ORGAN TOXICITY AND MECHANISMS

Protective effect of resveratrol in endotoxemia-induced acute phase response in rats

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Abstract Lipopolysaccharide (LPS), a glycolipid component of the cell wall of gram-negative bacteria can elicit a systemic inflammatory process leading to septic shock and death. Acute phase response is characterized by fever, leucocytosis, thrombocytopenia, altered metabolic responses and redox balance by inducing excessive reactive oxygen species (ROS) generation. Resveratrol (*trans*-3,5,4′ trihydroxystilbene) is a natural polyphenol exhibiting antioxidant and anti-inflammatory properties. We investigated the protective effect of resveratrol on endotoxemia-induced acute phase response in rats. When acutely administered by i.p. route, resveratrol (40 mg/kg b.w.) counteracted the effect of a single injection of LPS (4 mg/kg b.w.) which induced fever, a decrease in white blood cells (WBC) and platelets (PLT) counts. When i.p. administered during 7 days at 20 mg/kg per day (subacute treatment), resveratrol abrogated LPS-induced erythrocytes lipoperoxidation and catalase (CAT) activity depression to control levels. In the plasma compartment, LPS increased malondialdehyde (MDA) via nitric monoxide (NO) elevation and decreased iron level. All these deleterious LPS effects were reversed by a subacute resveratrol pre-treatment via a NO independent way. Resveratrol exhibited potent protective effect on LPS-induced acute phase response in rats.

Abbreviations

CAT	Catalase
LPS	Lipopolysaccharide
MDA	Malondialdehyde
NO	Nitric oxide
NOS	Nitric oxide synthase
PLT	Platelets
ROS	Reactive oxygen species
RVT	Resveratrol
WBC	White blood cells

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Introduction

The endotoxin lipopolysaccharide is a major glycolipid component of the cell wall of gram-negative bacteria. During infection, released endotoxin acts as a potent signaling molecule to elicit a systemic inflammatory process initiated by an acute phase response and eventually leading to sepsis and septic shock, which are the most causes of morbidity and mortality in intensive care units (Westphal et al. 2003). Acute inflammatory response is characterized by fever, leucocytosis, thrombocytopenia, changes in vascular permeability, altered metabolic responses, and organs dysfunction (Thiemermann et al. 1995). LPS also altered redox balance by inducing the generation of ROS, particularly NO (Kitajima et al. 1995). These in turn induce lipid

peroxidation and antioxidant enzymes inhibition, which are the basis of many toxicological and pathological processes (Ikeda et al. 2004). The therapeutic use of antioxidants to protect against cellular damage produced by ROS during infection may provide a pharmacological tool for interfering with these acute inflammatory processes and ameliorating the clinical manifestations of septic shock (Cadenas and Cadenas 2002).

Resveratrol (*trans*-3,5,4′ trihydroxystilbene), a naturally occurring phenolic phytoalexin abundantly found in grapes and red wine, possesses diverse biochemical and physiological actions including estrogenic and chemopreventive properties (Soleas et al. 1997). This polyphenol was shown to exhibit a wide range of beneficial health effects as cardioprotective (Das et al. 1999), renoprotective (Giovannini et al. 2001) or neuroprotective (Bastianetto et al. 2000). Resveratrol also exhibited anti-inflammatory properties partly by its antioxidant effect as assessed by decreased lipid peroxidation evaluated by MDA index. Moreover these resveratrol effects were shown in some cases to be mediated by NO (Chander et al. 2005; Hung et al. 2001). In the present work, we aimed to investigate the putative effect of resveratrol on the acute phase inflammatory process in experimentally-induced endotoxemia in rats.

