European Journal of Pharmaceutical Sciences 105 (2017) 144–149

Contents lists available at ScienceDirect

European Journal of Pharmaceutical Sciences

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

Treatment with sodium nitroprusside improves the endothelial function in aortic rings with endothelial dysfunction

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A R T I C L E I N F O

Keywords:
Sodium nitroprusside
Nitric oxide
Endothelial dysfunction
Superoxide

A B S T R A C T

Purpose: Verify if sodium nitroprusside (SNP) is able to improve endothelial function and if this effect is independent of nitric oxide (NO) release of the compound.

Methods: Normotensive (2K) and hypertensive (2K-1C) wistar rats were used. Intact endothelium aortas were placed in a myograph and incubated with SNP: 0.1 nM; 1 nM or 10 nM during 30 min. Cumulative concentration-effect curves for acetylcholine (Ach) were realized to measure the relaxing capacity. Intracellular NO were measured (by DAF-2DA probe) in HUVEC treated with SNP 0.1 nM or DETA/NO 0.1 μM. The detection of intracellular superoxide radical ($O_2^{\cdot-}$) was obtained by using DHE probe.

Results: Treatment of 2K-1C aortic rings with SNP (0.1; 1.0 and 10 nM) improved endothelium dependent relaxation induced by acetylcholine. This improvement induced by SNP was verified at the concentration of 0.1 nM, which does not release NO, suggesting that this effect was not induced due to NO release by SNP compound. Besides, we show that the cell treatment with 0.1 nM of SNP decreased the fluorescence intensity to DHE in cells stimulated with angiotensin II. These results indicate that SNP decreases the concentration of $O_2^{\cdot-}$ in HUVEC cells.

Conclusions: The SNP at a concentration that does not release NO inside the cells is able to attenuate endothelial dysfunction.

Drugs and chemicals: Acetylcholine (Ach) (PubChem CID:6060); angiotensin II human (Ang II) (PubChem CID: 16211177); diethylenetriamine/nitric oxide (DETA-NO) (PubChem CID 4518); dihydroethidium (DHE) (PubChem CID: 128682); phenylephrine (Phe) (PubChem CID: 5284443); sodium nitroprusside (SNP) (PubChem CID: 11963579); Thiazolyl Blue Tetrazolium Bromide (MTT) (PubChem CID: 64965); 4,5-diamino-fluorescein diacetate (DAF-2DA); 4-hidroxy-Tempo (Tempol) (PubChem CID: 137994), were purchased from Sigma–Aldrich (St. Louis, MO, USA).

1. Introduction

The endothelial cells play important functions in the modulation of vascular tonus, inflammation and coagulation, which are controlled by endothelial factors. Endothelial dysfunction is characterized by reduction of endothelium dependent vasodilation, mainly by decrease of endothelial cells nitric oxide (NO) release and/or an increase in endothelium-derived contracting factors (Vanhoutte et al., 2009; Mombouli and Vanhoutte, 1999). Endothelial dysfunction has been considered a marker for cardiovascular disease (Vanhoutte et al., 2009) since the decrease of bioavailability of NO contributes to the development and progression of hypertension and atherosclerosis (Yetik-Anacak and Catravas, 2006; Kawashima and Yokoyama, 2004; Harrison, 1997). According to the World Health Organization (2015), cardiovascular diseases are the main cause of global death.

Some studies have shown that the vascular relaxation in aorta from renal hypertensive rats is compromise due to many factors. Those factors include the caveolae number alteration (Rodrigues et al., 2007) and increased superoxide radical ($O_2^{\cdot-}$) generation (Rodrigues et al., 2008; Heitzer et al., 1999; Harrison and Ohara, 1995). The reactive oxygen species was found in high concentrations in aortic vascular smooth muscle cells from renal hypertensive rats (Rodrigues et al., 2008; Heitzer et al., 1999). Therefore, the NO bioavailability is decreased in presence of $O_2^{\cdot-}$, because it reacts with NO to form

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http://dx.doi.org/10.1016/j.ejps.2017.04.022
Received 28 November 2016; Received in revised form 27 March 2017; Accepted 25 April 2017
Available online 27 April 2017
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peroxynitrite ($ONOO^-$) (Cosentino et al., 1994; Auch-Schwelk et al., 1989). In this way, a compound with antioxidant properties for removal of $O_2^{\cdot -}$ and NO releasing would be desired to improve endothelial function.

Sodium nitroprusside (SNP) is an inorganic nitrous compound (nitroferricyanide) well known as NO donor which provides effects on arterial and venous beds. The SNP therapeutic potential in the treatment of hypertension was first suggested in 1928 and 1929 by Johnson (Tinker and Michenfelder, 1976) and its clinical use has been approved in 1974 (Friederich and Butterworth, 1995). The SNP gained acceptance for reducing intraoperative hypertension, decreasing afterload and improving cardiac output heart failure and inducing hypotension in the prevention of surgical blood loss (Hottinger et al., 2014). There are many limitations to its chronic use. High doses of SNP or its prolonged use has been associated with endothelial dysfunction (Fukatsu et al., 2007), as well as accumulation of cyanide and the metabolite thiocyanate (Arnold et al., 1984). However, it is not known the effects of low concentration of SNP on endothelial function and $O_2^{\cdot -}$ scavenging. Experimental evidence suggests that some of NO donors may reduce the bioavailability of reactive oxygen species (Oishi et al., 2015; Potje et al., 2014; Metzker et al., 2013).

In this way, the present study aimed to investigate whether low concentration of SNP can revert the endothelial dysfunction by inactivate $O_2^{\cdot -}$ by a NO release independent mechanism.

