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Inhibition of endoplasmic reticulum stress protected DOCA-salt hypertension-induced vascular dysfunction

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ABSTRACT

Hypertension has complex vascular pathogenesis and therefore the molecular etiology remains poorly elucidated. Endoplasmic reticulum stress (ERS), which is a condition of the unfolded/misfolded protein accumulation in the endoplasmic reticulum, has been defined as a potential target for cardiovascular disease. In the present study, the effects of ERS inhibition on hypertension-induced alterations in the vessels were investigated.

In male Wistar albino rats, hypertension was induced through unilateral nephrectomy, deoxycorticosterone-acetate (DOCA) injection (20 mg/kg, twice a week) and 1% NaCl with 0.2% KCl added to drinking water for 12 weeks. An ERS inhibitor, tauroursodeoxycolic acid (TUDCA) (150 mg/kg/day, i.p.), was administered for the final four weeks.

ERS inhibition in DOCA-salt induced hypertension was observed to have reduced systolic blood pressure, improved endothelial dysfunction, enhanced plasma nitric oxide (NO) level, reduced protein expressions of phosphorylated-double-stranded RNA-activated protein kinase-like endoplasmic reticulum kinase (pPERK), 78 kDa glucose-regulated protein (GRP78), Inositol trisphosphate receptor1 (IP_3R1) and Epidermal growth factor receptor (EGFR), increased expressions of endoplasmic reticulum Ca^{2+}-ATPase2 (SERCA2) and B cell lymphoma2 (Bcl2) in vessels. These findings suggest that the beneficial effects of ERS inhibition on hypertension may be related to protection of vessel functions through restoration of endoplasmic reticulum calcium homeostasis, and apoptotic and mitotic pathways.

1. Introduction

Hypertension is one of the most prevalent cardiovascular diseases characterized by elevated blood pressure [1]. Multifactorial pathogenesis of hypertension remains poorly understood, but studies of hypertension maintain its importance and priority. It is well known that vascular dysfunction is implicated in the pathophysiology of hypertension [2–5]. Vascular endothelium plays an important role in the regulation of vessel functions by releasing vasoconstrictor and vasodilator substances. Endothelial dysfunction has been mostly attributed to decreased bioavailability of NO in conditions of oxidative stress [6]. Improvement of vascular dysfunction is an important therapeutic approach to reduce blood pressure.

Endoplasmic reticulum (ER) is one of the calcium store of the cells and regulates cell functions through calcium release. In addition, protein folding in eukaryotes is constructed in ER and ERS occurs when unfolded or misfolded proteins accumulate in the lumen of ER. ERS induces an adaptive program, which is called unfolded protein response (UPR) of the cell [7]. UPR attempts to restore ER homeostasis by inhibiting protein synthesis and up-regulation of protein folding and degradation pathways. When ERS is prolonged and/or excessive, UPR triggers the inflammatory pathways and finally apoptotic cell death [8]. It has been previously reported that ERS plays a key role in the pathogenesis of hypertension [9]. Recent studies have revealed that ERS inhibition improved the vascular dysfunction in Angiotensin II induced hypertension [10], reduced systolic blood pressure and normalized aorta contraction in spontaneously hypertensive rats (SHR) [11]. However, the underlying mechanism of the role of ERS in hypertension has not been fully understood.

The aim of this study was to elucidate the effects of ERS inhibition on hypertension-induced alterations of the mechanical functions of the thoracic aorta. In a rat model of DOCA salt-induced hypertension,

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examinations were made of the expressions of ER calcium homeostasis proteins (SERCA2 and IP_3R1); ERS (PERK and GRP78); apoptosis (Bcl-2-associated X (Bax) and Bcl2) and mitogenesis (EGFR, protein kinase B (AKT), p-AKT, extracellular signal-regulated kinase (ERK), p-ERK, and inhibitor of kappa B (IκB-α)) markers in vessels and blood biomarkers related to endothelial function (NO and TAC).

2. Materials and methods

2.1. Animal care and surgical procedure

All experiments were carried out in compliance with the National Institute of Health Guide for the Care and Use of Laboratory Animals. The experimental procedure was approved by the Animal Ethics Committees of Ankara and Gazi University, Ankara. Male Wistar albino rats, aged 8 weeks and each weighing 240 g–260 g, were used in this study. All the rats were housed in a constant temperature of 24 ± 1 °C with a 12 h light/dark cycle, and free access to food and water during the experimental period. All animals were housed in pairs in standard laboratory cages and randomly divided into four groups. Blinding was not performed.

The DOCA-salt hypertension model that has been previously described in detail was used in this study [12]. In summary, the animals were anesthetized with a mixture of ketamine (60 mg/kg) and xylazine (10 mg/kg) and left unilateral nephrectomy was performed in hypertensive groups. After a one-week recovery period, the rats were administered DOCA subcutaneously at a dose of 15 mg/kg twice a week, and 1% NaCl and 0.2% KCl was added to the drinking water for 12 weeks. TUDCA, which is an ERS inhibitor, was administered intraperitoneally (150 mg/kg/day) to the normotensive and hypertensive animal groups for the last 4 weeks of the experimental period.

Systolic blood pressure was measured once a week on pre-warmed and restrained rats, using tail-cuff plethysmography (NIBP200A, COMMAT, Turkey). At least six measurements were made for each rat and the mean values were calculated.

2.2. Isolated tissue bath procedure

At the end of the treatment period, the rats were anesthetized with a mixture of ketamine (60 mg/kg) and xylazine (10 mg/kg) and blood samples were collected from bifurcation of the abdominal aorta. The thoracic and carotid artery were carefully dissected and cleaned from adventitial tissues. Four vessel rings (2–3 mm) of the thoracic aorta were used for functional studies and the endothelium of some rings was denuded using a cotton thread. Isolated rings of aorta were placed in organ baths containing Krebs solution (in mM: 112 NaCl, 5 KCl, 11.5 Dextrose, 25 $NaHCO_3$, 0.5 $MgCl_2$, 2.5 $CaCl_2$, 1.2 NaH_2PO_4, pH: 7.4) aerated with 95% O_2, and 5% CO_2 and warmed to 37 °C. Each ring was mounted between stainless-steel hooks and connected to a force displacement transducer (FT 03) under an initial tension of 2 g. The tissues were allowed to equilibrate for at least 40 min. Cumulative concentration–response curves of acetylcholine (ACh) and sodium nitroprusside (SNP) were performed in the endothelium-intact aorta rings pre-contracted by phenylephrine (Phe 10^{-6} M). The relaxations were expressed as a percentage of the pre-contraction.