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

Male Wistar rats (200–240 g) from Pasteur Institute of Tunis were used in these experiments in accordance with the local ethic 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 animal house at controlled temperature ($22 \pm 2°C$) with a 12 h light–dark cycle. In acute experiments, rats were divided into three groups of ten animals each: control, LPS and LPS + resveratrol. Resveratrol (40 mg/kg b.w.) and LPS (4 mg/kg b.w.) were simultaneously administered by intraperitoneal (i.p.) injection for 24 h. In subacute experiments, animals were divided into four groups: control, resveratrol, LPS, LPS + resveratrol. They were daily injected i.p. during 7 days either with vehicle (control 5% ethanol) or with 20 mg/kg b.w. resveratrol prepared as a stock solution of 20 mg/ml in 5% ethanol

(injected volume was 1 ml/kg b.w.). Twenty-four hours after the last resveratrol injection, endotoxemia was induced by single i.p. injection of LPS (4 mg/kg b.w.) for 24 h while control animals received vehicle (NaCl 9‰). Animals were regularly monitored for fever by measuring rectal temperature. In acute experiments, blood was collected by ocular ponction at different times after endotoxemia (3, 6, 12, 24 and 48 h) for blood cells count. In subacute experiments, blood was collected by decapitation for the determination of plasma and erythrocytes antioxidant parameters. Erythrocytes were isolated by gentle centrifugation (2,000g, 15 min at 4°C) resuspended in phosphate buffer pH 7.4, lysed with a hypotonic solution consisting of 20 mM Tris–HCl pH 7.2. After a second centrifugation at 20,000g during 40 min at 4°C, supernatant containing erythrocyte lysates was used for MDA and CAT activity determination.

Rectal temperature measurement

Rectal temperature was assessed with a digital thermometer (Typ91.04.01 model Testwert 31, 89 bis).

Lipoperoxidation measurement

Lipid peroxidation was determined by malondialdehyde (MDA) measurement according to the double heating method (Draper and Hadley 1990). Briefly aliquots from erythrocytes lysate or plasma were mixed with BHT-TCA solution containing 1% BHT (w/v) dissolved in 20% TCA (w/v) and centrifuged at 1,000g 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 using an extinction coefficient for MDA–TBA complex of $1.56 \times 10^5 \, M^{-1} \, cm^{-1}$.

CAT activity assay

CAT activity was assayed by measuring the initial rate of H_2O_2 disappearance at 240 nm (Aebi 1984). 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.

NO metabolites assessment

Plasma NO was 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. (1982).

Iron measurement

Plasma non haem iron was measured colorimetrically using ferrozine as described (Leardi et al. 1998).

Protein determination

Protein concentration was determined according to Hartree (1972), which is a slight modification of the Lowry method. Serum albumin was used as standard.

Statistics

Data expressed as mean ± standard error of the mean (SEM) were analyzed by unpaired Student's *t* test or one-way analysis of variance (ANOVA). Assays were done in triplicate. Statistical analyses were conducted using Graph-Pad InStat version 3.0a for MacIntosh (GraphPad Software, San Diego, CA, USA). All statistical tests were two-tailed, and a *P* value of 0.05 or less was considered significant.

Results

Rectal temperature measurement

From Fig. 1, one can see that acute administration of LPS (4 mg/kg b.w.) induced a rapid rise in body temperature, which culminated at 6–12 h after injection. Acute resveratrol treatment highly attenuated the LPS-induced increase in temperature occurring at 3 and 6 h and completely abolished it at 12 and 24 h.

Hematological parameters determination

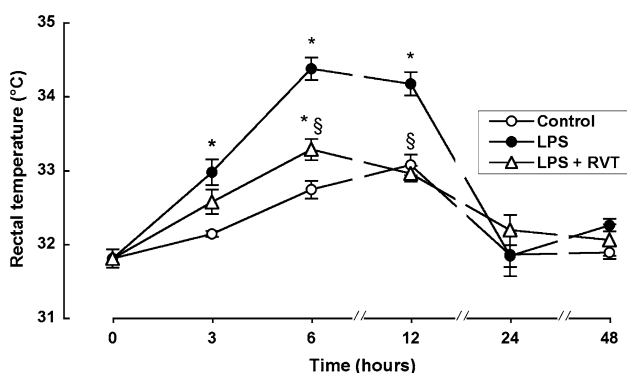We reported in Fig. 2a the effect of an acute injection of LPS (4 mg/kg b.w.) on WBC count versus time. Data

