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Leptin Induces Oxidative Stress Through Activation of NADPH Oxidase in Renal Tubular Cells: Antioxidant Effect of L-Carnitine

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ABSTRACT

Leptin is a protein involved in the regulation of food intake and in the immune and inflammatory responses, among other functions. Evidences demonstrate that obesity is directly associated with high levels of leptin, suggesting that leptin may directly link obesity with the elevated cardiovascular and renal risk associated with increased body weight. Adverse effects of leptin include oxidative stress mediated by activation of NADPH oxidase. The aim of this study was to evaluate the effect of L-carnitine (LC) in rat renal epithelial cells (NRK-52E) exposed to leptin in order to generate a state of oxidative stress characteristic of obesity. Leptin increased superoxide anion ($O_2^{\bullet}-$) generation from NADPH oxidase (via PI3 K/Akt pathway), NOX2 expression and nitrotyrosine levels. On the other hand, NOX4 expression and hydrogen peroxide (H_2O_2) levels diminished after leptin treatment. Furthermore, the expression of antioxidant enzymes, catalase, and superoxide dismutase, was altered by leptin, and an increase in the mRNA expression of pro-inflammatory factors was also found in leptin-treated cells. LC restored all changes induced by leptin to those levels found in untreated cells. In conclusion, stimulation of NRK-52E cells with leptin induced a state of oxidative stress and inflammation that could be reversed by preincubation with LC. Interestingly, LC induced an upregulation of NOX4 and restored the release of its product, hydrogen peroxide, which suggests a protective role of NOX4 against leptin-induced renal damage. J. Cell. Biochem. 9999: 1–8, 2016.

KEY WORDS: LEPTIN; L-CARNITINE; NADPH OXIDASE; OXIDATIVE STRESS

Obesity is one of the most serious health problems in industrialized countries. High weight is associated with enhanced risk of developing cardiovascular diseases such as heart failure, hypertension, atherosclerosis, diabetes and dyslipidemia [Ahima and Flier, 2000; Rahmouni and Haynes, 2004; Haynes, 2005]. In this sense, the Framingham Heart Study suggests that

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65–75% of the risk for hypertension is attributed to excess weight (Singh et al., 2010).

Leptin is a circulating, 16-kDa protein that belongs to the interleukin-6 family of pro-inflammatory cytokines. It is secreted from white adipocytes and has been implicated in the regulation of food intake, energy expenditure, immune and inflammatory responses, and energy balance [Houseknecht et al., 1998; Oswal and Yeo, 2010]. Clinical and experimental evidence suggests that the adipokine leptin may directly link obesity with the elevated cardiovascular risk associated with increased body weight. In animal experiments and in humans, circulating serum leptin levels correlate directly with body fat content, which suggest that obesity results in hyperleptinemia [Frederich et al., 1995]. Furthermore, leptin increases blood pressure, generation of reactive oxygen species (ROS), plasma renin activity, and serum angiotensinogen levels, all of them mechanisms postulated for leptin relating to increased cardiovascular risk [Koh et al., 2008; Yang and Barouch, 2007; Singh et al., 2010]. In the same sense, the obese gene product, leptin, activates endothelin-1 (ET-1) and NADPH oxidase-dependent pathway, leading to impaired endothelial and cardiac function. These data suggest a link between obesity, cardiovascular disease, leptin, and oxidative stress [Morawietz and Bornstein, 2006]. In the same way, there are studies suggesting that ET-1 upregulates NADPH oxidase after leptin administration. In addition, administration of leptin in neonatal rat cardiomyocytes induces intracellular superoxide anion generation and upregulates expression of p67phox/p47phox subunits of the NADPH oxidase [Yang and Barouch, 2007]. Different studies have demonstrated that obesity adverse effects are in part due to an enhancement in the generation of ROS produced by an increase in leptin levels.

Obesity has traditionally been considered as a risk factor for chronic kidney disease (CKD) and end-stage renal disease due to its link with diabetes mellitus and hypertension [Foster et al., 2008]. Two potential mechanisms underlying impaired renal function in obesity are glomerular hyperfiltration and leptin-mediated glomerular injury. Moreover, CKD is characterized by an increase in plasma leptin concentration that may be explained by defective renal clearance. Hyperleptinemia seems to play a key role in the pathogenesis of complications associated with CKD such as chronic inflammation, insulin resistance, and cardiovascular damage. Leptin is also involved in the progression of renal disease through its pro-fibrotic and pro-hypertensive actions [Pedone et al., 2015].

