Ebselen reduces nitrination and restores voltage-gated potassium channel function in small coronary arteries of diabetic rats

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Bubolz AH, Wu Q, Larsen BT, Gutterman DD, Liu Y. Ebselen reduces nitrination and restores voltage-gated potassium channel function in small coronary arteries of diabetic rats. Am J Physiol Heart Circ Physiol 293: H2231–H2237, 2007. First published August 3, 2007; doi:10.1152/ajpheart.00717.2007.—Small coronary arteries (SCA) from diabetic rats exhibit enhanced peroxynitrite (ONOO−) formation and concurrent impairment of voltage-dependent potassium (Kv) channel function. However, it is unclear whether ONOO− plays a causative role in this impairment. We hypothesized that functional loss of Kv channels in coronary smooth muscle cells (SMC) in diabetes is due to ONOO− with subsequent tyrosine nitration of Kv channel proteins. Diabetic rats and nondiabetic controls were treated with or without ebselen (Eb) for 4 wk. SCA were prepared for immunohistochemistry (IHC), immunoprecipitation (IP) followed by Western blot (WB), videomicroscopy, and patch-clamp analysis. IHC revealed excess ONOO− in SCA from diabetic rats. IP and WB revealed elevated nitration of the Kv1.2 α-subunit and reduced Kv1.2 protein expression in diabetic rats. Each of these changes was improved in Eb-treated rats. Protein nitration and Kv1.5 expression were unchanged in SCA from diabetic rats. Forskolin, a direct cAMP activator that induces Kv1 channel activity, dilated SCA from nondiabetic rats in a correolide (Cor; a selective Kv1 channel blocker)-sensitive fashion. Cor did not alter the reduced dilation to forskolin in diabetic rats; however, Eb partially restored the Cor-sensitive component of dilation. Basal Kv current density and response to forskolin were improved in smooth muscle cells from Eb-treated DM rats. We conclude that enhanced nitrosative stress in diabetes mellitus contributes to Kv1 channel dysfunction in the coronary microcirculation. Eb may be beneficial for the therapeutic treatment of vascular complications in diabetes mellitus.

IN DIABETES MELLITUS (DM), pathological changes occur in many tissues, including the retina, renal glomeruli, nerves, vasculature, and heart. Although DM induces changes in multiple organ systems, the major causes of morbidity and mortality in diabetic patients are due to cardiovascular complications involving both conduit arteries and microvessels (14, 17). Substantial evidence suggests that enhanced oxidative stress is a fundamental contributor in the development of these diabetic vascular complications (37). Elevated levels of glucose facilitate the formation of the superoxide (O2−•) anion (21, 38), which subsequently quenches nitric oxide (NO) at near-diffusion-limited rates, thereby reducing NO bioavailability and endothelium-dependent vasodilation (24, 29). As a result of O2−• interacting with NO, the highly toxic reactive oxidant peroxynitrite (ONOO−) is formed. ONOO− exerts its detrimental effects in part through nitration of tyrosine residues, resulting in the impaired function of multiple proteins including vascular K+ channels that are critical for vasodilation (8, 32).

Substantial evidence suggests that voltage-gated potassium (Kv) channels play a critical role in regulating resting membrane potential and participate in a variety of physiological responses, including coronary cAMP-mediated dilation (13, 33), pulmonary hypoxic constriction (2), vasomotor response to changes in pH (4), and H2-receptor mediated coronary constriction (25). Furthermore, studies using RT-PCR and Western blot analysis have demonstrated that Kv1.2 and Kv1.5 are highly expressed in rat cerebral (1), mesenteric (15, 16), and pulmonary arteries. A similar finding was also observed in human ventricular coronary arteries (unpublished data). These data indicate that Kv1 channels, most likely Kv1.2 and Kv1.5, play an essential role in mediating vasomotor function. Therefore, in the present study we focused on Kv1.2 and Kv1.5 channels.

Previous work in our laboratory (27) showed that production of ONOO−, induced by exposing rat small coronary arteries (SCA) to high glucose (23 mM) for 24 h in vitro, impairs Kv channel-mediated dilation to the direct cAMP agonist forskolin. However, two important consequences of this observation remain unresolved. First, it is unclear whether the above effects observed in acute in vitro hyperglycemic studies also occur in vivo in a chronic diabetic animal model, where numerous other factors may act to augment or diminish overall vascular function. Second, the impaired dilation is coincident with increased levels of Kv1 channel nitration (28). However, it is not known whether ONOO−-induced nitration is mechanistically responsible for the reduction in vascular function and, if so, whether it is reversible. We hypothesized that in diabetes, there is functional loss of Kv1 channel activity in coronary smooth muscle cells due to the overproduction of ONOO−, which leads to reversible tyrosine nitration and subsequent inhibition of channel proteins. This is the first study designed to systematically determine the mechanisms behind hyperglycemia-induced Kv channel dysfunction in vivo. Data derived from this study may suggest new strategies on how to prevent or improve vascular complications associated with DM.

