Resveratrol potently reduces prostaglandin E₂ production and free radical formation in lipopolysaccharide-activated primary rat microglia

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Abstract

Background: Neuroinflammatory responses are triggered by diverse ethiologies and can provide either beneficial or harmful results. Microglial cells are the major cell type involved in neuroinflammation, releasing several mediators, which contribute to the neuronal demise in several diseases including cerebral ischemia and neurodegenerative disorders. Attenuation of microglial activation has been shown to confer protection against different types of brain injury. Recent evidence suggests that resveratrol has anti-inflammatory and potent antioxidant properties. It has been also shown that resveratrol is a potent inhibitor of cyclooxygenase (COX)-1 activity. Previous findings have demonstrated that this compound is able to reduce neuronal injury in different models, both in vitro and in vivo. The aim of this study was to examine whether resveratrol is able to reduce prostaglandin E₂ (PGE₂) and 8-iso-prostaglandin F₂α (8-iso-PGF₂α) production by lipopolysaccharide (LPS)-activated primary rat microglia.

Methods: Primary microglial cell cultures were prepared from cerebral cortices of neonatal rats. Microglial cells were stimulated with 10 ng/ml of LPS in the presence or absence of different concentrations of resveratrol (1–50 µM). After 24 h incubation, culture media were collected to measure the production of PGE₂ and 8-iso-PGF₂α using enzyme immunoassays. Protein levels of COX-1, COX-2 and microsomal prostaglandin E synthase-1 (mPGES-1) were studied by Western blotting after 24 h of incubation with LPS. Expression of mPGES-1 at the mRNA level was investigated using reverse transcription-polymerase chain reaction (RT-PCR) analysis.

Results: Our results indicate that resveratrol potently reduced LPS-induced PGE₂ synthesis and the formation of 8-iso-PGF₂α, a measure of free radical production. Interestingly, resveratrol dose-dependently reduced the expression (mRNA and protein) of mPGES-1, which is a key enzyme
Resveratrol (trans-3,5,4′-trihydroxystilbene) is a polyphenolic compound present in relatively large amounts in grapes and red wine. In smaller quantities, resveratrol is also present in almost 70 plant species, where it has been found to act as an anti-fungicide and confer disease resistance in the plant kingdom [1]. Recently, this natural compound has received a great deal of attention due to its ability to serve as a potent antioxidant [2]. In addition, resveratrol has been proven to possess anti-inflammatory, immunomodulatory, chemopreventive, neuroprotective, and cardioprotective properties [3-10].

One of the most interesting properties of resveratrol is its ability to confer potent neuroprotection in several models of brain injury, both in vitro [10-12] and in vivo [7,8,13,14]. Resveratrol readily crosses the intact blood-brain barrier as demonstrated in previous studies [7,15]. There is much evidence from recent studies, which indicate that ischemic brain injury is potently reduced in resveratrol-treated animals. The first report suggesting that cerebral infarction is significantly diminished by systemic administration of resveratrol comes from Huang et al. [13], using an in vivo model of focal cerebral ischemia in rats. In another study, resveratrol increased the number of CA1 hippocampal neurons surviving a global cerebral ischemic insult [7]. Resveratrol not only reduced neuronal death but also reduced the number of reactive astrocytes and activated microglial cells [7]. The free radical scavenging ability seems to underlie the efficacy of resveratrol against neuronal demise in cerebral ischemia, as suggested in a recent study [16].

In order to explain at the molecular level the mechanisms responsible for resveratrol neuroprotection under ischemic conditions, in vitro models involving neuronal cultures as well as hippocampal slices subjected to oxygen-glucose deprivation have been employed. Nitric oxide-related toxicity to cultured hippocampal neurons was dramatically inhibited by resveratrol through a mechanism that seems to be at least partially related to its antioxidant effect [11]. Similarly, resveratrol attenuated cell death in organotypic hippocampal slice cultures exposed to oxygen-glucose deprivation through activation of the phosphoinositide-3-kinase (PI3-K)/Akt pathway [17].

