levels (Fig. 2, A and B). The inhibition of VDAC in diabetic MCECs decreases not only the resting [Ca\(^{2+}\)\(_{\text{mit}}\)] but also Ca\(^{2+}\) uptake into mitochondria when cytosolic Ca\(^{2+}\) levels are raised by inhibiting SERCA with CPA (10 μmol/l) (Fig. 2, C and D).

VDAC-shRNA expression attenuates mitochondrial O\(_{2}\) production and decreases the mPTP opening activity in diabetic MCECs. Ca\(^{2+}\) overload into mitochondria leads to cell apoptosis partially due to increasing mitochondrial O\(_{2}\) production as well as opening the mPTP, which subsequently releases pro-apoptotic molecules from the mitochondria to the cytosol. Figure 3 demonstrates that O\(_{2}\)[\(_{\text{mit}}\)] in MCECs isolated from diabetic mice is significantly higher than in control mice, whereas the inhibition of VDAC in diabetic MCECs restores the increased O\(_{2}\)[\(_{\text{mit}}\)] to the level observed in the control. In addition, diabetic MCECs exhibit a significant decrease in
calcein-AM fluorescence in the mitochondria compared with control MCECs, suggesting that the mPTP opening is more activated at the resting level in diabetic MCECs. VDAC-shRNA expression significantly increases the calcein-AM intensity in diabetic MCECs, suggesting that the activity of the mPTP opening was decreased by VDAC-shRNA over-expression in diabetic MCECs (Fig. 4).

**Hexokinase II protein expression in diabetic MCECs is significantly lower than control.** Hexokinases (HKs) serve as endogenous VDAC regulators with inhibiting VDAC activation. Figure 5 demonstrates that hexokinase II (HK2) is significantly decreased in diabetic MCECs compared with control, whereas there is no difference in HK1 protein levels between control and diabetic MCECs.

**DISCUSSION**

Hyperglycemia increases VDAC1 expression in β cells (1) and in the kidney (14), whereas VDAC levels are not altered or decreased in cardiac myocytes after high-glucose treatment (19, 24). It is, however, yet to be explored whether VDAC contributes to coronary endothelial dysfunction in diabetes. Here, we demonstrate that MCECs isolated from diabetic mice exhibit a significant increase in VDAC protein level when

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**Fig. 1.** Augmented endothelial cell (EC) apoptosis in the left ventricle (LV) and increased voltage-dependent anion channel (VDAC) protein level and increased mitochondrial Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_{\text{mit}}\)) in mouse coronary ECs (MCECs) isolated from diabetic mice. A: columns show summarized data of the percentage of apoptotic ECs (the number of apoptotic ECs divided by total number of ECs). Control (Cont), n = 5; diabetic (Dia), n = 6. Data are means ± SE. *P < 0.05 vs. control. B: representative images showing [Ca\(^{2+}\)]\(_{\text{mit}}\) in MCECs isolated from control and diabetic mice (left photomicrographs). Right columns show summarized [Ca\(^{2+}\)]\(_{\text{mit}}\) data (Rhod-2-fluorescence intensity from the cell minus background intensity). Cont, n = 25; Dia, n = 38. Data are means ± SE. *P < 0.05 vs. control. Bar = 50 μm. C: Western blots showing VDAC and actin protein levels (left). Actin was used as a loading control. Right columns show VDAC protein levels normalized by actin. Cont, n = 6; Dia, n = 6. Data are means ± SE. *P < 0.05 vs. control.

