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# L-Carnitine attenuates oxidative stress in hypertensive rats

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**Abstract**

The present study aimed to investigate whether L-carnitine (LC) protects the vascular endothelium and tissues against oxidative damage in hypertension. Antioxidant enzyme activities, glutathione and lipid peroxidation were measured in the liver and heart of spontaneously hypertensive rats (SHR) and Wistar-Kyoto (WKY) rats. Nitrite and nitrate levels and total antioxidant status (TAS) were evaluated in plasma, and the expression of endothelial nitric oxide synthase (eNOS) and p22phox subunit of NAD(P)H oxidase was determined in aorta. Glutathione peroxidase activity was lower in SHR than in WKY rats, and LC increased this activity in SHR up to values close to those observed in normotensive animals. Glutathione reductase and catalase activities, which were higher in SHR, tended to increase after LC treatment. No differences were found in the activity of superoxide dismutase among any animal group. The ratio between reduced and oxidized glutathione and the levels of lipid peroxidation were respectively decreased and increased in hypertensive rats, and both parameters were normalized after the treatment. Similarly, LC was able to reverse the reduced plasma nitrite and nitrate levels and TAS observed in SHR. We found no alterations in the expression of aortic eNOS among any group; however, p22phox mRNA levels showed an increase in SHR that was reversed by LC. In conclusion, chronic administration of LC leads to an increase in hepatic and cardiac antioxidant defense and a reduction in the systemic oxidative process in SHR. Therefore, LC might increase NO availability in SHR aorta by a reduction in superoxide anion production.


*Keywords:* Antioxidant enzymes; L-Carnitine; Nitric oxide; Oxidative stress; SHR

## 1. Introduction

There is increasing evidence indicating that oxidative stress plays an important role in the pathogenesis of hypertension [1]. Superoxide anions and other reactive oxygen species (ROS) contribute to the generation and/or maintenance of hypertension through several mechanisms, such as inactivation of endothelium-derived nitric oxide (NO) to produce peroxynitrite, a potent constrictor and lipid-oxidizing radical [2–4].

Many studies support a role for altered redox status in hypertension [5]. At the cellular level, an increased production of superoxide anions and activation of NAD(P)H oxidase, the major superoxide anion-generating enzyme in vascular cells, have been reported as an important mechanism involved in endothelial dysfunction observed in arterial hypertension [6,7]. In addition, hypertension is associated with changes in hepatic and heart redox system [8–10]. In view of these considerations, an ROS-decreasing treatment has been suggested in order to ameliorate the adverse effects of oxygen-derived free radicals [11,12], and dietary antioxidants such as vitamin C and E have reduced arterial blood pressure in experimental hypertension models such as spontaneously hypertensive or Dahl salt-sensitive rats [13].

L-Carnitine (β-hydroxy-γ-*N*-trimethylammonium-butyrate; LC) is a vital component in lipid metabolism for the production of ATP through the β-oxidation of long-chain fatty acids. An antioxidant promoting action has been suggested for this compound. Thus, LC acts as a free-radical scavenger [14,15] and protects cells from ROS [16]. Previous studies have described antioxidant properties of

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carnitine in aging [17] and atherosclerotic rats [18] and hypercholesterolaemic rabbits [19]. In this sense, recent studies in our laboratory have demonstrated an improvement in the aortic endothelial dysfunction of spontaneously hypertensive rats (SHR) after LC chronic treatment, and results obtained in the presence of superoxide dismutase (SOD) plus catalase (CAT) indicate that this improvement is related to the antioxidant properties of LC [20].

The present study therefore aimed to test the hypothesis that LC might somehow protect tissues against hypertension-induced oxidative damage. To this purpose, the specific activities of the antioxidant enzymes, glutathione peroxidase (GSH-Px), glutathione reductase (GSH-Red), SOD and CAT, as well as the ratio between reduced and oxidized glutathione (GSH/GSSG) and the levels of thiobarbituric acid-reactive substances (TBARS), were measured in liver and heart homogenates from SHR and normotensive Wistar-Kyoto (WKY) rats treated or untreated with LC. Plasma nitrite and nitrate levels ($NO_x$) and total antioxidant status (TAS) were also evaluated in all experimental groups. In addition, protein and mRNA expression of endothelial NO synthase (eNOS) and mRNA expression of the p22phox subunit of NAD(P)H oxidase were also studied in aorta from the same rats, in order to study the effect of LC on vascular endothelium.

