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Time-dependent increases in ouabain-sensitive Na^+, K^+-ATPase activity in aortas from diabetic rats: The role of prostanoids and protein kinase C

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ABSTRACT

Aims: Na^+, K^+-ATPase activity contributes to the regulation of vascular contractility and it has been suggested that vascular Na^+, K^+-ATPase activity may be altered during the progression of diabetes; however the mechanisms involved in the altered Na^+, K^+-ATPase activity changes remain unclear. Thus, the aim of the present study was to evaluate ouabain-sensitive Na^+, K^+-ATPase activity and the mechanism(s) responsible for any alterations on this activity in aortas from 1- and 4-week streptozotocin-pretreated (50 mg kg^{-1}, i.v.) rats.
Main methods: Aortic rings were used to evaluate the relaxation induced by KCl (1–10 mM) in the presence and absence of ouabain (0.1 mmol/L) as an index of ouabain-sensitive Na^+, K^+-ATPase activity. Protein expression of COX-2 and p-PKC-βII in aortas were also investigated.
Key findings: Ouabain-sensitive Na^+, K^+-ATPase activity was unaltered following 1-week of streptozotocin administration, but was increased in the 4-week diabetic aorta (27%). Endothelium removal or nitric oxide synthase inhibition with L-NAME decreased ouabain-sensitive Na^+, K^+-ATPase activity only in control aortas. In denuded aortic rings, indomethacin, NS-398, ridogrel or Gö-6976 normalized ouabain-sensitive Na^+, K^+-ATPase activity in 4-week diabetic rats. In addition, COX-2 (51%) and p-PKC-βII (59%) protein expression were increased in 4-week diabetic aortas compared to controls.
Significance: In conclusion, diabetes led to a time-dependent increase in ouabain-sensitive Na^+, K^+-ATPase activity. The main mechanism involved in this activation is the release of TxA_2/PGH_2 by COX-2 in smooth muscle cells, linked to activation of the PKC pathway.

Introduction

The sodium- and potassium-activated adenosine-triphosphatase, Na^+, K^+-ATPase, plays an important role in maintaining the membrane potential of the cell and through this role can contribute to the regulation of vascular tone and contractility (Skou and Esmann 1992; Blaustein 1993; Marin and Redondo 1999). Abnormalities in the distribution or function of Na^+, K^+-ATPase are thought to be involved in several pathological states, including hypertension and diabetes mellitus.

Human and experimental models of diabetes are characterized by a hypoinsulinemic and/or insulin resistant state, associated with abnormal smooth muscle and endothelial function (Durante et al. 1988; Tesfamariam et al. 1989, 1991; Abebe and MacLeod 1991; McNally et al. 1994; Pieper 1999; Xavier et al. 2003). It is well-established that Na^+, K^+-ATPase activity can be modulated by insulin, protein kinase C (PKC) as well as by endothelial factors such as nitric oxide (NO) and prostaglandins (Lowndes et al. 1990; Gupta et al. 1991; Skou and Esmann 1992; Pedemonte et al. 1997; Marin and Redondo 1999; Duran et al. 2004). It is therefore possible that the insulin resistance and endothelial dysfunction characteristic of diabetes could interfere with the activity of Na^+, K^+-ATPase (Simmons and Winegrad 1993; Sweeney and Klip 1998), although at present this theory remains controversial.

Ohara et al. (1991) observed a decrease in Na^+, K^+-ATPase activity in aortic vascular cells from animals following 2, 7 and 14 days of induced diabetes. In line with this, Michea et al. (2001) also observed a reduced activity of the Na^+, K^+-ATPase in aortic rings from rats following 14 days of induced diabetes. In contrast, Orie et al. (1993) found an enhanced potassium-induced relaxation in the aorta of 4-week diabetic rat, indicating an increase in Na^+, K^+-ATPase activity. Smith et al. (1997) further suggested that at more advanced stages of diabetes in rats (12 weeks), altered endothelial function could impair the physiological activity of the vascular Na^+, K^+-ATPase. These data suggests a time-dependent alteration in vascular Na^+, K^+-ATPase activity when diabetes is present; however the mechanisms involved in this remain unclear.

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In addition, it is well established that diabetes mellitus is linked to the development of vascular dysfunction in a time-dependent manner (Kakkar et al. 1996; Pieper 1999; Xavier et al. 2003). Our group has demonstrated that while there were no changes in the vasoconstrictor response to phenylephrine after 1-week of streptozotocin-induced diabetes, 4-weeks of induced diabetes enhanced the contraction of rat aorta to phenylephrine (Xavier et al. 2003). This was associated with an increased role of local vasoconstrictor prostanoids and enhanced extracellular calcium mobilization (Xavier et al. 2003). Moreover, increased cyclooxygenase-derived products such as PGH_2 and TxA_2 are found in experimental diabetes, which contribute to increased vascular contractility (Peredo et al. 1999; Xavier et al. 2003; Shi and Vanhoutte 2008) and impairment of endothelium-dependent relaxation (Tesfamariam et al. 1989; Akamine et al. 2006). Furthermore, alteration of PKC by hyperglycaemia is another pathway associated with micro- and macro-vascular complications observed in diabetic experimental models and patients (Lee et al. 1989; Nagareddy et al. 2009; Geraldes and King 2010). Among the existing PKC isoforms, the βII PKC is preferentially increased in aorta from diabetic animals (Inoguchi et al. 1992; Guo et al. 2003) and recent published results suggested that inhibition of PKC-βII may be a useful approach for correcting abnormal cardiovascular alterations present in diabetes (Nagareddy et al. 2009; Geraldes and King 2010).

