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Attenuated vascular responsiveness to K^+ channel openers in diabetes mellitus: the differential role of reactive oxygen species

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Abstract. The current study examined the responsiveness of blood vessels from diabetic rats to K^+ channel openers and explored whether ROS might be involved in any changes. Responses were measured in aortic rings isolated from four weeks streptozotocin (65 mg/kg)-induced diabetic rats. Relaxation to levcromakalim (ATP-sensitive potassium channel K_{ATP} opener, 10^{-9}–10^{-5} mol/l) and (+/–)-naringenin (large conductance calcium–activated channel BK_{Ca} opener, 10^{-8}–10^{-3} mol/l) were recorded in phenylephrine (1 μmol/l) pre-contracted segments in the absence and presence of superoxide dismutase (SOD, 100 μmol/l) and apocynin (an antioxidant and inhibitor of NADPH oxidase, 100 μmol/l). Contractions to phenylephrine (10^{-9}–10^{-5} mol/l) and relaxation to acetylcholine (ACh, 10^{-9}–10^{-5} mol/l) were also recorded. Relaxation curves for levcromakalim, naringenin and ACh for the diabetic group were shifted to the right ($p < 0.05$) compared with the control. Contractions to phenylephrine were enhanced in the diabetic group ($p < 0.01$). SOD restored the ACh response but not those of K^+ channel openers. On the other hand, apocynin restored the relaxation to naringenin but had no effect on both levcromakalim and ACh responses. The results suggest that both K_{ATP} and BK_{Ca} activities are attenuated in diabetes mellitus and that ROS appears to contribute only to the change in BK_{Ca} function.

Key words: Aorta — Apocynin — Diabetes mellitus — Levcromakalim — Naringenin — Superoxide dismutase

Abbreviations: Ach, acetylcholine; BK_{Ca}, large conductance calcium-dependent potassium channel; DM, diabetes mellitus; K_{ATP}, ATP-sensitive potassium channel; NADPH, nicotinamide adenine dinucleotide phosphate; NO, nitric oxide; PE, phenylephrine; ROS, reactive oxygen species; SOD, superoxide dismutase; STZ, streptozotocin; TBARS, thiobarbituric acid reactive substances.

Introduction

Vascular complications play a significant part in the mortality and morbidity of diabetes mellitus (DM) (Gu et al.1999; Young et al. 2009). The hyperglycaemia associated with DM facilitates the formation of reactive oxygen species (ROS) *via* protein kinase C (PKC)-dependent NADPH oxidase activation (Inoguchi et al. 2000), with the potential to disrupt the functions of cellular proteins including ion channels and enzymes. DM is associated with increased generation of ROS (Orie et al. 1999, 2000) which could adversely affect cellular functions including contractility (Pannirselvam et al. 2005). The major free radicals associated with DM are superoxide (Gutterman et al. 2005), hydrogen peroxide (Soto et al. 2002) and peroxynitrite (Bubolz et al. 2005) generated *via* NADPH-oxidase and nitric oxide synthase (NOS) pathways (Lassègue and Clempus 2003). There is evidence that both large conductance calcium-activated (BK_{Ca}) channel and ATP-sensitive potassium (K_{ATP}) channel functions are

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altered by ROS in diabetic and insulin resistant states (Soto et al. 2002; Erdos et al. 2004; Bubolz et al. 2005; McGahon et al. 2007). Increased production of ROS in the vasculature has also been linked to impairment of nitric oxide (NO)-mediated endothelium-dependent vasodilation (Olukman et al. 2010; Spitaler and Graier 2002) in both diabetic humans and animals (De Vriese et al. 2000; Pieper et al. 2002). Since antioxidants can prevent the generation of ROS in these circumstances (Hayashi et al. 2005; Liu et al. 2007) they provide a potential mechanism for ameliorating diabetic complications. It is, however, not clear whether changes in K^+ channel functions will benefit from such antioxidant treatment. The present study was undertaken to determine whether treatment with two chemically distinct antioxidants, superoxide dismutase (SOD) and apocynin, a free radical scavenger (Heumüller et al. 2008) would restore changes in K^+ channel mediated vascular reactivity in DM.