To investigate the functional capacity of the ER calcium store in the Phe-induced contraction, the previously described experiment design was followed [13]. Briefly, after equilibration and washing of endothelium-denuded aortic rings in the organ bath, the medium was replaced with Ca^{2+}-free Krebs solution containing EGTA (10^{-5} M) for 15 min then the Phe (10^{-5} M) was added. The response of rings to Phe in Ca^{2+}-free medium was recorded for 10 min to observe intracellular calcium release. Then, Ca^{2+} (2.5 mM) was added to the bath medium and extracellular calcium-induced contraction was determined in the presence of Phe.

2.3. Biochemical examinations

The plasma nitrite level as an indicator of NO production was measured using the spectrophotometric method based on the Griess reaction [14]. Total Antioxidant Capacity (TAC) of plasma was measured by the method previously described [15], based on the reduction of Cu^{+2} to Cu^{+1} by the antioxidants of plasma. Neocuproine (Nc) was used as a chromogenic agent and the color of the formed complex (Nc-Cu^{+1}) was detected spectrophotometrically at 455 nm.

2.4. Western Blot analysis

The isolated and cleaned thoracic aorta and right and left carotid arteries were placed in Krebs solution. One half of the right and left carotid arteries were incubated with Krebs solution and the other half of the carotid arteries were incubated with phenylephrine (10^{-6} M) in 37 °C for 10 min. Then, the carotid arteries and the remaining part of the thoracic aorta were immediately frozen (−80 °C). The thoracic aorta and carotid artery samples were homogenized in homogenization buffer containing 2 mM EDTA, 50 mM Tris, 1 mM PMSF, 1% NP40 (v/v), 10% Sucrose (w/v) and protease and phosphatase inhibitor tablets (Roche, USA) using Tissue Ruptor homogenizator (Qiagen, Venlo, Netherlands). Homogenates were centrifuged at 800*g* for 10 min at 4 °C and supernatants were collected. Total protein concentrations were quantified using the Lowry method. Equal amounts of protein (50 μg) samples were mixed with sample buffer, separated by SDS-polyacrylamide gel electrophoresis and electroblotted to PVDF membranes. The membranes were blocked by incubation in blocking buffer (3% BSA or 5% nonfat dried milk) and primary antibody incubation (1:100) was performed using the following antibodies: PERK, pPERK, GRP78, SERCA2, IP_3R1, Bcl2, Bax, EGFR, AKT, p-AKT, ERK, p-ERK, IκB-α, and GAPDH (1:2000) (Santa Cruz Biotec., CA, USA). Then, the membranes were incubated with horseradish peroxidase conjugated secondary antibodies (1:10.000, Santa Cruz Biotec., CA, USA) for 1 h. Signals were visualized by chemiluminescent detection using the ChemiDoc™ MP (Bio-Rad Laboratories, Hercules CA, USA) system. GAPDH was used as an internal control protein. The expression level of proteins relative to GAPDH was determined using the ImageLab 4.1 software.

2.5. Chemicals

TUDCA was obtained from Calbiochem (La Jolla, CA, USA). Acetylcholine, phenylephrine, DOCA and all other chemicals were obtained from Sigma Chemical Co (St Louis, MO, USA). TUDCA was prepared daily in serum physiologic. DOCA was dissolved in corn oil (20 mg/ml, *w/v*).

2.6. Statistical analysis

The results were expressed as mean ± standard error of the mean (SEM). Repeated-measures of two-way ANOVA and posthoc Bonferroni test (for ACh and SNP dose-response curves) and the Student's *t*-test were used to test differences among the groups for statistical analysis. A value of $p < 0.05$ was considered statistically significant.

3. Results

The body weights of all animals were similar at the beginning of experiment. At the end of the experiment, the body weights of the hypertensive animals were significantly lower than those of the control group ($p < 0.05$). Reduced body weight was increased by TUDCA treatment ($p < 0.05$). In normotensive animals, TUDCA treatment prevented the weight gain (Fig. 1A).

Systolic blood pressure of the animals was measured weekly using the tail-cuff method. In the DOCA-salt group, systolic blood pressure was significantly increased compared to the control group ($p < 0.05$).

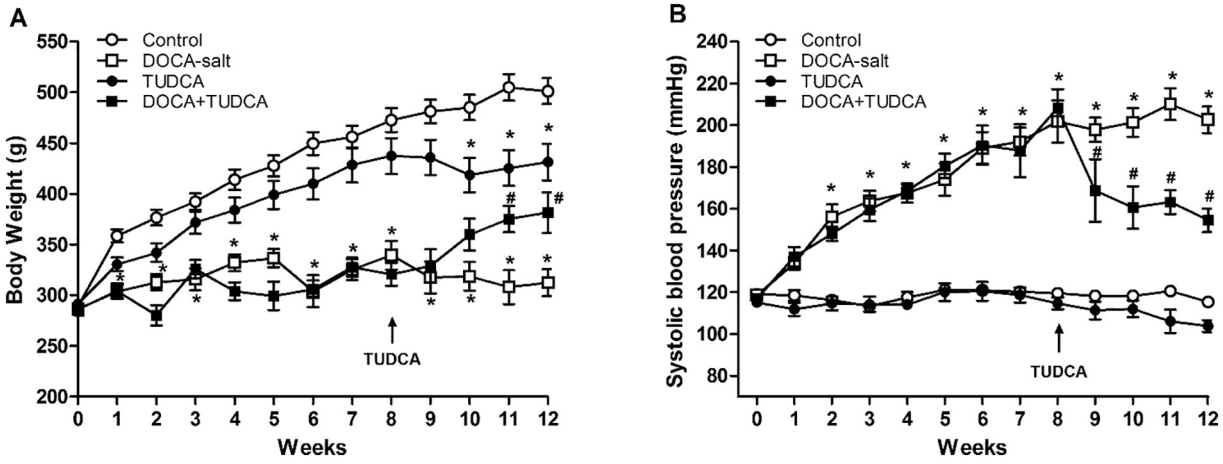

Fig. 1. The effect of ERS inhibition with TUDCA on the body weight and systolic blood pressure of the animals. TUDCA treatment enhanced body weight in hypertensive rats and decreased it in normal rats (A). Systolic blood pressure was increased with DOCA-salt administration and reduced with TUDCA treatment (B). Differences from *Control, #DOCA-salt (n = 6–8).

