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Protective effect of resveratrol on acute endotoxemia-induced nephrotoxicity in rat through nitric oxide independent mechanism

HICHEM SEBAI[1,2], MOSSADOK BEN-ATTIA[3], MAMANE SANI[3], EZZEDINE AOUANI[1], & NÉZIHA GHANEM-BOUGHANMI[1]

[1]*UR Ethnobotanie & Stress Oxydant, Département des Sciences de la Vie, Faculté des Sciences de Bizerte, 7021 Zarzouna, Tunisie,* [2]*INSERM UMR-911, Université de la Méditerranée, Faculté de Médecine-Timone, 27 Boulevard Jean Moulin, 13385 Marseille Cedex 05, France, and* [3]*Laboratoire de Biosurveillance de l'Environnement, Faculté des Sciences de Bizerte, 7021 Zarzouna, Tunisie*

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Abstract

Lipopolysaccharide (LPS) is a glycolipid component of the cell wall of gram negative bacteria inducing deleterious effects on the kidney. Endotoxemia-induced nephrotoxicity is characterized by disturbed intracellular redox balance and reactive oxygen species (ROS) accumulation leading to DNA, proteins and membrane lipid damages. Resveratrol (trans-3,5,4'-trihydroxystilbene) is a polyphenol displaying antioxidant and anti-inflammatory properties. This study investigated its effects on LPS-induced nephrotoxicity in rats. Resveratrol counteracted all LPS-induced changes in renal haemodynamic parameters. In the kidney resveratrol abrogated LPS-induced lipoperoxidation and antioxidant enzyme activities depletion as superoxide dismutase (SOD) and catalase (CAT) but not peroxidase (POD) activity. LPS increased plasma and urine nitric oxide (NO) level and resveratrol reversed them. More importantly, LPS-induced iron mobilization from plasma to kidney, which was also abolished by resveratrol treatment. All these results suggest that resveratrol exerted strong antioxidant properties against LPS-induced nephrotoxicity and that its mode of action seemed to involve iron shuttling proteins.

Keywords: *Resveratrol, lipopolysaccharide, kidney, oxidative stress, nitric oxide, iron.*

Abbreviations: *BHT, Butylated hydroxytoluene; CAT, Catalase; LPS, Lipopolysaccharide; MDA, Malondialdehyde; NO, Nitric oxide; NOS, Nitric oxide synthase; POD, Peroxidase; ROS, Reactive oxygen species; RVT, Resveratrol; SOD, Superoxide dismutase; TBA, Thiobarbituric acid; TCA, Trichloroacetic acid.*

Introduction

LPS, a glycolipid component of the cell wall of gram negative bacteria, is an endotoxin implicated in the triggering of sepsis and septic shock [1], which are the most important causes of morbidity and mortality in intensive care units [2]. Endotoxin has harmful effects on various organs including the kidney through the production and release of inflammatory mediators [3]. The increased expression of cytokines in response to acute administration of LPS, which plays a crucial role in protecting the host against infectious microorganisms, may also have profound detrimental consequences as disturbed intracellular redox balance also known as oxidative stress [4]. Excessive ROS accumulation in renal tissue leads to cellular injury initiated by impairment of vital macromolecules as DNA [5], protein [6] and lipid [7]. Lipid peroxidation is associated with a wide variety of

Correspondence: Mossadok Ben-Attia, Laboratoire de Biosurveillance de l'Environnement (LBE), Département des Sciences de la Vie, Faculté des Sciences de Bizerte, 7021 Zarzouna, Tunisia. Fax: +(216) 72 590 566. Email: benattia.mossa@gmail.com

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toxic effects, including decreased membrane fluidity, impaired mitochondrial functions and inhibition of antioxidant enzymes [8]. Resveratrol (trans-3,5,4′-trihydroxystilbene) is a polyphenol phytoalexin abundantly found in grapes that exhibits diverse biochemical and physiological effects including oestrogenic, anti-platelet and anti-inflammatory properties (for review see Soleas et al. [9]). Resveratrol was found to protect heart, brain and kidney from ischemia-reperfusion injury [10–12]. More recently, resveratrol was shown to protect against cyclosporine-induced nephrotoxicity in rats by its antioxidant properties through a NO-dependent mechanism [13]. In the present study we investigated the putative protective effect of resveratrol on nephrotoxicity induced by LPS treatment and discussed the mechanism involved in such reno-protection.

