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Pharmacological characterization of the mechanisms underlying the vascular effects of succinate

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ABSTRACT

We investigated the mechanisms underlying the vascular effects of succinate. Vascular reactivity experiments were performed in aortic rings isolated from male Wistar rats and C57BL/6 wild type (WT) or GPR91$^{-/-}$ mice. Nitrate/nitrite (NOx) was measured colorimetrically whereas 6-keto-prostaglandin $F_{1\alpha}$ (stable product of prostacyclin) was measured by enzyme immunoassay (EIA). Phosphorylation of endothelial nitric oxide synthase (eNOS) was assessed by western immunoblotting. Functional assays revealed that the direct effect of succinate in the vasculature is biphasic. At lower concentrations succinate induced relaxation while at higher concentrations succinate induced vascular contraction. Succinate concentration dependently relaxed rat aortic rings with intact endothelium. Endothelial removal reduced, but not abolished succinate-induced relaxation. Similarly, succinate relaxed endothelium-intact and endothelium-denuded aortas isolated from both C57BL/6 and GPR91$^{-/-}$ mice. Pre-incubation of endothelium-intact, but not endothelium-denuded rat aortic rings with L-NAME, indomethacin and tetraethylammonium (TEA) reduced succinate-induced relaxation. In endothelium-intact rings, succinate-induced relaxation was attenuated by ODQ, haemoglobin, Rp-8-Br-Pet-cGMPS, thapsigargin, wortmannin and SC-560. Blockade of K^+ channels with 4-aminopyridine, apamin and charybdotoxin reduced succinate-induced relaxation. Succinate increased the concentration of NOx and 6-keto-prostaglandin $F_{1\alpha}$ as well as eNOS phosphorylation at ser^{1177} residue. $CaCl_2$-induced contraction of endothelium-intact or endothelium-denuded aortas was not affected by succinate. The major finding of our study is that it first demonstrates a direct effect of succinate in the vasculature. Succinate displays a biphasic and concentration-dependent effect. The vascular relaxation induced by succinate is partially mediated by endothelial GPR91 receptors via the NO-cGMP pathway, a vasodilator cyclooxygenase (COX) product(s) and the opening of K^+ channels.

1. Introduction

Succinate is normally present in the mitochondria as part of the citric acid cycle (also called the Krebs cycle) to produce adenosine triphosphate and for this reason it plays a central role in cell biochemistry and bioenergetics. In the Krebs cycle, succinate is a substrate for succinate dehydrogenase, which promotes the dehydrogenation of succinate to fumarate. However, succinate is also found in the systemic circulation and increasing evidences show that its actions extend to signal transduction, paracrine modulation and inflammation (Tretter et al., 2016).

Succinate acts as a signaling molecule by binding to a G-protein-coupled receptor termed GPR91 (He et al., 2004). The succinate receptor GPR91 (or SUCNR1) is expressed in several tissues such as the spleen, kidney, liver, placenta, cardiomyocytes, retina and the aorta (Sapieha et al., 2008; Aguiar et al., 2010; Diehl et al., 2016). Plasma succinate concentrations vary from 6 to 20 μmol/l in rodents and from 2 to 20 μmol/l in humans (Kushnir et al., 2001; Sadagopan et al., 2007). The half-maximal response concentration for succinate-induced activation of mouse and human GPR91 receptor are 28 ± 5 and 56 ± 8 μmol/l, respectively (He et al., 2004).

GPR91 activation couples to a pertussis-toxin-insensitive G_q pathway and to a pertussis-toxin-sensitive G_i/G_o pathway (He et al., 2004). For that reason, GPR91 signaling is complex and involves the activation of several intracellular pathways. In cardiomyocytes, the increase in Ca^{2+} mobilization induced by succinate is mediated by adenylyl cyclase and protein kinase A (PKA) (He

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et al., 2004; Aguiar et al., 2010). Phospholipase C (PLC) is also activated by GPR91 receptors, resulting in cleavage of phosphatidylinositol 4,5-bisphosphate (PIP_2) to diacylglycerol (DAG) and inositol trisphosphate (IP_3). IP_3 and DAG induce Ca^{2+} mobilization and protein kinase C (PKC) activation, respectively, and subsequent nitric oxide (NO) and prostaglandin(PG) E_2 production as well as activation of mitogen-activated protein kinases (MAPK) such as ERK1/2 and p38MAPK (He et al., 2004; Toma et al., 2008; Li et al., 2014).