**Fig. 2.** Effect of VDAC inhibition by VDAC-short hairpin RNA (shRNA) adenovirus (Adv) (VDAC-shRNA) on mitochondrial Ca\(^{2+}\) uptake. A: mouse VDAC shRNA was designed and cloned into an adenoviral vector with a U6 promoter. B: infection of mouse ECs with VDAC-shRNA significantly decreases VDAC protein levels. Western blots showing VDAC and actin protein levels (left). Actin was used as a loading control. Right columns show VDAC protein level normalized to actin. ECs infected with control Adv (Cont-Adv), n = 2; ECs infected with VDAC-shRNA, n = 2. Data are means ± SE. *P < 0.05 vs. control. C and D: measurement of [Ca\(^{2+}\)]\(_{\text{mit}}\) in MCECs at rest and after treatment with the sarco-endoplasmic reticulum Ca\(^{2+}\)-ATPase (SERCA) inhibitor, cyclopiazonic acid (CPA), in the absence of extracellular Ca\(^{2+}\). C: time course of the [Ca\(^{2+}\)]\(_{\text{mit}}\) measurement experiments. Resting level indicates [Ca\(^{2+}\)]\(_{\text{mit}}\) without treatment in the presence of Ca\(^{2+}\) in the extracellular media. After changing the extracellular media to Ca\(^{2+}\)-free media, CPA (10 μmol/l) was added to test Ca\(^{2+}\) uptake by mitochondria. Area under the curve (AUC) and Δpeak were used to analyze Ca\(^{2+}\) uptake. D: summarized [Ca\(^{2+}\)]\(_{\text{mit}}\) data. Control ECs infected with control Adv (Cont-EC, Cont-Adv), n = 16; diabetic ECs infected with control Adv (Dia-EC, Cont-Adv), n = 19; diabetic ECs infected with VDAC-shRNA (Dia-EC, VDAC-shRNA), n = 22. Data are means ± SE. *P < 0.05 vs. Cont-EC, Cont-Adv. #P < 0.05 vs. Dia-EC, Cont-Adv.
compared with control (Fig. 1C). The experiments of Fig. 1A and our previous work showing decreased vascular density (25) are performed 10 wk after diabetic induction. In the current study, the increase of VDAC protein and $[\text{Ca}^{2+}]_{\text{mit}}$ could be seen 4 wk after diabetic induction, implying that EC apoptosis in the heart may be initiated byVDAC overexpression at an early stage.

Increased $[\text{Ca}^{2+}]_{\text{mit}}$ can facilitate ATP production to meet increased cellular ATP demands by activating Ca$^{2+}$-sensitive enzymes in the tricarboxylic acid cycle (21, 28). However, Ca$^{2+}$ overload in the mitochondria leads to apoptosis (16, 22), suggesting that maintaining $[\text{Ca}^{2+}]_{\text{mit}}$ within the physiological range is crucial to keep cells functional and viable. High-glucose treatment leads to decreased $[\text{Ca}^{2+}]_{\text{mit}}$ in the adipocyte (10) and cardiac myocyte (19), which may be the cause of insufficient energy production. Interestingly, $[\text{Ca}^{2+}]_{\text{mit}}$ is significantly increased in diabetic coronary ECs compared with control (Fig. 1B), and the inhibition of VDAC in diabetic MCECs decreases not only the resting $[\text{Ca}^{2+}]_{\text{mit}}$ level, but also mitochondrial Ca$^{2+}$ uptake, when cytosolic Ca$^{2+}$ levels are raised. These data suggest that in diabetic MCECs increased VDAC expression is responsible for augmented Ca$^{2+}$ uptake into mitochondria and increased resting $[\text{Ca}^{2+}]_{\text{mit}}$. VDAC overexpression increases mitochondrial Ca$^{2+}$ uptake by augmenting mitochondrial permeability and/or by increasing the number of contact sites between mitochondria and the endoplasmic reticulum (33). Mitochondrial Ca$^{2+}$ overload sequentially triggers the release of pro-apoptotic proteins from the mitochondria to the cytosol (29, 38) and increases $[\text{O}_2]^-$mit (32).

Increased $[\text{O}_2]^-$ production is implicated in the pathogenesis of diabetes-associated vascular complications (11, 13, 18). Figure 3 shows that VDAC inhibition significantly decreases $[\text{O}_2]^-$mit in diabetic MCECs, suggesting that overproduction of $[\text{O}_2]^-$ is induced by Ca$^{2+}$ overload in mitochondria via increased VDAC expression in diabetic coronary ECs.