Materials and Methods

Animals

Sprague-Dawley male rats (8–10 weeks old) weighing between 170–220 g were obtained from the Biological Services Unit of the University College London, UK. The rats were placed randomly into control and diabetic groups of twelve rats each and were housed in plastic cages at room temperature of 19–21°C with 12/12-h light/dark cycle. They had access to tap water and food *ad libitum*. All studies were conducted in accordance with the Guide for the Care and Use of Laboratory Animals of University College London and conformed to the UK Animal Scientific Procedures Act of 1986.

Induction of DM

DM was induced in Sprague Dawley rats by a single intraperitoneal injection of streptozotocin (STZ, 65 mg/kg body weight) prepared in citrate buffer (pH 4.5). Age-matched control rats were injected with the citrate buffer vehicle alone. Basal blood glucose levels were measured just prior to STZ injection using an automated glucose analyzer (glucometer Acucheck mini plus, Roche, Germany). A drop of blood sample was collected by a prick on the tail vein. DM was confirmed 48 h after STZ injection by the presence of high blood glucose greater than 10 mmol/l and polyuria. The animals were used for experiments four weeks after induction.

Artery segment preparation

Rats were sacrificed by cervical dislocation and their descending thoracic aorta rapidly removed and placed in cold (4°C) physiological salt solution (PSS) of the following composition (mmol/l): NaCl 112; KCl 5; $CaCl_2$ 1.8; $MgCl_2$ 1, $NaHCO_3$ 25; KH_2PO_4 0.5; NaH_2PO_4 0.5; glucose 10; pH 7.4. Each aorta was cleaned of connective tissues under a dissecting microscope and cut into rings (3 mm long). Each segment was mounted in a 20 ml organ bath containing PSS that was maintained at 37°C and gassed with 95% O_2 and 5% CO_2. The segments were then connected to isometric force transducers (Grass FT03), which were coupled to a Powerlab data acquisition unit (ADInstruments Ltd, Australia) and isometric contraction was recorded in a computer using AD Chart Software version 4.2.4 (ADInstruments Ltd). With an initial tension of 1 g, each segment was allowed to equilibrate for 90 min while being rinsed every 15 min. During the equilibration period, the aortic rings were challenged twice with 10 μmol/l phenylephrine, and once with 1 μmol/l phenylephrine followed by 10 μmol/l acetylcholine (Ach) to test endothelial integrity.

Test procedure

After the equilibration period, cumulative concentration-response curves were generated for phenylephrine (10^{-9}–10^{-5} mol/l) in both normal and diabetic rat aorta. In other sets of experiments, the contraction of aortic rings was achieved by phenylephrine (1 μmol/l). When the contraction reached a plateau, Ach (10^{-9}–10^{-5} mol/l), levcromakalim (10^{-9}–10^{-5} mol/l) or (+/–)-naringenin (10^{-8}–10^{-3} mol/l) was added cumulatively to rings from diabetic and control rats. To determine the role of reactive oxygen species, SOD (100 μmol/l) and apocynin (100 μmol/l) were applied to another set of aortic rings from each rat 30 min prior to pre-contraction with phenylephrine (PE) (1 μmol/l). The cumulative concentration-response curves for ACh, levcromakalim and naringenin were then constructed and compared with those obtained in rings treated with vehicle. Separate aortic rings from each rat were used for separate experimental procedures.

Measurements of lipid peroxidation

TBARS, a marker for lipid peroxidation, was measured in plasma using TBARS commercial kits (Calbiochem Chemicals, Ann Abbor, USA) according to the manufacturer's instructions. The level of TBARS was normalized to micromoles of malondialdehyde.

Drugs

Phenylephrine hydrochloride, acetylcholine chloride, (+/–)-naringenin, levcromakalim, apocynin, and SOD were purchased from Sigma-Aldrich (Poole, UK). Levcromakalim, naringenin and apocynin were initially dissolved in dimethyl

sulphoxide (DMSO) before subsequent dilutions were made in water to ensure that tissues were not exposed to more than 0.1% of DMSO which had no effect on tissue response.