## 2. Materials and methods

### 2.1. Animals and experimental design

Normotensive male Wistar-Kyoto (WKY) and spontaneously hypertensive rats (SHR) aged 4 weeks were obtained from Harlan IBERICA (Barcelona, Spain). Rats were kept under standard conditions (23±1°C, 12-h light/12-h dark cycles) and fed a standard pellet diet. WKY and SHR were divided at random into two groups. The control group had free access to tap water. In the other group, an L-carnitine (LC) solution was offered instead of water for 8 weeks. The amount of consumed LC was 0.2 g/kg body weight per day, adjusted from their daily water consumption. LC dosing was chosen from previous work [21] taking into account the normal oral doses in humans and the appropriate conversion factor from humans to rats [22]. Diastolic and systolic blood pressures were measured weekly by the indirect method of tail-cuff occlusion in conscious animals using a pressure recorder NIPREM 645 (Cibertec, Barcelona, Spain). The mean of three or four successive measurements was used as the estimate of blood pressure. Body weight was determined on the same day that blood pressure was measured. Studies were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

### 2.2. Blood sampling and tissue preparation

At the end of the experimental period, rats were fasted for 18 h before killing. They were anesthetized with pentobarbital (50 mg/kg, ip) and sacrificed by decapitation. To minimize diurnal variations, they were routinely killed between 0900 and 1000 hours. Blood samples were obtained by cardiac punction and collected into tubes containing lithium heparin. Plasma was separated by low-speed centrifugation at 1500×*g* at 4°C for 30 min and immediately analyzed for measurement of nitrite and nitrate and determination of TAS. Liver and heart were quickly removed, the blood being washed out with ice-cold 0.9% saline solution. The left ventricle was dissected from the rest of the heart and both tissues were weighed and stored at −70°C. For the determination of enzyme activities and thiobarbituric acid reactive substances (TBARS), tissues were homogenized in 0.25 mol/L sucrose, 1 mmol/L EDTA, 1 mmol/L DL-dithiothreitol and 15 mmol/L Tris/HCl (pH 7.4), using an all-glass Potter Elvehjem homogenizer (Selecta, Barcelona, Spain). After centrifugation at 800×*g* for 20 min, the resulting supernatant fraction was used. The protein concentration of the supernatant was determined by the method described by Lowry et al. [23]. For determining GSH/GSSG levels, liver and heart samples were prepared in 50 mmol/L potassium phosphate buffer (pH 7.5) containing 1 mmol/L EDTA and 1 mg/ml BSA.

### 2.3. Measurement of enzyme activities and GSH/GSSG levels

GSH-Px activity was assayed with an enzyme system in which oxidized glutathione (GSSG) reduction was coupled to NADPH oxidation by glutathione reductase [24]. GSH-Red activity was determined spectrophotometrically by measuring NADPH oxidation at 340 nm [25]. Superoxide dismutase activity was measured using the xanthine-oxidase-cytochrome *c* method as described by McCord and Fridovich [26]. CAT activity was assayed according to the method of Beers and Sizer [27]. All measurements were carried out in a Shimadzu160A ultraviolet spectrophotometer using 1 ml quartz cuvettes with a light path of 1 cm. Total GSH levels and GSSG and GSSG/GSH ratio were determined using a commercial kit (Bioxytech GSH/GSSH-412, Oxis Research).

### 2.4. Measurement of tissue lipid peroxidation levels

Tissue oxidative stress was determined by measuring the concentration of TBARS on homogenized tissues as described by Esterbauer and Cheeseman [28]. The amount of aldehydic products generated by lipid peroxidation was quantified by the thiobarbituric acid reaction using 3 mg of protein per sample. The TBARS values were expressed in nanomoles of malondialdehyde (MDA) equivalents.