Succinate acts as a signaling molecule in the cardiovascular system. Intravenous administration of succinate increases plasma renin activity and mean arterial blood pressure in rats (He et al., 2004). In cardiomyocytes, succinate increases global Ca^{2+} transient, an effect that is mediated by GPR91 receptors and PKA activation (Aguiar et al., 2010). Regarding its vascular effects, succinate acts as a mediator of vessel growth in normal retinal development and proliferative ischemic retinopathy, where it regulates the production of angiogenic factors, such as vascular endothelial growth factor (VEGF) (Sapieha et al., 2008). In rats, the expression of GPR91 receptors was previously described in vascular endothelial cells, in both terminal afferent arteriole and glomerulus (Toma et al., 2008). More recently, Diehl et al. (2016) demonstrated that GPR91 receptors are expressed in the mouse aorta.

Increased mobilizations of extracellular and intracellular Ca^{2+}, activation of PKA, PKC and MAPK and the generation of vasorelaxing mediators such as NO, PGI_2 and PGE_2 are some of the intracellular pathways activated by GPR91 receptors (He et al., 2004; Aguiar et al., 2010). These pathways play an important role in the maintenance of the vascular tonus (Touyz and Schiffrin, 2000; Hristovska et al., 2007; Wynne et al., 2009; Bauer and Sotníková, 2010). Since GPR91 receptors are expressed in the vasculature and succinate signaling involves the activation of intracellular pathways that are described to mediated vascular contraction and/or relaxation, we hypothesized that succinate would induce a direct effect in the vasculature. Although *in vitro* and *in vivo* studies have shown that succinate displays cardiovascular actions, there is no evidence on the effect of succinate on vascular responsiveness. In the present study, we aimed to investigate the vascular effects of succinate and the mechanisms underlying such effects.

2. Material and methods

2.1. Animals

All experiments were approved by the Ethical Animal Committee of the *Campus* of Ribeirão Preto - Universidade de São Paulo (#12.1.1348.53.5). Male Wistar rats (230–250 g) and C57BL/6 or GPR91$^{-/-}$ mice (20–25 g) were used for aorta isolation. GPR91$^{-/-}$ mice were generated in the C57BL/6 background by Deltagenon as previously described (Rubic et al., 2008) and were provided by Novartis (Novartis Institutes for Biomedical Research, Switzerland). Animals were housed in a temperature-controlled room under a 12-h light-dark cycle with free access to standard chow and water.

2.2. Vessel ring preparation

Rats and mice were anaesthetised intraperitoneally with urethane at 1.25 g/kg (Sigma-Aldrich, St. Louis, MO, USA). The thoracic aorta was removed, cleaned of adherent connective tissues and cut into rings (4–5 mm in length). Two stainless-steel stirrups were passed through the lumen of each ring. One stirrup was connected to an isometric force transducer (TRI201; Panlab, Spain) to measure tension in the vessels. The rings were placed in organ chambers containing Krebs solution at 37 °C and gassed with 95% O_2/5% CO_2. The composition of Krebs solution was as follows (mmol/l): NaCl, 118.0; KCl, 4.7; KH_2PO_4, 1.2; $MgSO_4$, 1.2; $NaHCO_3$, 15.0; Glucose, 5.5; $CaCl_2$, 2.5. The aortas from rats and mice were stretched until they reached a basal tension of 15 mN and 5 mN, respectively. The basal tension for the studies with the rat aorta was determined by length-tension relationship experiments carried in our laboratory. For studies with the mouse aorta, resting tension was based on a previous study (Russell and Watts, 2000). Then, the rings were allowed to equilibrate for 60 min and during this time, the bath fluid was changed every 15–20 min. For some experiments, the endothelium was removed mechanically by rolling the lumen vessel on a thin wire. Endothelial integrity was assessed quantitatively by the degree of relaxation caused by the muscarinic agonist acetylcholine (1 μmol/l) in the presence of contractile tone induced by phenylephrine (0.1 μmol/l). For studies of endothelium-intact vessels, a ring was discarded if relaxation with acetylcholine was not 70% or greater. For studies of endothelium-denuded vessels, a ring was discarded if there was any degree of relaxation. Agonist concentration-response curves were fitted using the nonlinear interactive fitting program Graph Pad Prism 3.0 (GraphPad Software Inc., San Diego, CA). Agonist maximal responses and potencies were expressed as E_{max} (maximum effect elicited by the agonist) and pD_2 ($-\log EC_{50}$), respectively.