Accordingly, it is possible to hypothesize that a combination of endothelial dysfunction, changes in the synthesis of vasoactive factors, alterations in calcium mobilization and increased PKC activity could be able to modulate Na^+, K^+-ATPase activity in the aorta of streptozotocin-diabetic animals. The aim of the present study was therefore to investigate ouabain-sensitive Na^+, K^+-ATPase activity in the aorta from diabetic rats at an early and an intermediate stage of the disease (1 and 4 weeks, respectively). In addition, we aimed to evaluate the mechanism(s) responsible for any alterations, focusing on the role of the endothelium, nitric oxide, cyclooxygenase-derived prostanoids and PKC.

Material and methods

Animals

Three month old male Wistar rats (270–370 g) were obtained from colonies maintained at the Animal Quarters of the Institute of Biomedical Sciences at the University of Sao Paulo. The animals were housed four to six per cage at a constant room temperature and light cycle (12:12 h light–dark). Food and water were allowed ad libitum to all animals. The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and with the guidelines of the Committee on Care and Use of Laboratory Animal Resources at the Institute of Biomedical Sciences at the University of Sao Paulo.

Diabetes was induced by a single venous injection of streptozotocin (50 mg kg^{-1}; diluted in 0.1 M citrate buffer solution, pH 4.5) in anaesthetized rats (ketamine, xilazine and acepromazine mixture, 64.9, 3.2 and 0.78 mg kg^{-1}, respectively, i.p.), as previously demonstrated (Davel et al. 2000; Xavier et al. 2003). In the control group, only citrate buffer solution (vehicle) was injected. Two main groups were used in this study: 1- and 4-week streptozotocin-treated rats and age-matched controls.

Blood glucose was measured using a hemoglucotest (OneTouch Ultra, Lifescan Inc., Johnson & Johnson, USA) after 1- or 4-weeks of streptozotocin treatment. Animals receiving the streptozotocin injection but with normal levels of glycaemia were removed from the study. In addition, rats were weighed both before and after 1- or 4-weeks of streptozotocin treatment. The streptozotocin injection increased glycaemia levels (1-week control: 159 ± 17 ($N = 7$) *vs.* 1-week diabetic rat: 496 ± 42 mg/dL ($N = 7$); *t*-test, $P < 0.05$ and 4-week control: 158 ± 13 ($N = 13$) *vs.* 4-week diabetic rat: 544 ± 13 mg/dL ($N = 11$); *t*-test, $P < 0.05$) and reduced the body weight in all diabetic animals used in this study, as previously demonstrated by our group (Davel et al. 2000; Xavier et al. 2003).

Tissue bath studies

At the end of each streptozotocin treatment period, animals were terminally anaesthetized with a ketamine, xilazine and acepromazine mixture, as described above. The thoracic aorta was dissected, freed of surrounding connective tissue and cut into rings 4 mm in length as previously described (Rossoni et al. 2002). In order to analyse the influence of the endothelium on vascular responses, the endothelial layer was mechanically removed in certain experiments by rubbing the lumen with a needle. The rings were mounted in an isolated organ bath system containing Krebs–Henseleit bicarbonate buffer (KHB). The buffer consisted of (in mmol/L): NaCl 118; KCl 4.7; $NaHCO_3$ 25; $CaCl_2$–$2H_2O$ 2.5; KH_2PO_4 1.2; $MgSO_4$–$7H_2O$ 1.2; glucose 11 and EDTA 0.01. Thoracic aorta segments were subjected to tension of 1.0 g, during a 45 minute equilibration period. Isometric tension was recorded using an isometric force transducer (Letica TRI 210, Barcelona, Spain) connected to an acquisition system (MP100 Biopac Systems, CA, USA).

Experimental protocols

After 45 min of stabilization, the contractile viability was determined following 30 min of exposure to KCl (75 mmol/L). Vasoconstrictor responses to this depolarizing solution were similar in all groups studied, as previously published by our group (Xavier et al. 2003). Afterwards, the integrity of the endothelium was established by observing the response to acetylcholine (10 μmol/L) in segments pre-contracted with phenylephrine (~10 μmol/L) at a concentration producing 50–70% of the contraction induced by KCl (75 mmol/L). The endothelium was considered intact if the aortic ring relaxed more than 80% to acetylcholine, while endothelial denudation was confirmed by less than 10% relaxation.

To evaluate the influence of diabetes on the functional activity of Na^+, K^+-ATPase, we measured activity using the method described by Webb and Bohr (1978). Thus, after a 30 minute equilibration period, aortic rings were incubated in a K^+-free medium for 30 min and then contracted with phenylephrine (~10 μmol/L) to obtain approximately 50–70% of the contraction induced by KCl. Once a plateau had been reached, KCl (1–10 mmol/L) was cumulatively added at intervals of 2.5 min. After a washout period, rings were exposed to 0.1 mmol/L of ouabain in K^+-free medium for 30 min and a second relaxation–response curve to KCl was then performed. The concentration–response curve to KCl did not change over time for both control and diabetic rats (results not shown). In addition, it is important to note that the phenylephrine-induced contraction was similar in all groups studied, since the concentrations used to obtain the plateau of pre-contraction elicited by phenylephrine were adjusted in all experimental groups.