Statistics

Values are presented as means ± SEM. Data were analysed using Graphpad Prism Software (version 5.0). Groups were compared by one-way analysis of variance (ANOVA) followed by *post hoc* Bonferroni's test or Student's unpaired *t*-test as appropriate. Maximum relaxation (R_{max}) to ACh, levcromakalim or (+/–)-naringenin was measured as a percentage of the initial tone by phenylephrine. The sensitivities of segments to agonists (pEC_{50} or pIC_{50}) and R_{max} values were obtained from the individual response curves using nonlinear regression and comparison between groups was made with ANOVA. The significance level was $p < 0.05$.

Results

Body weights, blood glucose and TBARS levels

The body weight, blood glucose and TBARS levels of the rats are shown in Table 1. Four weeks after treatment with STZ or vehicle, the body weight in diabetic rats was significantly ($p < 0.05$) lower than in control rats. The blood glucose and TBARS levels of diabetic rats were significantly greater than control rats. TBARS, a marker for lipid peroxidation, was significantly elevated in plasma from diabetic rats compared to control rats (Table 1). The plasma levels of glucose in diabetic groups were significantly ($p < 0.01$) higher than in control group.

Contraction to PE

Contractions to PE were significantly ($p < 0.05$) enhanced in the diabetic group compared with control, with a leftward shift of the cumulative concentration-response curve (Fig. 1). Maximum contractions (E_{max}, diabetic *vs.* control) were 2.0 ± 0.15 g *vs.* 1.34 ± 0.04 g ($p < 0.05$) and pEC_{50} 8.07 ± 0.05 *vs.* 7.39 ± 0.10 ($p < 0.05$). There was a significant ($p < 0.05$) reduction in contraction in DM+apocynin group when compared with control and DM. The enhanced contraction of diabetic aorta was reduced by the addition of apocynin with E_{max} of 1.15 ± 0.05 while the pEC_{50} was 6.96 ± 0.04. The E_{max} and pEC_{50} were significantly ($p < 0.05$) lower when compared with diabetic group.

Table 1. Body weight, blood glucose and thiobarbituric acid reacting substance (TBARS) levels of male Sprague-Dawley rats treated with either streptozotocin (DM group) or vehicle (Control group)

Parameter	Group	
	Control	DM
Body weight (g)	430 ± 26.2	286 ± 19.6*
Blood glucose (mmol/l)	6.9 ± 0.38	33 ± 0.57†
TBARS (μmol/l MDA)	2.87 ± 0.54	9.93 ± 1.0†

Results are presented as mean ± SEM ($n = 6$). * $p < 0.05$, † $p < 0.01$ significantly different from values in normal rats (Student's unpaired *t*-test, $p < 0.05$). DM, Diabetes mellitus.

Relaxation to acetylcholine

Relaxation to ACh was recorded as a measure of endothelium-dependent relaxation. The results showed a significant rightward shift of the cumulative concentration-response curves for the diabetic group compared with control (Fig. 2A and B, $p < 0.05$). The values of pIC_{50} (diabetic *vs.* control) were 7.3 ± 0.07 *vs.* 7.73 ± 0.11 ($p < 0.05$) and the R_{max} for the 2 groups were significantly different (R_{max}, diabetic, 90.04 ± 1.23 *vs.* control, 95.14 ± 1.59%, $p < 0.05$). There were variable effects of SOD (100 μmol/l) and apocynin (100 μmol/l) on the diabetic curves. SOD reversed the shift and sensitivity to ACh (Fig. 2B) while apocynin did not reverse the shift of curve or sensitivity (Fig. 2A, $p > 0.05$). The R_{max} in diabetic group treated with apocynin was 92.6 ± 0.88% which was comparable with DM group (90.04 ± 1.23%). The R_{max} value in diabetic group treated with SOD was 94.8 ± 0.19% which was significantly ($p < 0.05$) higher than the value in DM group.

