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Role of Endothelial Nitric Oxide Synthase in Glucocorticoid-Induced Hypertension: An Overview of Experimental Data

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Abstract

Imbalances in the synthesis or in the bioavailability of nitric oxide (NO), the freely diffusible vasodilator, in myocardial endothelial cells were demonstrated to be crucial in the development of hypertension. Glucocorticoids (GCs) are widely used as immunomodulators. One of the numerous side effects of GC therapy is hypertension arising from reduced release of the endothelium-derived NO. GCs can modulate NO synthesis by targeting the genes involved in it, like nitric oxide synthase (NOS) and guanosine triphosphate (GTP) cyclohydrolase-1 (GTPCH-1). This chapter will give an overview on the impact of GCs on NO synthesis and signalling in animal models as well as in in vitro cell culture models. Moreover, strategies for preventing or neutralizing side effects of long-term GC therapy will be discussed.

Keywords: myocardial endothelial cells, nitric oxide, nitric oxide synthase, glucocorticoids, glucocorticoid receptor

1. Introduction

Hypertension mediated by glucocorticoids (GCs) is a frequent clinical problem. Among others it results from the fact that GCs remain as one of the most commonly prescribed drugs for many conditions, such as inflammatory diseases, asthma, multiple sclerosis, chronic obstructive pulmonary disease (COPD) and many more. Either the pharmacological administration of GCs or intrinsic GC excess in humans (caused that is by Cushing syndrome) may result in hypertension. The problem was originally discussed to originate from sodium excess or from

an impairment of water reabsorption by the renal mineralocorticoid receptor [1, 2]. However, extensive experimental and clinical data begun to unravel the complex molecular mechanisms inducing the onset and leading to a persistence of pathologically high blood pressure induced by GCs. In addition, recent research pointed to the role of extra-renal tissues, such as the vascular endothelium, that have not been taken into account so far in the regulation of blood pressure. The importance of glucocorticoid receptor (GR) signalling became more and more obvious in this process [3, 4].

Effects of GCs on vessel vasodilatation and nitric oxide (NO) synthesis as well as imbalances between NO and superoxide were examined extensively in numerous in vitro and in vivo studies. Imbalances in the bioavailability of endothelium-derived NO play a crucial role in diverse cardiovascular diseases including hypertension, arteriosclerosis and hypercholesterolemia [5–11]. NO is also known to influence circulating platelets and white blood cells and modulates various cellular events, such as platelet activation, mitochondrial function, ion transport, inflammation, angiogenesis and cell proliferation, all processes being essential in cardiovascular homeostasis. It also contributes to the control of the heart rate and its contractility. Although its role in these processes is not fully known, the amount of bioactive NO has been suggested to be involved in the control of cardiac contractility. While lower NO levels seem to promote contractility, higher levels have the opposite effects [12]. It limits cardiac remodelling after ischemic injury. NO is known to be constitutively generated in cardiomyocytes by two different NO synthase (NOS) isoforms, the endothelial and neuronal one indicating different roles of these isoforms in cardiac function [13]. In this chapter, we summarized the state of knowledge from available experimental data considering the influence of externally administered GCs on the synthesis of the vasodilator NO in hypertension. Hypertension arising from intrinsic GC excess will not be discussed here.

2. Glucocorticoids and the glucocorticoid receptor

GCs are steroid hormones synthesized in the adrenal glands. They are essential for life and involved in various cellular processes, such as in the regulation of metabolic and immunological pathways, and the control of cellular homeostasis. Cortisol is the main form of GCs produced in the human body. Multiple naturally occurring as well as synthetic compounds, like dexamethasone, are being used in the treatment of diverse inflammatory and immunological diseases. The cellular effects mediated by GCs, including genomic and non-genomic ones, are exerted by binding to their receptor, the GR. In its inactive state, the GR is kept in the cytosol fraction by chaperon proteins, such as the heat shock protein 90 (HSP90). The lipophilic GCs diffuse through the cell membrane into the cytosol; they bind to the GR which in that moment is released by the HSPs and is then activated (**Figure 1**). After dimerization, the GR-GC complex enters the nucleus where it binds to so-called glucocorticoid response elements (GREs) located in the promoter regions of target genes. By this binding, the GR activates or represses the expression of its specific targets [14]. Moreover, by binding to other transcription factors, the GR can indirectly influence the expression of certain other genes [14].

Figure 1. Glucocorticoid receptor signalling. In its inactive state, the glucocorticoid receptor (GR) is kept in the cytosol by chaperon proteins, such as the heat shock protein 90 (HSP90). The lipophilic GCs diffuse through the cell membrane into the cytosol, bind to the GR resulting in release of GR from the HSP complex and its activation. After dimerization, the GR-GC complex enters the nucleus where it binds to so-called glucocorticoid response elements (GREs) located in the promoter regions of target genes. The protein amount of the GR correlates with its transcriptional activity. Under physiological conditions and excess GC treatment, the recycling process of receptor/transcriptional DNA complexes is regulated by the 26S proteasome, GR ubiquitination and degradation.

The GR is conserved among all species and its expression was confirmed in a wide range of tissues including the vascular endothelium. It was also shown to be expressed in microvascular endothelial cells of the myocardium and of the blood-brain barrier [15–19]. The protein amount of the GR correlates with its transcriptional activity. Under physiological conditions, the GC turnover and the recycling process of receptor/transcriptional DNA complexes is regulated by the 26S proteasome as well as by the degradation of the GR (**Figure 1**) [20, 21].

Although GCs are the most widely prescribed medicine, systemic GC administration, especially for a long period, is associated with numerous detrimental side effects [22, 23]. GC-induced

hypertension is one of the common undesirable effects. Tissue-specific GR knockout mice shed light on the molecular mechanisms behind GC-induced hypertension. Against expectations, a GR deletion in the distal nephron did not protect mice against hypertension caused by GC administration [24]. Also, the lack of GR in vascular smooth muscle cells has only delayed the onset of hypertension in comparison to wild-type mice [25]. Finally, mice with a tissue-specific GR knockout in the vascular endothelium were resistant to GC-induced hypertension [26]. These facts underlined the pivotal role of the GR expressed in vascular endothelial cells in generation and maintenance of GC-mediated hypertension demonstrating that cell-specific actions of the GR are responsible for the phenotype of a whole organism.

