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

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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 contributes to Kv1 channel dysfunction in the coronary microcirculation (8, 32).

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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 for 1 h. The supernatant was centrifuged at 100,000 g for 1 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 -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 lose tone were treated with U-46619 (10−8 M) to supplement constriction to 70% of the passive diameter. Corrodele (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 diluted 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 according to published methods (30). To eliminate interfering currents from Ca2+-sensitive (Kca) and ATP-sensitive K+ (KATP) channels, we added 100 nM ibetorin [a large-conductance Kca blocker (30)], 1 μM ampin [a small-conductance Kca blocker (26)], and 1 μM glibenclamide [a KATP blocker (5)] to the bath solution.

To minimize dialyzing of intracellular components, we performed the perforated whole cell recording by adding amphoterinin B (2.5 μg/ml) in the pipette solution. Recordings were obtained using standard pulse protocols and instrumentation as previously described (33). Briefly, families of K+ currents were generated by stepwise 10-mV depolarizing pulses (400-ms duration, 5-s intervals) from a holding potential of −60 mV in cells dialyzed with 100 nM ionized Ca2+.

Seal resistance was 2–10 GΩ. Peak current elicited at a single membrane potential was defined as the average of 500 sample points encompassing the maximal current point. Trials were performed in triplicate and averaged to estimate peak current amplitudes. K+ currents were normalized by cellular membrane area and expressed as K+ current density (pA/pF) (31). For each cell, 10-mV hyperpolarizing steps were averaged to account for capacitance and leak compensation values.

Chemicals. All chemicals were purchased from Sigma. All antibodies were obtained from Upstate. Cor was generously provided by Dr. Maria Garcia and Dr. Gregory Kaczorowski (Merck Research Laboratories, Rahway, NJ). Cor was prepared as a 20 mM stock in DMSO and stored at 4°C. Vehicle control experiments indicated no effect of DMSO on vascular reactivity.

Statistical analysis. All data are means ± SE. Percent dilation was calculated as the change from control internal diameter to maximal diameter measured in the presence of papaverine. Percent constriction was defined as the percent reduction from the diameter before administration of contractile agents. Data from Western blot, videomicroscopic, and patch-clamp measurements were compared using ANOVA two-way repeated measurements to detect differences between non-DM and DM rats with or without the treatment of ebselen. Dose-response curves before and after Cor treatment were compared using a one-way ANOVA. A Newman-Keuls test was performed to detect differences between individual doses. All differences were judged to be significant at P < 0.05.

RESULTS

Averaged (20 rats in each group) body weight, blood glucose, and HbA1c levels are summarized in Table 1. Blood glucose and HbA1c levels were increased four- and twofold, respectively, in DM compared with non-DM rats. Body weights were significantly reduced in DM rats. Treatment with Eb improved body weight and blood glucose level in the DM group, but these improvements did not reach non-DM (normal) levels.

Role of Eb in reducing ONOO− generation in diabetic coronary arteries. Immunohistochemical staining using nitrotyrosine antibodies, a marker of protein modification by ONOO−, revealed a marked increase in levels of ONOO− in SCA from DM rats (Fig. 1B) compared with SCA from DM non-DM rats. The presence of Eb significantly reduced ONOO− staining, particularly in SCA from DM+Eb rats, as compared with DM rats (Fig. 1B). These findings confirm that Eb can reduce ONOO− generation in diabetic coronary arteries.