TUDCA treatment reduced the systolic blood pressure in hypertensive animals ($p < 0.05$). In normal rats, systolic blood pressure was not altered by TUDCA treatment (Fig. 1B).

Vascular ERS was examined by quantifying the expression levels of ERS markers such as p-PERK and GRP78. While p-PERK expression in the thoracic aorta was significantly increased in the hypertensive group compared to the control group ($p < 0.05$), elevated p-PERK expression was decreased by TUDCA treatment ($p < 0.05$) but, total PERK expression was similar in all groups (Fig. 2A, B, C and D). Another ERS marker, GRP78 expression, was also enhanced by DOCA-salt hypertension and TUDCA treatment significantly reduced expression level in the carotid artery similar to in the thoracic aorta (Fig. 2E and F).

ACh-induced endothelium-dependent relaxations of the vessel were recorded against Phe-induced (10^{-6} M) pre-contraction in the thoracic aorta (Fig. 3A). Endothelium-dependent relaxations were significantly reduced in the DOCA-salt hypertensive rats when compared to the control rats ($p < 0.05$). The DOCA-salt induced decrease in the endothelium-dependent relaxations were improved by TUDCA treatment ($p < 0.05$). SNP-induced endothelium-independent relaxations were also decreased in the DOCA-salt group and improved by TUDCA treatment ($p < 0.05$) (Fig. 3B).

The plasma nitrite level, which reflects NO production, was not different in the DOCA-salt group from the control group (Fig. 4A). TUDCA treatment significantly increased the plasma nitrite levels in both hypertensive and normotensive animals ($p < 0.05$). The plasma TAC levels were significantly augmented in the DOCA-salt group compared to the control group ($p < 0.05$). Elevated plasma TAC levels were significantly decreased by TUDCA treatment ($p < 0.05$) (Fig. 4B).

Although the effect of ERS on protein synthesis and cell metabolism has been studied in detail, the functional situation of the intracellular ER calcium store has not been examined to date. To evaluate the contribution of Ca^{2+} release from ER store on the Phe-induced contraction of vascular smooth muscle, vessel response was obtained in the calcium-free and calcium-containing medium. Phe-induced contractions of the aortic rings isolated from DOCA-salt rats were attenuated in the calcium-free medium and additional contraction induced by calcium was also smaller than in the control group ($p < 0.05$) (Fig. 5). In hypertensive and normal rats, TUDCA treatment significantly elevated Phe-induced contractions in the calcium-free medium ($p < 0.05$). In the hypertensive group, TUDCA treatment enhanced the contractions in the calcium-containing medium ($p < 0.05$) (Fig. 5).

Regulation of ER calcium store was examined by aortic expressions of SERCA and IP_3R, which are responsible for calcium storage and the release of ER. Although expressions of SERCA2 protein in the thoracic aorta were significantly reduced, IP_3R1 expression was significantly increased in the DOCA-salt hypertensive rats compared to the control group ($p < 0.05$) (Fig. 6). TUDCA treatment enhanced expressions of SERCA2 and reversed expression of IP_3R1.

The relationship of ERS inhibition with apoptosis in the vessel was examined using marker expression. DOCA-salt hypertension significantly increased the expression level of Bax protein when compared to the control group ($p < 0.05$) (Fig. 7A and B). The expression of Bcl2 protein was significantly reduced in the aorta of DOCA-salt rats and was increased by TUDCA treatment ($p < 0.05$) (Fig. 7C and D).

Hypertension is associated with remodeling of resistance vessels. Hypertension-induced mitotic pathways in resting and Phe-stimulated conditions were investigated in the carotid artery. Enhanced EGFR expression in the DOCA-salt group was significantly lowered with TUDCA treatment ($p < 0.05$) (Fig. 8A, B). Expressions of AKT, p-AKT, ERK, p-ERK and IκB-α protein in the carotid artery, were similar in all the groups. However, p-AKT expression was significantly elevated by Phe stimulation in the carotid artery isolated from DOCA-salt hypertensive rats (Fig. 8D).

4. Discussion

The results presented in this study showed that DOCA-salt induced hypertension impaired vascular responsiveness and altered expressions of functional proteins in the vessel wall. These were seen as enhanced expressions of pPERK, GRP78, IP_3R1, Bax, EGFR and reduced expressions of SERCA2 and Bcl2. ERS inhibition with TUDCA decreased systolic blood pressure, improved vascular dysfunction, and reduced expressions of pPERK, GRP78, IP_3R1 and EGFR, increased expressions of SERCA2 and Bcl2 in the vessel.

The accumulation of misfolded or unfolded proteins in ER lumen is defined as ER stress which leads to the activation of the unfolded protein response (UPR) to restore homeostasis [16]. Young et al. first reported that ERS plays a pivotal role in chronic hypertension [9]. Since then, ERS has been the subject of studies on hypertension [10,11,17–20].

