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Inhibition of nitric oxide biosynthesis by anthocyanin fraction of blackberry extract

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Abstract

Anthocyanins are natural colorant belonging to the flavonoid family, widely distributed among flowers, fruits, and vegetables. Some flavonoids have been found to possess anticarcinogenic, cytotoxic, cytostatic, antioxidant, and anti-inflammatory properties. Since increased nitric oxide (NO) plays a role in inflammation, we have investigated whether the pharmacological activity of the anthocyanin fraction of a blackberry extract (cyanidin-3-*O*-glucoside representing about 88% of the total anthocyanin content) was due to the suppression of NO synthesis. The markedly increased production of nitrites by stimulation of J774 cells with lipopolysaccharide (LPS) for 24 h was concentration-dependently inhibited by the anthocyanin fraction (11, 22, 45, and 90 μg/ml) of the extract. Moreover, this inhibition was dependent on a dual mechanism, since the extract attenuated iNOS protein expression and decreased the iNOS activity in lungs from LPS-stimulated rats. Inhibition of iNOS protein expression appeared to be at the transcriptional level, since the extract and similarly cyanidin-3-*O*-glucoside (10, 20, 40, and 80 μg/ml, amounts corresponding to the concentrations present in the extract) decreased LPS-induced NF-κB activation, through inhibition of IκBα degradation, and reduced ERK-1/2 phosphorylation in a concentration-dependent manner. In conclusion, our study demonstrates that at least some part of the anti-inflammatory activity of blackberry extract is due to the suppression of NO production by cyanidin-3-*O*-glucoside, which is the main anthocyanin present in the extract. The mechanism of this inhibition seems to be due to an action on the expression/activity of the enzyme. In particular, the protein expression was inhibited through the attenuation of NF-κB and/or MAPK activation.

Keywords: Cyanidin-3-*O*-glucoside; Blackberry extract; iNOS; NF-κB; ERK-1/2; Macrophages

Anthocyanins, glycosylated polyhydroxy, and polymethoxy derivatives of flavilium salts, are natural colorant belonging to the flavonoid family. They are widely distributed among flowers, fruits, and vegetables. Epidemiological studies have shown that the consumption of vegetables, fruits, and tea is associated with a decreased risk of cancer and flavonoids are believed to play an important role in delaying or preventing carcinogenesis [1–4]. Some flavonoids have been found to possess anticarcinogenic [4,5], anti-inflammatory [6], antiviral [7], cytotoxic [8], cytostatic [9], and antioxidant [10] properties. For example, cyanidin-3-*O*-glucoside exhibits free radical scavenging activity [11], protects against endothelial dysfunction [12], and decreases myocardium damage [13]. Thus, a plant-derived diet might contain certain flavonoids that exert these healthy effects, thereby protecting from some diseases.

Nitric oxide (NO) is known to participate in the physiological and pathological functions of many organs [14], in fact it plays a major role in regulating vascular tone, neurotransmission, killing of microorganisms and tumour cells, and other homeostatic mechanisms [15]. High levels of NO have been described in a variety of pathophysiological processes including various forms of circulatory shock [16], inflammation [17], and carcino-

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genesis [18]. NO is synthesized by nitric oxide synthases (NOS) [14]. To date, three NOS isoforms have been identified: neuronal NOS (nNOS or NOS I), inducible NOS (iNOS or NOS II), and endothelial NOS (eNOS or NOS III) [19]. The nNOS and eNOS isoforms are constitutively expressed in neuronal and endothelial cells, respectively, and are Ca^{2+}/calmodulin-dependent. The iNOS isoform is not constitutively present, it can be rapidly induced by inflammatory stimuli, including toxins such as lipopolysaccharide (LPS) and cytokines, and its activation is Ca^{2+}/calmodulin-independent [20]. NO production by iNOS is mainly regulated at the transcriptional level [21,22]. In macrophages, LPS activates the transcription factor nuclear factor-κB (NF-κB), which leads to induction of expression of many immediate early genes [23]. Exposure of cells to LPS causes rapid phosphorylation of inhibitor κB (IκB) by IκB kinase (IKK), with subsequent degradation by proteosomes. Dissociation of IκB from NF-κB allows the activated free dimer to translocate to the nucleus [24], where it induces the gene transcription through the *cis*-acting κB element. The NF-κB activation is regulated by mitogen-activated protein kinases (MAPK) [25]. In fact, the extracellular signal-regulated kinases (ERK) 1/2 signalling pathway have been often implicated in NF-κB activation through phosphorylation and subsequent degradation of the inhibitory subunit IκBα [26,27].

In this study, we have examined, in murine macrophages J774 cell line stimulated with LPS, the effects of anthocyanins present in a blackberry extract and cyanidin-3-*O*-glucoside on NO production, iNOS protein expression and signal pathways involved in its regulation. To this aim, we have utilised a blackberry extract, whose content in anthocyanins was mainly due (by about 88%) to cyanidin-3-*O*-glucoside. Small amounts of other anthocyanins, some of them only recently identified [28], were also present.

Experimental procedures

Anthocyanins contained in blackberry extract

Blackberry fruits were obtained as commercial products. Fruit extract (43.6 g) was obtained by pounding in a mortar about 1 kg of fruits. The extract was centrifuged at 6000 rpm for 20 min, the juice filtered on glass wool, and the pulps extracted overnight with about 1 L of ethyl alcohol (EtOH)/HCl (99:1, v/v) at room temperature. The filtered extract was concentrated under reduced pressure and diluted with water (1:1, v/v). Both the filtered juice and the pulp extract were applied to a C18 silica column previously activated with 10 ml of EtOH and 10 ml of water. Anthocyanins were extracted with 1 L of EtOH/HCl (99:1, v/v) and the solvent removed under reduced pressure. The anthocyanin fraction so obtained was used for chemical characterisation [28] and in vitro studies. The anthocyanin content was determined spectrophotometrically by absorbance at 518 nm and by HPLC analysis with photodiode array detector using a calibration curve obtained by analysing different concentrations of cyanidin-3-*O*-glucoside (Extrasynthese) (Fig. 1A) standard (external standard method). The curve was used to obtain quantitative data for all the components of the anthocyanin fraction. This is possible because anthocyanins present in blackberry extract have similar spectroscopic properties and a comparable molecular mass and consequently concentrations can be correctly expressed in terms of cyanidin-3-*O*-glucoside, available as pure standard. In HPLC analysis, the areas of the five peaks were summed and measured against cyanidin-3-*O*-glucoside curve (Fig. 1B). The extract contained 331 mg of anthocyanins expressed as cyanidin-3-*O*-glucoside. Cyanidin-3-*O*-glucoside was the main component of the fraction (88.0%); pelargonidin-3-*O*-glucoside, cyanidin-3-*O*-xyloside, malvidin-3-*O*-glucoside, and unknown anthocyanins were also present at a level of 2.5, 7.1, 1.2, and 1.2% respectively. Values reported are the average of three

