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L-carnitine and its propionate: Improvement of endothelial function in SHR through superoxide dismutase-dependent mechanisms

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Abstract

To clarify the mechanism underlying the antioxidant properties of L-carnitine (LC) and propionyl-L-carnitine (PLC) on spontaneously hypertensive (SHR) and normotensive WKY, animals were treated with either PLC or LC (200 mg kg^{-1}). Aorta was dissected and contraction to (R)-(−)-phenylephrine (Phe) and relaxation to carbachol (CCh) were assessed in the presence or not of the NO synthase (NOS) inhibitor, L-NAME. $O_2^{\cdot-}$ production was evaluated by lucigenin-enhanced chemiluminescence and its participation on relaxation was observed after incubation with superoxide dismutase (SOD) plus catalase. Protein expressions of eNOS, Cu/Zn-SOD and Mn-SOD were studied by western blot. Both LC and PLC treatments improved endothelial function of SHR through increasing NO participation and decreasing $O_2^{\cdot-}$ probably involving higher Cu/Zn-SOD expression. PLC treatment augmented eNOS expression in SHR. Surprisingly, LC increased $O_2^{\cdot-}$ produced by aorta from WKY and thus diminished NO and damaged endothelial function. Conversely, PLC did not affect CCh-induced relaxation in WKY. These results demonstrate that LC and PLC prevent endothelial dysfunction in SHR through an antioxidant effect.

Keywords: *L-carnitine, propionyl-L-carnitine, eNOS, superoxide, Cu/Zn-SOD*

Introduction

Cardiovascular diseases, including hypertension, atherosclerosis, cardiac hypertrophy, heart failure and diabetes mellitus, are associated with increased reactive oxygen species (ROS) formation in the vascular wall. In those pathologies, ROS contribute to endothelial dysfunction and vascular remodelling through oxidative damage and impair endothelium-dependent vasodilatation by reducing the bioavailability of NO [1]. Furthermore, ROS stimulate endothelial cell migration and activate adhesion and proinflammatory molecules that contribute to endothelial dysfunction [2].

The role played by ROS in hypertension has been widely studied using the spontaneously hypertensive rat (SHR) model. The increased vascular generation of superoxide ($O_2^{\cdot-}$) anions observed in these studies [3] rapidly inactivates endothelial NO and thereby alters vascular tone [4]. Recently, it has been shown that either the enhanced expression of the $O_2^{\cdot-}$ generating enzyme, NAD(P)H oxidase subunits nox1, nox2, nox4 and p22phox [5] or the increased activity of this system [4] are involved in the generation of vascular oxidative stress. Together with these facts, a decrease in the activity and expression of the antioxidant enzymes Mn-SOD, Cu/Zn-SOD and

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catalase has also been reported in SHR [6]. Accordingly, it is well-known that treatment with antioxidants improves endothelial function and even prevents development of hypertension in SHR [7,8].

The antioxidant action of L-carnitine (LC) and of its most potent naturally occurring derivate, propionyl-L-carnitine (PLC) is thought to be responsible for some of their described effects [9–11]. The protective effect of these drugs has been demonstrated in several models of ischemia-reperfusion, where the increased ROS production could be the major cause of damage [12,13]. In addition, in patients with peripheral arterial disease, treatment with PLC was effective in improving treadmill exercise performance and functional status [14]. This effect may be partly explained by a reduction of ROS and an enhancement of NO participation [15]. Nevertheless, the mechanism underlying the antioxidant effects of LC and PLC is still unclear.

Recent studies from our laboratory have shown that chronic treatment either with LC or PLC improve the endothelial dysfunction in SHR through mechanisms involving enhanced NO production as well as enhanced PGI_2 liberation in LC-treated animals [16]. Although we found that these effects could be ascribed to a reduction of ROS, the mechanism underlying the antioxidant properties of LC and PLC remained unclear. Thus, we designed the present study with the aim to assess the role of ROS in endothelial and vascular function of SHR after treatment with LC and PLC and to evaluate the role of the antioxidant enzyme superoxide dismutase (SOD) in that effect.

