Role of EDHF in type 2 diabetes-induced endothelial dysfunction

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Park Y, Capobianco S, Gao X, Falck JR, Dellersperger KC, Zhang C. Role of EDHF in type 2 diabetes-induced endothelial dysfunction. Am J Physiol Heart Circ Physiol 295: H1982–H1988, 2008.—Endothelium-derived hyperpolarizing factor (EDHF) is observed in numerous blood vessels from different species (12). Different candidates have been proposed, such as potassium ions (K⁺), epoxyeicosatrienoic acids (EETs), and hydrogen peroxide (H₂O₂), to function as EDHFs (12). However, the specific identity of EDHF involved in the coronary microcirculation in type 2 diabetes is unknown.

The reduction in NO bioavailability impairs endothelium-dependent relaxation in diabetic vascular beds (7, 12). However, EDHF may play an important role in regulating the vascular tone and reactivity, especially in small resistant vessels when NO-mediated control is compromised (7, 35). Despite some studies in diabetic rats (13, 24, 38) and humans (1), many issues remain unresolved.

Here we present data that increased TNF-α and other cytokines in type 2 diabetes induce the activation of reactive oxygen species (ROS), leading to endothelial dysfunction in diabetic coronary arteries, but no direct study has been conducted to show the role of IL-6 in type 2 diabetes-induced endothelial dysfunction (6, 16, 31). Moreover, the mechanisms of EDHF-mediated endothelial dysfunction in type 2 diabetic coronary arteries have not been investigated. To test this we 1) evaluated the pivotal contribution of EDHF in type 2 diabetes-induced endothelial dysfunction, 2) evaluated the identity of EDHF candidates in coronary microcirculation in normal and type 2 diabetic mice, and 3) determined the role of IL-6 in EDHF-induced vasodilation in type 2 diabetes.

METHODS

Animal models. The procedures followed were in accordance with approved guidelines set by the Laboratory Animal Care Committee at University of Missouri. Our animal use protocols were approved by Animal Care and Use Committee of the University of Missouri in Columbia. Wild-type (WT, C57BL/6J) controls, type 2 diabetic (db/db, BKS.Cg-m+/−/Leprdb/J) mice, and db/db mice null for TNF (dbTNF−/−/dbTNF−/−) were purchased from Jackson Laboratory (Bar Harbor, Me) and maintained on a normal rodent chow diet. Our studies used 24–30-wk-old, 27–31 g WT and 55–66 g db/db and dbTNF−/−/dbTNF−/− mice of either sex.

mRNA expression of IL-6 by real-time PCR. Total RNA was extracted from isolated coronary arteries using TRIzol reagent (Life Technologies) and was processed directly to cDNA synthesis using the SuperScript III Reverse Transcriptase (Life Technologies). cDNA was amplified using qRT-PCR Kit with SYBR Green (Life Technologies). The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
Treatments with IL-6 neutralization. Neutralizing antibody to IL-6 (anti-IL-6) is a functionally purified anti-mouse IL-6 (eBioscience). At 24–30 wk of age, both WT and db/db mice received the neutralizing anti-IL-6 (0.28 μg·ml⁻¹·kg⁻¹·day⁻¹ ip for 3 days); dosage was based on our estimates of IL-6 expression (in the low ng or pg range), and this is able to neutralize 10–100-fold of this amount of IL-6.