### 2.5. Total antioxidant status and nitrite and nitrate determination

The TAS was assayed using a commercial kit (Randox Lab) based on the method by Miller et al. [29]. Nitrite and nitrate levels were determined with a commercial kit (Nitric Oxide Colorimetric Assay, Roche Diagnostics) based on the Griess reaction [30] on microtiter plates.

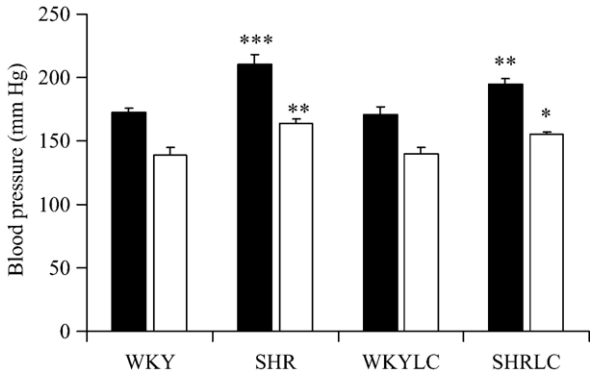


Fig. 1. Systolic (closed bars) and diastolic (open bars) blood pressures in WKY and SHR treated and untreated with LC. Values represent means± S.E.M. of 10 animals per group. *$P<.05$, **$P<.01$ and ***$P<.001$ compared with WKY rats.

### 2.6. Western blot analysis for eNOS

Aorta tissues were prepared for measurement of endothelial NOS (eNOS) protein abundance by Western blot analysis. Homogenates (10% w/v) of rat aortas were prepared in 50 mM phosphate buffer, pH 7, containing 0.01 mM EDTA, leupeptin, aprotinin and pepstatin (1 μg/ml each), and 1 mM phenylmethylsulfonylfluoride (PMSF) at 0–4°C with a polytron homogenizer. The homogenate was centrifuged at 2000×$g$ for 10 min at 4°C, and the supernatant was then used for determination of total protein concentration [23]. Aorta tissue protein preparations were solubilized in Laemmli sample buffer and resolved by 8% SDS-PAGE [31]. Proteins were electrotransferred onto nitrocellulose membranes and immunoblotted as previously described [32] using monoclonal anti-eNOS antibodies (Transduction Laboratories, Madrid, Spain), diluted at 1:2500. The anti-eNOS antibody was detected by the enhanced chemiluminiscence (ECL) method according to the supplier's protocol and using a peroxidase-conjugated antimouse IgG as a secondary antibody (1:2000 dilution).

### 2.7. Aortic eNOS and p22phox mRNA expression. Reverse transcription-polymerase chain reaction (RT-PCR)

Whole RNA was extracted from frozen rat aortas after homogenization with 1 ml of Tripure Isolation Reagent (Roche Diagnostics Corp., Indianapolis, USA) as described by Chomczynski and Sacchi [33]. RT was carried out in a