Materials and methods

Chemicals

LPS (055B5 from E. coli) was from Sigma-Aldrich s.r.l. (Milano, Italy). Resveratrol was from Orchid Chemicals & Pharmaceuticals Ltd (Nungambakkam, Chennai, 600034, India). All other chemicals were of analytical grade.

Animals and treatment

Forty male wistar rats (200–240 g) from Pasteur Institute of Tunis were used in these experiments in accordance with the local ethics committee of Tunis University for use and care of animals in conformity with the NIH recommendations. They were provided with food and water *ad libitum* and maintained in an animal house at controlled temperature ($22 \pm 2^{\circ}C$) with a 12 h light–dark cycle. Rats were divided into four groups of 10 animals each: control, resveratrol, LPS and resveratrol plus LPS. Animals were daily intraperitoneally injected (*i.p.*) during 7 days with either vehicle (control 5% ethanol) or with 20 mg/kg *b.w.* resveratrol prepared as a stock solution of 20 mg/mL in 5% ethanol (injection volume was 1 mL/kg *b.w.*). Twenty-four hours after the last resveratrol injection, endotoxemia was induced by a single *i.p.* injection of LPS (8 mg/kg *b.w.*) or not (control NaCl 9%) and animals housed in metabolic cages in order to collect urines. Twenty-four hours later animals were sacrificed, their kidney rapidly excised and either processed for histological study or homogenized in phosphate buffer saline pH 7.4 with a Potter–Elvehjem homogenizer using a Teflon pestle driven by a motor. After centrifugation at 10 000 *g* for 10 min at 4°C, supernatant was used for biochemical determination of protein, MDA, antioxidant enzyme activities, iron and NO. Blood was also collected and plasma processed for creatinine, urea, iron, NO and protein determination. Proteinuria in 24 h cumulated urine was also determined.

Assessment of renal function

Plasma and urine samples were assayed for blood urea, urea clearance, blood creatinine and creatinine clearance by using commercially available diagnostic kits supplied by Randox laboratories (Ardmore, Northern Ireland, UK).

Lipid peroxidation measurement

Lipid peroxidation was determined by MDA measurement according to the double heating method [14]. Briefly, aliquots from kidney tissue homogenate were mixed with BHT-TCA solution containing 1% BHT (w/v) dissolved in 20% TCA (w/v) and centrifuged at 1000 g for 5 min at 4°C. Supernatant was blended with 0.5 N HCl and 120 mM TBA in 26 mM Tris and then heated at 80°C for 10 min. After cooling, absorbance of the resulting chomophore was determined at 532 nm using a UV-visible spectrophotometer (Beckman DU 640B). MDA levels were determined by using an extinction coefficient for MDA-TBA complex of $1.56 \times 10^5\ M^{-1}cm^{-1}$.

Antioxidant enzyme activities assay

All spectrophotometric analyses were performed with a Beckman DU 640B spectrophotometer. SOD activity was determined by using modified epinephrine assay [15]. At alkaline pH, superoxide anion O_2^- causes the auto-oxidation of epinephrine to adenochrome; while competing with this reaction, SOD decreased the adenochrome formation. One unit of SOD is defined as the amount of the extract that inhibits the rate of adenochrome formation by 50%. Enzyme extract was added in 2 mL reaction mixture containing 10 μL of bovine catalase (0.4 U/μL), 20 μL epinephrine (5 mg/mL) and 62.5 mM sodium carbonate/bicarbonate buffer pH 10.2. Changes in absorbance were recorded at 480 nm. CAT activity was assayed by measuring the initial rate of H_2O_2 disappearance at 240 nm [16]. The reaction mixture contained 33 mM H_2O_2 in 50 mM phosphate buffer pH 7.0 and CAT activity was calculated using the extinction coefficient of 40 $mM^{-1}\ cm^{-1}$ for H_2O_2. POD activity was measured at 25°C using guaiacol as hydrogen donor. The reaction mixture contained 9 mm guaiacol, 19 mM H_2O_2 in 50 mM phosphate buffer pH 7.0 and 50 μL of enzyme extract in 1 mL final volume. The reaction was initiated by the addition of H_2O_2 and monitored by measuring the increase of absorbance at 470 nm. POD activity was expressed in nmol of guaiacol oxidized per min with a molecular extinction coefficient of 26.2 $mM^{-1}\ cm^{-1}$ for calculation [17].