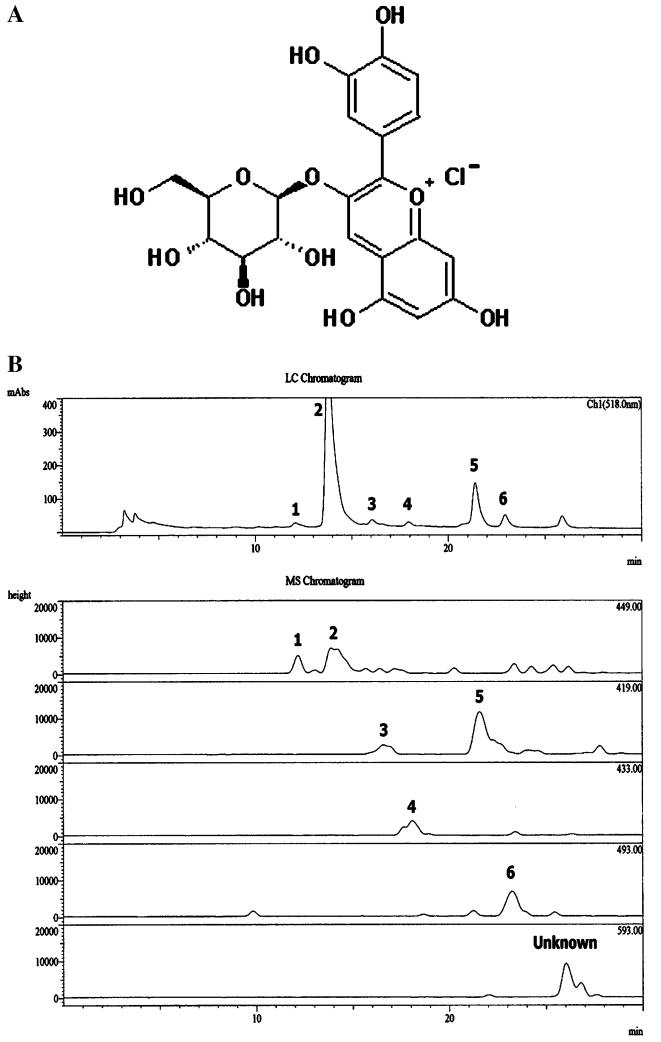

Fig. 1. (A) Structure of cyanidin-3-*O*-glucoside (as chloride). (B) HPLC-UV and MS chromatograms, (see Experimental procedures), of anthocyanin fraction of blackberry extract. 1. cyanidin-3-*O*-galactoside, 2. cyanidin-3-*O*-glucoside, 3. cyanidin-3-*O*-arabinoside, 4. pelargonidin-3-*O*-glucoside, 5. cyanidin-3-*O*-xyloside, and 6. malvidin-3-*O*-glucoside.

replicates, and coefficients of variation (CV%) were lower than 3.5% for both spectrophotometric and HPLC methods.

Cell culture

The murine monocyte/macrophage J774 cell line was grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 2 mM glutamine, 25 mM Hepes, penicillin (100 U/ml), streptomycin (100 μg/ml), 10% foetal bovine serum (FBS), and 1.2% Na pyruvate.

Cells were plated to a seeding density of in P60 well plates and 2.5×10^5 in 24 multiwell. Cells were pre-treated (for 2 h) with increasing concentration of test compounds, and stimulated with LPS from *Escherichia coli*, Serotype 0111:B4, (10 μg/ml). Treatment with test compounds and/or LPS was carried out under serum-free conditions.

Cell viability

Cell respiration, an indicator of cell viability, was assessed by the mitocondrial-dependent reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan [29]. After stimulation with LPS in the absence or presence of anthocyanins of the blackberry extract (11, 22, 45, and 90 μg/ml) for 24 h, cells were incubated in 96-well plates with MTT (0.2 mg/ml), for 1 h. Culture medium was removed by aspiration and the cells were solubilized in DMSO (0.1 ml). The extent of reduction of MTT to formazan within cells was quantified by the measurement of OD_{550}.

Nitrite assay

Cells were pre-treated with test compounds for 2 h and further incubated for 24 h with LPS (10 μg/ml). At the end of the incubation, the supernatants were collected for the nitrite measurement. The nitrite concentration in the samples was measured by the Griess reaction, by adding 100 μl of Griess reagent (0.1% naphthylethylenediamide dihydrochloride in H_2O and 1% sulphanilamide in 5% concentrated H_2PO_4; vol. 1:1) to 100 μl samples. The optical density at 550 nm (OD_{550}) was measured using ELISA microplate reader (SLT-Labinstruments Salzburg, Austria). Nitrite concentration was calculated by comparison with OD_{550} of standard solutions of sodium nitrite prepared in culture medium.

Animals

Male Wistar rats (Charles River), weighting 250–300 g, were used after 1 week for adaptation to the housing conditions. Rats were housed in a controlled environment and provided with standard rodent chow and water. Animal care was in compliance with Italian regulations (DM 116192) on protection of animals used for experimental and other scientific purpose, as well as with the EEC regulations (OJ of EC L 358/1 18/12/1986).

