

Anti-inflammatory effects of resveratrol occur via inhibition of lipopolysaccharide-induced NF- κ B activation in Caco-2 and SW480 human colon cancer cells

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Abstract

Resveratrol, a polyphenol abundantly found in grapes and red wine, exhibits beneficial health effects due to its anti-inflammatory properties. In the present study, we evaluated the effect of resveratrol on inflammatory responses induced by lipopolysaccharide (LPS) treatment of human intestinal Caco-2 and SW480 cell lines. In the LPS-treated intestinal cells, resveratrol dose-dependently inhibited the expression of inducible NO synthase (iNOS) mRNA as well as protein expression, resulting in a decreased production of NO. In addition, Toll-like receptor-4 expression was significantly diminished in LPS-stimulated cells after resveratrol pre-treatment. To investigate the mechanisms by which resveratrol reduces NO production and iNOS expression, we examined the activation of inhibitor of κ B (I κ B) in LPS-stimulated intestinal cells. Results demonstrated that resveratrol inhibited the phosphorylation, as well as the degradation, of the I κ B complex. Overall, these results show that resveratrol is able to reduce LPS-induced inflammatory responses by intestinal cells, interfering with the activation of NF- κ B-dependent molecular mechanisms.

Key words: Resveratrol: Intestinal cells: Lipopolysaccharide: Nitric oxide: NF- κ B

Resveratrol (3,4,5-trihydroxy-trans-stilbene; Fig. 1) is a natural polyphenol present in a variety of medicinal plants, in grapes and in red wine. Evidence suggests that resveratrol exhibits beneficial pleiotropic health effects, being recognised as one of the most promising natural molecules in the prevention and treatment of chronic inflammatory disease⁽¹⁾. The first evidence of the beneficial effects of resveratrol on human health was revealed by its ability to protect against CHD⁽²⁾ and, more recently, numerous cancer-chemopreventive properties of resveratrol have been demonstrated⁽³⁾. In this respect, resveratrol has been shown to scavenge free radicals and to regulate many enzymes involved in inflammation, such as cyclooxygenase (COX), inducible NO synthase (iNOS), lipoxygenase, protein kinase C and others^(4,5). Resveratrol is able to inhibit lipid peroxidation^(4,6) and to suppress iNOS expression and subsequent NO production in cultured macrophages⁽⁷⁾.

Recently, it was reported that polyphenols may be able to modulate bowel inflammation, thus reducing or delaying the development of inflammatory bowel disease (IBD) in humans⁽⁸⁾. Inflammatory bowel disease is a common disturbance characterised by an uncontrolled reaction of the intestinal immune system against the normal enteric microflora,

causing mucosal damage, abdominal pain and chronic diarrhoea. IBD greatly increases the risk of colon cancer⁽⁹⁾.

The bowel mucosa is an important route of entry for microbial pathogens, since enteric epithelial cells are the initial sites of attack by entero-invasive micro-organisms, including Gram-negative bacteria⁽¹⁰⁾. Immune and non-immune cells are involved in the production of mediators (cytokines, growth factors, adhesion molecules, etc.) which promote and amplify the inflammatory response. Lipopolysaccharide (LPS), the principal component of the outer membrane of Gram-negative bacteria, plays a pivotal role in triggering an early inflammatory response, that constitutes the first mechanism of defence by the host to fight infection⁽¹¹⁾.

The effects of LPS are mediated through the interaction of several receptors for microbial products. Among these, Toll-like receptors (TLR) are a group of transmembrane proteins that function as pattern-recognition receptors for detecting and responding to microbial ligands termed pathogen-associated molecular patterns, present on bacteria, and bacterial products. The best-studied member of this family of receptors is TLR-4, involved in the recognition of endotoxins or bacterial LPS⁽¹²⁾.

Abbreviations: COX-2, cyclo-oxygenase-2; iNOS, inducible nitric oxide synthase; I κ B, inhibitor of κ B; LPS, lipopolysaccharide; MTT, 3,4,5-dimethylthiazol-2-yl-2,5-diphenyltetrazolium bromide; NOS, nitric oxide synthase; TLR-4, Toll-like receptors 4.

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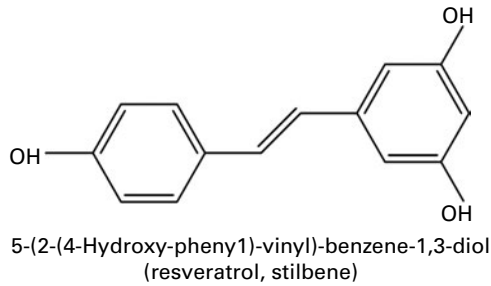


Fig. 1. Structure of resveratrol.

The host response to LPS is characterised by the production of various proinflammatory mediators and microbicidal molecules, including NO^(13,14).

The transcription factor NF- κ B is activated by a variety of stimuli and regulates diverse gene expression and biological responses. NF- κ B, a latent cytoplasmic transcriptional factor complexed with an inhibitor of κ B (I κ B), is composed of relA (p65) and p50 subunits, while I κ B- α , I κ B- β and I κ B- γ are the most abundant inhibitors⁽¹⁵⁾. After stimulation by a variety of agents, such as LPS, I κ B is phosphorylated and degraded; so free NF- κ B translocates into the nucleus to regulate the expression of multiple NF- κ B-dependent genes, such as acute-phase response proteins and inflammatory enzymes, including iNOS^(16,17). The promoter of iNOS contains two consensus NF- κ B binding sites that mediate LPS-inducibility⁽¹⁸⁾.

The purpose of the present study was to investigate the anti-inflammatory properties of resveratrol in two human colon cancer cell lines, Caco-2 and SW480, submitted to LPS treatment as a pro-inflammatory stimulus. These cancer cell lines represent the most commonly used *in vitro* model for studies of structural and functional properties of human differentiated enterocytes. Therefore, we investigated the potential targets of resveratrol in the inflammatory responses of LPS-stimulated intestinal cells, examining NO production, iNOS and TLR-4 expression. The regulation of the signalling NF- κ B activation pathway by resveratrol was also investigated.