3. Glucocorticoid effects on the NO synthesis and signalling pathway

3.1. Influence on eNOS

At cellular level, GCs negatively influence the synthesis of NO causing endothelial dysfunction. NOS is expressed in three isoforms: neuronal NOS (nNOS, NOS1), inducible NOS (iNOS, NOS2) and endothelial NOS (eNOS, NOS3) [27, 28]. Under physiological circumstances, all NOS isoforms can catalyse the conversion of L-arginine to L-citrulline and NO. Endothelial NOS constitutively produces NO in vascular endothelial cells. Cofactors involved in this process, such as 5,6,7,8-tetrahydrobiopterin (BH_4), are limiting elements in the synthesis of NO. It has been observed that the absence of BH_4 or L-arginine leads to eNOS uncoupling and to the production of reactive oxygen species (ROS) instead of NO. In consequence, this results in endothelial dysfunction followed by severe vascular disorders, that is, of cardiovascular nature [29]. The pivotal role of eNOS and its product NO in regulating the blood pressure and blood flow was demonstrated in eNOS knockout and eNOS over-expressing mice. While mice lacking eNOS were hypertensive, animals over-expressing eNOS in the vascular tissue became hypotensive and suffered from decreased vasoreactivity [30–32]. These mice models confirmed that blood pressure is regulated by eNOS-derived NO. Therefore, first efforts to explain the molecular mechanism of GC-mediated hypertension focussed on the regulation of eNOS.

GCs lead to a down-regulation of eNOS mRNA and protein in cultured endothelial cells, in the aorta and in organs such as kidney and liver of GC-treated rats [33]. In vivo, disruption of the eNOS gene preserved mice from hypertension [34]. In vitro studies with human umbilical vein endothelial cells (HUVECs) have demonstrated that endogenous GR acts as a negative regulator of both eNOS and iNOS. It was shown that a GR knockdown in HUVECs increased eNOS mRNA and protein levels [35]. An endothelial deletion of the GR in mice resulted in an accelerated expression of eNOS in septic mice [35]. Moreover, other animal studies demonstrated that following GC treatment, the expression of eNOS was significantly down-regulated [33, 36]. The observed eNOS reduction could be further confirmed at promoter level after identification of a potential GRE in the promoter region of this gene [37]. This study which has been performed in HUVECs demonstrated the suppression of the eNOS promoter activity in response to cortisol application. In line with these results, the binding of the GR to the suppressive GRE at –111 to –105 bp located on the NOS promoter was tested

by chromatin immunoprecipitation [37]. Recently, a dexamethasone-mediated regulation of the eNOS promoter in microvascular myocardial endothelial cells derived from mice has been shown. Concordantly with previous results, a GC-mediated suppression of the eNOS promoter could be measured [19]. The GR can inhibit eNOS and other forms of NOS by trans-repressive interactions with other transcription factors, such as NF κ B or AP-1 [38, 39]. Moreover, recent reports revealed the meaning of the molar ratio between BH $_4$ and eNOS rather than the absolute amounts of the cofactor and its enzyme. As we know by now, GCs cause a reduction of both BH $_4$ and eNOS. They also inhibit NOS phosphorylation without an evident uncoupling of eNOS after GCs treatment [40].

3.2. Effects on L-arginine and BH $_4$

GCs are known to alter various molecules in the NOS-mediated biosynthesis of NO. L-arginine is an essential substrate for all NOS isoforms. In GC-induced hypertension, systemic levels of L-arginine and L-citrulline have been observed to be diminished reflecting the importance of upstream NOS regulators in this clinical condition [41]. L-arginine supplementation could partially reverse hypertension in GC-treated rats [41, 42]. BH $_4$ is an essential cofactor of eNOS in the biosynthesis of NO [43]. It can be produced by two different strategies. The first is via the conversion from GTP catalysed by the GTP cyclohydrolase 1 (GTPCH-1), which is the rate-limiting enzyme involved in its synthesis. The second way is the salvage production pathway, where the precursor protein sepiapterin is converted into the intermediate molecule BH $_2$ and then into BH $_4$ [44]. The importance of the bioavailability of BH $_4$ was validated in ex vivo studied vessel pieces. Here, a 30-fold increase of NOS activity in the presence of BH $_4$ compared with BH $_4$ -free preparations could be determined [45, 46]. Limited amounts of BH $_4$ can lead to an uncoupling of eNOS and further to endothelial dysfunction [47, 48]. Uncoupled eNOS overproduces ROS which in turn enhance the oxidation of BH $_4$ to BH $_2$ resulting in BH $_4$ deficiency [49–52]. BH $_2$ can be reconverted to BH $_4$ by dihydrofolate reductase (DHFR) [53]. A GC supplementation of cultured human endothelial cells was shown to reduce mRNA and protein levels of DHFR [40]. Furthermore, administration of external BH $_4$ led to an increase of NO levels following GC-treatment, as it was demonstrated in animal studies as well as in coronal endothelial cells [51, 54]. However, BH $_4$ application or treatment with sepiapterin, a precursor of BH $_4$, did not alter systolic blood pressure although total concentrations of plasma BH $_4$ were elevated in GC-induced hypertensive rats [55, 56]. GC effects on BH $_4$ and NO down-regulation have been proven to be genomic by the usage of specific GR antagonists. One of such antagonistic compounds is RU486, the application of which could reverse the decreasing effects on NO synthesis caused by GC treatment [34, 57].

3.3. Control of GTP cyclohydrolase 1

As the rate-limiting enzyme involved in the de novo biosynthesis of BH $_4$, GTPCH-1 shows a decreased expression in response to GC treatment. As a result of its decrease, a significantly lowered BH $_4$ and NO production contributed to impaired endothelium-dependent vaso-relaxation [55, 58, 59]. In vitro studies with human and mouse endothelial cells demonstrated genomic effects of GCs on GTPCH-1 by GC-mediated down-regulation of GTPCH-1 mRNA and promoter activity [19, 40]. It was also shown that dexamethasone mediated a

dense down-regulation of both GTPCH-1 and eNOS after a long-term treatment period [19]. Elsewhere it was shown that a gene transfer of human GTPCH-1 restored vascular BH_4 concentrations and positively influenced endothelial function in hypertonic rats [60]. The summary of this data clearly displays that GCs influence the whole multistep cascade of NO production mediated by eNOS.

