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Lycopene, quercetin and tyrosol prevent macrophage activation induced by gliadin and IFN-γ

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Abstract

Oxidative stress plays an important role in inflammatory process of celiac disease. We have studied the effect of the lycopene, quercetin and tyrosol natural antioxidants on the inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) gene expression in RAW 264.7 macrophages stimulated by gliadin in association with IFN-γ. The IFN-γ plus gliadin combination treatment was capable of enhancing iNOS and COX-2 gene expression and nuclear factor-κB (NF-κB), interferon regulatory factor-1 (IRF-1) and signal transducer and activator of transcription-1α (STAT-1α) activation induced by reactive oxygen species generation at 24 h. Lycopene, quercetin and tyrosol inhibited all these effects. The results here reported suggest that these compounds may represent non toxic agents for the control of pro-inflammatory genes involved in celiac disease.

Keywords: Celiac disease; Gliadin; Interferon-gamma; Transcription factors; RAW 264.7 macrophage

1. Introduction

Celiac disease, an enteropathy caused by permanent intolerance to gluten/gliadin, is characterized by a complex interplay between genetic and environmental factors (Sollid, 2005). The disease in its typical form is histologically characterized by villous atrophy, crypt cell hyperplasia, and increased number of intra-epithelial lymphocytes (Sollid, 2005). The mechanisms by which gluten/gliadin damages the intestinal mucosa of celiac patients remain unclear. A large body of evidence indicates a dysregulated immune response to gluten-derived peptides in celiac patients (Sollid, 2005). However, beside the immunologic pathway, the direct cytotoxic action of gliadin peptides against intestinal mucosa has been postulated as one of the mechanisms underlying the pathogenesis and the progression of celiac disease (Maiuri et al., 1996; Maiuri et al., 2003; Gianfrani et al., 2005). It is well known that toxic gluten peptides are presented by macrophages in the lamina propria and recognised by gliadin antigen specific CD4+T cells (Sollid, 2005). As a result, secreted mediators, such as IFN-γ, may cause activation of macrophages which, in turn, produce pro-inflammatory cytokines contributing to the damage of the mucosal matrix (Kontakou et al., 1995; Pender et al., 1996; Ciccocioppo et al., 2005). Although the molecular mechanisms involved in the inflammatory process of celiac disease have not been completely elucidated, a large body of data suggests the involvement of transcription factors such as nuclear factor-κB (NF-κB), signal transducer and activator of transcription-1α (STAT-1α) and interferon regulatory factor-1 (IRF-1) (Salvati et al., 2003; De Stefano et al., 2006). These transcription factors are dependent on the intracellular redox state (Pahl, 1999; Ramana et al., 2000; Kroger et al., 2002) and can cooperate in order to promote synergistically transcriptional activity of pro-inflammatory genes (Ohmori and Hamilton, 1993; Kinugawa et al., 1997). Binding of IFN-γ to its receptor induces activation of STAT-1α which binds to a specific consensus sequence, termed the IFN-γ activation site (GAS), of IRF-1 promoter and induces IRF-1 (Seidel et al., 1995; Decker et al., 1991). Furthermore, the IRF-1 gene promoter has been described as containing a composite GAS/κB element (Sims et al., 1993; Harada et al., 1994) and cooperative regulation of transcription by IRF-1 and NF-κB has been previously described (Garoufalis et al., 1994;

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Neish et al., 1995). The promoter regions of iNOS and COX-2 contain consensus sequences for NF-κB, IRF-1 and STAT-1α (Fletcher et al., 1992; Lowenstein et al., 1993; Sirois et al., 1993; Kamijo et al., 1994; Kosaka et al., 1994; Gao et al., 1997). Several investigations have reported the involvement of free radicals in the progression of a number of inflammatory bowel diseases, such as celiac disease (Sido et al., 1998). It has been demonstrated that gliadin alters oxidative balance in Caco-2 cells (Dolfini et al., 2003) and that glutathione peroxidase levels decrease in biopsies from celiac disease patients (Stahlberg et al., 1988). A common feature of patients with inflammatory bowel diseases is a general depletion of antioxidant levels such as ascorbate, β-carotene, α-tocopherol (Sido et al., 1998). Actually, the only available treatment of celiac disease is a strict gluten-exclusion diet. Lycopene is the most abundant carotenoid in tomatoes (Heber and Lu, 2002). Quercetin is a flavonoid mostly found in onions, extra-virgin olive oil and broccoli (de la Puerta et al., 2001). Tyrosol is a polyphenol from white wine and extra-virgin olive oil (Di Carlo et al., 1999). All these compounds have been demonstrated to inhibit, *in vitro* and *in vivo*, pro-inflammatory gene expression by scavenging reactive oxygen species (Heber and Lu, 2002; de la Puerta et al., 2001; Di Carlo et al., 1999); natural and synthetic antioxidants have been demonstrated to ultimately block the activation of NF-κB, IRF-1 and STAT-1α (Hecker et al., 1996; Epinat and Gilmore 1999; Faure et al., 1999; Kim et al., 2003). We have recently reported that gliadin increases iNOS gene expression in IFN-γ-stimulated RAW 264.7 cells through a mechanism involving NF-κB, IRF-1 and STAT-1α. These effects were prevented by synthetic and natural antioxidants (De Stefano et al., 2006). In the present study we examined the effect of other natural antioxidants, such as lycopene, quercetin and tyrosol, on iNOS and COX-2 gene expression induced by gliadin in RAW 264.7 macrophages stimulated with IFN-γ for 24 h.