Table 1. Body weight, blood glucose, and hemoglobin levels

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<tr>
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<th>Non-DM</th>
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<th>Non-DM + Eb</th>
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<tr>
<td>Body weight, g</td>
<td>350±8</td>
<td>326±9*</td>
<td>349±9</td>
<td>297±8†</td>
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<tr>
<td>Blood glucose, mg/dl</td>
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<td>502±2*</td>
<td>133±9</td>
<td>410±5†</td>
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<tr>
<td>HbA1c, %</td>
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<td>10±0.5*</td>
<td>4.7±0.1</td>
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Values are means ± SE (n = 20 in each group) of body weight, serum glucose level, and HbA1c level for non diabetic (non-DM) and diabetic (DM) rats with and without ebselen (Eb) treatment. *P < 0.05 vs. non-DM, †P < 0.05 vs. DM.
Reversal effects of ebselen on protein expression and nitration of Kv1 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 Kv1 channel expression and ONOO\(^-\)-induced nitration, focusing on Kv1.2 and Kv1.5. As shown in sample images (Fig. 2A) and the summarized data (Fig. 2B), overall protein expression of Kv1.2 was decreased in DM SCA (DM vs. non-DM: 0.4 ± 0.1 vs. 1 ± 0.2, \(n = 4\), \(P < 0.05\) vs. non-DM). Treatment with Eb normalized Kv1.2 protein expression levels in DM SCA (0.9 ± 0.3). Figure 2C reveals a notable increase in nitrotyrosine immunoprecipitates of Kv1.2 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, \(n = 4\), \(P < 0.05\) 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 Kv1.2, Kv1.5 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 Kv1.5 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 Kv channel subtypes to nitrosative stress is different even within the Kv1 channel family.

Effect of ebselen on K\(_{v1}\) channel function in coronary arteries from diabetic rats. Based on the above initial findings, we hypothesized that the altered protein nitration and expression of Kv1 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 Kv channels (27), in the absence and presence of Cor. As shown in Fig. 4A, Cor significantly inhibited dilation in non-DM SCA (maximal dilation at 10\(^{-6}\) M: 76 ± 4 vs. 33 ±

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**Fig. 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).

**Fig. 2.** A: representative immunoblots indicating expression of Kv1.2 proteins in SCA from non-DM, DM, non-DM+Eb, and DM+Eb rats. B: summary of immunoband densities normalized to α-actin. Kv1.2 channel expression was reduced in SCA from DM rats compared with SCA from non-DM rats. Treatment with Eb restored the expression of Kv1.2 channels in SCA from DM rats and had no effect on SCA from non-DM rats. C: sample image of immunoprecipitation (IP) and Western blots examining the nitration (NT) of Kv1.2. D: averaged Kv1.2 nitration level normalized to protein expression. Nitration of Kv1.2 was increased 3-fold in DM SCA relative to non-DM SCA. Eb treatment greatly attenuated nitration of Kv1.2 proteins in DM SCA but had little effect on non-DM SCA.
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.

Effect of ebselen treatment on Kv channel currents in diabetic coronary smooth muscle cells. Our group (9) has previously reported that forskolin profoundly enhances Kv currents 50% in coronary smooth muscle cells from nondiabetic rats. However, basal Kv currents in coronary smooth muscle from diabetic rats were decreased and did not respond to forskolin (peak current density, control vs. forskolin: 11.5 ± 0.9 vs. 12.3 ± 1.2 pA/pF) (9). To determine whether this impaired responsiveness to forskolin in DM is reversible, we tested whole cell Kv current in coronary smooth muscle cells from DM+Eb rats. As shown in sample traces (Fig. 5A) and summarized data of Kv current density as a function of membrane potential (Fig. 5B), treatment of DM rats with Eb not only improved basal Kv currents but also partially restored forskolin-stimulated Kv current (control vs. forskolin: 20 ± 3 vs. 28 ± 3 pA/pF) in coronary smooth muscle cells, similar to what was previously observed in non-DM animals (9). These results indicated that Eb directly improves Kv channel function in the rat coronary microcirculation. Based on the functional study in which the treatment with Eb had little effect on forskolin-induced dilation in SCA from non-DM+Eb rats, we did not anticipate a significant change in Kv current in this group of rats compared with non-DM rats without Eb treatment.

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...
with Eb. These findings provide the first evidence that elevated levels of ONOO\(^-\) in DM have a detrimental effect on K\(_v\)1 channel-related vascular function, which can be improved through the therapeutic effects of a scavenger of the reactive nitrogen species.

**Alteration of K\(_v\)1 channel protein expression and nitration in diabetes.** Our previous in vitro studies have demonstrated the ability of authentic ONOO\(^-\) to directly inhibit K\(_v\)1 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 K\(_v\)1 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 K\(_v\)1 channel subtypes through immunoprecipitation and Western blot analysis showed an augmentation of nitration in K\(_v\)1.2. The enhanced nitration of K\(_v\)1.2 was not due to an increase in overall channel protein expression in DM, since the protein expression of K\(_v\)1.2 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 K\(_v\) 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 K\(_v\)1.2 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 K\(_v\)1.2 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 K\(_v\)1.2 channel in DM remains to be explored.

