A Polysaccharide from Tomato (*Lycopersicon esculentum*) Peels Affects NF-κB Activation in LPS-Stimulated J774 Macrophages

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We investigated the effect of PS (**1**) on nitrite and reactive oxygen species (ROS) production in J774 macrophages stimulated with bacterial lipopolysaccharide (LPS) for 24 h. PS (**1**) inhibited in a concentration-dependent manner nitrite and ROS production as well as inducible nitric oxide synthase (iNOS) protein expression induced by LPS. Incubation of cells with PS (**1**) determined a significant decrease of nuclear factor-κB (NF-κB)/DNA binding activity which was correlated with a marked reduction of iNOS mRNA levels. These results show that PS (**1**) inhibits NF-κB activation and iNOS gene expression by preventing the reactive species production and suggest a role for this compound in controlling oxidative stress and/or inflammation.

Plant extracts are an attractive source of additives for the food and drug industry. Most plant extracts contain important vitamins, flavonoids, and polyphenols that are of great interest for use in complementary medicine supplements.[1] However, it is intriguing to observe that an emerging class of bioactive compounds from plant extracts consists of polysaccharides exhibiting a large range of applications in the industrial field.[2] High molecular weight polysaccharides from *Opuntia ficus-indica* accelerate re-epithelization in a model of dermal wound[3] and those from *Salvia chinensis* elicit B lymphocytes *in vivo*.[4] It has been reported that plant polysaccharides possess mainly antioxidant activities *in vitro*.[5] In addition, agaro-oligosaccharides can exert *in vitro* and *in vivo* hepatoprotective effects by scavenging oxidative damage induced by ROS.[6,7] Free radicals are formed in pathological events, such as oxidative stress during inflammatory processes, which are often correlated with increased levels of iNOS-derived nitric oxide.[8] Nitrite and nitrate, nitric oxide stable metabolites, can react with superoxide radical, thus generating peroxynitrites which, in turn, may activate NF-κB.[9] NF-κB is a member of the *Rel* family proteins and is typically a heterodimer of p50 and p65 subunits. In quiescent cells, NF-κB resides in the cytosol in latent form bound to inhibitory proteins, IκBs. Different stimuli trigger signal pathway activating several redox-sensitive kinases which phosphorylate IκB resulting in its degradation. NF-κB, free to translocate to nucleus, stimulates the transcription of target genes such as iNOS.[9] A recent finding reports that polysaccharides from *Saururus chinensis* inhibit iNOS mRNA expression in LPS-stimulated RAW 264.7 macrophages by blocking NF-κB activation.[10] Tomato is an important worldwide fruit crop whose ingestion has been recognized to confer antioxidant and chemopreventive properties, always correlated to lycopene.[11] Recently, we have isolated and characterized, from solid wastes of the tomato-processing industry, a new polysaccharide PS (**1**).[12] In the present study we have focused our attention on PS (**1**), demonstrating, for the first time, its ability to prevent NF-κB activation.

We obtained 1.5 g of lyophilized PS (**1**) with high carbohydrate content and 20% of uronic acids from 20 g of lyophilized biomass. The biopolymer showing a high molecular weight (>1000000 Da) possessed a negative specific rotation ($[\alpha]^{25}_{D} = -0.189$). After hydrolysis (2 M TFA), the sugar components were identified by both TLC and HPAE-PAD Dionex.[12] The polymer presented a glucoxylan structure. The rheological properties were further characterized by studying the specific viscosity η of the PS (**1**) fraction. The viscosity did not change drastically with respect to increasing pH, and the maximum value of viscosity was obtained at pH 3.0 ($\eta = 3.290$). PS (**1**) (10 mg) was degraded at 250 °C in 20 min, leaving a residue of about 5 mg.