Functional assessment of isolated coronary arterioles. The techniques for identification and isolation of coronary microvessels were previously described in detail (40, 41). Hearts were excised and immediately placed in cold (4°C) saline solution. Each coronary artery (50 to 100 μm in internal diameter) from WT, db/db, and db/db + Anti-IL-6 mice was carefully isolated and then used in the functional studies. To determine the response of coronary arterioles to EDHF stimulation, the vessels were cannulated with glass micropipettes and pressurized to 60 cmH₂O intraluminal pressure without flow. After baseline tone was developed, the experimental interventions were performed. The concentration–diameter relationships for an activator of endothelium-dependent NO-mediated vasodilation, ACh (0.1 μmol/l to 10 μmol/l), and NO donor sodium nitroprusside (SNP, 0.1 μmol/l to 10 μmol/l) were then established. To determine EDHF-induced vasodilation, ACh-dependent vasodilation was performed in the presence of NO synthase inhibitor N⁶-nitro-L-arginine methyl ester (l-NAME, 10 μmol/l) and cyclooxygenase inhibitor indomethacin (Indo, 10 μmol/l) incubated for 30 min before beginning the protocols. In some experiments, a higher dose of l-NAME (100 μmol/l) was given with Indo to evaluate whether the full inhibition was achieved.

To distinguish which EDHF candidate(s) play a significant role in coronary microcirculation in WT and db/db mice, we studied 1) K⁺ channel blockade using a combination of a nonselective blocker of intermediate-conductance Ca²⁺-activated K⁺ channels (IKCa), charybdotoxin (CTX, 10 μmol/l), and a selective blocker of small-conductance Ca²⁺-activated K⁺ channels (SKCa), apamin (Apa, 50 μmol/l), incubated for 30 min; 2) epoxyeicosatrienoic acids (EETs) synthesis blocker, 14,15-epoxyeicos-5(Z)-enoic acid (14,15-EEZE, 10 μmol/l) incubated for 30 min; or 3) catalase, an enzyme that selectively dismutates H₂O₂ to water and oxygen (1,000 U/ml, 60-min incubation). Following the incubation, the responses to EDHF-induced vasodilation were examined. We studied the role of IL-6 (incubated for 30 min with IL-6, 5 ng/ml) in ACh-induced vasodilation in WT mice. All drugs were administered extraluminally in the chamber bathing solution during these functional experiments.

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) except for 14,15-EEZE (from Dr. John R. Falck, University of Texas Southwestern Medical School, Dallas, TX).

Protein expression of IL-6 by Western blot analyses. Hearts were separately homogenized and sonicated in lysis buffer (Cellytic MT Mammalian Tissue Lysis/Extraction Reagent, Sigma). Protein concentrations were assessed (BCA Protein Assay Kit; Pierce), and equal amounts of protein (40 μg) were separated by SDS-PAGE and transferred to nitrocellulose membranes (Hybond, Amersham). Horseradish peroxidase-conjugated goat anti-mouse was used as the secondary antibody (1:2,000 dilution) (Abcam). Signals were visualized by enhanced chemiluminescence (Amersham) and quantified by Quantity One (Bio-Rad Versadoc imaging system).

Data analysis. At the end of each experiment, the vessel was exposed to 100-μmol/l SNP to obtain its maximal diameter at 60 cmH₂O intraluminal pressure (41). All diameter changes to pharmacological agonists were normalized to the control diameter. All data are presented as means ± SE, except as specifically stated (e.g., as means ± SD for molecular studies). Statistical comparisons of vasomotor responses under different treatments were made with one-way or two-way ANOVA, and intergroup differences were tested with a least significance difference test. Significance was accepted at P < 0.05.

RESULTS

Body weight, abdominal girth, and glucose concentration. Table 1 shows that body weight, abdominal girth, and glucose concentration were significantly higher in db/db mice, dbTNF⁻/⁻/dbTNF⁻⁻ mice, and db/db mice treated with anti-IL-6 compared with WT mice.

Role of EDHF-mediated vasodilation in type 2 diabetes. To measure EDHF-dependent dilation, we studied ACh-induced vasodilation in the presence of l-NAME and Indo. Vasodilation to ACh was significantly attenuated following the administration of l-NAME and Indo in WT mice, whereas ACh-induced vasodilation was resistant to l-NAME and Indo in db/db mice (Fig. 1A). A higher dose of l-NAME (100 μmol/l) did not affect this response (data not shown). This result indicates that EDHF-induced vasodilation is preserved in diabetic mice. ACh-induced vasodilation was significantly lower in db/db mice than that in WT control mice, and ACh-induced EDHF-dependent vasodilation was also attenuated in db/db mice compared with WT mice (Fig. 1A). However, the endothelium-independent vasodilator SNP-induced vasodilation was identical in db/db versus WT mice (Fig. 1B). The incubation with l-NAME and Indo did not affect the basal tone in these functional studies.

