Research Article

Dexamethasone, tetrahydrobiopterin and uncoupling of endothelial nitric oxide synthase

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Abstract

Objective To find out whether dexamethasone induces an uncoupling of the endothelial nitric oxide synthase (eNOS).

Methods & Results A major cause of eNOS uncoupling is a deficiency of its cofactor tetrahydrobiopterin (BH4). Treatment of human EA.hy 926 endothelial cells with dexamethasone decreased mRNA and protein expression of both BH4-synthesizing enzymes: GTP cyclohydrolase I and dihydrofolate reductase. Consistently, a concentration- and time-dependent reduction of BH4, dihydrobiopterin (BH2) as well as BH4: BH2 ratio was observed in dexamethasone-treated cells. Surprisingly, no evidence for eNOS uncoupling was found. We then analyzed the expression and phosphorylation of the eNOS enzyme. Dexamethasone treatment led to a down-regulation of eNOS protein and a reduction of eNOS phosphorylation at serine 1177. A reduction of eNOS expression may lead to a relatively normal BH4: eNOS molar ratio in dexamethasone-treated cells. Because the BH4:eNOS stoichiometry rather than the absolute BH4 amount is the key determinant of eNOS functionality (i.e., coupled or uncoupled), the down-regulation of eNOS may represent an explanation for the absence of eNOS uncoupling. Phosphorylation of eNOS at serine 1177 is needed for both the NO-producing activity of the coupled eNOS and the superoxide-producing activity of the uncoupled eNOS. Thus, a reduction of serine 1177 phosphorylation may render a potentially uncoupled eNOS hardly detectable.

Conclusions Although dexamethasone reduces BH4 levels in endothelial cells, eNOS uncoupling is not evident. The reduction of NO production in dexamethasone-treated endothelial cells is mainly attributable to reduced eNOS expression and decreased eNOS phosphorylation at serine 1177.

J Geriatr Cardiol 2015; 12: 528–539. doi:10.11909/j.issn.1671-5411.2015.05.013

Keywords: Dexamethasone; Endothelial cells; eNOS uncoupling; Nitric oxide synthase; Reactive oxygen species; Tetrahydrobiopterin

1 Introduction

Glucocorticoids are steroid hormones that are synthesized in the adrenal cortex. Glucocorticoids play an essential role in physiological processes like immune response, energy metabolism, electrolyte balance, and stress response. Chronic excessive activation of glucocorticoid receptor induces obesity, insulin resistance, glucose intolerance, dyslipidemia and hypertension.[1] Abnormalities of the hypothalamic-pituitary-adrenal axis and/or of tissue sensitivity to glucocorticoids are also associated with these cardiovascular risk factors in patients with the metabolic syndrome. Furthermore, glucocorticoids have direct effect on heart and blood vessels, mediated by both glucocorticoid and mineralocorticoid receptors.[2,3] These effects influence vascular function, atherogenesis and vascular remodeling following intra-vascular injury or ischemia.

Synthetic glucocorticoids, such as dexamethasone (Dex), play a fundamental role in the treatment of inflammatory disease. One of the side effects of Dex treatment is an elevation of blood pressure. Our group has previously demonstrated that a major mechanism underlying the Dex-induced hypertension is a reduced nitric oxide (NO) production from the endothelium.[4]

Under physiological conditions, vascular NO is mainly produced by endothelial nitric oxide synthase (eNOS).[5] Endothelial NO relaxes blood vessels and reduces blood pressure. It diffuses from endothelial cells into the underlying smooth muscle cells and induces vasodilation by stimulating NO-sensitive guanylyl cyclase. NO produced by the endothelial eNOS also diffuses into the blood and inhibits platelet aggregation and adhesion. In addition to these anti-
hypertensive and antithrombotic actions, eNOS-derived NO also possesses multiple anti-atherosclerotic properties, including prevention of leukocyte adhesion to vascular endothelium and leukocyte migration into the vascular wall; inhibition of low-density lipoprotein oxidation; and inhibition of vascular smooth muscle cell proliferation. Genetic depletion of eNOS elevates blood pressure, and exacerbates diet-induced atherosclerosis in mouse models.