It has been reported that ERS affects the lipid, glucose and energy metabolism [21–23]. In addition, TUDCA treatment has been shown to inhibit weight gain in mice fed with a high-fat diet [23]. In the current study, the body weights of hypertensive rats were significantly lower than those of the control group rats. TUDCA treatment increased the body weight of hypertensive animals and inhibited weight gain in normotensive animals. The effect of ERS inhibition by TUDCA on body weight may be altered by hypertension-induced regulation of lipid, glucose and energy metabolism. However, further examinations are needed to elucidate the mechanism of hypertension and the effects of

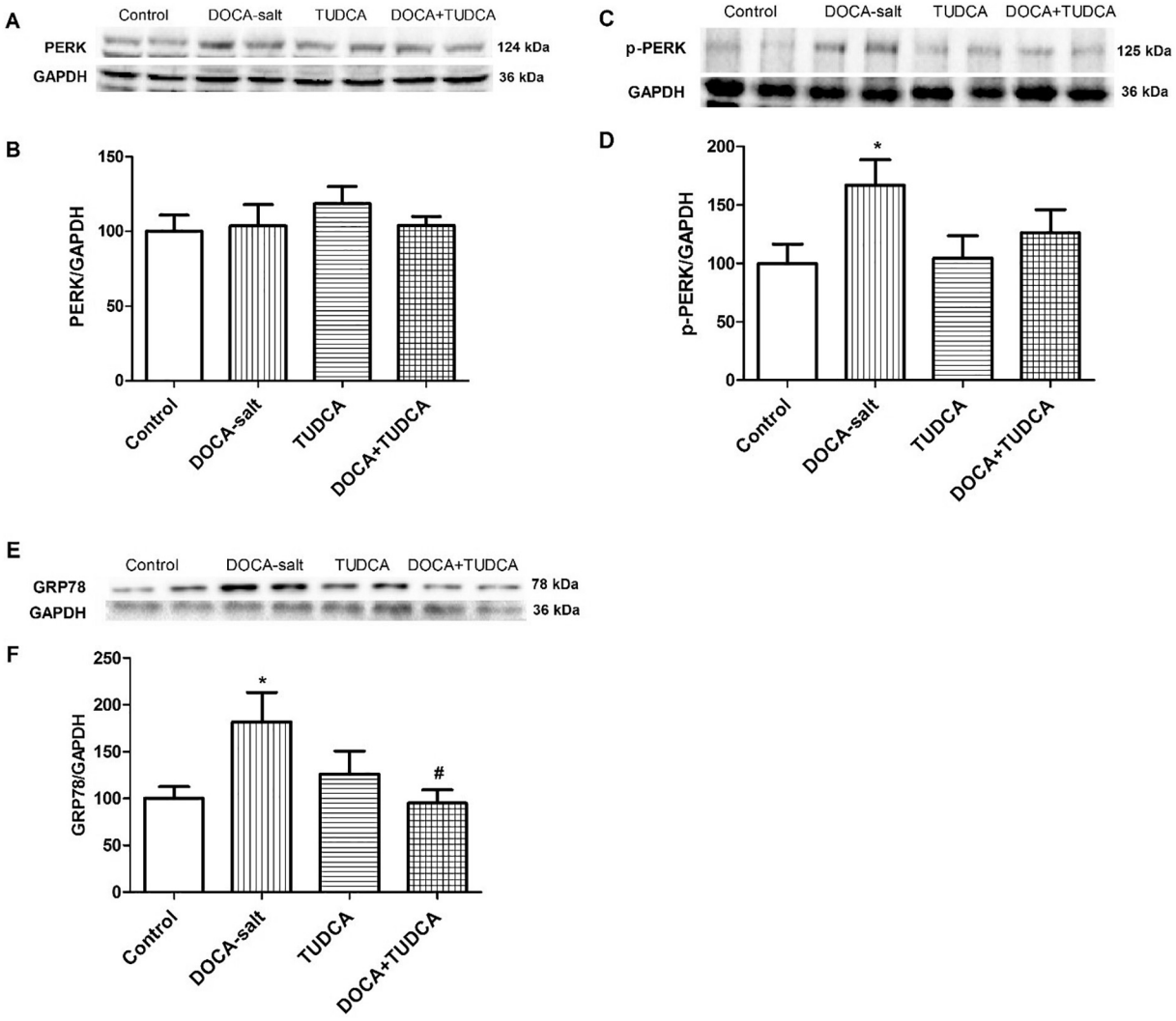

Fig. 2. The effect of ERS inhibition with TUDCA on PERK, p-PERK and GRP78 protein expressions. Total PERK expressions of the thoracic aortas were similar in all groups (A and B). Aortic p-PERK expression level was enhanced in the DOCA-salt group and was reduced with TUDCA treatment (C and D). Increased GRP78 protein expression of the carotid artery isolated from hypertensive animals was reduced with TUDCA treatment (E and F). Difference from *Control (n = 6).

TUDCA on weight regulation.

It has been shown that ERS inhibition with TUDCA lowers the blood pressure in Angiotensin II-induced hypertension [10,18] and SHR [17]. Similar to a previous study, ERS inhibition with TUDCA in the current study was seen to reduce the systolic blood pressure of the DOCA-salt hypertensive rats, suggesting that TUDCA affected the main mechanisms of hypertension.

Eukaryotic cells respond to ERS through three sensors that are bound to the ER membrane mediating the UPR signaling: PERK, activating transcription factor 6 (ATF6) and inositol requiring kinase 1 (IRE1) [24]. Under ERS, the GRP78/PERK complex is separated and PERK is activated by autophosphorylation [25]. It has been shown that

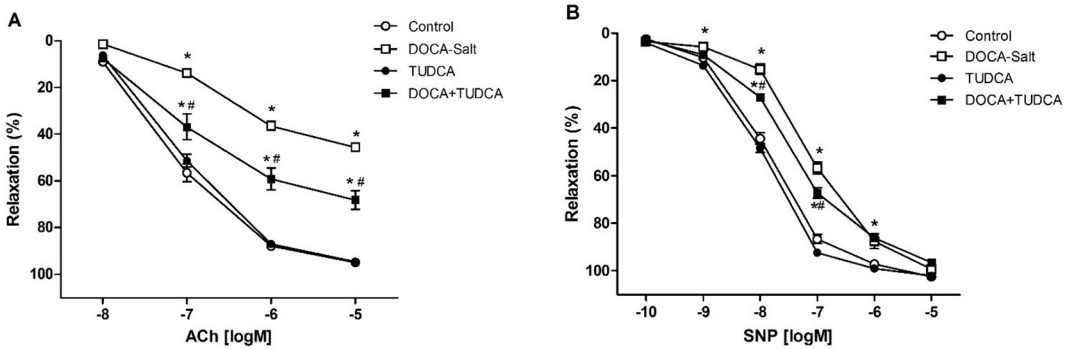

Fig. 3. The effect of ERS inhibition with TUDCA on endothelium-dependent and endothelium-independent relaxations. ACh-induced endothelium-dependent relaxations were decreased in hypertensive rats and ameliorated with TUDCA treatment (A). SNP-induced endothelium-independent relaxations were decreased in the DOCA-salt group (B). Differences from *Control, #DOCA-salt (n = 11–24).