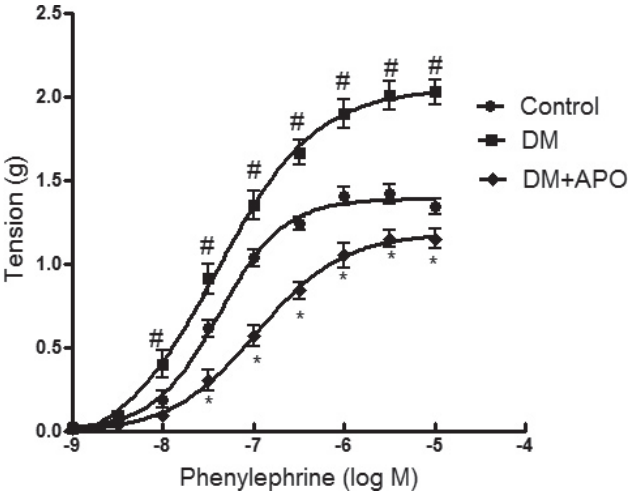

Figure 1. Effect of apocynin on phenylephrine-induced contraction in diabetic rat aorta.* $p < 0.05$ compared with control and DM groups, # $p < 0.05$ DM group compared with control, $n = 5$ in control and DM+apocynin groups and $n = 6$ in DM group. DM, diabetes mellitus; APO, apocynin.

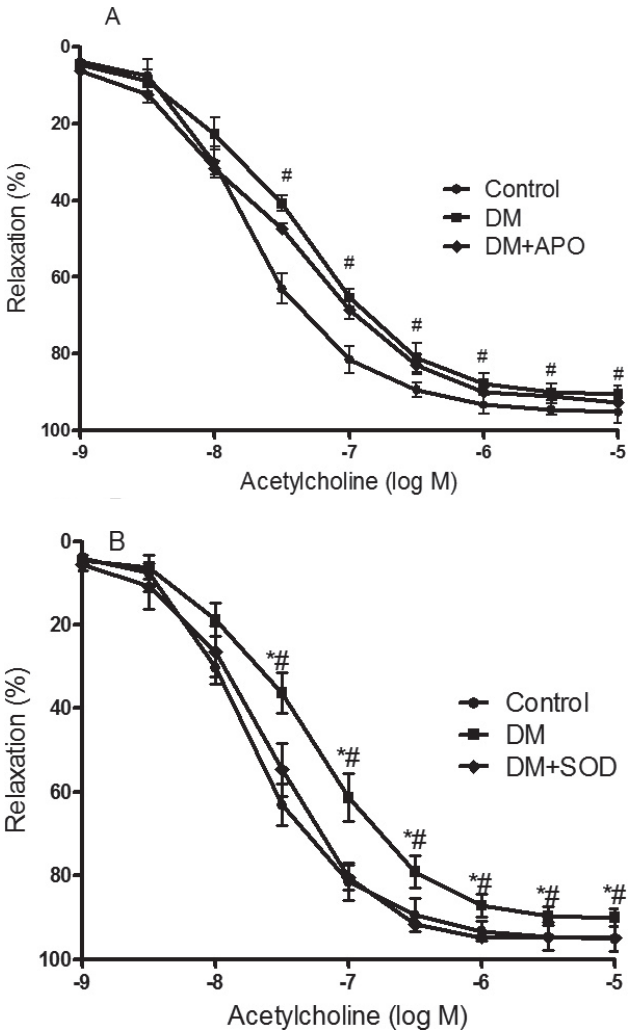

Figure 2. Cumulative concentration-response curves for acetylcholine in aortic rings from diabetic and control groups in the absence and presence of 100 μmol/l apocynin (**A**) and 100 μmol/l superoxide dismutase SOD (**B**). * $p < 0.05$ comparison between DM and DM+SOD groups, $^{\#}$ $p < 0.05$ comparison between DM and control group; $n = 5$ in control and DM+apocynin groups; $n = 6$ in DM group. DM, diabetes mellitus; APO, apocynin; SOD, superoxide dismutase.