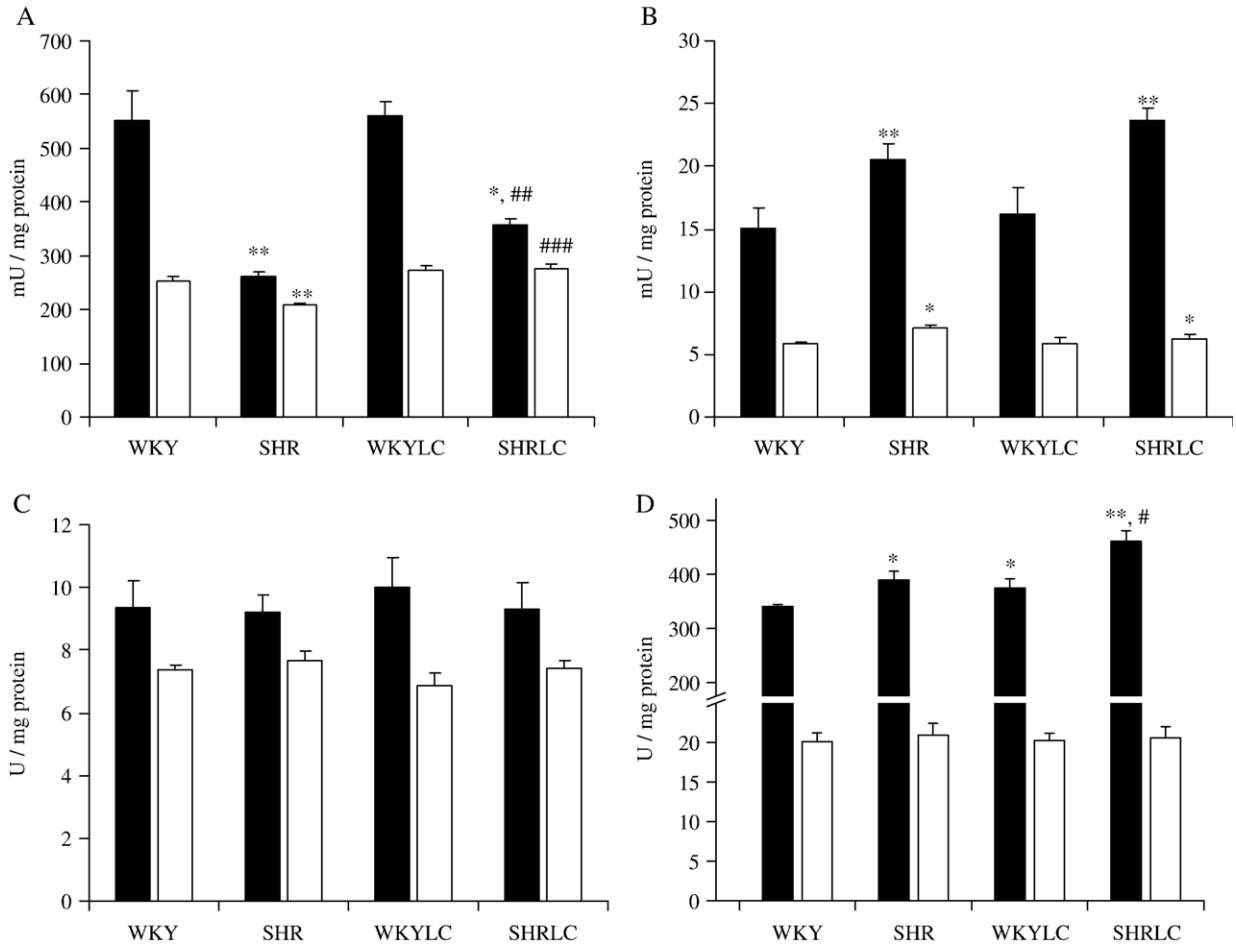


Fig. 2. Glutathione peroxidase (A), glutathione reductase (B), superoxide dismutase (C) and catalase (D) activities in liver (closed bars) and heart (open bars) homogenates from WKY and SHR treated and untreated with LC. Values represent means±S.E.M. of at least six different preparations. *$P<.05$ and **$P<.01$ compared with WKY rats; $^{\#}P<.05$, $^{\#\#}P<.01$ and $^{\#\#\#}P<.001$ compared with SHR.

Table 1
GSH/GSSG ratio and TBARS[a] levels in liver and heart of normotensive (WKY) and hypertensive (SHR) animals untreated or treated with LC

| | WKY | SHR | WKYLC | SHRLC |
|---|---|---|---|---|
| GSH/GSSG (liver) | 138±14 | 78±6* | 116±7 | 125±16† |
| TBARS (liver) | 0.32±0.03 | 0.43±0.02** | 0.32±0.01 | 0.33±0.03† |
| GSH/GSSG (heart) | 147±3 | 72±2*** | 148±7 | 108±4***,††† |
| TBARS (heart) | 0.29±0.03 | 0.39±0.02* | 0.24±0.03 | 0.27±0.03†† |

Values are expressed as means±S.E.M. of al least five animals per group.
[a] Thiobarbituric acid-reactive substances (nmol/mg protein per 30 min).
* $P<.05$ vs. WKY.
** $P<.01$ vs. WKY.
*** $P<.001$ vs. WKY.
† $P<.05$ vs. SHR.
†† $P<.01$ vs. SHR.
††† $P<.001$ vs. SHR.