Experimental methods

Cell cultures and treatments

The Caco-2 cell line (ICLC HTL 97023-Interlab Cell Line Collection) was grown in minimum essential medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 μ g/ml streptomycin, L-glutamine (2 mM), 1% non-essential amino acids, referred to as complete medium (all reagents were purchased from Life Technologies-Invitrogen).

The human colon adenocarcinoma cell line SW480 (ICLC HTL99017-Interlab Cell Line Collection) was cultured on Leiboviz-15 medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 μ g/ml streptomycin and L-glutamine (2 mM; Life Technologies-Invitrogen).

Cultures were maintained at 37°C in a humidified atmosphere containing 5% CO₂ and expanded in tissue culture flasks (75 cm²; BD Biosciences), changing the medium daily.

The cells were seeded in six-well cell culture plates at 5×10^5 cells/well and cultured to reach 80% confluency.

For the experiments, cells were treated with *Salmonella enterica* serotype *typhimurium* LPS (Sigma). Preliminary experiments were performed in order to establish the optimal dose (1 μ g/ml) of LPS and time of exposure to LPS (48 h). Before LPS stimulation, some wells were pre-treated with different concentrations (30, 40, 50 μ M) of resveratrol (Sigma). After 1 h of incubation at 37°C, cell cultures were then stimulated with LPS as previously indicated. Untreated cells were used as the control.

Cell viability assay

The viability of the cells was assessed by the 3,4,5-dimethylthiazol-2-yl-2,5-diphenyltetrazolium bromide (MTT) assay, which is based on the reduction of MTT by the mitochondrial dehydrogenase of intact cells to a purple formazan product. Cells (2.5×10^4) were seeded in a ninety-six-well plate (BD Biosciences). After the treatment previously described, culture media were carefully removed by aspiration. Following this, 100 μ l of 0.5 mg/ml MTT in cell culture medium were added to each well and incubated for 2 h. Then, 100 μ l of 10% SDS, 0.01 M-HCl solution were added to each well to dissolve the formazan crystals formed. The plates were covered with aluminium foil and kept in an incubator for 12 h for dissolution of the formed formazan crystals. The amount of formazan was determined by measuring the absorbance at 560 nm using a microplate reader.

Nitric oxide production

The stable nitrite (NO₂⁻) concentration, being the end product of NO generation, was determined by the method described by Ding *et al.*⁽¹⁹⁾. Briefly, intestinal cells, cultured as indicated previously, were exposed to LPS for 48 h. At the end of treatment, culture supernatants were collected and incubated (1:1, v/v) with the Griess reagent (1% sulphanilamide, 0.1% N-1-naphthylethylenediamine dihydrochloride and 2.5% phosphoric acid) for 10 min at room temperature. Absorbance was measured at 570 nm after incubation for 10 min. The NO₂⁻ concentration was determined by extrapolation from a NaNO₂ standard curve and expressed as nmol/ml. To avoid interference by nitrites possibly present in the medium, in each experiment the absorbance of the unconditioned medium was assumed as the 'blank'.

Electrophoresis and Western blotting

After treatments, cells were lysed with lysis buffer (1% Triton X-100, 20 mM-Tris-HCl, 137 mM-NaCl, 10% glycerol, 2 mM-EDTA, 1 mM-phenylmethylsulphonyl fluoride, 20 μ M-leupeptin hemisulphate salt, 0.2 U/ml aprotinin (all from Sigma-Aldrich)) for 30 min on ice. The lysate was vortexed for 15–20 s and then centrifuged at 12800 g for 20 min; the protein concentration in the supernatant was spectrophotometrically determined by Bradford's protein assay⁽²⁰⁾. Protein samples were diluted with a sample buffer (0.5 M-Tris-HCl pH 6.8,

10% glycerol, 10% w/v SDS, 5% β 2-mercaptoethanol, 0.05% w/v bromophenol blue) and then boiled for 3 min. Proteins (25 μ g/lane) and pre-stained standards (Bio-Rad Laboratories) were loaded on 7% SDS precast polyacrylamide gels (Bio-Rad Laboratories).

After electrophoresis, the resolved proteins were transferred from the gel to nitrocellulose membranes. A blotting buffer (20 mM-Tris/150 mM-glycine, pH 8, 20% (v/v) methanol) was used for gel and membrane saturation and blotting. A blocking solution (bovine serum albumin, 0.2%–5% (w/v), Tween-20 (0.05–0.1%), non-fat dry milk (0.5–5%), casein (1%), all from Bio-Rad Laboratories) was used in order to prevent non-specific binding of unoccupied membrane sites. Then, the membranes were incubated in the dark with (1:250 diluted) primary antibody (anti-human TLR-4, anti-human NOS II, anti-human $\text{I}\kappa\text{B-}\alpha$, anti-human phosphorylated $\text{I}\kappa\text{B-}\alpha$ (all from Santa Cruz Biotechnology)), for 60 min at room temperature. The membranes were washed with T-PBS (phosphate-buffered saline with Tween 20) (for 20 min, three times) and then incubated with the secondary antibody (anti-human IgG diluted 1:2000, horseradish peroxidase-conjugate (Santa Cruz Biotechnology)) for 60 min. Bands were visualised by the chemiluminescence method (Bio-Rad Laboratories).