Material and methods

Animals

Male SHR and normotensive Wistar Kyoto rats (WKY) were housed at 24 ± 2°C with 60 ± 20% relative humidity, on a 12/12 light/dark cycle. Rats were fed a standard diet and water *ad libitum*, and were periodically weighed. Systolic blood pressure (SBP) was evaluated before starting and at the end of the treatments by tail-cuff method with a pressure meter (Niprem 645, Cibertec, Madrid, Spain). Both SHR and WKY were divided into three groups at the age of 4 weeks-old (80–100 g weight) and were treated for the following 8 weeks. The first group received vehicle (water); the second and the third were treated with 200 mg kg^{-1} per day of LC and PLC, respectively. Sigma-Tau, Spain, kindly provided both compounds. The selected dose was chosen taking into account previous reports that found that either LC or PLC at 200 mg kg^{-1} given to rats was able to significantly increase plasma free LC concentration [17,18]. Treatments were administered in the drinking water and the amount of either LC or PLC was adjusted as previously described [16]. All experiments were performed according to the guidelines of the European Union for the ethical treatment of animals and were approved by the Institutional Animal Investigation Committee (University of Seville, Spain).

Aortic ring preparation and functional studies

The descending thoracic aorta was placed in a modified Krebs-Henseleit solution (KHS) containing (mmol l^{-1}), NaCl (118), KCl (4.75), $NaHCO_3$ (25), $MgSO_4$ (1.2), $CaCl_2$ (1.8), KH_2PO_4 (1.2) and glucose (11). After excess fat and connective tissue were removed, the aortae were cut into 2–3-mm rings and mounted in organ baths containing KHS as previously described [19]. The endothelial and vascular functions were assessed by the relaxation of precontracted aortic rings by carbachol (CCh, 10^{-8}–10^{-4} mol l^{-1}) and the contraction induced by (R)-(−)-phenylephrine (Phe, 10^{-9}–10^{-4} mol l^{-1}), respectively. The involvement of NO was evaluated by the effect of the nitric oxide synthase inhibitor N-nitro-arginine-methyl-ester (L-NAME, 3×10^{-4} mol l^{-1}) on concentration-response curves to Phe and CCh. Endothelial function was also studied in the presence of the antioxidant enzymes superoxide dismutase (SOD, 150 UI ml^{-1}) plus catalase (1000 UI ml^{-1}).

Vascular $O_2^{\cdot-}$ anion production

$O_2^{\cdot-}$ anion levels were measured using lucigenin chemiluminescence, a method that allows comparison of relative rates of $O_2^{\cdot-}$ production between various vessels [20]. Briefly, aortic segments were rinsed in KHS for 20 min, equilibrated for 30 min at 37°C in HEPES buffer (pH 7.4) of the following composition (mmol l^{-1}): NaCl 119, HEPES 20, KCl 46, $MgSO_4$ 1, $NaHPO_4$ 0.15, KH_2PO_4 40, $NaHCO_3$ 1, $CaCl_2$ 1.2 and glucose 5.5. In some arteries, vascular production of $O_2^{\cdot-}$ was stimulated by addition of NADPH (10^{-4} mol l^{-1}) and luminescence was collected during 200 s after addition of lucigenin (5×10^{-6} mol l^{-1}) using a luminometer (TD 20/20 Turner Designs, Sunnyvale, California). At this concentration, lucigenin failed to stimulate $O_2^{\cdot-}$ production from vascular segments, as detected by electron parametric resonance [21]. Some assays were performed in the presence of either the $O_2^{\cdot-}$ scavenger SOD (100 UI ml^{-1}) or the inhibitor of NADPH oxidase, diphenyleneiodonium chloride (DPI 10^{-5} mol l^{-1}) to ensure the specificity of the method.

Western blot detection of eNOS, Cu/Zn-SOD and Mn-SOD

Frozen aortic rings were homogenized in lysis buffer and 30 or 5 μg of protein fractions were loaded into

7 and 12% SDS-polyacrylamide gel to separate eNOS and Cu/Zn-SOD or Mn-SOD as previously described [22,23]. Immunoblotting was achieved using a specific monoclonal mouse anti-eNOS (1:2500, BD Transduction Laboratories, San Jose, CA, USA) and polyclonal rabbit anti-Mn-SOD and anti-Cu/Zn-SOD (Stressgen, Ann Arbor, MI, USA), and then with the appropriate secondary peroxidase-conjugated antibody. The blots were detected using an enhanced chemiluminiscence assay (Pierce Chemical Company, Rockford, IL, USA) and evaluated by densitometry. The sample loading was verified by staining membranes with Ponceau red and by immunostaining of smooth muscle α-actin with monoclonal mouse anti-α-actin (1:5000, Sigma, St Louis, Mo, USA).

Statistical analysis

Data represented are means ± SEM of $n = 5–7$ rats. Analysis of variance (ANOVA) and Fisher's Multiple Comparison test were used for statistical analysis. Differences were considered significant when $P < 0.05$. Concentration-response curves to CCh were fitted by a non-linear regression equation and whole curves were compared using the software Statview 5.0 (SAS Institute Inc., NY, USA). If no differences were found in the comparison of the curves, the effects at high concentration of CCh ($10^{-5}–10^{-4}$ mol l^{-1}) were compared. Areas under concentration-response curves to Phe (AUC_{Phe}) and to CCh (AUC_{CCh}) were calculated in the absence and in the presence of L-NAME and then the subtraction AUC in the presence of L-NAME minus AUC in control conditions was calculated.