Inducible NO synthase preparation

The inducible isoform of NO synthase was prepared from lungs of rats treated with LPS (6 mg/kg, i.p.) or vehicle (saline). Six hours after treatment, animals were anaesthetized with urethane (1.3 g/Kg), the right ventricle was cannulated and the lung, perfused through the pulmonary artery for 2–3 min with 0.9% NaCl (5 ml/min, outflow via the pulmonary vein), was explanted and frozen in liquid nitrogen. Lungs were homogenized at 4 °C in 4 volumes of Hepes buffer 20 mM, pH 7.2, containing 320 mM sucrose, 1 mM DL-dithiothreitol, 10 μg/ml soybean trypsin inhibitor, 2 μg/ml aprotinin, and 10 μg/ml leupeptin. The homogenate was centrifuged at 100,000*g* (L8-70 ultracentrifuge, Beckman) for 30 min at 4 °C. The supernatants, i.e., the cytosolic fractions containing NO synthase activity, were stored at −80 °C until use. Protein concentration in the cytosolic fraction was measured spectrophotometrically using the Bio-Rad protein assay kit (Bio-Rad Laboratories) and bovine serum albumin as standard.

Assay of NO synthase activity

NOS activity was evaluated by measuring the rate of conversion of L-[U-^{14}C]arginine to citrulline, according to Salter [30]. Briefly, an aliquot of the cytosolic fraction was pre-incubated for 5 min at 37 °C in 50 mM potassium phosphate buffer, pH 7.2 in the presence or absence of anthocyanins of the blackberry extract (11, 22, 45, and 90 μg/ml). Samples were then incubated for 10 min at 37 °C with L-[U-^{14}C]arginine (150,000 dpm, specific activity 313 mCi/mmol, Perkin-Elmer Life Sciences) and 20 μM L-arginine (Sigma). The reaction was stopped by the addition of 1.0 ml of a mixture of H_2O/Dowex-50W 1:1 v/v (Sigma). The Na^+-form of Dowex-50W was prepared by washing four times the H^+-form of resin with 1 M NaOH and then with bi-distilled water until the pH was less than 7.5. The resin was settled by centrifugation (11,000*g* for 3 min) in a microfuge and an aliquot of the supernatant was taken for scintillation counting. The activity of inducible Ca^{2+}/independent enzyme was determined from the difference between the labelled citrulline produced by samples containing 1 mM EGTA and samples containing 1 mM EGTA plus 1 mM N^G-monomethyl-L-arginine (L-NMMA), a NOS inhibitor. The activity was expressed as pmol/min/mg of protein.

Western blot analysis

The analysis of pERK-1/2, ERK-2, and iNOS in J774 macrophages was performed on whole cell lysates. After stimulation with LPS for 15 min (pERK-1/2, ERK-2) or 24 h (iNOS), cells were washed with cold PBS and lysed for 10 min at 4 °C with lysis buffer (50 mM Tris, pH 7.4, 0.5% Nonidet P-40, and 0.01% SDS) containing complete protease inhibitor cocktail (Roche Applied Science). Lysates from adherent cells were collected by scraping and centrifuged at 12,000*g* for 15 min at 4 °C. The supernatants were

collected and protein concentration in cell lysates was determined by Bio-Rad Protein Assay (Bio-Rad). The analysis of IκBα was performed on cytosolic extract. Thirty minutes after LPS stimulation, cells were washed with cold PBS, harvested in cold hypotonic lysis buffer (10 mM Hepes, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM phenylethylsulphonylfluoride, 1.5 μg/ml soybean trypsin inhibitor, 7 μg/ml pepstatin A, 5 μg/ml leupeptin, 0.1 mM benzamidine, and 0.5 mM DTT) and incubated on ice for 15 min. The cells were lysed by rapid passage through a syringe needle for five times and the cytoplasmic fraction was then obtained by centrifugation at 13,000*g* for 1 min at 4 °C. The supernatants were collected and protein concentration was determined.

Equal amounts of protein (50 μg) were mixed with gel loading buffer (50 mM Tris, 10% SDS, 10% glycerol, 10% 2-mercaptoethanol, and 2 mg/ml of bromophenol) in a ratio of 1:1, boiled for 3 min and centrifuged at 10,000*g* for 10 min. Each sample was loaded and electrophoresed on a 10% SDS–polyacrylamide gel. The proteins were transferred on to nitrocellulose membranes (Hybond ECL Nitrocellulose, Amersham). The membranes were blocked with 0.1% PBS-Tween containing 5% non-fat dry milk for ERK-2, IκBα, iNOS and β-actin, whereas with 0.1% PBS-Tween containing 5% non-fat dry milk and 50 mM NaF for pERK-1/2. After the blocking, the membranes were incubated with the relative primary antibody overnight at 4 °C. Mouse monoclonal antibodies anti ERK-2, IκBα (Santa Cruz Biotechnology) and anti iNOS (BD Transduction Laboratories) were diluted 1:1000 in 0.1% PBS-Tween, 5% non-fat dry milk; mouse monoclonal antibody anti pERK-1/2 (Santa Cruz Biotechnology) was diluted 1:1000 in 0.1% PBS-Tween, 5% non-fat dry milk, 50 mM NaF; mouse monoclonal antibody anti β-actin (Sigma–Aldrich) was diluted 1:10000 in 0.1% PBS-Tween, 5% BSA. After the incubation, the membranes were washed six times with 0.1% PBS-Tween and were incubated for 1.5 h at room temperature with horseradish peroxidase-conjugated anti-mouse secondary antibodies (Dako Cytomation) diluted 1:1000 in 0.1% PBS-Tween containing 5% non-fat dry milk. The membranes were washed and protein bands were detected by an enhanced chemiluminescence system (Amersham Pharmacia). Densitometric analysis was performed with a Fluor S quantitative imaging system (Bio-Rad Laboratories).