4. Strategies to reduce glucocorticoid-induced hypertension

Since mechanisms behind GC-induced side reactions, such as hypertension, are complex and in part controversial, a basic knowledge of the pharmacology, clinical guidelines and the adverse effects of these substances is imperative. Once increased blood pressure as an unwanted effect of GC treatment has been detected, the use of appropriate anti-hypertensive compounds, such as NO-generating compounds and direct vasodilators and hybrid drugs possessing a NO-releasing moiety including diuretics, calcium channel blockers, angiotensin-converting enzyme (ACE) inhibitors, angiotensin receptor blockers, β -blockers, α -adrenergic receptor antagonists, centrally acting α -2 receptor agonists, should be considered. There are different strategies and natural as well as synthetic compounds that have been discussed to reduce or prevent GC-induced imbalances occurring in the production of NO. A brief overview on the most important ones that directly influence NO availability or signalling will be given below.

4.1. Reducing glucocorticoid toxicity

First of all, the side effects of a drug administration should be considered in terms of the drug duration/dosage, which in turn should take into account the risk/benefit ratio. GC-induced side effects are usually more severe following systemic than topical application. As an important parameter, a definite indication to start GC treatment should be considered. Moreover, short- and intermediate-acting ones should be preferred over longer-acting GCs and a minimum necessary dose and duration of treatment should be chosen. Furthermore, a once-a-day morning administration should be preferred over a divided dose therapy. Necessarily, the body weight, blood and ocular pressure, the development of cataracts, serum lipids, blood and urine glucose concentration should be monitored during the complete GC therapy.

4.2. NO-generating agents

Several drugs act as NO-releasing agents. Well known are organic nitrates and nitrites, including nitroglycerin acting as anti-anginal drug. However, the use of these agents has been associated with some limitations and some of the compounds are still tested in clinical trials.

Pulmonary rather than cardiac hypertension can be reduced by a direct delivery of NO into the lung through inhalation. Reaching well-ventilated areas of the lungs, inhaled NO increases blood oxygenation and improves ventilation-perfusion. However, its effect on the systemic arterial tone is minor since NO is rapidly scavenged and acutely inactivated after reacting with circulating haemoglobin [61]. Therefore, effects of distal NO rather occur due to the

formation of other bioactive nitrogen oxides, including inorganic nitrate and S-nitrosothiols acting in an endocrine manner [62].

Nitrite therapy also contributes to the regulation of blood flow and blood pressure through its reduction to NO catalysed by metalloprotein oxidoreductases. The bioconversion of nitrite into NO in the vascular system is regulated by a reductive reaction with deoxyhaemoglobin and couples hypoxic sensing with NO production and therefore augmented under physiological or pathological hypoxia [63–65]. Inhaled nitrite causes pulmonary vasodilatation and induces protective remodelling in animal studies [66]. Moreover, nitrite reduces blood pressure and improves cell and organ viability after ischemia reoxygenation as it was shown in the heart, liver and lungs [67, 68]. Either oral or inhaled delivery of nitrite has been shown to exhibit a rapid and efficient systemic absorption and is still tested for further effects in various clinical studies.

Nitrate can be considered as a pro-drug that is further metabolized to nitrite and other nitrogen oxides after application. Due to its long half-life, the enterosalivary circulation and reformation, nitrite ensures a low-grade NO-like bioactivity over a longer period of time [69]. Nitrate has been shown to possess a robust NO-like activity in the cardiovascular system and can be assimilated from nitrate-rich diet, especially in green leafy vegetables, or as a salt [70]. Animal models revealed compensating effects of dietary nitrite and nitrate on the lack of eNOS expression [71]. Similar to nitrite, nitrate has also cardio- and vascular- and renoprotective properties and both molecules act as a blood pressure-lowering and anti-inflammatory agent in different animal models of cardiovascular diseases [72–74].

Regulatory functions for the cardiovascular system have also been demonstrated for nitrated fatty acids [75]. These electrophilic compounds can be generated in the reaction of unsaturated fatty acids with NO-derived reactive species and act via NO-dependent and NO-independent pathways [76]. Besides positive effects on vascular relaxation, nitrated fatty acids have shown anti-oxidative and anti-inflammatory effects in blood vessels [77, 78]. Despite the cardio-protective action of nitrated fatty acids proven in animal models, the first human clinical trial is still ongoing and safety is being tested.

4.3. L-arginine and L-citrulline administration

A direct and very simple way to enhance NO availability is L-arginine delivery. As a substrate for all NOSs, this amino acid is present in sufficiently high concentrations in most cells. However, L-arginine administration was observed to even increase NO generation [79]. Dietary L-arginine possesses protective effects on the cardiac vascularity, as it was shown in a number of studies and is therefore frequently supplemented in food. However, mixed results of L-arginine supplementation have been shown in multiple clinical trials, that is, no significant effects of L-arginine on NO availability and on various cardiovascular parameters followed by even detrimental effects, such as a higher incidence of myocardial infarction after L-arginine administration, were shown in clinical trials [80]. However, studies focusing on effects on blood pressure have shown blood pressure-lowering effects [81]. The partially converse effects may partly result from the unspecific binding of this amino acid to all three NOS isoforms. Moreover, the molecule mediates many other effects than those associated with

NO metabolism, that is, it is a precursor of other amino acids or is a substrate for arginase, a key enzyme in the urea cycle [82]. In this context, it was identified that L-arginine also interacts with the asymmetric dimethylarginine (ADMA) that acts as an endogenous NOS inhibitor. Since high ADMA levels are generated in cardiovascular diseases, L-arginine supplementation seems to normalize but not improve NO levels and endothelial function in such individuals [83].

A problem arising from L-arginine administration might also be that this amino acid is extensively metabolized by intestinal bacteria and arginase in the liver before reaching the circulation [84]. Compared with L-arginine, L-citrulline has been shown to be effectively transported into endothelial cells where it is enzymatically converted into L-arginine [84]. However, despite potential advantages shown for this molecule, no clinical trial has been initiated to study the effects of L-citrulline on cardiovascular function.