The neuroprotective effect of resveratrol is not only restricted to cerebral ischemia. This natural compound also reduced oxidative stress and lesion volume in a model of traumatic brain injury [18] and spinal cord injury [19,20] in rats. Furthermore, resveratrol protected against excitotoxicity induced by kainic acid [8], and oxidative stress and behavioral changes in a rat model of Huntington's disease [21]. In addition, it has been recently demonstrated that resveratrol promotes intracellular degradation of amyloid β peptide via a mechanism that involves the proteasome [22].

Although mounting evidence convincingly demonstrates the potential of resveratrol to provide significant protection against different types of brain injury, the exact molecular mechanisms responsible for these beneficial effects are not fully elucidated. Its antioxidant ability alone can not give an explanation to the wide array of pharmacological properties of this compound.

Microglial cells are important protagonists in the cascade of events leading to tissue injury following neurodegeneration and other types of cerebral damage [23-28]. Very few studies have investigated the effects of resveratrol on microglial activation during neuroinflammation. In an earlier study, resveratrol was found to produce a potent suppressive effect on tumor necrosis factor α (TNFα) and nitric oxide production induced by lipopolysaccharide (LPS) in the mouse microglial cell line N9 [29]. These effects are mediated through inhibition of nuclear factor κB (NF-κB) activation and p38 MAPK phosphorylation [29].

Based on these previous reports, the present study was undertaken to investigate the effects of resveratrol on neuroinflammatory events associated to the production of prostaglandin E2 and reactive oxygen species (ROS) in activated primary microglial cells. Our findings indicate for the first time that resveratrol is a potent inhibitor of PGE2 production in microglia through a mechanism that involves reduction in the expression of the key enzyme

**Background**

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involved in PGE₂ formation, namely, microsomal prostaglandin E synthase-1 (mPGES-1). Direct inhibitory effects of resveratrol on cyclooxygenase activity as well as reduction in 8-isoprostane formation are other beneficial effects that contribute to the potent attenuation of microglial activation, and possibly to the neuroprotective efficacy of this natural compound in different models of brain injury.

**Methods**

**Reagents and Antibodies**

Trans-resveratrol, Trolox C, α-tocopherol, and LPS (from *Salmonella typhimurium*) were obtained from Sigma-Aldrich (Taufkirchen, Germany). LPS was resuspended in sterile phosphate buffered saline (PBS; Cell Concepts, Umkirch, Germany) as 5 mg/ml stock, and was used at a final concentration of 10 ng/ml in the culture. The COX-1 inhibitors SC-560 and Valeroyl salicylate (VAS) were obtained from Cayman Chemical Co. (Ann Arbor, USA). Resveratrol, Trolox C, and α-tocopherol were dissolved in absolute ethanol. SC-560 and VAS were dissolved in DMSO and water, respectively. Solvent concentration in the culture media was maintained at less than 0.1%. All agents, used at the given concentrations, do not affect the viability of the cells as observed through a luminescent kit (Promega, Madison, WI, USA), which measures metabolic ATP levels (data not shown). Antibodies against COX-1 (M-20) and COX-2 (M-19) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rabbit polyclonal antibody against mPGES-1 was obtained from Cayman Chemical Co. (Ann Arbor, MI, USA), while the antibody against actin was from Sigma (Saint Louis, MO, USA).

**Primary rat microglial cell culture**

Primary mixed glial cell cultures were established from cerebral cortices of one-day neonatal Sprague-Dawley rats as described in details in our previous reports [30-34]. Briefly, forebrains were minced and gently dissociated by repeated pipetting in PBS and filtered through a 70-µm cell strainer (Falcon). Cells were collected by centrifugation (1000 rpm, 10 min), resuspended in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal calf serum (Biochrom AG, Berlin, Germany) and antibiotics (40 U/ml penicillin and 40 µg/ml streptomycin, both from PAA Laboratories, Linz, Austria), and cultured on 10-cm cell culture dishes (5 × 10⁵ cells/plate, Falcon) in 5% CO₂ at 37°C. Medium was prepared taking extreme care to avoid LPS contamination [35]. Floating microglia were harvested every week (between 2–7 week) and reseeded into 75 cm² culture flask (for RNA extraction and Western blots) or 24-well plates (for 8-isoprostane and PGE₂ estimation) to give pure microglial cultures. The following day, cultures were washed to remove non-adherent cells, and fresh medium was added. The purity of the microglial culture was >98% as previously determined by immunofluorescence and cytochemical analysis [35].

