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Hydrogen sulfide is involved in dexamethasone-induced hypertension in rat

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ABSTRACT

Glucocorticoid (GC)-induced hypertension is a common clinical problem still poorly understood.

The presence of GC receptor (GR) in vascular smooth muscle and endothelial cells suggests a direct role for GC in vasculature. In response to hemodynamic shear stress, endothelium tonically releases nitric oxide (NO), endothelial-derived hyperpolarizing factor (EDHF) and prostacyclin contributing to vascular homeostasis. Recently, hydrogen sulfide (H_2S) has been proposed as a candidate for EDHF. H_2S is endogenously mainly formed from L-cysteine by the action of cystathionine-β-synthase (CBS) and cystathionine-γ-lyase (CSE). It plays many physiological roles and contributes to cardiovascular function. Here we have evaluated the role played by H_2S in mesenteric arterial bed and in carotid artery harvested from rats treated with vehicle or dexamethasone (DEX; 1.5 mg/kg/day) for 8 days. During treatments systolic blood pressure was significantly increased in conscious rats. EDHF contribution was evaluated in ex-vivo by performing a concentration–response curve induced by acetylcholine (Ach) in presence of a combination of indomethacin and L-NG-Nitroarginine methyl ester in both vascular districts. EDHF-mediated relaxation was significantly reduced in DEX-treated group in both mesenteric bed and carotid artery. EDHF-mediated relaxation was abolished by pre-treatment with both apamin and charybdotoxin, inhibitors of small and big calcium-dependent potassium channels respectively, or with propargylglycine, inhibitor of CSE.

Western blot analysis revealed a marked reduction in CBS and CSE expression as well as H_2S production in homogenates of mesenteric arterial bed and carotid artery from DEX-treated rats. In parallel, H_2S plasma levels were significantly reduced in DEX group compared with vehicle.

In conclusion, an impairment in EDHF/H_2S signaling occurs in earlier state of GC-induced hypertension in rats suggesting that counteracting this dysfunction may be beneficial to manage DEX-associated increase in blood pressure.

1. Introduction

Chronic administration of glucocorticoids (GCs), as well as an endogenous increase of GCs, such as in Cushing's syndrome, leads to hypertension [1]. This form of hypertension is generally ascribed to the activation of the mineral corticoid receptor [2], although a growing body of evidence indicates that GCs also elevate blood pressure independently of this receptor in both human and animal models [3–5]. Moreover, an increase in endogenous levels of vasoconstrictor agents i.e. norepinephrine and angiotensin II, as well as in calcium influx, has been reported as plausible mechanisms of steroid-induced hypertension [6–9]. The presence of glucocorticoid receptor (GR) in vascular smooth muscle and endothelial cells implies a direct role for GCs in vasculature tone control [10–13]. Genetic and molecular studies have demonstrated that suppression of GR in vascular smooth muscle attenuates, but does not prevent, the development of dexamethasone (DEX)-induced hypertension [14]. Conversely, deletion of GR in endothelial cells completely abrogates this effect [15]. Even though these studies indicate a key role for the endothelium, the mechanism(s) involved are still unclear. GCs can interfere with vascular reactivity through

Abbreviations: GC, glucocorticoid; GR, glucocorticoid receptor; DEX, dexamethasone; SK_{Ca}, small calcium-dependent potassium channel; BK_{Ca}, big calcium-dependent potassium channel; APA, apamin; ChTX, charybdotoxin; PE, phenylephrine; INDO, indomethacin; L-NAME, L-NG-nitroarginine methyl ester; PAG, propargylglicine.

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a variety of effects, including the increase of vasoconstrictor agents and the decrease of vasodilating mediators, such as prostacyclin and nitric oxide (NO). However, also in this case, conflicting data are reported in the current literature [16,17]. Of note, at the present stage the possible involvement of endothelial-derived hyperpolarizing factor (EDHF) in GC-induced hypertension has not been addressed as yet.

