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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.

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

Healthcare (Madrid, Spain). All other chemicals were obtained from Sigma-Aldrich, unless otherwise stated. L-carnitine was acquired from Fagron (Terrasa, Barcelona, Spain, PubChem CID: 10917).

2.2. Cell culture

NRK-52E cells (rat renal proximal tubular cells) were grown in Dulbecco's Modified Eagle Medium (DMEM) 1X supplemented with 10% foetal bovine serum and 1% streptomycin/penicillin. When the monolayer became confluent, NRK-52E cells were incubated in minimum medium (0.5% foetal bovine serum) for at least 8 h. Then, cells were incubated with Ang II (0.1–100 nM) with or without pre-incubation with LC (0.001–10 mM). Unless otherwise indicated, further experiments were performed using 100 nM Ang II (24 h) and/or 1 mM LC, the latter being always preincubated for 30 min before the addition of Ang II, where appropriate.

To determine H_2O_2 production, culture medium was used for Amplex Red assay. For protein analyses, cell lysates were homogenized in sucrose buffer (sucrose 150 mM, EGTA 1 mM, KH_2PO_4 50 mM) containing a protease inhibitor cocktail. mRNA was obtained as described below. Phosphorylation of Akt, p38 MAPK, $ERK_{1/2}$, NF-κB and IκB was estimated at different time intervals, as indicated in the corresponding figures. All drugs were initially prepared as 1000-fold concentrated stock solutions in appropriate diluents and added into the cell culture medium directly. LC was dissolved in PBS. Control cells were treated with the corresponding vehicle in all experiments.

2.3. siRNA experiments

NRK-52E cells were transfected with siRNA at a final concentration of 17.5 nM (NOX4 or Scramble), using Lipofectamine®2000 reagent according to the manufacturer's instructions. Cells were incubated for 24 h under serum- and antibiotic-free conditions. To examine the effect of si-RNA on NADPH oxidase activity and H_2O_2 production, cells were stimulated with Ang II and preincubated with LC as indicated above. NOX4 isoform depletion by siRNA was confirmed by reverse transcription – polymerase chain reaction (RT-PCR) (data not shown).

2.4. NADPH oxidase activity (NADPH oxidase-dependent $O_2^{\cdot-}$ production)

To determine NADPH oxidase activity, a lucigenin-enhanced chemiluminescence (CL) assay was used. The reactions were initiated by the addition of lucigenin (5 μM) into appropriate luminescence test tubes containing NADPH (0.1 mM) and samples (equivalent to 5 μg of protein homogenates), in a final volume of 300 μL. CL intensity was quantified by means of a CL reader (Junior LB9509, Berthold, Germany) for 4 min and reported in relative arbitrary units. The results are expressed as percentages of the control group, after subtracting a buffer blank from each reading. The responses to Ang II were measured in the absence or the presence of oxypurinol (an inhibitor of xanthine oxidase), rotenone (inhibitor of mitochondrial electrons chain), diphenyleneiodonium (DPI, inhibitor of flavoproteins), and superoxide dismutase (SOD, a superoxide anion scavenger). Specific inhibitors such as wortmannin, SB203580, PD98059 and bisindolylmaleimide I were also used to explore relevant signalling pathways. In some experiments, cells were preincubated for 30 min with a specific inhibitor of NOX1 (2-acetylphenothiazine), or NOX2 (gp91 ds-tat), as indicated in the corresponding figures.

2.5. Amplex red colorimetric assay

H_2O_2 production was measured in the cell media using the Amplex Red Hydrogen Peroxide/Peroxide Assay Kit according to the manufacturer's instructions. Previously, the culture media were diluted 1:1 in 1X Reaction Buffer. 50 μL of Amplex Red reaction mixture (i.e., 50 μL Amplex Red 10 mM and 100 μL horseradish peroxidase 10 U/mL diluted in 5 mL of reaction buffer) were added to 50 μL of samples. All reactions were performed in a final volume of 100 μL. Absorbance was measured on a microplate reader (Asys UVM 340, Biochrom, UK) at a wavelength of 560 nm.

2.6. Reverse-transcriptase polymerase chain reaction (RT-PCR)

Total RNA was extracted and retro-transcribed from cultured NRK-52E cells as previously described (Zambrano, Blanca, Ruiz-Armenta, Miguel-Carrasco, Arévalo, et al., 2013). The cDNA obtained was diluted in DEPC water and used for PCR (polymerase chain reaction) amplification. The primers used in this study were (5′–3′; forward – reverse): NOX1 (TTCACCAATTCCCAGGATT GAAGTGGATGGTC GACCTGTCACGATGTCAGTGGCCTTGTCAA); NOX2 (CCCTTTGGTACAGCCAGTGAAGAT–CAATCCCACGTCCCAC TAACATCA); NOX4 (TTGCTTTTGTATCTTC – CTTACCTTCGTCACAG); GAPDH (GCCAAAAGGGTCATCATCTCCGC – GGATGACCTTGCCCA-CAGCCTTG). Each specific gene product was amplified by the Light-Cycler® 480 Real-Time PCR System. Sybr Green™ reactions and the amplification data were collected by the sequence detector and analysed with sequence detection software. For each assay, a standard curve was constructed using increasing amounts of cDNA. In all cases, the slopes of the curves presented values between 3.2 and 3.4, which ensured appropriate PCR conditions. Glyceraldehyde-3-phosphate dehydrogenase (GADPH) mRNA was used as a housekeeping gene, and the quantitative fold changes in mRNA expression were calculated using the $2^{-\Delta\Delta Ct}$ method.

2.7. Western blotting

Total proteins from cell lysates were subjected to SDS-PAGE (4–20% gradient gel), and transferred into a nitrocellulose membrane. Membranes were blocked with 5% fat-free milk diluted in Tris-Buffered Saline with Tween® 20 (TBS-T) 1X, except for phosphorylation studies, in which they were blocked with 5% albumin in TBS-T 1X. For NADPH oxidase expression and nitrosative stress estimation, rabbit polyclonal anti-NOX1, rabbit polyclonal anti-NOX2, rabbit polyclonal anti-NOX4, and mouse monoclonal anti-nitrotyrosine were used. For phosphorylation studies, membranes were incubated with rabbit polyclonal (anti-p-Akt, anti-Akt, anti-p-p38 MAPK and anti-p38 MAPK), and mouse monoclonal (anti-p-$ERK_{1/2}$ and anti-$ERK_{1/2}$) antibodies. To estimate NF-κB nuclear translocation, rabbit polyclonal anti-NF-kB and mouse monoclonal p-α-IκB antibodies were employed. All antibodies were used at 1:1000 dilution, except for anti-NOX2 (1:2000). Mouse monoclonal anti-β-actin (1:5000) antibody was also used as a protein loading control. After incubation with the appropriate secondary antibody, signals were revealed with an enhanced chemiluminescence kit, and detected with a LAS-3000 mini System (Fujifilm, Japan).