Electrophoretic mobility shift assay

To analyze NF-κB DNA binding activity, total cell extracts were prepared by using a detergent lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 50 mM NaF, 1 mM Na_3VO_4, 0.5% Nonidet P-40, 0.5 mM dithiothreitol, and complete protease inhibitors mixture (Roche Applied Science)). After LPS stimulation, cells were harvested by centrifugation at 4 °C for 10 min at 180*g*, washed on ice in cold PBS, and resuspended in detergent lysis buffer (30 μl/5×10^6 cells). The cell lysate was incubated on ice for 30 min and then centrifuged at 4 °C for 5 min at 10,000*g*. The protein content of the supernatant was determined, and equal amounts of protein (10 μg) were added to a reaction mixture containing 20 μg of bovine serum albumin, 2 μg of poly(dI–dC), 10 μl of binding buffer (20 mM Hepes, pH 7.9, 10 mM $MgCl_2$, 20% glycerol, 100 mM KCl, 0.2 mM EDTA, 0.5 mM dithiothreitol, and 0.5 mM phenylmethylsulfonylfluoride), and 150,000 dpm of a ^{32}P-labeled consensus NF-κB binding oligonucleotide (Santa Cruz Biotechnology) in a final volume of 20 μl. The specificity of the DNA–protein binding was determined by competition reactions in which a 50-fold molar excess of unlabelled oligonucleotide was added to the binding reaction. Samples were incubated at room temperature for 30 min and run on a non-denaturing 4% polyacrylamide gel. Autoradiography was done for 18 h on Kodak XAR 5 film.

Statistical analysis

The results are expressed as means ± standard error (SEM) of the mean of n observations, where n represents the number of experiments performed in different days. Triplicate wells were used for the various treatment conditions. The results were analysed by one-way ANOVA followed by a Bonferroni post hoc test for multiple comparisons. A p-value less than 0.05 was considered significant.

Results

Effect of anthocyanin fraction of blackberry extract on LPS-induced nitrite production

To asses whether the anthocyanin fraction of the blackberry extract was able to reduce NO production we utilized the Griess assay to measure nitrite, the stable end-product of NO, in supernatants of the murine macrophage cell line J774 stimulated with LPS (10 μg/ml). A marked increase of nitrite production in the cell medium was observed at 24 h (23.10 ± 0.55 nmol/10^6 cells; $p < 0.001$, $n = 3$) in respect to unstimulated macrophages (3.12 ± 0.40 nmol/10^6 cells). When the anthocyanin fraction of the blackberry extract (0, 11, 22, 45, and 90 μg/ml) was added to J774 macrophages, 2 h before LPS stimulation, a significant ($p < 0.001$, $n = 3$) and concentration-dependent decrease of nitrite production in cell medium was observed (Fig. 2A). In fact, the observed inhibition was 23, 34, 60, and 70% at 11, 22, 45, and 90 μg/ml respectively ($p < 0.001$, $n = 3$). The IC_{50} was 36 μg/ml (95% IC 24–53 μg/ml; r^2 0.976). The aminoguanidine (10 μg/ml), a well known inhibitor of iNOS enzyme, inhibited the nitrite production by 84% ($p < 0.001$, $n = 3$).

To exclude the possibility that the inhibitory effect of the anthocyanin fraction of the blackberry extract on LPS-induced NO production was due to cytotoxicity, J774 cells, stimulated for 24 h with LPS after pre-treatment with test compound, were tested for cell viability using MTT assay. No significant difference was observed between the viability

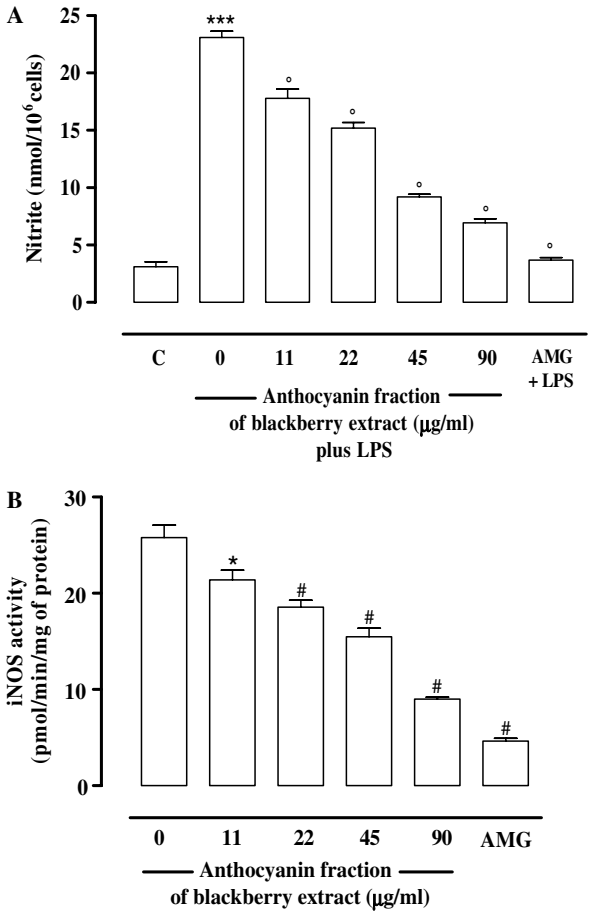

Fig. 2. Effect of anthocyanin fraction of blackberry extract on LPS-induced nitrite production (A) and iNOS activity (B). (A) J774 cells were pre-treated for 2 h with test compound (0, 11, 22, 45, and 90 μg/ml), prior to LPS stimulation (10 μg/ml) for 24 h. Aminoguanidine (AMG: 10 μg/ml), a known inhibitor of NOS enzyme, acted as a positive control. Unstimulated J774 cells acted as a negative control (C). Nitrites, stable end-products of NO, were measured by the Griess reaction. (B) iNOS enzyme was prepared from lungs of LPS-treated rats (6 mg/kg, i.p) 6 h after stimulation and iNOS activity was evaluated by measuring the rate of conversion of L-[U-^{14}C]arginine to [U-^{14}C]citrulline (see Experimental procedures). Data are expressed as means ± SEM from three separate experiments performed in triplicate. ***$P < 0.001$ vs unstimulated cells (C), °$p < 0.001$ vs LPS alone; *$p < 0.05$; #$p < 0.001$ vs iNOS activity (0 μg/ml anthocyanin fraction blackberry extract).

of cells treated with LPS and cells treated with test compound plus LPS, at any of the concentrations examined (data not shown).