The EDHF-mediated vasodilatation is endothelium-dependent, insensitive to combination of NO-synthase (NOS) and cyclooxygenase (COX) inhibitors, results in hyperpolarization of vascular smooth muscles cells controlling vascular resistance tone. In the vasodilatatory mechanism(s) are involved both small (SK_{Ca}) and big (BK_{Ca}) conductance calcium-activated potassium channels, as reported by pharmacological modulation studies performed with apamin (APA) and charybdotoxin (ChTX), inhibitors of SK_{Ca} and BK_{Ca}, respectively [18–21]. Noteworthy, EDHF is not a single molecule but, more likely, it represents a group of molecules [20]. A number of putative chemical mediators, having EDHF like activity, have been identified such as H_2O_2, cytochrome P450 products such as the epoxyeicosatrienoic acids, C-type natriuretic peptide as well as the gases carbon monoxide (CO) and more recently hydrogen sulfide (H_2S) [20–23]. H_2S has emerged as a critical vascular signaling molecule similar to NO and CO with a profound impact on heart and circulation [24]. H_2S is endogenously produced from L-cysteine and/or L-methionine mainly by two enzymes, cystathionine-β-synthase (CBS) and cystathionine-γ-lyase (CSE) [25]. Interestingly, recent evidence reported that DEX suppresses both CSE expression and H_2S production in neutrophils and primary macrophages isolated from rats [26,27]. These findings suggest that DEX may have an impact on H_2S pathway. On this basis we investigated the involvement of EDHF/H_2S in GC-induced hypertension by using isolated mesenteric plexus and carotid artery harvested from rats treated *in vivo* with DEX or vehicle.

2. Material and Methods

2.1. Animals

Male Wistar rats (200-250g, Harlan, Udine, Italy) were used for in vivo and ex vivo experiments. Rats were randomly divided in two groups ($n = 12$ for each group) and treated subcutaneously with DEX (1.5 mg/kg/day, Sigma-Aldrich, Milan, Italy) or vehicle (saline 0.9% NaCl, 1 ml/kg/day) for 8 days, as previously described [28]. Animals were kept under temperature 23 ± 2 °C, humidity range of 40–70%, and 12-h light/dark cycles. Food and water were fed ad libitum. The present study was approved by the Animal Ethics Committee of the University of Naples "Federico II" (Italy), in agreement with both Italian and European guidelines for animal care.

2.2. Measurement of arterial blood pressure in conscious rat

The systolic blood pressure (SBP) was recorded in conscious rats by a tail cuff connected to a blood pressure recorder (Blood Pressure Recorder, Ugo Basile Apparatus, Comerio, Italy). After a week of training period, rats were treated as described above and blood pressure was monitored every 2 days [29]. Data were expressed as mmHg and calculated as mean ± SE. Results were analyzed by Student's *t*-test. A value of $p < 0.05$ was considered significant.

Rats were sacrificed after 8 days of treatment and mesenteric plexus, carotid artery and blood were harvested.

2.3. Ex vivo experiments: isolated and perfused mesenteric vascular bed

Mesenteric bed preparation was performed as previously described [30]. Briefly, in anesthetized rats the superior mesenteric artery was cannulated to perfuse the whole vascular bed with Krebs' buffer containing heparin (10 IU/ml; Sigma-Aldrich) for 5 min at 2 ml/min. The mesenteric bed, separated from intestine and connected to a pressure transducer (Bentley 800 Trantec; Ugo Basile, Comerio, Italy), was perfused by warmed (37 °C) and gassed (95% O_2 and 5% CO_2) Krebs' solution (2 ml/min) composed of NaCl (115.3 mM), KCl (4.9 mM), $CaCl_2$ (1.46 mM), $MgSO_4$ (1.2 mM), KH_2PO_4 (1.2 mM), $NaHCO_3$ (25.0 mM) and glucose (11.1 mM) (Carlo Erba Reagents, Milan, Italy) with INDO. In order to evaluate the contribution of EDHF, that hyperpolarizes vascular smooth muscle cells by COX- and NOS-independent relaxation, all the experiments were performed with Krebs' solution medicated with indomethacin (INDO; 10 μM; Sigma-Aldrich) *plus* L-NG-Nitro arginine methyl ester (L-NAME, 100 μM, Sigma-Aldrich), inhibitors of COX and NOS respectively. Thereafter, U-46619 (0.1 μM, Sigma-Aldrich), a stable thromboxane-A_2 analogue, was added to the Krebs' solution to obtain a stable contraction. A concentration–response curve of acetylcholine (Ach, 1–100 μM; Sigma-Aldrich) was performed on stable tone by U-46619 in presence of INDO plus L-NAME. APA (5 μM, Sigma-Aldrich) *plus* ChTX (100 nM, Sigma-Aldrich), inhibitors of SK_{Ca} and BK_{Ca} channels respectively, i.e. EDHF targets [30], or propargylglicine (PAG, 10 mM, Sigma-Aldrich), CSE inhibitor, were used. The Ach-induced relaxation was calculated as area under the curve (mmHg × min). Basically, during all the experiments the tissue was continuously perfused with INDO and L-NAME that were added to the Krebs' solution. Data were expressed as mean ± SE. Results were analyzed by two-way ANOVA followed by Bonferroni's post-test. A value of $p < 0.05$ was considered significant.