4.4. BH₄ supplementation

BH₄ is an important factor controlling the activity of all NOS isoforms. Concerning the salvage pathway by which this factor can be produced endogenously, the external supplementation of BH₄ has been considered as a strategy improving NO synthesis and reducing eNOS uncoupling. Intracellular levels of BH₄ can be restored by exposing cells to sepiapterin, being converted to BH₄ through sepiapterin reductase. Satisfying results on superoxide formation and vascular function have been achieved by the administration of sepiapterin in various models of hypertension, that is, in spontaneously hypertensive or nephrectomised animals [85, 86]. Endothelial dysfunction has also been restored by sepiapterin in dexamethasone-incubated aorta rings *ex vivo* [55]. There are also *in vitro* studies applying aortic endothelial cells showing an improvement of NO release and decrease of ROS production [58]. There is one main difference between a direct BH₄ and a sepiapterin administration. While sepiapterin is absorbed by cells and converted to intracellular BH₄, a direct BH₄ administration acts extracellularly [43]. Despite this seemingly important difference, there is only one report describing sepiapterin administration, whereas a direct BH₄ administration has not been described in the context of GC-induced hypertension [56]. The low number of experimental data might be due to the fact that sepiapterin did not affect dexamethasone-mediated hypertension in rats although plasma levels of BH₄ were significantly increased. As the authors explained, the uncoupling of eNOS seems not to play a major role in the pathogenesis of GC-induced hypertension [56].

4.5. Response to antioxidants and anti-inflammatory agents

Antioxidants and anti-inflammatory agents such as tempol, apocynin, N-acetylcysteine, folic acid and atorvastatin can prevent and partially reverse GCs-induced hypertension *in vivo* [87–92]. Therefore, these substances represent promising candidates for prevention and treatment of increased systolic blood pressure induced by long-term and high-dosage GC treatment.

Tempol, 4-hydroxy-2,2,6,6-tetramethyl piperidinoxyl, is a superoxide scavenger with either water-soluble or membrane-permeable characteristics. It is known to normalize blood pressure, as it was shown in numerous animal models of differently induced hypertension [90, 93–95].

Interesting findings have been reported by Zhang et al. They showed that tempol did not have an influence on oxidative stress measured in rat plasma. However, they reported tempol to have prevented and partially reversed dexamethasone-induced hypertension independently of systemic ROS.

The hydrophilic antioxidant N-acetylcysteine is already widely used in the clinic, that is, as a mucolytic agent or in ischemia-reperfusion injury [96, 97]. In dexamethasone-induced hypertension among rats, this agent was shown to lower systolic blood pressure as well as restore plasma NO levels, without any effect on oxidative markers in the plasma. Similar experimental settings revealed N-acetylcysteine treatment having partially preventing but not reversing properties on GC-induced hypertension in comparison to what has been shown for tempol [91].

The same laboratory tested folic acid in hypertension induced by synthetic GCs [87]. Folic acid is a B group vitamin that is known from its ROS decreasing properties [98]. It also lowers the concentration of homocysteine that reduces intracellular BH_4 levels, thereby increasing BH_4 bioavailability [99]. Patients suffering from Cushing syndrome tend to have higher systemic homocysteine and reduced folate levels than healthy controls [100]. It is therefore not surprising that the supplementation of folic acid might prevent and partially revers GC-induced hypertension by increasing BH_4 amounts, although the detailed mechanism remains elusive [87].

The same group has demonstrated that antioxidant apocynin, which is a specific NAD(P)H oxidase inhibitor, is able either to reverse or to prevent dexamethasone-caused hypertension [101]. Interestingly, the same study has shown that the NO precursor L-arginine did not have the same effects as those being observed for apocynin in dexamethasone-induced hypertensive rats.

Among its pleiotropic functions, atorvastatin has been reported to improve endothelial function through increased bioavailability of NO and ameliorated ROS and pro-inflammatory cytokine production in different models of hypertension [92, 102, 103]. Atorvastatin could lower the blood pressure of dexamethasone-treated hypertensive rats due to its positive influence on the expression of eNOS measured in aortic samples and due to the lowered levels of superoxide in plasma. However, there was no significant effect of this compound on vasorelaxation although the endothelial function was markedly improved [92].

4.6. Influencing the GC signalling pathway

As it was shown in a transgenic animal model, an endothelial GR knockout was protected against GC-induced hypertension [26]. Targeting GR in endothelial cells could therefore constitute a preventive treatment strategy directed against GC-induced hypertension. A recent study by Blecharz et al. provided new insights into the GC-mediated disturbances of NO production in the mouse microvascular endothelial cell line MyEND [19]. They observed negative effects on NO-synthesis either after a short treatment period of 24 and 48 h or after a long-term GC administration persisting 30 days. These effects were accompanied by lowered NO concentrations and eNOS activity in response to dexamethasone application. However, instead of reduced eNOS protein expression, a decrease of GTPCH-1 protein they documented

a decrease of GTPCH-1 protein. Interestingly, the observed changes in enzymes and cofactors involved in NO synthesis were accompanied by a significantly lowered immune reactivity of the GR in MyEND cells. Ligand-dependent down-regulation of the GR, associated with the loss of functional GC responsiveness, was referred in numerous publications [20, 21, 104, 105]. It was also shown that the expression of the GR is lower in endothelial cells derived from the mouse myocardium than in those isolated from the blood-brain barrier [106]. Although the degradation of the receptor by the proteasome can be observed in capillaries from both organs, brain endothelial cells remain responsive to GCs due to a sufficient expression of the GR [17]. The complete loss of GC responsiveness verified in MyEND cells in contrast to endothelium of the central nervous system hinders the translocation of the GC-GR complex into the nucleus, thereby hindering the transactivation of the GC target gene GTPCH-1 (as depicted in **Figure 1**) [19]. As it was shown, blocking the proteasomal GR degradation by calpain inhibitor I or by over-expressing a nondegradable GR isoform rescued either NO or BH₄ levels, as well as eNOS activity in MyEND cells after GC treatment. Hence, these data provide the biochemical evidence that GC-induced disturbed vasodilatation may result from the involvement of the proteasome in the failure of GC responsiveness rather than from GC-mediated trans-repression of NO-synthesizing molecules, as it was thought before [19, 57]. On the other hand, together with data gained in brain endothelial cells, the results support the endocrine heterogeneity of different vascular beds and a blockage of the proteasomal signalling pathway may be a potential future option for neutralizing or reducing unfavourable effects of long-term GC therapy [17, 107].