Results

Systolic blood pressure

At the age of 4 weeks (before starting the treatments) SHR have not yet developed hypertension and in consequence, SBP was not different from WKY (128.53 ± 9.83 and 123.03 ± 16.63 mm Hg, respectively). Twelve weeks-old vehicle-treated SHR were hypertensive (205.71 ± 7.45 mm Hg) when compared to WKY (159.73 ± 5.33 mm Hg, $P < 0.01$). Oral administration of LC or PLC to SHR during 8 weeks did not prevent hypertension (205.81 ± 4.29 and 209.16 ± 7.12 mm Hg for LC- and PLC-treated SHR, respectively). Neither of the treatments affected SBP in WKY (data not shown).

Implication of endothelial NO-dependent pathways on the effects of LC and PLC

Participation of NO in vasoconstriction to Phe was expressed as the difference between area under the concentration-response curves to Phe (AUC_{Phe}) in the absence and in the presence of L-NAME. As illustrated in Figure 1(a), treatment of SHR with LC significantly increased NO involvement in the response to Phe ($P < 0.05$). On the contrary, administration of LC to WKY did not affect this value. When examining the effect of PLC treatment, a higher contribution of NO to Phe response was found in both WKY and SHR ($P < 0.001$; Figure 1(a)).

Contribution of NO to endothelial function was also evaluated by the effect of L-NAME on concentration-response curves to CCh and expressed as the subtraction $AUC_{L\text{-}NAME + CCh}$-$AUC_{CCh}$. Those experiments showed that NO involvement in endothelium-dependent relaxations was reduced in non-treated SHR when compared to WKY (Figure 1(b); $P < 0.001$). Interestingly, LC treatment decreased NO participation in endothelial function of aortas from WKY ($P < 0.001$; Figure 1(b)) but increased the same parameter in SHR ($P < 0.01$). In contrast, PLC administration did not change NO contribution to endothelial responses in aorta from WKY (Figure 1(b)) and elicited higher NO participation in aortas from SHR (Figure 1(b); $P < 0.05$).

The mechanism involved in these effects was firstly studied by determination of eNOS protein expression in aortic homogenates. Our results did not show any differences among eNOS protein expression in aortas from WKY and vehicle-treated SHR (Figure 1(c),(d)). Treatment with LC decreased expression of this protein in WKY, whereas it did not have any effect on eNOS in aortas from SHR ($P < 0.05$; Figure 1(c),(d)). On the other hand, administration of PLC increased eNOS protein in SHR ($P < 0.01$; Figure 1(c),(d)) but did not alter the expression of this enzyme in WKY.

Involvement of $O_2^{\cdot-}$ production in endothelial LC and PLC effects

The role of oxygen free radicals in endothelial function was evaluated using the combination of enzymatic scavengers SOD plus catalase in arterial organ bath preparations. In the absence of any inhibitor, the concentration-response curves to CCh reached a similar extent of relaxation in both PLC and vehicle-treated WKY rats, while administration of LC significantly impaired the relaxant response induced by CCh compared to the mentioned groups ($P < 0.001$; Figure 2(b)). Vasorelaxation to CCh in aortic segments from vehicle-treated WKY rats was not modified by incubation with SOD plus catalase (Figure 2(a)). Contrarily, after chronic administration of LC to WKY, the presence of these antioxidant enzymes enhanced endothelial-dependent relaxation to CCh ($P < 0.001$; Figure 2(b)). In aortic rings from PLC-treated WKY rats, vasorelaxation induced by CCh was only increased at higher doses (from $10^{-5}–10^{-4}$ mol l^{-1}) of the muscarinic agonist ($P < 0.01$; Figure 2(c)).