2.4. Ex vivo experiments: carotid artery rings

Carotid artery rings were performed as previously described [31]. Briefly, after treatment, carotid arteries were excised and carefully cleaned from connective tissue and cut in rings 2–3 mm long. The rings were filled with thermostated (37 °C) and gassed (95% O2 and 5% CO2) Krebs' solution, composed as reported above, connected to isometric force transducers (FORT10, 2Biological Instruments, Varese, Italy) and changes in tension continuously recorded using a computerized system (PowerLab ADInstrument, 2Biological Instruments). After equilibration, contribution of EDHF was evaluated in presence of Krebs' solution medicated with INDO (10 μM) *plus* L-NAME (100 μM), inhibitors of COX and NOS respectively, and a concentration–response curve of Ach (10 nM–10 μM) was performed on phenylephrine (PE; 0.3 μM, Sigma-Aldrich) stable tone. APA (5 μM) *plus* ChTX (100 nM), inhibitors of SK_{Ca} and BK_{Ca} channels respectively, or PAG (10 mM), CSE inhibitor, were also used. All the experiments were performed in presence of INDO and L-NAME that were added to the organ bath. Relaxing response was calculated as percentage of maximal contraction to PE. Data were expressed as mean ± SE and results were analyzed by two-way ANOVA followed by Bonferroni's post-test. A value of $p < 0.05$ was considered significant.

2.5. Western blot analysis

Western blot study was performed as previously described [32]. Briefly, frozen mesenteries or carotids were homogenized in a modified RIPA buffer (Tris–HCl 50 mM, pH 7.4, Triton 1%, sodium deoxycholate 0.25%, NaCl 150 mM, EDTA 1 mM, phenylmethylsulphonyl fluoride 1 mM, aprotinin 10 μg/ml, leupeptin 20 μM, NaF 1 μM, sodium orthovanadate 1 μM, Sigma-Aldrich). Protein concentration was determined by the Bradford assay using bovine serum albumin as standard. Equal amounts of protein (60 μg) from tissue lysates were separated on 8% sodium dodecyl sulfate polyacrylamide gels and transferred to a polyvinylidene fluoride membrane. Membranes were blocked by incubation in

phosphate-buffered saline (PBS) containing 0.1% v/v Tween 20, non fat dry milk (5%) and NaF (50 mM) for 1 h, followed by overnight incubation at 4 °C with the following antibodies: polyclonal rabbit anti-CBS (1:1000; Santa Cruz, Heidelberg, Germany), monoclonal mouse anti-CSE (1:1000; Abnova, Milan, Italy) and monoclonal mouse anti-β-actin (Sigma-Aldrich). Results were normalized with β-actin expression, as referential protein. Densitometric analyses were performed by using a gel imaging digital system (Image Quant-400 Capture; GE Healthcare, Milan, Italy). Data were expressed as mean ± SE. Results were analyzed by Student's *t* test. A value of $p < 0.05$ was considered significant.

2.6. H_2S measurement in vessel homogenates and in plasma

H_2S production was evaluated as previously described [33]. Mesenteries or carotids were treated with lysis buffer containing potassium phosphate buffer (100 mM, pH 7.4), sodium orthovanadate (10 mM) and proteases inhibitors. Homogenate samples were added for 40 min in a reaction mixture (total volume 500 µl) containing piridoxal-5′-phosphate (2 mM, 20 µl) and saline (20 µl) or L-cysteine (10 mM, 20 µl, Sigma-Aldrich, Milan, Italy) to measure enzyme activity i.e. H_2S generation. The reaction was performed in parafilmed Eppendorf tubes and initiated by transferring tubes from ice to a water bath at 37 °C and carried out for 40 min. After incubation, zinc acetate (1%, 250 µl, Sigma-Aldrich) and trichloroacetic acid (10%, 250 µl) were added. Subsequently, N,N-dimethyl-p-phenylendiamine sulfate (DPD, 20 mM, Sigma-Aldrich) in HCl (7.2 M) and iron chloride ($FeCl_3$, 30 mM, Sigma-Aldrich) in HCl (1.2 M) were added and optical absorbance of the solutions was measured after 20 min at a wavelength of 667 nm. All samples were assayed in duplicate and H_2S concentration was calculated against a calibration curve of NaHS (3.9–250 µM, Sigma-Aldrich).