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MATERIALS AND METHODS

Chemical induction and ebselen treatment of diabetic rats. Age-matched male Sprague-Dawley rats (250–300 g) were divided into four groups: DM, DM plus ebselen (DM+Eb), non-DM, and non-DM+Eb. Streptozotocin freshly dissolved in sterilized saline was injected (60 mg/kg ip) into DM and DM+Eb rats with the same volume of saline given to non-DM and non-DM+Eb rats. In Eb treatment groups, Eb was administered orally to non-DM+Eb or DM+Eb rats (5 mg/kg, twice a day) for a duration of 4 wk beginning on the second day after developing frank DM (serum fasting glucose >250 mg/dl before insulin treatment). Changes in blood glucose and body weight were monitored daily. Hemoglobin A1c (HbA1c) levels were measured weekly. DM rats were treated with 1–3 U/day of ultralente insulin adjusted to the degree of hyperglycemia to prevent ketoacidosis (39) and weight loss (23, 34). Animals were studied between 4–6 wk after developing frank DM. All rat protocols were approved by the Animal Care Committee at the Medical College of Wisconsin in accordance with the recommendations of the American Association for the Accreditation of Laboratory Animal Care to ensure animal welfare.

Preparation of rat small coronary arteries. DM, DM+Eb, non-DM, or non-DM+Eb rats were anesthetized with pentobarbital sodium (60 mg/kg ip), and hearts were removed. SCA (internal diameter 100–200 μm) were dissected from the left ventricle and prepared for immunohistochemical, immunoprecipitation, Western blot, videomicroscopic, and patch-clamp studies.

Immunohistochemical detection of nitrotyrosine. SCA from respective groups were embedded with optimal tissue compound (OTC) and kept at −80°C. Frozen sections (5 μm) were cut and fixed onto slides. Immunohistochemistry using an antibody to nitrotyrosine was performed as described previously (28).

Immunoprecipitation and Western blot analysis. Immunoprecipitation and Western blots were performed as described previously (28). Briefly, SCA were homogenized and centrifuged at 1,000 g followed by 14,000 g (10-min each step). Total protein (~100 μg/reaction) was immunoprecipitated with anti-nitrotyrosine antibodies (4 μl/sample) for 2 h at room temperature. The nitrotyrosine antigen-antibody complex was then bound to protein A (10 mg/sample) for 1 h. The immunoprecipitates were centrifuged and washed three times with ice-cold lysis buffer, and Western blots were performed using monoclonal antibodies specific for K1.2 or polyclonal antibodies specific for K1.5 (Upstate Biotechnology, Lake Placid, NY) as described previously (28). The housekeeping protein β-actin was also blotted and used as a control for protein loading. In some experiments, immunoprecipitates were obtained with K1.2 or 1.5 antibody and then Western blotting was performed using the nitrotyrosine antibody.

Videomicroscopy. SCA were cannulated on glass micropipettes in an organ chamber filled with physiological salt solution (PSS) containing (in mM) 118 NaCl, 4.7 KCl, 2.5 CaCl2, 1.2 KH2PO4, 1.2 MgSO4, 20 NaHCO3, 0.026 Na2EDTA, and 11 dextrose, pH 7.4 (33). The PSS was warmed to 37°C, continuously circulated, and bubbled with 21% O2, 5% CO2, and 74% N2. Vessels were equilibrated for 1 h at an intraluminal pressure of 60 mmHg. Most SCA developed spontaneous tone that reduced diameter to an average of 70% of the passive diameter. Those that did not were treated with U-46619 (10−8 M) to supplement constriction to 70% of the passive diameter. Correolide (Cor; 1 μM), a specific blocker of K1 channels (12, 35), was used to ascertain the overall effect that DM inflicted on the physiological function of these channels. Concentration-response curves to the direct cAMP activator forskolin (10−10 to 10−6 M) were compared with and without Cor. At the end of each experiment, vessels were maximally dilated with papaverine (10−4 M), and the percent dilation to agonists was normalized to this passive diameter (27).