2.8. PKC activity assay

PKC activity was assayed by a nonradioactive ELISA kit (Enzo Life Sciences, Belgium). Cells were centrifuged at 2000*g* for 10 min (4 °C). Then, the pellets were resuspended and incubated for 10 min in lysis buffer (MOPS 20 mM, beta-glycerophosphate 50 mM, sodium fluoride 50 mM, sodium orthovanadate 1 mM, EGTA 5 mM, EDTA 1 mM, NP-50 1%, DTT 1 mM, benzamidine 1 mM, PMSF 1 mM, leupeptin 10 μg/mL, and aprotinin 10 μg/mL).

Cytosolic fractions were obtained by centrifugation at 16,200*g* for 15 min and used for measuring PKC activity.

2.9. NF-kB p65 subunit translocation

Cells were centrifuged for 5 min at 1000*g* (4 °C). The resulting pellet was resuspended and incubated for 15 min in 150 μL of cold buffer A (Hepes 5 mM, $MgCl_2$ 1.5 mM, KCl 10 mM, IGEPAL 0.5%, dithiothreitol 0.5 mM, NaF 50 mM, and protease inhibitor cocktail), then centrifuged again for 10 min at 3000 g. The supernatant thus obtained corresponded to the cytoplasmic fraction. Afterwards, the pellet was washed with 1 mL of cold buffer B (i.e., cold buffer A without IGEPAL) and centrifuged for 10 min at 3000 g. This new pellet was resuspended and incubated for 30 min in 100 μL of cold buffer C (Hepes 20 mM, glycerol 25%, NaCl 0.5 M, $MgCl_2$1.5 mM, EDTA 0.5 mM, dithiothreitol 0.5 mM, NaF 50 mM, and protease inhibitor cocktail), and then centrifuged again for 30 min at 15,000 g. The resulting supernatant corresponded to the nuclear fraction. Finally, translocation of p65 from the cytosol to the nucleus was estimated by Western blotting as described above.

2.10. Statistical analysis

Data were analysed using one-way analysis of variance (ANOVA) followed by a Tukey multiple comparison test when differences were significant (Statgraphics Centurion XVI.I, Madrid, Spain). The null hypothesis was rejected when the p value was lower than 0.05.

3. Results

3.1. Dose-response relationship and time course of angiotensin II-induced superoxide anion production

Incubation of NRK-52E cells in the presence of Ang II resulted in an induction of $O_2^{\cdot-}$ production in a dose- and time-dependent manner (Fig. 1A and B). The highest level of stimulation was reached when using Ang II at 100 nM and 1-h incubation time, then remained constant up to 24 h (Fig. 1A and B). This dose of Ang II (100 nM) was selected for all subsequent experiments.

In order to characterize the enzymatic source responsible for $O_2^{\cdot-}$ release in response to Ang II, we analysed $O_2^{\cdot-}$ generation in NRK-52E cells in the presence of oxypurinol, rotenone and DPI. DPI completely abolished Ang II-induced $O_2^{\cdot-}$ production, whereas rotenone and oxypurinol were ineffective; these observations identify NADPH oxidase as the enzymatic source of $O_2^{\cdot-}$. In addition, the chemiluminescence signal was completely abolished in the presence of SOD, which confirmed $O_2^{\cdot-}$ as the reactive oxygen species detected by lucigenin (Fig. 1C). Therefore, any subsequent determination of superoxide anions is referred to as "NADPH oxidase activity".

3.2. L-Carnitine reduces AngII-induced NADPH oxidase hyperactivity, and hydrogen peroxide and nitrotyrosine excess production in NRK-52E cells

LC inhibited Ang II-induced NADPH oxidase activity in a dose-dependent manner, with the maximal inhibition rate obtained from 0.1 mM (Fig. 1D). The dose of 1 mM was chosen for subsequent experiments. As seen in Fig. 2A, no changes were observed when LC was added to control, unstimulated cells.

Stimulation with Ang II led to 1.7- and 1.5-fold increases in the production of hydrogen peroxide (Fig. 2B) and in the levels of nitrotyrosine (Fig. 2C), respectively. LC was able to restore these parameters up to those measured in control, unstimulated cells.