2. Materials and methods

2.1. Cell culture

The mouse monocyte/macrophage cell line RAW 264.7 was cultured at 37 °C in humidified 5% CO_2/95% air in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% foetal bovine serum, 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 25 mM Hepes and 5 mM sodium pyruvate. The cells were plated in 24 culture wells at a density of 2.5×10^5 cells/ml per well or 10 cm diameter culture dishes at a density of 3×10^6 cells/ml per dish and allowed to adhere for 2 h. Thereafter the medium was replaced with fresh medium and cells were activated with IFN-γ (25 U/ml). Gliadin (800 μg/ml), lycopene (5, 10 and 20 μM), quercetin (0.1, 1 and 10 μM) and tyrosol (1, 2 and 4 mM), were added to the cells 10 min before IFN-γ challenge. IFN-γ stocks were dissolved in 0.5% BSA in PBS and stored at −20 °C. Contaminations of gliadin where evaluated by Limulus Polyphemus test; gliadin was prepared at needing, dissolved in 50% (v/v) EtOH and sterilized by 30 minutes of UV irradiation. Lycopene stocks were dissolved in DMSO and stored at 4 °C away from light. Tyrosol and quercetin stocks were dissolved in EtOH 60% and stored at −20 °C. The highest concentrations of vehicles in the samples were 0.6% EtOH and 0.6% DMSO, therefore both vehicles alone were added to the cells to verify if they may affect cell viability. The cell viability was determined by using 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) conversion assay as previously described (De Stefano et al., 2006).

2.2. Nitrite determination

NO was measured as nitrite (NO_2^-, nmol/10^6 cells) accumulated in the incubation medium after 24 h. A spectrophotometric assay based on the Griess reaction was used (De Stefano et al., 2006). Briefly, Griess reagent (1% sulphanilamide, 0.1% naphthylethylenediamine in phosphoric acid) was added to an equal volume of cell culture supernatant and the absorbance at 550 nm was measured after 10 min. The nitrite concentration was determined by reference to a standard curve of sodium nitrite.

2.3. PGE_2 determination

The accumulation of PGE_2 in the culture medium, 24 h after treatments, was measured by an ELISA kit, according to the manufacturer's instructions (TEMA Ricerche, Milan, Italy).

2.4. Measurement of reactive oxygen species

The formation of reactive oxygen species was evaluated by means of the probe 2′,7′-dichlorofluorescin-diacetate (H_2DCF-DA, Sigma) as described elsewhere (Santamaria et al., 2004). Briefly, RAW 264.7 cells were grown in DMEM containing 10% (v/v) foetal bovine serum, then were plated at a density of 1.5×10^4 cells/well and then incubated in the growth medium containing 5 μM H_2DCF-DA for 2 h at 37 °C. H_2DCF-DA is a nonfluorescent permeant molecule that passively diffuses into cells, where the acetates are cleaved by intracellular esterases to form H_2DCF and thereby traps it within the cell. In the presence of intracellular reactive oxygen species, H_2DCF is rapidly oxidised to the highly fluorescent 2′,7′-dichlorofluorescein (DCF). Cells were washed twice with PBS buffer; thereafter, the medium was replaced with fresh medium and cells were stimulated with IFN-γ (25 U/ml) and gliadin (800 μg/ml) for 24 h. Lycopene (20 μM), quercetin (10 μM) or tyrosol (2 mM) were added to the cells 10 min before IFN-γ + gliadin challenge. After treatment, cells were washed twice with PBS buffer and plates were placed in a fluorescent microplate reader (Perkin Elmer LS55 Luminescence Spectrometer; Perkin Elmer, Beaconsfield, UK). Fluorescence was monitored using an excitation wavelength of 485 nm and an emission wavelength of 538 nm. In each experiment, fluorescence increase was measured in ten replicate cultures ($n = 10$) for each treatment.

2.5. Cytosolic and nuclear extracts

Cytosolic and nuclear extracts of macrophages stimulated for 24 h with IFN-γ (25 U/ml) and incubated in the presence or absence of gliadin (800 μg/ml), lycopene (20 μM), quercetin (10 μM) and tyrosol (2 mM) were prepared as previously

described with some modifications (De Stefano et al., 2006). Briefly, harvested cells (3×10^6) were washed two times with ice-cold PBS and centrifuged at 180 ×*g* for 10 min at 4 °C. The cell pellet was resuspended in 100 μl of ice-cold hypotonic lysis buffer (10 mM Hepes, 10 mM KCl, 0.5 mM phenylmethylsulphonyfluoride, 1.5 μg/ml soybean trypsin inhibitor, 7 μg/ml pepstatin A, 5 μg/ml leupeptin, 0.1 mM benzamidine, 0.5 mM dithiothreitol) and incubated on ice for 15 min. The cells were lysed by rapid passage through a syringe needle for five or six times and the cytoplasmic fraction was then obtained by centrifugation for 1 min at 13,000 ×*g*. The supernatant containing the cytosolic fraction was removed and stored at −80 °C. The nuclear pellet was resuspended in 60 μl of high salt extraction buffer (20 mM Hepes pH 7.9, 10 mM NaCl, 0.2 mM EDTA, 25% v/v glycerol, 0.5 mM phenylmethylsulphonyfluoride, 1.5 μg/ml soybean trypsin inhibitor, 7 μg/ml pepstatin A, 5 μg/ml leupeptin, 0.1 mM benzamidine, 0.5 mM dithiothreitol) and incubated with shaking at 4 °C for 30 min. The nuclear extract was then centrifuged for 15 min at 13,000 ×*g* and supernatant was aliquoted and stored at −80 °C. Protein concentration was determined by Bio-Rad (Milan, Italy) protein assay kit.