NO metabolites assessment

Tissue, plasma and urine NO were measured by quantification of NO metabolites nitrite and nitrate, determined colorimetrically using a commercially available kit from Roche Diagnostics France, according to Green et al. [18].

Iron measurement

Tissue and plasma non-haem iron were measured colorimetrically using ferrozine as described [19].

Protein determination

Tissue, plasma or urine protein concentrations were determined according to Hartree [20], which is a slight modification of the Lowry method using serum albumin as standard.

Statistical analysis

The data were analysed by unpaired Student's *t*-test or one-way analysis of variance (ANOVA) and are expressed as means ± standard error of the mean (SEM). Data are representative of 10 independent experiments carried out in triplicate. Statistical analyses were conducted using GraphPad InStat version 3.0a for MacIntosh (GraphPad Software, San Diego, CA). All statistical tests were two-tailed and a *p*-value of 0.05 or less was considered statistically significant.

Results

Renal function

We studied the effect of resveratrol on LPS-induced renal dysfunction (Figure 1). LPS *per se* increased significantly plasma creatinine (Figure 1A) and urea (Figure 1C) levels. Resveratrol *per se* decreased creatinine (Figure 1A) but had no significant effect on urea (Figure 1C). However resveratrol pre-treatment totally abolished LPS effect on either plasma creatinine or urea till control level. There was a significant decrease in creatinine (Figure 1B) and urea clearance (Figure 1D) in LPS treated animals, which was markedly improved by resveratrol treatment. Endotoxemia generally induced proteinuria. LPS provoked a decrease in plasma protein (Figure 2A), an increase in urine level (Figure 2B) and resveratrol pre-treatment mitigated the LPS-induced proteinuria.

Renal lipoperoxidation and antioxidant enzyme activities

LPS *per se* highly increased MDA level (3-fold) and this effect was completely reversed by resveratrol pre-treatment (see Figure 3). Resveratrol *per se* had no significant effect vs control. We further looked at tissular antioxidant enzymes (Figure 4). LPS treatment significantly decreased SOD (Figure 4A), CAT (Figure 4B) and POD (Figure 4C) activities. Resveratrol *per se* had no significant effect on SOD or CAT activities, but reduced POD activity. When concomitantly administered with LPS, resveratrol abrogated LPS-induced decrease in SOD and CAT activities to near control level. However LPS-induced decrease in POD activity was not reversed by resveratrol treatment.

Plasma, tissue and urine NO levels

We then assessed the NO mediated effect of LPS (Figure 5). LPS *per se* had no significant effect on renal tissue NO level, nor resveratrol nor the combination of the two molecules (Figure 5A). As expected, LPS alone highly increased plasma NO, while resveratrol slightly decreased it. When concomitantly administered with LPS, resveratrol abrogated LPS effect to near control level (Figure 5B). Moreover, urine NO levels followed the same profile as plasma, i.e. increased after LPS, decreased after resveratrol treatment and abrogated to control level when co-treated with both molecules (Figure 5C).

Plasma and tissue iron levels

We further sought whether LPS was able to modulate free labile iron levels. LPS significantly decreased plasma iron while resveratrol alone slightly but significantly increased it (see Figure 6A). When concomitantly administered with LPS, resveratrol reversed LPS effect to near control level. LPS alone significantly increased tissue iron level while resveratrol *per se* decreased it. Moreover, resveratrol pre-treatment reversed LPS-induced increase in tissue iron to near control level (see Figure 6B).

Discussion

The present investigation reveals that acute administration of LPS (8 mg/kg *i.p.* for 24 h) resulted in an overt nephrotoxicity, as evidenced by the marked elevation of plasma urea and creatinine and decrease in both clearances. Nephrotoxicity was also reflected by proteinuria, as evidenced by a decrease in plasma and a concomitant increase in urine proteins. As expected too, LPS induced renal dysfunction by alteration of the diuretic process resulting in polyuria (data not shown). In the kidney nephrotoxicity was assessed by significant elevation of lipid peroxidation level and depletion of CAT, SOD and POD activities. No evidence of LPS-induced renal tissue damage was obtained from histological study (not illustrated), which is in disagreement with a previous report [21]. Such discrepancy might be explained by differential LPS serotype used, i.e. commercial *E. coli* 055 B5 serotype in the present study and extracted *E. coli* 0157 H7 serotype in Kanter et al. [21]. LPS-induced