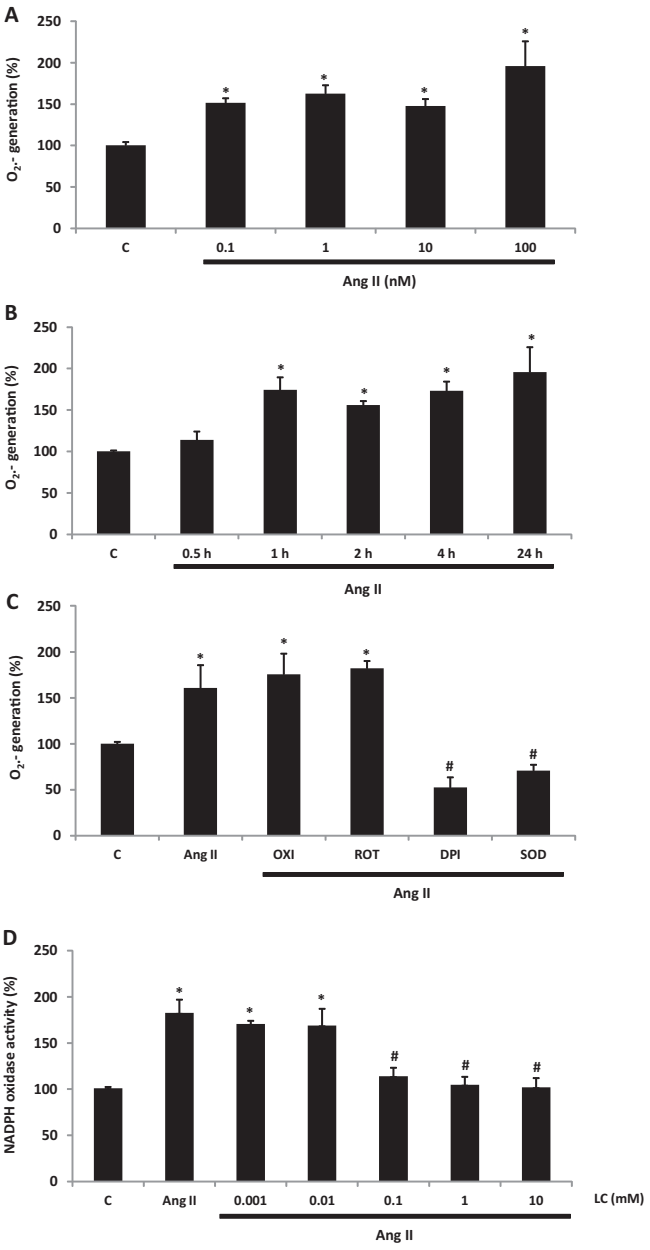

Fig. 1. Effect of angiotensin II (Ang II) and L-carnitine (LC) on $O_2^{\cdot-}$ production. (A) Dose-response curve for Ang II-dependent superoxide anion production. NRK-52E cells were incubated with Ang II (0.1–100 nM) for 24 h. (B) Time-response curve for Ang II-dependent superoxide anion production. Cells were incubated with Ang II (100 nM) for the indicated incubation times (h = hours). (C) Characterization of superoxide anion source in NRK-52E cells stimulated with Ang II. NRK-52E cells were incubated with Ang II (100 nM, 24 h) in the presence of 0.1 mM oxypurinol (OXI), rotenone (ROT) or diphenyleneiodonium (DPI), or 100 U/mL superoxide dismutase (SOD). (D) Dose-response curve for LC in NRK-52E cells treated with Ang II. Cells were incubated with Ang II (100 nM) in the presence of LC at the indicated doses. Histograms represent means ± SEM from four independent experiments. C = control, non-stimulated cells. $^{*}p < 0.05$ vs. C; $^{\#}p < 0.05$ vs. Ang II.

On the other hand, treatment of control cells with LC had no effect on these parameters (Fig. 2B and C).

In order to know which isoforms of NADPH oxidase were involved in the modulatory effects of LC on AngII-induced NADPH oxidase activity, experiments were performed in the presence of specific inhibitors of NOX1 (2-acetylphenothiazine) and NOX2 (gp91 ds-tat); this was complemented with gene silencing experiments for NOX4 isoform. Studies with NOX1 inhibitor indicated a

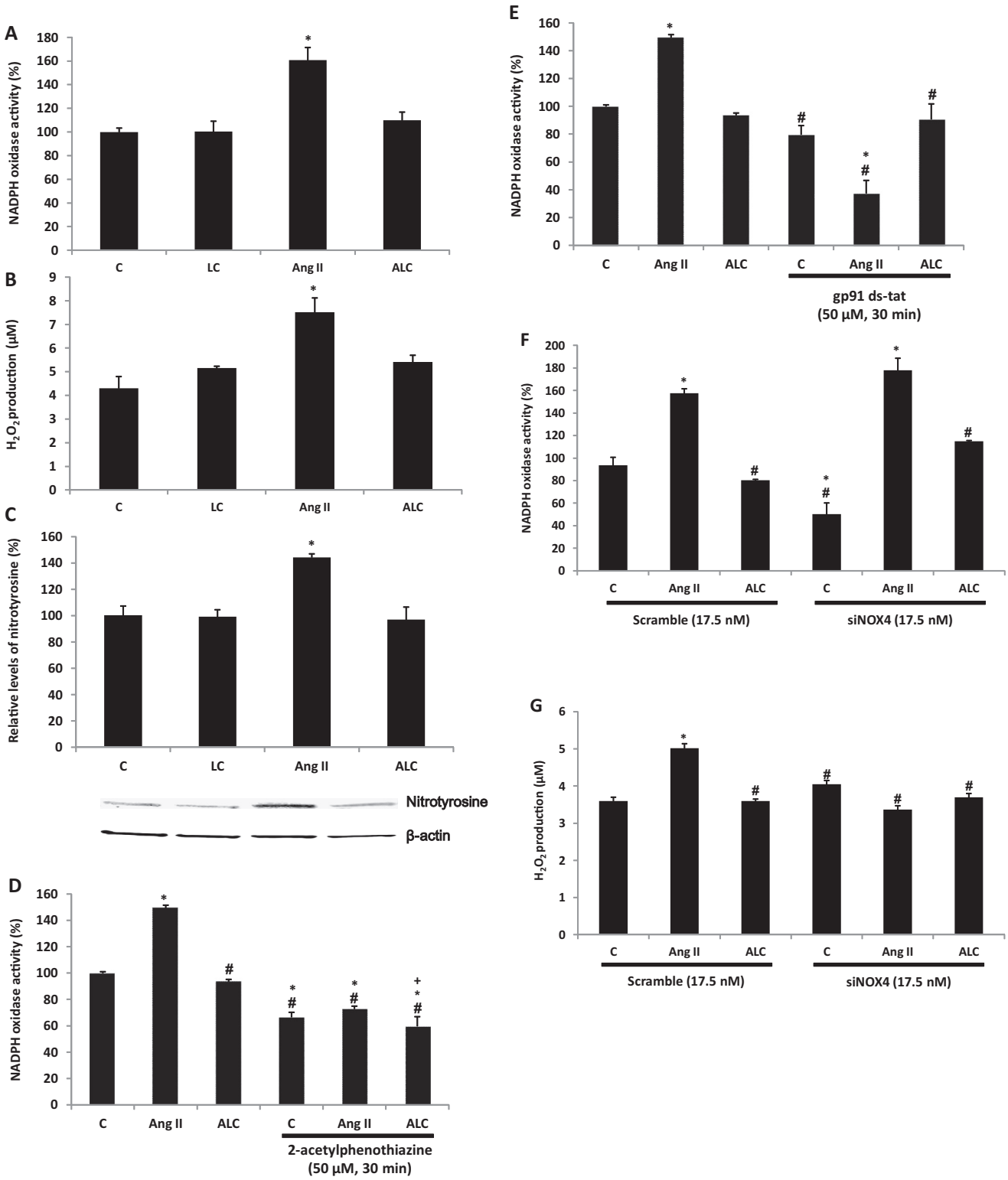

Fig. 2. Effect of angiotensin II (Ang II) and L-carnitine (LC) on nadph oxidase activity (A), hydrogen peroxide production (B) and levels of nitrotyrosine (C), and role of nadph oxidase isoforms in LC effect (D–G). NRK-52E cells were treated with 1 mM LC (LC), 100 nM Ang II (Ang II), or both (ALC), in the absence or the presence of the specific inhibitors for NOX1 (2-acetylphenothiazine; D), NOX2 (gp91 ds-tat; E), and Scramble (Scr) or NOX4 siRNA (F, G). Histograms represent means ± SEM from four independent experiments. C = control, non-stimulated cells. $^{*}p < 0.05$ vs. C; $^{\#}p < 0.05$ vs. Ang II; $^{+}p < 0.05$ vs. ALC.