LC (β-hydroxy-γ-N-trimethylammonium-butyrate) is a vital component in lipid metabolism for the production of ATP through the beta-oxidation of long-chain fatty acids. LC is a natural compound that is widely distributed in nature, especially in red meats and dairy products [Mate et al., 2010]. It has been demonstrated to be effective in cardiovascular and renal diseases [Mate et al., 2010], and we have previously described hypotensive, anti-inflammatory, antioxidant, and anti-fibrotic effects of LC in rat models of arterial hypertension [Miguel-Carrasco et al., 2008; Mate et al., 2010; Zambrano et al., 2013, 2014].

Several studies have shown the beneficial effects of LC in obesity. Su et al. [2015] demonstrated the cardiovascular and liver protection of LC in high-fat diet fed mice, and a reduction in the level of serum leptin has also been observed in the same animals [Kang et al., 2011; Jang et al., 2014]. Derosa et al. [2011], when comparing orlistat (an inhibitor of pancreatic lipases used to treat obesity) plus LC and orlistat alone, found that the former resulted in better outcomes in terms of body weight, glycemic and lipid profile, and inflammatory parameters. Little is known, however, on the mechanisms involved in the beneficial effects of LC in the context of obesity.

The aim of this study was to evaluate the effects of LC in rat renal epithelial cells incubated with leptin, and to study the mechanisms involved in LC action. To this purpose, the activity and expression of NADPH oxidase, as well as generation of hydrogen peroxide, were determined in NRK-52E stimulated leptin, with/without simultaneous administration of LC. Oxidative stress parameters (namely, levels of nitrotyrosine and mRNA expression of antioxidant enzymes, superoxide dismutase, and catalase) and gene expression of pro-inflammatory cytokines, IL-1β and IL-6, have also been evaluated.

MATERIALS AND METHODS

CELL CULTURE

NRK-52E cells (rat renal proximal tubular cells) obtained from the American Type Culture Collection were grown in DMEM 1X supplemented with 10% fetal bovine serum and 1% streptomycin/penicillin, until the monolayer became confluent. The culture medium was then replaced by minimum medium (0.5% fetal bovine serum), and cells were incubated overnight (at least 8 h) before the experiment, to arrest at quiescent state. Then, cells were treated with leptin (10 nM) with or without a 30-min pre-incubation with LC (1 mM). Cell lysates were homogenized either in sucrose buffer, for determination of superoxide anion production, or in RIPA buffer containing a protease inhibitor cocktail (Roche Diagnostics, Madrid, Spain), for Western blotting. The protein concentration was determined by the Bradford method [Bradford, 1976].

DETERMINATION OF SUPEROXIDE ANION PRODUCTION

Chemiluminescence (CL) assay with 5 μM lucigenin (Sigma–Aldrich, Madrid, Spain) was used to measure superoxide anion in cell homogenates (5 μg protein). The reaction was started by the addition of lucigenin to cell samples in a final volume of 300 μL. Photon emission was measured for 4 min with a CL reader (Junior LB 9509, Berthold Technologies, Bad Wildbad, Germany). The responses to leptin were studied in the absence and in the presence of diphenylene iodonium (DPI, an inhibitor of flavoproteins), superoxide dismutase (SOD, superoxide anion scavenger), and the PI3 K/Akt pathway inhibitor, wortmannin (Sigma–Aldrich).

REAL-TIME PCR

Total RNA was isolated from cultured NRK-52E cells using TRIzol® (Life Technologies, Madrid, Spain) and retro-transcribed as described [Chomczynski and Sacchi, 2006]. cDNA was diluted in sterile water and used as the template for amplification by the polymerase chain reaction (PCR). Forward and reverse primers used in this study are shown in Table I, and were obtained from Biomedal (Seville, Spain). Each specific gene product was amplified by real-time PCR System

TABLE I. Primers Used for Real-Time PCR

Gene	Forward primer (5′–3′)	Reverse primer (5′–3′)
NOX2	CCCTTTGGTACAGCCAGTGAAGAT	CAATCCCACGTCCCACTAACATCA
NOX4	TTGCTTTTGTATCTTC	CTTACCTTCGTCACAG
CAT	GCGGATTCCTGAGAGAGTGG	GACTGTGGAGAATCGGACGG
SOD	CGTCATTCACTTCGAGCAGAAGG	GTCTGAGACTCAGACCACATA
IL-1β	GAGGCTGACAGACCCCAAAAGAT	GCACGAGGCATTTTTGTTGTTCA
IL-6	GAAATACAAAGAAATGATGGATGCT	TTCAAGATGAGTTGGATGGTCT

(Roche Diagnostics, Madrid, Spain). Amplification data were collected by the sequence detector and analyzed with sequence detection software. For each assay, a standard curve was constructed using increasing amounts of cDNA. In all cases, the slope of the curves indicated adequate PCR conditions (slopes of 3.2–3.4). The RNA concentration in each sample was determined from the threshold cycle (Ct) values and calculated with the sequence detection software supplied by the manufacturer. The quantitative fold changes in mRNA expression were determined relative to glyceraldehyde-3-phosphate dehydrogenase (GADPH) mRNA levels in each corresponding group and calculated using the $2^{-\Delta\Delta Ct}$ method [Livak and Schmittgen, 2001].

