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# Endothelial nitric oxide synthase impairment is restored by clofibrate treatment in an animal model of hypertension

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ABSTRACT

Adequate production of nitric oxide (NO) by endothelial nitric oxide synthase (eNOS) requires eNOS coupling promoted by tetrahydrobiopterin ($BH_4$). Under pathological conditions such as hypertension, $BH_4$ is diminished, avoiding eNOS coupling. When eNOS is "uncoupled", it yields a superoxide anion instead of NO. Peroxisome proliferator activated receptors (NR1C) are a family of nuclear receptors activated by ligand. Clofibrate, a member of a hypolipidemic class of drugs, acts by activating the alpha isoform of NR1C. To determine the participation of NR1C1 activation in $BH_4$ and dihydrobiopterin ($BH_2$) metabolism and its implications on eNOS coupling in hypertension, we performed aortic coarctation (AoCo) at inter-renal level on male Wistar rats in order to have a hypertensive model. Rats were divided into the following groups: Sham + vehicle (Sham-V); AoCo + vehicle (AoCo-V); Sham + clofibrate (Sham-C), and AoCo + clofibrate (AoCo-C). Clofibrate (7 days) increased eNOS coupling in the AoCo-C group compared with AoCo-V. Clofibrate also recovered the $BH_4$:$BH_2$ ratio in control values and prevented the rise in superoxide anion production, lipoperoxidation, and reactive oxygen species production. In addition, clofibrate increased GTP cyclohydrolase-1 (GTPCH-1) protein expression, which is related with $BH_4$ recovered production. NR1C1 stimulation re-establishes eNOS coupling, apparently through recovering the $BH_4$:$BH_2$ equilibrium and diminishing oxidative stress. Both can contribute to high blood pressure attenuation in hypertension secondary to AoCo.

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## 1. Introduction

Nitric oxide (NO) is yielded as co-product of the enzymatic conversion of L-arginine into L-citrulline in the presence of the following co-factors: NADP; FAD; FMN; calcium; calmodulin, and tetrahydrobiopterin ($BH_4$) by a family of enzymes denominated nitric oxide synthases (NOS). Three NOS isoforms comprise the family: neuronal; inducible, and endothelial (eNOS). In order for eNOS to produce NO, homodimerization and stabilization by $BH_4$ are required in order to expose the catalytic site for L-arginine (Alderton et al., 2001). $BH_4$ is also responsible for electron transport among subunits, allowing L-arginine oxidation to produce NO. Therefore, NO production is $BH_4$-dependent for maintaining vascular physiological conditions. Moreover, it has been reported that under vascular pathological conditions, $BH_4$ plasma levels are reduced and eNOS is uncoupled, leading to eNOS-deficient activity and its unavailability to produce NO. Instead, eNOS produces superoxide anion ($O_2^{\bullet -}$), a powerful reactive oxygen species. Therefore, the reaction becomes the most important source of the generation of oxidant stress (Hein et al., 2009; Sasaki et al., 2008; Takaya et al., 2007), which promotes an oxidant environment, causing vasoconstriction and inflammatory signaling. Thus, $BH_4$ metabolism represents an important mechanism in the maintenance of cardiovascular homeostasis. Synthesis of $BH_4$ is mainly carried out by GTP cyclohydrolase-1 (GTPCH-1). The relevance of GTPCH-1 in cardiovascular pathology is supported by studies in which changes in GTPCH-1 are related with changes in blood pressure: for example, gene silencing (siRNA) of GTPCH-1 (knock-down mice) raises blood pressure compared with control siRNA (Wang et al., 2008). Moreover, pharmacologic inhibition of GTPCH-1 with 2,4-diamine-6-hydroxypyrimidine (DAHP) in rats yielded a 30-mm Hg rise in blood pressure in these DAHP-treated rats compared with control rats (Mitchell et al., 2003). Overexpression of GTPCH-1 attenuated the rise in blood pressure in mice with salt-sensitive hypertension (Du et al., 2008). In another study, it was demonstrated that GTPCH-1 gene transfer reversed $BH_4$ deficiency in diabetic rats

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and increased the synthesis of NO (Meininger et al., 2004), improving endothelial dysfunction, thus supporting the relevance of $BH_4$ in blood pressure regulation.