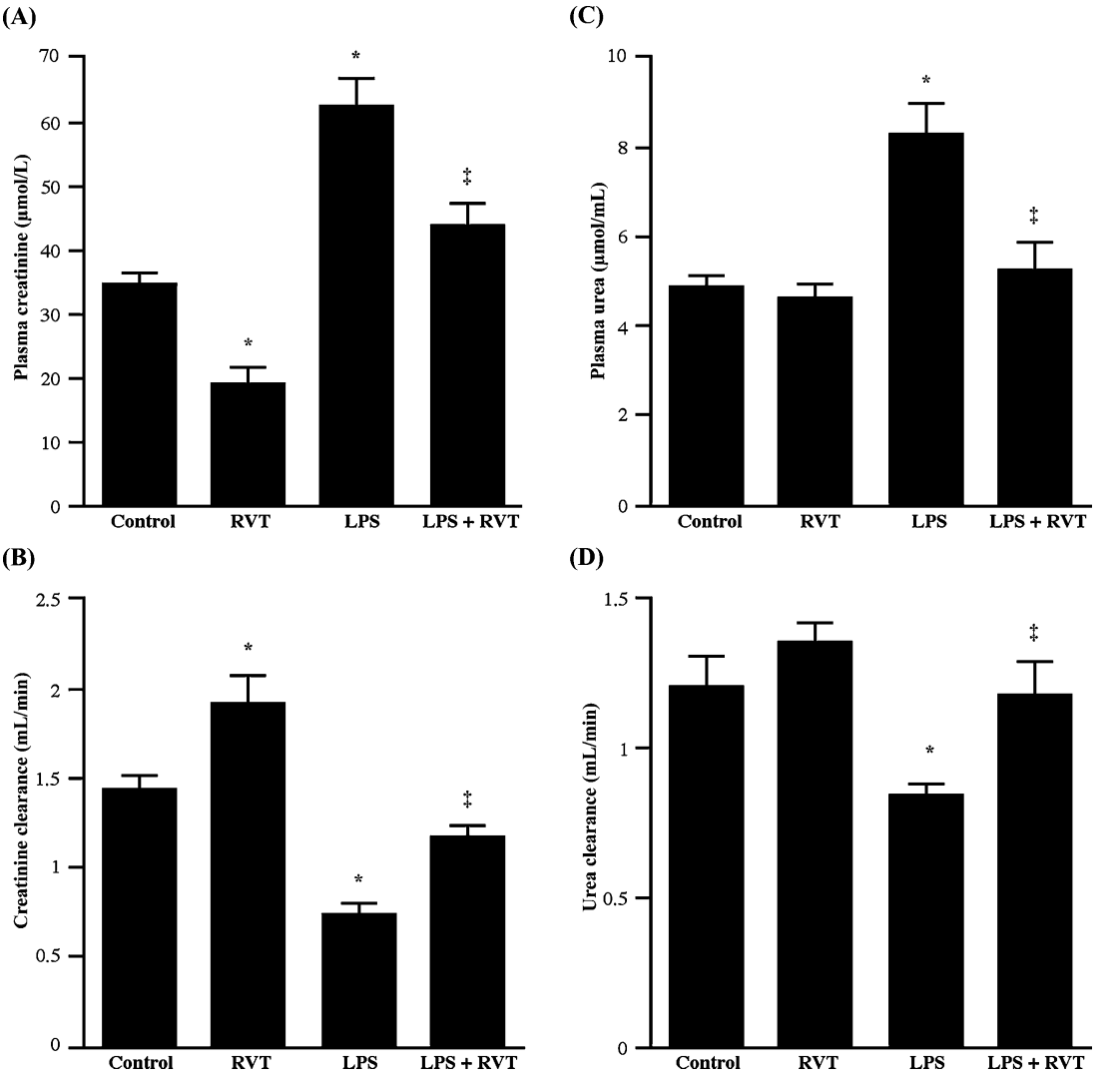

Figure 1. Effect of resveratrol pre-treatment (20 mg/kg *b.w.* 7 days) on endotoxemia-induced changes in plasma creatinine (A), creatinine clearance (B), urea (C) and urea clearance (D). LPS (8 mg/kg *b.w.*) was administered by a single injection. * $p < 0.05$ compared to control group and ‡ $p < 0.05$ compared to LPS group.

renal dysfunction was further assessed by increased tissue iron accumulation, concomitant depletion from plasma, marked elevation in plasma NO and no significant effect in tissue level.