**Prostaglandin E₂ (PGE₂) Enzyme Immunoassay**

Supernatants were harvested, centrifuged at 10,000 × g for 10 min and levels of PGE₂ in the media were measured by enzyme immunoassay (EIA) (Assay Designs Inc., Ann Arbor, MI, USA; distributed by Biotrend, Cologne, Germany) according to the manufacturer’s instructions. Standards from 39 to 2500 pg/ml were used. The sensitivity of this assay is 36.2 pg/ml.

**Cyclooxygenase Activity Assay**

To determine any direct inhibitory effect of resveratrol on COX-1 and COX-2 enzymatic activity, an arachidonic acid assay was performed as described [36,37]. Briefly, primary rat microglial cells were plated in 24-well cell culture plates, and pre-incubated with LPS (10 ng/ml) for 24 h. Medium was then removed, and cells washed in serum-free medium. Resveratrol (5–50 µM) was added, and after 15 min pre-stimulation, 15 µM arachidonic acid was supplemented for another 15 min. Supernatants were then used for determination of PGE₂ [36]. We also investigated the effects of resveratrol on microglial COX-1 enzymatic activity. Under unstimulated conditions, primary microglial cells only express the COX-1 isoform [34]. Thus, the COX-1 activity assay was conducted exactly as mentioned before without pre-incubation with LPS.

**RNA Isolation and Reverse Transcription-Polymerase Chain Reaction (RT-PCR)**

Total RNA was extracted using the guanidine isothiocyanate method according to Chomczynski and Sacchi [38]. For RT-PCR, 2 µg of total RNA was reverse transcribed using Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase (Promega, Mannheim, Germany), RNase Inhibitor rRNasin® (Promega), dNTP master mix (Invitrek, Berlin, Germany) and random hexamer primers (Promega). PCR was carried out using Taq DNA polymerase (Promega), dNTP master mix (Invitrek, Berlin, Germany) and the following primers: rat microsomal prostaglandin E synthase-1, mPGES-1 (forward: 5'-ATG ACT TCC CTG GGT TTG GTG AGA -3', reverse: 5'- ACA GAT GGT GGG CCA CTT CCC AGA -3', annealing temperature 65°C, 35 cycles, amplicon size: 459 bp). Primers for rat COX-2 were as follows: forward, 5'-TGC GAT CCT CTT CCC AGC TGT GCT -3' and reverse, 5'- TCA GGA AGT TCC TTA TIT CTC CCC AGA -3', annealing temperature 55°C, 35 cycles, amplicon size: 479 bp). Primers used for amplifying a fragment of 426 bp from rat COX-1 mRNA were: forward, 5'-CGG CCT CGA CCA CTA CCA ATG -3' and reverse, 5'- TGC GGG GCG GGA ATG AACT T-3', annealing temperature 60°C, 30 cycles. Equal equilibration was determined using rat β-actin primers (forward: 5'- ATG GAT GAC GAT ATC GCT -3', reverse: 5'-
ATG AGG TAG TCT GTC AGG T -3', 48°C, 30 cycles, product length: 569 bp) or primers for S12 from rat (forward: 5'-AGC TCA ACA CTG CCT TAC A -3', reverse: 5'-CTT TGC CAT AGT CCT TAA C -3', 56°C, 30 cycles, product length: 312 bp). PCR products were separated electrophoretically on a 2% agarose gel. Potential contamination by genomic DNA was controlled by omitting reverse transcriptase and using primers for the housekeeping genes (β-actin or S12) in the subsequent PCR amplification. Only RNA samples showing no bands after this procedure were used for further investigation. Primers were designed using the Primer3 software developed by the Whitehead Institute for Biomedical Research [39], and synthesized through an in-house facility (Dr. Gabor Igloi, Institute for Biology III, Freiburg, Germany). PCR analysis was performed after 4 h of stimulation with LPS.