Effect of anthocyanin fraction of blackberry extract on iNOS catalytic activity

To evaluate the effect of anthocyanin fraction of the blackberry extract on inducible enzyme activity, we utilized as a source of the enzyme the lung of LPS-treated rats. The inducible NO synthase activity from lungs of LPS-treated rats was 25.8 ± 1.29 pmol/min/mg of protein ($n = 3$). No activity was present in the lungs of vehicle treated rats. The anthocyanin fraction of the blackberry extract (0, 11, 22, 45, and 90 μg/ml) significantly inhibited in a concentration-dependent manner the activity of the enzyme (Fig. 2B). In fact, the observed inhibition was 17 ($p < 0.05$; $n = 3$), 28, 40, and 65% ($p < 0.001$, $n = 3$) at 11, 22, 45, and 90 μg/ml respectively. The IC_{50} was 56 μg/ml (95% IC 37 to 84 μg/ml; r^2 0.977). The aminoguanidine (10 μg/ml) inhibited the iNOS catalytic activity by 83% ($p < 0.001$, $n = 3$).

Effect of anthocyanin fraction of blackberry extract on LPS-induced iNOS expression

To determine whether the inhibition of LPS-induced nitrite production by test compound was mediated by the regulation of iNOS protein expression, next we evaluated the levels of iNOS protein in whole lysates from J774 cells stimulated with LPS and pre-treated with test compound, as described above. As shown in Fig. 3, LPS (10 μg/ml) caused, at 24 h, a significant increase of iNOS protein levels compared to unstimulated cells (control). When cells were pre-treated with the anthocyanin fraction (0, 11, 22, 45, and 90 μg/ml) for 2 h before LPS stimulation, iNOS protein expression was inhibited in concentration-dependent manner in respect to LPS-stimulated cells, but only from the concentration of 22 μg/ml ($p < 0.001$, $n = 3$). In fact, the observed inhibition was 13%, at the lowest concentration

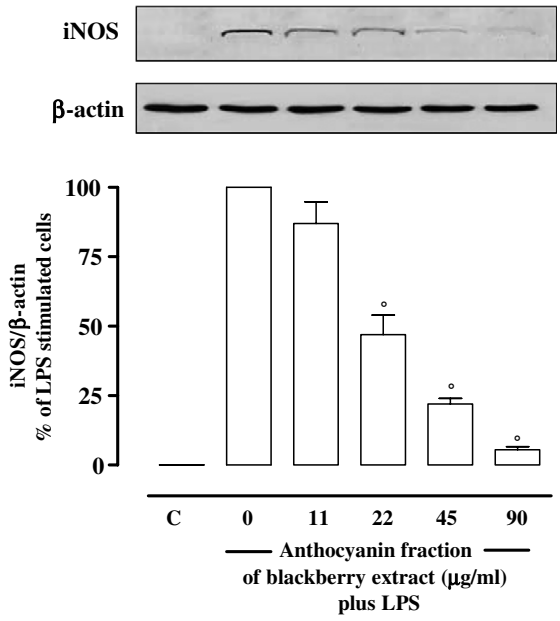

Fig. 3. Effect of anthocyanin fraction of blackberry extract on LPS-induced iNOS expression. J774 cells, pre-treated (for 2 h) with increasing concentration of test compound (0, 11, 22, 45 and 90 μg/ml), were stimulated with LPS (10 μg/ml) for 24 h. Equal amounts of whole lysates, containing 50 μg of proteins, were fractionated by 10% SDS–PAGE and subjected to Western blot analysis using 1:1000 diluted iNOS antiserum. iNOS protein was visualized by chemoluminescence Western blotting detection reagents. The bands corresponding to iNOS protein were quantified by densitometric analysis and the results are expressed as a percentage of LPS-stimulated cells. Densitometric analysis of iNOS protein expression represents the mean ± SEM from three separate experiments. Data were normalized on the basis of β-actin levels. °$p < 0.001$ vs LPS alone.

tested (11 μg/ml, $p > 0.05$, $n = 3$), 53, 78, and 94.5% at 22, 45, and 90 μg/ml, respectively ($p < 0.001$, $n = 3$) (Fig. 3). Test compound given alone to J774 cells did not influence iNOS protein expression in respect to control cells (data not shown).

Effect of anthocyanin fraction of blackberry extract on NF-κB activation

Because activation of NF-κB is critical for the induction of iNOS by LPS, we determined whether the anthocyanin fraction of the blackberry extract might suppress NF-κB activation in LPS-activated macrophages. To this aim, we performed an electrophoretic mobility shift assay (EMSA) on extract from J774 pre-treated (2 h before LPS) with the highest concentration (90 μg/ml) of test compound. LPS treatment caused nuclear translocation of NF-κB and induced its DNA binding activity in a time dependent manner, which peaked between 0.5 and 1 h (data not shown). As shown in Fig. 4A, the induction of specific NF-κB binding activity by LPS was reduced by anthocyanin fraction of the blackberry extract. The specificity of the protein-DNA complex was confirmed by a competition assay. Binding was competed by the addition of non-radioactive κB oligonucleotide. Since it has been well documented that activation of NF-κB correlates with rapid proteolytic degradation of IκBα, the levels of this protein were investigated as an index of NF-κB inhibition by test compound. As shown in Fig. 4B, following cell stimulation with LPS for 30 min, a degradation of IκBα was observed, whereas pre-treatment (2 h before LPS) with test compound (0, 11, 22, 45, and 90 μg/ml) prevented IκBα degradation in a concentration-dependent manner, but only from the concentration of 22 μg/ml ($p < 0.001$, $n = 3$). In fact, the observed inhibition of IκBα degradation was 11%, at the lowest concentration tested (11 μg/ml, $p > 0.05$, $n = 3$), 48, 65, and 83% at 22, 45, and 90 μg/ml, respectively ($p < 0.001$, $n = 3$).