decrease in NADPH oxidase activity in cells stimulated with Ang II in the absence and presence of LC, when compared with their corresponding control groups without the inhibitor; although, the highest reduction was observed in cells stimulated with Ang II. In addition, the inhibition of NOX1 led to diminished basal NADPH oxidase activity (Fig. 2D). The incubation with the inhibitor of NOX2 resulted in a decrease in NADPH oxidase activity only in Ang II-stimulated cells; on the other hand, no changes were found

in non-stimulated cells nor in those preincubated with LC, compared with their corresponding groups without the inhibitor (Fig. 2E). Meanwhile, the depletion of NOX4 did not produce changes in NADPH oxidase activity in any experimental groups, except for a reduction in unstimulated cells (Fig. 2F). However, when H_2O_2 production was studied in the same cells, we observed a decrease in Ang II-stimulated cells but no modifications in those incubated with both Ang II and LC (Fig. 2G).

3.3. L-Carnitine restores Ang II-induced changes in gene and protein expression of NADPH isoforms NOX1, NOX2 and NOX4

Exposure to Ang II resulted in twofold and threefold increases for NOX1 (Fig. 3A) and NOX2 (Fig. 3B) gene expression, respectively; this was accompanied by a 1.5-fold increase in the protein expression of both NADPH oxidase subunits (Fig. 3D and E). On the other hand, the expression of NOX4 decreased by 50%, approximately (Fig. 3C and F). Interestingly, when Ang II-treated cells were preincubated with LC, the protein and mRNA expressions of these three NADPH isoforms were normalized and therefore reached values similar to those observed in control cells (Fig. 3A–F). In all cases, no effect of LC *per se* was observed in unstimulated cells (i.e., in the absence of Ang II).

3.4. Effect of wortmannin, SB203580, PD98059 and bisindolylmaleimide I on Ang II-stimulated NADPH oxidase activity

To examine the signalling pathways that might be involved in the effect of LC on Ang II-induced NADPH oxidase stimulation, cells were preincubated with 1 μM wortmannin, SB203580, PD98059 or bisindolylmaleimide I for 1 min. As shown in Fig. 4A, all these inhibitors prevented the excess in NADPH oxidase activity in response to Ang II, which indicates that Ang II can activate the enzyme through the PI3K/p38MAPK/$ERK_{1/2}$/PKC signalling pathways. Interestingly, no changes in superoxide production were observed in Ang II-induced cells preincubated with SB203580 and PD98059

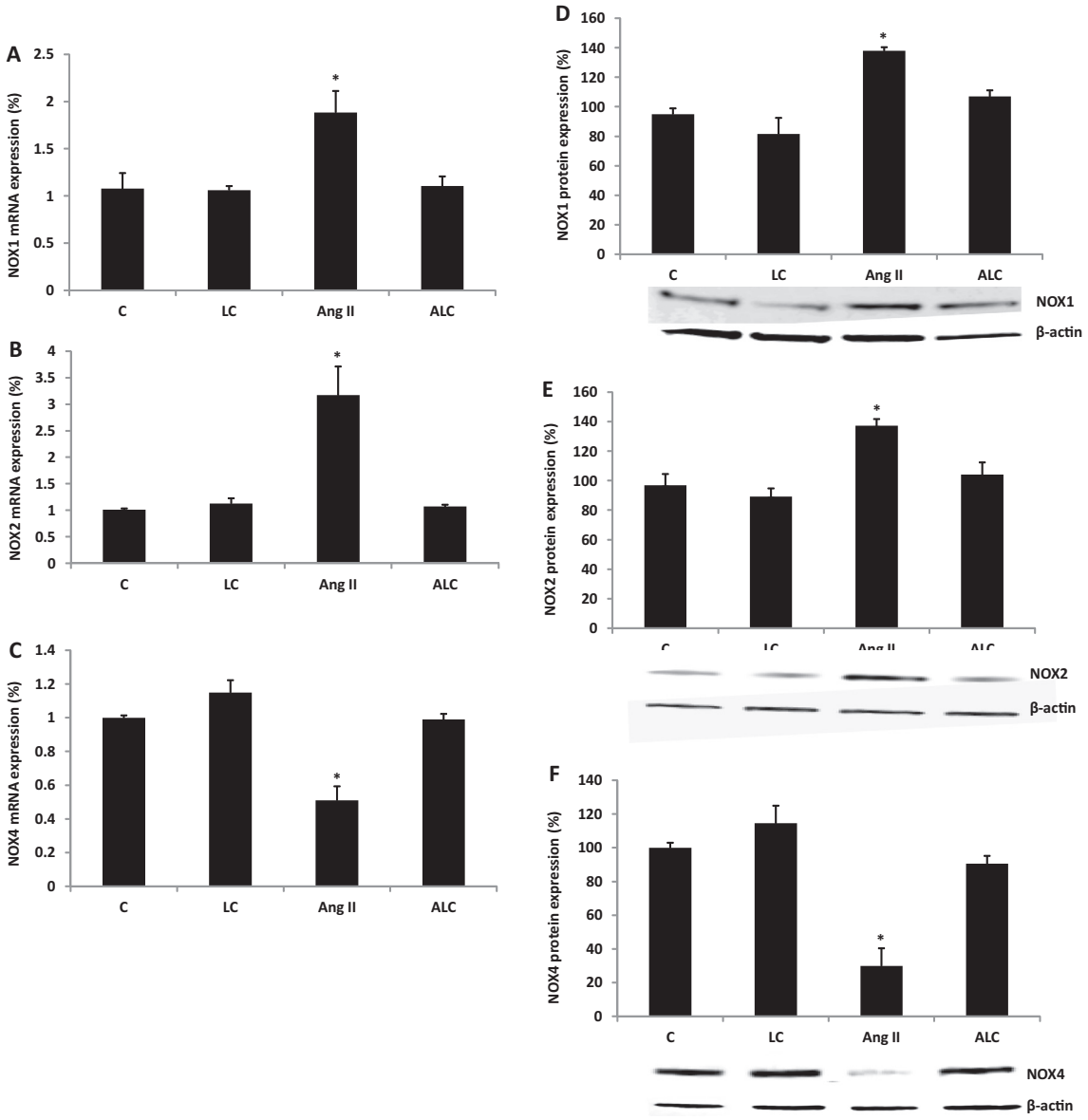

Fig. 3. Effect of angiotensin II (Ang II) and L-carnitine (LC) on NADPH oxidase gene and protein expression. Effect of Ang II and LC on gene/protein expression of NOX1 (A, D), NOX2 (B, E) and NOX4 (C, F). NRK-52E cells were treated with 1 mM LC (LC), 100 nM Ang II (Ang II), or both (ALC). Histograms represent means ± SEM from four (gene expression) and three (protein expression) independent experiments. C = control, non-stimulated cells. *$p < 0.05$ compared with all other groups.