Role of EDHF in coronary arterioles from diabetic mice. At 10 μmol/l of ACh, ~50% of ACh-induced vasodilation is dependent and the other 50% is NO and PGI₂-induced vasodilation in WT coronary arterioles (Fig. 2A), whereas in diabetic coronary arterioles, the portion of EDHF-dependent vasodilation is significantly increased to ~81% of total endothelium-dependent vasodilation (Fig. 2B).

Identity of EDHF in type 2 diabetes-induced endothelial dysfunction. To establish the identity of EDHF, we administered the inhibitors of each EDHF pathway: 1) K⁺ channel blockade combined incubation of a specific blocker of IKCa, CTX and a selective blocker of SKCa, Apa; 2) EETs synthesis blocker, 14,15-EEZE; or 3) catalase, an enzyme that selectively dismutates H₂O₂ to water and oxygen. A higher dose of l-NAME (100 μmol/l) was given with Indo to evaluate whether the full inhibition was achieved.
bination of CTX and Apa significantly attenuated the EDHF-dependent vasodilation, indicating that K+ and H2O2, but not EETs, are involved in the EDHF-induced vasodilation in type 2 diabetes (db/db mice, Fig. 3B). No significant differences in the basal tones of the isolated coronary arterioles incubated with the inhibitors were found.

Role of IL-6 in type 2 diabetes-induced vascular dysfunction. The incubation of arterioles isolated from WT mice with IL-6 impaired EDHF-induced vasodilation, whereas the administration of anti-IL-6 in diabetic db/db mice partially restored EDHF-mediated vasodilation comparable with the vasodilation in WT control mice. However, anti-IL-6 did not affect EDHF-mediated vasodilation in WT and db/db mice. *P < 0.05 vs. WT; #P < 0.05 vs. db/db.

Expression of IL-6 in type 2 diabetes. The mRNA expression of IL-6 in the heart tissue of WT, db/db, db/db mice treated with anti-TNF, and dbTNF−/dbTNF− mice was significantly elevated in db/db mice, but it was markedly attenuated in db/db mice treated with anti-IL-6 or in dbTNF−/dbTNF− mice (Fig. 5). Likewise, the protein expression of IL-6 was higher in db/db mice, but the anti-IL-6 treatment attenuated the protein expression of IL-6 in db/db mice. The protein expression of IL-6 is normal in dbTNF−/dbTNF− mice versus WT mice (Fig. 6).

DISCUSSION

Our results indicate that endothelium-mediated vasodilation is NO dependent in coronary arterioles in WT mice. However, we found that a portion of the NO-dependent, endothelium-dependent vasodilation is significantly reduced in db/db mice, supporting the view that EDHF plays a pivotal role in type 2 diabetes-induced endothelial dysfunction. Also, three EDHF candidates, H2O2, K+, and EETs, may play roles in dilating the coronary arterioles in response to ACh in WT control mice. The impairment of the H2O2 response and/or the abnormalities in K+ channels in advanced diabetes may be the possible mechanisms for the reduction in EDHF-mediated vascular function in db/db mice. Our studies indicated that the EETs were not involved in the endothelium-dependent, EDHF-mediated vasodilation in db/db mice. Our findings also support the concept that IL-6 plays a pivotal role in the EDHF-dependent endothelial dysfunction in type 2 diabetes, based on observing...
normalized coronary vascular function in the presence of anti-IL-6-neutralizing antibody. Also, the addition of IL-6 to the bath of WT coronary vessels produced a similar degree of dysfunction as seen in the db/db mice. The molecular evidence also supports our findings in that the expression of IL-6 was significantly increased in db/db mice. The administration of anti-IL-6 attenuated IL-6 expression in db/db mice compared with WT mice. Lastly, the expression of IL-6 was similar in dbTNF/−/dbTNF/− versus WT mice. These findings provide a further understanding of the mechanism(s) that contribute to the role of EDHF in endothelial dysfunction in type 2 diabetes in the coronary microcirculation.