The functional activity of ouabain-sensitive Na^+, K^+-ATPase was expressed as "differences" of area under the concentration–response curves (dAUC) to KCl in the absence and presence of 0.1 mmol/L ouabain. AUC were calculated from the graphs of individual concentration–response curves (GraphPad Prism Software, San Diego, CA) and the differences were expressed as a percentage of the difference in the AUC of the corresponding control experiment.

The possible roles of the endothelium, nitric oxide (NO), cyclooxygenase-(COX) derived prostanoids and PKC activity in ouabain-sensitive Na^+, K^+-ATPase activity were evaluated in aortic rings from 4-week diabetic rats and their respective controls. Endothelium intact rings were preincubated with either N^G-nitro-L-arginine methylester (L-NAME; nitric oxide synthase (NOS) inhibitor, 100 μmol/L) or indomethacin (INDO; a cyclooxygenase inhibitor, 10 μmol/L). In addition, rings without endothelium were preincubated with indomethacin (10 μmol/L), N-[2-(cyclohexyloxy-4-nitrophenyl]-methansulfonamide (NS-398; selective COX-2 inhibitor, 10 μmol/L), ridogrel (RIDO; TxA_2 synthase inhibitor and TxA_2/PGH_2 receptor antagonist, 1 μmol/L) or Gö-6976 (GO; inhibitor of constitutive isoforms of PKC, 50 μmol/L). Drugs were added 30 min before the KCl-induced relaxation curve and were then present throughout the experiment. It is important to emphasize that all drugs used in the present study did not modify the basal tonus of the arteries or the phenylephrine-induced contraction.

In order to confirm our hypothesis that TxA_2/PGH_2 pathway is involved on the increase of ouabain-sensitive Na^+, K^+-ATPase activity in aortic rings from 4-week diabetic rats, we assessed the Na^+, K^+-ATPase activity in endothelium-denuded aorta of control rats preincubated with the thromboxane mimetic 9,11-dideoxy-11α,9α-epoxy methanoprostaglandin (U46619, 10 nmol/L). The U46619 was added 30 min before the KCl-induced relaxation curve and was then present throughout the experiment.

Western-blot analysis

Aortas were homogenized in a buffer containing sodium metavanadate (1 mmol/L), SDS 10% and Tris–HCl (10 mmol/L, pH 7.4), at 99 °C, and protein concentration measured by the Lowry method (Lowry et al. 1951). The total protein extracts of aorta (50 μg of protein) were electrophoretically separated on a 7.5% SDS-PAGE and then transferred to polyvinyl difluoride membranes overnight at 4 °C by using a Mini Trans-Blot Cell system (Bio-Rad) containing 25 mmol/L Tris, 190 mmol/L glycine, 20% methanol, and 0.05% SDS.

After blockade of non specific sites with 5% nonfat dry milk, the membranes were incubated overnight at 4 °C with the primary antibody anti-COX-2 (0.5 μg/mL, Upstate Biotechnology, Temeluca, U.S.A.), anti-p-PKCα (1:400, Santa Cruz Biotechnology, California, U.S.A.) or anti-p-PKC βII (1:750, Santa Cruz Biotechnology, California, U.S.A.). After washing (10 mM Tris, 100 mM NaCl, and 0.1% Tween 20), membranes were incubated with peroxidase-conjugated IgG antibody anti-rabbit (1:3000, Bio-Rad, U.S.A.) for p-PKC βII, or anti-goat (1:4000, Santa Cruz Biotechnology, California, U.S.A.) for COX-2 and p-PKCα. Membranes were then thoroughly washed and the immunocomplexes detected using an enhanced horseradish peroxidase/luminal chemiluminiscence system (ECL Plus, Amersham International plc, Little Chalfont, U.K.) and subjected to autoradiography (Hyper®lm ECL, Amersham International plc). Signals on the immunoblot were quantified with a Scion Image computer program. Homogenates from rat lungs exposed to LPS were used as positive control for COX-2.

In the same membrane, α-actin protein expression was detected using a monoclonal antibody anti α-actin (1:3000 dilution; Sigma, Steinheim, Germany), and used as an internal control for the experiments.

Drugs

All drugs were obtained from Sigma (St. Louis, USA), excluding NS-398 that was obtained from Cayman Chemical (Ann Arbor, USA). Stock solutions (10 mmol/L) were prepared in distilled water, except for indomethacin, which was dissolved in 0.1 M TRIS (hydroximethyl aminomethane) buffer, NS-398 which was dissolved in DMSO, ridogrel, which was dissolved in saline containing Na_2CO_3 (2%) plus NaOH (40 mmol/L) and U46619, which was dissolved in ethanol. Stock solutions were kept at −20 °C and appropriate dilutions made on the day of the experiment.