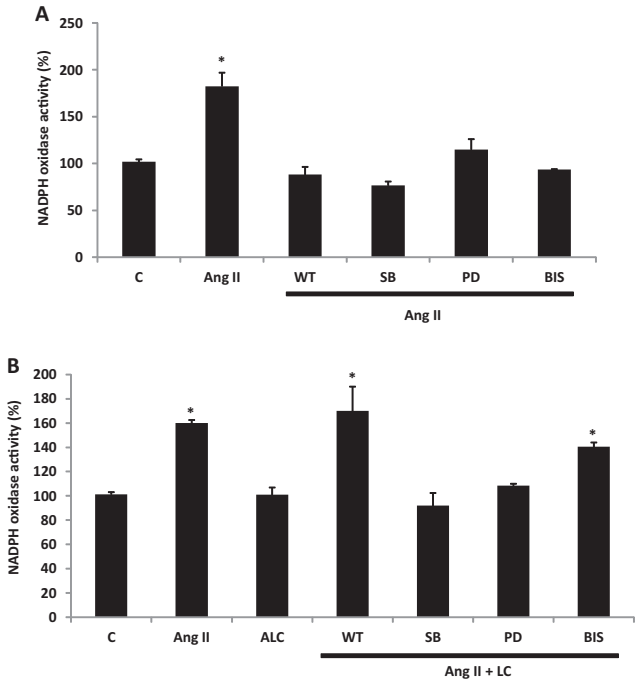

Fig. 4. Characterization of the signaling pathways involved in the activation of NADPH oxidase. (A) Pathways involved in the activation of NADPH oxidase by angiotensin II (Ang II). B) Pathways involved in the effect of L-carnitine (LC) on Ang II-mediated NADPH oxidase activation. NRK-52E cells were treated with 1 mM LC (LC), 100 nM Ang II (Ang II), or both (ALC and Ang II + LC). Specific inhibitors used (at 1 μM; 1 min preincubation) were, wortmannin (WT, PI3K inhibitor); SB203580 (SB, p38MAPK inhibitor); PD98059 (PD, MEK-1 inhibitor); and bisindolylmaleimide I (BIS, PKC inhibitor). Histograms represent mean ± SEM from three independent experiments. C = control, non-stimulated cells. $^{*}p < 0.05$ compared with all other groups.

plus LC when compared with Ang II-stimulated cell preincubated with LC alone (i.e., with the ALC group in Fig. 4B). However, wortmannin and bisindolylmaleimide I did reverse the inhibition of NADPH oxidase activity observed in this ALC group (Fig. 4B).

3.5. L-Carnitine does not affect Ang II-stimulated phosphorylation of Akt, p38 MAPK and $ERK_{1/2}$

To further study the signalling pathways involved in the inhibitory effect of LC on Ang II-induced NADPH oxidase activity, we examined the degree of phosphorylation of Akt, p38 MAPK and $ERK_{1/2}$. Incubation of NRK-52E cells with Ang II resulted in 1.5–1.6-fold increases in the relative levels of phosphorylation of Akt and p38 MAPK (Fig. 5A and C). Meanwhile, $ERK_{1/2}$ presented maximal phosphorylation (50%) after 45 min of stimulation with Ang II (Fig. 5E).

Incubations in the presence of LC did not modify Ang II-induced Akt (Fig. 5B), p38 MAPK (Fig. 5D) or $ERK_{1/2}$ (Fig. 5F) phosphorylation levels. In additional experiments, preincubation of cells with LC in the absence and in the presence of wortmannin yielded a 1.6-fold increase in Akt phosphorylation (Fig. 5G).

3.6. L-Carnitine inhibits Ang II-mediated NF-kB activation and PKC activity

Exposure of NRK-52E cells to Ang II led to a 1.5-fold increase in the activity of PKC, which was reverted after preincubation with LC (Fig. 6A). In addition, Ang II induced the activation of NF-kB at 20 min (80%) and 40 min (190%) of incubation time (Fig. 6B); this effect was also abolished by LC preincubation, with lower translocation of the complex NF-kB into the nucleus (Fig. 6C). As for NF-kB inhibitory protein, IkB, Ang II-dependent phosphorylation of IkB (70% increase) was completely abrogated by LC preincubation (Fig. 6D). No effect in any of these parameters was observed in control cells treated with LC (Fig. 6A, C, and D).

4. Discussion

The main findings of this study indicate that L-carnitine (LC) was able to counteract the increase in NADPH oxidase activity induced by Ang II *in vitro*, in rat renal proximal tubular cells (namely, NRK-52E cells). LC effect was implemented by modulating the expression/activity of NADPH oxidase through a reduction in the activity of protein kinase C and in the translocation of NF-kB to the nucleus.

As expected from previously published data, Ang II induced an increase in the production of superoxide anion via activation of NADPH oxidase, an effect that was accompanied by upregulation of the protein and gene expression of NOX1 and NOX2 isoforms of the enzyme (Dikalov et al., 2008; Qin et al., 2013). Nitrotyrosine levels were also elevated in cells treated with this peptide hormone, which might have resulted from NO/superoxide interaction following Ang II exposure (Mate et al., 2010).

Contrary to the effects on NOX1 and NOX2, Ang II downregulated the expression of NOX4 while increasing the release of hydrogen peroxide in our rat renal epithelial cell line, as discussed below. Interestingly, this latter effect of Ang II was abolished by NOX4 silencing. Other groups have also reported a reduction of NOX4 expression and higher production of hydrogen peroxide following *in vitro* exposure to Ang II; for instance, using rat aortic smooth muscle cells (Dikalov et al., 2008). On the other hand, our results differ from prior studies reporting an increase in NOX4 mRNA in NRK-52E cells stimulated with Ang II for 24 h (Peng et al., 2009), although in this case the authors did not measure NOX4 protein abundance.