We have shown that Dex reduce eNOS mRNA and protein expression in human endothelial cells by transcriptional inhibition as well as mRNA destabilization. The observed down-regulation of eNOS could be also demonstrated in vivo. Oral Dex treatment diminished eNOS mRNA level in several endothelium-rich organs in rats. Furthermore, plasma levels of NO\textsubscript{2}/NO\textsubscript{3} (NO oxidation products) were reduced as well as endothelium-dependent vasodilation. In addition, a significant increase in blood pressure could be observed in Dex-treated animals. All these results suggest that Dex-induced blood pressure elevation is attributable to a reduced NO production by the eNOS enzyme. Consistent with this concept, our group further demonstrated that glucocorticoids had no effect on the blood pressure in eNOS\textsuperscript{-} mice.

Recent studies have shown that eNOS can be converted from a NO-producing enzyme to a molecule that generates superoxide under pathological conditions. This is referred to as eNOS uncoupling. Uncoupling of eNOS is known to be the major cause of endothelial dysfunction in a number of cardiovascular diseases, including hypertension, atherosclerosis, and diabetes mellitus.

Therefore, we conducted the present study to find out whether Dex treatment also causes eNOS uncoupling.

2 Methods

2.1 Cell culture

Human EA.hy 926 endothelial cells were grown under 10 % CO\textsubscript{2} in Dulbecco’s modified Eagle’s medium (DMEM, Sigma-Aldrich) supplemented with 10% fetal bovine serum, 2 mmol/L L-glutamine, 1 mmol/L sodium pyruvate, 100 U/mL penicillin, 100 µg/mL streptomycin and 1× HAT (hypoxanthine, aminopterin and thymidine) (Invitrogen). U/mL penicillin, 100 µg/mL streptomycin and 1× HAT (hypoxanthine, aminopterin and thymidine) (Invitrogen). Cells were treated with dexamethasone or its solvent dimethyl sulfoxide (DMSO) as control.

2.2 Real-time PCR for mRNA expression analyses

Gene expression at mRNA level was analyzed with quantitative real-time PCR (qPCR) using an iCycler IQ\textsuperscript{TM} System (Bio-Rad Laboratories). Total RNA was isolated from cultured endothelial cells using peqGOLD TriFast (PEQLAB Biotechnologie GmbH). cDNA was generated using High-Capacity cDNA Reverse Transcription Kit (Life Technologies) and 40 ng of cDNA was used for qPCR analysis. The mRNA expression levels of the target genes were normalized to TATA box binding protein (TBP) mRNA. The qPCR primer sequences were as follows: eNOS\textsubscript{forward}: 5'-CAC CAG GAA GAA GAC CTT TAA AGA-3', eNOS\textsubscript{reverse}: 5'-TCA CTC GCT TCG CCA TCA C-3'; GCH1\textsubscript{forward}: 5'-TCA GTG TGT GTC TGC AGA ACC A-3', GCH1\textsubscript{reverse}: 5'-TGT CTT CTA CCA CCG TCA GTT CAT T-3'; DHFR\textsubscript{forward}: 5'-TCC TCC CGC TGC TGT CA-3', DHFR\textsubscript{reverse}: 5'-GCC GAT GCC CAT GTT CTG-3'; TBP\textsubscript{forward}: 5'-GCC GGC GCC TGT TTA ACT-3', TBP\textsubscript{reverse}: 5'-ACG CCA AGA AAC AGT GAT GCT-3'.

2.3 Western blotting for protein analyses

Confluent EA.hy 926 cells were incubated with dexamethasone and then total protein was isolated. Western blotting was performed using 30 µg of protein sample and antibodies against dihydrofolate reductase (DHFR, BD Biosciences), GTP cyclohydrolase I (GCH1, Abnova), eNOS (BD Biosciences), eNOS phospho-serine1177 (Cell Signaling) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH, Santa Cruz Biotechnology). Protein samples were separated on 10% SDS polyacrylamide gels and transferred onto nitrocellulose membranes. Blots were blocked for 1 h at room temperature with 5% powdered milk in TBS (10 mmo/L Tris HCl, pH 7.4, 150 mmo/L NaCl) with 0.1% Tween 20 and the incubated with the primary antibodies in 5 % powdered milk. Blots were washed three times in TBS/Tween 20 (0.1 %) and then incubated with a horseradish peroxidase conjugated secondary antibody in 5% powdered milk and 0.1% Tween 20 in TBS for 1 h at room temperature. After washing, immune complexes were developed using an enhanced horseradish peroxidase/luminol chemiluminescence reagent (PerkinElmer Life Science) according to the manufacturer’s instructions.