Plasma determination of H_2S was performed as described by d'Emmanuele di Villa Bianca et al. [34]. Briefly, 200 µl of plasma were added in Eppendorf tubes containing trichloroacetic acid (10%, 300 µl), after centrifugation (10,000 rpm for 10 min, at 4 °C) zinc acetate (1% w/v, 150 µl) was added to each sample. Subsequently, DPD (20 mM, 100µl, in 7.2 M HCl) and $FeCl_3$ (30 mM, 133 µl, in 1.2 M HCl) were added to the reaction mixture and then H_2S determination followed the same protocol described above. Data were calculated as nmol/mg protein/min or µM respectively, and expressed as mean ± SE of delta increase (with or without the substrate L-cysteine). The results were analyzed by Student's *t* test. A value of $p < 0.05$ was considered significant.

3. Results

3.1. Measurement of blood pressure in conscious rats

SBP was measured in all animals prior to starting treatment with vehicle or DEX. SBP values were not significantly different in the two experimental groups (101.3 ± 2.6 mmHg *vs* 99.3 ± 7.7 mmHg, $n = 12$). Treatment with vehicle did not affect SBP values throughout the experiment. Rats treated daily with DEX (1.5 mg/kg, for 8 days) showed a significant rise in SBP (Fig. 1). Noteworthy, a significant increase in SBP was already evident after 2 days of treatment reaching a maximal value at day 8 ($p < 0.05$ at days 2 and 4, $p < 0.01$ at day 6, $p < 0.001$ at day 8, Fig. 1). After 8 days of treatment the SBP values were 98.5 ± 6.0 and 179.0 ± 6.7 mmHg for vehicle and DEX group, respectively.

3.2. EDHF-induced relaxation in mesenteric plexus from DEX- or vehicle-treated rats

In mesentery harvested from vehicle-treated rats, basal arterial perfusion pressure was 15.8 ± 1.3 mmHg. In mesentery harvested

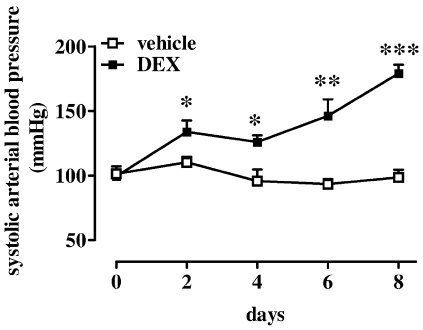

Fig. 1. Dexamethasone increases systolic blood pressure in rat. Evaluation of systolic blood pressure (SBP) in conscious rats treated with vehicle or DEX during 8 days of treatment. DEX treatment (1.5 mg/kg i.p.) caused a significant increase in SBP compared with vehicle ($^*p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$). Data were expressed as mean ± SE ($n = 12$) and analyzed by Student's *t*-test.

from DEX-treated rats the basal perfusion pressure was significantly increased, reaching 22.3 ± 2.3 mmHg ($p < 0.05$). Once the mesenteric beds obtained either from vehicle or DEX-treated rats were challenged with U-46619 (0.1 µM), the maximal increase in perfusion pressure achieved was not statistically different between the two groups (49.0 ± 8.3 mmHg *vs* 60.4 ± 4.4 mmHg for vehicle- and DEX-treated rats, respectively). The addition of L-NAME on U46619 stable tone produced a further increase in perfusion pressure of mesenteric arteries that was similar in both groups (92.9 ± 11.3 mmHg for the vehicle and 85.1 ± 4.6 mmHg ($n = 9$) for DEX-treated rats).

In order to evaluate the EDHF contribution, once stable tone was achieved upon U-46619 administration, Ach concentration–response curve was performed in presence of INDO (10 µM) *plus* L-NAME (100 µM). Ach caused a concentration-dependent vasodilatation in mesenteries of both groups (Fig. 2A). Interestingly, Ach-induced relaxation was significantly reduced in DEX-treated animals ($p < 0.001$, Fig. 2A). To further confirm EDHF involvement, the mesentery was perfused with Krebs' solution medicated with INDO and L-NAME *plus* a combination of APA (5 µM) and ChTX (100 nM), using the widely accepted protocol to evaluate the EDHF contribution (Fig. 2B). The drug association abolished Ach-induced effect in mesenteric bed of either vehicle or DEX-treated rats ($p < 0.001$, Fig. 2B). In order to determine whether or not H_2S was involved in EDHF impairment observed in DEX-treated group we used PAG, a CSE inhibitor, instead of the combination of APA/ChTX. The addition of PAG (10 mM) to Krebs' solution, medicated with INDO *plus* L-NAME, abolished Ach-induced vasodilatation in both vehicle and DEX groups ($p < 0.001$, Fig. 2C).