Relaxation to K^+ channel openers

Levcromakalim

Relaxation to the K_{ATP} opener, levcromakalim was significantly ($p < 0.05$) impaired in the diabetic group compared with control (Fig. 3A and 3B). Maximum relaxation (R_{max}) was significantly ($p < 0.05$) lower in the diabetic group (92.6 ± 1.1%) compared with the control (97.6 ± 1.1%), and the sensitivities of the 2 groups to levcromakalim were significantly ($p < 0.05$) different from control (pIC_{50}: 7.05 ± 0.12 *vs.* 7.4 ± 0.12, diabetic *vs.* control). Both apocynin and SOD failed to reverse the changes in levcromakalim response. The R_{max} in diabetic treated with apocynin and SOD were 91.8 ± 1.6% and 90.7 ± 1.2%, respectively, which was not statistically different from DM group.

Naringenin

Naringenin was less potent than levcromakalim in relaxing segments from both diabetic and control groups. The response to this BK_{Ca} opener was attenuated following 4

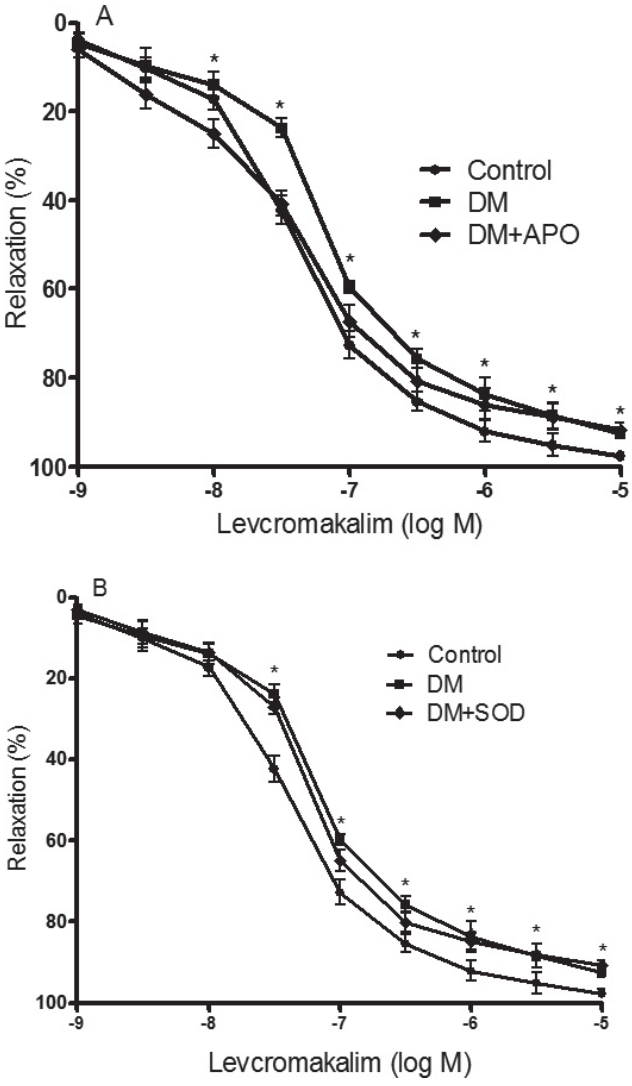

Figure 3. Cumulative concentration-response curves for levcromakalim in segments from diabetic and control groups in the absence and presence of 100 μmol/l apocynin (**A**) and 100 μmol/l SOD (**B**), * $p < 0.05$ comparison between control and DM groups; $n = 5$ in control and DM+apocynin groups; $n = 6$ in DM group. DM, diabetes mellitus; APO, apocynin; SOD, superoxide dismutase.

weeks of STZ diabetes compared with control. As shown in Fig. 4A, there was a significant ($p < 0.05$) decrease in relaxation induced by (+/–)-naringenin in DM when compared with control. The R_{max} of 77.8 ± 2.2% in DM group was significantly ($p < 0.05$) lower than that of control (88.9 ± 3.5%). The pIC_{50} in DM (3.7 ± 0.15) was significantly ($p < 0.05$) lower compared with control (4.6 ± 0.4). The relaxation of aorta in the DM group was significantly ($p < 0.05$) enhanced in the presence of apocynin with a pIC_{50} of 4.7 ± 0.1 that was comparable with control. However, as shown in Fig. 4B, the relaxation of the aortic rings from DM group in the presence of SOD (78.9 ± 2.7%) was not enhanced compared with control and DM.