2.4 Detection of biopterins with HPLC

EA.hy 926 cells were plated in 6-well plates and treated with Dex. After treatment, the cells were washed twice with ice-cold PBS (50 mL PBS + 50 µL 1,4-dithioerythritol + 10 µL Pefabloc\textsuperscript{®}). Cells were scraped off the wells with ice-cold PBS\textsuperscript{+} (PBS + 5 nmol/L Neopterin) and identical samples were pooled. HPLC analyses were performed with phosphate buffer (50 mmol/L, pH 3.0) as eluent in appropriate columns (125/4 Nucleosil SA 100-5 mm, Macherey-Nagel) at 25°C and the flow rate of 1.2 mL/min.
Equipment from Bischoff was used as well as the Hitachi fluorescence detector 8470 (excitation: 350 nm, emission: 450 nm). Evaluation was done with the help of MCDAcq Integrator software from Bischoff.\[16,17\]

2.5 Detection of reactive oxygen species (ROS)

ROS levels were measured with H$_2$DCFDA fluorescence,\[18\] and fluorescence-activated cell sorting (FACS) analyses.\[19\]

For H$_2$DCFDA fluorescence, cells were washed with Hanks balanced salt solution (HBSS) followed by a 30 min incubation step with the H$_2$DCFDA (2.5 µmol/L) at 37°C. Cells were again washed with HBSS (37°C) and covered with pure HBSS or HBSS in combination with a substance like L-NAME for the measurement, depending on the experiment. ROS development was monitored up to 12 h within the plate reader (excitation: 485 nm, emission: 530 nm).\[18\]

For FACS analyses, the fluorescent dye H$_2$DCFDA was used again. EA.hy 926 cells were seeded into 24-well plates to 80% confluence before treatment. After incubation with an experiment-specific substance, cells were washed with PBS. Subsequently, cells were incubated with H$_2$DCFDA (2.5 µmol/L) in HBSS for 30 min at 37°C in the dark. Afterwards, cells were washed again with PBS and trypsinized. Medium was added to stop the enzymatic reaction and cells were collected in a 1.5 mL tube. After a 4-min centrifugation at 350 r/min and 4°C, the supernatant was discarded. The cell pellet was resuspended in 300 µL PBS and transferred to a FACS-tube for analysis using the FACSCalibur™ flow cytometer (BD Biosciences).\[19\]

2.6 RFL-6 reporter cell assay for NO measurement

NO production by EA.hy 926 cells was bioassayed with RFL-6 rat lung fibroblasts as reporter cells as previously described.\[20,21\] EA.hy 926 cells were grown to confluence and then incubated with Dex for 24 h or 48 h. For determination of NO production, EA.hy cells and confluent RFL-6 cells were washed twice with Locke’s solution. Then EA.hy cells were incubated with Locke’s solution containing 200 U/mL SOD and 100 µmol/L L-arginine, and RFL-6 cells with Locke’s solution containing 0.6 mmol/L IBMX for 30 min. After the pre-incubation, EA.hy cells were incubated for 2 min at 37°C in 1 mL of Locke’s solution containing 200 U/mL SOD, 0.3 mmol/L IBMX, 100 µmol/L L-arginine, and 10 µmol/L calcium ionophore A23187. Then the conditioned media containing the NO released from the EA.hy cells were transferred to the RFL-6 cells, and another incubation of 2 min at 37°C was performed. The reaction was stopped by aspiration of the solution, adding 1 mL of ice-cold 50 mmol/L sodium acetate, pH 4.0, and rapidly freezing the cells with liquid nitrogen. The cGMP content of the RFL-6 samples was determined by radioimmunoassay.\[20,21\]

2.7 Statistics

Results are expressed as mean ± SE. Statistical differences between mean values were determined by analysis of variance (ANOVA) followed by Dunnett’s test for multiple comparisons to a single control group. Differences were considered statistically significant at \(P < 0.05\).