Impaired NO-dependent coronary control and contribution of EDHF in type 2 diabetes. In the present study, both NO- and EDHF-mediated vasodilation are attenuated in coronary arterioles from db/db mice compared with WT controls, whereas the endothelium-independent vasodilation is not altered. Our data support previous studies that diabetes-induced endothelial dysfunction is mainly NO-dependent in the coronary arterioles of diabetic mice (16) and prediabetic rat coronary resistance vessels (31). Inflammatory cytokine TNF-α plays an important role in the impaired NO-mediated vasodilation in diabetic coronary arterioles by attenuating endothelial NO synthase expression and increasing peroxynitrite production. These actions can result in decreasing NO bioavailability. Our findings provide the first evidence that EDHF-mediated vasodilation is impaired in coronary arterioles from advanced type 2 diabetic db/db mice. This is consistent with previous studies showing that EDHF-induced vasodilation is impaired in diabetic rat mesenteric arteries (13, 24).

Although NO-dependent vascular responses were not altered in diabetic femoral and mesenteric arteries (38), accumulating data have confirmed that decreased NO bioavailability is the major mechanism of endothelial dysfunction in diabetic coronary microcirculation. Endothelial dysfunction in advanced diabetes is due to the decreased endothelial NO synthase expression and is exaggerated by a diabetes-induced increase in intracellular sources for oxygen free radicals [e.g., mito-
The increased intracellular Ca$^{2+}$ by blocker of SKCa) to investigate the role of the K$^{+}$ in the smooth muscle membrane (5). H$_2$O$_2$ is released from the muscle through the activation of inward-rectifying K$^{+}$ both SKCa and IKCa in the endothelial cell. The increase in antioxidants and PGI$_2$-mediated mechanisms are diminished or eliminated. a crucial role in endothelium-dependent vasodilation when NO- and PGI$_2$-mediated vascular function in diabetic small mesenteric arteries (30). In contrast, the K$^{+}$-mediated, EDHF response is impaired in small mesenteric arteries in Zucker diabetic fatty rats (3). This discrepancy may be due to the different vascular beds (arteries vs. small resistance vessels) and/or different species or strains of the animals.

H$_2$O$_2$ is an interesting factor in endothelium-dependent vasodilation. Our laboratory (16) previously reported that H$_2$O$_2$ was involved in the impairment of endothelium-dependent vasodilation since catalase, which dismutates H$_2$O$_2$ to water and oxygen, partially protected impaired vasodilation induced.

Fig. 5. mRNA expression of IL-6 was higher (3.5-fold) in db/db vs. WT mice. However, mRNA expression of IL-6 was attenuated in db/db mice treated with anti-IL-6 and in db$^{TNF-\alpha}$/db$^{TNF-\alpha}$ vs. db/db mice. *$P < 0.05$ vs. WT; #$P < 0.05$ vs. db/db ($n = 4$).