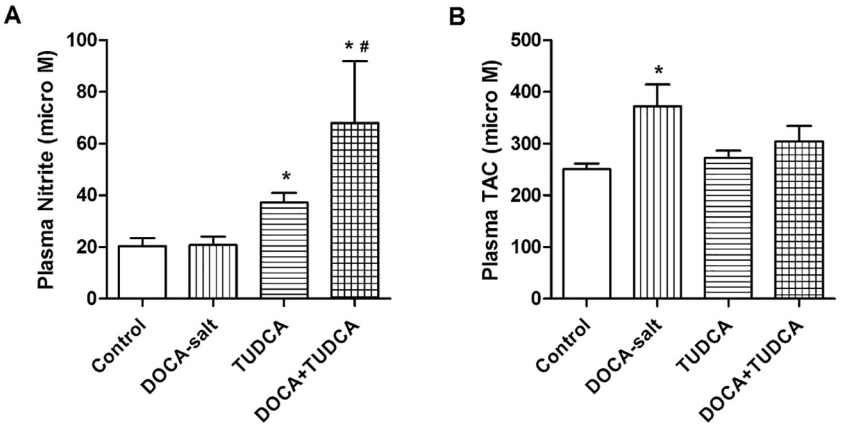

Fig. 4. The effect of ERS inhibition with TUDCA on plasma levels of nitrite and TAC. TUDCA treatment enhanced plasma nitrite levels in both hypertensive and normotensive rats (A). TAC levels were augmented in the hypertensive group and decreased with TUDCA treatment (B). Differences from *Control, #DOCA-salt (n = 6–8).

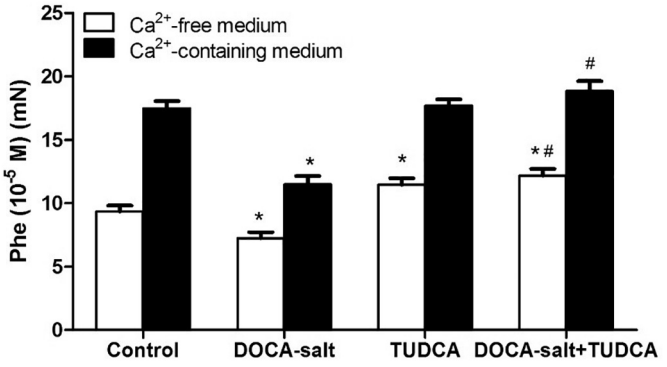

Fig. 5. The effect of ERS inhibition with TUDCA on Phe-stimulated contractions in Ca^{2+}-free and Ca^{2+}-containing medium. Intracellular calcium release and calcium entry from the extracellular medium were enhanced with TUDCA treatment in the aortic smooth muscle stimulated by Phe. Differences from *Control, #DOCA-salt (n = 11–23).

total PERK expression was not changed in the aorta of hypertensive animals [26]. In the current study, total PERK expressions were also similar in all experimental groups as seen in the previous study. However, the aortic expression level of activated PERK (pPERK) was higher in the hypertensive group compared to the control group and TUDCA treatment reversed this increment. GRP78 is a major endoplasmic reticulum chaperone which plays a pivotal role in the regulation of UPR [27]. In conditions of ERS, GRP78 binds to misfolded proteins and separates from and activates the transmembrane sensors [28]. In the current study, GRP78 expression in the vessel wall was also increased by DOCA-salt hypertension and TUDCA treatment reduced the expression level. These results posited that hypertension caused ERS of the vessel and TUDCA treatment inhibited it.

Hypertension induced endothelial dysfunction has been reported in both human and experimental models of hypertension [2–5,12,29]. In addition, there is accumulating evidence to indicate the relationship between ERS and endothelial dysfunction [26,30,31]. It has been shown that ERS inhibition with TUDCA restored the endothelial dysfunction in angiotensin II induced hypertension [10]; inhibited tunicamisin induced endothelial dysfunction in mice aorta [32]; and enhanced endothelium-dependent relaxation in mesenteric arteries of SHR [33]. In addition, Galan et al. showed that ERS decreased endothelial NOS expression and activation in endothelial cells [26]. In accordance with previous studies, ACh-induced endothelium-dependent relaxations of hypertensive rat aortas were decreased when compared to the control group in the current study. This is the first study to have observed that ERS inhibition with TUDCA improved DOCA-salt hypertension induced endothelial dysfunction. Therefore, it can be suggested that the antihypertensive action of TUDCA may be due to its beneficial effect on the endothelial function in DOCA-salt hypertension.