AMPLEX RED COLORIMETRIC ASSAY

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

WESTERN BLOTTING

Cell lysates containing 10–25 μg of protein were subjected to SDS–PAGE electrophoresis and immunoblotted with specific antibodies. Rabbit monoclonal anti-NOX2 (1:2,000 dilution; Epitomics-Abcam, Burlingame, CA), rabbit polyclonal anti-NOX4 (1:1,000; Santa Cruz Biotechnology, Heidelberg, Germany), and mouse monoclonal anti-nitrotyrosine (1:1,000; Millipore, Temecula, CA) were used. Mouse monoclonal anti-β-actin (1:5,000; Sigma–Aldrich) was also used for protein loading control.

STATISTICAL ANALYSIS

All results were subjected to one-way analysis of variance, and represent mean ± SEM. Differences in mean values between groups were assessed using the Turkey multiple comparison test with a GraphPad InStat software and were considered statistically different at $P < 0.05$.

RESULTS

CONCENTRATION–RESPONSE AND TIME–RESPONSE RELATIONSHIP OF LEPTIN-INDUCED SUPEROXIDE ANION PRODUCTION

Incubation of NRK-52E cells with leptin resulted in an induction of superoxide anion ($O_2^{\bullet}-$) production in a dose-dependent (Fig. 1A) and time-dependent (Fig. 1B) manner. The highest stimulation of $O_2^{\bullet}-$ production was reached with leptin at 10 nM and 30 min incubation time (60%), then remained constant up to 24 h. This dose of leptin (10 nM) was selected for all subsequent experiments.

In order to characterize the enzymatic source of $O_2^{\bullet}-$ in response to leptin, we analyzed $O_2^{\bullet}-$ generation in NRK-52E cells in the presence of DPI (NADPH oxidase inhibitor). DPI completely prevented leptin-induced $O_2^{\bullet}-$ production, thus identifying NADPH oxidase as the enzymatic source of $O_2^{\bullet}-$. In addition, the chemiluminescence signal induced by leptin was completely inhibited by SOD, which confirmed that $O_2^{\bullet}-$ was the reactive oxygen species detected by lucigenin (Fig. 1C).

L-CARNITINE REDUCED NADPH OXIDASE HYPERACTIVITY, AND REVERSED HYDROGEN PEROXIDE AND NITROTYROSINE LEVELS ALTERED BY LEPTIN IN NRK-52E CELLS

LC inhibited leptin-induced NADPH oxidase activity by 70% approximately, and no changes were observed when LC was added to unstimulated cells (Fig. 2A). To examine whether the PI3 K/Akt pathway is involved in the effect of leptin on NADPH oxidase activity stimulation, cells were preincubated with 1 μM wortmannin for 1 min. As shown in Figure 2A, wortmannin prevented the excess $O_2^{\bullet}-$ production stimulated by leptin. Leptin decreased the production of hydrogen peroxide by 35% (Fig. 2B) and increased nitrotyrosine levels by 80% (Fig. 2C). Pretreatment with LC reverted these changes in hydrogen peroxide and nitrotyrosine levels induced by leptin. On the other hand, treatment of control cells with LC had no effect on any of these parameters (Fig. 2A–C).

L-CARNITINE ABOLISHED LEPTIN-INDUCED CHANGES IN GENE AND PROTEIN EXPRESSION OF NADPH OXIDASE ISOFORMS NOX2 AND NOX4

NRK-52E cells treated with leptin resulted in a five-fold increase in NOX2 gene expression (Fig. 3A), and a 1.4-fold increase in the corresponding protein expression (Fig. 3C). Regarding NOX4, leptin-treated cells showed reductions of approximately 50% (gene) and 40% (protein) expression of NOX4 (Fig. 3B and D). In all cases, incubation with LC restored those changes caused by leptin incubation (Fig. 3A–D).