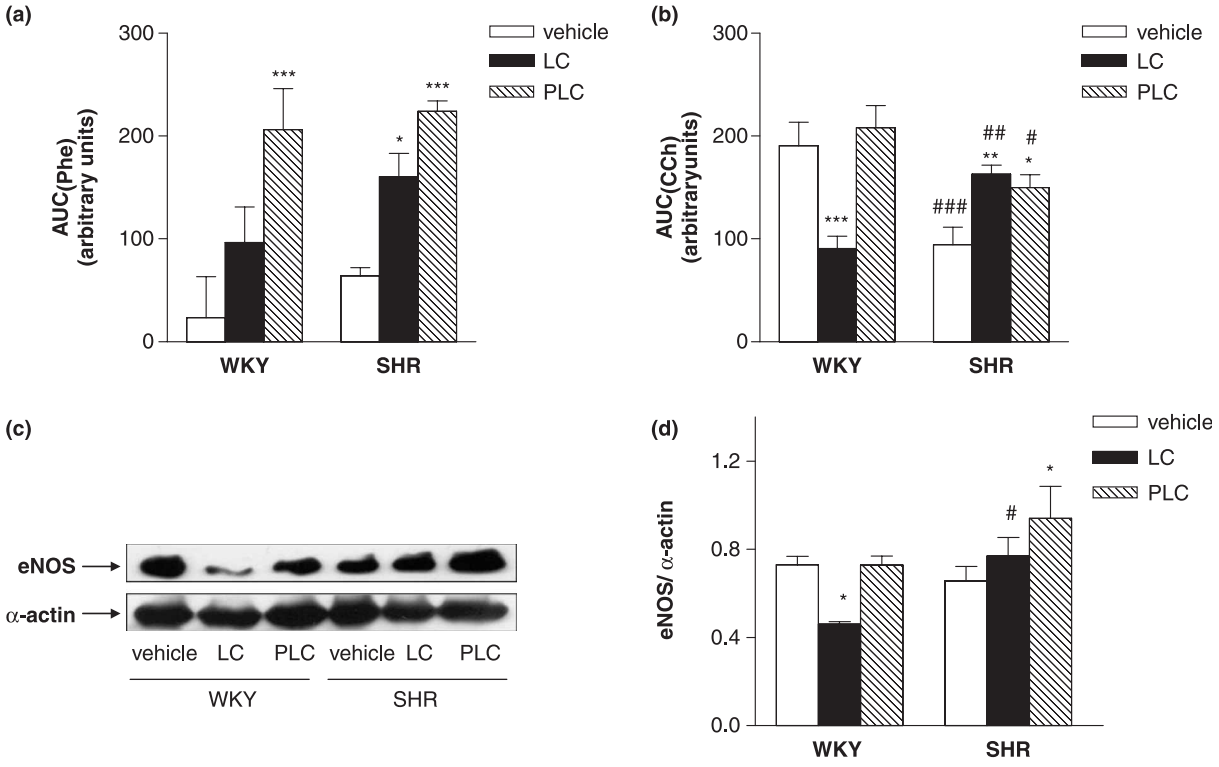

Figure 1. (a, b) Bars showing contribution of NO represented as area under the concentration-response curves to (R)-(−)-phenylephrine (Phe, 10^{-9}–10^{-4} mol l^{-1}) (a) or carbachol (CCh, 10^{-8}–10^{-4} mol l^{-1}) (b) in intact aortic rings from WKY and SHR treated with either vehicle, L-carnitine (LC, 200 mg kg^{-1}) or propionyl-L-carnitine (PLC, 200 mg kg^{-1}) for 8 weeks in drinking water. Data were obtained from the subtraction: $AUC_{L\text{-}NAME+Phe}$-AUC_{Phe} (a); $AUC_{L\text{-}NAME+CCh}$-AUC_{CCh} (b). $\star P < 0.05$, $\star\star P < 0.01$; $\star\star\star P < 0.001$ vs. vehicle; $\#P < 0.05$, $\#\#P < 0.01$, $\#\#\#P < 0.001$ vs. treatment-matched WKY. (c) Representative Western blot of eNOS from aorta treated with either vehicle, LC or PLC. (d) Bars showing the ratio eNOS/α-actin determined by optic densitometry of $n = 3$ blots. $\star P < 0.05$ vs. vehicle; $\#P < 0.05$ vs. treatment-matched WKY.

As expected, SHR exhibited impaired endothelium-dependent relaxation when compared to WKY ($P < 0.01$; Figure 2(d)). LC and PLC were both able to enhance the CCh-induced response in aortic rings from SHR ($P < 0.05$ and $P < 0.01$, respectively; Figure 2(e),(f)). CCh-evoked relaxation was markedly improved by the action of SOD plus catalase in intact aortic rings from the vehicle group ($P < 0.01$; Figure 2(d)). Conversely, the combination of ROS scavengers did not alter the relaxant profile evoked by CCh in SHR aorta treated with LC (Figure 2(e)). In rings from PLC-treated SHR, the incubation with SOD plus catalase slightly increased the response to CCh. Nevertheless, no statistical difference was found between these curves (Figure 2(f)).