RT-PCR

Briefly, total tissue RNA was extracted from treated and untreated cells by the Trizol isolation reagent (Invitrogen) according to the manufacturer's instructions. Reverse transcription was performed in a final volume of 20 μ l containing 3 μ g of total RNA, 40 U of RNase Out (Invitrogen), 40 mU of oligo dT with 0.5 mM-deoxyribonucleotide triphosphate (PCR Nucleotide Mix; Roche Diagnostics) and 40 U of Moloney Murine Leukemia Virus RT (Roche Diagnostics). The reaction tubes were incubated at 37°C for 59 min, and then at 95°C for 5 min and at 4°C for 55 min. Complementary DNA obtained was then amplified by a thermal cycler (Eppendorf) under the following conditions: 95°C for 1 min, 55°C for 1 min and 72°C for 1 min (thirty cycles of amplification). The reaction tube contained, in a final volume of 50 μ l, 2 μ l of complementary DNA, 200 μ M-deoxyribonucleotide triphosphate (PCR Nucleotide Mix; Roche Diagnostics), 4 U TaqDNA Polymerase (Roche Diagnostics), 5 μ l of MgCl_2 buffer stock solution and 50 pmol of the specific primers.

The primers used for amplification were: for iNOS (NCBI reference sequence NM_000625.4) forward primer 5'-CGGCC-ATCACCGTGTTC-3'; reverse primer, 5'-TGCAGTCGAGT-GGTGGTCCA-3'; and for β -actin forward primer, 5'-GGCGGC-ACCACCATGTACCCT-3'; reverse primer, 5'-AGGGGCCGGAC-TCGTCATACT-3'.

Densitometric analysis

The visualised bands obtained after immunoblotting and RT-PCR experiments were submitted to densitometric analysis using 1D image analysis software (Kodak Digital Science). β -Actin was used for normalisation of immunoblotting and

RT-PCR products, respectively. Results were expressed as relative optical measured density.

Microscopic analysis

To investigate the surface expression of TLR-4, intestinal cells were plated onto four-well tissue culture plates stimulated with LPS as indicated. Briefly, 2.5×10^5 cells/cm² adherents on 13 mm diameter coverslips (Celloclate; Eppendorf) were kept at 37°C, 5% CO₂ for 8 d. After three washes with PBS, the cells were fixed with 4% paraformaldehyde for 15 min. After repeated washes, they were blocked for 45 min with goat normal serum (Sigma-Aldrich) at 37°C and incubated with mouse monoclonal anti-human TLR-4 (1:100, Santa Cruz Biotechnologies) in 1% bovine serum albumin overnight at 4°C. After incubation, the cells were kept for 1 h at room temperature; and then washed and treated with goat anti-mouse IgG TRITC conjugate (1:200; Molecular Probes) for 2 h at room temperature in the dark.

After washes with PBS for 10 min at room temperature, coverslips were mounted and observed using a Nikon microscope equipped for fluorescence (Leica) with a 40x optical lens. Alexa Fluor 488 and TRITC were excited at 488 and 540 nm, respectively, and then detected between 506 and 538 nm and 570 and 573 nm, respectively.

Statistical analysis

Data are presented as the means and standard deviations. Statistical comparisons of the differences between means were performed using one-way ANOVA followed by the Tukey *post hoc* test (software MINITAB release 15.1). *P* values <0.05 were considered statistically significant and those <0.01 considered statistically very significant.

Results

Preliminary tests

The potential toxicity of resveratrol (1–100 μ M) to Caco-2 and SW480 cells was assessed by the MTT assay at different times of incubation (6–72 h). Cell viability was not affected by the presence of resveratrol concentrations of 1–100 μ M (data not shown). We used a 1–50 μ M range concentration in our experiments, which elicited an effective anti-inflammatory activity.

Preliminary tests in order to verify cell viability in the presence of LPS and different resveratrol concentrations were performed. In this respect, we observed that cell viability was not changed when LPS (1 μ g/ml) was used in combination with resveratrol (1–50 μ M; data not shown).

Maximal cell responses (NO release, iNOS and TLR-4 expression) in our experimental conditions were reached at 1 μ g/ml LPS, as also reported by others^(21,22). Moreover, we exposed cell cultures to LPS for different times of incubation (6–72 h) and the maximal response, in terms of NO production, was observed at 48 h incubation, and so this incubation time was chosen for all experiments.

Effect of resveratrol on nitric oxide production by the intestinal cells

NO production by Caco-2 and SW480 cells stimulated by LPS in the presence or absence of resveratrol was measured. For Caco-2 cells, LPS (1 µg/ml) significantly increased the level of NO as compared to untreated cells (control). A significant reduction ($P < 0.05$) of LPS-inducible NO production (20–60%) was observed after 1 h pre-treatment with resveratrol; this was dose-dependent, with the maximal reduction ($P < 0.01$) occurring at 40 µM, as shown in Fig. 2(a) and (c).

For SW480 cells, LPS (1 µg/ml) significantly increased the level of NO in comparison to untreated cells (control). Also in this case, 1 h pre-treatment with resveratrol reduced LPS-inducible NO production (17–82%), in a dose-dependent manner, and again the maximal reduction ($P < 0.01$) was observed at a concentration of 40 µM (Fig. 2(b) and (c)).

Effect of resveratrol on inducible nitric oxide synthase protein and mRNA expression in intestinal cells

In order to assess whether the inhibitory effect of resveratrol on NO production was exerted via the inhibition of the corresponding inducible isoform of NO synthase (NOS), both protein and mRNA expressions of iNOS after the different treatments were determined by Western blot and RT-PCR analysis, respectively.

Western blotting analysis revealed the presence of a 130 kDa band corresponding to the iNOS molecular weight, which appeared more intense after LPS stimulation (1 µg/ml, 48 h) of both Caco-2 and SW480 cells in comparison with unstimulated cells. Densitometric analysis of immunoblots showed that 48 h LPS treatment of intestinal cells resulted in significantly ($P < 0.05$) enhanced expression of iNOS in comparison with untreated cells (Fig. 3(a) and (b)). In both Caco-2 and SW480 cells, iNOS levels were maximally reduced ($P < 0.01$) by a concentration of 40 µM-resveratrol, as shown in Fig. 3(a) and (b)).