An increased mPTP opening activity is the hallmark of mitochondria-induced cell apoptosis (30). The mPTP is composed of VDAC in the outer mitochondrial membrane (OMM), adenosine nucleotide translocator (ANT) in the inner mitochondrial membrane, and cyclophilin D in the mitochondrial matrix (6, 17). Excess Ca$^{2+}$ influx triggers the increase of ANT conductivity followed by an inward flux of protons and ions through the ANT. The increase in matrix osmolality leads to water influx, mitochondrial swelling, and apoptogenic protein release from the mitochondrial storage to the cytosol though the mPTP opening. BAX/BAK-VDAC channel, and/or ruptured OMM (3, 29, 38). There are reports showing that in an ex vivo study acute high-glucose treatment increases the mPTP opening in ECs (9, 20). We demonstrate that the mPTP opening is augmented at the resting level in diabetic MCECs compared with control MCECs, and VDAC-shRNA Adv infection significantly inhibits the mPTP opening in diabetic MCECs, implying that increased VDAC protein expression in diabetic MCECs leads to endothelial apoptosis via augmented mPTP opening.

![Fig. 4](http://ajpcell.physiology.org/)

**Fig. 4.** Activity of the mitochondrial permeability transition pore (mPTP) opening is significantly increased in diabetic MCECs, and VDAC-shRNA expression decreases the activity toward the control level. The fluorescence intensity of calcine-AM in mitochondria was measured to assess the activity of the mPTP opening. The decrease in calcine-AM intensity indicates the increase of the mPTP opening. Cont-EC and Cont-Adv, n = 108; Dia-EC and Cont-Adv, n = 133; Dia-EC and VDAC-shRNA, n = 98. Data are means ± SE. $^*P < 0.05$ vs. Cont-EC, Cont-Adv. $^#P < 0.05$ vs. Dia-EC, Cont-Adv.

![Fig. 5](http://ajpcell.physiology.org/)

**Fig. 5.** Hexokinase II (HK2), but not hexokinase I (HK1), is significantly decreased in diabetic MCECs. Western blots showing HK1, HK2, and actin protein levels (left). Actin was used as a loading control. Right columns show HK1 and HK2 protein expression levels normalized by actin. Cont, n = 4; Dia, n = 4. Data are means ± SE. $^*P < 0.05$ vs. control.
VDAC has been reported to display the binding sites for HK, glycerol kinase, creatine kinase, as well as pro- and anti-apoptotic proteins of the Bcl-2 family (e.g., BAX, Bcl-2, Bcl-xI), and these bindings regulate the mPTP opening, which in turn modulate cell apoptosis (31, 35). Vertebrates have four isoforms of HK, and HK1 and HK2 exhibit their anti-apoptotic effect by directly binding to VDAC, followed by the channel closure (2), as well as by indirect mechanisms including interfering with the megachannel formation, which consists with VDAC and BAX (31). We found that MCECs isolated from diabetic mice have significantly decreased HK2 protein expression compared with control MCECs, and enhanced due to decreased HK2 protein expression.

To summarize, diabetic MCECs exhibit a significant increase in VDAC protein expression compared with control MCECs, and VDAC inhibition restores the increased [Ca^{2+}]_{mit}, [O_2]_{mit} and mPTP opening activity in diabetic MCECs to control levels. These data suggest that normalizing VDAC protein levels may decrease endothelial cell apoptosis, increase capillary density in the heart, and subsequently decrease the incidence of cardiac ischemia in diabetes.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: K.S., R.D., B.T.S., M.H., Y.-E.C., and A.M. approved final version of the article; B.T.S. and A.M. performed experiments; K.S., R.D., M.H., Y.-E.C., and A.M. analyzed data; K.S., R.D., and A.M. interpreted results of experiments; K.S. and A.M. prepared figures; K.S., R.D., B.T.S., M.H., Y.-E.C., and A.M. approved final version of manuscript; R.D., B.T.S., and A.M. edited and revised manuscript; A.M. conceived and directed research; A.M. drafted manuscript.

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