Patch-clamp recording of K+ currents. Enzymatic isolation of single vascular smooth muscle cells (VSMC) was performed accord-
non-DM rats (Fig. 1A). Treatment with Eb markedly reduced immunostaining in SCA from DM rats (Fig. 1D). A slight reduction in ONOO$^-$ was also observed in non-DM+Eb SCA (Fig. 1C). Specificity of the antibody was confirmed by lack of brown staining when the primary antibody (anti-nitrotyrosine) was eliminated (data not shown). Isotype studies showing no positive staining ruled out nonspecific binding of goat IgG (data not shown). These data suggest that ONOO$^-$ is increased in SCA upon exposure to prolonged hyperglycemia and that this augmentation can be effectively reversed in vivo with oral Eb treatment.

**Figure 1.** Immunohistochemical detection of nitrotyrosine residues in small coronary arteries (SCA) from nondiabetic (non-DM; A) and diabetic rats (DM; B). Nitrotyrosine staining was greatly diminished in non-DM and DM SCA after anti-nitrosative treatment with ebselen (Eb; C and D). Antibody specificity was demonstrated by elimination of the primary antibody (not shown). Isotype IgG staining was also negative for nonspecific binding (not shown).

Reversal effects of ebselen on protein expression and nitration of K$_{\text{v}1}$ channels in diabetic coronary arteries. Based on the observation of a global increase in ONOO$^-$ generation in DM SCA, we next examined the levels of K$_{\text{v}1}$ channel expression and ONOO$^-$-induced nitration, focusing on K$_{\text{v}1.2}$ and K$_{\text{v}1.5}$. As shown in sample images (Fig. 2A) and the summarized data (Fig. 2B), overall protein expression of K$_{\text{v}1.2}$ was decreased in DM SCA (DM vs. non-DM: 0.4 ± 0.1 vs. 1 ± 0.2, *p < 0.05 vs. non-DM). Treatment with Eb normalized K$_{\text{v}1.2}$ protein expression levels in DM SCA (0.9 ± 0.3). Figure 2C reveals a notable increase in nitrotyrosine immunoprecipitates of K$_{\text{v}1.2}$ in DM SCA, which were reduced by treatment with Eb. Immunoband densities resulting from nitrotyrosine immunoprecipitates were normalized to their respective protein level and summarized in Fig. 2D. A significant augmentation of nitration was seen in SCA from DM rats (3.5 ± 0.1, *p < 0.05 vs. non-DM) compared with SCA from non-DM rats (1 ± 0.1). Eb treatment (1 ± 0.2) reversed the enhanced nitration seen in DM SCA.

In contrast to K$_{\text{v}1.2}$, K$_{\text{v}1.5}$ protein levels in DM SCA were not different from levels observed in non-DM vessels (Fig. 3, A and B). Similarly, protein nitration (Fig. 3, C and D) of K$_{\text{v}1.5}$ was not altered in DM SCA. Eb had little effect on protein expression or nitration levels in either group of rats. Similar findings were observed when antibodies used for immunoprecipitation and Western blotting were reversed. These data suggest that the susceptibility of K$_{\text{v}}$ channel subtypes to nitrosative stress is different even within the K$_{\text{v}1}$ channel family.

**Effect of ebselen on K$_{\text{v}1}$ channel function in coronary arteries from diabetic rats.** Based on the above initial findings, we hypothesized that the altered protein nitration and expression of K$_{\text{v}1}$ channels with and without Eb treatment would have functionally relevant effects on vasomotor responsiveness. To test this hypothesis, we compared responses to forskolin, which mediates the dilation of SCA via opening of K$_{\text{v}}$ channels (27), in the absence and presence of Cor. As shown in Fig. 4A, Cor significantly inhibited dilation to forskolin in non-DM SCA (maximal dilation at 10$^{-6}$ M: 76 ± 4 vs. 33 ± 10.220.32.247 on June 26, 2017 http://ajpheart.physiology.org/ Downloaded from 10.220.32.247 on June 26, 2017 http://ajpheart.physiology.org/ Downloaded from 10.220.32.247 on June 26, 2017 http://ajpheart.physiology.org/ Downloaded from 10.220.32.247 on June 26, 2017
5%, n = 6, P < 0.05), suggesting an important role for Kv1 channels. In contrast, the dilation to forskolin was markedly decreased in DM SCA (Fig. 4B; maximal dilation at 10^{-6} M: 39.5 ± 2%, n = 6). In DM SCA, Cor had little effect on forskolin-induced dilation (maximal dilation at 10^{-6} M in DM + Cor vs. DM: 39 ± 8 vs. 40 ± 4%, n = 6, P = nonsignificant), indicating that the reduced dilation in DM results from impairment of Kv1 channel function. Importantly, treatment with Eb partially restored Cor-sensitive dilation to forskolin in DM SCA (Fig. 4C) but had no effect on forskolin-induced dilation in non-DM SCA (data not shown). Together, these data indicate that DM is associated with a functionally significant impairment of Kv1 channels that may be partially restored in vivo through oral Eb administration.