2.6. Electrophoretic mobility shift assay (EMSA)

Double stranded oligonucleotides containing NF-κB (5′-CAA CGGCAGGGGAATCTCCCTCTCCTT-3′), IRF-1 (5′GGAAGC-GAAAATGAAATTGACT-3′) and STAT-1α (5′-CATGTTATG-CATATTCCTGTAAGTG-3′) recognition sequences were endlabelled with ^{32}P-γ-ATP. Nuclear extracts containing 5 μg protein were incubated for 15 min with radiolabeled oligonucleotides ($2.5–5.0 \times 10^4$ cpm) in 20 μl reaction buffer containing 2 μg poly dI-dC, 10 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1 mM EDTA, 1 mM DTT, 10% (v/v) glycerol. The specificity of the DNA/protein binding was determined by competition reaction in which a 50-fold molar excess of unlabeled wild-type, mutant or Sp-1 oligonucleotide was added to the binding reaction 15 min before addition of radiolabeled probe. In supershift assay, antibodies reactive to p50, p65, IRF-1 and STAT-1α proteins were added to the reaction mixture 15 min before the addition of radiolabeled NF-κB, IRF-1 and STAT-1α probe. Nuclear protein–oligonucleotide complexes were resolved by electrophoresis on a 6% non-denaturing polyacrylamide gel in 1× TBE buffer at 150 V for 2 h at 4 °C. The gel was dried and autoradiographed with intensifying screen at −80 °C for 20 h. Subsequently, the relative bands were quantified by densitometric scanning of the X-ray films with a GS-700 Imaging Densitometer (Bio-Rad, Milan, Italy) and a computer program (Molecular Analyst; IBM).

2.7. Western blot analysis

Immunoblotting analysis of anti-iNOS, anti-COX-2, anti-p50 and anti-p65, anti-IRF-1 and STAT-1α was performed on cytosolic or nuclear fraction. Cytosolic and nuclear fraction proteins were mixed with gel loading buffer (50 mM Tris, 10% SDS, 10% glycerol, 10% 2-mercaptoethanol, 2 mg/ml of bromophenol) in a ratio of 1:1, boiled for 3 min and centrifuged at 10,000 ×*g* for 5 min. Protein concentration was determined and equivalent amounts (30 μg) of each sample were electrophoresed in a 8% or 12% discontinuous polyacrylamide minigel. The proteins were transferred onto nitro-cellulose membranes, according to the manufacturer's instructions (Bio-Rad, Milan, Italy). The membranes were saturated by incubation at 4 °C overnight with 10% non-fat dry milk in PBS and then incubated with (1:1000) anti-iNOS, anti-COX-2, anti-p50, anti-p65, anti-IRF-1 and anti-STAT-1α for 1 h at room temperature. The membranes were washed three times with 0.01% Tween^{20} in PBS and then incubated with anti-rabbit or anti-mouse immunoglobulins coupled to peroxidase (1:1000). The immunocomplexes were visualised by the ECL chemiluminescence method (Santa Cruz, Milan, Italy). The membranes were stripped and re-probed with β-actin or histone 1 antibodies to verify equal loading of proteins. Subsequently, the relative expression of iNOS, COX-2, p50, p65, IRF-1 and STAT-1α in cytosolic or nuclear fraction was quantified by densitometric scanning of the X-ray films with a GS 700 Imaging Densitometer (Bio-Rad) and a computer programme (Molecular Analyst, IBM).

2.8. Reverse transcription-polymerase chain reaction

RAW 264.7 cells were plated in a six-well plate at a density of 3×10^6/well, where they were allowed to adhere for 2 h. Thereafter, the cells were treated with lycopene (10 μM), quercetin (10 μM) or tyrosol (2 mM) in the presence of gliadin (800 μg/ml) and, 10 min after treatment, were stimulated with IFN-γ (25 U/ml) for 24 h. Total RNA extraction, by using TRIzol (Invitrogen, Milan, Italy) was performed as described elsewhere (Maiuri et al., 2005). The levels of iNOS and COX-2 mRNA were evaluated by using PCR amplification of reverse-transcribed mRNA. The housekeeping gene β-actin was used as an internal control. Five micrograms of total RNA was reverse-transcribed into cDNA by using oligo (dT)12–18 primer (Invitrogen) and MMLV-Reverse Transcriptase (Invitrogen). 1 μl of cDNA was amplified by PCR using Taq Polymerase (Invitrogen) according to the manufacturer's instructions. The primers were:

COX-2: sense 5′-CCGGGTTGCTGGGGGAAGA-3′,
antisense 5′-GTGGCTGTTTTGGTAGGCTGTGGA-3′;
iNOS: sense 5′-TGGGAATGGAGACTGTCCCAG3′;
antisense: 5′-GGGATCTGAATGTGATGTTTG-3′;
β-actin: sense 5′-AT GAAGATCCTGACCGCGCGT-3′;
antisense: 5′-AACGCAGCTCAGTAACAGTCCG-3′.