EFFECT OF L-CARNITINE ON CHANGES IN mRNA EXPRESSION OF ANTIOXIDANT ENZYMES INDUCED BY LEPTIN

In order to further examine the effect of LC on leptin-induced oxidative stress in NRK cells, mRNA expression of two antioxidant enzymes were performed: superoxide dismutase (SOD), an enzyme that converts the high-reactive radical $O_2^{\bullet}-$ to the less reactive compound H_2O_2; and catalase (CAT), enzyme which removes the

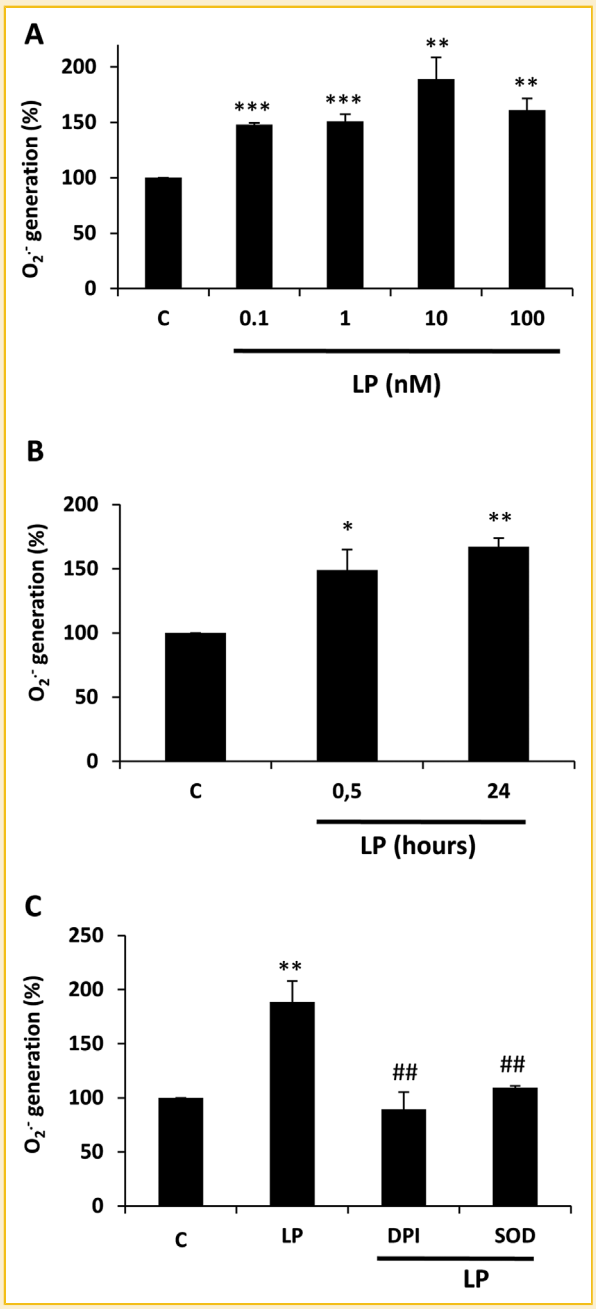

Fig. 1. Effect of leptin (LP) on $O_2^{\bullet}-$ production. (A) Concentration–response for leptin-dependent superoxide anion production. NRK-52E cells were incubated with leptin (0.1–100 nM) for 24 h. (B) Time–response for leptin-dependent superoxide anion production. Cells were incubated with leptin (10 nM) for the indicated incubation times. (C) Characterization of superoxide anion source in NRK-52E cells stimulated with leptin. NRK-52E cells were incubated with leptin (10 nM, 24 h) in the presence of diphenyliodonium (DPI), or 100 U/mL superoxide dismutase (SOD). Histograms represent means ± SEM from three independent experiments. C = non-stimulated cells. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus C; $^{\#\#}P < 0.01$ versus LP.