Statistical analysis

Data are presented as the mean results ± SEM. The data were analysed using an unpaired *t*-test or 1- or 2-way ANOVA. When ANOVA indicated a significant effect of the treatment, Tukey's *post-hoc* test was used to compare means. $P<0.05$ was considered significant.

Results

The effect of diabetes on ouabain-sensitive Na^+, K^+-ATPase activity

Potassium-induced relaxation was observed in aortic rings from 1- (data not shown) and 4-week diabetic rats (Fig. 1B) and in arteries from age-matched control animals (Fig. 1A). In all groups, this response was attenuated by ouabain (0.1 mmol/L) (Fig. 1A and B). There were no differences in ouabain-sensitive Na^+, K^+-ATPase activity between 1-week diabetic rats and age-matched control (dAUC%, 1-week Control: 59.5 ± 4.50 ($N=7$) *vs.* 1-week Diabetic rat: 59.5 ± 5.20 ($N=7$); *t*-test, $P>0.05$). However, aortas from 4-week diabetic rats showed a significant increase (27%) in ouabain-sensitive Na^+, K^+-ATPase activity compared with their respective controls (Fig. 1C). As the ouabain-sensitive Na^+, K^+-ATPase activity was not statistically different in aortic rings from 1-week diabetic rat in comparison to controls, the following experiments were performed only with vessels from 4-week diabetic rats.

Removal of the endothelium and inhibition of NO synthesis (L-NAME, 100 μmol/L) similarly decreased the ouabain-sensitive Na^+, K^+-ATPase activity in aorta from control rats, but had no effect in 4-week diabetic rats (Fig. 2A and B).

In control endothelium-intact and -denuded aortas, pre-incubation with indomethacin (10 μmol/L) did not modify ouabain-sensitive Na^+, K^+-ATPase activity (Fig. 2C and D). In contrast, the enhanced ouabain-sensitive Na^+, K^+-ATPase activity found in the 4-week diabetic aorta was restored to control levels by indomethacin (Fig. 2C and D). It is important to highlight that this effect was independent of the endothelium (Compare Fig. 2C and D). Results obtained in the presence of NS-398 (a selective inhibitor of COX-2, 10 μmol/L) or ridogrel (a TxA_2 synthase inhibitor and TxA_2/PGH_2 receptor antagonist, 1 μmol/L) were comparable to those obtained in the presence of indomethacin (Figs. 3A and 4A, respectively).

In endothelium-denuded aortic rings from 4-week diabetic rats, incubation with Gö-6976 (an inhibitor of the constitutive isoforms of PKC, 50 μmol/L) restored the enhanced ouabain-sensitive Na^+, K^+-ATPase activity to control levels, whilst in control endothelium-denuded rings no significant effect was observed in presence of this inhibitor (Fig. 5A). Indomethacin, NS-398, ridogrel and Gö-6976 all exhibited a similar magnitude of effect on ouabain-sensitive Na^+, K^+-ATPase activity in arteries from 4-week diabetic rats (compare Figs. 2D, 3A, 4A and 5A).

The acute incubation of endothelium-denuded aortic rings from control rats with TxA_2/PGH_2 receptor (TP receptor) agonist U46619 (10 nmol/L) induced a significant increase (57%) in ouabain-sensitive Na^+, K^+-ATPase activity (Fig. 4B). Moreover, it is important to emphasize that phenylephrine-induced contractions were similar in endothelium-denuded aortic rings from control rats in the absence or presence of U46619 (10 nmol/L) in both conditions before and after 30 min ouabain (0.1 mmol/L) incubation (E−, before ouabain: 1.91 ± 0.05 *vs.* after ouabain: 2.01 ± 0.06 g ($N=5$); U46619, before ouabain: 2.21 ± 0.14 *vs.* after ouabain: 2.18 ± 0.15 g ($N=7$); 1-way ANOVA; $P>0.05$).

Western blot analysis

COX-2 protein levels were significantly increased in aortas from 4-week diabetic rats compared to controls (Fig. 3B). In addition, the phosphorylation of PKC-βII also increased in 4-week diabetic rats (Fig. 5B). In the present study, the phosphorylation of PKC-α was not detected in aortas from either control or diabetic rats (data not shown).