Discussion

The present study examined the responsiveness of blood vessels to K^+ channel openers in diabetes and whether ROS might be involved in any changes. The results show a shift to the right of the response curves for levcromakalim, a K_{ATP} opener (Pérez-Vizcaino et al. 1999) and naringenin, a BK_{Ca} channel opener (Saponara et al. 2006) after four weeks of STZ diabetes. Apocynin reversed the shift in naringenin curve consistent with the involvement of excess ROS notably O_2^- generated *via* NADPH oxidase activity (Brandes and Kreuzer 2005). However, both apocynin and SOD failed to reverse the shift in levcromakalim response, suggesting that ROS played no major role in the reduced activity of K_{ATP} channel in this model. In line with previous reports (Utkan et al. 1998; Ajay and Mustafa 2006), enhanced contractile response to PE and attenuated relaxation to ACh were also recorded. The increase in PE response suggests an alteration in the adrenergic pathway at the early stages of DM in agreement with previous reports (Tang et al. 2011; Owu et al. 2013). The reduction in ACh response indicates endothelial dysfunction. However, it is important to note that there was a difference between groups in the magnitude of pre-contraction and this effect cannot be ruled out in the relaxing effect. The normal endothelium produces both vasodilators e.g. NO and vasoconstrictors e.g. endothelin-1 that are secreted in such a balance as to maintain normal vascular tone (Mather et al. 2004). Since ACh relaxation in the aorta is mediated by the release of endothelial NO (Furchgott and Zawadzki 1980), the current data is consistent with a reduction in endothelial NO availability in this model (Natali et al. 2005; Leo et al. 2011). The reversal of this decrease in ACh response by SOD is also consistent with previous reports (Orie et al. 1999, 2000; Pérez-Vizcaino et al. 1999; Pieper et al. 2002; Pannirselvam et al. 2005; Rafiq et al. 2011). SOD more specifically catalyses the conversion of superoxide radical into H_2O_2 and molecular oxygen (Senejoux et al. 2012) within cell membrane extracellular matrix (Shao et al. 2004; Shi et al. 2007). On the other hand, apocynin which is an inhibitor of NADPH oxidase, that is responsible for the production of superoxide in the vasculature, did not reverse the reduction in ACh response. The differential effects of SOD and apocynin may be related to differences in their mechanisms of antioxidant actions described above. Interestingly, apocynin reversed the reduction in the response to the BK_{Ca} channel opener, naringenin. The reason for this differential effectiveness of apocynin is not clear, but could be related to the fact that both naringenin (Zygmunt et al. 2010) and apocynin (Fraga et al. 2010) are flavonoids and could have acted in synergy. Synergistic interactions are important in phytomedicines and