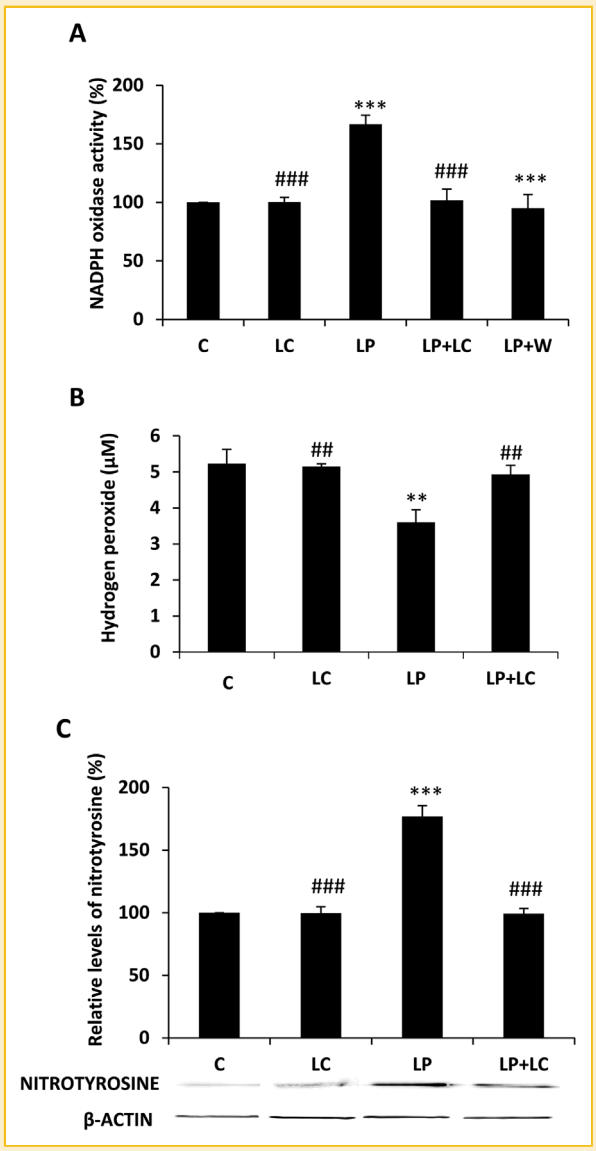

Fig. 2. Effect of leptin (LP) and L-carnitine (LC) on NADPH oxidase activity (A), hydrogen peroxide production (B), and levels of nitrotyrosine (C). NRK-52E cells were treated with 1 mM L-carnitine (LC), 10 nM leptin (LP), leptin plus L-carnitine (LP + LC), or leptin plus 1 μM wortmannin (LP + W). Histograms represent means ± SEM from three (A and C) or five (B) independent experiments. C = non-stimulated cells. $^{**}P < 0.01$, $^{***}P < 0.001$ versus C; $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ versus LP.

excess of H_2O_2 to H_2O. A 40% decline together with a 2.7-fold rise in the mRNA expression of SOD and CAT, respectively, were observed in leptin-induced cells. However, pretreatment with LC was able to reverse these alterations (Fig. 4A and B).

L-CARNITINE NORMALIZED THE UPREGULATION OF IL-1β AND IL-6 INDUCED BY LEPTIN

Leptin produced an increase in the gene expression of IL-1β and IL-6 (2.3- and 1.9-fold, respectively). Again, LC restored these values back to those found in unstimulated cells (Fig. 5A and B).

DISCUSSION

Clinical and experimental studies have demonstrated that obesity is directly associated with hyperleptinemia, indicating that high levels