Studies were extended to determine whether the iNOS protein expression paralleled mRNA expression. RT-PCR analysis confirmed that LPS maximally increased iNOS mRNA at 48 h and that resveratrol reduced the LPS-inducible increase in the iNOS mRNA (Fig. 3(c) and (d)). RT-PCR induced the expression of a band of the predicted size (622 bp) in both Caco-2 and SW480 cells submitted to LPS treatment. In this context, we observed that upon LPS treatment, iNOS mRNA expression was markedly increased in comparison to control. However, pre-treatment of the cells with 40 µM-resveratrol significantly reduced mRNA expression. As demonstrated by densitometric analysis of the bands obtained on the agarose gel, when 40 µM-resveratrol were added to Caco-2 cells, the iNOS mRNA levels were significantly reduced ($P < 0.01$) in comparison to the levels obtained in LPS-treated cells. Similar results were observed in SW480 cells, in which resveratrol diminished iNOS mRNA levels to a concentration of 40 µM (Fig. 3(c) and (d)).

Overall, these data suggest that resveratrol can down-regulate LPS-induced iNOS expression at the transcription level.

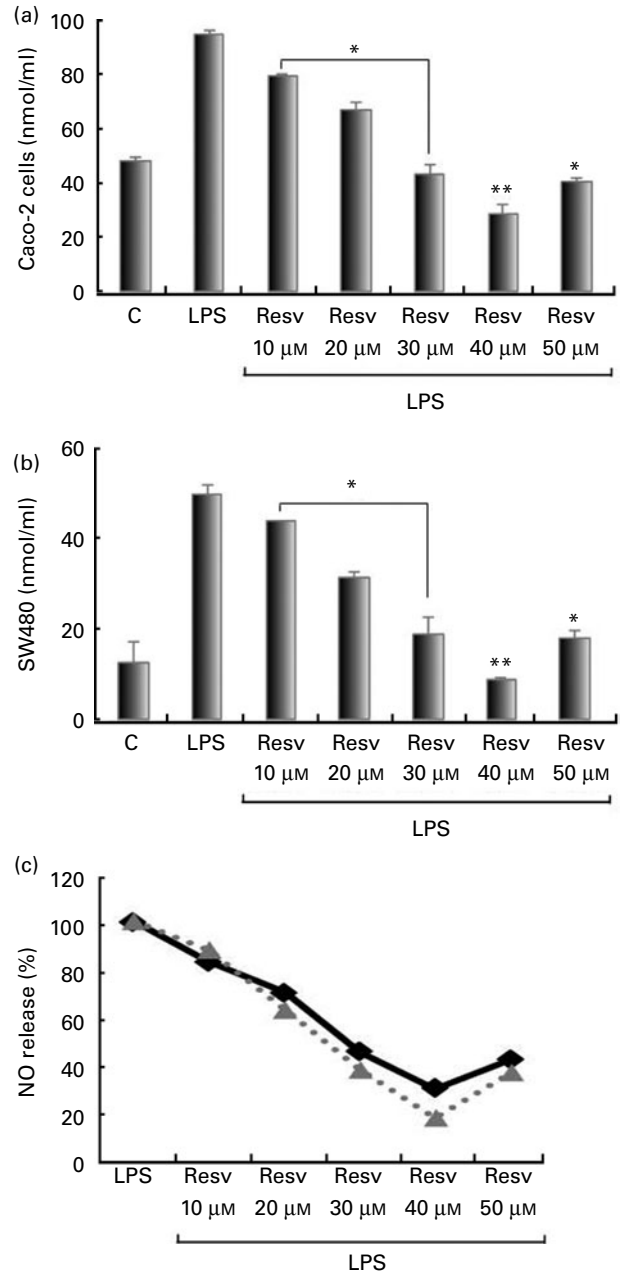


Fig. 2. Effects of resveratrol (Resv) on lipopolysaccharide (LPS)-induced nitric oxide (NO) production in intestinal cells. Intestinal cells were pre-incubated in medium containing various concentrations of Resv for 1 h and then treated with LPS (1 µg/ml) for 48 h. The amount of nitrite in the medium was measured for (a) Caco-2 cells and for (b) SW480 cells. (c) Percentage of NO production measured in LPS-stimulated Caco-2 (—◆—) and SW480 (---▲---) cells in the presence of the indicated concentrations of Resv. Data were normalised (LPS = 100%). Values are means of five separate experiments, with standard deviations represented by vertical bars. Mean values were significantly different from those of LPS: * $P < 0.05$, ** $P < 0.01$.

Expression of Toll-like receptors 4 on the intestinal cells

The regulation of TLR-4 expression by resveratrol was investigated in the two cell lines. Immunoblot analysis (Fig. 4(a) and (b)) demonstrated that both Caco-2 and SW480 cells express