**DISCUSSION**

The key findings of this study are threefold. First, in DM, nitration of Kv1.2 was enhanced and expression was reduced in SCA, whereas no changes were seen in Kv1.5. Second, the diabetes-induced nitrosative stress on Kv1.2 in the coronary vasculature was reversed by treatment with Eb. Third, Kv1 channel activity and vasodilatory function were impaired in SCA from diabetic rats, and both could be partially restored...
The enhanced nitration of Kv1.2 was not due to an increase in protein expression, as blot analysis showed an augmentation of nitration in Kv1.2. Although the baseline levels of nitration were similar between channel subtypes, Kv1.5 channel protein expression was not reduced in SCA. One possible explanation may be that the Kv1.5 channel is more protected from nitrosative stress, suggesting differential susceptibility to nitrosative stress within the Kv channel family, possibly influencing the relative role of these various channels in DM, where vasodilation is impaired by increased oxidative stress.

Reversal effect of ebselen on impaired Kv1 channel function. Our group (9) has previously reported that cAMP-mediated dilation in rat coronary arteries is reduced in DM, which is largely due to impairment of Kv channel function by enhanced oxidative stress. However, this study did not determine whether the reduced dilation or Kv channel activity could be reversed. Kv1 channels are highly expressed in SCA, since Cor inhibits a large Kv current in rat coronary smooth muscle cells (28). In the present study, the Cor-sensitive component of Kv1 current was significantly reduced in SCA from DM rats compared with SCA from non-DM rats. Treatment with Eb restored a large portion of the Cor-sensitive component, indicating that the impaired Kv1 channel function seen in DM is indeed reversible. The restoration of Kv1 channel function was further confirmed through patch-clamping studies, where Kv current elicited by forskolin was greater in smooth muscle cells from Eb-treated DM rats than the Kv current generated in cells from DM rats without Eb treatment (28). These findings are consistent with the results from other investigators. Studies by Brodsky et al. (7) suggest that a 4-wk treatment with Eb can restore reduced aortic dilator responses to acetylcholine and NO production in Zucker diabetic rats. Similarly, Eb prevents nephropathy, increases renal capillary density, and improves NO-mediated dilation in renal microvessels of Zucker diabetic rats (11, 26). We show an additional benefit on vascular function in that Eb not only effectively attenuates both accumulation of nitrotyrosine and Kv1 channel-specific nitration but also reverses impairment of Kv1 channel-mediated vasodilation. These data suggest a mechanistic link between the vascular nitrosative stress of DM and impaired Kv1.2 channel function. Although this study did not provide direct evidence that tyrosine nitration is the cause for the impaired dilator response to forskolin, it supports this hypothesis and provides compelling rationale for further mechanistic studies at the

with Eb. These findings provide the first evidence that elevated levels of ONOO\(^-\) in DM have a detrimental effect on Kv1 channel-related vascular function, which can be improved through the therapeutic effects of a scavenger of the reactive nitrogen species.

Alteration of Kv1 channel protein expression and nitration in diabetes. Our previous in vitro studies have demonstrated the ability of authentic ONOO\(^-\) to directly inhibit Kv1 channel function (28). Similar pathological changes can be achieved through exposure of SCA of rats to a high-glucose medium (23 mM) for a period of 24 h. Scavenging of ONOO\(^-\) with in vitro application of Eb or urate results in a clear improvement of Kv1 channel function. Since in vitro studies cannot adequately reproduce the complexity and chronicity of DM, it is important to extend these findings to a pathophysiologically relevant model of DM. Therefore, in this study, nitrosative stress induced by hyperglycemia was examined in a chemically induced diabetic rat model. Accumulation of the hallmark for nitrosative stress, nitrotyrosine, was observed on immunohistochemical staining in small coronary arteries from diabetic rats. Further examination linking nitrosative stress to Kv1 channel subtypes through immunoprecipitation and Western blot analysis showed an augmentation of nitration in Kv1.2. The enhanced nitration of Kv1.2 was not due to an increase in overall channel protein expression in DM, since the protein expression of Kv1.2 was actually diminished in DM. These results are consistent with our group’s in vitro study (28) and are also supported by other studies where enhanced ONOO\(^-\) has been reported in other diabetic models (10, 11). Eb is an effective scavenger of ONOO\(^-\) (3, 18). Four-week treatment with Eb resulted in marked prevention of nitrotyrosine accumulation in DM SCA. A similar observation was reported by Kruger et al. (26), where an increase in the level of heme oxygenase protein was paradoxically coupled to a decrease in its activity in the Zucker diabetic rat. These alterations were also reversed through the administration of Eb to scavenge ONOO\(^-\) (26). The present study provides evidence for the pathogenic role of ONOO\(^-\) as well as a potential therapeutic role of Eb in DM.