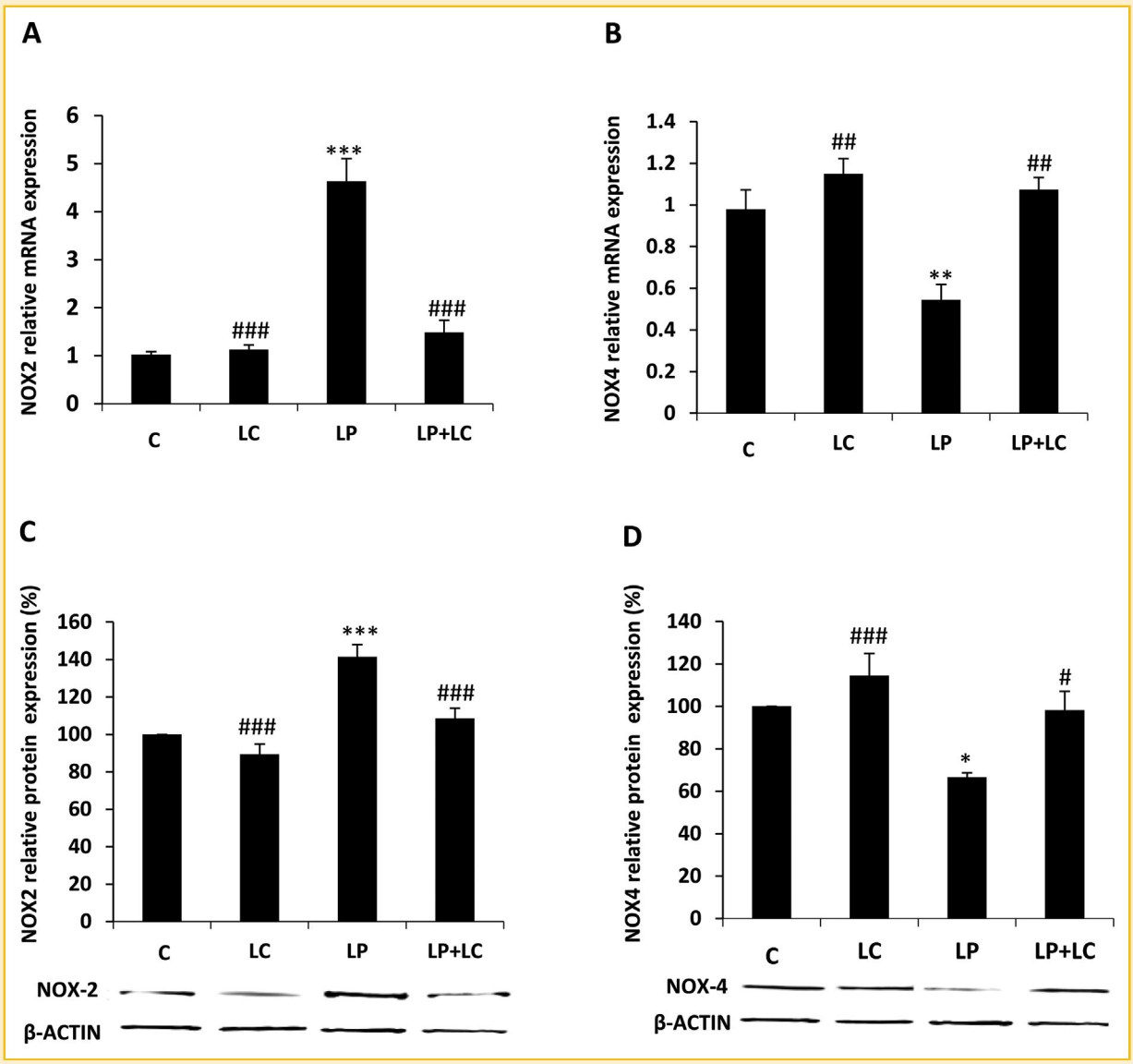

Fig. 3. Effect of leptin (LP) and L-carnitine (LC) on gene and protein expression of NADPH oxidase subunits NOX2 (A and C) and NOX4 (B and D). NRK-52E cells were treated with 1 mM LC (LC), 10 nM leptin (LP), or leptin plus L-carnitine (LP + LC). Histograms represent means ± SEM from four (A and B) or three (C and D) independent experiments. C = non-stimulated cells. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus C; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ versus LP.

of leptin in the blood are of particular relevance in linking obesity with an increased risk of developing cardiovascular/renal diseases and type II diabetes [Hanson et al., 1995]. Among the mechanism involved in the negative effect of leptin is the increase in ROS generation [Yang and Barouch, 2007; Koh et al., 2008; Singh et al., 2010], mainly due to an activation of NADPH oxidase enzyme [Morawietz and Bornstein, 2006; Yang and Barouch, 2007].

In the current study, using the renal cell line NRK-52E, we demonstrate that treatment with leptin resulted in an increase in $O_2^{\bullet}-$ production at concentrations of 0.1–100 nM, thus leading to a higher release of $O_2^{\bullet}-$ (either using 30 min or 24 h of incubation time). Furthermore, this increase was NADPH oxidase-dependent, as shown by the inhibition of $O_2^{\bullet}-$ production with DPI. Additionally, the increase in NADPH oxidase activity was accompanied by an upregulation of NOX2 (catalytic subunit of the enzyme) at both mRNA and protein levels. This increment in the activity and expression of NADPH oxidase led to higher oxidative stress, as reflected by elevated nitrotyrosine levels (a marker of oxidative stress) following leptin treatment. Moreover, the use of wortmannin indicated, as previously shown, that PI3 K/Akt pathway is involved in the activation of NADPH oxidase by leptin (Fortuño et al., 2010; Uddin et al., 2010; Chen et al., 2011). Although a critical role for PI3 K/PKC-dependent p47phox and p67phox phosphorylation has been described specifically for the activation of phagocytic NADPH oxidase [Fortuño et al., 2010], the possibility that other kinases might be involved in leptin-mediated NADPH oxidase activation in non-phagocytic cells (such as the epithelial NRK-52E cell line) cannot be excluded.