Fig. 1 Acute resveratrol effect on endotoxemia-induced fever in rats. Animals were treated either with resveratrol (40 mg/kg b.w., i.p.) or with vehicle (5% ethanol) and challenged with a single dose of LPS (4 mg/kg b.w., i.p.). Rectal temperature was monitored each 3 h untill 48 h. * *P* < 0.05 versus control and § *P* < 0.05 versus LPS group
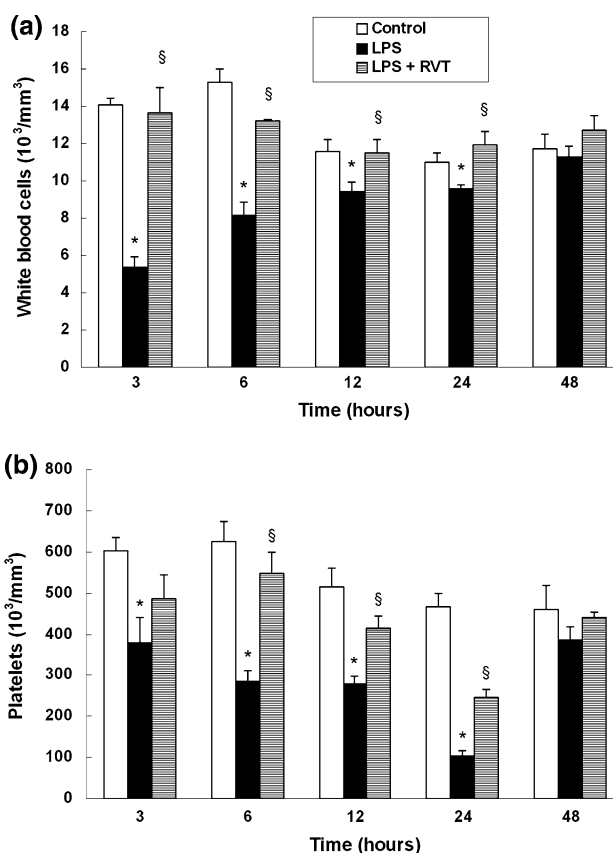
Fig. 2 Acute resveratrol effect on endotoxemia-induced changes in WBC (**a**) and PLT (**b**) counts. Animals were treated with resveratrol (40 mg/kg b.w., i.p.) or vehicle (5% ethanol) and challenged with single dose of LPS (4 mg/kg b.w., i.p.). At each time indicated, blood was collected and WBC and PLTs were counted. * *P* < 0.05 versus control and § *P* < 0.05 versus LPS group

clearly showed a rapid decrease in circulating leucocytes (as soon as 3 h after injection), which progressively returned to control values at 48 h. Resveratrol (40 mg/kg per day b.w.) co-treatment abolished all LPS effects, especially the high decrease in WBC observed at 3 h. Figure 2b dealt with the effect of LPS on platelets count. LPS also induced a time-dependent decrease in platelets as seen for WBC but with a different time-course, i.e., the highest decrease in PLTs occurred at 24 h and returned to near basal values after 48 h. Resveratrol co-treatment counteracted LPS effect on PLTs number although its effect appeared more delayed than for WBC, i.e., significantly protective at 6 h (Fig. 2).