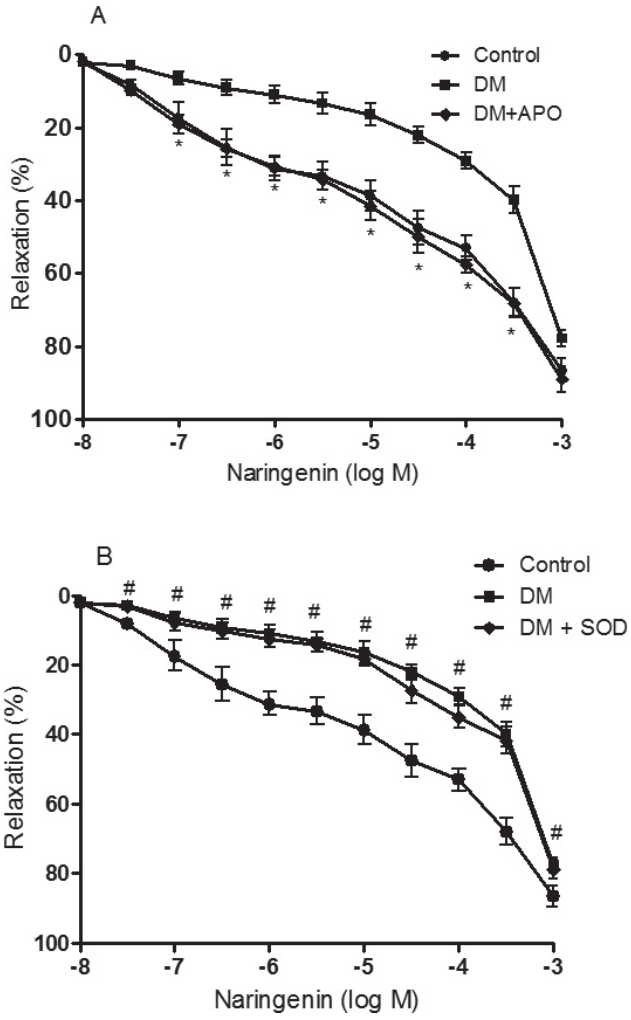

Figure 4. The cumulative concentration-response curves for naringenin in aortic rings from diabetic and control groups in the absence and presence of 100 µmol/l apocynin (**A**) and 100 µmol/l SOD (**B**). * $p < 0.05$ comparison between DM *vs.* DM+APO and control groups. $^\#$ $p < 0.05$ comparison between DM and DM+SOD groups *vs.* control; $n = 5$ in control and DM+apocynin groups; $n = 6$ in DM group. DM, diabetes mellitus; APO, apocynin; SOD, superoxide dismutase.

such interactions by phytochemicals increase the efficacy of active ingredients in plants (Williamson 2001). For instance, two plant extracts were shown to elicit a synergistic vasorelaxant and antihypertensive effect on isolated rat aorta (Kim and Rhyu 2010). This could have been the case between apocynin and naringenin in this study. In addition, apocynin's effectiveness could depend on its reported interaction with other vascular mechanisms. For instance, apocynin is known to activate the voltage-gated K (K_V) channel expressed in both the endothelium and vascular smooth muscle cells (Han et al. 2010). Other reports suggest that apocynin could directly inhibit ROS-induced signalling in vascular cells (Chan et al. 2007). These potential additional interactions could determine the net vascular impact of apocynin.

DM is characterised by concurrent depletion of antioxidant enzymes such as SOD, catalase and gluthathione (Ceriello 2006; Jay et al. 2006) and formation of peroxynitrite (Tang et al. 2004). This ultimately leads to impaired function of multiple proteins including vascular potassium ion channels that are important for vasodilation (Brzezinska et al. 2000; Liu et al. 2002). In this study, lipid peroxidation end product, TBARS were elevated in plasma. Elevated levels of lipid peroxides in plasma are consequences of increased production and liberation into the circulation of tissue lipid peroxides due to pathological changes such as associated with DM (Turk et al. 2002).

There is evidence that BK_{Ca} and K_{ATP} channel functions are inhibited by ROS in diabetic and insulin resistant states (Soto et al. 2002; Erdos et al. 2004; Bubolz et al. 2005; McGahon et al. 2007). The BK_{Ca} channel inhibition could involve decreases in both number of active channels and the open state probability of the channel (Soto et al. 2002) probably *via* the down regulation of the channel beta 1 subunit (McGahon et al. 2007). In Type-2 DM, expression of BK_{Ca} channel β1, but not α-subunits is markedly reduced at both of mRNA and protein levels in cerebral arteries resulting in reduced activity of BK_{Ca} channel, increased vascular tone and blood pressure (Wang et al. 2010). The picture is less clear for vascular K_{ATP} channel, with both inhibition (Erdos et al. 2004) and activation (Gutterman et al. 2005) by ROS reported. What the current data suggest is that vascular K_{ATP} channel is less susceptible to ROS-induced changes than the BK_{Ca}, at least in the early stages of DM.

In summary, both K_{ATP} and BK_{Ca} channel activities are attenuated in STZ DM after four weeks, and ROS appear to contribute to the change in BK_{Ca} activation and not to the change in K_{ATP} activation.

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