Western blot analysis
For COX-1, COX-2 and mPGES-1 immunoblotting, microglial cells were left untreated or treated with LPS (10 ng/ml) in the presence or absence of resveratrol (1–50 µM) for 24 h. Cells were washed with phosphate buffered saline (PBS) and lysed in 1.3x SDS (sodium dodecyl sulfate)-containing sample buffer without DTT or bromophenol blue containing 100 µM orthovanadate [40]. Lysates were homogenized by repeated passage through a 26-gauge needle. Protein contents were measured using the bicinchoninic acid method (BCA protein determination kit from Pierce, distributed by KFC Chemikalien, Munich, Germany) according to the manufacturer's instructions. Bovine serum albumin (BSA, Sigma) was used as a standard. Before electrophoresis, bromophenol blue and DTT (final concentration, 10 mM) were added to the samples. For Western blotting, 60 µg of total protein from each sample were subjected to SDS-PAGE (polyacrylamide gel electrophoresis) under reducing conditions. Proteins were then transferred onto a polyvinylidene fluoride (PVDF) membrane (Millipore, Bedford, MA, USA) by semi-dry blotting. The membrane was blocked overnight at 4°C using RotiBlock (Roth, Karlruhe, Germany) and for another hour at room temperature before incubation with the primary antibody. Primary antibodies were goat anti-COX-2, goat anti-COX-1 and rabbit anti-mPGES-1. Primary antibodies were diluted 1:500 in Tris-buffered saline (TBS) containing 0.1% Tween 20 (TBST) and 1% bovine serum albumin (BSA, Sigma). Membranes were incubated with the corresponding primary antibody for 2 h at room temperature. After extensive washing (three times for 15 min each in TBST), proteins were detected with horseradish peroxidase-coupled rabbit anti-goat IgG (Santa Cruz, 1:100,000 dilution) or goat anti-rabbit IgG (Amersham, 1:25,000 dilution) using chemiluminescence (ECL) reagents (Amersham Pharmacia Biotech, Freiburg, Germany). Quantification of the Western blots was performed using ScanPack 3.0 software (Biometra, Göttingen, Germany). Equal protein loading and transfer were assessed by subtraction of each sample to a Western blot for actin (rabbit anti-actin IgG, diluted 1:5000). All western blot experiments were carried out at least three times.

Determination of 8-iso-prostaglandin F_{2α} (8-iso-PGF_{2α})
8-iso prostanes are formed in response to free radical attack on arachidonic acid on membrane phospholipids and are considered as a reliable and highly sensitive measure of free radical formation [41]. Microglial cells were pre-treated for 30 min with different concentrations of resveratrol, Trolox C, α-tocopherol, SC-560, or VAS (see Results and Figures). After 30 min pre-stimulation, cells were added 10 ng/ml LPS for 24 h. Control experiments consisted of cells treated only with the solvent of each compound without LPS. Supernatants were harvested and the levels of 8-iso-PGF_{2α} (IUPAC nomenclature: 15-F_{2α}-IsoP) were measured by an enzyme immunoassay according to the manufacturer's instructions (Cayman Chemicals, Ann Arbor, MI, USA). The standards were used in the range of 3.9 to 500 pg/ml (detection limit of 5 pg/ml).

Data analysis
Data from at least 3 experiments were used for data analysis. Original data were converted into %-values of LPS control and mean ± S.E.M. were calculated. Values were compared using t-test (two groups) or one-way ANOVA with post-hoc Student-Newman-Keuls test (multiple comparisons).