Identity of EDHF in type 2 diabetes-induced endothelial dysfunction. Several EDHF candidates have been proposed: 1) K$^{+}$, 2) EETs, a product of cytochrome P-450 oxygenase, and/or 3) H$_2$O$_2$. These major candidates for EDHF play a crucial role in endothelium-dependent vasodilation when NO- and PGII$_2$-mediated mechanisms are diminished or eliminated. The increased intracellular Ca$^{2+}$ stimulation by ACh activates both SKCa and IKCa in the endothelial cell. The increase in extracellular K$^{+}$ hyperpolarizes and relaxes vascular smooth muscle through the activation of inward-rectifying K$^{+}$ channels and the Na$^{+}$/K$^{+}$-ATPase (9). We used a combination of CTX (a nonselective blocker of IKCa) and Apa (a selective blocker of SKCa) to investigate the role of the K$^{+}$ as an EDHF candidate in the endothelial cell. Others have shown that these combined inhibitors block the efflux of K$^{+}$ from the intracellular compartment to the extracellular space in the endothelial cell and that the relaxation of vascular smooth muscle was inhibited (18, 19, 27). EETs are synthesized in the endothelium from arachidonic acid via the action of cytochrome P-450 epoxygenase hyperpolarize vascular smooth muscle through activating the large-conductance Ca$^{2+}$-activated K$^{+}$ channels in the smooth muscle membrane (5). H$_2$O$_2$ is released from the endothelial cell and has been shown to hyperpolarize smooth muscle via IKCa activation (26). Catalase selectively dismutates H$_2$O$_2$ to water and oxygen and exogenous catalase inhibits the effects of exogenous H$_2$O$_2$ (33, 36). However, the limitation of the present study is that we do not know how catalase affects ROS or whether the ROS is acting from within the cell.

Our data show that the blockade of each pathway results in a significant reduction in the EDHF-mediated vasodilation from the coronary arterioles in WT mice, suggesting that each mechanism is involved in vasodilation in the normal healthy coronary microcirculation. In coronary arterioles isolated from db/db mice, EDHF-mediated vasodilation is not altered by the inhibition of EETs synthesis, the product of cytochrome P-450 oxygenase, whereas the antagonism of other factors, namely K$^{+}$ and H$_2$O$_2$, results in a significant decrease in EDHF-dependent vasodilation in the same vascular beds. These results suggest that vasodilation induced by cytochrome P-450 oxygenase-derived EETs synthesis may be totally impaired in advanced type 2 diabetes. H$_2$O$_2$ and K$^{+}$ have been introduced as the primary EDHFs, especially in the coronary circulation, and our results support this view (2, 4, 26). A highly selective and competitive inhibitor of EETs synthesis, 14,15-EEZE, was used in this study since nonspecific inhibitors of cytochrome P-450 may affect other vasoactive factors such as action of the K$^{+}$ channel blocker (17). Previous studies reported that bradykinin-induced, EDHF-dependent relaxation in small mesenteric arteries from diabetic mice is regulated through cytochrome P-450. However, ACh-induced, EDHF-mediated relaxation involves neither cytochrome P-450 product nor H$_2$O$_2$, suggesting that a K$^{+}$-dependent EDHF response may play a role in the endothelium-dependent vasodilation in diabetic small mesenteric arteries (14, 15-E5EZE, was used in this study since nonspecific inhibitors of cytochrome P-450 may affect other vasoactive factors such as action of the K$^{+}$ channel blocker (17). Previous studies reported that bradykinin-induced, EDHF-dependent vascular function in diabetic small mesenteric arteries (30). In contrast, the K$^{+}$-mediated, EDHF response is impaired in small mesenteric arteries in Zucker diabetic fatty rats (3). This discrepancy may be due to the different vascular beds (arteries vs. small resistance vessels) and/or different species or strains of the animals.

H$_2$O$_2$ is an interesting factor in endothelium-dependent vasodilation. Our laboratory (16) previously reported that H$_2$O$_2$ was involved in the impairment of endothelium-dependent vasodilation since catalase, which dismutates H$_2$O$_2$ to water and oxygen, partially protected impaired vasodilation induced.