Peroxisome proliferator activated receptors (NR1C) are nuclear receptors that act as transcription factors. There are three isoforms (alpha, beta/delta, and gamma). The alpha isoform (NR1C1) is stimulated by a family of hypolipidemic drugs known as fibrates. Once activated, NR1C heterodimerize with retinoic X receptors (NR2B) and these translocate to the nucleus and bind DNA to an NR1C-specific response element (PPRE), modulating gene expression. NR1C1 stimulation upregulates eNOS expression in bovine endothelial cells, suggesting that NR1C1 activation benefits endothelial functions (Wang and Wang, 2006). Hence, it has been proposed that NR1C1 participates in vascular tone regulation through the NO-pathway. Because eNOS lacks a PPRE, it has been hypothesized that NR1C1 stimulation stabilizes eNOS mRNA and consequently increases eNOS protein (Goya et al., 2004). However, it is possible that NR1C1 activators can modulate eNOS activity probably by affecting $BH_4$ metabolism. Thus, we hypothesized that NR1C1 activation with clofibrate would stimulate GTPCH-1 expression promoting $BH_4$ synthesis and its bioavailability, favoring eNOS coupling and reducing anion superoxide.

## 2. Material and methods

### 2.1. Animals

Male Wistar rats (weight, 250–300 g) were divided into two groups: Sham-operated, and aortic coarctated (AoCo). Aortic coarctation was performed as previously described (Fernandes et al., 1976). Briefly, under deep anesthesia with isoflurane, animal subjects underwent abdominal laparotomy to expose aorta at the inter-renal level and were partially ligated with silk (3–0). Reproducibility was achieved by tying the silk temporarily to the artery with a 19-gauge needle. The rats were sutured in layers and allowed to recover. These groups were subdivided into the following treatment groups: Sham-vehicle (Sham-V); Sham-clofibrate (100 mg/kg/day, intraperitoneal [i.p.], Sham-C); AoCo-vehicle (AoCo-V), and AoCo-clofibrate (100 mg/kg/day, i.p., AoCo-C). Treatment was administered for 7 days. The animals were maintained at standard conditions of light and temperature and with water and food *ad libitum*. Once the pharmacological treatment was accomplished, the rats were anesthetized with pentobarbital (50 mg/kg, i.p.) and intracarotid blood pressure was measured as previously described (Cervantes-Pérez et al., 2010). Serum, liver, right kidney, and aorta were obtained, frozen in liquid nitrogen, and maintained at −80 °C for subsequent experiments.

### 2.2. NOS activity

Tissues were homogenized at 0 °C in 500 µl of a buffer (50 mM Tris–HCl, 0.1 mM EGTA, β-mercaptoethanol 0.1%, pH 7.5) containing a cocktail of protease inhibitors (100 µM leupeptin, 1 mM phenylmethylsulfonyl fluoride, 2 µg/ml aprotinin, 10 µg/ml soybean trypsin inhibitor), and Nonidet P-40 0.1% (v/v). The samples containing 500 µg of protein were incubated for 30 min at 37 °C in the presence of 10 µM L-arginine-HCl, 1 mM NADP, 100 nM calmodulin, 30 µM tetrahydrobiopterin, 2.5 mM $CaCl_2$, and 0.2 µCi [$^3$H]-L-arginine (Amersham, Buckinghamshire, UK). Maximum volume for each reaction was 100 µl. Reactions were stopped by the addition of 1 ml of ice-cold stop buffer containing 2 mM EGTA, 2 mM EDTA, and 20 mM HEPES at pH 5.5. The reaction mixture was applied to a cation exchange resin column (Dowex-50 W) that had been previously equilibrated with stop buffer. The amount of labeled L-citrulline was measured using a Beckman LS6500 scintillation counter. Data were expressed as ng L-citrulline/500 µg protein/30 min.