The amplified fragments were 479 bp, 305 bp and 584 bp respectively. The PCR reaction was performed under the following conditions: a first cycle of denaturation at 94 °C for 1 min 40 s, then 25 cycles of denaturation at 94 °C for 40 s, annealing at 56 °C for 40 s, extension at 72 °C for 1 min and one additional cycle of extension at 72 °C for 8 min. The PCR products were run on a 1% agarose gel and visualised by Sybr Safe staining (Invitrogen, Milan, Italy).

2.9. 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 $P<0.05$.

2.10. Reagents

DMEM, foetal bovine serum, glutamine, penicillin, streptomycin, Hepes, sodium pyruvate and PBS were from BioWhittaker (Caravaggio, BG, Italy). Recombinant mouse interferon-γ was from Vinci-Biochem (Florence, Italy). ^{32}P-γ-ATP was from Amersham (Milan, Italy). Poly dI-dC was from Boehringer-Mannheim (Milan, Italy). Anti-p50, anti-p65, anti-IRF-1, anti STAT-1α, anti-iNOS, anti-COX-2, anti-β-actin and anti-histone 1 antibodies were from Santa Cruz (Milan, Italy). Phosphate buffer saline was from Celbio (Milan, Italy). Oligonucleotide synthesis was performed to our specifications by Tib Molbiol (Boehringer-Mannheim, Genova, Italy). Non-fat dry milk was from Bio-Rad (Milan, Italy). Sybr Safe, MMLV-Reverse Transcriptase, TRIzol, dNTPs were from Invitrogen (Milan, Italy). DL-dithiothreitol, pepstatin A, leupeptin, benzamidine, phenylmethylsulfonilfluoride were from Applichem (Darmstadt, Germany). Gliadin, lycopene, quercetin, tyrosol and MTT as well as other reagents were from Sigma (Milan, Italy).

3. Results

3.1. Effect of lycopene, quercetin and tyrosol on the increase of nitrite and PGE_2 production

The nitrite and PGE_2 production by unstimulated cells was undetectable. Stimulation of cells with IFN-γ (25 U/ml) for 24 h resulted in an accumulation of nitrite and PGE_2 in the medium. Gliadin (800 μg/ml) added to the cells together with IFN-γ caused a significant increase in production of nitrite and PGE_2 as compared with IFN-γ alone. Treatment of cells with lycopene (5, 10 and 20 μM), quercetin (0,1, 1 and 10 μM) or tyrosol (1, 2 and 4 mM) inhibited significantly and in a concentration-manner the increase of nitrite (42.7±0.95%, 51.8±1.42% and 75.09±1.39%; 4.13±0.81%, 46.7±1.32% and 73.7±2.01%; 33.84±0.93%, 63.6±1.38% and 26.6±1.66%, respectively; $n=7$) (Fig. 1A) and PGE_2 (21.9±1.4%, 41.5±1.6% and 60.6±1.9%; 11.7±2.3%, 59.5±1.5% and 80.7±1.4%; 34.9±1.2%, 54.8±1.1% and 75.5±0.9%, respectively; $n=3$) (Fig. 1B) production induced by gliadin+IFN-γ. Lycopene, quercetin, tyrosol, gliadin and IFN-γ did not affect cell viability (>90%; data not shown).

3.2. Effect of lycopene, quercetin and tyrosol on the increase of iNOS and COX-2 expression

Upon stimulation with IFN-γ (25 U/ml) for 24 h, cells expressed high level of iNOS and COX-2 protein as compared with untreated cells. Gliadin (800 μg/ml), added to the cells together with IFN-γ (25 U/ml), increased iNOS and COX-2 protein expression compared with IFN-γ alone. Lycopene (20 μM), quercetin (10 μM) and tyrosol (2 mM) significantly inhibited the increase of iNOS (70.6±0.19%, 65.1±0.10% and 61.38±0.32%, respectively; $n=5$) and COX-2 (53.0±0.27%, 48.8±0.13% and 45.8±0.13%, respectively; $n=5$) protein expression induced by gliadin+IFN-γ (Fig. 2). Gliadin alone failed to augment the iNOS and COX-2 protein expression level (data not shown). In addition, to explore whether the reduced level either of iNOS or COX-2 protein observed in cells treated with lycopene, quercetin or tyrosol could be attributed to a reduced gene transcription, we analysed the expression either of iNOS and COX-2 mRNA. The IFN-γ and gliadin combination treatment led to a significantly higher increase of iNOS and COX-2 mRNA levels compared with IFN-γ alone. Incubation of cells with lycopene, quercetin and tyrosol significantly reduced iNOS (by 44.5±0.7%, 40.8±0.9% and 24.4±1.0%, respectively; $n=3$) and COX-2 (by 39.6±0.5%, 35.8±0.8% and 21.4±0.7%; respectively; $n=3$) mRNA levels (Fig. 3). β-actin mRNA levels are reported as an internal control.