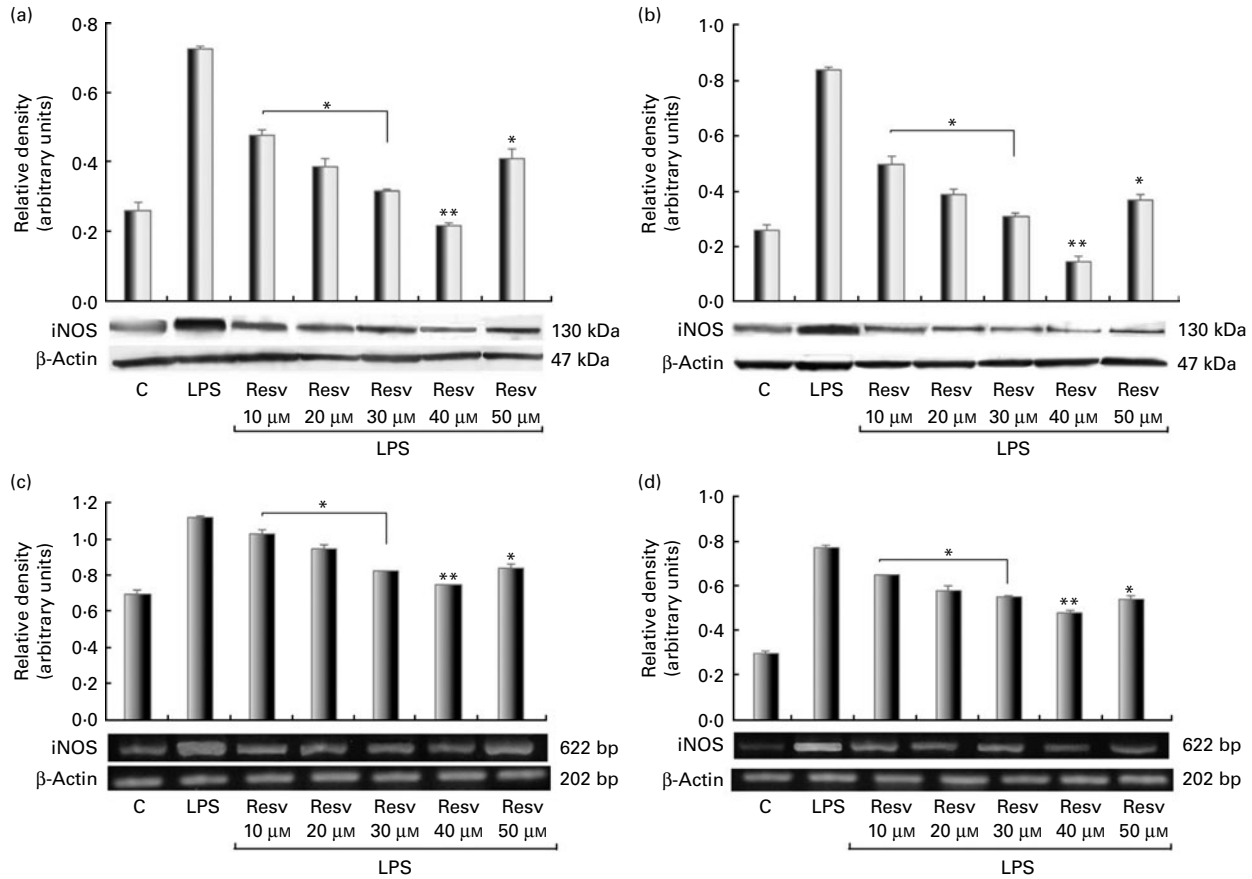


Fig. 3. Effects of resveratrol (Resv) on lipopolysaccharide (LPS)-induced inducible nitric oxide synthase (iNOS) expression in intestinal cells. iNOS protein levels in (a) Caco-2 and in (b) SW480 cells determined by Western blot analysis (see Experimental methods section). Expression of mRNA transcripts for iNOS was detected by RT-PCR in (c) Caco-2 and (d) SW480 cells (see Experimental methods section). Protein and mRNA levels were determined by measuring band intensities by scanning densitometry. Values are means of five separate experiments, with standard deviations represented by vertical bars. Mean values were significantly different from those of LPS: * $P < 0.05$, ** $P < 0.01$. C, control (unstimulated cells); LPS, endotoxin-treated cells; Resv, LPS-treated cells (pre-incubated with various concentrations of Resv).

this receptor, as revealed by the presence of a 90 kDa protein band corresponding to TLR-4. We also observed that the expression of TLR-4 in intestinal cells was modulated by LPS treatment because the TLR-4 protein levels were significantly increased after cell treatment with LPS. Resveratrol pre-treatment before LPS stimulation led to a dose-dependent decrease in the expression of TLR-4, reaching a maximal reduction at 40 μM -resveratrol. These data suggest a negative modulation by this flavonoid of endotoxin receptor expression.

Morphological examination by fluorescence microscopy confirmed that both Caco-2 and SW480 express TLR-4. Because Caco-2 cells feature poor expression of the TLR-4 complex, they serve as an excellent control when evaluating the possible modulations of TLR-4 expression by endotoxin treatment. In Fig. 4(c), Caco-2 cells pre-treated with anti-human TLR-4 and subsequently incubated with anti-human TRITC labelled IgG exhibited a fluorescent signal, positive for the presence of the TLR-4 receptor. The fluorescent signal was detected both in LPS-stimulated cells and in unstimulated cells, although a more intense signal appeared in the LPS-treated cells. In order to morphologically verify the effect of resveratrol, we used the optimal dose of resveratrol found to maximally reduce the expression of TLR-4 in

immunoblot assays. Interestingly, in resveratrol (40 μM) pre-treated cells, the fluorescent signal was visibly reduced in comparison to LPS-treated cells, as shown in Fig. 4(c). Similar results were obtained in SW480 cells, in which a fluorescent signal appeared on the membrane surface after treatment with anti-human Alexa Fluor 488 labelled IgG both with or without LPS treatment, although much higher fluorescence was detectable in the LPS-treated cells. Also in this case, 40 μM of resveratrol reduced the intensity of the fluorescent signal (Fig. 4(d)). Overall, these data lead us to conclude that resveratrol is able to down-regulate the level of TLR-4.

Negative controls, represented by intestinal cells treated with anti-mouse IgG alone, without the primary anti-TLR-4 antibody, showed no fluorescent signal (data not shown).