3.3. EDHF-induced relaxation in carotid arteries from DEX- or vehicle-treated rats

The carotid arteries harvested from DEX- or vehicle-treated animals showed no significant difference in contraction to PE (827 ± 105 and 1274 ± 227 dyn/mg). In order to evaluate the contribution of EDHF in carotid artery, a concentration–response curve to Ach (10 nM–10 µM) was performed on PE induced contraction in Krebs' solution medicated with INDO (10 µM) *plus* L-NAME (100 µM). In this experimental condition PE-induced contraction was similar between DEX and vehicle group (1182 ± 108. and 1441 ± 161.3 dyn/mg, $n = 10$). Conversely, Ach-induced relaxation was significantly reduced in carotids harvested from DEX-treated rats compared with vehicle group ($p < 0.001$, Fig. 3A). The combination of APA and ChTX markedly reduced Ach vasorelaxant effect in both groups ($p < 0.001$, Fig. 3B). In an another set of experiments, addition of PAG (10 mM), to Krebs' solution, already medicated with INDO *plus*

L-NAME, abolished Ach-induced relaxation in both vehicle- and DEX-treated animals ($p < 0.001$, Fig. 3C).

3.4. H_2S pathway in mesenteric plexus from DEX- or vehicle-treated rats

Based on *ex vivo* results, we next evaluated the expression of CBS and CSE by western blot analysis in homogenate samples of mesenteric beds harvested from rats treated with DEX or vehicle. Western blot data clearly showed that DEX treatment significantly reduced both CBS and CSE expression in mesenteries ($p < 0.05$, Fig. 4A and B). In order to further confirm these data we used homogenate samples from both treatment groups as enzymatic source. Mesenteric homogenates from DEX-treated rats displayed a significant lower production of H_2S compared with vehicle group ($p < 0.05$, Fig. 4C).

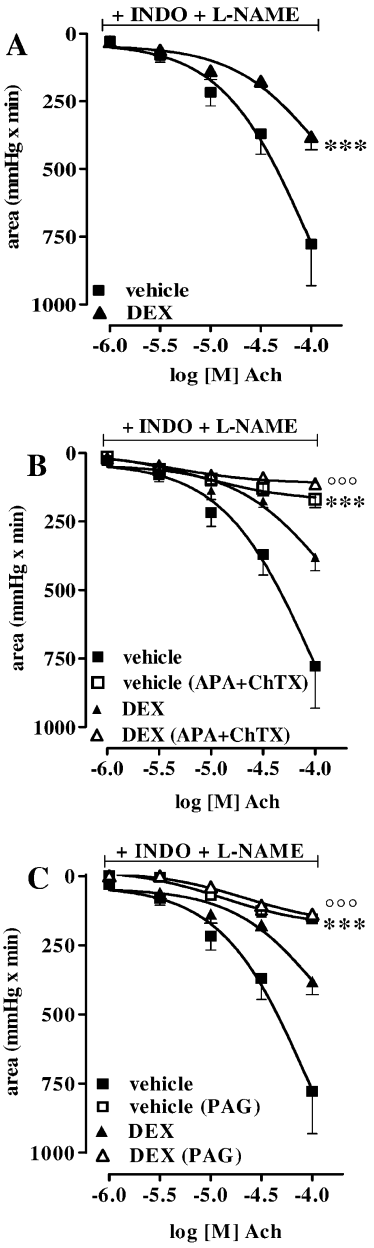

Fig. 2. Dexamethasone impairs EDHF-mediated vasodilatation in mesenteric vascular bed. Concentration–response curve to Ach (1–100 μM) on stable tone induced by U-46619 (0.1 μM) in mesenteric vascular bed in presence of INDO (10 μM) *plus* L-NAME (100 μM). (A) Ach-induced relaxation was significantly reduced in DEX group (***$p < 0.001$ *vs* vehicle). (B) The combination of APA (5 μM) *plus* ChTX (100 nM) abolished Ach-induced relaxation in both groups (***$p < 0.001$ *vs* vehicle and °°°$p < 0.001$ *vs* DEX). (C) PAG (10 mM), abrogated Ach-induced relaxation in both groups (***$p < 0.001$ *vs* vehicle and °°°$p < 0.001$ *vs* DEX). The Ach-induced relaxation was calculated as area under the curve (mmHg × min). Data were expressed as mean ± SE ($n = 4$–8) and analyzed by two-way ANOVA followed Bonferroni as post test.