Furthermore, measurements of vascular $O_2^{\cdot-}$ anion production were carried out in endothelium-intact aortic ring. As illustrated in Figure 3, the augmented oxidative stress attributed to SHR was confirmed by higher $O_2^{\cdot-}$ production after stimulation with NADPH in aortas from these animals compared with WKY ($P < 0.01$; Figure 3). The amount of $O_2^{\cdot-}$ released after stimulation of SHR aortic rings with NADPH was notably attenuated in arteries from either LC- or PLC-treated SHR ($P < 0.001$). Furthermore, aortas from LC and PLC groups, also exhibited lower basal $O_2^{\cdot-}$ concentrations ($P < 0.01$; Figure 3). On the other hand, $O_2^{\cdot-}$ production in aortic rings from LC-treated WKY after stimulation with NADPH was significantly higher than that found in arteries from vehicle- and PLC-treated WKY rats ($P < 0.01$).

Effect of LC and PLC on SOD protein expression

According to these results, it was interesting to analyse the effect of long-term administration of LC or PLC on SOD aortic expression by using the specific antibody for Cu/Zn-SOD and Mn-SOD. A decrease in Cu/Zn-SOD expression levels was detected in vehicle-treated SHR compared to its treatment-matched WKY rats ($P < 0.05$; Figure 4(a),(b)). Both LC and PLC treatments increased Cu/Zn-SOD expression ($P < 0.05$) resembling that found in aorta from vehicle-treated WKY (Figure 4(a),(b)). Nevertheless, the expression level of this protein in normotensive WKY remained unchanged after treatment with LC or PLC (Figure 4(a),(b)). Finally, Mn-SOD expression levels were not modified by any treatment vs. vehicle-treated rats from both strains (Figure 4(c),(d)).