Additional studies using different experimental approaches *in vivo* (e.g., in rats with aldosterone- or L-NAME-induced hypertension) have also found increased or unaltered expression of NOX4 (Bayorh, Rollins-Hairston, Adiyiah, Lyn, & Eatman, 2011; Zambrano, Blanca, Ruiz-Armenta, Miguel-Carrasco, Arévalo, et al., 2013; Zambrano, Blanca, Ruiz-Armenta, Miguel-Carrasco, Revilla, et al., 2013). In fact, there is contradictory information concerning the role of NOX4 in the pathophysiology of arterial hypertension, since both deleterious and protective effects have been attributed to this NOX isoform (Ray et al., 2011). Thus, although NOX4-derived ROS have been implicated in hypertension (Bayorh et al., 2011), recent studies claim that the NOX4 isoform of NADPH oxidase would generate mainly H_2O_2 instead of superoxide anion (in contrast to NOX1 and NOX2 isoforms that do generate superoxide). An elevation in hydrogen peroxide production might then have a protective effect in hypertension, as this molecule may act as an endothelium-derived vasodilator (Dikalov et al., 2008). In the present study, the lower expression of NOX4 derived from Ang II incubation was accompanied by an increase in the production of H_2O_2 and superoxide anion, as mentioned above. This suggests that, under our experimental conditions, the bulk of H_2O_2 would be generated from intermediate superoxide anion production rather than by activation of NOX4 (Helmcke, Heumüller, Tikkanen, Schröder, & Brandes, 2009).

Preincubation of NRK-52E cells with LC significantly reduced Ang II-induced NADPH oxidase activity and the release of superoxide anion and hydrogen peroxide. Using cardiac fibroblasts, Chao et al. (2010) observed a similar effect of LC against Ang II-increased NADPH oxidase activity and superoxide anion formation. Therefore, there seem to be enough evidence of the ability of LC to

FIGURE 5

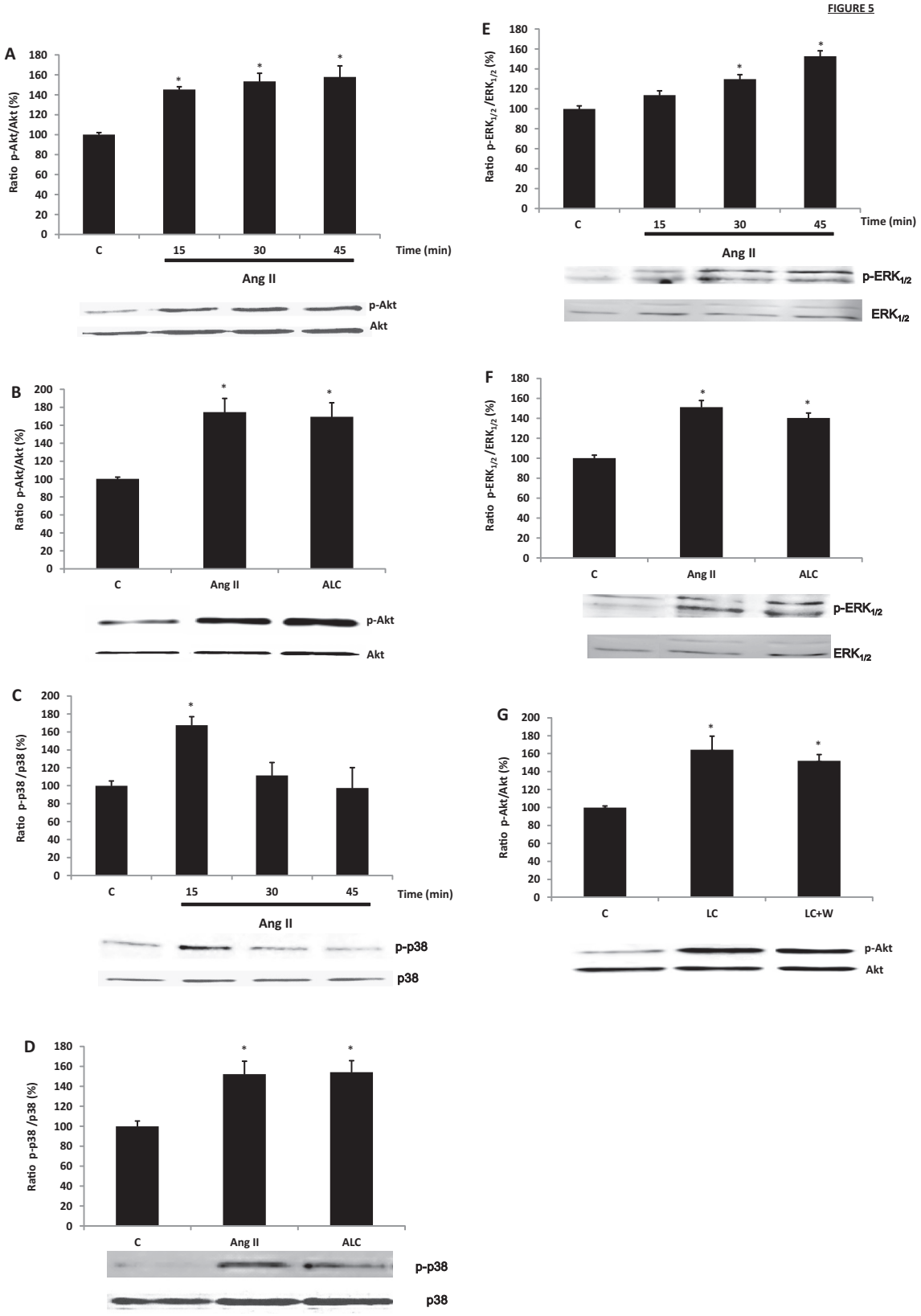

Fig. 5. Effect of L-carnitine (LC) on angiotensin II (Ang II)-increased AKT, P38 MAPK and $ERK_{1/2}$ phosphorylation. (A, C and E) Time-dependent activation of Akt, p38 MAPK and $ERK_{1/2}$ signalling pathways, respectively, induced by Ang II. Cells were incubated with Ang II (100 nM) for the indicated incubation times. (B, D and F) Effect of LC on Ang II-increased Akt, p38 MAPK and $ERK_{1/2}$ phosphorylation rate (Ang II incubation times = 15 min for Akt and p38 MAPK, and 45 min for $ERK_{1/2}$). (G) Effect of wortmannin (W) on Akt phosphorylation after LC incubation. NRK-52E cells were treated with 100 nM Ang II (Ang II), 100 nM Ang II plus 1 mM LC (ALC), 1 mM LC (LC), or 1 mM LC plus 1 μM wortmannin (LC + W). Histograms represent means ± SEM from three independent experiments. C = control, non-stimulated cells. In Fig. A, B, D, F, G, $^{*}p < 0.05$ vs. C; In Fig. C, E, $^{*}p < 0.05$ when compared with all other groups.