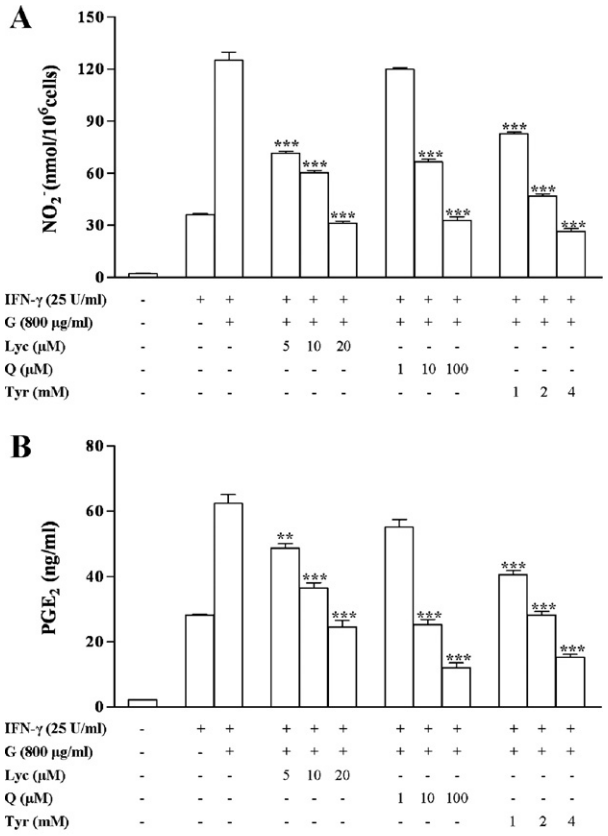

Fig. 1. Effect of lycopene (Lyc), quercetin (Q) and tyrosol (Tyr) on the increase of nitrite (A) and PGE_2 (B) production by RAW 264.7 macrophages stimulated with IFN-γ and gliadin for 24 h. Data are expressed as mean±S.E.M. of three-seven experiments in triplicate. $^{**}P<0.001$, $^{***}P<0.0001$ *vs.* IFN-γ and gliadin.

3.3. Effect of lycopene, quercetin and tyrosol on NF-κB, IRF-1 and STAT-1α activation

The effects of lycopene (20 μM), quercetin (10 μM), and tyrosol (2 mM) on NF-κB, IRF-1 and STAT-1α DNA/binding activity induced by gliadin (800 μg/ml) in RAW 264.7 macrophages stimulated with IFN-γ (25 U/ml) for 24 h were evaluated by EMSA using the specific binding elements for each transcription factor. A

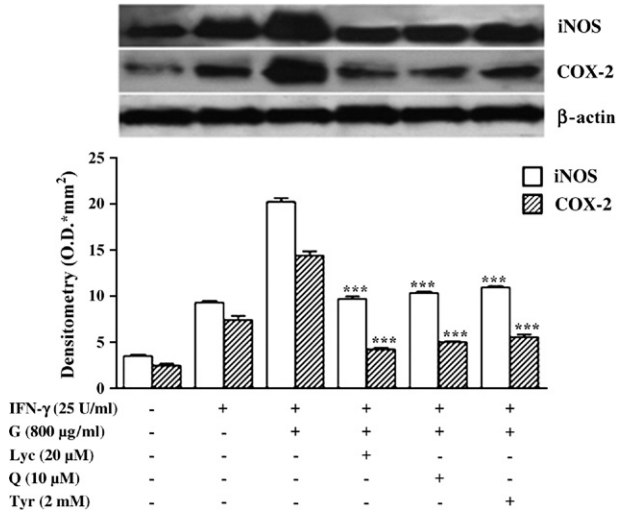

Fig. 2. Representative Western blot as well as relative densitometric analysis show the effect of lycopene (Lyc), quercetin (Q) and tyrosol (Tyr) on the increase of iNOS and COX-2 protein expression in RAW 264.7 macrophages stimulated with IFN-γ and gliadin for 24 h. β-actin expression is shown as a control. Data are from a single experiment and are representative of five separate experiments. Densitometric data are expressed as mean±S.E.M. of five experiments. ***$P<0.0001$ *vs*. IFN-γ and gliadin.

low basal level of NF-κB as well as IRF-1 and STAT-1α/DNA binding activity was detected in nuclear proteins from unstimulated macrophages. Conversely, a retarded band was clearly detected following stimulation with IFN-γ, the intensity of which was markedly increased in extracts from cells exposed to gliadin and IFN-γ. Treatment of cells with lycopene as well as quercetin or tyrosol caused a significant reduction of specific protein-DNA complexes induced by gliadin and IFN-γ (by 74.6±1.99%, 62.9±1.6% and 57.2±2.6%, respectively for NF-κB; 63.3±0.7%, 60.2±

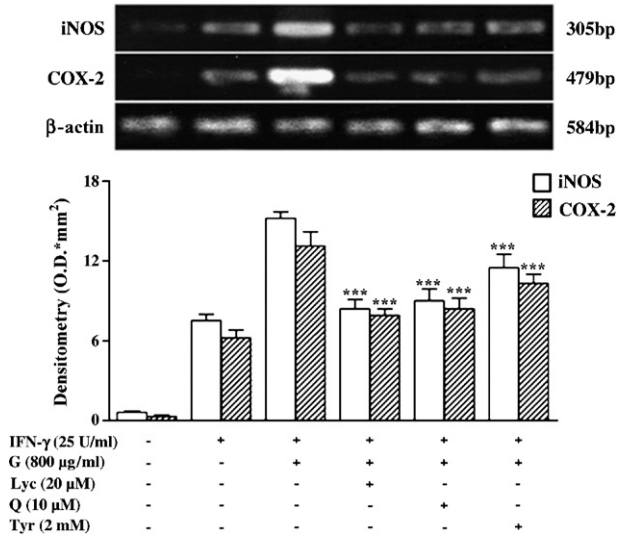

Fig. 3. Effect of lycopene (Lyc), quercetin (Q) and tyrosol (Tyr) on the increase of iNOS and COX-2 mRNA expression induced by IFN-γ and gliadin in RAW 264.7 cells at 24 h. mRNA levels were measured by RT-PCR as described under "Materials and methods". β-actin mRNA levels are shown as control. Data are from a single experiment and representative of three separate experiments. Densitometric data are reported are expressed as mean±S.E.M. of three separate experiments. ***$P<0.0001$ *vs*. IFN-γ and gliadin.