final volume of 33 μl. Ready-To-Go You-Prime First-Strand Beads (Amersham Biosciences, Madrid, Spain) were used according to the supplier's protocol and using 5 μg of primer random $p(dN)_6$ (Roche, Madrid, Spain) and 2 μg of whole RNA as a template. After RT, cDNA was purified using a commercial kit (GFX DNA purification kit, Amersham Biosciences). PCR for eNOS and p22-phox was performed in a final volume of 50 μl in the presence of 25–150 ng cDNA as a template, 1.5 U Taq DNA polymerase in the supplier's buffer (Amersham Biosciences, Madrid, Spain), 1.5 mmol/L $MgCl_2$, 30 μmol/L dNTPs and 0.15 μmol/L each of the following sets of primers: 5′-GGGCCAGGGTGATGAGCTCTG-3′ (sense) and 5′-CCCTCCTGGCTTCCAGTGTCC-3′ (antisense) for eNOS, product size 324 bp; and 5′GCTCATCTGTCTGCTGGAGTA-3′(sense) and 5′ACGACCTCATCTGTCACTGGA-3′ (antisense) for p22-phox, product size 434 bp. After an initial denaturation at 94°C for 4 min, 36 cycles of denaturation (94°C for 60 s), annealing (60°C for 60 s) and elongation (72°C for 60 s) were performed, followed by a final elongation period of 5 min at 72°C. GAPDH was used as the internal standard, with a sense primer, 5′GCCAAAAGGGTCATCATCTCCGC-3′ and an antisense primer, 5′-GGATGACCTTGCCCACAGCCTTG-3′ (product size 319 bp). The conditions of PCR for GAPDH were similar to those shown for eNOS and p22phox except for the number of cycles, 26.

### 2.8. Reagents

Unless otherwise specified, all reagents were obtained from Sigma (Madrid, Spain). Reagents used in Western blot analysis were from Bio-Rad (Barcelona, Spain), and the ECL reagent was from Amersham Biosciences (Madrid, Spain). Primers for RT-PCR analysis were synthesized by Tib Molbiol (Berlin, Germany). L-Carnitine was a kind gift of Sigma Tau (Madrid, Spain).

### 2.9. Statistical methods

All results were subjected to one-way analysis of variance (ANOVA) and represent means±S.E.M. Differences in mean values between groups were assessed by the Student–Newman–Keuls multiple comparison test and considered statistically different at $P<.05$.

## 3. Results

### 3.1. Measurement of body weight and blood pressure

Both diastolic and systolic blood pressures were higher in SHR than in WKY rats. In normotensive rats, no significant changes were observed in blood pressure values after the treatment with LC. However, the administration of LC in hypertensive rats showed a slight, although not significant, decrease in both blood pressures when compared with untreated SHR control rats (Fig. 1). Body weight did not change between WKY and SHR (313±7 and 295±6 g for control WKY and SHR, respectively). Treatment with LC did not affect the body weight of the animals from the two strains (307±5 and 295±4 g for treated WKY and SHR rats, respectively).

### 3.2. Measurement of antioxidant enzymes, GSH and lipid peroxidation levels

GSH-Px activities in the liver and heart were significantly lower (53% and 18%, respectively) in SHR than in WKY rats. LC treatment increased the activity of GSH-Px seen in SHR, reaching, in the case of the heart, values similar to those found in untreated WKY rats. No effect was observed in WKY rats treated with LC (Fig. 2A).

GSH-Red activities are shown in Fig. 2B. A significant increase was observed in the liver and heart of hypertensive rats (36% and 22%, respectively) when compared with WKY rats. LC treatment did not modify this enzyme activity in either SHR or WKY rats.

The activities of SOD in all four groups of experimental rats are shown in Fig. 2C. No significant changes in SOD activities were observed between SHR and WKY rats, and LC treatment did not modify those values.

