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L-Carnitine ameliorates the oxidative stress response to angiotensin II by modulating NADPH oxidase through a reduction in protein kinase c activity and NF-κB translocation to the nucleus

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ABSTRACT

L-Carnitine (LC) exerts beneficial effects in arterial hypertension due, in part, to its antioxidant capacity. We investigated the signalling pathways involved in the effect of LC on angiotensin II (Ang II)-induced NADPH oxidase activation in NRK-52E cells. Ang II increased the generation of superoxide anion from NADPH oxidase, as well as the amount of hydrogen peroxide and nitrotyrosine. Co-incubation with LC managed to prevent these alterations and also reverted the changes in NADPH oxidase expression triggered by Ang II. Cell signalling studies evidenced that LC did not modify Ang II-induced phosphorylation of Akt, p38 MAPK or $ERK_{1/2}$. On the other hand, a significant decrease in PKC activity, and inhibition of nuclear factor kappa B (NF-kB) translocation, were attributable to LC incubation. In conclusion, LC counteracts the pro-oxidative response to Ang II by modulating NADPH oxidase enzyme via reducing the activity of PKC and the translocation of NF-kB to the nucleus.

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1. Introduction

Arterial hypertension is one of the main risk factors for development of cardiovascular disease. The pathogenesis of hypertension is associated with enhanced production of reactive oxygen species (ROS) that participate in the initiation, maintenance and progression of hypertensive disease via different molecular mechanisms, including inflammatory and fibrotic processes. This eventually leads to endothelial dysfunction, cardiovascular and renal remodelling, and increased peripheral resistance with subsequent elevated blood pressure (Mate, Miguel-Carrasco, & Vázquez, 2010; Zambrano, Blanca, Ruiz-Armenta, Miguel-Carrasco, Arévalo, et al., 2013; Zambrano, Blanca, Ruiz-Armenta, Miguel-Carrasco, Revilla, et al., 2013).

The enzyme, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, is the most important source of ROS in hypertension-related organ damage. Moreover, angiotensin II (Ang II), which is known to be a major contributor to hypertension via systemic, vascular and renal effects, can also act as a potent inducer of oxidative stress, since Ang II-dependent hypertension is particularly sensitive to NADPH oxidase activation (Montezano & Touyz, 2012). Five different NADPH oxidase homologues, namely NOX1-NOX5, and two related oxidases, Duox 1 and Duox 2, have been characterized so far (Rodiño-Janeiro et al., 2013). Of special relevance in the cardiovascular and renal systems, NOX2/gp91 phox is localized in phagocytes, vascular cells, heart, kidney, neurons, myocytes and hepatocytes. It consists of two membrane-bound subunits, p22phox and gp91phox (also called NOX2), which form the cytochrome b558 complex; and four cytosolic subunits, p40phox, p47phox, p67phox, and Rac1 or 2, which upon stimulation translocate to b558 and lead to assembly and activation of the enzymatic complex. NOX1 is mainly located in epithelial cells of colon, prostate, uterus, endothelial cells, adventitial fibroblasts and smooth muscle cells. NOX4 is abundantly represented in the kidney (hence its equivalent term, Renox), and is also expressed in endothelial cells, fibroblasts, osteoclasts, vascular smooth mus-

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cle cells (VMSCs) and cardiac myocytes (Ago, Kuroda, Kamouchi, Sadoshima, & Kitazono, 2011).

The activity of NOX1 and NOX2 can be stimulated by Ang II through interaction with Ang II type 1 (AT-1) receptor. Following the action of AT-1 receptor, several signalling cascades are affected, including the activation of protein kinase C (PKC) and phosphoinositide 3-kinase (PI3K)/Akt pathways; this results in the phosphorylation of p47phox and its translocation to membrane for NADPH oxidase activation (San José et al., 2009). Additional pathways involved in Ang II-mediated NADPH oxidase activation include mitogen-activated protein (MAP) kinases (p38 MAPK or extracellular signal-regulated kinase (ERK)½) and the nuclear factor kappa B (NF-kB) (Wei et al., 2006).

L-Carnitine (β-hydroxy-γ-*N*-trimethylammonium-butyrate; LC) is a natural compound that can be incorporated into the body from dietary sources and also via endogenous biosynthesis. From a physiological point of view, carnitine has long been recognised as a vital component in lipid metabolism for the production of ATP through the beta-oxidation of long-chain fatty acids (Mate et al., 2010). In addition, supplementation with LC and its short-chain esters, such as acetyl L-carnitine and propionyl-L-carnitine, has also proven effective in different pathologies, including cardiovascular diseases (Lee, Lin, Lin, & Lin, 2014), diabetes (Flanagan, Simmons, Vehige, Willcox, & Garrett, 2010), renal failure (Higuchi et al., 2014), neurodegenerative diseases (Traina, 2016) and ulcerative colitis (Scioli et al., 2014). Recently, Higuchi et al. (2016) have reported the role of LC in the improvement of cardiac function in haemodialysis patients with left ventricular hypertrophy. These and other studies have suggested that the beneficial effects of LC and its short-chain ester derivatives might arise from their antioxidant capacity, especially. However, studies reporting precise molecular mechanisms of carnitine action are scarce.