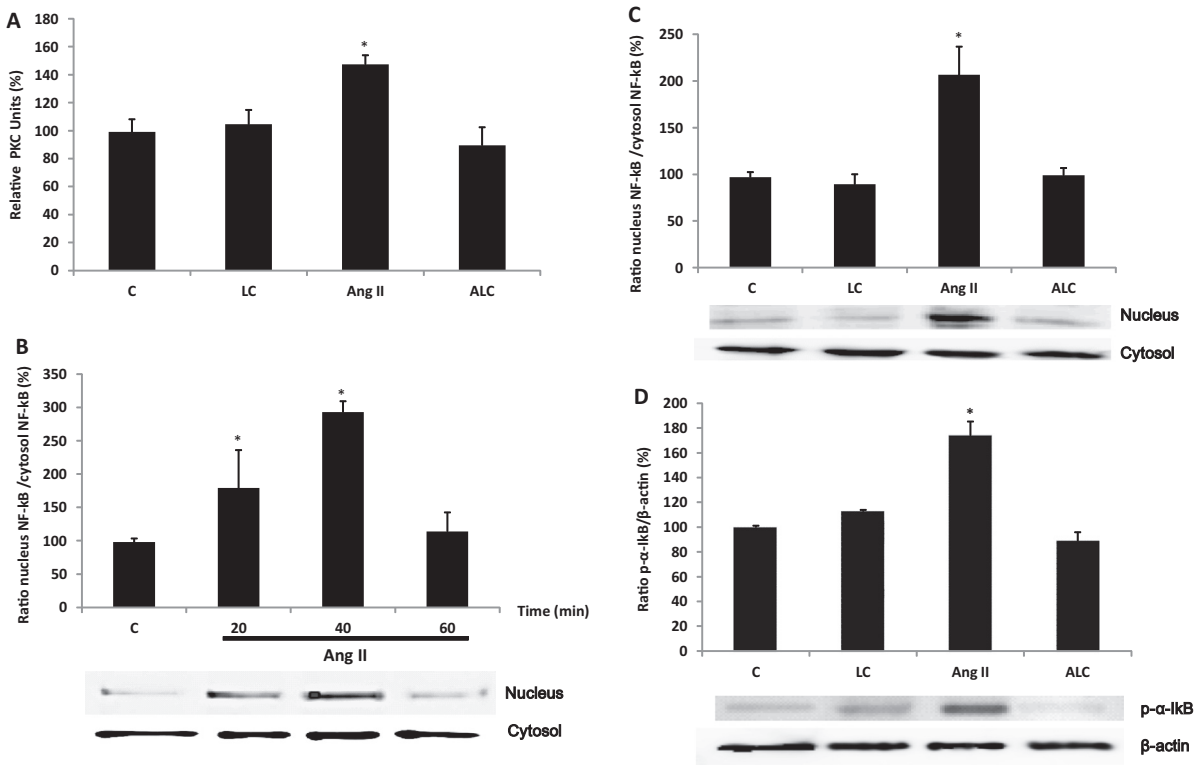

Fig. 6. Effect of L-carnitine (LC) on angiotensin II (ANG II)-induced PKC activity and NF-KB translocation to the nucleus. (A) Effect of LC on PKC activity induced by Ang II, (B) Time-dependent activation of NF-kB signalling pathways induced by Ang II. (C) Effect of LC on Ang II maximal peak activation of NF-kB signalling pathways. (D) Effect of LC on Ang II-induced IkB phosphorylation. NRK-52E cells were treated with 1 mM LC (LC), 100 nM Ang II (Ang II), or both (ALC). Ang II incubation times = 30 min for PKC, and 40 min for NF-kB (except for time-dependence, as indicated in the corresponding figure) and IkB experiments. Histograms represent mean ± SEM from three independent experiments. C = control, non-stimulated cells. $^{*}p < 0.05$when compared with all other groups.

reduce Ang II-induced ROS production *in vitro*. Antihypertensive drugs, such as losartan, showed similar counteracting effects after exposure of skeletal muscle cells to Ang II (Wei et al., 2006). Since the production of ROS induced by Ang II (mainly superoxide anion) is directly related to the increase in nitrotyrosine levels (Guo et al., 2003), it is not surprising that preincubation with LC in our study resulted in a reduction of nitrotyrosine levels up to values similar to those observed in control, unstimulated cells. Prior studies using losartan and/or the well-known antioxidant tempol yielded similar results in a rat model of renal hypertension (Dobrian, Schriver, & Prewitt, 2001).

We also found that the presence of LC in the incubation medium led to a decrease in the protein and gene expression of NOX1 and NOX2 isoforms of NADPH oxidase, while NOX4 isoform was upregulated. Thus, LC was able to restore the expression of all three isoforms of the enzyme that are present in NRK-52E cells, thus counteracting the alterations caused by Ang II in this regard. Previous *in vivo* studies carried out in our laboratory demonstrated a decrease in NADPH oxidase activity in the heart and kidney of hypertensive animals treated with LC chronically (Zambrano, Blanca, Ruiz-Armenta, Miguel-Carrasco, Arévalo, et al., 2013; Zambrano, Blanca, Ruiz-Armenta, Miguel-Carrasco, Revilla, et al., 2013); in these cases, the effect on enzyme activity was accompanied by a decrease in the protein/gene expression of NOX2 and NOX4. In studies carried out in platelets, other authors reported that LC inhibited superoxide anion formation elicited by arachidonic acid-mediated stimulation of NADPH oxidase (Pignatelli et al., 2003). Moreover, the short-chain carnitine ester, propionyl-L-carnitine, has been demonstrated to reduce the expression of NOX4 in human umbilical vascular endothelial cells (Stasi et al., 2010). Taken together, the effects of LC on NADPH oxidase function/expression might be dependent on the experimental design, e.g., *in vitro* vs. *in vivo* studies and/or the cell type used in the experiment.