### 2.3. Determination of nitrites and nitrates

Kidney homogenates and plasma samples were analyzed according to Miranda et al. (2001) using Griess reagents by means of a spectrophotometric technique UV–vis Spectrometer DW-2000 (SLM-Aminco Co., Urbana, IL, USA).

### 2.4. Determination of superoxide anion

Aortas were cut into 5-mm segments, embedded in Tissue-Tek® Optimum cutting temperature (OCT) compound and immediately frozen using liquid nitrogen. Unfixed frozen aortic rings and transversally sliced kidneys were cut into 10-µm sections and placed on a glass slide. Slides were incubated with dihydroethidium (1 µmol/l) in a light-protected, humidified chamber at 37 °C for 30 min and cover-slipped. Tissue sections were visualized with a Zeiss 210 confocal microscope with fluorescence detected with a 780-nm long-pass filter, and images were collected and stored digitally. Red nucleus in tissue sections represents $O_2^{\bullet -}$ level (Fernandes et al., 2007).

### 2.5. Reactive oxygen species determination

Reactive oxygen species were evaluated according to a previously reported method (Pérez-Severiano et al., 2004). Five microliters of homogenized kidney was mixed with 1.945 ml TRIS–HEPES (18:1) (TRIS pH 7.4: HEPES solution, in mM: NaCl 120, KCl 2.5, $NaH_2PO_4$ 1.2, $MgCl_2$ 0.1, $NaHCO_3$ 5.0, glucose 6.0, $CaCl_2$ 1.0, HEPES 10.0) and 50 µl of 2′-7′-dichlorodihydrofluorescein diacetate (DCFH-DA) 10 mM. Samples were incubated for 1 h at 37 °C with constant shaking. The amount of fluorescence was determined in a fluorescence spectrophotometer (Perkin-Elmer LS3) at 488-nm excitation and at 525-nm emission. Values were obtained interpolating the readings with a standard DCF curve (Sigma Chemical Co., St. Louis, MO, USA). Data is expressed as DCF nmol/mg protein/min.

### 2.6. Lipid peroxidation determination

The formation of lipid-soluble fluorescent compounds was measured according to Pérez-Severiano et al. (2004). One third of hypertrophic kidney was homogenized in 3 ml of saline solution (0.9% NaCl). One-milliliter aliquots were added to 4 ml of chloroform–methanol (2:1 v/v), vortexed, and placed on ice for 30 min in the dark. The upper phase was discarded and fluorescence was measured from the chloroformic phase by means of a fluorescence spectrophotometer at 370-nm excitation and 430-nm emission wavelengths in a Perkin-Elmer LS50-B luminescence spectrometer. The sensitivity of the equipment was adjusted to a fluorescent signal of 140 fluorescence units (FU) with a standard quinine solution (quinine, 0.001 mg/ml of $H_2SO_4$ 0.05 M). The results are expressed as FU per mg of protein.

### 2.7. Malondialdehyde determination

Serum malondialdehyde (MDA) levels were determined by capillary zone electrophoresis using ultraviolet (UV) detection by diode arranged according to Claeson et al. (2000). Briefly, serum was deproteinized with methanol (1:1) and centrifuged at 15,000×*g* for 15 min. Then, supernatant was filtered through a 0.22-µm nitrocellulose membrane and diluted 1:10 with NaOH 0.1 M. The sample was passed through Sep-Pak Classic $NH_2$ Waters-cartridge and analyzed directly. Analysis was performed by a P/ACE™ MDQ system from Beckman Coulter, which had a capillary preconditioned with NaOH 1 M for 10 min, distilled water for 10 min and finally work buffer containing Tris 0.1 M – boric acid 0.1 M – EDTA 2 mM, pH 8.75 for 10 min. Samples were injected under 0.5 psi/10 s hydrodynamic pressure. Sample separation was performed applying 30 kV for 10 min at

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