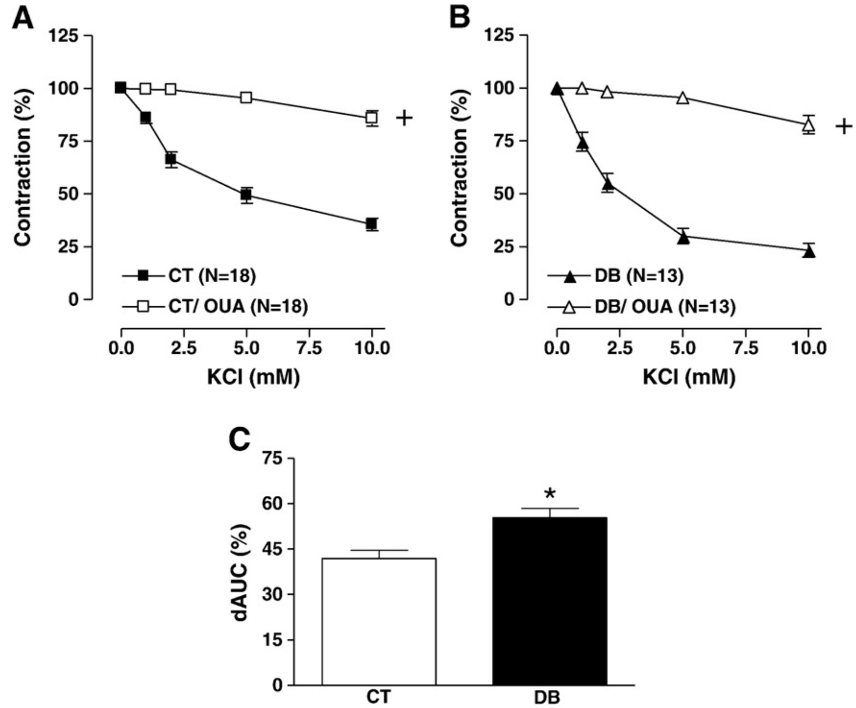

Fig. 1. Relaxation curves to potassium in aortic rings with intact endothelium of A) control (CT) and B) 4-week streptozotocin-induced diabetic rats (DB), before and after 30 min of ouabain incubation (OUA, 0.1 mmol/L). Two-way ANOVA, $^{+}P<0.05$ *vs.* before OUA. The bar graphic (C) represents the functional ouabain-sensitive Na^{+}, K^{+}-ATPase activity expressed by the difference between the area under the concentration–response curve (dAUC%) to KCl in aortas of CT and DB, before and after ouabain. *t*-test, $^{*}P<0.05$ *vs.* CT.

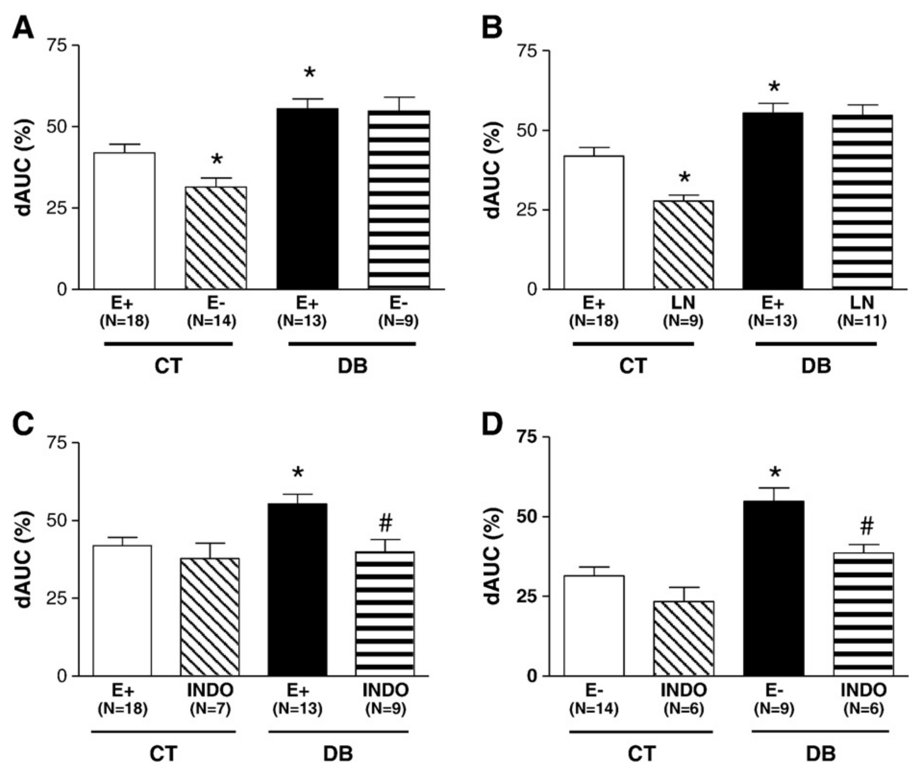

Fig. 2. Functional ouabain-sensitive Na^{+}, K^{+}-ATPase activity of aortic rings from control (CT) and 4-week streptozotocin-induced diabetic rats (DB). Bar columns represent the difference between the area under the concentration–response curve (dAUC%) to KCl in aortas of CT and DB, before and after ouabain (0.1 mmol/L) incubation. The effects of: A) the presence (E+) or absence (E−) of endothelium, B) NOS inhibition with L-NAME (LN, 100 µmol/L), C) cyclooxygenase inhibition with indomethacin (INDO, 10 µmol/L) in aortic rings E+ or D) E− of CT and DB rats were evaluated. 1-way ANOVA, $^{*}P<0.05$ *vs.* CT E+ or E−; $^{\#}P<0.05$ *vs.* DB E+ or E−.