3 Results

3.1 Dexamethasone down-regulates the expression of GCH1 and DHFR

As the rate-limiting enzyme of the de novo pathway in the biosynthesis of BH$_4$, GCH1 has a crucial function. Another important enzyme is DHFR, which can regenerate BH$_4$ from BH$_2$. To measure the impact of Dex on these two enzymes, human EA.hy 926 endothelial cells were treated with different concentrations of Dex (1, 10 or 100 nmol/L) for time periods up to 72 h. qPCR analyses showed a concentration- and time-dependent reduction of GCH1 mRNA expression in response to Dex treatment (Figure 1). GCH1 protein expression was found down-regulated after 72 h Dex treatment (Figure 1). Dex treatment also led to a down-regulation of DHFR expression at both mRNA and protein levels (Figure 2).

3.2 Dexamethasone reduces tetrahydrobiopterin content

As a crucial cofactor for eNOS, BH$_4$ plays an important role for eNOS functionality and coupling. A significant time- and concentration-dependent decrease in total biop- terin, BH$_4$ and BH$_2$ after Dex treatment was observed (Figure 3). Since BH$_4$: BH$_2$ ratio is even more important than the absolute BH$_4$ concentration for eNOS functionality, BH$_4$: BH$_2$ ratio was calculated. With increasing time and concentration of Dex treatment, the BH$_4$: BH$_2$ ratio declined (Figure 3).

3.3 No evidence for dexamethasone-induced eNOS uncoupling

The results in ROS experiments were inconsistent. With the H$_2$DCFDA fluorescence experiment as an example, we performed totally eight experiments and each experiment showed different results (Figure 4A). Generally, no sig-
Figure 1.  Dexamethasone down-regulates GCH1 expression. (A): Human EA.hy 926 endothelial cells were treated with either Dex (1, 10 or 100 nmol/L) or with the solvent control DMSO for 24, 48 or 72 h. mRNA expression of GCH1 was analyzed with qPCR; (B & C): protein expression was studied with Western blot analyses. β-tubulin was shown for normalization. Columns represent mean ± SE, n = 12–24. *P < 0.05, **P < 0.01 compared with Ctl. Ctl: control; Dex: dexamethasone; GCH1: GTP cyclohydrolase I; NS: not significant.

A significant increase in ROS was observed in EA.hy 926 endothelial cells in response to Dex treatment (Figure 4). The eNOS inhibitor L-NAME did not reduce ROS levels in Dex-treated cells, indicating that eNOS was not producing ROS, i.e., eNOS was not uncoupled (Figure 4). We also measured ROS production with L-012 chemiluminescence,[16] but found no evidence for eNOS uncoupling either (data not shown).

Treatment of EA.hy 926 cells with Dex led to a down-regulation of the ROS-producing enzyme NADPH oxidase 4 (Nox4) and a down-regulation of the antioxidant enzyme thioredoxin-2 (TNX2) (Figure 5). However, no significant effects of Dex were observed on redox enzymes such as xanthine dehydrogenase, catalase, superoxide dismutase 1, glutathione peroxidase 1 (GPx1) (Figure 5), the major redox enzymes in this cell type.[22]

3.4  Dex down-regulates eNOS expression, decreases eNOS phosphorylation and reduces endothelial NO production

Treatment of EA.hy 926 endothelial cells led to a concentration- and time-dependent down-regulation of eNOS at both mRNA and protein levels (Figures 6A and 6B). A much greater effect was seen on eNOS phosphorylation, with a marked decrease in eNOS phosphorylation at serine 1177 (Figure 6C). The down-regulation of eNOS and reduc-
Figure 2. Dexamethasone down-regulates DHFR expression. (A): EA.hy 926 endothelial cells were treated with either Dex (1, 10 or 100 nmol/L) or with the solvent control DMSO for 24, 48 or 72 h. mRNA expression of DHFR was analyzed with qPCR; (B & C): protein expression was studied with Western blot analyses. GAPDH was shown for normalization. Columns represent mean ± SE, n = 12–24. *P < 0.05, **P < 0.01 compared with Ctl. Ctl: control; Dex: dexamethasone; DHFR: dihydrofolate reductase; NS: not significant.