To check the specific contributions of NOX isoforms towards LC-mediated inhibition of NADPH oxidase, we used selective NOX inhibitors and gene silencing approaches. Our studies indicate that NOX1 and NOX2, but not NOX4, seem to be involved in Ang II-stimulated NADPH oxidase activity, and that the effect of LC is not directly mediated by NOX isoforms. In contrast, depletion of NOX4 produced a decrease in H_2O_2 production in Ang II-stimulated cells, indicating that this isoform would be involved in the generation of H_2O_2. Moreover, NOX1 and NOX4 seem to be involved also in the basal activity of NADPH oxidase. Similar results were found by Dikalov et al. (2008) using rat aortic smooth muscle cells stimulated with Ang II. On the other hand, Stasi et al. (2010) observed that the beneficial effect of LC on intermittent claudication was NOX4-dependent and that NOX2 was not involved in this effect. Therefore, our study suggests, as previously demonstrated (Gülçin, 2006), that LC might act via superoxide anion and hydrogen peroxide scavenging, which in turn would reduce the generation of ROS and modulate the activity and expression of NADPH oxidase enzyme.

It is well documented that Ang II has a pivotal role as a pathogenic factor in the origin, development and maintenance of arterial hypertension. In particular, Ang II-dependent hypertension is especially sensitive to ROS derived from the activity of NADPH oxidase. In agreement with previous observations (Luengo-Blanco et al., 2008), we found that kinases such as PI3K/Akt, p38 MAPK, $ERK_{1/2}$ and PKC, as well as the activation of NF-κB, were all involved in the stimulation of NADPH oxidase mediated by Ang II. Interestingly, the normalization of NADPH oxidase activity resulting from

Ang II and LC co-incubation was abrogated in the presence of wortmannin and bisindolylmaleimide I, whereas no changes were observed in the presence of SB203580 and PD98059. These findings indicate that PI3K/Akt and PKC pathways are likely to be involved in the attenuation of NADPH oxidase activity mediated by LC. In addition, LC also counteracted the activation of NF-kB following exposure to Ang II. To further confirm these observations, we measured the degree of phosphorylation of Akt, p38 MAPK and $ERK_{1/2}$, as well as the activity of PKC. Our results indicate that incubation in the presence of LC did not modify Ang II-induced Akt, p38 MAPK or $ERK_{1/2}$ phosphorylations; on the contrary, we did observe a significant decrease in PKC activity after preincubation of NRK-52E cells with LC, so that the activity of this kinase returned to basal levels.

Studies concerning the underlying signalling pathways involved in the effects of LC and its short-chain esters indicate that these compounds can act by inducing phosphorylation of Akt, $ERK_{1/2}$ and p38 MAPK proteins. For instance, it has been reported that acetyl-L-carnitine exerts neuroprotective effects through activation of PI3K/Akt and $ERK_{1/2}$ (Mohmmad Abdul & Butterfield, 2007); it can also block TNF-α-induced insulin resistance by means of ERK and Akt phosphorylation (Zhang et al., 2009). Meanwhile, LC favoured p38 MAPK pathway and senescence in glioblastoma cells (Yamada, 2012), and activated IGF-1/PI3K/Akt signalling pathway in skeletal muscle of rats (Keller et al., 2013). Propionyl-L-carnitine has recently been found to activate eNOS via PI3K and Akt kinases (Ning & Zhao, 2013). In the present study, interestingly, Akt phosphorylation did not seem to parallel LC-mediated attenuation of NADPH oxidase activity (see Fig. 5B). It is also remarkable that direct Akt phosphorylation by LC was insensitive to wortmannin (Fig. 5G); this suggests that, in our experimental conditions, Akt phosphorylation was independent of PI3K. In fact, former studies carried out by Zhang et al. (2009) suggested that Akt might be phosphorylated by a PI3K-independent protein.

The importance of PKC in the activation of NADPH oxidase has been widely reported in the literature. According to our results, LC was capable of reducing the activity of this enzyme (thus reversing the stimulatory effect of Ang II) up to those values measured in control, unstimulated cells. This might result in an inhibition of p47phox phosphorylation that in turn would inactivate NADPH oxidase activity and reduce excessive ROS formation derived from the presence of Ang II. These findings are in agreement with Rajasekar, Palanisamy, and Anuradha (2007), who observed a reduction in the protein expression of PKC β II in fructose-fed hyperinsulinemic rats treated with LC. The PKC-inhibiting action of LC might be related to the decrease in ROS and free fatty acid levels that are observed in hypertensive animals after treatment with LC (Baek et al., 2010; Inoguchi et al., 2000). Nevertheless, the mechanisms by which LC affects the activity of PKC are not fully clear, and additional studies are warranted to establish the interactions between these compounds.

Finally, LC also reduced the amount of active NF-kB at the nuclear level, thus establishing an additional mechanism by which carnitine might normalize the expression of NADPH oxidase enzyme. These latter results were supported by a parallel effect of LC on IkB, a well-known inhibitory molecule whose phosphorylation causes its dissociation from NF-kB with subsequent activation and translocation of this transcription factor into the nucleus. Noteworthy, when studying the possible mechanisms involved in the effect of fatty-acid-bearing albumin on tubulointerstitial injury, other authors demonstrated that LC suppressed the activation of NF-kB via inhibition of c-SRC kinase (Baek et al., 2010). As a consequence, the decrease in both reactive oxygen species and in free fatty acids induced by LC might have been a hindrance to NF-kB (and eventually NADPH oxidase) activation.

Taken together, the findings in this study suggest that the antioxidant capacity of LC is not due to a direct effect on NOX isoforms but might rather arise from its activity as a free radical scavenger. This results in a modulation of the activity and expression of NADPH oxidase by reducing protein kinase C activity and NF-κB translocation to the nucleus, and by inducing the phosphorylation of Akt via PI3K-independent mechanism(s).

5. Conclusion

The increase in Ang II-induced NADPH oxidase in epithelial renal cells seems to be mediated by phosphorylation of kinases such as PI3K/Akt, p38 MAPK, $ERK_{1/2}$ and PKC, and by activation of the transcription factor NF-κB. L-Carnitine was able to reverse angiotensin II-induced oxidative stress by modulating the activity/expression of NADPH oxidase through reduction of both protein kinase C and NF-kB translocation to the nucleus.

Author contribution

Study design and interpretation of data: ER, AM, CMV; analysis and acquisition of data: AJB, MVR-A SZ, JLM-C; drafting and/or revising the article for important intellectual content: AJB, FMG-R, AF, ER, AM, CMV; final approval of the version: all authors.

Conflict of interest

On behalf of all authors, the corresponding author states that there is no conflict of interest.

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