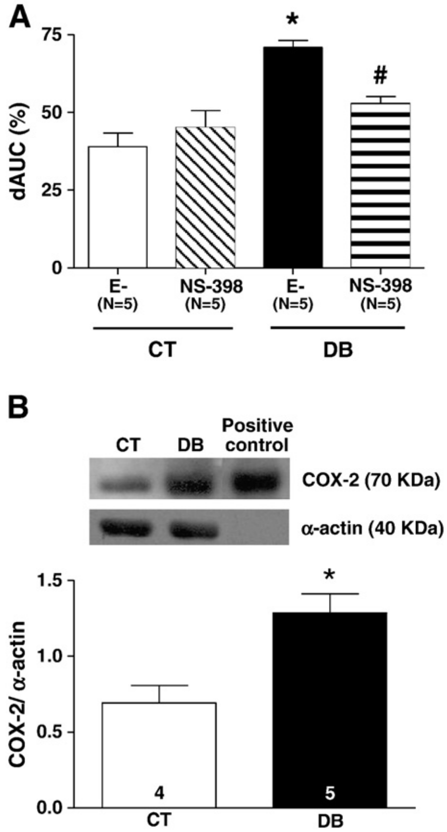

Fig. 3. A) The role of cyclooxygenase-2 inhibition with NS-398 on the functional ouabain-sensitive Na^+, K^+-ATPase activity of endothelium-denuded (E−) aortic rings from control (CT) and 4-week streptozotocin-induced diabetic rats (DB). Bar columns represent the difference between the area under the concentration–response curve (dAUC%) to KCl in aortas of CT and DB incubated with or without NS-398 (10 μmol/L), before and after ouabain (0.1 mmol/L) incubation. 1-way ANOVA, $^*P<0.05$ *vs.* CT E−; $^\#P<0.05$ *vs.* DB E−. B) Representative Western blot autoradiographies (top) and densitometric analysis (bottom) of cyclooxygenase-2 (COX-2) protein expression in aortas from control (CT) and 4-week streptozotocin-induced diabetic rats (DB). α-actin was used as internal control. Bars are mean ± SEM. Number of animals is indicated in the bars. *t*-test, $^*P<0.05$ *vs.* CT.

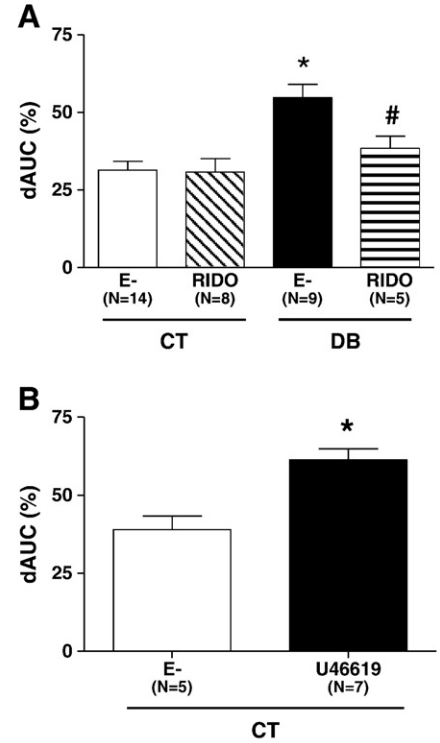

Fig. 4. The possible involvement of TxA_2/PGH_2 pathway on the functional ouabain-sensitive Na^+, K^+-ATPase activity of endothelium-denuded (E−) aortic rings from control (CT) and 4-week streptozotocin-induced diabetic rats (DB). Bar columns represent the difference between the area under the concentration–response curve (dAUC%) to KCl in aortas of CT and DB, before and after ouabain (0.1 mmol/L) incubation. The effects of: A) TxA_2 synthase inhibitor and TxA_2/PGH_2 receptor antagonist with ridogrel (RIDO, 1 μmol/L) in aortic rings from CT and DB rats or B) TP receptor agonist U46619 (10 nmol/L) in aortic rings from CT rats were evaluated. 1-way ANOVA, $^*P<0.05$ *vs.* CT E−; $^\#P<0.05$ *vs.* DB E− and *t*-test, $^*P<0.05$ *vs.* E−, respectively.

Discussion

The present study demonstrates a time-dependent increase in ouabain-sensitive Na^+, K^+-ATPase activity in aortic rings from rats with streptozotocin-induced type I diabetes. Moreover, our study shows a role of cyclooxygenase-2 products and activation of PKC in the increased ouabain-sensitive Na^+, K^+-ATPase activity observed in aorta from 4-week diabetic rats.

It is well established that diabetes mellitus is linked to the development of vascular dysfunction in a time-dependent manner (Kakkar et al. 1996; Pieper 1999; Xavier et al. 2003). This can include changes in ionic balance, increased vascular contractility and impaired endothelium dependent-relaxation (Tesfamariam et al. 1991; Gupta et al. 1992; Xavier et al. 2003; Guo et al. 2005). It is well known that each of these vascular alterations can influence Na^+, K^+-ATPase activity in certain cardiovascular diseases, including hypertension, heart failure and diabetes (Gupta et al. 1992; Marin and Redondo 1999; dos Santos et al. 2003).