Erythrocytes lipoperoxidation and CAT activity determination

We further sought to determine the effect of a single injection of LPS on erythrocytes redox balance. Figure 3a showed the MDA erythrocytes content of rats subacutely
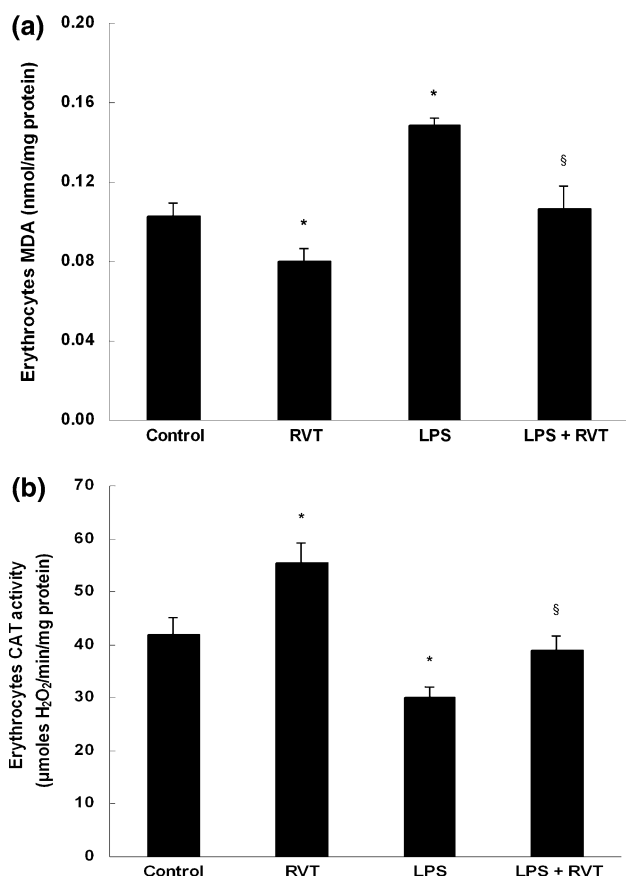
Fig. 3 Subacute effect of resveratrol on endotoxemia-induced changes in erythrocytes MDA (**a**) and CAT activity (**b**). Animals were pre-treated during 7 days with resveratrol (20 mg/kg b.w., i.p.) or vehicle (5% ethanol) and challenged with a single i.p. injection of LPS (4 mg/kg b.w.) or vehicle (NaCl 9‰) for 24 h. Erythrocytes were then collected and processed for MDA content and CAT activity. Assays were carried out in triplicate.* $P < 0.05$ versus control and § $P < 0.05$ versus LPS group

treated with resveratrol (20 mg/kg per day b.w.) and challenged with LPS. Resveratrol per se significantly decreased MDA level while LPS per se increased it. Subacute pre-treatment (7 days) with resveratrol abolished LPS deleterious effect on MDA level till control values. Figure 3b dealt with the erythrocyte CAT activity from the same experiment. Resveratrol alone increased CAT activity and LPS decreased it; resveratrol pre-treatment counteracted LPS effect and restored CAT activity to near basal values.