Effect of anthocyanin fraction of blackberry extract on LPS-induced ERK-1/2 activation

To investigate whether the inhibition of iNOS expression and IκBα degradation, observed in our experimental conditions, corresponded to a modulation of MAPK pathway, one of the signal transduction pathways involved in the regulation of iNOS expression, we analysed ERK-1/2 activation 15 min after LPS treatment, in the presence or absence of anthocyanins of the blackberry extract (2 h before LPS). In fact ERK signalling pathway has been often implicated in NF-κB activation through phosphorylation and subsequent degradation of the inhibitory subunit IκBα [26,27]. As shown in Fig. 5, stimulation of the cells in the presence of LPS-induced ERK-1/2 phosphorylation. In the presence of increasing concentrations of the anthocyanin fraction of the blackberry extract (0, 11, 22, 45, and 90 μg/ml) a concentration-dependent and significant inhibition of ERK-1/2 phosphorylation was observed, but

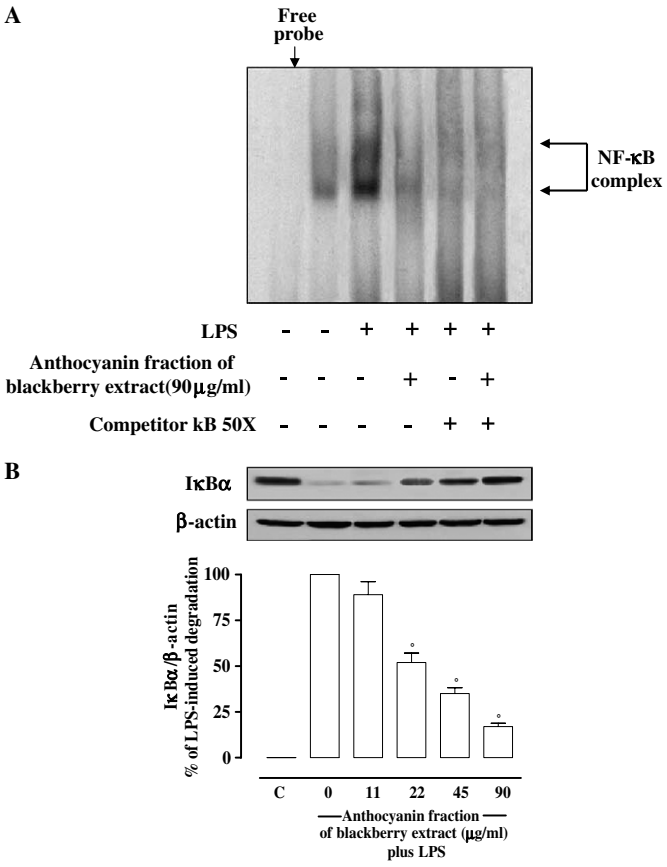

Fig. 4. Effect of anthocyanin fraction of blackberry extract on NF-κB activation (A) J774 cells, pre-treated (for 2 h) with test compound at the concentration of 90 μg/ml, were stimulated with LPS (10 μg/ml) for 30 min. EMSA was performed on total cell lysates containing 10 μg of proteins in the presence of a ^{32}P-labeled NF-κB oligonucleotide alone or with a 50-fold molar excess of analogous unlabeled oligonucleotide as competitor. Results are representative of three independent experiments. (B) J774 cells, pre-treated (for 2 h) with increasing concentration of test compound (0, 11, 22, 45, and 90 μg/ml), were stimulated with LPS (10 μg/ml) for 30 min. Equal amounts of cytosolic extract, containing 50 μg of proteins, were fractionated by 10% SDS–PAGE and subjected to Western blot analysis using 1:1000 diluted IκBα antiserum. IκBα protein was visualized by chemoluminescence Western blotting detection reagents. The bands corresponding to IκBα were quantified by densitometric analysis and the results are expressed as a percentage of LPS-induced degradation. Densitometric analysis of IκBα protein expression represents the mean ± SEM from three separate experiments. Data were normalized on the basis of β-actin levels. °$p < 0.001$ vs LPS alone.

only from the concentration of 22 μg/ml (71%, $p < 0.001$, $n = 3$). At 45 and 90 μg/ml the observed inhibition was 79 and 83%, respectively ($p < 0.001$, $n = 3$).

Effect of cyanidin-3-O-glucoside on LPS-induced iNOS expression, IκBα degradation and ERK-1/2 activation

Since cyanidin-3-*O*-glucoside accounted for the 88% of the anthocyanin fraction of the blackberry extract utilized, we investigated the effect of this anthocyanin on LPS-induced iNOS expression in J774 cells. Similarly to what observed with the extract, pre-treatment of the LPS-stimulated cells with increasing concentrations of the

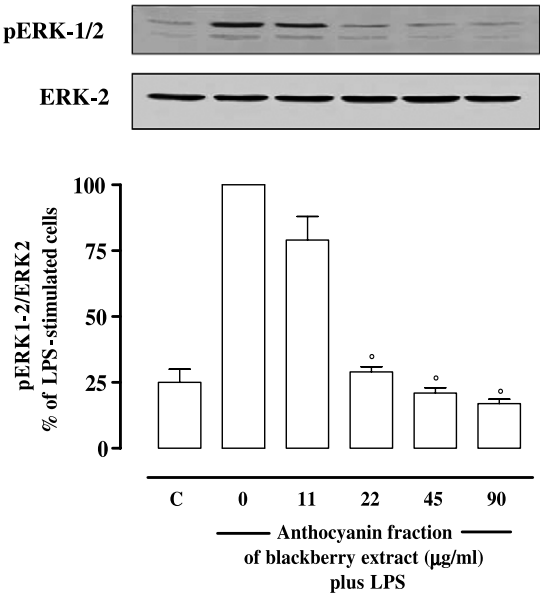

Fig. 5. Effect of anthocyanin fraction of blackberry extract on LPS-induced ERK-1/2 activation J774 cells, pre-treated (for 2 h) with increasing concentration of test compound (0, 11, 22, 45, and 90 μg/ml), were stimulated with LPS (10 μg/ml) for 15 min. Equal amounts of lysates containing 50 μg of proteins were fractionated by 10% SDS–PAGE and subjected to Western blot analysis using 1:1000 diluted pERK-1/2 antiserum. pERK-1/2 protein was visualized by chemoluminescence Western blotting detection reagents. The bands corresponding to pERK-1/2 protein were quantified by densitometric analysis and the results are expressed as a percentage of LPS-stimulated cells. Densitometric analysis of pERK-1/2 protein expression represents the mean ± SEM from three separate experiments. Data were normalized on the basis of ERK-2 levels. $^{\circ}p < 0.001$ vs LPS alone.