Na^+, K^+-ATPase activity is an important mechanism contributing to the maintenance of vascular tone and membrane potential in vascular smooth muscle cells (Blaustein 1993; Marin and Redondo 1999). Previously, we have demonstrated that in a time-dependent manner, streptozotocin-induced type I diabetes increased contraction to phenylephrine in rat aorta, which was mediated by an increase of TxA_2/PGH_2 release and enhanced Ca^{2+} influx and/or sensitivity of the vascular smooth muscle cells (Xavier et al. 2003). As the Na^+, K^+-pump regulates vascular tone (Blaustein 1993; Ponte et al. 1996; Marin and Redondo 1999; Rossoni et al. 2002, 2003; dos Santos et al. 2003), the diabetic animals exhibit changes in endothelial and smooth muscle function (Durante et al. 1988; Tesfamariam et al. 1989, 1991; Abebe and MacLeod 1991; Pieper 1999; Xavier et al. 2003; Akamine et al. 2006), and vascular changes associated with diabetes are time dependent (Pieper 1999; Xavier et al. 2003), the current study was designed to evaluate the probable changes in ouabain-sensitive Na^+, K^+-ATPase activity in aortic rings from diabetic animals over two different periods of diabetes.

One-week of induced diabetes had no detectable effect on ouabain-sensitive Na^+, K^+-ATPase activity in rat aorta, while arteries from 4-week diabetic rats displayed an increase in ouabain-sensitive Na^+, K^+-ATPase activity. These results corroborate previous reports (Orie et al. 1993) indicating that aortas from 4-week diabetic rats exhibit increased ouabain-sensitive Na^+, K^+-ATPase activity and that these effects are time-dependent. Additionally, Cohen and Klepser (1988) reported decreased kidney glomerular Na^+, K^+-ATPase activity in animals with 2 weeks of streptozotocin-induced diabetes, but a significant increase in the enzyme activity in rats with 4 weeks of diabetes, which reinforces that the time-course of diabetes is critical for the influence on Na^+, K^+-ATPase activity. Other investigators, however, have demonstrated that Na^+, K^+-ATPase activity is unmodified (Simmons and Winegrad 1993; Smith et al. 1997) or even diminished (Ohara et al. 1991; Tesfamariam et al. 1993; Davel et al. 2000) in arteries of diabetic animals. The reasons for these differences are not clear, although they could be explained by differences in the vascular bed studied and the duration of diabetes.

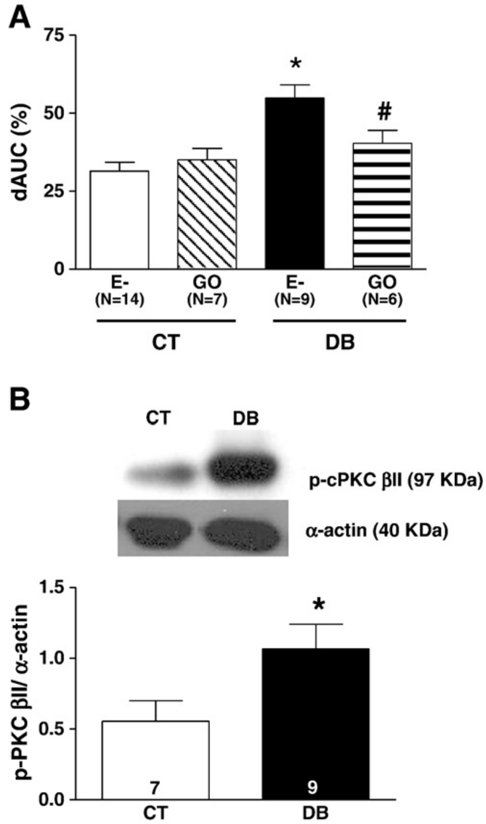

Fig. 5. A) The role of constitutive isoforms of PKC inhibition with Gö-6976 on the functional ouabain-sensitive Na^+, K^+-ATPase activity of endothelium-denuded (E−) aortic rings from control (CT) and 4-week streptozotocin-induced diabetic rats (DB). Bar columns represent the difference between the area under the concentration–response curve (dAUC%) to KCl in aortas of CT and DB incubated with or without Gö-6976 (GO, 50 µmol/L), before and after ouabain (0.1 mmol/L) incubation. 1-way ANOVA, $^*P<0.05$ *vs.* CT E−; $^\#P<0.05$ *vs.* DB E−. B) Representative Western blot autoradiographies (top) and densitometric analysis (bottom) of phosphorylated protein kinase C βII (p-PKC βII) protein expression in aortas from control (CT) and 4-week streptozotocin-induced diabetic rats (DB). α-actin was used as internal control. Bars are mean ± SEM. Number of animals is indicated in the bars. *t*-test, $^*P<0.05$ *vs.* CT.

It is well established in the literature that the endothelium exerts a positive effect on Na^+, K^+-pump activity, which seems to be mediated mainly by nitric oxide (Marin and Redondo 1999). The present results support previous studies (Rossoni et al. 2002, 2003; dos Santos et al. 2003) that have demonstrated that removal of the endothelium or preincubation with L-NAME reduces ouabain-sensitive Na^+, K^+-ATPase activity in aortic segments from control rats, indicating a stimulatory effect of endothelium-derived nitric oxide on Na^+, K^+-ATPase activity. However, neither endothelium removal or preincubation with L-NAME significantly affected ouabain-sensitive Na^+, K^+-ATPase activity in aortic rings of diabetic rats, suggesting a lack of modulation by endothelium-derived nitric oxide on Na^+, K^+-ATPase activity in this group. One important consequence of diabetes is impaired bioavailability of nitric oxide, due to an excess of vascular superoxide anions (Pandoffi et al. 2003; Bojunga et al. 2004; Akamine et al. 2006). Thus, it is possible to speculate that the lack of nitrergic modulation on ouabain-sensitive Na^+, K^+-ATPase activity observed in segments from 4-week diabetic rats could be associated with diminished bioavailability of nitric oxide. In addition, it is important to observe that the effect of diabetes on Na^+, K^+-ATPase activity was similarly observed in de-endothelized vessels, from which one might infer that the endothelium is not the "source" of increased ouabain-sensitive Na^+, K^+-ATPase activity.