Plasma MDA, NO and iron measurement

We reported in Fig. 4 the effect of LPS treatment on plasma MDA Fig. 4a, NO Fig. 4b and iron Fig. 4c levels in rats, subacutely pre-treated or not with 20 mg/kg per day polyphenol. Resveratrol per se slightly decreased plasma MDA and LPS per se had an opposite effect although the combi-
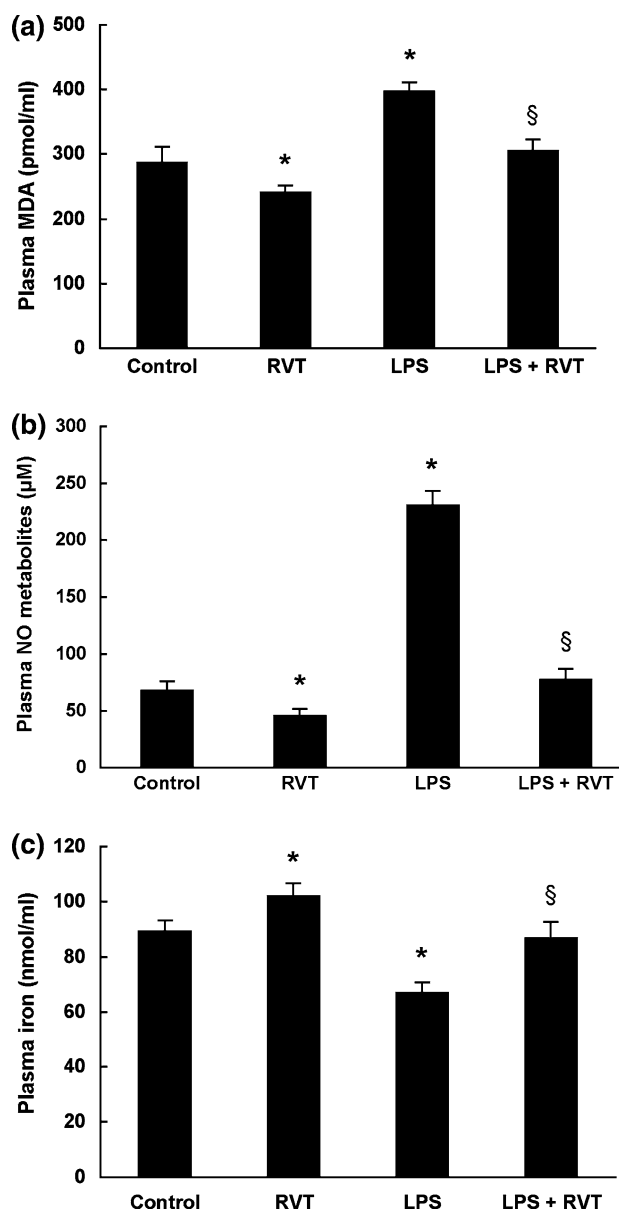

Fig. 4 Subacute effect of resveratrol on endotoxemia-induced changes in plasma MDA (**a**), NO (**b**) and iron (**c**). Animals were pre-treated during 7 days with resveratrol (20 mg/kg b.w., i.p.) or vehicle (5% ethanol) and challenged with a single i.p. injection of LPS (4 mg/kg b.w.) or vehicle (NaCl 9‰) for 24 h. Plasma was used for MDA, NO and iron determinations. Assays were carried out in triplicate. * $P < 0.05$ versus control and § $P < 0.05$ versus LPS group

nation of the two molecules restored MDA level to basal values (Fig. 4a). As expected, resveratrol per se decreased plasma NO level while LPS per se highly increased it. Resveratrol pre-treatment abolished LPS-induced increase in plasma NO to control levels (Fig. 4b). Resveratrol per se significantly increased iron level versus control while LPS significantly decreased it. Subacute treatment with resveratrol restored iron basal levels.

Discussion

In the present investigation, acute administration of LPS to healthy rats resulted in a multifaceted and already well established acute phase response characterized by fever, reduction in the number of circulating WBC and PLTs and increase in oxidative status (Baumann and Gauldie 1994). We also showed that at least for some parameters, LPS-induced sepsis followed a differential kinetic, i.e., fever culminating 6 h after infection although WBC and PLTs counts were mostly affected at 3 and 24 h, respectively, which fully corroborated previous work (Kitajima et al. 1995). LPS had no effect on erythrocytes number or Hb content nor Ht (data not shown), which is also in agreement with previous studies (Kitajima et al. 1995). However, LPS altered erythrocytes redox balance by inducing lipoperoxidation as assessed by increased MDA and reduced CAT activity corroborating previous data observed for other cell types as macrophages (Halliwell and Gutteridge 1999). LPS-induced an increase in plasma MDA, which could be assimilated at a first glance as cumulative MDA from all LPS responsive organs. Furthermore LPS clearly increased plasma NO and decreased plasma iron. Overall these data confirmed the immunomodulatory role of LPS (Cadenas and Cadenas 2002; Freudenberg and Galanos 1990). They further showed that erythrocytes, which responded to LPS, express receptors to pathogen-associated molecular patterns (PAMPs), which could suggest their implication in host defense mechanism acting as circulating pathogen sentinels to initially alert cells of the innate immune system as recently suggested for PLTs (Aslam et al. 2006).