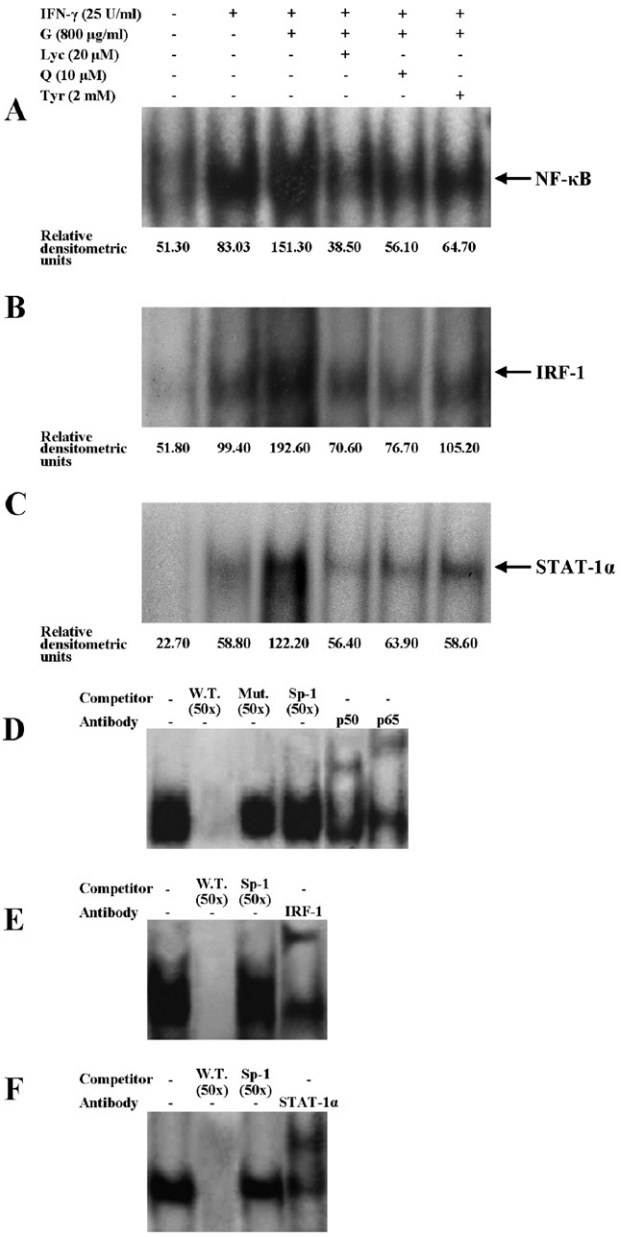

Fig. 4. Representative EMSA as well as relative densitometric analysis shows the effect of lycopene (Lyc), quercetin (Q) and tyrosol (Tyr) on the increase of (A) NF-κB, (B) IRF-1 and (C) STAT-1α/DNA binding activity in RAW 264.7 stimulated with gliadin and IFN-γ for 24 h. Data are from a single experiment and are representative of five separate experiments. Densitometric data are expressed as units of optical density (mm^2) and reported under "Results" as percentage of the mean±S.E.M. of six separate experiments. (D) In competition reaction nuclear extracts were incubated with radiolabeled NF-κB probe in absence or presence of: identical but unlabeled oligonucleotides (W.T., 50×), mutated non-functional NF-κB probe (Mut., 50×) or unlabeled oligonucleotide containing the consensus sequence for Sp-1 (Sp-1, 50×). (E) In competition reaction nuclear extracts were incubated with radiolabeled IRF-1 probe in absence or presence of: identical but unlabeled oligonucleotides (W.T., 50×) or unlabeled oligonucleotide containing the consensus sequence for Sp-1 (Sp-1, 50×). (F) In competition reaction nuclear extracts were incubated with radiolabeled STAT-1α probe in absence or presence of: identical but unlabeled oligonucleotides (W.T., 50×) or unlabeled oligonucleotide containing the consensus sequence for Sp-1 (Sp-1, 50×). Data in (D, E and F) are from a single experiment and are representative of three separate experiments.