Fig. 6. The protein expression of IL-6 was higher in db/db vs. WT mice, but anti-IL-6 attenuated protein expression of IL-6 in db/db mice. IL-6 protein expression was attenuated in db$^{TNF-\alpha}$/db$^{TNF-\alpha}$ vs. db/db mice. Moreover, the protein expression of IL-6 was lower in db/db mice treated anti-IL-6 and db$^{TNF-\alpha}$/db$^{TNF-\alpha}$ mice compared with WT mice. *$P < 0.05$ vs. WT ($n = 10$); #$P < 0.05$ vs. db/db ($n = 10$).
by ACh in type 2 diabetes. However, our present study shows that H₂O₂ is one of the EDHF candidates to induce vasodilation in coronary arteries in WT and db/db mice when NO is blocked by l-NAME and Indo as supported by others (34, 39). Based on these results, we suggest that H₂O₂ plays a role in the stimulation of vasodilation as EDHF when NO is absent.

**IL-6 and endothelial dysfunction in type 2 diabetes.** Accumulating evidence shows that endothelial dysfunction is associated with inflammation. Our laboratory has reported that TNF-α plays a pivotal role in endothelial dysfunction in type 2 diabetes through activating the advanced glycation end (AGE) products/receptor of AGE (RAGE) and nuclear factor-κB (NF-κB) signaling pathway (16). Like TNF-α, another proinflammatory cytokine, the interleukin family is important and is also closely associated with type 2 diabetes. Elevated plasma concentration of IL-6 is an indicator of the development of type 2 diabetes (8, 32), and the chronic administration of IL-6 causes insulin resistance (22). Moreover, the neutralization of IL-6 reduces hepatic insulin resistance in obese (Lepr-db) mice (21). However, no studies have reported a direct effect of IL-6 on endothelial function. The present results provide direct evidence that IL-6 plays a key role in EDHF-mediated endothelial dysfunction in type 2 diabetes. We found that EDHF-mediated vasodilation was impaired with the incubation of IL-6 in coronary arterioles from WT control mice, whereas this was restored with the treatment of IL-6-neutralizing antibody in coronary arterioles from db/db mice. These findings indicate that IL-6 plays a pivotal role in the diabetes-induced endothelial dysfunction. The present molecular data also support this view, since mRNA and protein expression of IL-6 are significantly higher in the db/db mice heart and the neutralization of IL-6 decreases both mRNA and protein expression in db/db mice heart, resulting in a lower level of expression than that in WT control mice.

Although the relationship between TNF-α and IL-6 has not been clearly established, Turner et al. (37) recently reported that IL-6 mRNA expression was stimulated via TNF-α receptor I and mediated through the p38 mitogen-activated protein kinase, phosphoinositide 3-kinase/Akt, and NF-κB pathway. Our current results also suggest that TNF-α is a mediator of IL-6, since our molecular data indicate that IL-6 mRNA and protein expressions are significantly reduced in dbTNF−/−dbTNF−/− mice. Moreover, the functional data show that the EDHF-mediated vasodilatory function is restored in dbTNF−/−dbTNF−/− mice to the level of WT control mice and that the EDHF-mediated vasodilation was impaired with the incubation of IL-6 in dbTNF−/−dbTNF−/− mice.

In conclusion, EDHF contributes to endothelial-dependent vasodilation in maintaining coronary blood flow when the bioavailability of NO is substantially reduced in type 2 diabetes. Three EDHF candidates, K⁺, EETs, and/or H₂O₂, are involved in the EDHF-mediated vasodilation in normal coronary circulation, but the EETs are not involved in the diabetic condition. We also found that the overexpression of IL-6 (protein and mRNA) impairs the EDHF-mediated vasodilation in coronary arterioles in type 2 diabetic mice, and this impaired EDHF-mediated endothelial function can be restored to the level of normal control by the administration of neutralizing antibody to IL-6. These findings provide important new insights into the identity and mechanisms of EDHF-mediated vasodilation in the coronary circulation and may help identify novel therapeutic targets for cardiovascular disease associated with elevated levels of IL-6.

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Role of EDHF in Diabetes