In order to clarify whether PS (**1**) was capable of blocking the oxidant species induced by LPS in J774 macrophages, we evaluated nitrite and intracellular ROS production. Stimulation of cells with LPS (10 μg/mL) for 24 h caused an accumulation of nitrite in the medium, compared with unstimulated cells. Incubation of cells with PS (**1**) (10, 1, and 0.1 μg/mL) or PDTC (10 μM) inhibited in a significant and concentration-dependent manner the nitrite (57.6 ± 2.1%, 41.6 ± 2.9% and 3.14 ± 2.7%, 69.3 ± 3.5%, respectively; $n = 4$ experiments in triplicate) and ROS production (Figure 1A). Exposure of J774 macrophages to LPS for 24 h also resulted in an increased intracellular ROS production compared with unstimulated cells. Addition of PS (**1**) (1 μg/mL) as well as pyrrolidine dithiocarbamate (PDTC) (10 μM) to the cells caused a significant reduction of intracellular ROS generation (by 37.8 ± 0.04% and 42.2 ± 0.07%, respectively; $n = 10$) (Figure 1B). PS (**1**) did not affect cell viability (>95% vs untreated cells) and proliferation (>90% vs untreated cells) up to 72 h, evaluated by MTT and crystal violet assays, respectively, and using daunorubicin as a reference antineoplastic drug (data not shown).

Upon stimulation with LPS (10 μg/mL) for 24 h, cells showed high level of iNOS protein expression as compared with untreated cells. Incubation of cells with PS (**1**) or PDTC inhibited iNOS protein expression compared with LPS alone (52.0 ± 0.2% and 60.0 ± 0.3%, respectively; $n = 3$) (Figure 2). In addition, to explore whether the reduced level of iNOS protein observed in cells treated with the PS (**1**) could be attributed to a reduced gene transcription, we analyzed the expression of iNOS mRNA by RT-PCR. Stimulation of cells with LPS (10 μg/mL) led to a significantly higher increase of iNOS mRNA levels. Incubation of cells with PS (**1**) or PDTC significantly reduced iNOS mRNA levels (54.4 ± 0.1% and 63.9 ± 0.3%, respectively; $n = 3$) (Figure 3).

To explore whether the reduced iNOS gene expression by PS (**1**) could be related to NF-κB/DNA binding activity, we performed EMSA experiments. A low basal level of NF-κB/DNA binding activity was detected in nuclear proteins from unstimulated macrophages. Conversely, a retarded band was clearly detected following stimulation with LPS. Treatment of cells with PS (**1**) or PDTC caused a significant reduction of specific protein–DNA

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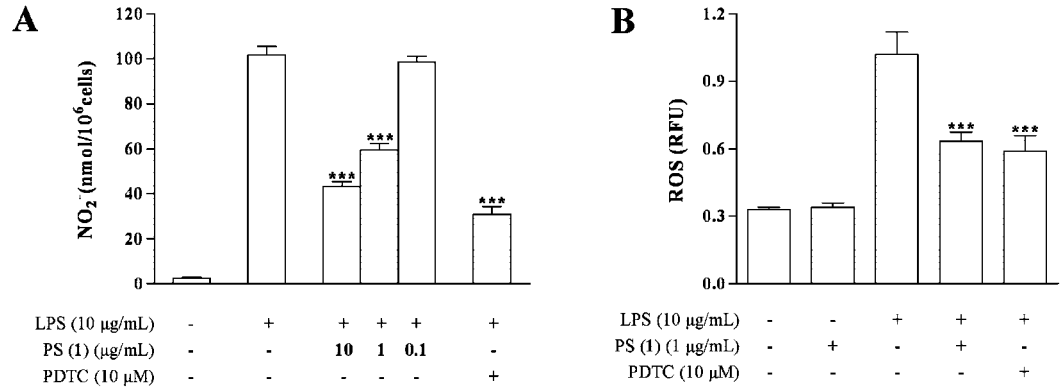

Figure 1. Effects of PS (**1**) or PDTC on nitrite (A) and ROS (B) production in LPS-stimulated J774 macrophages. Data are expressed as mean ± SEM of four experiments in triplicate. (B) Data are expressed as mean ± SEM of 10 experiments in triplicate (RFU = relative fluorescence units). $^{**}P < 0.001$, $^{***}P < 0.0001$ vs LPS.