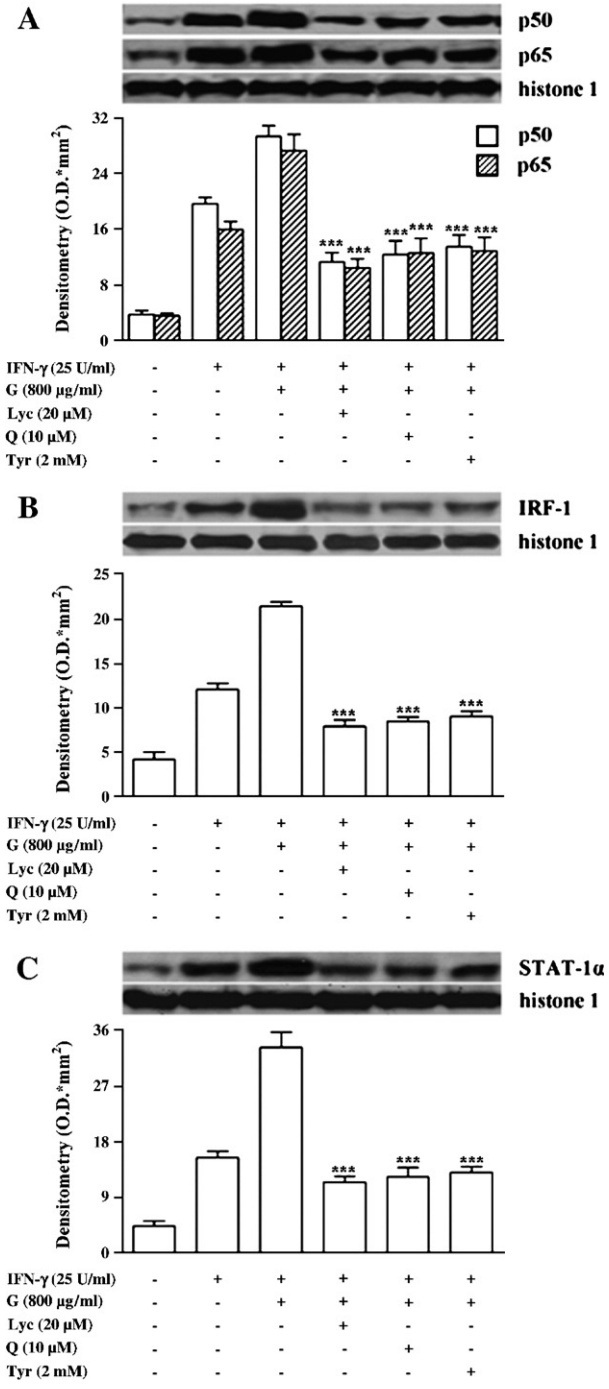

Fig. 5. Representative Western blot shows the effect of lycopene (Lyc), quercetin (Q) and tyrosol (Tyr) on the increase of (A) p50 and p65, (B) IRF-1, (C) STAT-1α protein expression induced by IFN-γ and gliadin in RAW 264.7 macrophages at 24 h. Histone 1 expression is shown as a control. Data are from a single experiment and are representative of three separate experiments. Densitometric data are reported are expressed as mean±S.E.M. of three separate experiments. ***$P<0.0001$ *vs.* IFN-γ and gliadin.

1.6% and 45.3±2.8%, respectively for IRF-1; by 55.3±1.9%, 47.7±2.1% and 52.0±5.5%, respectively for STAT-1α; $n=5$) (Fig. 4A, B and C). The composition of protein-DNA binding complexes of respective transcription factors was determined by competition assay. In the reaction of 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 complex, suggesting that the NF-κB complex contained p50 and p65 dimers (Fig. 4D). Also the specificity of IRF-1 or STAT-1α/DNA binding complexes was evident by the complete displacement of protein-DNA binding in the presence of a 50-fold molar excess either of unlabeled IRF-1 and STAT-1α probe, respectively. In contrast a 50-fold molar excess either of unlabeled mutated IRF-1 or STAT-1α probe (data not shown) or Sp-1 oligonucleotide had no effect on DNA/binding activity. Addition of anti-IRF-1 or anti-STAT-1α antibody to the binding reaction clearly gave rise to a characteristic supershift of the retarded complex, respectively (Fig. 4E and F). Moreover, the presence of p50 and p65 as well as IRF-1 and STAT-1α subunits in nuclear fraction was examined by immunoblotting analysis. Unstimulated cells expressed basal levels of p50 and p65, IRF-1 and STAT-1α. Upon stimulation with IFN-γ, cells exhibited p50 and p65, IRF-1 and STAT-1α high nuclear levels which were increased by gliadin. Lycopene as well as quercetin or tyrosol prevented the nuclear translocation of respective subunits (by 61.7±1.3%, 53.9±2.1% and 52.9±2.0% for p50; by 61.4±1.3%, 57.9±2.0% and 54.0±1.7% for p65; by 63.1±0.7%, 57.7±0.6% and 63.0±1.5% for IRF-1;) by 60.4±0.5%, 65.6±1.0% and 60.5±1.0% for STAT-1α, respectively; $n=3$) (Fig. 5A, B and C).

3.4. Effect of lycopene, quercetin and tyrosol on intracellular reactive oxygen species production

In order to clarify whether the inhibitory effect of lycopene, quercetin and tyrosol on NF-κB, STAT-1α and IRF-1 activation as well as iNOS and COX-2 expression was mediated through the inhibition of reactive oxygen species generation induced by gliadin, we measured intracellular reactive oxygen species production in RAW 264.7 macrophages stimulated with IFN-γ. Exposure of

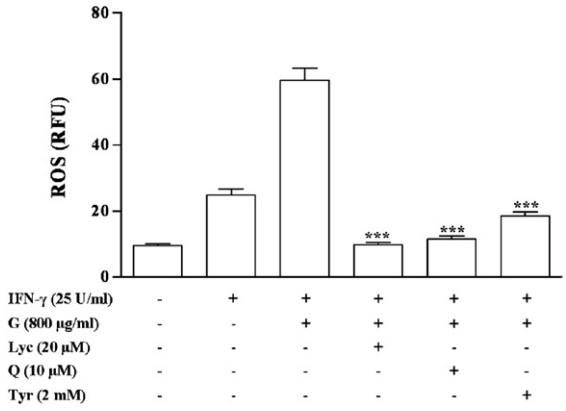

Fig. 6. Effect of lycopene (Lyc), quercetin (Q) and tyrosol (Tyr) on intracellular reactive oxygen species production by RAW 264.7 macrophages stimulated with IFN-γ and gliadin for 24 h. Data are expressed as means±S.E.M. of three independent experiments ($n=10$). ***$P<0.001$ *vs.* IFN-γ and gliadin. RFU, relative fluorescence unit.