Although the therapeutic indications of LC are primarily limited to those pathologies presenting with a clear deficiency of this compound, recent evidence supports the notion that carnitine is not a mere cofactor in beta-oxidation but rather possess many undiscovered functions in the pathophysiology of different diseases, including arterial hypertension (Mate et al., 2010). In this regard, several experimental approaches have tried to deepen our understanding of those signalling pathways involved in LC effects. For instance, Mohmmad Abdul and Butterfield (2007) demonstrated that pretreatment of neurons with acetyl-L-carnitine confers protection against 4-hydroxynonenal (HNE)-induced neurotoxicity via activation of PI3K/Akt and $ERK_{1/2}$ pathways. Zhang et al. (2009) reported that acetyl-L-carnitine can block tumour necrosis factor alpha (TNF-α)-induced insulin resistance through ERK and Akt phosphorylation. Similarly, propionyl-L-carnitine stimulated eNOS phosphorylation in cultured human aortic endothelial cells via activation of PI3K and Akt pathways (Ning & Zhao, 2013). In addition, it has been demonstrated that L-carnitine is also able to: i) induce senescence in glioblastoma cells by activation of p38 MAPK pathway (Yamada, 2012); ii) exert antifibrotic effects through an inhibition in angiotensin II-induced ERK phosphorylation in cardiac fibroblasts (Chao et al., 2010); and iii) activate insulin-like growth factor (IGF)-1/PI3K/Akt signalling pathway in rat skeletal muscle (Keller, Couturier, Haferkamp, Most, & Eder, 2013).

In the last years, the role of LC and its short-chain esters in the pathogenesis of arterial hypertension has had considerable attention. Experimental and clinical data support that LC treatment exerts beneficial effects on arterial and pulmonary hypertension (Arduini, Bonomini, Savica, Amato, & Zammit, 2008). Our research group has previously reported hypotensive, antioxidant, anti-inflammatory and antifibrotic effects of LC in different rat models of arterial hypertension (Mate et al., 2010; Miguel-Carrasco, Monserrat, Mate, & Vázquez, 2010; Zambrano, Blanca, Ruiz-Armenta, Miguel-Carrasco, Revilla, et al., 2013). In humans, a sequential off-on-off pilot study demonstrated a significant decrease in blood pressure after oral administration of acetyl-L-carnitine (Ruggenenti et al., 2009). On the other hand, a paradoxical association between elevated blood pressure and high serum levels of LC and long-chain acylcarnitines in African and Caucasian men was recently reported (Mels et al., 2013). The authors suggested that higher serum carnitine levels might come from enhanced biosynthesis as an adaptive response to increased oxidative stress, or be the result of reduced tissue uptake and/or renal excretion of LC.

Although the antioxidant capacity of LC might also been responsible for its ability to lower arterial blood pressure, the intracellular signalling pathways involved in this hypotensive effect of LC have not been described so far. Therefore, and taking into account the widely accepted relationship between the renin-angiotensin system, oxidative stress, hypertension and renal damage, the aims of the present study were to evaluate the effects of LC on Ang II-induced oxidative stress in epithelial tubular cells (namely, normal rat kidney (NRK)-52E cells), and to explore potentially relevant intracellular mechanisms of LC action. To this purpose, gene and protein expression of NADPH oxidase isoforms (NOX1, NOX2 and NOX4), as well as generation of ROS and nitrosylation of proteins, were determined in NRK-52E cells following exposure to Ang II and/or LC. In addition, gene silencing and selective inhibition studies were performed in order to know which NOX isoforms are targeted by LC. Finally, we have also studied the signalling pathways involved in the induction of NADPH oxidase by Ang II, as well as the potential counteracting effects of LC in this regard.

2. Materials and methods

2.1. Materials and reagents

Cell-culture media/supplements and Amplex Red colorimetric assay for hydrogen peroxide were purchased from Invitrogen (Alcobendas, Madrid, Spain). Angiotensin II (PubChem CID: 172198), oxypurinol (PubChem CID: 4644), rotenone (PubChem CID: 6758), diphenyleneiodonium (PubChem CID: 3101), lucigenin (PubChem CID: 16219691), superoxide dismutase, wortmannin (PI3K inhibitor, PubChem CID: 312145), SB203580 (p38MAPK inhibitor, PubChem CID: 176155), PD98059 (MEK-1 inhibitor, PubChem CID: 4713), bisindolylmaleimide I (PKC inhibitor, PubChem CID: 2396) and 2-acetylphenothiazine (PubChem CID: 81131) were all obtained from Sigma-Aldrich (Madrid, Spain). Lipofectamine®2000, Opti-MEM® Reduced Serum Medium, Silencer Selected Negative Control siRNA (s4390844), Silencer Selected pre-designed siRNA from NOX4 (s136995), TRIzol® reagent for RNA isolation and reverse transcription kit were acquired from Life Technologies (Alcobendas, Madrid, Spain). gp91 ds-tat was purchased from Anaspec (Fremont, CA). SYBR Green PCR Master Mix for quantitative PCR was purchased from Roche Diagnostics (Spain), and forward and reverse primers were supplied by Biomedal (Seville, Spain). anti-NOX1, anti-NOX4, anti-p-$ERK_{1/2}$, anti-$ERK_{1/2}$, anti-NF-kB and anti-p-α-IkB primary antibodies, as well as anti-rabbit and anti-mouse secondary antibodies, were acquired from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-NOX2 was obtained from Epitomics-Abcam (Burlingame, CA); anti-p-Akt, anti-Akt, anti-p-p38 MAPK and anti-p38 MAPK were purchased from Cell Signalling Technology (Beverly, MA); anti-nitrotyrosine was obtained from Millipore (Temecula, CA), and anti-β-actin was purchased from Sigma-Aldrich (Madrid, Spain). Protein assay reagent and bovine gamma globulin, as a protein standard, were obtained from Bio-Rad (Madrid, Spain). Protease inhibitor cocktail was acquired from Roche Diagnostics (Madrid, Spain), and ECL Prime Western Blotting Detection Kit was obtained from GE

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