2. Methods

2.1. Experimental animals

Male wistar rats were used weighing 180–200 g. Animals were maintained on a light-dark cycle with free access to both food (standard rat chow) and water. Renovascular hypertension was induced in rats using the 2K-1C model proposed by Goldblatt et al. (1934), and adapted to small animals, where only one renal artery is constricted to reduce chronic renal perfusion. Before renovascular hypertension induction, all animal were acclimated for 2 weeks in biotery. Animals were anesthetized with tribromoethanol (2.5 mg/kg, i.p.) and after a midline laparotomy a silver clip with an internal diameter of 0.20 mm was placed around the left renal artery. Normotensive two-kidney rats (2K) were only submitted to laparotomy. The systolic blood pressure (SBP) was measured by an indirect tail-cuff method (MLT125R pulse transducer/pressure cuff coupled to the PowerLab 4/S analog-to-digital converter; AD Instruments Pty Ltd., Castle Hill, Australia) weekly in non-anesthetized animals. Rats were considered hypertensive when the SBP was higher than 160 mm Hg, six weeks after surgery. The SBP was higher in 2K-1C rats (183.0 ± 4.1 mm Hg, n = 17) as compared with 2K rats (132.0 ± 4.7 mm Hg, n = 18, $p < 0.001$), six weeks after surgery. Experimental protocols followed standards and politics of the Animal Care and Use Committee of the Federal University of São Carlos (CEUA no. 5990210915).

2.2. Vascular reactivity

Rats were anesthetized with isoflurane and after euthanized by decapitation six weeks after surgery and the thoracic aortas were dissected and cut into 4 mm in length rings and placed in bath chambers containing Krebs solution at 37 °C, pH 7.4, continuously bubbled with 95% O_2 and 5% CO_2, in an isometric myograph (Mulvany-Halpern-model 610 DMT-USA, Marietta, GA) and recorded by a PowerLab8/SP data acquisition system (ADInstruments Pty Ltd., Colorado Springs, CO).

The aortic rings were submitted to a tension of 1.5 g before addition of the given drug. Endothelial integrity was assessed by the degree of relaxation induced by 1 μmol/L acetylcholine after contraction of the aortic ring by phenylephrine (0.1 μmol/L). The ring was discarded when relaxation with acetylcholine was lower than 80% in 2K and 60% in 2K-1C rat aortas. After the endothelial integrity test, aortic rings were pre-contracted with phenylephrine (0.1 μM) and then were constructed concentration–effect curves to acetylcholine (0.1 nM to 0.1 mM).

To relaxation induced by NO donor Deta-NO, the endothelium was mechanically removed by gently rolling the lumen of the vessel on a thin wire. Endothelial integrity was assessed by relaxation induced by 1 μmol/L acetylcholine after contraction of the aortic ring by phenylephrine (0.1 μmol/L). The aortic rings were discarded if there was any sign of relaxation.

Aortic rings from 2K and 2K-1C were treated for 30 min with SNP (at concentrations: 0.1; 1.0 or 10 nM) or PBS (control). After incubation, aortic rings were washed 3 times to remove drugs, than pre-contracted with phenylephrine (0.1 μM) and concentration-effect curves to acetylcholine or to Deta-NO were constructed. The potency values (pD2) and maximum relaxant effect (ME) were analyzed. Each experiment was performed on rings prepared from different rats.

2.3. Cell culture

Immortalized human umbilical vein endothelial cells (HUVEC) were maintained in DMEM medium (Dulbecco Modified Earle's balanced salt solution) supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/mL) and streptomycin (100 μg/mL). Cells were grown in humidified incubator containing 5% CO_2 at 37 °C.

2.4. Nitric oxide production

HUVEC were seeded in 96 well plates at the concentration of 5×10^4 cells per well and maintained at 37 °C in humidified incubator containing 5% CO_2. The experiments were performed after 24 h. The detection of intracellular NO was performed by incubation with selective fluorescent probe 4,5-diaminofluorescein (DAF-2DA - 10 μM) during 30 min, to react with dinitrogen trioxide (N_2O_3) (oxidation product of NO) and produces the fluorescent compound DAF-2T (Nakatsubo et al., 1998). The reading was held in SpectraMax GeminiXS fluorometer (Molecular Devices) at 485 nm excitation and 538 nm emission wavelength pair, respectively. In addition, the NO production was examined in a fluorescence microscope (Axiovert, Zeiss) at 40 × objective lens magnification.

2.5. Detection of superoxide radical

HUVEC were seeded in 96 well plates at a concentration of 5×10^4 cells per well and maintained at 37 °C in humidified incubator containing 5% CO_2. The experiments were performed after 24 h. Cells were treated with SNP 0.1; 1 or 10 nM during 30 min followed by treatment with angiotensin II (Ang II) 0.1 μM for 1 h. The detection of intracellular superoxide radical ($O_2^{\cdot -}$) was performed with 50 μM Dihydroethidium (DHE) added to the samples and the reads were conducted after 20 min. The increase in fluorescence intensity was monitored with a fluorescence microplate reader (SpectraMax GeminiXS, Molecular Devices) at 510 nm and 595 excitation wavelength pair.

2.6. Cell viability

Cell viability was determined by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay (Mosmann, 1983). Cells were seeded in 96 well plates at a concentration of 5×10^4 cells per well and were maintained at 37 °C in humidified incubator containing 5% CO_2. After 24 h, cells were treated with SNP (0.1 nM, 1 nM or 10 nM) with and without Ang II 0.1 μM during 4 h. The same protocol was performed with PBS. After that, 5 mg/mL of MTT was added, followed by 4 h incubation at 37 °C, 5% CO_2. Later, 100 μL of dimethyl sulfoxide was added and remained on the plate shaker for 10 min. The formazan crystals were measured as absorbance at 540 nm using a microplate

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