Increased COX-derived products such as PGH_2 and TxA_2 are found in experimental diabetes, which contribute to increased vascular contractility (Xavier et al. 2003) and impairment of endothelium-dependent relaxation (Tesfamariam et al. 1989; Akamine et al. 2006). Recently, Shi and Vanhoutte (2008) have demonstrated that smooth muscle-derived prostanoids also contribute to the vascular dysfunction of diabetic rats. In addition, Lockette et al. (1980) demonstrated that COX-derived prostanoids significantly enhanced the magnitude of K^+-induced relaxation, which is consistent with a stimulatory effect of prostaglandins on Na^+, K^+-ATPase activity. In the present study, incubation of endothelium-denuded arteries with indomethacin, an COX-1 and 2 inhibitor, or with NS-398, a selective COX-2 inhibitor, reduced ouabain-sensitive Na^+, K^+-ATPase activity in diabetic rats, but not in control animals, suggesting the involvement of a non-endothelial COX-2-derivative mechanism on increased ouabain-sensitive Na^+, K^+-ATPase activity in the diabetic aorta. Supporting these results, COX-2 is overexpressed in the aorta of diabetic rats, reaching approximately a 2-fold increase compared to control values. In line with the present results, previous studies have demonstrated an up-regulation of COX-2 in the vasculature, in type I and II diabetes (Quilley and Chen 2003; Okon et al. 2007; Shi and Vanhoutte 2008). Similarly, the TxA_2 synthase inhibitor and TxA_2/PGH_2 receptor (TP) antagonist ridogrel also normalized the ouabain-sensitive Na^+, K^+-ATPase activity in endothelium-denuded diabetic aortas, suggesting that PGH_2 and/or TxA_2 play a role in stimulating ouabain-sensitive Na^+, K^+-ATPase activity in these animals. In line with this result, pretreatment of the endothelium-denuded control aortas with the TP agonist U46619 was able to increase the ouabain-sensitive Na^+, K^+-ATPase activity, which strongly reinforce our hypothesis that the increased ouabain-sensitive Na^+, K^+-ATPase activity in 4-week diabetic animals is mediated by an increment on PGH_2 and/or TxA_2 pathway.

TxA_2/PGH_2, via activation of TP receptors is able to stimulate the diacylglycerol (DAG)/inositol triphosphate (IP_3) pathway, increasing free intracellular calcium and PKC activity (Brass et al. 1987). Various studies have demonstrated increased PKC activity with diabetes (Lee et al. 1989; Inoguchi et al. 1992; Hattori et al. 1999) or acute hyperglycaemia (Tesfamariam et al. 1991). In the present study, the non-selective conventional PKC isoform inhibitor Gö-6976, restored the increased activity of the ouabain-sensitive Na^+, K^+-ATPase in aorta of diabetic animals to control levels, indicating the involvement of PKC in this effect. Multiple PKC isoforms have been identified and include a family of closely related serine/threonine kinases (Nishizuka 1995). The most ubiquitous group, however, are the conventional, Ca^{2+}-dependent PKCs (α, βI, βII, and γ). Inoguchi et al. (1992) identified the expression of only the α and βII constitutive PKC isoforms in rat aorta, and it has been shown that the βII isoform is involved in the diabetes-induced vascular complications. Guo et al. (2003) also demonstrated an increase in mRNA and protein expression of PKC βII in aorta from 4- to 8-week streptozotocin-induced diabetic pigs. In line with these results, we only observed increased phosphorylation of PKC βII in the diabetic aorta, suggesting a major role of this isoform of PKC in the aorta of type I diabetic rats. It has been demonstrated that activation of PKC may stimulate Na^+, K^+-ATPase activity (Lowndes et al. 1990; Pedemonte et al. 1997; Duran et al. 2004). Gupta et al. (1991) demonstrated that endothelin increases Na^+, K^+-ATPase activity by a PKC-dependent pathway. In this way, p-PKC βII could exert a key role in the modulation of ouabain-sensitive Na^+, K^+-ATPase activity in the diabetic state.

Conclusion

In conclusion, diabetes led to a time-dependent increase in ouabain-sensitive Na^+, K^+-ATPase activity. The main mechanism involved in this activation is the release of TxA_2/PGH_2 by COX-2 in smooth muscle cells, linked to activation of the PKC probably via βII isoform. Therefore, we hypothesize that the increased ouabain-sensitive Na^+, K^+-ATPase activity present in the 4-week diabetic aorta could be a counter-regulatory mechanism to the vascular dysfunction present in this artery.

Conflict of interest statement

The authors do not have potential conflicts of interest.

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