Effect of resveratrol on lipopolysaccharide-induced inhibitor of κB - α phosphorylation and degradation in intestinal cells

To account for the effects of resveratrol, it has been suggested that its biological activities induce the down-regulation of proinflammatory markers expression by reducing the activities of NF- κB . Since the phosphorylation and degradation of I κB - α

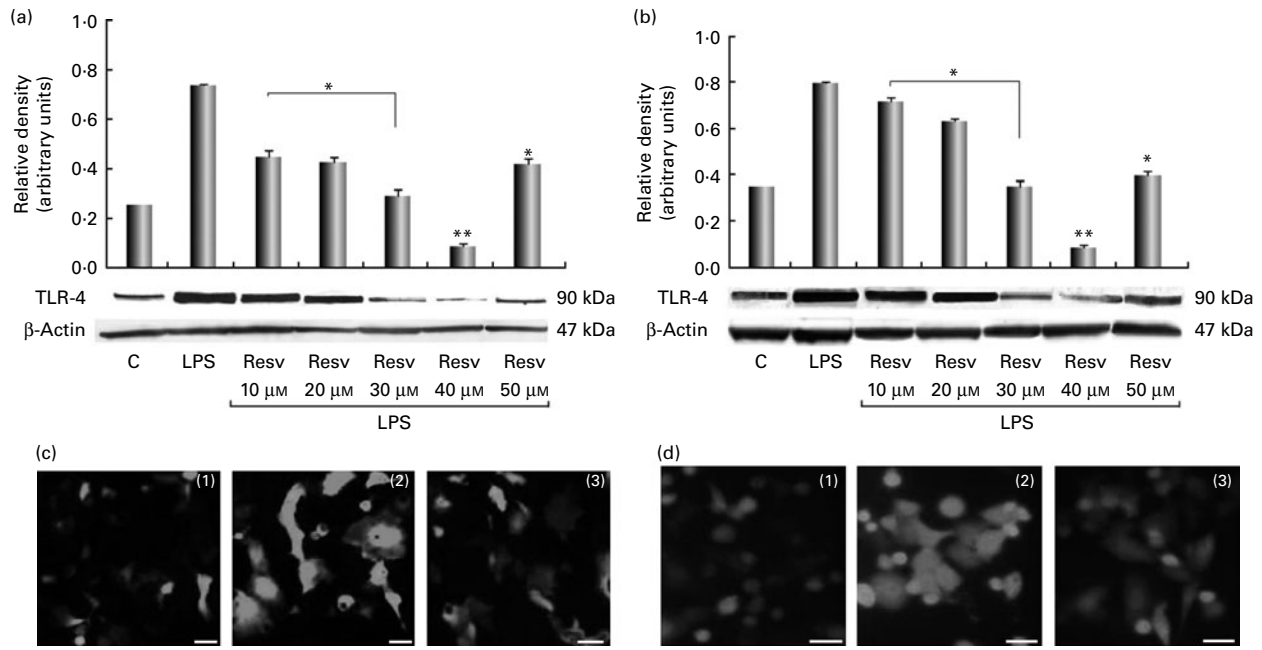


Fig. 4. Effects of resveratrol (Resv) on Toll-like receptors 4 (TLR-4) expression in intestinal cells. TLR-4 protein levels in (a) Caco-2 and (b) SW480 cells determined by Western blot analysis (see Experimental methods section). C, control (unstimulated cells); lipopolysaccharide (LPS), endotoxin-treated cells; Resv, LPS-treated cells (pre-incubated with various concentrations of Resv). Protein levels were determined by measuring immunoblot band intensities by scanning densitometry. Values are means of five separate experiments, with standard deviations represented by vertical bars. Mean values were significantly different from those of LPS: * $P < 0.05$, ** $P < 0.01$. Microscopic expression of TLR-4 receptor in (c) Caco-2 and (d) SW480 cells. (1) Unstimulated cells; (2) LPS-treated cells; (3) LPS-treated cells, pre-incubated with Resv (40 μM). Scale bars: 10 μm.

are an essential step in the translocation of NF-κB p65, we determined the effect of resveratrol on LPS-induced degradation and phosphorylation of IκB-α protein.

Therefore, we evaluated the expression of phospho-IκB in cell lysates obtained from LPS-treated Caco-2 and SW480 cells. In this context, we observed that cells exposed to LPS exhibited a significant increase of phosphorylated IκB expression as compared to unstimulated cells, with a maximal expression after 48 h of cell stimulation and a simultaneous reduction of unphosphorylated IκB form. Little phosphorylation of IκB was observed in unstimulated cells, as revealed by densitometric analysis (Fig. 5(a) and (b)).

Pre-treatment with resveratrol inhibited IκB-α phosphorylation and the degradation of IκB-α, reaching a maximal reduction at 40 μM in both Caco-2 (Fig. 5(a)) and SW480 (Fig. 5(b)) LPS-stimulated cells.

Discussion

In the present study, we demonstrate that a moderate concentration of resveratrol (40 μM) counteracts the inflammatory response of both Caco-2 and SW480 cells to LPS challenge. In this respect, we observed that concentrations higher than 40 μM resulted ineffective in order to increase anti-inflammatory action in tested cells. This effect may be due to the biotransformation of resveratrol making the most ineffective of resveratrol at higher concentration as just reported by other authors⁽²³⁾. This biotransformation may be due to, for example, the saturation of the UDP-glucuronosyltransferases UGT1A7 and UGT1A10, both expressed in the

human gastrointestinal tract and able to catalyse resveratrol glucuronidation⁽²⁴⁾ or, alternatively, to the sulphate conjugation as reported by Kaldas *et al.*⁽²⁵⁾. Moreover, in our study, the concentration range 10–50 μM used is correspondent to the normal concentration of dietary polyphenols present in the bowel^(26,27). We show that both intestinal cell lines are able to release significant levels of NO after exposure to LPS. NO is a free radical generated from L-arginine by NOS. Here, three isoforms of NOS have been identified: two constitutive isoforms of NOS produce low levels of NO, which plays a physiological role in gut function, including the modulation of intestinal water and electrolyte transport and mucosal permeability, whereas the iNOS is induced after LPS stimulation, as well as in response to proinflammatory cytokines^(28–30). Generally, an increased expression of iNOS is associated with inflammatory responses and also with serious disorders such as septic shock⁽³¹⁾. At the intestinal level, the sustained release of NO resulting from iNOS up-regulation after an attack, including endotoxaemia, may lead to cellular injury and gut barrier failure^(29,30,32,33).

In order to demonstrate a possible involvement of iNOS in NO release by Caco-2 and SW480 intestinal cell lines exposed to LPS, we monitored iNOS protein and gene expression. Western blot and RT-PCR analyses revealed that intestinal cells exposed to LPS show augmented levels of iNOS protein and mRNA.