cyanidin-3-*O*-glucoside (0, 10, 20, 40, and 80 μg/ml, amounts corresponding to the concentrations present in the blackberry extract) inhibited iNOS expression in a concentration-dependent manner ($p < 0.001$, $n = 3$), but only from the concentration of 20 μg/ml (Fig. 6A). In fact, the observed inhibition was 23% at 10 μg/ml ($p > 0.05$, $n = 3$), the lowest concentration tested, 69, 81, and 99% at 20, 40, and 80 μg/ml, respectively ($p < 0.001$, $n = 3$). Moreover, similarly to what observed with the blackberry extract, cyanidin-3-*O*-glucoside (0, 10, 20, 40, and 80 μg/ml) prevented IκBα degradation and inhibited ERK-1/2 phosphorylation in a concentration-dependent manner ($p < 0.001$, $n = 3$), but only from the concentration of 20 μg/ml (44 and 48%, respectively). At the highest concentrations utilized the inhibition of IκBα degradation was 62% at 40 μg/ml and 80% at 80 μg/ml (Fig. 6B; $p < 0.001$, $n = 3$), whereas the inhibition of ERK-1/2 activation was 70% at 40 μg/ml and 74% at 80 μg/ml (Fig. 6C; $p < 0.001$, $n = 3$).

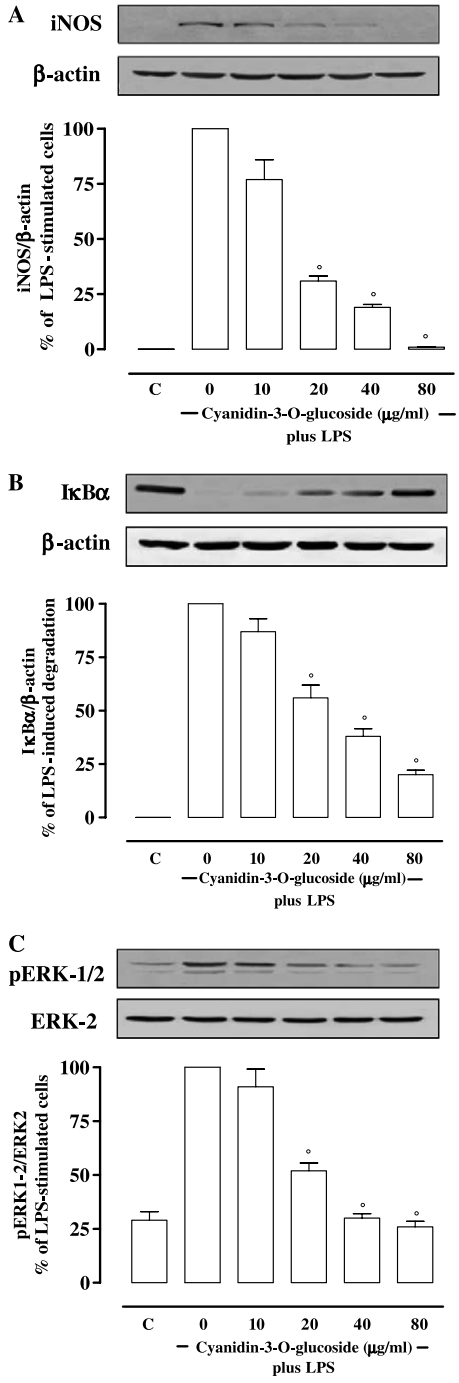

Fig. 6. Effect of cyanidin-3-*O*-glucoside on LPS-induced iNOS expression (A), IkBα degradation (B), and ERK-1/2 activation (C). J774 cells, pre-treated (for 2 h) with increasing concentration of cyanidin-3-*O*-glucoside (0, 10, 20, 40, and 80 μg/ml, amounts corresponding to the concentrations present in the blackberry extract), were stimulated with LPS (10 μg/ml) for 24 h (iNOS), 30 min (IκBα) and 15 min (pERK-1/2). Cell lysates and western blot analysis were performed as described (see Experimental procedures). The bands were quantified by densitometric analysis and the results are expressed as a percentage of LPS-stimulated cells (iNOS, pERK-1/2) and as a percentage of LPS-induced degradation (IκBα). Densitometric analysis of protein expression represents the mean ± SEM from three separate experiments. Data were normalized on the basis of β-actin (iNOS, IκBα) and ERK-2 (pERK-1/2) levels. $^{\circ}p < 0.001$ vs LPS alone.

Discussion

Recently, much attention has focused on the protective function of naturally occurring antioxidants in biological systems, and on the mechanisms of their action. Phenolic

compounds, which are widely distributed in plants, are considered to play an important role as dietary antioxidants for the prevention of oxidative damage caused, in living systems, by active oxygen radicals.