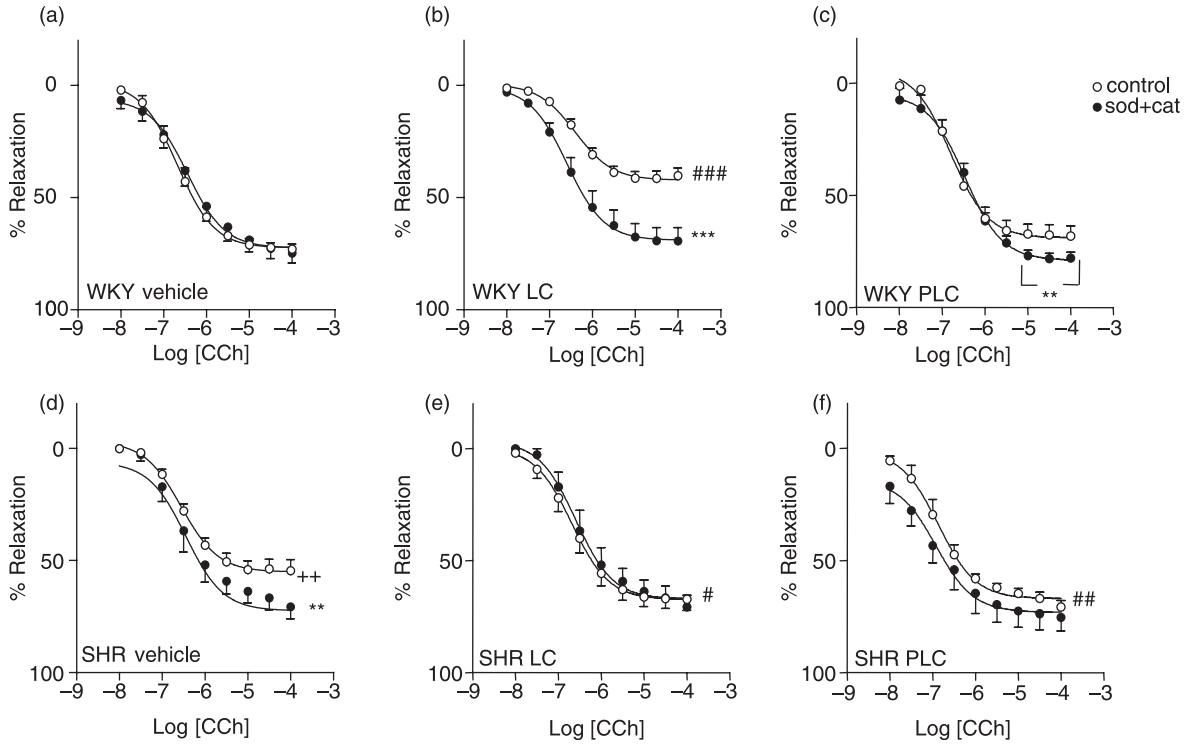

Figure 2. Endothelium-dependent relaxation to carbachol (CCh, 10^{-8}–10^{-4} mol l^{-1}) in endothelium-intact aorta in the absence (control, ○) or in the presence of SOD (150 UI ml^{-1}) plus catalase (cat, 1000 UI ml^{-1}) (•) from WKY (a–c) and SHR (d–f) rats treated with vehicle (a and d), L-carnitine (LC, 200 mg kg^{-1}) (b and e) or propionyl-L-carnitine (PLC, 200 mg kg^{-1}) (c and f) for 8 weeks. Curve in the absence of inhibitors was taken as control and whole concentration-response curves were compared except for panel c where the effect at three last concentrations of CCh was statitically analysed. $**P < 0.01$, $***P < 0.001$ vs. control; $\#P < 0.05$, $\#\#P < 0.01$, $\#\#\#P < 0.001$ vs. vehicle-treated control; $++P < 0.01$ vehicle-treated SHR vs. vehicle-treated WKY.

Discussion

This study provides evidence that the protective effects exerted by LC or its propionate, PLC, on endothelial function of hypertensive rats, are related to a decrease in ROS production by aortic rings probably through increasing expression of Cu/Zn-SOD and thus enhancing antioxidant defence of vascular wall. In addition, we have confirmed that NO participation in endothelial and vascular functions of SHR was improved after chronic treatment with PLC. This observation could be attributed to the antioxidant actions of PLC, but also to the increase in eNOS expression observed in arteries from SHR. Conversely, we have found that LC elicits opposite effects on the endothelium of normotensive WKY to those described in SHR. In arteries from WKY, this compound impaired endothelial function because of an enhanced production of $O_2^{\cdot-}$ associated to lower expressions of eNOS and Cu/Zn-SOD.

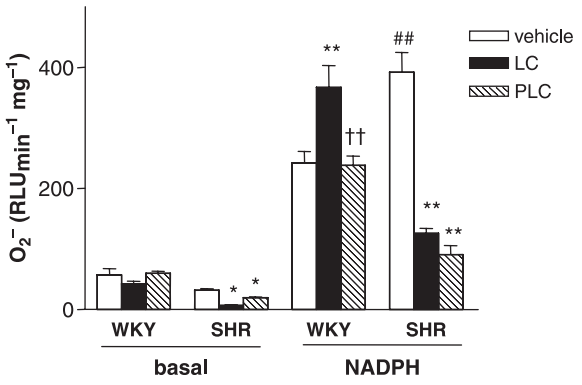

Figure 3. Superoxide ($O_2^{\cdot-}$) release quantified by lucigenin-enhanced chemiluminescence, in aorta from WKY and SHR treated with either vehicle, L-carnitine (LC, 200 mg kg^{-1}) or propionyl-L-carnitine (PLC, 200 mg kg^{-1}), under basal and NADPH (10^{-4} mol l^{-1})-stimulated conditions. Data represented are mean ± SEM of $n = 4$. $*P < 0.05$, $**P < 0.01$ vs. vehicle; $\#\#P < 0.01$ vs. treatment-matched WKY; $\dagger\dagger P < 0.01$ LC vs. PLC treatment.

LC is a natural aminoacid whose major physiological role is to transport long-chain fatty acids into the mitochondrial matrix for β-oxidation in order to provide energy in form of ATP. It has been demonstrated that both LC and its derivative PLC reduce myocardial injury after ischemia-reperfusion [24] and improve exercise performance in patients with peripheral artery disease [14]. Prevention of the accumulation of long-chain acyl-CoA has been involved in those effects; also, the improvement of repair mechanisms for oxidative-induced damage and the reduction of ischemia-induced apoptosis may account for the therapeutic application of LC and