Results
In our initial experiments, we investigated the effects of resveratrol on LPS-induced PGE_{2} production in microglia. As shown in Fig. 1A, resveratrol potently diminished PGE_{2} synthesis starting at 1 µM and showing a dose-dependent inhibitory effect between 1 and 10 µM. The higher doses of resveratrol tested in this study did not further decrease PGE_{2} formation, maintaining PGE_{2} at basal levels (not different from unstimulated control cells). Since resveratrol is a potent antioxidant compound, we decided to examine if other antioxidants were also able to modify PGE_{2} production by activated microglia. In contrast to resveratrol, the antioxidants α-tocopherol and its synthetic analogue Trolox C only slightly reduced PGE_{2} biosynthesis at relatively high doses (100 and 250 µM respectively; Fig. 1B). These antioxidant compounds were unable to reduce PGE_{2} formation when added to the microglial cultures at lower doses (data not shown).

These findings prompted us to investigate the ability of resveratrol to reduce COX enzymatic activity in activated microglia. Results from this experiment are shown in Fig. 2. Significant inhibition of COX-1 activity was observed when microglial cells were pre-incubated for 30 min with...
25 and 50 µM of resveratrol (Fig. 2A). The effect of resveratrol on total COX activity (COX-1 + COX-2) was also investigated. In this in vitro assay, cells were pre-incubated with LPS for 24 h before resveratrol was added for 30 min, and PGE2 levels were measured in the supernatant. The addition of resveratrol produced a significant inhibition of COX activity starting at 10 µM and showing a 50% inhibition with the highest dose of 50 µM (Fig. 2B).

Since there are significant changes in gene expression in LPS-activated microglia, including a dramatic upregulation of the PGE2 synthesizing enzymes COX-2 and mPGES-1 [31,34,42], we decided to investigate the effects of resveratrol on the expression of these key enzymes responsible for PGE2 production in LPS-stimulated microglia, at both the mRNA and protein levels. RT-PCR analysis showed that control microglial cells do not express COX-2 or mPGES-1 mRNA under control conditions, but the expression of these enzymes is dramatically induced by addition of LPS to the cultures (Fig. 3A). Of great interest is our finding that resveratrol produced a significant reduction in the expression of mPGES-1 mRNA, without modifying COX-2 mRNA expression (Figs. 3A and 3B). Similar results were observed when investigating the effects of resveratrol on mPGES-1 protein levels as assessed by Western blotting (Fig. 4A). It is important to emphasize that resveratrol did not modified basal COX-1 or LPS-induced COX-2 expression in microglia. A densi-
This reduction in 8-iso-PGF$_2\alpha$ that was seen at very low doses (starting at 1 µM) potently reduced LPS-mediated formation of ROS, an immunoassay (Fig. 5). More importantly, resveratrol significantly reduced free radical production by activated microglia. LPS produced a very significant increase in the formation of 8-iso-PGF$_2\alpha$, a very sensitive marker of cellular free radical generation, as assessed by enzyme immunoassay (Fig. 5). More importantly, resveratrol potentely reduced LPS-mediated formation of ROS, an effect that was seen at very low doses (starting at 1 µM). This reduction in 8-iso-PGF$_2\alpha$ by resveratrol showed a concentration-dependent response between 1 and 10 µM (Fig. 5A), an effect also seen in our first experiment evaluating PGE$_2$ levels (Fig. 1A). We then tested the ability of two well-known antioxidants, Trolox C and α-tocopherol, to reduce free radical formation in LPS-activated microglia. These antioxidants significantly reduced 8-iso-PGF$_2\alpha$ as well, but not as potent as resveratrol (Fig. 5B).

Since resveratrol has been proven to have potent antioxidant properties, we aimed to determine the efficacy of this compound in reducing free radical production by activated microglia. LPS produced a very significant increase in the formation of 8-iso-PGF$_2\alpha$, a very sensitive marker of cellular free radical generation, as assessed by enzyme immunoassay (Fig. 5). More importantly, resveratrol potentely reduced LPS-mediated formation of ROS, an effect that was seen at very low doses (starting at 1 µM). This reduction in 8-iso-PGF$_2\alpha$ by resveratrol showed a concentration-dependent response between 1 and 10 µM (Fig. 5A), an effect also seen in our first experiment evaluating PGE$_2$ levels (Fig. 1A). We then tested the ability of two well-known antioxidants, Trolox C and α-tocopherol, to reduce free radical formation in LPS-activated microglia. These antioxidants significantly reduced 8-iso-PGF$_2\alpha$ as well, but not as potent as resveratrol (Fig. 5B).