Anthocyanin pigments are widely distributed in the human diet through crops, beans, fruits, and vegetables, suggesting that we ingest considerable amounts of anthocyanin pigments from plant based daily diets. The protective role and safety suggest that they can be used as drugs for some oxidative-damage-induced diseases. Moreover, several flavonoids are biochemically active compounds with known anti-inflammatory, anticarcinogenic, and free radical scavenging properties. In fact in previous studies, we have demonstrated that anthocyanins from blackberry extract reduce acute lung inflammation [31], circulatory failure and multiple organ dysfunction caused by endotoxin in the rat [6]. Several studies have demonstrated that anthocyanins protect against endothelial dysfunction and inhibit endothelial cell apoptosis [11,13,32]. In this regard the extract utilized in this study has been shown to exert antioxidant activity through the inhibition of the peroxynitrite-induced oxidative damage of the endothelium [12]. Moreover, some studies have reported that dietary anthocyanins, such as cyanidin-3-*O*-glucoside, are directly absorbed in rats and humans, appear in the blood, and are excreted intact in urine [33,34], suggesting that the glucoside form of cyanidin is important.

During infection and inflammation, high production of NO has been shown to cause DNA damage as well as mutations in vivo [35]. The formation of carcinogenic *N*-nitrosamines, resulting from elevated NO formation, has been also demonstrated in cell cultures and in vivo [36]. Recently, it has been suggested that iNOS over-expression may be intimately involved in the pathogenesis of many diseases, such as colon cancer, multiple sclerosis, neurodegenerative diseases, and heart infarction. The inducible NOS derived from macrophages is predominantly responsible for the overproduction of NO in injured tissues and inflammation processes [37]. Thus, we have performed our studies in murine macrophage cell line J774, which express iNOS, following stimulation with LPS.

We utilized a blackberry extract whose content in anthocyanins was mainly due (by about 88%) to cyanidin-3-*O*-glucoside and we compared its anti-inflammatory activity to the one obtained with cyanidin-3-*O*-glucoside standard. First of all, our results demonstrate that the anti-inflammatory activity of the anthocyanin fraction of the blackberry extract is mainly due to the cyanidin-3-*O*-glucoside, since all the parameters tested were inhibited to the same extent by both anthocyanin fraction and cyanidin-3-*O*-glucoside. Moreover, we suggest a molecular basis for this anti-inflammatory effect.

Our results demonstrate that LPS-induced NO production in J774 cells is susceptible to inhibition by blackberry extract through dual mechanism, since it attenuated iNOS protein expression and concomitantly decreased iNOS activity. Moreover, a direct inhibition of iNOS activity in lungs from LPS-stimulated rats was observed in agreement with our previously published data [6], although the mechanism of this action has not been investigated.

The action of cyanidin-3-*O*-glucoside on NO production has been particularly studied with regard to eNOS. In fact it has been demonstrated that, in bovine vascular endothelial cells, the compound induces eNOS expression and NO production [38] and increases vascular eNOS activity [39]. Thus, cyanidin-3-*O*-glucoside seems to display opposite effects on different isoforms of NOS, since in our study it exerts an inhibitory activity on iNOS. This could be due to different roles exerted by the two isoforms in different pathophysiological contest. In fact, it must be considered that while the generation of NO by eNOS plays a major role in maintaining cardiovascular homeostasis, on the contrary the chronic production of NO by iNOS, such as in heart failure and ischemia-reperfusion, exerts deleterious effects on circulatory function [40,41]. Similarly to cyanidin-3-*O*-glucoside, other polyphenolic compounds have been shown to exert, in different experimental models [42], opposite effects on NO production: an increase in NO production via eNOS and an inhibition of NO production via iNOS enzyme. Thus, the flavonoids may contribute to the balance between iNOS and eNOS in different pathophysiological systems.

NO produced by iNOS in J774 macrophages is regulated at the transcriptional level, predominantly via NF-κB-mediated pathway. It is interesting to note that NF-κB is involved in the induction of iNOS also in humans [43,44]. Our results demonstrate that anthocyanin fraction of the blackberry extract, and similarly cyanidin-3-*O*-glucoside, decreases iNOS protein by inhibition of LPS-induced NF-κB activation.

Accumulating evidence indicates that NF-κB activation, which is essential for gene transcription regulation, is modulated by MAPK/ERK kinase kinase-1 (MEKK-1). In fact, the activation of ERK-1 and -2 has been shown to be involved, in murine macrophages [45], in stimulation of NF-κB activity and iNOS expression through phosphorylation and subsequent degradation of the inhibitory subunit IκBα [25]. Moreover, it has been demonstrated, in mouse macrophages and in human colon epithelial cells [46], that the inhibition of ERK-1/2 directly suppresses endotoxin-induced NO synthesis. Our results demonstrate that anthocyanin fraction of the blackberry extract, and particularly the major anthocyanin present in the extract, cyanidin-3-*O*-glucoside, inhibited in a concentration-dependent manner both IκBα degradation and ERK-1/2 activation. Thus blackberry extract and cyanidin-3-*O*-glucoside could inhibit iNOS expression by attenuating IκBα degradation via ERK-1/2 or by inhibiting directly ERK-1/2 activation or by both mechanisms.

In conclusion, our study demonstrates that at least some part of the anti-inflammatory activity of the anthocyanin fraction of blackberry extract is due to suppression of NO production by cyanidin-3-*O*-glucoside, which is the main anthocyanin present in the extract. The mechanism of this

inhibition seems to be due to an action on the expression/activity of the enzyme. In particular, protein expression was inhibited through the attenuation of NF-κB and/or MAPK activation.

Thus, since up-regulation of iNOS has been associated with pathophysiology of certain types of human cancer as well as inflammatory disorders, our results suggest that anthocyanins of blackberry extract, and particularly cyanidin-3-*O*-glucoside, could exert beneficial action as iNOS inhibitors. Moreover, additional important therapeutic implications are suggested by the modulation of NF-κB, an ubiquitous transcription factor which controls the gene expression of numerous enzymes, cytokines, cell adhesion molecules, and growth factors involved in inflammation, carcinogenesis, and other immunological disorders. More generally since NF-κB and iNOS are deeply involved in the inflammatory process our findings, together with the reported antioxidant activity as peroxynitrite scavenger [12] and according to others [47], could suggest that anthocyanin dietary daily intake might have beneficial implications in the prevention of inflammatory diseases.

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