2.3. Effect of succinate on aortic rings contracted with phenylephrine

In order to establish a concentration-response curve for succinate, the aorta was exposed to initial concentrations of succinate that are within those found physiologically in plasma. Steady tension was evoked by phenylephrine (0.1 or 1 μmol/l for rat and mouse aortas, respectively), and succinate was then added in a stepwise fashion in rat aortic rings (1 μmol/l to 10 mmol/l) or endothelium-intact and denuded aortic rings isolated from C57BL/6 or GPR91$^{-/-}$ mice (0.1 fmol/l to 1 mmol/l).

To investigate the mechanisms underlying succinate-induced relaxation, endothelium-intact or denuded rat aortic rings were contracted with phenylephrine (0.1 μmol/l and 0.03 μmol/l, respectively) 30 min after being incubated with one of the following drugs: N^G-Nitro-L-arginine methyl ester (non-selective NO synthase inhibitor, L-NAME, 100 μmol/l), 1 H-[1,2,4]Oxadiazolo[4,3-*a*] quinoxalin-1-one (selective guanylyl cyclase inhibitor, ODQ, 1 μmol/l), indomethacin (non-selective cyclooxygenase [COX] inhibitor, 10 μmol/l), tiron (superoxide anion scavenger, 100 μmol/l), PEG-catalase (analogue of endogenous catalase, 250 /ml), tetraethylammonium (non-selective K^+ channel blocker, TEA, 1 mmol/l), 9-(Tetrahydro-2-furanyl)-9H-purin-6-amine (adenylate cyclase inhibitor, SQ22536, 100 μmol/l) or *N*-[2-[[3-(4-Bromophenyl)-2-propen-1-yl]amino]ethyl]-5-Isoquinolinesulfonamide dihydrochloride (cAMP-dependent protein kinase inhibitor, H89, 1 μmol/l). Concentration of the inhibitors was based on previous studies (Rees et al., 1990; Nelson and Quayle, 1995; Chinellato et al., 1998; Yogi et al., 2010). Because L-NAME and ODQ enhanced phenylephrine-induced contraction, the rings with intact endothelium exposed to these compounds were pre-contracted with phenylephrine (0.03 μmol/l) to induce a magnitude of contraction similar to that found in the intact rings not exposed to the inhibitors.

In another set of experiments, we examined the mechanisms underlying the endothelial response to succinate. Endothelium-intact rings were pre-incubated for 30 min with the following drugs: 7-nitroindazole (selective nNOS inhibitor, 100 μmol/l), haemoglobin (extracellular NO scavenger, 10 μmol/l), Rp-8-Br-Pet-cGMPS (cGMP-dependent protein kinase inhibitor, 3 μmol/l), thapsigargin (sarcoplasmic reticulum Ca^{2+}-ATPase inhibitor, 1 μmol/l), wortmannin (inhibitor of phosphoinositide (PI) 3-kinase, 1 μmol/l), SC-560 (selective COX-1 inhibitor, 1 μmol/l),