On the other hand, it has been demonstrated that SNP-induced

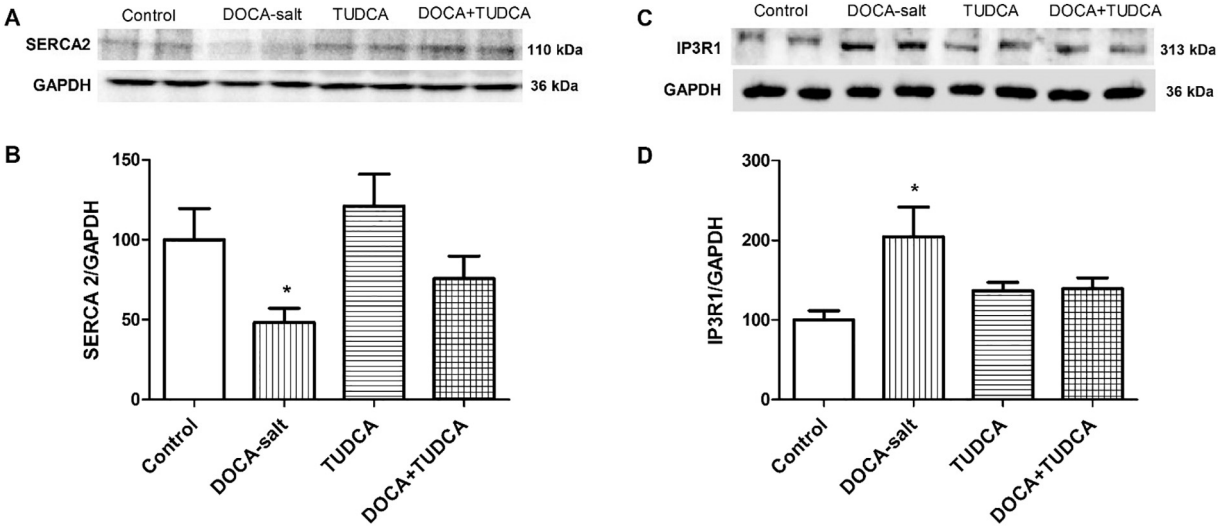

Fig. 6. The effect of ERS inhibition with TUDCA on SERCA2 and IP_3R protein expressions in the aorta. The SERCA2 expression level was reduced in hypertension and enhanced with TUDCA treatment (A, B). The IP_3R expression level was enhanced in the DOCA-salt group and TUDCA treatment inhibited this increment (C, D). Difference from *Control (n = 6).

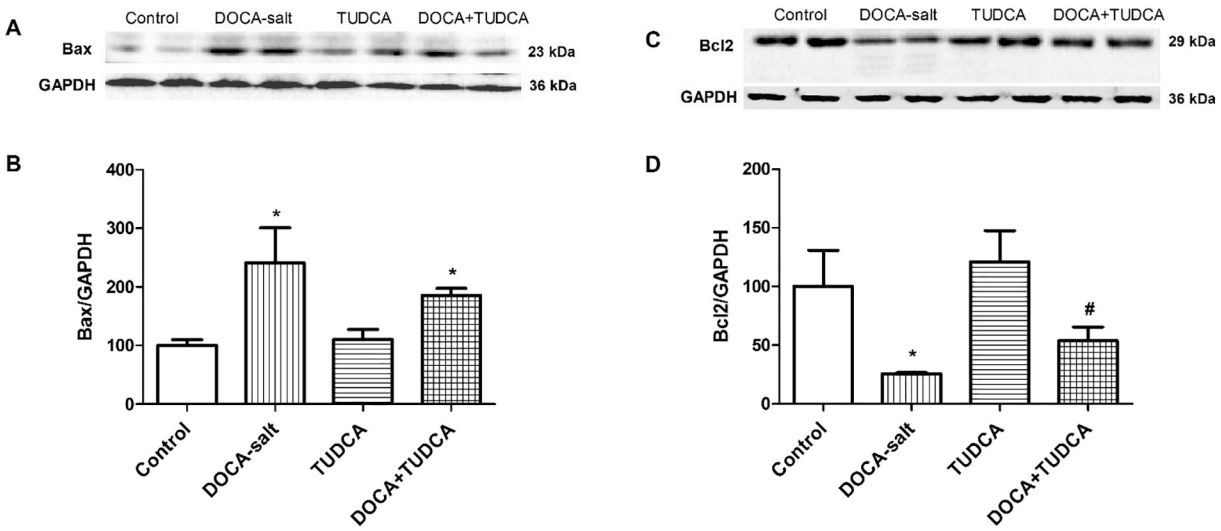

Fig. 7. The effect of ERS inhibition with TUDCA on Bax and Bcl2 protein expressions. Bax expression was enhanced in the DOCA-salt group (A, B). Bcl2 expression was decreased in hypertensive rats and enhanced with TUDCA treatment (C, D). Differences from *Control, #DOCA-salt ($n = 6$).

endothelium-independent relaxations decreased in animal models of hypertension including SHR [34], renovascular hypertension [35] and high-salt diet induced hypertension [36]. In the present study, SNP-induced endothelium-independent relaxation was also attenuated in the aorta of DOCA-salt hypertensive animals and was improved with TUDCA treatment. It has been suggested that reduced SNP-induced relaxations are caused by impairment in the NO-cGMP signaling pathway in the vascular smooth muscle cells [36].

The endothelium-derived NO level is regarded as an indicator of endothelial function. However, NO may be synthesized in other cells in case of inflammation which is involved in the pathophysiology of hypertension [37]. Attenuated or unchanged plasma levels of NO in hypertension have been reported in previous studies [12,38]. In the current study, it was observed that plasma levels of NO were not changed by DOCA-salt hypertension. Thus, it was hypothesized that a reduced level of endothelium-derived NO production in hypertension might be balanced by NO secreted from immune cells. In the present study, TUDCA treatment enhanced plasma levels of NO in both hypertensive and normotensive rats. These results suggest that the effect on NO levels of ERS inhibition with TUDCA may be another important mechanism to improve endothelial function in hypertension.

Oxidative stress is another important factor which is involved in the pathophysiology of hypertension [39–41]. Enhanced production or impaired inactivation of reactive oxygen species (ROS) lead to a decrease in NO bioavailability [6]. TAC measurement is a method which allows the examination of different components of the antioxidant defense system together. In the current study, plasma levels of TAC increased in DOCA-salt hypertensive rats in consistent with previous studies [42,43] and returned to the control values with TUDCA treatment. It has been argued that reduced or increased TAC levels occur based on the progression of the pathology and the duration of exposure to oxidative stress [43]. On the one hand, long term oxidative stress may be lead to depletion of the antioxidant system. On the other hand, in the earlier stages of hypertension, a reactive TAC increment may be a consequence of enhanced activity of the antioxidant defense system in response to sustained oxidative stress.