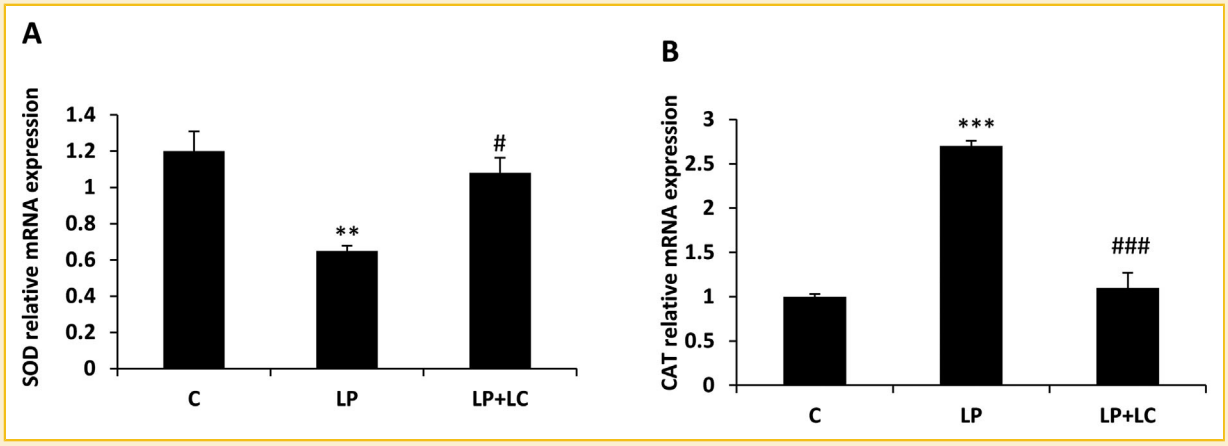

Fig. 4. Effect of leptin (LP) and L-carnitine (LC) on gene expression of superoxide dismutase (SOD) (A) and catalase (CAT) (B). NRK-52E cells were treated with 1 mM LC (LC), 10 nM leptin (LP), or leptin plus L-carnitine (LP + LC). Histograms represent means ± SEM from four independent experiments. C = non-stimulated cells. $^{**}P < 0.01$, $^{***}P < 0.001$ versus C; $^{\#}P < 0.05$, $^{\#\#\#}P < 0.001$ versus LP.

Previous studies have reported that leptin can stimulate the formation of reactive oxygen species in phagocytic [Caldefie-Chezet et al., 2001] and non-phagocytic cells [Yamagishi et al., 2003; Xu et al., 2004; Singh et al., 2007; Chetboun et al., 2012; Schroeter et al., 2013]. However, few studies have concluded that this increase is due to an excessive generation of $O_2^{\bullet}-$ via NADPH oxidase activation. In phagocytic cells, leptin stimulated $O_2^{\bullet}-$ generation in an NADPH oxidase-dependent manner, which was accompanied by an increase in the expression of NOX2 [Fortuño et al., 2010]. In ventricular myocytes from mice, an increase in intracellular production of $O_2^{\bullet}-$ was also found after treatment with leptin; interestingly, this was not accompanied by changes in NOX2 expression, although it was observed an enhancement in the expression of p47phox and p67phox (also subunits of NADPH oxidase) [Dong et al., 2006]. In addition, obesity subsequent to high-fat diet resulted in an increase in $O_2^{\bullet}-$ formation that was paralleled by an upregulation of p47 phox [Chen and Stinnett, 2008].

In this study, we also analyzed the expression of NOX4 isoform at gene/protein levels, and we observed a reduction in the group of cells subjected to treatment with leptin. Hydrogen peroxide, which is currently considered as the main product of NOX4 catalytic action [Von Lohneysen et al., 2010], also diminished after exposure of NRK-52E cells to leptin. Our results are not in agreement with the study by Chetboun et al. [2012], who reported an increase in the levels of hydrogen peroxide in pancreatic beta cells treated with leptin. In fact, the role of NOX4 in cardiovascular pathophysiology is currently contradictory in the literature, since both protector and adverse effects have been described [Ray et al., 2011]. The notion that NOX4 mainly generates H_2O_2 [Dikalov et al., 2008] instead of $O_2^{\bullet}-$ (in contrast to NOX2, which produces $O_2^{\bullet}-$), would support a protective role for NOX4 in cardiovascular pathophysiology, since it would act

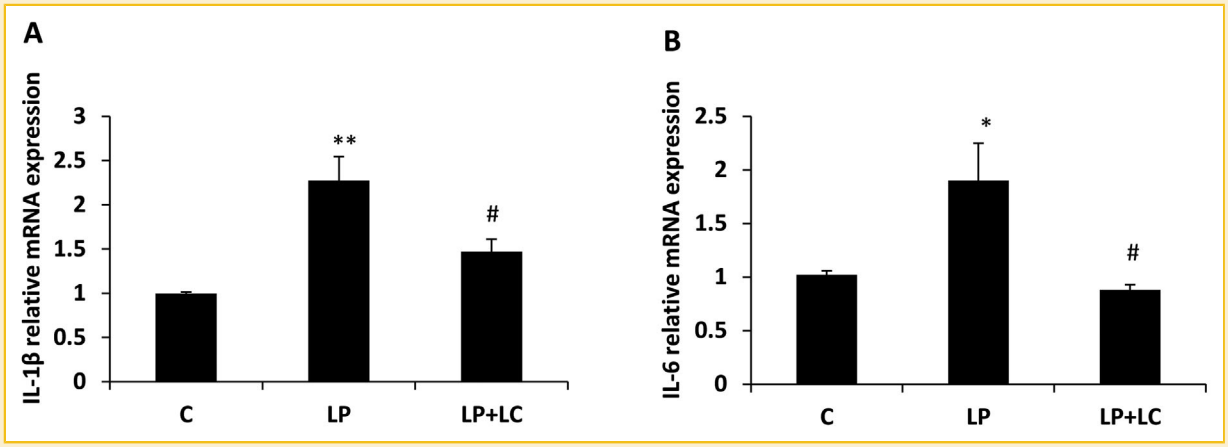

Fig. 5. Effect of leptin (LP) and L-carnitine (LC) on gene expression of IL-1β (A) and IL-6 (B). NRK-52E cells were treated with 1 mM LC (LC), 10 nM leptin (LP), or leptin plus L-carnitine (LP + LC). Histograms represent means ± SEM from four Independent experiments. C = non-stimulated cells. $^{*}P < 0.05$, $^{**}P < 0.01$ versus C; $^{\#}P < 0.05$ versus LP.