Liver from hypertensive animals expressed higher CAT activities than that from WKY rats (14%). LC treatment increased liver CAT activities in both WKY and SHR-treated rats (10% and 15%, respectively) when compared with control, untreated ones. However, CAT activities in the heart were similar between SHR and WKY rats, and these activities were not modified by LC chronic administration (Fig. 2D).

Table 2
Nitrite and nitrate determination and TAS in normotensive (WKY) and hypertensive (SHR) animals untreated or treated with LC

| | WKY | SHR | WKYLC | SHRLC |
|---|---|---|---|---|
| $NO_2$+$NO_3$ (μmol/L) | 21±1 | 14±1*** | 26±0.5** | 18±0.6*,†† |
| TAS (μmol/L) | 1150±12 | 1013±36* | 1139±26 | 1170±35†† |

Values are expressed as means±S.E.M. of at least five animals per group.
* $P<.05$ vs. WKY.
** $P<.01$ vs. WKY.
*** $P<.001$ vs. WKY.
†† $P<.01$ vs. SHR.

GSH/GSSG ratios and lipid peroxidation status in liver and heart for all experimental groups are shown in Table 1. There was a significant reduction in the ratio of GSH/GSSG in hypertensive rats when compared with normotensive ones (41% and 52% in the liver and heart, respectively). The treatment with LC did not modify these values in normotensive rats; however, in SHR the treatment enhanced this ratio in the liver up to values similar to those of untreated, normotensive rats. Likewise, significant increases in the levels of TBARS were also observed in liver and heart from SHR when compared with those of normotensive ones (34% and 35%, respectively). LC treatment managed to reverse these values in both tissues in hypertensive rats, and no modifications were observed in WKY rats after the treatment.

### 3.3. Total antioxidant status and nitrite and nitrate production

The levels of $NO_2$+$NO_3$ ($NO_x$) were significantly lower (33%) in SHR than in WKY rats. LC treatment significantly increased $NO_x$ production in hypertensive and normotensive rats (Table 2). Similarly, a 12% decrease was observed in the total antioxidant status of plasma in SHR when compared with WKY rats. The treatment with LC increased this capacity in hypertensive rats, which reached the same values found in untreated WKY rats.

### 3.4. Western blot for eNOS

Measurements of eNOS relative protein abundance are shown in Fig. 3. The relative abundance of eNOS was similar between normotensive and hypertensive rats, and no changes were observed after the treatment with LC.

### 3.5. Aortic eNOS and p22phox mRNA expression

Expression of aortic eNOS mRNA was similar between normotensive and hypertensive rats after normalizing to GAPDH mRNA levels, and the treatment with LC did not modify this expression in any animal group (Fig. 4A). On the other hand, an increase in the expression of p22phox mRNA was observed in SHR when compared with WKY rats, which was prevented by the treatment with LC (Fig. 4B). No effect was observed in normotensive rats after the treatment.

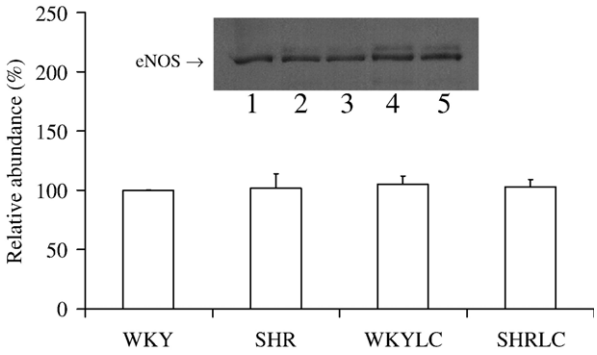


Fig. 3. Relative abundance of eNOS protein in aorta from WKY and SHR treated and untreated with LC. Values represent means±S.E.M. of five different preparations. 1=eNOS-positive control; 2=WKY group; 3=SHR group; 4=WKYLC group; 5=SHRLC group.