RAW 264.7 macrophages to IFN-γ for 24 h resulted in an increased reactive oxygen species production compared with unstimulated cells. Addition of gliadin to the cells caused a further accumulation of reactive oxygen species which was significantly reduced by lycopene, quercetin and tyrosol (by 83.5±0.7%, 80.7±1.0% and 68.8±1.1%, respectively; $n=10$) (Fig. 6).

4. Discussion

The molecular mechanisms by which gluten/gliadin induces damage in celiac disease are not completely known. Actually, there is overwhelming evidence that mucosal lesion in gluten sensitivity is initiated by activated macrophages which, in turn, produce high levels of proinflammatory mediators (Marsh, 1992). It has been reported that chronic inflammation results in a dramatic increase of free radical production in celiac disease (Pavlick et al., 2002). Excessively produced NO is known to act as a free radical and cause tissue damage (Grisham et al., 2002). High levels of NO are present in serum and urine of children with celiac disease and correlated with an increased iNOS expression in the small intestine (ter Steege et al., 1997; Beckett et al., 1998; Holmgren Peterson et al., 1998; Beckett et al., 1999; van Straaten et al., 1999). Moreover, it has been reported that in homogenized small bowel biopsy specimens from patients with active celiac disease, PGE_2 levels are increased and involved in the pathophysiology of diarrhoea (Lavö et al., 1990). Previous studies have shown that iNOS is expressed more in enterocytes and COX-2 in the cells of lamina propria (Kainulainen et al., 2002). These enzymes have been shown to be involved in disease induction and maintenance (Lavö et al., 1990; ter Steege et al., 1997; Beckett et al., 1999; van Straaten et al., 1999). We have previously reported that gliadin increases iNOS gene expression in IFN-γ-stimulated RAW 264.7 cells through a mechanism involving NF-κB, IRF-1 and STAT-1α and these effects were prevented by synthetic and natural antioxidants (Maiuri et al., 2003, De Stefano et al., 2006). The results of the present study show that other strong antioxidants from food, namely lycopene, quercetin and tyrosol inhibit iNOS and COX-2 expression in RAW 264.7 stimulated with IFN-γ and gliadin for 24 h. First, we found that lycopene, quercetin and tyrosol reduced in a concentration-dependent manner nitrite and PGE_2 production which was correlated and quantitatively comparable with a reduction either of iNOS and COX-2 mRNA and protein expression. The inhibition of iNOS and COX-2 gene expression by lycopene, quercetin and tyrosol occurred at transcriptional level by preventing NF-κB, IRF-1 and STAT-1α activation. Moreover, EMSA experiments using antibodies against p50 and p65 as well as IRF-1 and STAT-1α allowed us to confirm that the formation of specific protein-DNA complexes induced by gliadin and IFN-γ involved NF-κB, IRF-1 and STAT-1α. Lycopene, quercetin and tyrosol drastically reduced NF-κB, IRF-1 and STAT-1-α/DNA binding activity as well as respective nuclear subunit level. Moreover, it is well known that NF-κB, IRF-1 and STAT-1α activation is dependent on the intracellular redox state (Pahl 1999; Ramana et al., 2000; Kroger et al., 2002). Natural and synthetic antioxidants have been reported to have anti-inflammatory properties; a likely target for these compounds seems to be the signal transduction cascade leading to the activation of transcription factors (Hecker et al., 1996; Epinat and Gilmore 1999; Faure et al., 1999; D'Acquisto et al., 2001). We have found that blockade of NF-κB, IRF-1 and STAT-1α by lycopene, quercetin and tyrosol was correlated with the inhibition of reactive oxygen species generation induced by gliadin and IFN-γ. Some observations report that naturally occurring antioxidants may be overwhelmed during active episodes of celiac disease (Pavlick et al., 2002). It has been reported that administration of antioxidants in drinking water restores physiological scavenger levels thus attenuating colonic inflammation (Pavlick et al., 2002). Furthermore, dietary supplementation with antioxidants has been proved to be very effective in treating a wide kind of mucosal injuries (Pavlick et al., 2002). Recently, it has been proved that oral administration of sulfasalazine, a common drug used in the treatment of many inflammatory bowel diseases, is very effective because of its metabolite 5-aminosalicylic acid (5-ASA). This metabolite has been demonstrated to be a very potent antioxidant and free radical scavenger (Pavlick et al., 2002). Basing on these observations it has been raised the hypothesis that in celiac disease, besides the current clinical approach mainly based on the adoption of a strict gluten exclusion from diet, both a supplementation of diet with antioxidants and administration of drugs having antioxidant properties could be effective for celiac disease treatment. Further studies are needed to evaluate the possibility to prevent/counteract gliadin citotoxicity by dietary intake of antioxidants. In conclusion, our results suggest that lycopene, quercetin and tyrosol, by preventing NF-κB, IRF-1 and STAT-1α activation and pro-inflammatory related genes, may represent potential non toxic agents for the control of intestinal inflammation in celiac disease.

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