5. Conclusions

GC-induced hypertension remains a severe clinical problem, since GCs are the widest prescribed drugs by clinicians. Despite numerous basic and clinical studies, the complexity of the induction and persistence of hypertension remains not entirely understood. A robust explanation for GC hypertension is that GCs reduce the activity and expression of eNOS. Moreover, enzymes and cofactors upstream of eNOS are negatively influenced. The effects mediated by GCs on these molecules can be genomic or non-genomic. Therefore, further examinations are necessary to learn about this very frequent and serious clinical issue. On one hand, a detailed comprehension of the multistep GC/GR signalling cascade in the vasculature as well as in other non-renal tissues is required. Moreover, the regulation of molecules upstream and downstream of eNOS by GCs in a genomic and non-genomic manner should be clarified, given the fact that these effects might differ depending on the tissue. This might be explained by the evidence that the expression of the GR differs in a tissue-specific manner. In addition, it will be necessary to develop new in vivo models mimicking this complex disease and helping to understand the interplay between organs such as the kidney, the liver, the cardiovascular and the central nervous system. These animal models should be supported by single- or multi-tissue culture models in vitro. They would accelerate and promote the development of new compounds, such as synthetic and natural antioxidants lowering GC-induced pathologically increased blood pressure, and enable the identification of potential therapeutic targets.

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References

- [1] Whitworth, J.A., G.J. Mangos, and J.J. Kelly, *Cushing, cortisol, and cardiovascular disease. Hypertension*, 2000. **36**(5): pp. 912–6.
- [2] Baid, S. and L.K. Nieman, *Glucocorticoid excess and hypertension. Curr Hypertens Rep*, 2004. **6**(6): pp. 493–9.
- [3] Ullian, M.E., *The role of corticosteroids in the regulation of vascular tone. Cardiovasc Res*, 1999. **41**(1): pp. 55–64.
- [4] Pimenta, E., M. Wolley, and M. Stowasser, *Adverse cardiovascular outcomes of corticosteroid excess. Endocrinology*, 2012. **153**(11): pp. 5137–42.
- [5] Gounarides, J.S., et al., *Effect of dexamethasone on glucose tolerance and fat metabolism in a diet-induced obesity mouse model. Endocrinology*, 2008. **149**(2): pp. 758–66.
- [6] Hadoke, P.W., J. Iqbal, and B.R. Walker, *Therapeutic manipulation of glucocorticoid metabolism in cardiovascular disease. Br J Pharmacol*, 2009. **156**(5): pp. 689–712.
- [7] Panoulas, V.F., et al., *Long-term exposure to medium-dose glucocorticoid therapy associates with hypertension in patients with rheumatoid arthritis. Rheumatology (Oxford)*, 2008. **47**(1): pp. 72–5.
- [8] Shinozaki, K., et al., *Oral administration of tetrahydrobiopterin prevents endothelial dysfunction and vascular oxidative stress in the aortas of insulin-resistant rats. Circ Res*, 2000. **87**(7): pp. 566–73.
- [9] Laursen, J.B., et al., *Endothelial regulation of vasomotion in apoE-deficient mice: implications for interactions between peroxynitrite and tetrahydrobiopterin. Circulation*, 2001. **103**(9): pp. 1282–8.
- [10] Zerr-Fouineau, M., et al., *Progestins overcome inhibition of platelet aggregation by endothelial cells by down-regulating endothelial NO synthase via glucocorticoid receptors. FASEB J*, 2007. **21**(1): pp. 265–73.
- [11] Widder, J.D., et al., *Regulation of tetrahydrobiopterin biosynthesis by shear stress. Circ Res*, 2007. **101**(8): pp. 830–8.

- [12] Tang, L., H. Wang, and M.T. Ziolo, *Targeting NOS as a therapeutic approach for heart failure*. Pharmacol Ther, 2014. **142**(3): pp. 306–15.
- [13] Seddon, M., A.M. Shah, and B. Casadei, *Cardiomyocytes as effectors of nitric oxide signalling*. Cardiovasc Res, 2007. **75**(2): pp. 315–26.
- [14] Oakley, R.H. and J.A. Cidlowski, *The biology of the glucocorticoid receptor: new signaling mechanisms in health and disease*. J Allergy Clin Immunol, 2013. **132**(5): pp. 1033–44.
- [15] Tischner, D. and H.M. Reichardt, *Glucocorticoids in the control of neuroinflammation*. Mol Cell Endocrinol, 2007. **275**(1–2): pp. 62–70.
- [16] Schweingruber, N., et al., *Mechanisms of glucocorticoids in the control of neuroinflammation*. J Neuroendocrinol, 2012. **24**(1): pp. 174–82.
- [17] Forster, C., et al., *Occludin as direct target for glucocorticoid-induced improvement of blood-brain barrier properties in a murine in vitro system*. J Physiol, 2005. **565**(Pt 2): pp. 475–86.
- [18] Forster, C., et al., *Differential effects of hydrocortisone and TNFalpha on tight junction proteins in an in vitro model of the human blood-brain barrier*. J Physiol, 2008. **586**(7): pp. 1937–49.
- [19] Blecharz, K.G., et al., *Inhibition of proteasome-mediated glucocorticoid receptor degradation restores nitric oxide bioavailability in myocardial endothelial cells in vitro*. Biol Cell, 2014. **106**(7): pp. 219–35.
- [20] Wallace, A.D. and J.A. Cidlowski, *Proteasome-mediated glucocorticoid receptor degradation restricts transcriptional signaling by glucocorticoids*. J Biol Chem, 2001. **276**(46): pp. 42714–21.
- [21] Bellingham, D.L., M. Sar, and J.A. Cidlowski, *Ligand-dependent down-regulation of stably transfected human glucocorticoid receptors is associated with the loss of functional glucocorticoid responsiveness*. Mol Endocrinol, 1992. **6**(12): pp. 2090–102.
- [22] Ciriaco, M., et al., *Corticosteroid-related central nervous system side effects*. J Pharmacol Pharmacother, 2013. **4**(Suppl 1): p. S94–8.
- [23] Poetker, D.M. and D.D. Reh, *A comprehensive review of the adverse effects of systemic corticosteroids*. Otolaryngol Clin North Am, 2010. **43**(4): pp. 753–68.
- [24] Goodwin, J.E., et al., *The glucocorticoid receptor in the distal nephron is not necessary for the development or maintenance of dexamethasone-induced hypertension*. Biochem Biophys Res Commun, 2010. **394**(2): pp. 266–71.
- [25] Goodwin, J.E., J. Zhang, and D.S. Geller, *A critical role for vascular smooth muscle in acute glucocorticoid-induced hypertension*. J Am Soc Nephrol, 2008. **19**(7): pp. 1291–9.
- [26] Goodwin, J.E., et al., *Knockout of the vascular endothelial glucocorticoid receptor abrogates dexamethasone-induced hypertension*. J Hypertens, 2011. **29**(7): pp. 1347–56.
- [27] Tsutsui, M., et al., *Significance of nitric oxide synthases: Lessons from triple nitric oxide synthases null mice*. J Pharmacol Sci, 2015. **127**(1): pp. 42–52.
- [28] Ignarro, L.J., C. Napoli, and J. Loscalzo, *Nitric oxide donors and cardiovascular agents modulating the bioactivity of nitric oxide: an overview*. Circ Res, 2002. **90**(1): pp. 21–8.