Importantly, our data showed that sub-acute (7 days) treatment with resveratrol (20 mg/kg *b.w.*) abolished almost all parameters of LPS-induced renal dysfunction. Our data fully corroborated several previous reports in the field, demonstrating resveratrol protective and healing effects on nephrotoxicity induced by damaging molecules as gentamicin [22–24], cyclosporine [13], cisplatin [25], glycerol [26], $KBrO_3$ [27] or after ischemia/reperfusion injury [12,28,29]. We also found that resveratrol pre-treatment counteracted LPS-induced body temperature elevation and diarrhea (data not shown) and it corrected LPS-induced nephrotoxicity by reversal of renal haemodynamic markers as plasma creatinine, urea, their clearances and proteinuria. Resveratrol prevented the rise in tissue lipid peroxides and greatly ameliorated CAT and SOD activities, but curiously not POD activity. Previous studies have demonstrated the antioxidant ability of resveratrol [30]. This amphipathic molecule is capable of scavenging free radicals as hydroxyl and superoxide anion [10,31] and to induce antioxidant enzyme activities in the brain [32] and kidney [13]. In our present study, resveratrol was unable to up-regulate POD activity. Organ differences as recently described for SOD activity could be evoked [33]. In addition, resveratrol treatment counteracted all LPS effects on plasma, tissue or urine iron (data not shown) distribution. As LPS-induced a decrease in both plasma and urine iron it had no effect on iron excretion. On the other hand, LPS increased renal iron level (and other organs too as liver, heart and brain data not shown), which allow us to reasonably suppose an increased iron mobilization from plasma to kidney. Interestingly this result fits rather well with the LPS-induced pro-oxidant effect, having in mind

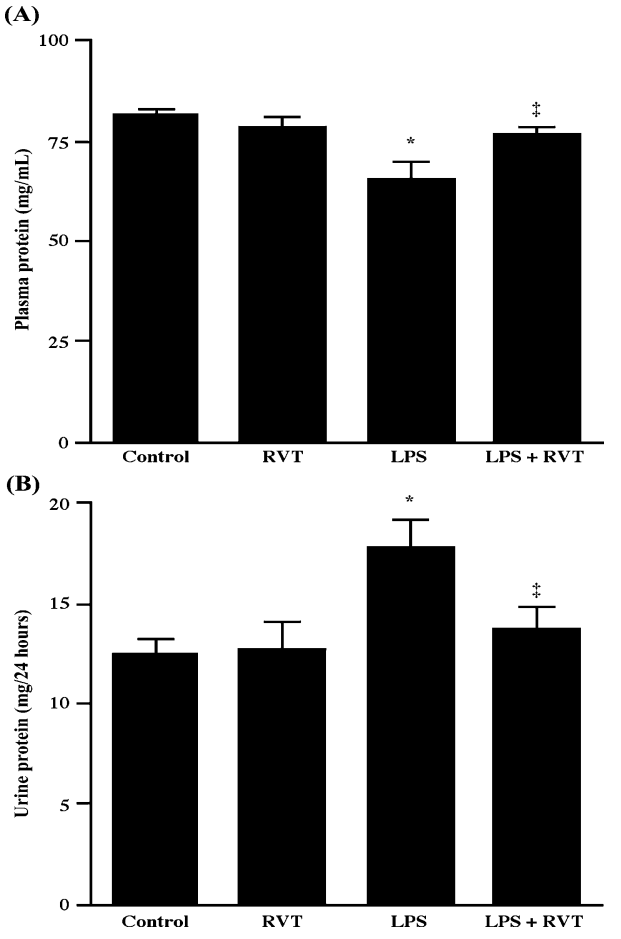

Figure 2. Effect of resveratrol pre-treatment (20 mg/kg *b.w.*7 days) on endotoxemia-induced changes in plasma (A) and urinary (B) proteins. LPS (8 mg/kg *b.w.*) was administered by a single injection. ⋆ $p<0.05$ compared to control group and ‡ $p<0.05$ compared to LPS group.