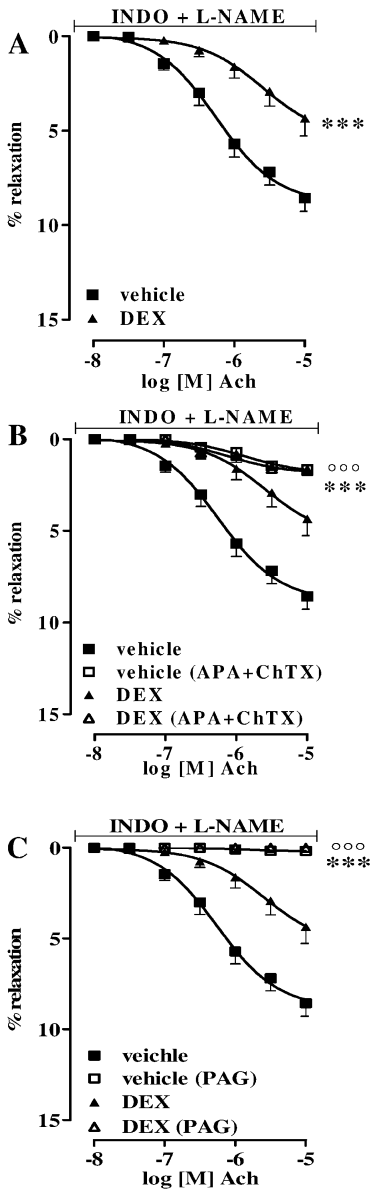

Fig. 3. Dexamethasone impairs EDHF-mediated vasodilatation in carotid artery. Concentration–response curve to Ach (10 nM–10 μM) on stable contraction induced by PE (0.3 μM) in carotid arteries in presence of INDO (10 μM) *plus* L-NAME (100 μM). (A) Ach-induced relaxation was significantly reduced in DEX group (***$p < 0.001$). (B) The combination of APA (5 μM) *plus* ChTX (100 nM) completely abolished Ach-induced relaxation in both groups (***$p < 0.001$ *vs* vehicle and °°°$p < 0.001$ *vs* DEX). (C) PAG (10 mM) abrogated Ach-induced relaxation in both groups (***$p < 0.001$ *vs* vehicle and °°°$p < 0.001$ *vs* DEX). The Ach-induced relaxation was calculated as % of relaxation. Data were expressed as mean ± SE ($n = 6$) and analyzed by two-way ANOVA followed by Bonferroni as post test.

3.5. H_2S pathway in carotid arteries from DEX- or vehicle-treated rats

Western blot analysis clearly showed that DEX treatment significantly reduced both CBS and CSE expression in carotid arteries

($p < 0.05$, Fig. 5A and B). Homogenates of carotid arteries obtained from DEX-treated rats displayed a significant reduction in H_2S production in comparison with vehicle ($p < 0.05$, Fig. 5C).

3.6. H_2S plasma levels in DEX- or vehicle-treated rats

In order to confirm the role of H_2S pathway in GC-induced hypertension, we also measured H_2S plasma levels in animals after 8-days of treatment in both vehicle and DEX groups. Rats treated with DEX showed H_2S circulating levels significantly lower than those observed in vehicle-treated rats ($p < 0.05$, Fig. 6).

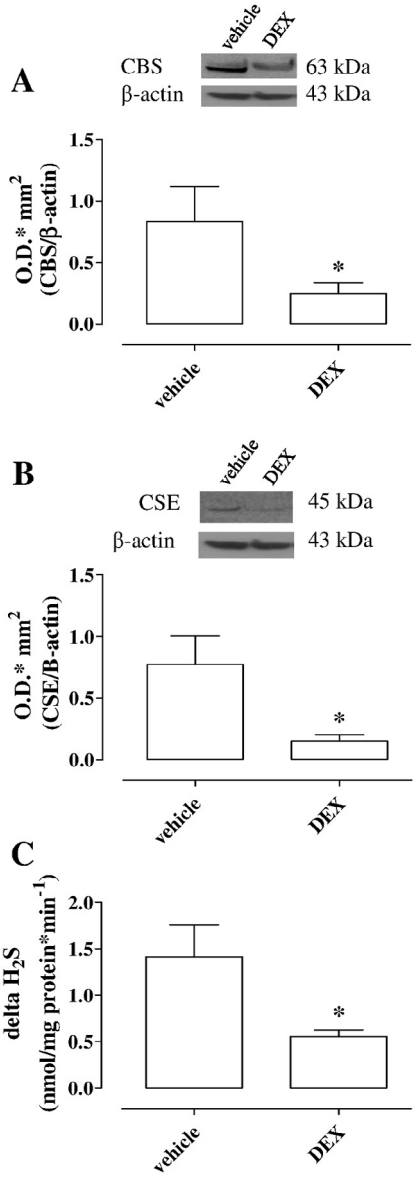

Fig. 4. Dexamethasone impairs H_2S signaling in rat mesenteric vascular bed. Representative western blot and optical densitometric (O.D.) analysis for CBS (A) and CSE (B). CBS and CSE expression were markedly reduced by DEX treatment ($^*p < 0.05$ vs vehicle). Data were normalized against β-actin as a housekeeping protein, expressed as mean ± SE ($n = 4$) and analyzed by Student's t-test. (C) The activity of both CBS and CSE was evaluated as delta of increase in H_2S production. H_2S production was significantly lower in DEX group ($^*p < 0.05$ vs vehicle). Data were expressed as mean ± SE ($n = 4$) and analyzed by Student's t-test.