Intracellular Ca^{2+} homeostasis plays an important role in vascular excitation-contraction coupling. Contraction of the arterial smooth muscle by stimulation of G_q coupled receptors is dependent on the elevation of intracellular free calcium concentration due to the release of Ca^{2+} from the ER store and Ca^{2+} influx from the extracellular space [44]. IP_3-mediated Ca^{2+} release from ER due to IP_3R and Ca^{2+} entry through membrane Ca^{2+} channels is involved in the alpha-1 adrenoreceptor-stimulated contraction of smooth muscle. In the current study, Phe-induced contractions stimulated by Ca^{2+} release from ER and extracellular influx were attenuated in the aortic rings isolated from the DOCA-salt hypertensive rats. In the hypertensive and normotensive rats, TUDCA treatment elevated Phe stimulated Ca^{2+} release-induced contraction in the calcium-free medium. In the hypertensive group, TUDCA treatment also increased the contractions induced by extracellular Ca^{2+} influx. These results indicate that TUDCA treatment enhanced the contraction induced by Ca^{2+} release from intracellular stores in both hypertensive and normotensive animals. In addition, TUDCA increased the contraction mediated by extracellular Ca^{2+} entry in the hypertensive rats. This is the first study to have obtained this functional data that alpha-1 adrenoreceptor-stimulated Ca^{2+} release and influx diminished by hypertension and ERS inhibition with TUDCA treatment reversed the hypertension-induced disruption of the functional reflection of calcium homeostasis in the vascular smooth muscle cells.

On the other hand, increased intracellular free calcium concentration after cell stimulation is reversed by calcium transporters. The most important transporter is SERCA, which is a calcium ATPase and carries calcium in the ER lumen and is responsible for filling the ER calcium store. SERCA regulates the ER calcium store and has an important role in contraction-relaxation coupling, apoptosis and proliferation [45]. It has been shown that an alteration in SERCA activity diminished intracellular Ca^{2+} homeostasis, which was related to enhancement of vascular tone in hypertensive animals [46]. Decreased endothelial SERCA2b expression in SHR has also been reported [47]. In addition, depletion of the ER calcium store by SERCA2 inhibition has been used in an ERS induction model [48]. It has been reported that attenuation of SERCA2a activity caused ER Ca^{2+} store depletion and consequently, ERS [49]. It has been reported that SERCA functions are disrupted but the expression level is not changed under oxidative stress [45]. Regulation of SERCA activity and expression seem to be related to many factors but the relationship with hypertension has not been fully elucidated. In the present study, it was observed for the first time that aortic expression of SERCA2 decreased in DOCA-salt hypertension and enhanced with TUDCA treatment. This result also supported the Phe-induced contraction of the aorta in the calcium free medium that was reduced by hypertension and enhanced after TUDCA treatment. These findings suggest that the effect of ERS inhibition with TUDCA on SERCA2 expression may be another important mechanism in the beneficial effect on hypertension.