The impaired vasodilator response and reduced Kv current in DM may derive from both a reduction in protein expression and changes in channel gating properties. Although not shown, we observed in preliminary studies using site-directed mutagenesis that substituting for tyrosine residues near the pore-forming region of Kv1.2 prevented the inhibition of K\(^+\) currents by application of authentic ONOO\(^-\), suggesting the inhibitory effect of tyrosine nitration on channel gating ability. The mechanism for the reduced Kv1.2 channel protein expression in DM rats is not clear. It may be due to augmentation of protein degradation resulting from oxidation-induced proteolysis as suggested by Grune et al. (22). Whether the same type of oxidative modification of protein also applies to Kv1.2 channel in DM remains to be explored.

Interestingly, Kv1.5 appears to be more resistant to the enhanced nitrosative stress seen in DM compared with Kv1.2. Although the baseline levels of nitration were similar between channel subtypes, Kv1.5 channel protein expression was not reduced in SCA. One possible explanation may be that the Kv1.5 channel is more protected from nitrosative stress, suggesting differential susceptibility to nitrosative stress within the Kv channel family, possibly influencing the relative role of these various channels in DM, where vasodilation is impaired by increased oxidative stress.
single-channel level to specifically determine the role of tyrosine nitration on $K_v$ channel function.

**Study limitations.** Eb rapidly decomposes ONOO$^-$ to nitrate, a redox-inactive compound (19). However, Eb, an organoselenium compound, is not specific and also has been shown to act as a glutathione peroxidase mimic (6, 18, 36), which also confers an anti-inflammatory action. This could have a confounding influence when Eb is used as an anti-ONOO$^-$ agent. However, we believe that nonspecific effects of Eb, if any, cannot fully account for the present results for two reasons: 1) a notable reduction in nitrotyrosine residues, a protein modification that is specific for ONOO$^-$, was seen in SCA from Eb-treated DM rats (Fig. 1); and 2) the restorative effect of Eb on forskolin-induced dilation was similar to the effect of a chemically distinct ONOO$^-$ scavenger, urate, in restoring $K_v$ function in SCA exposed to high-glucose media in vitro (28). In addition, preliminary experiments applying $N^\omega$-nitro-$L$-arginine methyl ester acutely to diabetic SCA to eliminate NO formation and subsequent ONOO$^-$ generation revealed a tendency toward improvement in the dilation to forskolin.

We cannot exclude a contribution of other $K_v$ family channels, such as $K_v2$ and $K_v4$, to forskolin-induced dilation and whole cell $K_v$ current. However, our group’s previous study (28) indicated that Cor blocks the majority of $K_v$ current in coronary VSMC, suggesting that a contribution of other $K_v$ family members, if any, is small. Cor blocks all $K_v$ family channels, and selective $K_v1.2$ or $K_v1.5$ channel blockers are not available. For this reason, we were not able to identify specifically which subtype within the $K_v1$ family is responsible for the reduced $K_v$ channel function but are confident that the majority of the relevant current is generated by $K_v1$. Future studies are necessary to pinpoint the specific subtype(s) of $K_v1$ channels involved in dysfunction of the diabetic coronary vasculature.

**Summary.** In this study, we demonstrated that chronic treatment with Eb can greatly reduce nitrotyrosine accumulation and modification of $K_v1$ channel proteins in SCA from diabetic rats. Reduction of nitrosative stress by Eb resulted in an ameliorated Cor-sensitive dilator response and $K_v$ current to forskolin. These results provide strong evidence that enhanced nitrosative stress on $K_v1$ channels in DM may be one of the primary culprits resulting in vascular dysfunction of the coronary microcirculation. Peroxynitrite scavenging compounds may be beneficial for the therapeutic treatment of vascular complications in patients with DM.

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