The ability of resveratrol to inhibit the peroxidase activity of COX-1 is a well-known pharmacological effect of this compound [43,44]. It was then very important to investigate the ability of other COX-1 inhibitors to reduce PGE$_2$ and 8-iso-PGF$_2\alpha$ production in microglia activated with LPS. Interestingly, two structurally different highly selective COX-1 inhibitors (SC-560 and VAS) potently reduced the formation of COX-1, COX-2, mPGES-1, and β-actin mRNAs. Microglial cells were treated with LPS (10 ng/ml) in the absence or presence of different concentrations of resveratrol. The mRNA expression levels were tested for each condition. There is a constitutive COX-1 mRNA expression that is observed under all conditions. However, COX-2 and mPGES-1 are undetectable in untreated microglial cells, while their expression is dramatically increased in the presence of LPS. Resveratrol treatment significantly reduced mPGES-1, but not COX-2 expression. RT-PCR analysis was performed after 4 h of incubation with LPS. Resveratrol was added to the cultures 30 min before LPS. This reduction in 8-iso-PGF$_2\alpha$ by resveratrol showed a concentration-dependent response between 1 and 10 µM (Fig. 5A), an effect also seen in our first experiment evaluating PGE$_2$ levels (Fig. 1A). We then tested the ability of two well-known antioxidants, Trolox C and α-tocopherol, to reduce free radical formation in LPS-activated microglia. These antioxidants significantly reduced 8-iso-PGF$_2\alpha$ as well, but not as potent as resveratrol (Fig. 5B).

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PGF2
ANOVA followed by Student-Newman-Keuls
iments. Statistical analysis was performed using one-way
histograms represent mean ± S.E.M. of 4 independent exper-
and ***p < 0.001 with respect to LPS alone. For both panels,
oxidants Trolox C and
independent experiments (Fig. 7B). In another control
expression. Trolox C slightly reduced mPGES-1 expres-
diers in activated microglia [31,45], we studied the
effects of Trolox C on LPS-induced COX-2 and mPGES-1
Since previous studies have shown the ability of antioxi-
mology of microglial cells with LPS alone, or in combination
resveratrol at the given concentrations. B: Moderate
formation by the anti-
tors and Trolox C on the expression of both COX iso-
zymes. No effect of these compounds was observed in our
in vitro model, as shown in Fig. 7. Furthermore, we also
vestigated if other COX inhibitors might have an effect on
the expression of mPGES-1 by activated microglia. These
compounds included the non-selective COX inhibitors indomethacin and ibuprofen, as well as the highly
selective COX-2 inhibitor SC-58125. None of these com-
modify the LPS-induced mPGES-1 expression (data not shown).

**Discussion**

Scientific literature on natural compounds has been
recently accumulating an enormous amount of reports
describing the neuroprotective ability of natural polyphen-
lolic substances in different models of brain injury both
in vitro and in vivo. Resveratrol is one of the most promis-
compounds, considering recent evidence indicating its
potent ability to reduce cerebral damage after ischemia/
hypoxia [7,13,17], trauma [18], excitotoxicity [8], and
other conditions leading to neuronal demise [19,21].

It is widely accepted that neuroinflammation is a key
player in various pathological events associated with
brain injury. More specifically, microglial activation and
the subsequent release of pro-inflammatory cytokines,
ROS, and prostaglandins play a role of paramount import-
ance in cerebral damage. Inhibition of COX-2 induction
and/or activity has been proven to reduce brain injury
after ischemia [46-50], excitotoxicity [47,51], and MPTP-
duced neurodegeneration [52-54]. The most important
mechanisms associated with the toxic effects of enhanced
COX activity during neuroinflammation include produc-
tion of PGE2 [55-59] and formation of free radicals lead-
ing to oxidative stress [34,37,60,61].