4 Discussion

In the present study, we demonstrate that treatment of endothelial cells with Dex leads to a down-regulation of BH₄-synthesizing enzymes and a reduction of intercellular BH₄ content. At the same time, Dex decreases eNOS expression, eNOS phosphorylation at serine 1177, and NO production. An uncoupling of eNOS, however, is not evident.

In our previous studies,[4,11] we have provided evidence that a reduction in endothelial NO production represents a major mechanism for Dex-induced hypertension. In these studies, we proposed that the reason for the reduction NO production is a down-regulation of the eNOS enzyme.[4,11] The objective of the present study is to test the hypothesis that the decreased NO production in response to Dex treatment may also involve eNOS uncoupling, a common
mechanism seen in many pathological conditions including hypertension, atherosclerosis and diabetes. Uncoupling of eNOS is a crucial mechanism contributing significantly to endothelial dysfunction. It not only reduces NO production, but also potentiates the pre-existing oxidative stress. The overproduction of reactive oxygen species (e.g., superoxide and subsequently peroxynitrite) by uncoupled eNOS in turn enhances oxidation of BH4, leading to BH4 deficiency, and thus creating a vicious circle. A number of mechanisms are implicated in eNOS uncoupling. Among these, BH4 deficiency is likely to represent a major cause of eNOS uncoupling. BH4 is biosynthesized from GTP with GCH1 acting as the rate-limiting enzyme. Under conditions associated with oxidative stress, superoxide can modestly and peroxynitrite strongly oxidize BH4 to BH2, leading to BH4 deficiency. BH2 can...
Figure 4. No evidence for dexamethasone-induced eNOS uncoupling. (A): EA.hy 926 cells were treated for either Dex (1, 10 or 100 nmol/L) or with the solvent control DMSO for 24 h. Then, cells were washed, and ROS measured with H2DCFDA fluorescence in presence (gray lines) or absence (red lines) of the eNOS inhibitor L-NAME (500 µmol/L). Totally 8 experiments (each row represents one experiment) were performed (n = 12 each), with no consistent results. (B): Cells were treated for 24 h and ROS measured with H2DCFDA FACS in presence (gray columns) or absence (red columns) of the eNOS inhibitor L-NAME (500 µmol/L). The graph shows representative results of one experiment. No significant differences were found. Integrated lines demonstrate the tendency of ROS development with increasing dexamethasone concentration in absence (red) or presence (gray) of L-NAME. Ctl: control; Dex: dexamethasone; ROS: reactive oxygen species.
be reduced back to BH$_4$ by the enzyme DHFR.$^{[26,27]}$ Thus, BH$_4$ deficiency can be caused by enhanced oxidation due to oxidative stress, by reduced de novo synthesis due to down-regulation/inhibition of GCH1 or by reduced recycling from BH$_2$ due to down-regulation of DHFR.$^{[28]}$

An important mechanism of eNOS uncoupling in response to cardiovascular risk factors is oxidative stress caused by NADPH oxidases, the major ROS-producing enzymes in the vasculature.$^{[29]}$ An upregulation of NADPH oxidase expression and/or activity may cause BH$_4$ oxidation and eNOS uncoupling. The predominant NADPH oxidase isoform in human endothelial cells (EA.hy 926 cells as well as human umbilical vein endothelial cells) is Nox4.$^{[30]}$ Dex treatment reduces Nox4 expression in EA.hy 926 cells (Figure 5). This is likely to be the reason why Dex does not induce oxidative stress and BH$_4$ oxidation.

We did, however, observe a reduced BH$_4$ level in EA.hy 926 cells after Dex treatment, which can be explained by the down-regulation of BH$_4$-synthesizing enzymes GCH1 and DHFR. Recent studies have shown that BH$_4$ and BH$_2$ have similar $K_m$ for eNOS, but BH$_2$ is without eNOS cofactor activity. BH$_1$ may compete with BH$_4$ for binding to eNOS, or even displace BH$_2$ prebound to eNOS, thus promoting eNOS uncoupling and superoxide production.$^{[26,31,32]}$ Therefore, BH$_4$: BH$_2$ ratio rather than the absolute amount of BH$_4$ is the key determinant eNOS functionality (i.e., NO or superoxide production by eNOS).$^{[32]}$ As shown in Figure 3, the calculated BH$_4$: BH$_2$ ratio was reduced by Dex treatment as well.