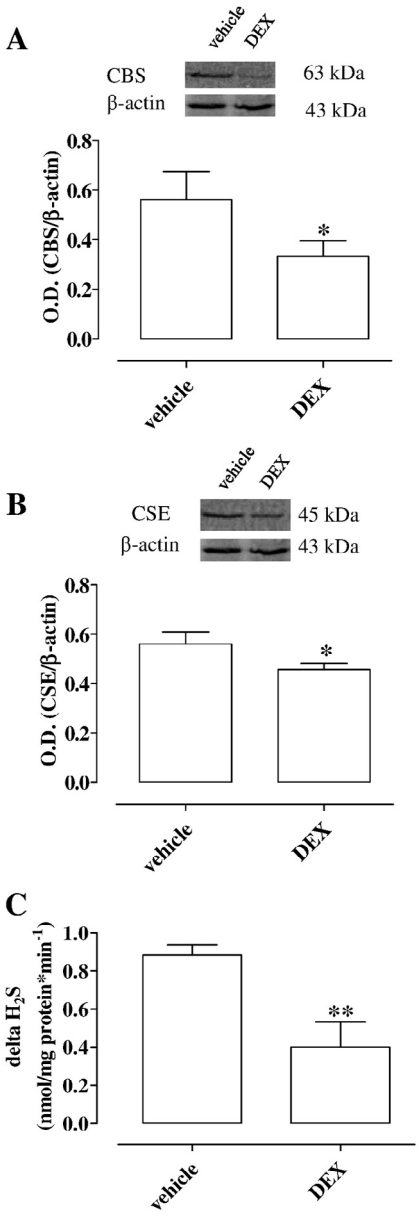

Fig. 5. Dexamethasone impairs H_2S signaling in rat carotid artery. Representative western blot and optical densitometric (O.D.) analysis for CBS (A) and CSE (B). CBS and CSE expression were markedly reduced by DEX treatment ($^*p < 0.05$ vs vehicle). Data were normalized against β-actin as a housekeeping protein, expressed as mean ± SE ($n = 4$) and analyzed by Student's t test. (C) The activity of both CBS and CSE was evaluated as delta of increase in H_2S production. H_2S production was significantly lower in the DEX group ($^*p < 0.05$ vs vehicle). Data were expressed as mean ± SE ($n = 4$) and analyzed by Student's t-test.

4. Discussion

Prolonged glucocorticoid therapy produces highly significant elevation in blood pressure in man and experimental animal models [17]. Despite of several studies, the mechanism of GC-induced hypertension remains poorly understood. Sodium and water retention contributes, but are not essential, to the development of GC-induced hypertension [35,36]. Indeed, different synthetic GCs raise blood pressure in human, without any increase in sodium retention and volume expansion [35]. It has also been shown that GC-induced hypertension occurs independently by the level of salt intake [37]. Conversely, cortisol-induced hypertension is accompanied by substantial but not significant sodium retention [38]. In general, the

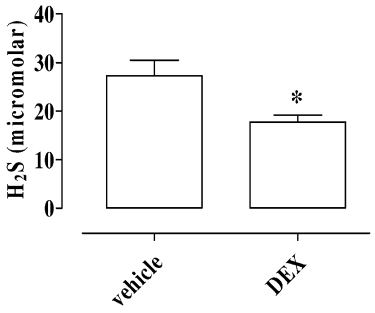

Fig. 6. Dexamethasone impairs H_2S plasma levels. Circulating H_2S levels were significantly reduced in DEX group compared with vehicle ($^*p < 0.05$). Data were expressed as mean ± SE ($n = 12$) and analyzed by Student's t-test.

relative quick onset of GC-induced hypertension is inconsistent with a mechanism based solely on renal sodium re-absorption. These findings imply the presence of a direct effect of GC on vascular tone. Consistent with literature, we found that a daily treatment with DEX leads to an increase in SBP that becomes significant within 48 h. In order to understand the contribution of the vessel reactivity to this phenomenon we harvested mesenteric and carotid arteries from rats treated with either DEX or vehicle. In mesenteric vascular bed in DEX-treated rats there was a clear increase in the basal perfusion pressure as compared with vehicle indicating, that DEX treatment increased the basal tone, altering the vascular homeostasis. However, when the mesentery was challenged with U-46619, to elicit a stable contraction, DEX group reached the same level of contraction of control group implying, that the mesenteric bed keeps intact its ability to respond to the exogenous contractile stimulus applied. This phenomenon is not restricted to the mesentery bed since isolated carotid arteries showed a similar PE-induced contraction profile in both control and DEX group.