- [29] Feletou, M. and P.M. Vanhoutte, *Endothelial dysfunction: a multifaceted disorder (The Wiggers Award Lecture)*. Am J Physiol Heart Circ Physiol, 2006. **291**(3): p. H985–1002.
- [30] Huang, P.L., et al., *Hypertension in mice lacking the gene for endothelial nitric oxide synthase*. Nature, 1995. **377**(6546): pp. 239–42.
- [31] Shesely, E.G., et al., *Elevated blood pressures in mice lacking endothelial nitric oxide synthase*. Proc Natl Acad Sci U S A, 1996. **93**(23): pp. 13176–81.
- [32] Ohashi, Y., et al., *Hypotension and reduced nitric oxide-elicited vasorelaxation in transgenic mice overexpressing endothelial nitric oxide synthase*. J Clin Invest, 1998. **102**(12): pp. 2061–71.
- [33] Wallerath, T., et al., *Down-regulation of the expression of endothelial NO synthase is likely to contribute to glucocorticoid-mediated hypertension*. Proc Natl Acad Sci U S A, 1999. **96**(23): pp. 13357–62.
- [34] Wallerath, T., et al., *Dexamethasone lacks effect on blood pressure in mice with a disrupted endothelial NO synthase gene*. Nitric Oxide, 2004. **10**(1): pp. 36–41.
- [35] Goodwin, J.E., et al., *Endothelial glucocorticoid receptor is required for protection against sepsis*. Proc Natl Acad Sci U S A, 2013. **110**(1): pp. 306–11.
- [36] Schafer, S.C., et al., *Dexamethasone suppresses eNOS and CAT-1 and induces oxidative stress in mouse resistance arterioles*. Am J Physiol Heart Circ Physiol, 2005. **288**(1): pp. H436–44.
- [37] Liu, Y., et al., *Glucocorticoid response elements and 11 beta-hydroxysteroid dehydrogenases in the regulation of endothelial nitric oxide synthase expression*. Cardiovasc Res, 2009. **81**(1): pp. 140–7.
- [38] Biddie, S.C. and G.L. Hager, *Glucocorticoid receptor dynamics and gene regulation*. Stress, 2009. **12**(3): pp. 193–205.
- [39] Radomski, M.W., R.M. Palmer, and S. Moncada, *Glucocorticoids inhibit the expression of an inducible, but not the constitutive, nitric oxide synthase in vascular endothelial cells*. Proc Natl Acad Sci U S A, 1990. **87**(24): pp. 10043–7.
- [40] Tobias, S., et al., *Dexamethasone, tetrahydrobiopterin and uncoupling of endothelial nitric oxide synthase*. J Geriatr Cardiol, 2015. **12**(5): pp. 528–39.
- [41] Wen, C., et al., *L-arginine partially reverses established adrenocorticotrophin-induced hypertension and nitric oxide deficiency in the rat*. Blood Press, 2000. **9**(5): pp. 298–304.
- [42] Turner, S.W., et al., *L-arginine prevents corticotropin-induced increases in blood pressure in the rat*. Hypertension, 1996. **27**(2): pp. 184–9.
- [43] Thony, B., G. Auerbach, and N. Blau, *Tetrahydrobiopterin biosynthesis, regeneration and functions*. Biochem J, 2000. **347**(Pt 1): pp. 1–16.
- [44] Nichol, C.A., G.K. Smith, and D.S. Duch, *Biosynthesis and metabolism of tetrahydrobiopterin and molybdopterin*. Annu Rev Biochem, 1985. **54**: pp. 729–64.
- [45] Schmidt, K., et al., *Tetrahydrobiopterin-dependent formation of endothelium-derived relaxing factor (nitric oxide) in aortic endothelial cells*. Biochem J, 1992. **281** (Pt 2): pp. 297–300.