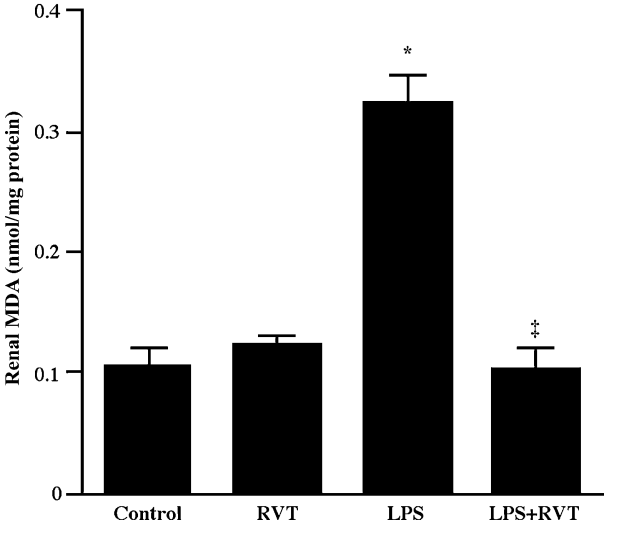

Figure 3. Effect of resveratrol pre-treatment (20 mg/kg *b.w.* 7 days) on endotoxemia-induced changes in kidney MDA level. LPS (8 mg/kg *b.w.*) was administered by a single injection. ⋆ $p<0.05$ compared to control group and ‡ $p<0.05$ compared to LPS group.

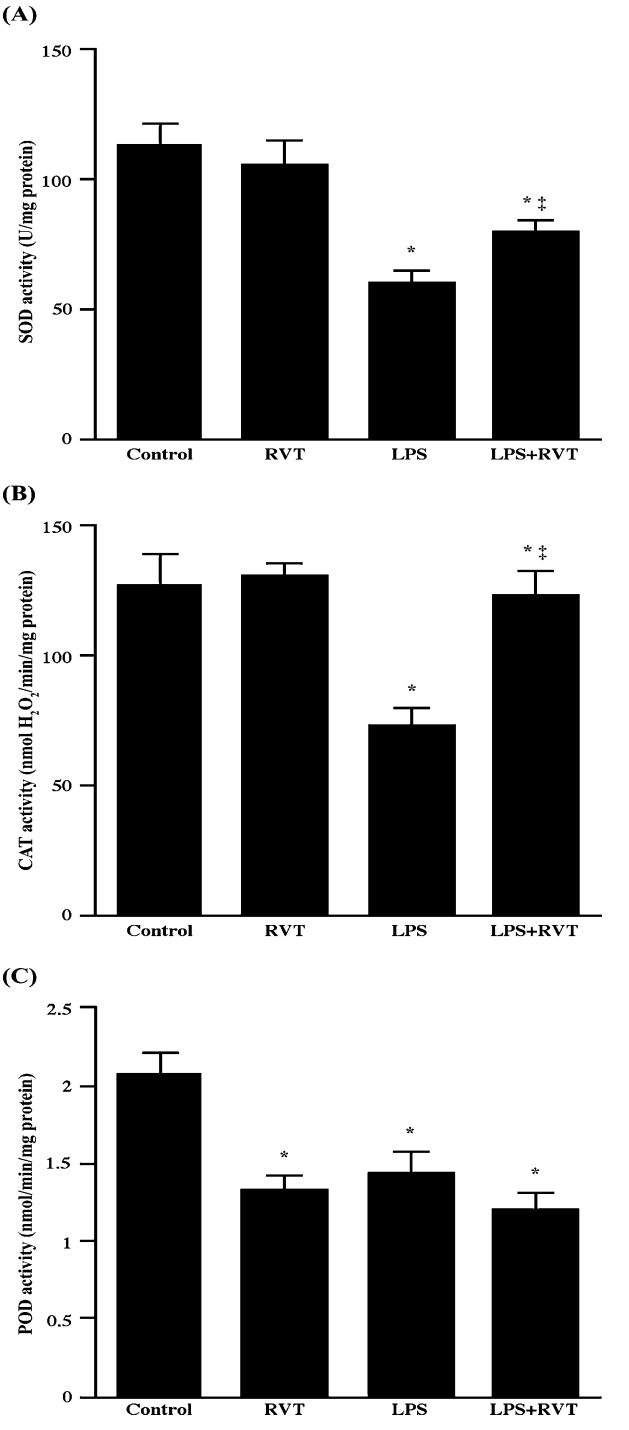

Figure 4. Effect of resveratrol pre-treatment (20 mg/kg *b.w.* 7 days) on endotoxemia-induced changes in kidney antioxidant enzymes SOD (A), CAT (B) and POD (C). LPS (8 mg/kg *b.w.*) was administered by a single injection. ⋆ $p<0.05$ compared to control group and ‡ $p<0.05$ compared to LPS group.