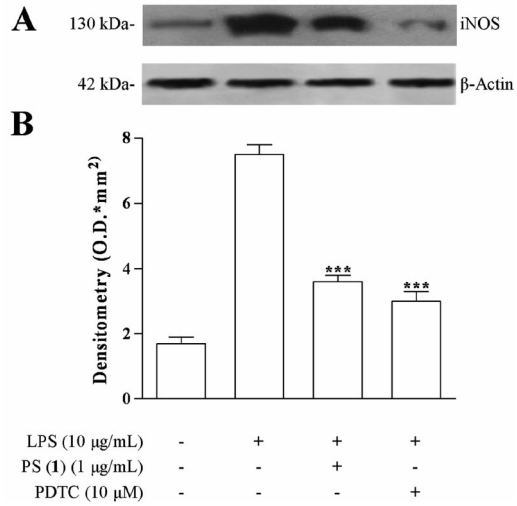

Figure 2. Representative Western blot (A) as well as relative densitometric analysis (B) show the effect of PS (**1**) or PDTC on iNOS protein expression in LPS-stimulated J774 macrophages. β-Actin expression is shown as a control. Data are from a single experiment and representative of five separate experiments. Densitometric data are expressed as mean ± SEM of three experiments. $^{***}P < 0.0001$ vs LPS.

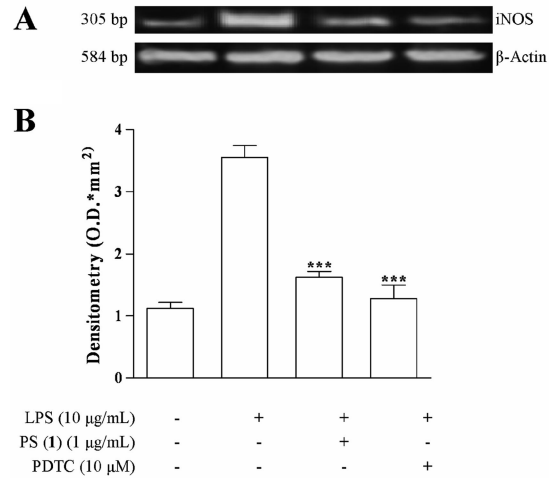

Figure 3. Representative PCR (A) as well as densitometric analysis (B) show the effect of PS (**1**) or PDTC on iNOS mRNA expression in LPS-stimulated J774 macrophages. (A) Data are from a single experiment and representative of three separate experiments. (B) Densitometric data are expressed as mean ± SEM of three separate experiments. $^{***}P < 0.001$ vs LPS. β-actin mRNA levels are reported as a control.

complexes induced by LPS (by 37.5 ± 2.3% and 61.7 ± 1.7%; respectively; $n = 3$) (Figure 4A). The composition of protein–DNA binding complexes was determined by competition and supershift experiments. In response to competition, the specificity of NF-κB/DNA binding complexes was evident by the complete displacement of protein–DNA binding in the presence of a 50-fold molar excess of unlabeled NF-κB probe. In contrast, a 50-fold molar excess of unlabeled mutated NF-κB probe or Sp-1 oligonucleotide had no effect on DNA binding activity. Addition of either anti-p50 and anti-p65 antibodies to the binding reaction clearly gave rise to a characteristic supershift of the retarded band, suggesting that the NF-κB complex contained p50 and p65 dimers (Figure 4C).

In summary, our findings address PS (**1**) as a nontoxic agent for the control of oxidative stress and/or inflammation. In addition, we also would like to suggest an interesting environmental implication of this study. Previously, we have observed that polysaccharidic fractions from tomato waste industry are good candidates to obtain biopolymers with a wide range of industrial applications.[12] It is interesting to observe that one of these polysaccharidic fractions, PS (**1**), may represent a protective and nontoxic class of biopolymers.