It is well known that mesenteric vascular bed, together with carotid artery, markedly contributes to total peripheral resistance controlling blood pressure [39]. At the present stage it is well accepted that in small resistance arteries EDHF plays a key role in the response to vasoactive substances and thus in the regulation of vascular tone. EDHF is a diffusible factor that causes smooth muscle hyperpolarization and thus vasodilatation, independent by NO and prostaglandins [40,41]. Several mediators have been shown to account for EDHF activity, such as arachidonic acid metabolites derived from cytochrome P450 pathway or other molecules as H_2O_2, CO [20–23]. More recently, H_2S has been proposed as a possible candidate for EDHF(s) [23,30]. H_2S is a member of the gasotransmitter family implicated in several physiological and pathological processes within the body [38–42], particularly, in regulation of vascular tone [23]. The deficiency of CSE leads to an increase in blood pressure and an impairment of endothelium-dependent vasorelaxation in mice [43]. Moreover, it has been shown that knocking-out the expression of CSE in mice, methacholine-induced endothelium-dependent relaxation of mesenteric arteries is abrogated [43]. In addition, it has also been reported that the reduction in endogenous H_2S production is essential for the development of spontaneous hypertension [44].

Therefore, we have evaluated the involvement of EDHF-like activity in DEX-induced hypertension. In our experimental condition the Ach-induced relaxation mediated by EDHF, e.g. in presence of INDO *plus* L-NAME, was significantly reduced in pre-constricted mesenteric arterial bed and carotid artery of DEX-treated rats. Furthermore, the vasodilating effect was abrogated in both mesenteric and carotid arteries by blocking SK_{Ca} and BK_{Ca} channels, i.e. EDHF targets [41]. Thus, an impairment in EDHF-mediated relaxation concurs to GC-induced hypertension. Since the recent literature hypothesizes that H_2S is a candidate for EDHF [23], we have addressed this issue. Arterial mesenteric bed or carotid arteries in presence of PAG, a selective CSE inhibitor, abolished EDHF-dependent vasodilatation. The inhibition of EDHF-dependent vasodilatation paralleled the reduction of endogenous production of H_2S. These findings taken together confirm a key role for H_2S in DEX-induced hypertension and support the concept that H_2S is a feasible candidate for EDHF-like activity [23,30]. To further extend our findings, we evaluated the expression and activity of CBS and CSE in DEX-induced hypertensive rats. The expression of both CBS and CSE was significantly reduced in both vascular district of DEX-treated animals and their enzymatic activity was impaired. Indeed, H_2S biosynthesis, following incubation with the substrate L-cysteine, was markedly reduced in both mesenteric and carotid tissue homogenate harvested from DEX-treated rats compared with vehicle group. Therefore, DEX treatment inhibits H_2S biosynthesis through the impairment of CBS and CSE expression contributing to the increase in blood pressure. This hypothesis is further corroborated by the fact that rats treated with DEX and showing hypertension, displayed also a significant reduction in H_2S circulating levels. Our findings are in line with other literature data showing that inhibition of H_2S pathway occurs following DEX treatment [26,27]. Indeed, DEX (i) directly inhibits CSE expression and H_2S production *in vitro* [26], (ii) abolishes LPS-induced rise in H_2S concentration in both plasma and tissue *in vivo* [27], (iii) inhibits CSE expression in LPS-challenged neutrophils and in human fetal liver cells [27].

In conclusion a significant impairment in H_2S generation occurs in carotid and mesenteric bed of rats treated with DEX and this contributes to the development of hypertension. We have also shown that H_2S mimics EDHF activity and it is impaired in DEX-induced hypertension. Our results indicate that the H_2S pathway plays an important role in the homeostasis of vascular resistance vessels. Based on these findings, the development of GC-H_2S-releasing compounds could represent a useful therapeutic tool to manage the GC-induced hypertension. In addition, the measurement of H_2S plasma levels could be considered as bio-marker in order to monitor the GC therapy.

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