In the present study, we have found that resveratrol is a
potent inhibitor of PGE2 and free radical formation
activated microglial cells. These findings add significant
information on the molecular mechanisms involved in
the neuroprotective effect of this compound. The ability
of resveratrol to reduce PGE2 production comes from the
modulation of multiple events in the COX/PGE2 pathway:
1) resveratrol is a potent inactivator of the peroxidase
reaction of COX-1 [43], and thus is considered a relatively
selective inhibitor of this isozyme; 2) resveratrol signifi-
cantly diminished LPS-induced expression of mPGES-1 (Figs. 3 and 4), the most important terminal synthase
responsible for PGE2 synthesis in activated microglia [42];
and 3) production of 8-iso-PGF2α, a reliable indicator of
free radical generation, is dramatically reduced by low
concentrations of resveratrol (Fig. 5).

Next, we examined the potential effects of SC-560 and
VAS on LPS-mediated mPGES-1 expression, which could
help to explain if the effects of resveratrol on the reduction
in mPGES-1 were mediated by a COX-1 mechanism. RT-
PCR analysis reveals that COX-1 inhibition by SC-560 or
vas failed to reduce mPGES-1 expression in LPS-activated
primary microglia (Fig. 7A and 7B).

Since previous studies have shown the ability of antioxi-
dants to modify gene expression of pro-inflammatory
mediators in activated microglia [31,45], we studied the
effects of Trolox C on LPS-induced COX-2 and mPGES-1
expression. Trolox C slightly reduced mPGES-1 expres-
sion, although this effect did not reach statistical signi-
ificance, as found in the densitometric analysis of three
independent experiments (Fig. 7B). In another control

PGE2 production (Fig. 6A and 6C) and free radical forma-
tion (Fig. 6B and 6D) in activated microglia in a dose-
dependent manner.
The relative contribution of each of these mechanisms to the overall reduction in PGE\textsubscript{2} by resveratrol is difficult to address based on present data. However, some issues deserve further discussion. The ability of resveratrol to inhibit the peroxidase activity of COX-1 is a well-known pharmacological effect of this compound [43,44]. COX-1 is constitutively expressed in microglia under resting conditions, and its expression is not induced by LPS as shown in Figs. 3A and 4A, and reported by us before [34]. However, according to present results, there is a significant contribution of COX-1 to PGE\textsubscript{2} formation by microglia upon LPS challenge. This is supported by a previous study performed in LPS-stimulated human adult microglial cells, in which selective inhibition of COX-1 was also very effective in reducing PGE\textsubscript{2} production [62]. Results from control experiments using other highly selective COX-1 inhibitors (SC-560 and VAS), in addition to resveratrol, indicate that COX-1 isoform is not only important in LPS-induced PGE\textsubscript{2} synthesis, but it is also a key source of free radicals in microglia. This is an unexpected observation, and represents the first evidence that microglial COX-1 activity is a significant source of free radicals during neuroinflammation.

It has been previously shown that increased COX activity is associated with oxidative damage following different types of brain injury, including excitotoxicity [61,63,64], ischemia [60,65,66], and traumatic brain injury [67]. Furthermore, treatment with COX inhibitors has been proven to significantly reduce IL-1\textbeta- and LPS-induced oxidative damage in neuronal and microglial cells, respectively [34,37].

The interesting pharmacological properties of resveratrol in terms of inhibition of COX-1 and direct antioxidant ability, may underlie the dramatic attenuation of PGE\textsubscript{2} and free radical production by LPS-activated microglia. It has long been known that free radicals directly increase COX activity and conversely antioxidants reduce COX catalytic activity [68-70]. Based on these reports, one may speculate that maintenance of microglial redox status by resveratrol contributes to the reduction in COX activity and PGE\textsubscript{2} production in these cells. This is further supported by our finding that other antioxidants (Trolox C and \alpha-tocopherol) are also able to reduce PGE\textsubscript{2} formation (Fig. 1B). Antioxidants have been shown to modulate microglial activation [71-74]. In an earlier study, vitamin
E was found to attenuate nitric oxide production and the induction of IL-1α and TNFα expression through suppression of signaling events necessary for microglial activation [71]. Furthermore, inhibition of ROS generation in LPS-activated microglia can reduce PGE2 production as reported previously by Wang et al [73].