Experimental Section

Tomato Wastes. The raw materials (*Lycopersicon esculentum* "Hybrid Rome") have been kindly supplied by "Fontanella S.p.A., Mercato San Severino, Salerno, Italy", a food packaging industry. The wastes were frozen with liquid nitrogen and then lyophilized under vacuum.

Extraction and Chemical Characterization of PS (1). The lyophilized biomass was treated with 5 N KOH under stirring for 3 days and then centrifuged at 10000*g* for 40 min. The supernatant was precipitated with cold EtOH (v/v) at −20 °C overnight. The pellet, after centrifugation, was dissolved in hot H_2O, dialyzed against tap water for 3 days, and dried under vacuum. The lyophilized material was utilized for further analysis performed as previously described.[12–14]

Rheological Properties. The η as functions of concentration of aqueous solutions of polysaccharide were carried out using Cannon-Ubbelohde 75 suspended level viscometers at 30 °C. The rheological properties were characterized by studying the η of sample (1%) in the pH range 2.32–8.36 by using 50 mM citrate–phosphate buffer at 30 °C.

Cell Culture. The mouse monocyte/macrophage cell line J774 was cultured as previously described.[15] The cells were plated in 24 culture wells (2.5×10^5 cells/mL per well) or 10 cm diameter culture dishes (3×10^6 cells/mL per dish) and allowed to adhere for 2 h. The medium was then replaced with fresh medium, and cells were stimulated with LPS (10 μg/mL). PS (**1**) (10, 1, and 0.1 μg/mL) or PDTC (10 μM), a synthetic antioxidant used as a