Interestingly, K\(_v\)1.5 appears to be more resistant to the enhanced nitrosative stress seen in DM compared with K\(_v\)1.2. Although the baseline levels of nitration were similar between channel subtypes, K\(_v\)1.5 channel protein expression was not reduced in SCA. One possible explanation may be that the K\(_v\)1.5 channel is more protected from nitrosative stress, suggesting differential susceptibility to nitrosoative 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 K\(_v\)1 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 K\(_v\) channel function by enhanced oxidative stress. However, this study did not determine whether the reduced dilation or K\(_v\) channel activity could be reversed. K\(_v\)1 channels are highly expressed in SCA, since Cor inhibits a large K\(_v\) current in rat coronary smooth muscle cells (28). In the present study, the Cor-sensitive component of K\(_v\)1 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 K\(_v\)1 channel function seen in DM is indeed reversible. The restoration of K\(_v\)1 channel function was further confirmed through patch-clamping studies, where K\(_v\) current elicited by forskolin was greater in smooth muscle cells from Eb-treated DM rats than the K\(_v\) 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, 20). We show an additional benefit on vascular function in that Eb not only effectively attenuates both accumulation of nitrotyrosine and K\(_v\)1 channel-specific nitration but also reverses impairment of K\(_v\)1 channel-mediated vasodilation. These data suggest a mechanistic link between the vascular nitrosative stress of DM and impaired K\(_v\)1.2 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

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**Fig. 5.** A: sample traces of whole cell recording showing the effect of forskolin on whole cell K\(_v\) current density in vascular smooth muscle cells (VSMC) from an Eb-treated DM rat. B: summary data of K\(_v\) current density as a function of membrane potential. Forskolin increased K\(_v\) channel activity in VSMC from rats that had been treated with Eb. *P < 0.05 vs. control.
single-channel level to specifically determine the role of tyrosine nitration on Kv channel function.

Study limitations. Eb rapidly decomposes ONOO\textsuperscript{−} 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\textsuperscript{−} 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\textsuperscript{−}, 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\textsuperscript{−} scavenger, urate, in restoring Kv function in SCA exposed to high-glucose media in vitro (28). In addition, preliminary experiments applying N\textsuperscript{+)\textsuperscript{−}}-nitro-L-arginine methyl ester acutely to diabetic SCA to eliminate NO formation and subsequent ONOO\textsuperscript{−} generation revealed a tendency toward improvement in the dilation to forskolin.

We cannot exclude a contribution of other Kv family channels, such as Kv\textsubscript{2} and Kv\textsubscript{4}, to forskolin-induced dilation and whole cell Kv current. However, our group’s previous study (28) indicated that Cor blocks the majority of Kv current in coronary VSMC, suggesting that a contribution of other Kv family members, if any, is small. Cor blocks all Kv\textsubscript{1} family channels, and selective Kv\textsubscript{1.2} or Kv\textsubscript{1.5} channel blockers are not available. For this reason, we were not able to identify specifically which subtype within the Kv\textsubscript{1} family is responsible for the reduced Kv channel function but are confident that the majority of the relevant current is generated by Kv\textsubscript{1}. Future studies are necessary to pinpoint the specific subtype(s) of Kv\textsubscript{1} 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 Kv\textsubscript{1} channel proteins in SCA from diabetic rats. Reduction of nitrosative stress by Eb resulted in an ameliorated Cor-sensitive dilator response and Kv current to forskolin. These results provide strong evidence that enhanced nitrosative stress on Kv\textsubscript{1} channels in DM may be one of the two reasons:

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- Effect of Eb on forskolin-induced dilation was similar to the effect of a chemically distinct ONOO\textsuperscript{−} scavenger, urate, in restoring Kv function in SCA exposed to high-glucose media in vitro (28). In addition, preliminary experiments applying N\textsuperscript{+)\textsuperscript{−}}-nitro-L-arginine methyl ester acutely to diabetic SCA to eliminate NO formation and subsequent ONOO\textsuperscript{−} generation revealed a tendency toward improvement in the dilation to forskolin.

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