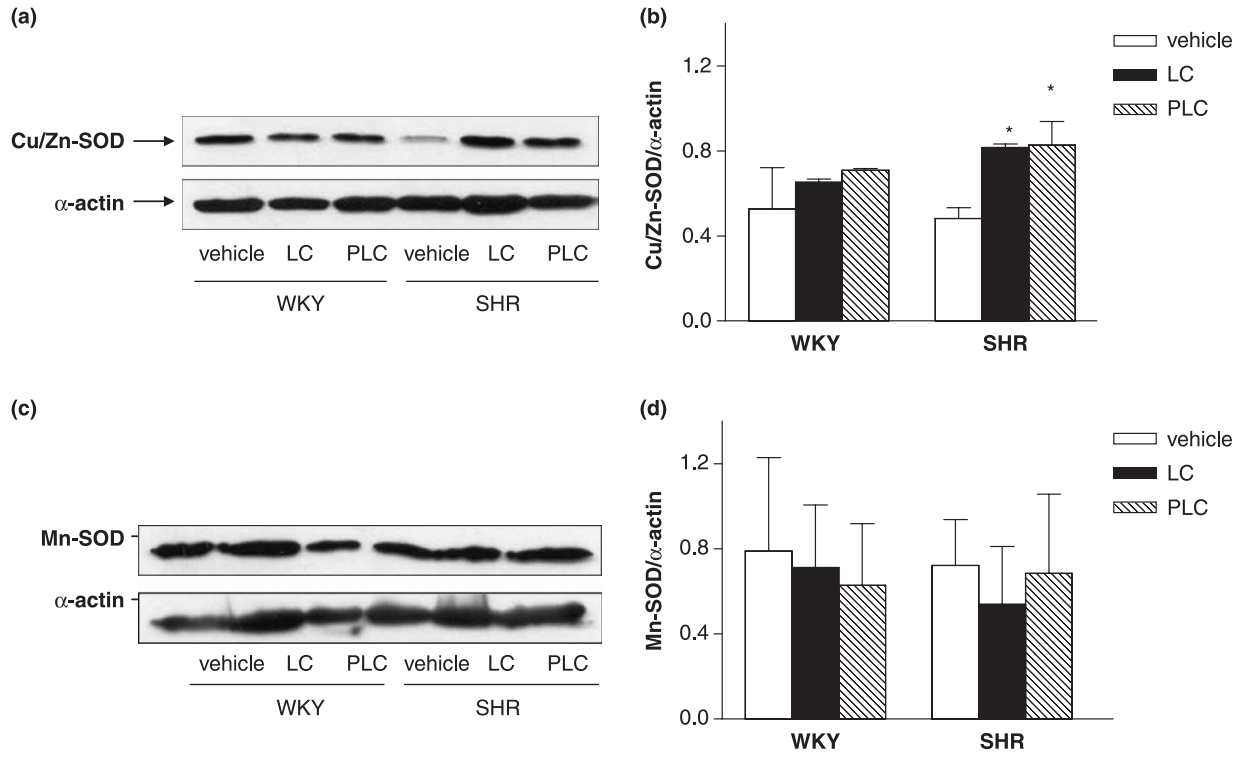

Figure 4. Representative Western blot of Cu/Zn-SOD (a) and Mn-SOD (c) from aorta treated with either vehicle, L-carnitine (LC, 200 mg kg^{-1}) or propionyl-L-carnitine (PLC, 200 mg kg^{-1}). Bars showing the ratio Cu/Zn-SOD/α-actin (b) and Mn-SOD/α-actin (d) determined by optic densitometry of $n = 3$ blots.

PLC (for review see [25]). The antioxidant properties of both compounds have been pointed out and their effects on pathologies characterised by enhanced ROS production have been explored. In this way, it has been reported that LC reduces lipid peroxidation in gastric mucosa [12], protects against acute renal failure in rats [26] and improves mitochondrial function [27] and antioxidant status in aged rat brain [28].

According to a previous report of our group [16], our results confirm that treatment with either LC or PLC clearly reverses endothelial dysfunction in SHR, which is known to be related to the higher vascular $O_2^{\cdot-}$ production [3–5,29]. The fact that the presence of the scavengers SOD plus catalase enhances endothelium-dependent relaxations of aortic rings from control SHR, but not in those from LC- or PLC-treated SHR, led us to conclude that the antioxidant properties of these compounds are involved in the restoration of endothelial function. Since $O_2^{\cdot-}$ is the ROS that plays a major role in this model of hypertension, production of this species was measured in aortic rings. Our results confirmed the well-known fact of the higher amount of $O_2^{\cdot-}$ produced by SHR [4] and showed that after treatment with either LC or PLC, $O_2^{\cdot-}$ reached values even lower than those found in vehicle-treated normotensive WKY.

Many hypotheses can be launched in order to explain these results: decrease in activity and/or expression of $O_2^{\cdot-}$-generating enzymes such as NADPH oxidase and/or increase of antioxidant defence in the arterial wall. We have studied the expression of two isoforms of the enzyme SOD: Mn-SOD and Cu/Zn-SOD. The former is located in mitochondrial matrix and the latter has been classically described as a cytosolic enzyme but also localizes bound to the inner membrane of mitochondria [30]. Since many of the effects of LC and PLC are related to mitochondrial activity, we have focused our attention in the expression of those isoenzymes of SOD. Previous reports showed that their expressions in SHR were lower [6], remained unchanged [31] or even increased [29] depending on the tissue and on the age of the animal. Our results showed that Cu/Zn-SOD expression decreased with hypertension and that treatment with either LC or PLC restored protein levels towards that observed in WKY. By contrast, Mn-SOD expression remained unaltered in all groups. Thus, the mechanism underlying the effects of LC and PLC on endothelial function might include upregulation of Cu/Zn-SOD expression that could contribute to scavenge $O_2^{\cdot-}$ and consequently decrease NO breakdown. At least in part, this mechanism could explain the enhanced endothelium-dependent

response of SHR after treatments. Nevertheless, we cannot confirm that hypothesis without knowing if the increased Cu/Zn-SOD were active.