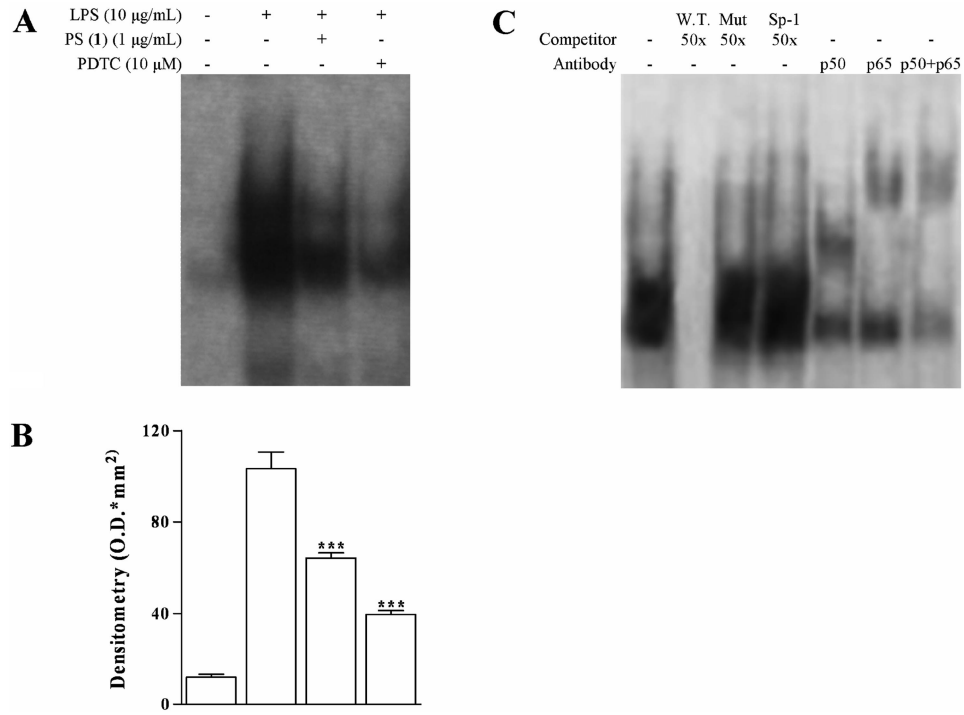

Figure 4. Representative EMSA (A) as well as densitometric analysis (B) show the effects of PS (**1**) and PDTC on NF-κB/DNA binding activity in LPS-stimulated J774 macrophages. (A) Data are from a single experiment and representative of four separate experiments. (B) Densitometric data are expressed as mean ± SEM of three experiments. ***P < 0.0001 vs LPS. (C) In competition reaction nuclear extracts from LPS-stimulated macrophages were incubated with radio-labeled NF-κB probe with W.T. (50×) unlabeled oligonucleotides, Mut. (50×), or unlabeled oligonucleotide sequence for Sp-1 (50×). In supershift experiments nuclear extracts were incubated with anti-p50 or anti-p65 15 min before incubation with radio-labeled NF-κB probe.

reference drug, was added to the cells 10 min before LPS challenge. LPS was dissolved in PBS and stored at −20 °C. Polysaccharide solutions were prepared as needed, dissolved in saline, and sterilized by UV irradiation for 30 min. Cell viability was determined by using 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) conversion assay as previously described.[15] The cell proliferation assay was performed as described elsewhere.[16]

Nitrite and ROS Determination. NO was measured as nitrite (NO_2^-, nmol/10^6 cells) accumulated in the incubation medium after 24 h as previously described.[15] ROS formation was evaluated by means of the probe 2′,7′-dichlorofluorescin diacetate as described elsewhere.[15]

Cytosolic and Nuclear Extracts. Cytosolic and nuclear extracts of macrophages stimulated for 24 h with LPS (10 μg/mL) and incubated in the presence or absence of PS (**1**) (1 μg/mL) or PDTC (10 μM) were prepared as previously described.[15] Protein concentration was determined by the Bio-Rad (Italy) protein assay kit.

Electrophoretic Mobility Shift Assay (EMSA). Double-stranded oligonucleotides containing NF-κB (5′-CAA CGG CAG GGG AAT CTC CCT CTC CTT-3′) recognition sequences were end-labeled with ^{32}P-γ-ATP as previously described.[15]

Western Blot Analysis. Immunoblotting analysis of anti-iNOS was performed on equivalent amounts of cytosolic extract (30 μg) which were electrophoresed in a 12% discontinuous polyacrylamide minigel as previously described.[15]

Reverse Transcription–Polymerase Chain Reaction. J774 cells were plated in a six-well plate (3×10^6/well) and allowed to adhere for 2 h. Thereafter, the cells were treated with PS (**1**) (1 μg/mL) or PDTC (10 μM) and, 10 min after treatment, were stimulated with LPS (10 μg/mL) for 24 h. Total RNA extraction by using TRIzol (Invitrogen, Milan, Italy) was performed as described elsewhere.[15]

Statistics. Results are expressed as the means ± SEM of n experiments. Statistical significance was calculated by one-way analysis of variance (ANOVA) and Bonferroni-corrected P value for multiple comparison test. The level of statistically significant difference was defined as $P < 0.05$.

Reagents. DMEM, foetal bovine serum, glutamine, penicillin, streptomycin, Hepes, sodium pyruvate, and PBS were from BioWhittaker (Caravaggio, BG, Italy). ^{32}P-γ-ATP was from Amersham (Milan, Italy). Poly dI-dC was from Boehringer-Mannheim (Milan, Italy). Anti-p50 and anti-p65 antibodies were from Santa Cruz (Milan, Italy). Oligonucleotide synthesis was performed to our specifications by Tib Molbiol (Boehringer-Mannheim, Genova, Italy). Nonfat dry milk was from Bio-Rad (Milan, Italy). Sybr Safe, MMLV-Reverse Transcriptase, TRIzol, and dNTPs were from Invitrogen (Milan, Italy). DL-Dithiothreitol, pepstatin A, leupeptin, benzamidine, and phenylmethylsulfonil fluoride were from Applichem (Darmstadt, Germany). MTT, LPS from *Salmonella tiphosa* (Code L-6386, protein concentration <3%), crystal violet, and all other reagents were from Sigma (Milan, Italy).

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