- [46] Werner-Felmayer, G., G. Golderer, and E.R. Werner, *Tetrahydrobiopterin biosynthesis, utilization and pharmacological effects*. *Curr Drug Metab*, 2002. **3**(2): pp. 159–73.
- [47] Kuzkaya, N., et al., *Interactions of peroxynitrite, tetrahydrobiopterin, ascorbic acid, and thiols: implications for uncoupling endothelial nitric-oxide synthase*. *J Biol Chem*, 2003. **278**(25): pp. 22546–54.
- [48] Thum, T., et al., *Endothelial nitric oxide synthase uncoupling impairs endothelial progenitor cell mobilization and function in diabetes*. *Diabetes*, 2007. **56**(3): pp. 666–74.
- [49] Li, H. and U. Forstermann, *Uncoupling of endothelial NO synthase in atherosclerosis and vascular disease*. *Curr Opin Pharmacol*, 2013. **13**(2): pp. 161–7.
- [50] Xia, Y., et al., *Superoxide generation from endothelial nitric-oxide synthase. A Ca²⁺/calmodulin-dependent and tetrahydrobiopterin regulatory process*. *J Biol Chem*, 1998. **273**(40): pp. 25804–8.
- [51] Cosentino, F. and T.F. Luscher, *Tetrahydrobiopterin and endothelial nitric oxide synthase activity*. *Cardiovasc Res*, 1999. **43**(2): pp. 274–8.
- [52] Cosentino, F. and Z.S. Katusic, *Tetrahydrobiopterin and dysfunction of endothelial nitric oxide synthase in coronary arteries*. *Circulation*, 1995. **91**(1): pp. 139–44.
- [53] Crabtree, M.J., et al., *Critical role for tetrahydrobiopterin recycling by dihydrofolate reductase in regulation of endothelial nitric-oxide synthase coupling: relative importance of the de novo biopterin synthesis versus salvage pathways*. *J Biol Chem*, 2009. **284**(41): pp. 28128–36.
- [54] Meininger, C.J., et al., *Impaired nitric oxide production in coronary endothelial cells of the spontaneously diabetic BB rat is due to tetrahydrobiopterin deficiency*. *Biochem J*, 2000. **349**(Pt 1): pp. 353–6.
- [55] Johns, D.G., et al., *Glucocorticoids inhibit tetrahydrobiopterin-dependent endothelial function*. *Exp Biol Med (Maywood)*, 2001. **226**(1): pp. 27–31.
- [56] Thida, M., et al., *Effects of sepiapterin supplementation and NOS inhibition on glucocorticoid-induced hypertension*. *Am J Hypertens*, 2010. **23**(5): pp. 569–74.
- [57] Mitchell, B.M., et al., *Glucocorticoids decrease GTP cyclohydrolase and tetrahydrobiopterin-dependent vasorelaxation through glucocorticoid receptors*. *J Cardiovasc Pharmacol*, 2004. **43**(1): pp. 8–13.
- [58] Mitchell, B.M., A.M. Dorrance, and R.C. Webb, *GTP cyclohydrolase 1 downregulation contributes to glucocorticoid hypertension in rats*. *Hypertension*, 2003. **41**(3 Pt 2): pp. 669–74.
- [59] Simmons, W.W., et al., *Glucocorticoids regulate inducible nitric oxide synthase by inhibiting tetrahydrobiopterin synthesis and L-arginine transport*. *J Biol Chem*, 1996. **271**(39): pp. 23928–37.
- [60] Zheng, J.S., et al., *Gene transfer of human guanosine 5'-triphosphate cyclohydrolase I restores vascular tetrahydrobiopterin level and endothelial function in low renin hypertension*. *Circulation*, 2003. **108**(10): pp. 1238–45.

- [61] Frostell, C., et al., *Inhaled nitric oxide. A selective pulmonary vasodilator reversing hypoxic pulmonary vasoconstriction*. Circulation, 1991. **83**(6): pp. 2038–47.
- [62] Cannon, R.O., 3rd, et al., *Effects of inhaled nitric oxide on regional blood flow are consistent with intravascular nitric oxide delivery*. J Clin Invest, 2001. **108**(2): pp. 279–87.
- [63] van Faassen, E.E., et al., *Nitrite as regulator of hypoxic signaling in mammalian physiology*. Med Res Rev, 2009. **29**(5): pp. 683–741.
- [64] Cosby, K., et al., *Nitrite reduction to nitric oxide by deoxyhemoglobin vasodilates the human circulation*. Nat Med, 2003. **9**(12): pp. 1498–505.
- [65] Liu, C., et al., *Mechanisms of human erythrocytic bioactivation of nitrite*. J Biol Chem, 2015. **290**(2): pp. 1281–94.
- [66] Hunter, C.J., et al., *Inhaled nebulized nitrite is a hypoxia-sensitive NO-dependent selective pulmonary vasodilator*. Nat Med, 2004. **10**(10): pp. 1122–7.
- [67] Shiva, S., et al., *Nitrite augments tolerance to ischemia/reperfusion injury via the modulation of mitochondrial electron transfer*. J Exp Med, 2007. **204**(9): pp. 2089–102.
- [68] Webb, A., et al., *Reduction of nitrite to nitric oxide during ischemia protects against myocardial ischemia-reperfusion damage*. Proc Natl Acad Sci U S A, 2004. **101**(37): pp. 13683–8.
- [69] Omar, S.A., E. Artime, and A.J. Webb, *A comparison of organic and inorganic nitrates/nitrites*. Nitric Oxide-Biol Chem, 2012. **26**(4): pp. 229–240.
- [70] Lundberg, J.O., E. Weitzberg, and M.T. Gladwin, *The nitrate-nitrite-nitric oxide pathway in physiology and therapeutics*. Nat Rev Drug Discov, 2008. **7**(2): pp. 156–67.
- [71] Carlstrom, M., et al., *Dietary inorganic nitrate reverses features of metabolic syndrome in endothelial nitric oxide synthase-deficient mice*. Proc Natl Acad Sci U S A, 2010. **107**(41): pp. 17716–20.
- [72] Carlstrom, M., et al., *Dietary nitrate attenuates oxidative stress, prevents cardiac and renal injuries, and reduces blood pressure in salt-induced hypertension*. Cardiovasc Res, 2011. **89**(3): pp. 574–85.
- [73] Bryan, N.S., et al., *Dietary nitrite supplementation protects against myocardial ischemia-reperfusion injury*. Proc Natl Acad Sci U S A, 2007. **104**(48): pp. 19144–9.
- [74] Hendgen-Cotta, U.B., et al., *Dietary nitrate supplementation improves revascularization in chronic ischemia*. Circulation, 2012. **126**(16): pp. 1983–92.
- [75] Trostchansky, A., et al., *Nitro-fatty acids: formation, redox signaling, and therapeutic potential*. Antioxid Redox Signal, 2013. **19**(11): pp. 1257–65.
- [76] O'Donnell, V.B., et al., *Nitration of unsaturated fatty acids by nitric oxide-derived reactive species*. Methods Enzymol, 1999. **301**: pp. 454–70.
- [77] Delmastro-Greenwood, M., B.A. Freeman, and S.G. Wendell, *Redox-dependent anti-inflammatory signaling actions of unsaturated fatty acids*. Annu Rev Physiol, 2014. **76**: pp. 79–105.