The detrimental effects of sustained NO production in gut inflammation have also been shown in human subjects. In this context, various studies have described strategies

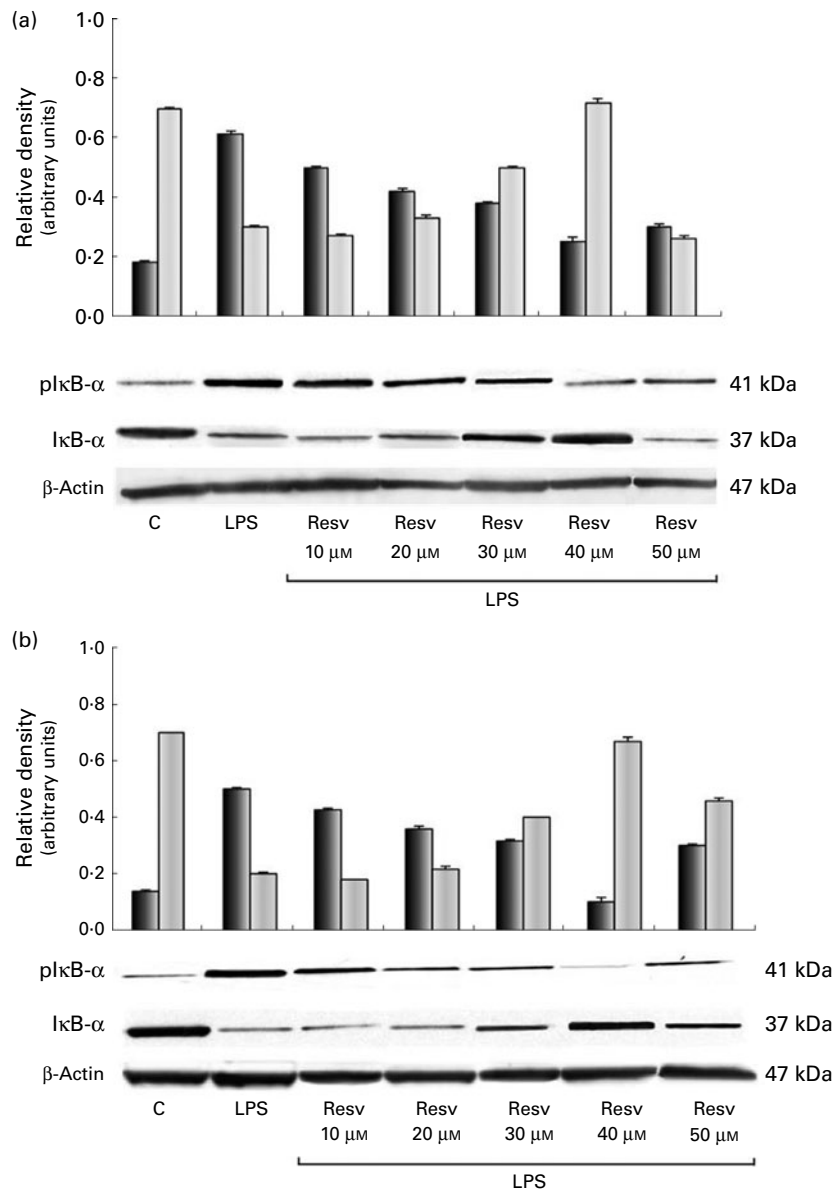


Fig. 5. Effects of resveratrol (Resv) on the phosphorylation and degradation of inhibitor of κ B- α (I κ B- α ; \square). Total cell lysates were prepared for Western blot analysis for the content of I κ B- α and phosphorylated I κ B- α (pI κ B- α ; \blacksquare) protein in (a) Caco-2 and (b) SW480 cells. Protein levels were determined by measuring immunoblot band intensities by scanning densitometry. Values are means of five separate experiments, with standard deviations represented by vertical bars. C, control; lipopolysaccharide (LPS), endotoxin-treated cells; Resv, LPS-treated cells, pre-incubated with different concentrations of Resv.

attempting to combat the deleterious effects of high levels of NO and to control inflammation^(34,35).

Interestingly, our results demonstrate that resveratrol was able to significantly reduce the induction of iNOS. In addition, the suppression of iNOS expression by this polyphenol was paralleled by a comparable inhibition of NO production. Similar results have been reported for resveratrol in different types of cells of other animal species, including mouse macrophages⁽³⁶⁾, murine 3T6 fibroblasts⁽³⁷⁾ and in rat liver⁽³⁸⁾. Moreover, it has recently been observed in mice that resveratrol has a significant beneficial effect in chronic experimentally induced colitis and that this protective effect seems to be related to a modulation of proinflammatory mediators, including a reduction of iNOS expression in colonic mucosa⁽³⁹⁾.

TLR are able to distinguish a broad range of both commensal and pathogenic bacteria with a different specificity^(40,41). Among these, TLR-4 specifically binds to LPS⁽⁴²⁾. The responsiveness to LPS of intestinal epithelial cell lines, including Caco-2 cells, is positively correlated with TLR-4 expression⁽⁴³⁾. In our experiments, we observed an up-regulation of TLR-4 in both Caco-2 and SW480 cells after LPS treatment, suggesting that under some circumstances this receptor may mediate LPS function(s). This result is in agreement with other studies reporting that LPS concentrations as low as 0.01 μ g/ml are enough to significantly up-regulate TLR-4 mRNA after 24 h in a bovine epithelial cell line⁽⁴⁴⁾. The same authors reported that increasing the concentration of LPS did not change TLR-4 mRNA amount, except for a slight but not significant

increase at 10.0 $\mu\text{g/ml}$, indicating that a very low concentration of LPS is enough to trigger an optimal TLR-4 signalling response. A positive regulation of TLR-4 by LPS was also reported by Gatti *et al.*⁽⁴⁵⁾ who demonstrated that a prostate epithelial cell line expresses enhanced levels of TLR-4 after 24 h of treatment with LPS, which remain elevated until 48 h.