In addition, stimulation of Phe causes IP_3-mediated activation of

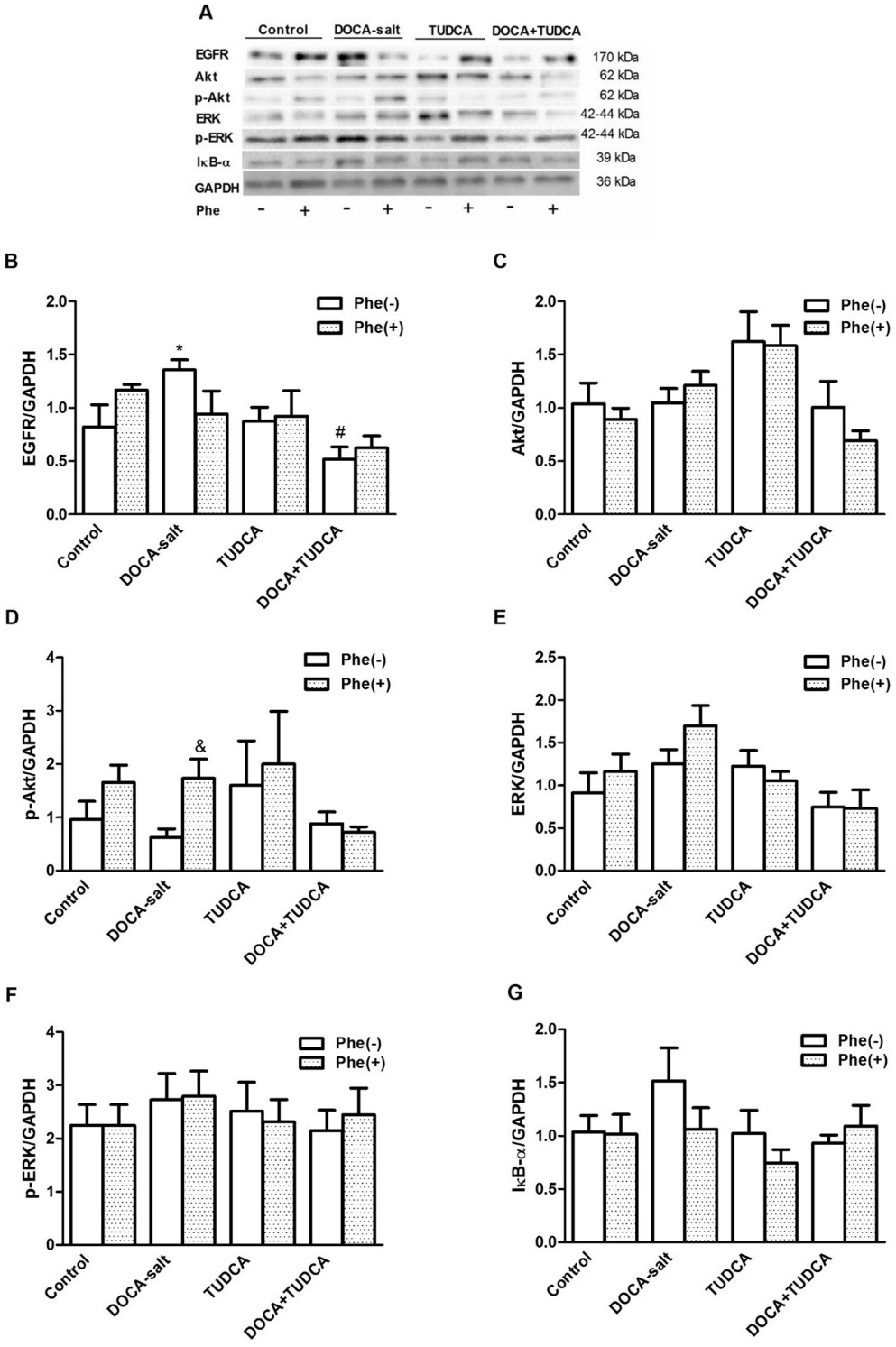

Fig. 8. The effect of ERS inhibition with TUDCA on EGFR, Akt, p-Akt, ERK, p-ERK and IκB-α protein expressions in the carotid artery. EGFR expression was increased in the DOCA-salt group and lowered with TUDCA treatment. The expression of p-AKT was elevated by Phe-incubation in the carotid artery isolated from the hypertensive rats. Differences from *Control, #DOCA-salt, & Phe incubation ($n = 6$).

IP_3R, which is a Ca^{2+} channel allowing Ca^{2+} release from ER into the cytoplasm. IP_3R1 isoform has been assumed to be the fourth ERS sensor [50]. The ER chaperone, GRP78, specifically binds IP_3R1 and acts together against ERS [51]. In the current study, it was shown for the first time that aortic IP_3R1 expression was augmented in DOCA-salt hypertension and decreased to control values with TUDCA treatment. Enhanced IP_3R1 expression in hypertension would be a compensation mechanism of the insufficient ER calcium store.

Enhanced apoptotic activity has been reported in several hypertension models [52,53]. In addition, exposure to chronic ERS can impair cellular function and result in apoptosis [54]. The Bcl2 family proteins including the anti-apoptotic (Bcl2, Mcl1: myeloid cell leukemia 1, BclxL: B-cell lymphoma extra-large, Bclw: Bcl2-like protein 2 and Bfl1: Bcl2-related protein A1) and the pro-apoptotic (Bax, Bak: Bcl2 homologous antagonist/killer and Bok: Bcl2-related ovarian killer) members, play an important role in the regulation of ERS-induced

apoptosis [55]. Pro-apoptotic Bcl2 family members, such as Bax, are the main steps for the induction of apoptosis in mitochondria. These proteins cause cytochrome C release and caspase activation and then cells undergo apoptosis [54]. Previous studies have shown that ERS inhibition produced anti-apoptotic activity in the aorta of SHR [17], inhibited apoptosis in the pulmonary arterial smooth muscle cells [56] and reduced apoptosis in the aorta of angiotensin II treated rats [18]. Spitler et al. reported that ERS inhibition with TUDCA reduced Bax protein expression and enhanced Bcl2 protein expression in the aorta of SHR [17]. In the current study, it was shown that the pro-apoptotic Bax protein expression level increased and the anti-apoptotic Bcl2 protein expression level decreased in the aorta of DOCA-salt hypertensive rats. In addition, ERS inhibition with TUDCA did not change Bax expression but enhanced Bcl2 expression in hypertensive animals. These results suggest that the anti-apoptotic action of ERS inhibition with TUDCA may play an important role in the antihypertensive effect.

Resistance arteries play an important role in the regulation of blood pressure [57]. Hypertension is related with a remodeling of the resistance vessels [58] and vascular remodeling is associated with increased proliferation and migration of vascular smooth muscle cells in response to enhanced production of growth factors [59]. Epidermal growth factor (EGF) is the one of the mitogenic growth factors for the vascular smooth muscle cells [60]. EGFR stimulation leads to activation of ERK, AKT and NH_2-terminal c-Jun kinase (JNK) pathways [61]. EGF/EGFR is involved in enhanced vascular smooth muscle proliferation and leads to hypertrophic remodeling [62]. The elevated level of EGF and the enhanced protein tyrosine kinase activity associated with the EGFR has been reported in the spontaneously hypertensive Lyon rat aorta [63]. However, the relationship between EGFR and ERS has been revealed in type 1 diabetes mellitus [64]. Takayanagi et al., showed that ERS inhibition prevented the EGFR activation by AngII in vascular cells [20]. In the present study, prevention of increased expression of EGFR with TUDCA treatment in the carotid artery was observed for the first time in the DOCA-salt hypertensive rats. It seems that one of the molecular mechanisms of ERS inhibition may be associated with mitogenic EGF/EGFR pathway, which leads to vascular remodeling in hypertension.

The AKT signaling pathway plays an important role in cardiovascular system regulation through involving angiogenesis and apoptosis [65]. It has been suggested that impairment of AKT activity contributed to endothelial dysfunction in hypertension [66]. ERK and Nuclear Factor kappa B (NF-κB) pathways are also involved in the proliferation process [67,68]. In the current study, the expression levels of AKT, p-AKT, ERK, p-ERK and a NF-κB inhibitor IκB-α were not altered by hypertension or TUDCA treatment in the carotid artery. Vascular remodeling is an active process that develops in response to long-standing changes in the hemodynamic situation. Hypertension-induced changes are dependent on interactions between local growth factors, vasoactive substances and hemodynamic stimuli therefore it may vary in different periods of the pathological process. Further studies are needed to investigate the long term effects of ERS inhibition on vascular mitogenic pathways in hypertension.

In conclusion, ERS inhibition with TUDCA treatment was observed to reduce systolic blood pressure and improve vessel functions in the DOCA-salt hypertension model. These effects of ERS inhibition were accompanied by enhancement of the plasma NO level, and restoration of vascular pPERK, GRP78, SERCA2, IP_3R1, Bcl2 and EGFR protein expressions. These findings will help to elucidate the molecular mechanisms of the beneficial effect of ERS inhibition on hypertension and can be suggested as potential targets for novel therapies.

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