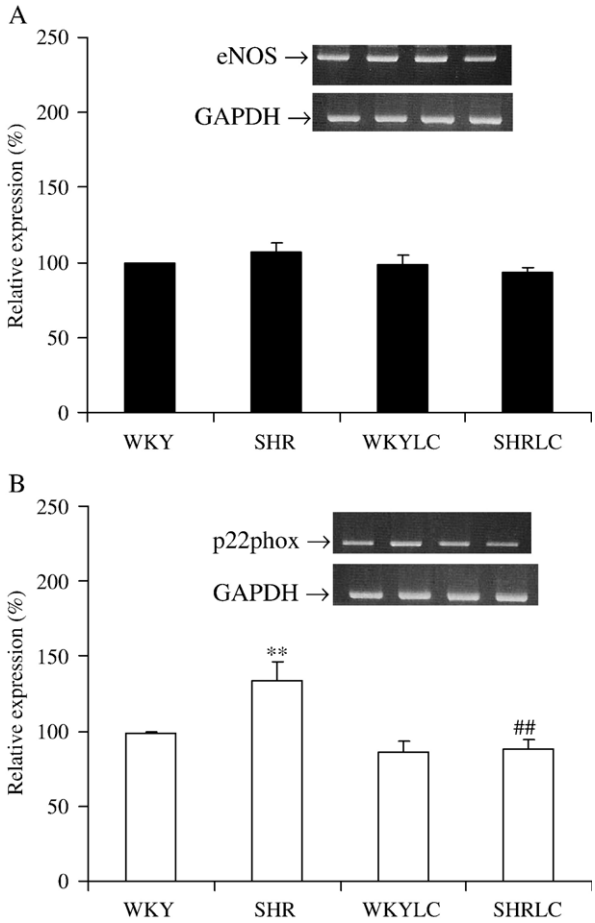


Fig. 4. mRNA expression of eNOS (A, closed bars) and p22phox (B, open bars) in aorta from WKY and SHR treated and untreated with LC. Values represent means±S.E.M. of five different preparations. $^{**}P<.01$ compared with WKY rats; $^{\#\#}P<.01$ compared with SHR.

## 4. Discussion

This study shows that treatment with LC ameliorated oxidative stress in liver, heart and plasma of SHR, together with a decrease in aortic p22phox mRNA expression. Nonetheless, these changes were not accompanied by alterations in either aortic eNOS relative protein abundance and mRNA expression or arterial blood pressure.

It has been reported that enhanced production of ROS in vascular endothelium, mainly superoxide anions, is responsible for endothelial dysfunction in SHR, because these anions produce inactivation of NO and, consequently, a decrease in NO availability [3,4,34]. Although we have not measured the production of superoxide anions in this study, the enhanced expression of aortic p22phox, a subunit of NAD(P)H oxidase; the elevated lipid peroxidation levels in liver and heart; and the decrease in TAS in the plasma of SHR compared with normal WKY rats support previous reports. In addition, the decrease in both GSH-Px activity

and GSH/GSSG ratio observed in SHR when compared with WKY rats could be responsible for an increase in ROS, since this enzyme catalyzes the reduction of $H_2O_2$ to $H_2O$ and $O_2$ at the expense of reduced glutathione (GSH).

When we measured $NO_x$ as an indirect technique to detect NO availability [35], we found a decrease in SHR, which was not due to a reduced expression of eNOS protein and mRNA in SHR, as determined in aorta by Western blotting and RT-PCR, respectively. However, since we do not know whether the results observed in protein and mRNA levels of eNOS correlate with the activity of this enzyme, the possibility of changes at this level cannot be ruled out.

There are previous studies in the literature that report changes in the oxidative status in SHR in vessels, liver, heart and plasma that indicate modifications in both local and systemic oxidative process [9,36–38]. Based on these reports, an antioxidant therapy has been proposed in order to ameliorate the oxidative stress associated with arterial hypertension [38–40]. The antioxidant activity of LC has been demonstrated in aging [14,15,17] and atherosclerotic [18] rats, and hypercholesterolaemic rabbits [19], which are all conditions producing an increase in the systemic oxidative process. Furthermore, previous studies in our laboratory demonstrated the antioxidant activity of propionyl-L-carnitine (PLC) in liver and heart of SHR [41], as well as an improvement in the aortic endothelial dysfunction of SHR with LC and PLC chronic treatment [20], suggesting that this improvement is related to the antioxidant properties of these compounds. Therefore, in the present work, we evaluated whether LC might somehow protect the liver and heart against hypertension-induced oxidative damage and the effect of LC on vascular endothelium. Our results show that the reduction in GSH-Px activity found in the liver and heart of SHR is reversed by long-term administration of LC. On the other hand, the increase observed in GSH-Red activity in the same rats was not modified by the LC treatment.