Recent evidence suggests the involvement of TLRs in the pathogenesis of human chronic diseases, including inflammatory pathologies^(46–48). In this respect, Youn *et al.*⁽⁴⁹⁾ observed that resveratrol suppressed NF- κB activation and COX-2 expression in RAW264.7 cells following TLR-3 and TLR-4 stimulation. Identifying molecular targets by which pharmacological or dietary factors modulate TLR-mediated signalling pathways and target gene expression would provide a new opportunity to manage the dysregulation of TLR-mediated inflammatory responses leading to acute and chronic inflammatory diseases. Interestingly, we observed that resveratrol treatment diminished the amount of this LPS receptor in LPS-treated Caco-2 and SW480 intestinal cells. To our knowledge, this is the first report describing resveratrol as a negative regulator of TLR-4 expression in the human intestinal cell lines.

Moreover, our data clearly showed how, in both cell lines, LPS treatment induces the activation of NF- κB , as confirmed by I κB phosphorylation and p65 nuclear translocation. In this respect, activation of this nuclear transcription factor seems to be correlated with iNOS activation, since NF- κB inhibition by its specific inhibitor caused a significant reduction of both iNOS expression and NO release (data not shown). Interestingly, resveratrol pre-treatment of Caco-2 cells before LPS stimulation prevented NF- κB activation, and

the consequent reduction of NO production as well as the protein and mRNA expression of iNOS. In this context, several polyphenols, including resveratrol, have been reported to modulate NF- κB activation *in vivo*, showing anti-inflammatory properties related to the inhibition of the NF- κB signalling cascade⁽⁵⁰⁾. Pure polyphenols have also been shown to modulate the expression, at mRNA and/or protein levels, of inflammatory mediators produced in damaged intestinal tissue of animals with experimentally induced inflammation. Among these, it was demonstrated that resveratrol can reduce several proinflammatory mediators, such as IL-1 β , TNF- α , or proinflammatory enzyme activities, such as COX-2 and iNOS⁽⁵¹⁾. There have since been many reports of resveratrol suppressing NF- κB activation induced by several agents, including LPS, in a variety of cell lines, including U-937, Jurkat and HeLa cells⁽⁵²⁾. Moreover, in mice^(7,53,54) and in humans⁽⁵⁵⁾, the action of resveratrol on the NOS/COX-2 gene and protein expression has been described to be mediated by inhibiting NF- κB activation, mainly as a result of inhibiting the degradation of I κB - α . In short, there seems to be no doubt that resveratrol can inhibit NF- κB activation.

In summary, our results demonstrate that resveratrol down-regulates: (1) the expression of the LPS receptor TLR-4, and (2) LPS-induced expression of iNOS at mRNA and protein levels, and hence NO production, through inhibiting I κB - α degradation and NF- κB activation. How resveratrol reduces the inflammatory response to LPS in intestinal cells is not known, but based on our experimental results, we propose a double mechanism elicited by resveratrol, in which NF- κB inhibition may be due to a direct action on the nuclear transcription factor via phosphorylation inhibition,

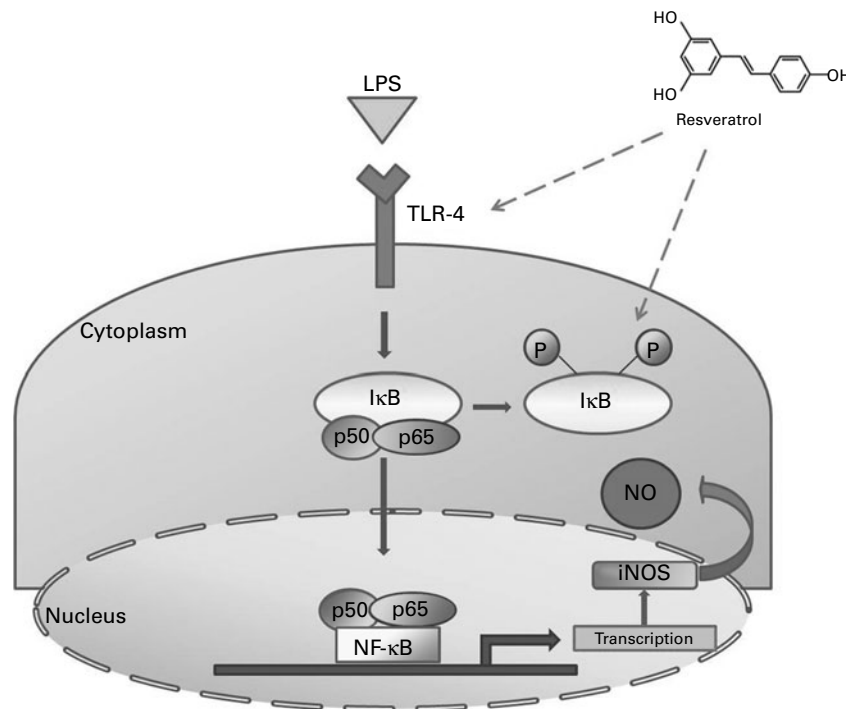


Fig. 6. Possible molecular mechanism of resveratrol-induced inhibition of cell activation by lipopolysaccharide (LPS) challenge in intestinal cell lines. TLR-4, Toll-like receptor 4; I κB , inhibitor of κB ; NO, nitric oxide; iNOS, inducible NO synthase.

or, alternatively, by TLR-4 down-regulation, leading to reduced NF- κ B activation (Fig. 6). Future investigation may focus on the molecular mechanism by which TLR-4 is down-regulated by resveratrol. Overall, our findings seem to show that resveratrol significantly attenuates several components of the intestinal cells' response to proinflammatory stimuli, thus suggesting a potential therapeutic effect in the treatment of inflammatory bowel diseases.

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