Importantly, our data also showed that resveratrol abrogated almost all LPS-induced deleterious effects. However, protection offered by resveratrol was partial while the polyphenol was administered as a single dose and was more efficient when subacutely administered during 7 days. Differential level of resveratrol reached in the blood might explain this discrepancy (Juan et al. 1999). Noteworthy that quercetin, a structurally related polyphenol, was recently shown to protect human leucocytes from oxidative damage caused by H_2O_2 (Wilms et al. 2008) but was without effect on LPS-induced fever in rats (Kanashiro et al. 2008). This last result outlined the efficiency of resveratrol as antioxidant and antipyrogenic polyphenol and opened the way to future studies aimed to explore which cytokines and regulatory way is involved in the anti-inflammatory activity of resveratrol (Zheng et al. 1995). We also found that when subacutely administered (7 days) resveratrol reversed LPS-induced erythrocytes lipoperoxidation and CAT activity inhibition. These results are in agreement with previous studies demonstrating antioxidant and protective properties of resveratrol on several cell types including platelets (Olas and Wachowicz 2002). Resveratrol exhibited a similar antioxidant effect on erythrocytes than described for a cocktail of vitamin A and vitamin C by Kanter et al. (2005). However in this latter case, no antioxidant enzyme activity was studied, although in our present work, not only resveratrol per se was able to up-regulate erythrocyte CAT activity as recently found in whole brain (Mokni et al. 2007a) but also to alleviate LPS depression in CAT activity. Similar protective effect of resveratrol on cyclosporine-induced renal damage especially on CAT activity depression has recently been described (Chander et al. 2005).

In the plasma compartment, resveratrol exerted potent antioxidant and protective properties against LPS-induced oxidative stress. In fact, when comparing to plasma NO and MDA, LPS and resveratrol had just opposite effects. As a confirmation, LPS mode of action is NO mediated (Vallance and Moncada 1993). It is now well recognized that resveratrol effects are independent of NO (Bi et al. 2005; Mokni et al. 2007b) and the present work further confirmed these results. 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 (Hobbs et al. 1999). However further work is needed to assess which type of NOS is the resveratrol target as it was the case for the selective iNOS inhibitor aminoguanidine on LPS-induced reduction in plasma NO (Tunctan et al. 1998).

Moreover, resveratrol counteracted LPS depressive effect on plasma iron level. LPS-induced decrease in plasma iron did not correspond to increased excretion of iron into urines (data not shown) but rather to an increased iron accumulation in tissues as kidney, liver, heart and brain (data not shown). As resveratrol per se slightly increased plasma and decreased tissue iron without modifying the urine level (data not shown), the polyphenol can either increase iron absorption from intestine and/or increase its extrusion from tissues. It is tempting to speculate that LPS acts by inducing iron overload from plasma to tissue compartments and that resveratrol prevents such effects. In accordance with such data, LPS was recently shown to regulate lipocalin 2 in lung and liver (Sunil et al. 2007) and hepcidin in macrophages (Ganz 2005), two acute phase proteins implicated in tissue iron retention. Moreover in our present study, LPS-induced plasma iron depletion was observed within 3 h after LPS administration (time-course study, not shown) as it was recently the case for LPS-induced hepcidin mRNA expression (Theurl et al. 2008). Moreover, lactoferrin another iron binding protein exerted protective effects against LPS-induced sepsis in mice by modulating inflammatory mediators such as TNFα and NO release into circulation (Kruzel et al. 2002). These effects were demonstrated in prophylactic and therapeutic protocols but are still mechanistically unresolved.

In conclusion, resveratrol should be envisaged as a preventing and healing natural compound in endotoxemia-induced sepsis due to its high efficiency and low toxicity.

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