the central role played by iron as a ROS inducing agent [34,35]. LPS seemed to act at least partly by inducing iron overload resulting from intracellular iron sequestration and decreased plasma levels [36]. These effects were recently linked to hepcidin induction and ferroportin reduction [37]. Furthermore, a time-course study (data not shown) showed that LPS-induced tissue iron overload always preceded

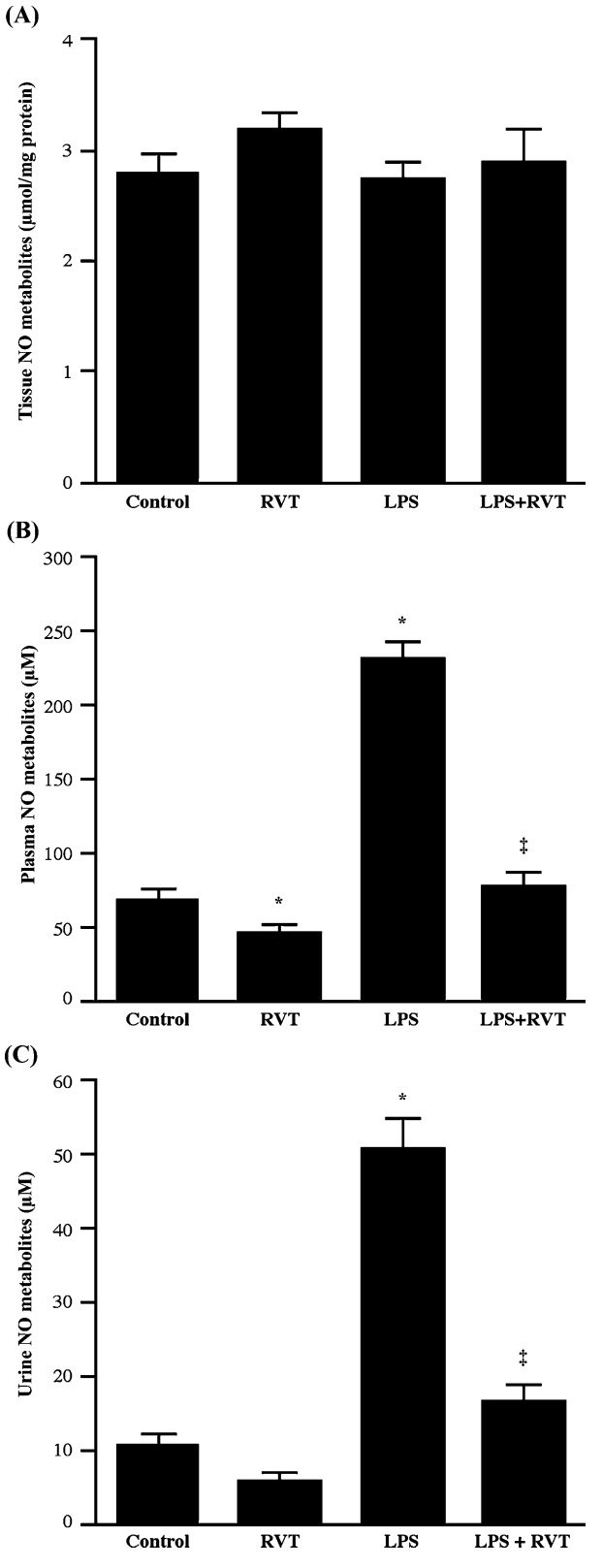

Figure 5. Effect of resveratrol pre-treatment (20 mg/kg *b.w.* 7 days) on endotoxemia-induced changes in tissue (A), plasma (B) and urine (C) NO metabolites levels. LPS (8 mg/kg *b.w.*) was administered by a single injection. * $p < 0.05$ compared to control group and ‡ $p < 0.05$ compared to LPS group.