An important new observation of the present study is the dramatic reduction in LPS-mediated expression of mPGES-1 in cells treated with resveratrol (Figs. 3 and 4). Based on very recent and convincing data, production of PGE2 in microglia following LPS treatment is almost entirely dependent on the activity of mPGES-1 [42]. Thus, blockade of mPGES-1 expression by resveratrol is an additional effect of this compound that contributes to its potent ability to block PGE2 synthesis in microglia. Resveratrol effects on mPGES-1 expression seem to be independent on its ability to reduce COX activity/PGE2 formation since other COX inhibitors were unable to modify LPS-induced mPGES-1 upregulation in microglial cells despite their potent inhibitory effects on PGE2 production (Figs. 6 and 7).

To the best of our knowledge, the present study is the first to document the ability of resveratrol to reduce mPGES-1 expression, as shown here in activated microglia. It is of great relevance that resveratrol reduced mPGES-1, but not COX-2 expression (Fig. 4). This suggests that LPS-induced microglial expression of mPGES-1 proceeds through molecular mechanisms which are different from the ones involved in COX-2 induction, providing for the first time evidence that the expression of mPGES-1 and COX-2 are not always coupled as suggested by other authors [75,76].

It has been recently reported that resveratrol reduces COX-2 expression in mouse BV-2 microglial cells through a mechanism that possibly involves inhibition of NF-κB activation [77]. Our present findings are not in line with these previous observations. The reasons for these discrepancies are not known, but might be related to different cell types and species (mouse BV-2 versus primary rat microglia).

At this point we have not yet identified the exact signal transduction pathway involved in resveratrol’s effects on mPGES-1 expression. However, present findings will inspire new investigations in order to elucidate the differential signal transduction pathways responsible for the expression of mPGES-1 and COX-2 in microglia. This is of importance since this could lead to the discovery of new targets for attenuating microglial activation and PGE2 synthesis.

Because of the downstream position of mPGES-1 in the PGE2-synthesizing cascade, selective pharmacological blockade of its expression, as shown here for resveratrol, would affect only the pro-inflammatory PGE2, and would not decrease the production of other physiologically important prostanoids. Although COX-2 inhibitors have been highly marketed in the last five years including clinical trials in Alzheimer's disease, there are also important side effects associated with this new group of drugs [78,79]. Thus, it is of paramount importance our finding that resveratrol specifically reduced mPGES-1 without affecting COX-2 levels. Recent evidence indicates that enhanced expression and activity of mPGES-1 is a critical pathological event during inflammation, both in the CNS.
and in the periphery. Mice lacking the mPGES-1 gene are protected against stroke-induced injury [80] and display reduced pain hypersensitivity and inflammation [81,82].

Conclusion
In summary, we are proposing here that significant attenuation of PGE$_2$ and free radical production by activated microglia might contribute to the neuroprotective effects of resveratrol. This study gives further support to the potential use of resveratrol as a therapeutic agent to reduce microglial activation following different types of brain injury.

Abbreviations
LPS (lipopolysaccharide); ROS (reactive oxygen species); PGE$_2$ (prostaglandin E$_2$); COX (cyclooxygenase); mPGES-1 (microsomal prostaglandin E synthase-1); EIA (enzyme immunoassay); DTT (1,4-Dithio-DL-threitol); BSA (bovine serum albumin); ANOVA (analysis of variance); 8-iso-PGF$_{2a}$ (8-iso-prostaglandin F$_{2a}$); VAS (Valeroyl Salicylate).

Competing interests
The author(s) declare that they have no competing interests.

Authors' contributions
ECJ designed the study, performed 8-iso-PGF$_{2a}$ assay, Western blot and RT-PCR analysis, partly directed the work, reviewed the data, and wrote the manuscript. ACPO, SG and HSB performed Western blot and RT-PCR analysis. MH and EM provided consultation and reviewed the data. BLF directed the work, contributed to the design of the study, reviewed the data and helped to write the manuscript. All authors read and approved the final manuscript.

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