LC administration has been shown to raise glutathione levels in ischemia [16] and aged rats [14,17], which in turn increased GSH-Px activity [17,18]. Also, the role of GSH-Px in protecting endothelium from oxidative damage has recently been demonstrated [42]. Therefore, LC might enhance the levels of GSH in SHR that in turn would increase their GSH-Px activity; the increase in the ratio of GSH/GSSG observed in hypertensive rats treated with LC confirmed this hypothesis. In addition, the higher CAT activity found in SHR after the treatment with LC might indicate an improvement in the removal of hydrogen peroxides before they produce new ROS.

A decreased aortic production of superoxide anion in LC-treated SHR may be suggested due to the normalization in p22phox expression observed in these rats. In addition, the reduced production of lipid peroxides together with the increase in both GSH-Px activity and GSH/GSSG ratio observed in the liver and heart of SHR after the treatment with LC might contribute to the enhancement in the total antioxidant status (TAS) found in these rats. Therefore, all these effects of LC would lead to an amelioration of oxidative status in SHR, which might result in both an increase in NO availability and an improvement in endothelial dysfunction. According to our results, the observed increase in $NO_x$ levels found in SHR after the treatment with LC would not be explained by a higher production of NO, since we found no significant changes in aortic protein and mRNA expression of eNOS; it may be related to a reduction in ROS formation resulting in a higher NO availability. However, it is also possible that the reduction in ROS by LC increases the enzymatic production of NO from eNOS, due to a decreased oxidative uncoupling of the enzyme. In the current study, this possibility cannot be discussed, since the activity of eNOS was not measured. On the other hand, $NO_x$ levels were also increased in WKY treated with LC, which might indicate a direct effect of LC on NO availability irrespective of the presence of oxidative stress. Taken together, all these results indicate that chronic treatment with LC leads to an enhancement in hepatic and cardiac antioxidant defense and a reduction in the systemic oxidative process in SHR.

The antioxidant capacity of LC was not accompanied by significant changes in arterial blood pressure, which indicates that, in our experimental conditions, LC did not prevent the development of hypertension in young SHR. In contrast, Rauchova et al. [21] found a decrease in both systolic and mean arterial blood pressure in SHR aged 8 weeks when LC (200 mg/kg body weight) was administered for 6 weeks, although no changes in diastolic arterial blood pressure were found. In addition, a slight decrease in diastolic and systolic arterial blood pressure was observed in hypertensive humans subjected to long-term LC treatment [43]. Changes in the experimental conditions such as the amount and duration of the treatment or the use of old rats with fully established hypertension might be responsible for these differences. In this sense, preliminary results in our lab have demonstrated that the administration of a higher dose of LC (300 mg/kg body weight per day) for 12 weeks produced a significant decrease in blood pressure in rats with established hypertension. Nonetheless, the amelioration of oxidative damage could be somehow relevant to a later reduction in arterial blood pressure. In fact, using the same experimental conditions as in this study, we have shown an improvement in the aortic endothelial dysfunction of SHR after LC chronic treatment [20].

The mechanism(s) responsible for the effect of LC on ROS production in arterial hypertension is not clear. Nonhemodynamic factors, such as angiotensin II and aldosterone, might be involved, since p22phox mRNA expression is elevated in rats receiving an angiotensin II infusion [44], an effect that is inhibited by blocking AT1 [45] and aldosterone [46] receptors. In this regard, preliminary results in our laboratory have found an increase in plasma levels of aldosterone in SHR which was prevented by the treatment with LC, reaching values similar to those

found in normotensive rats. Therefore, more studies are necessary in order to elucidate the mechanism(s) involved in the effect of LC on arterial hypertension.

## Acknowledgments

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