Interestingly, in WKY, where we have confirmed a lower participation of ROS, PLC neither altered production of $O_2^{\cdot-}$ nor increased relaxant response to CCh. Moreover, LC showed a paradoxical effect in normotensive WKY characterized by enhanced $O_2^{\cdot-}$ production and inhibition of CCh-induced relaxation and even a decrease of eNOS expression. The fact that the presence of SOD plus catalase restored the response to the muscarinic agonist revealed that the increase of $O_2^{\cdot-}$ impaired the endothelial function of these animals. Although most of the reports studying the effect of LC on different conditions with increased free radical production have found that this compound may have antioxidant properties, it has also been described that LC decreases NO synthase (NOS) activity and the antioxidant status of fibroblasts. Moreover, in those cells, LC was able to elicit the opposite effect depending on the peroxisomal function of fibroblasts [32]. Peroxisomes play an indispensable role in several metabolic pathways such as β-oxidation of fatty acids and contain several systems such as catalase, glutathione peroxidase, Cu/Zn-SOD and Mn-SOD that bring about the H_2O_2 and $O_2^{\cdot-}$ generated by the activity of this organelles (for review see [33]). Interestingly, peroxisome proliferation has been found in hearts from SHR compared to WKY and this fact has been attributed to the higher oxidative stress found in this model of hypertensive rats [34]. Whether the paradoxical effect of LC on ROS production in arteries from WKY is related or not to the peroxisomal function remains unknown. Nevertheless, it is interesting to point out that it is not the first time that LC negatively affects antioxidant defence and that peroxisomes were involved in such a heterogeneous response.

In addition to the action on $O_2^{\cdot-}$ and Cu/Zn-SOD, we have studied the effect of our treatments on aortic eNOS expression. Although reduced NO participation in endothelial function of SHR is well established, the data concerning eNOS regulation in this model are somewhat conflicting because either a decreased or increased eNOS expression has been reported [35]. This discrepancy has been related to the use of SHR in different hypertensive stages and to the studied tissue. Our results confirmed that in SHR, there is a lower NO component of CCh-induced relaxation, but no difference in eNOS expression was detected. In spite of the fact that chronic treatment of SHR with LC restored NO participation, the expression of eNOS was not altered. On the contrary, administration of PLC to SHR increased the expression of this protein in aorta. This increased eNOS protein expression could be involved in the enhancement of NO participation in the response to either Phe or CCh, which was found in aortic rings from SHR treated with PLC. In agreement with our results, previous observations in patients with peripheral arterial disease reported that PLC was able to improve NO generation [15]. Moreover, *in vitro* experiments carried out in human endothelial cells demonstrated that eNOS expression increased after incubation with PLC [11]. Nevertheless, we have also found that PLC enhanced NO generation in response to Phe in WKY and in this case, neither eNOS up-regulation nor decreased oxidative stress could be observed. Thus, we cannot exclude that an increase in eNOS activity could occur after treatment with PLC.

Finally, we have found that LC treatment decreases eNOS protein expression in aortic rings from WKY. This change could also be involved in the diminished relaxant response to CCh observed in arteries from these animals. Interestingly, in LC-treated WKY an increased aortic $O_2^{\cdot-}$ was also found. The fact that antioxidants not only increase NO availability, but also enhance eNOS expression [36] led us hypothesize that the effects of LC and PLC on eNOS protein expression could be related to their actions on vascular O2·− production.

In conclusion, we have shown that PLC improves endothelial dysfunction of SHR through increasing NO participation. The mechanism involved in such effect may include both the decrease of NO breakdown because of the increase of antioxidant defence and the higher NO generation probably due to the enhanced eNOS expression. Surprisingly, we have also pointed out the heterogeneous effects of LC on ROS production depending on the hypertensive strain of the animal: antioxidant properties that protect endothelium for SHR, but all the opposite actions for arteries from normotensive WKY. Finally, these results may contribute to explain the benefits of PLC on peripheral arterial diseases, which involve both increased ROS generation and a decrease in NO participation.

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