- [78] Rudolph, V., et al., *Endogenous generation and protective effects of nitro-fatty acids in a murine model of focal cardiac ischaemia and reperfusion*. *Cardiovasc Res*, 2010. **85**(1): pp. 155–66.
- [79] Wu, G. and S.M. Morris, Jr., *Arginine metabolism: nitric oxide and beyond*. *Biochem J*, 1998. **336**(Pt 1): pp. 1–17.
- [80] Schulman, S.P., et al., *L-arginine therapy in acute myocardial infarction: the vascular interaction with age in myocardial infarction (VINTAGE MI) randomized clinical trial*. *JAMA*, 2006. **295**(1): pp. 58–64.
- [81] Wilson, A.M., et al., *L-arginine supplementation in peripheral arterial disease: no benefit and possible harm*. *Circulation*, 2007. **116**(2): pp. 188–95.
- [82] Pernow, J. and C. Jung, *Arginase as a potential target in the treatment of cardiovascular disease: reversal of arginine steal?* *Cardiovasc Res*, 2013. **98**(3): pp. 334–43.
- [83] Boger, R.H., *The pharmacodynamics of L-arginine*. *J Nutr*, 2007. **137**(6 Suppl 2): pp. 1650S–1655S.
- [84] Solomonson, L.P., et al., *The caveolar nitric oxide synthase/arginine regeneration system for NO production in endothelial cells*. *J Exp Biol*, 2003. **206**(Pt 12): pp. 2083–7.
- [85] Podjarny, E., et al., *Effect of chronic tetrahydrobiopterin supplementation on blood pressure and proteinuria in 5/6 nephrectomized rats*. *Nephrol Dial Transplant*, 2004. **19**(9): pp. 2223–7.
- [86] Hong, H.J., et al., *Supplementation with tetrahydrobiopterin suppresses the development of hypertension in spontaneously hypertensive rats*. *Hypertension*, 2001. **38**(5): pp. 1044–8.
- [87] Miao, Y., et al., *Folic acid prevents and partially reverses glucocorticoid-induced hypertension in the rat*. *Am J Hypertens*, 2007. **20**(3): pp. 304–10.
- [88] Krug, S., et al., *N-Acetylcysteine prevents but does not reverse dexamethasone-induced hypertension*. *Clin Exp Pharmacol Physiol*, 2008. **35**(8): pp. 979–81.
- [89] Zhang, Y., et al., *Apocynin but not allopurinol prevents and reverses adrenocorticotrophic hormone-induced hypertension in the rat*. *Am J Hypertens*, 2005. **18**(7): pp. 910–6.
- [90] Zhang, Y., Y. Miao, and J.A. Whitworth, *Aspirin prevents and partially reverses adrenocorticotrophic hormone-induced hypertension in the rat*. *Am J Hypertens*, 2007. **20**(11): pp. 1222–8.
- [91] Zhang, Y., et al., *The anti-oxidant Tempol reverses and partially prevents adrenocorticotrophic hormone-induced hypertension in the rat*. *J Hypertens*, 2003. **21**(8): pp. 1513–8.
- [92] Mondo, C.K., et al., *Anti-oxidant effects of atorvastatin in dexamethasone-induced hypertension in the rat*. *Clin Exp Pharmacol Physiol*, 2006. **33**(11): pp. 1029–34.
- [93] Beswick, R.A., et al., *Long-term antioxidant administration attenuates mineralocorticoid hypertension and renal inflammatory response*. *Hypertension*, 2001. **37**(2 Pt 2): pp. 781–6.
- [94] Ortiz, M.C., et al., *Antioxidants block angiotensin II-induced increases in blood pressure and endothelin*. *Hypertension*, 2001. **38**(3 Pt 2): pp. 655–9.

- [95] Schnackenberg, C.G. and C.S. Wilcox, *Two-week administration of tempol attenuates both hypertension and renal excretion of 8-Iso prostaglandin f₂alpha*. *Hypertension*, 1999. **33**(1 Pt 2): pp. 424–8.
- [96] Arstall, M.A., et al., *N-acetylcysteine in combination with nitroglycerin and streptokinase for the treatment of evolving acute myocardial infarction. Safety and biochemical effects*. *Circulation*, 1995. **92**(10): pp. 2855–62.
- [97] Smilkstein, M.J., et al., *Efficacy of oral N-acetylcysteine in the treatment of acetaminophen overdose. Analysis of the national multicenter study (1976 to 1985)*. *N Engl J Med*, 1988. **319**(24): pp. 1557–62.
- [98] Das, U.N., *Folic acid says NO to vascular diseases*. *Nutrition*, 2003. **19**(7–8): pp. 686–92.
- [99] Topal, G., et al., *Homocysteine induces oxidative stress by uncoupling of NO synthase activity through reduction of tetrahydrobiopterin*. *Free Radic Biol Med*, 2004. **36**(12): pp. 1532–41.
- [100] Terzolo, M., et al., *Hyperhomocysteinemia in patients with Cushing's syndrome*. *J Clin Endocrinol Metab*, 2004. **89**(8): pp. 3745–51.
- [101] Hu, L., et al., *Apocynin but not L-arginine prevents and reverses dexamethasone-induced hypertension in the rat*. *Am J Hypertens*, 2006. **19**(4): pp. 413–8.
- [102] Wassmann, S., et al., *Cellular antioxidant effects of atorvastatin in vitro and in vivo*. *Arterioscler Thromb Vasc Biol*, 2002. **22**(2): pp. 300–5.
- [103] Sarath, T.S., et al., *Atorvastatin ameliorates arsenic-induced hypertension and enhancement of vascular redox signaling in rats*. *Toxicol Appl Pharmacol*, 2014. **280**(3): pp. 443–54.
- [104] Silva, C.M., et al., *Regulation of the human glucocorticoid receptor by long-term and chronic treatment with glucocorticoid*. *Steroids*, 1994. **59**(7): pp. 436–42.
- [105] Wallace, A.D., et al., *Lysine 419 targets human glucocorticoid receptor for proteasomal degradation*. *Steroids*, 2011. **75**(12): pp. 1016–23.
- [106] Forster, C., et al., *Glucocorticoid effects on mouse microvascular endothelial barrier permeability are brain specific*. *J Physiol Lond*, 2006. **573**(2): pp. 413–425.
- [107] Aird, W.C., *Endothelial cell heterogeneity*. *Crit Care Med*, 2003. **31**(4 Suppl): pp. S221–30.