as a vasodilator and compensate for the deleterious vasoconstrictor effect of $O_2^{\bullet}-$ [Edwards et al., 2008; Morawietz, 2011; Ray et al., 2011]. Therefore, our findings suggest that leptin might induce oxidative stress in NRK-52E cells not only by increasing $O_2^{\bullet}-$ production from NOX2, but also by decreasing H_2O_2 release via NOX4 inhibition.

To further examine the effects of leptin on oxidative status of NRK-52E cells, we measured the mRNA expression of two antioxidant enzymes, namely SOD and CAT. The downregulation of SOD might have contributed to the excess of $O_2^{\bullet}-$ observed after the incubation with leptin, since SOD is the enzyme responsible for the conversion of $O_2^{\bullet}-$ to H_2O_2. On the other hand, leptin treatment resulted in an increase in the gene expression of CAT; this might also account for the presence of low levels of H_2O_2 in leptin-treated cells, besides NOX4 downregulation. A decrease in SOD mRNA expression has been previously observed in pancreatic cells incubated with leptin, and in animals subjected to a high-fat diet [Chetboun et al., 2012; Su et al., 2015]. However, no changes or a decrease in CAT have been reported in cells treated with leptin and in animals fed a high-fat diet, respectively [Chetboun et al., 2012; Su et al., 2015].

It is well known the relationship between leptin and inflammation [Lau et al., 2005]. In fact, the link between cardiovascular–renal risk and leptin is associated with increased levels of inflammatory cytokines, such as TNFα and IL-1β, and with activation of NF-κB pathways [Lappas et al., 2005]. Moreover, an excessive production of superoxide anion might lead to an activation of NF-κB that eventually would produce an increase in pro-inflammatory cytokines, such as IL-1β and IL-6 [Zhang et al., 2001; Filippin et al., 2008]. In accordance with these studies, our results showed an increase in the gene expression of two pro-inflammatory cytokines, IL-1β and IL-6, in NRK-52E cells stimulated with leptin. Indra et al. [2013] demonstrated an activation of NF-κB and TNF-α in leptin-induced human umbilical vein endothelial cells (HUVEC), although higher concentrations of leptin were used in that case. On the contrary, leptin was found to inhibit gentamicin-induced activation of NF-κB [Chen et al., 2011], and no changes in serum content of IL-1β were found in mice fed a high-fat diet [Su et al., 2015]. Therefore, further research is warranted to get a deeper understanding of the behavior of pro-inflammatory cytokines following exposure to leptin.

Several studies have shown the beneficial effects of LC in obesity [Derosa et al., 2011; Kang et al., 2011; Jang et al., 2014; Su et al., 2015], as well as in cardiovascular and renal diseases [Mate et al., 2010]. In the present study, not only was LC able to decrease leptin-mediated stimulation of NADPH oxidase, but it also decreased the expression of NOX2 subunit and the levels of protein nitrosylation. Similar to LC, another study reported the antioxidant effects of pigment epithelium-derived factor (PEDF), an effect that was achieved by counteracting leptin-induced ROS generation [Yamagishi et al., 2003]. Moreover, LC normalized the levels of hydrogen peroxide and mRNA expression of NOX4, SOD, CAT, IL-1β, and IL-6 (i.e., all the parameters that were altered after incubation with leptin), thus confirming the antioxidant and anti-inflammatory effects of LC, as previously reported [Miguel-Carrasco et al., 2008; Zambrano et al., 2013]. This effect of LC also supports a protective role of NOX4, due to the ability of LC to reduce oxidative stress not only by decreasing the elevated expression of NOX2, but also by increasing those of NOX4 (which were attenuated under leptin treatment).

In conclusion, stimulation of NRK-52E cells with leptin induced oxidative stress and a pro-inflammatory status characterized by: (i) a stimulation of NADPH oxidase activity (through PI3 K/Akt signaling); (ii) an increase in the expression of NOX2, IL-1β, IL-6, and CAT, together with elevated levels of protein nitrosylation; and (iii) a downregulation of SOD and NOX4 (with lower production of hydrogen peroxide), which suggests a protective role for NOX4 in leptin-treated renal tubular cells. In addition, all the alterations subsequent to leptin incubation could be counteracted by preincubation with LC.

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