Despite the reduced intercellular BH$_4$ content and BH$_4$: BH$_2$ ratio, we found no evidence for eNOS uncoupling in Dex-treated endothelial cells. We propose that two mechanisms may be responsible for this paradoxical phenomenon: eNOS-BH$_4$ stoichiometry and eNOS phosphorylation.

Overexpression of eNOS in apolipoprotein E-knockout (eNOS-Tg/ApoE-KO) mice paradoxically results in increased vascular superoxide production at least in part from uncoupled eNOS.$^{[13]}$ The superoxide production induced by eNOS overexpression can be prevented by BH$_4$ supplementation or by endothelium-specific GCH1 overexpression.$^{[34]}$
Figure 6. Dexamethasone down-regulates eNOS expression and decreases eNOS phosphorylation. Human EA.hy 926 endothelial cells were treated with either Dex (1, 10 or 100 nmol/L) or with the solvent control DMSO for 24, 48 or 72 h. mRNA expression of eNOS was analyzed with qPCR (A); protein expression of eNOS (B) as well as eNOS-P at serine 1177 (C) were studied with Western blot analyses. GAPDH and β-tubulin were shown for normalization. Columns represent mean ± SE, \( n = 12–24 \). *\( P < 0.05 \), **\( P < 0.01 \) compared with Ctl. Ctl: control; Dex: dexamethasone; eNOS: endothelial nitric oxide synthase; eNOS-P: endothelial nitric oxide synthase phosphorylation; NS: not significant.
These data suggest that eNOS: BH4 ratio determines eNOS function. Indeed, in eNOS-overexpressing (eNOS-Tg) mice where BH4 levels are limiting (eNOS-Tg/ApoE-KO), superoxide production is markedly elevated compared with wild type and eNOS-Tg littermates, whereas eNOS is not uncoupled in the presence of saturating levels of BH4 (eNOS-Tg and eNOS/GCH-Tg).[35] Recently, the quantitative regulation of eNOS coupling by BH4-eNOS stoichiometry has been elegantly demonstrated in a model cell system.[32] Quantitative analysis of cellular BH4 and eNOS levels has revealed a striking linear relationship between eNOS protein and cellular BH4 stoichiometry, with eNOS uncoupling at eNOS: BH4 molar ratio > 1.[32]

In the present study, Dex treatment led to a reduction of BH4 and BH4: BH2 ratio (Figure 3). Meanwhile, we observed a down-regulation of eNOS protein (Figure 7). Although the precise quantitative stoichiometry of eNOS and BH4 could not be measured in our study, it is possible that the low BH4 level after Dex treatment is still sufficient for the eNOS enzyme, whose expression level is also reduced. This may be one reason for the absence of eNOS uncoupling in Dex-treated endothelial cells.

The second reason may be the reduced eNOS phosphorylation at serine 1177. Phosphorylation is an important mechanism regulating eNOS enzymatic activity.[36] The activation of eNOS catalytic function by serine 1177 phosphorylation is due to inhibition of calmodulin dissociation from eNOS and enhancement of the internal rate of eNOS electron transfer.[37] This phosphorylation is required for both the NO-producing activity of the coupled eNOS and the superoxide-producing activity of the uncoupled eNOS. Therefore, even if the reduction of BH4 is sufficient to induce eNOS uncoupling, the massive dephosphorylation at serine 1177 may inhibit the activity of the uncoupled eNOS enzyme, thus rendering the superoxide-producing activity of eNOS undetectable.

In conclusion, we found no evidence of eNOS uncoupling in endothelial cells treated with Dex despite a down-regulation of BH4-producing enzymes and a reduction of BH4 levels. The reduced NO production in Dex-treated endothelial cells is attributable to a down-regulation of the eNOS protein and eNOS dephosphorylation at serine 1177.

Acknowledgements

Tobias S and Siuda D were members of the Neuroscience Graduate School at the Johannes Gutenberg University of Mainz supported by the DFG (Deutsche Forschungsgemeinschaft) Research Training Group (GRK 1044). Siuda D was also supported by the Center for Thrombosis and Hemostasis (CTH, funded by the Federal Ministry of Education and Research, BMBF 01EO1003) of Johannes Gutenberg University Medical Center, Mainz, Germany.

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