Nitric Oxide ■■ (2014) ■■–■■

Contents lists available at ScienceDirect

Nitric Oxide

journal homepage: www.elsevier.com/locate/yniox

# Hydrogen sulfide is involved in dexamethasone-induced hypertension in rat

Roberta d'Emmanuele di Villa Bianca [a,1], Emma Mitidieri [a,1], Erminia Donnarumma [a], Teresa Tramontano [a], Vincenzo Brancaleone [b], Giuseppe Cirino [a,*], Mariarosaria Bucci [a], Raffaella Sorrentino [a]

[a] Department of Pharmacy, University of Naples, Federico II, Via D. Montesano, 49, 80131 Naples, Italy
[b] Department of Science, University of Basilicata, Via dell'Ateneo Lucano, 85100 Potenza, Italy

ARTICLE INFO

*Article history:*
Received 14 October 2014
Revised 14 November 2014
Accepted 21 November 2014
Available online

*Keywords:*
Glucocorticoid-induced hypertension
EDHF
Hydrogen sulfide
Mesentery
Carotid artery
Rat

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.

* Corresponding author. Department of Pharmacy, Via D. Montesano, 49, 80131 Naples, Italy. Fax: +39/081678403.
*E-mail address:* cirino@unina.it (G. Cirino).

[1] The authors have equally contributed.

http://dx.doi.org/10.1016/j.niox.2014.11.013
1089-8603/

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

Please cite this article in press as: Roberta d'Emmanuele di Villa Bianca, et al., Hydrogen sulfide is involved in dexamethasone-induced hypertension in rat, Nitric Oxide (2014), doi: 10.1016/j.niox.2014.11.013