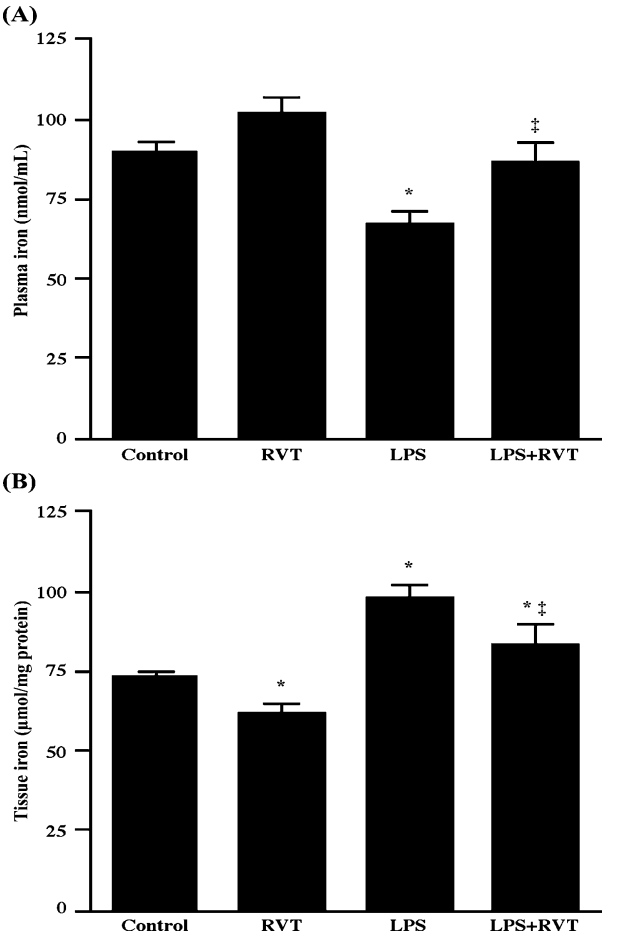

Figure 6. Effect of resveratrol pre-treatment (20 mg/kg *b.w.* 7 days) on endotoxemia-induced changes in plasma (A) and tissue (B) iron level. LPS (8 mg/kg *b.w.*) was administered by a single injection. * $p < 0.05$ compared to control group and ‡ $p < 0.05$ compared to LPS group.

lipoperoxidation. As resveratrol *per se* slightly increased plasma and decreased tissue iron without interfering with urine level, the polyphenol can either increase iron absorption from intestine and/or increase its extrusion from tissues as the kidney. We speculate that in the present endotoxemia model, resveratrol acts on the highly complex iron network in preventing LPS-induced tissular iron overload by modulation of proteins implicated in the maintenance of proper labile iron and homeostasis [38]. More specifically, we suggest resveratrol acting either on lipocalin 2 and its cell surface receptor complex involved in labile iron shuttling and apoptosis or on the acute phase protein hepcidin involved in iron homeostasis [39]. It is noteworthy that acute endotoxemia was recently shown to up-regulate lipocalin 2 in lung and liver [40] and hepcidin in liver and myeloid cells [37]. Thus, by its ability to modulate iron shuttling, resveratrol could represent a promising

alternative, being therapeutic in several iron metabolism disorders and even iron defects linked to cancers [19,41]. Interestingly, malignant progression of the oesophageal carcinoma has recently been shown to be associated with over-expression of cellular iron import proteins [42].

As expected, LPS effect is accompanied by an elevation in plasma NO [43] while resveratrol mode of action seemed independent from NO, as recently shown in activated microglia [44] or in ischemic heart [45]. Moreover, resveratrol totally abrogated LPS-induced plasma and urine NO elevation and to a lesser extent tissue NO level. Our data support the putative use of resveratrol as a NO synthase (NOS) inhibitor and in a therapeutic approach for the treatment of endotoxin-induced sepsis, particularly renal dysfunction (reviewed in Hobbs et al. [46]). However, further work is needed to assess which type of NOS might be the resveratrol target, as was the case for the selective iNOS inhibitor aminoguanidine on LPS-induced reduction in plasma NO [47].

Resveratrol dosage used in the present study (20 mg/kg *b.w.*) appeared non-toxic [48], as it had no deleterious effect on either weight loss, transaminases nor lactate deshydrogenase release (data not shown). Moreover it is yet far less from the recently proposed optimal concentration of 300 mg/kg *b.w.* [49].

In conclusion, resveratrol should be envisaged as a healing agent in endotoxemia-induced nephrotoxicity due to its high efficiency and low toxicity. Further work should investigate the involvement of iron shuttling proteins in resveratrol mode of action.

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