Biochemical Pharmacology, Vol. 59, pp. 865–870, 2000.

ISSN 0006-2952/00/$–see front matter
PII S0006-2952(99)00380-9

Effect of Resveratrol, a Natural Polyphenolic Compound, on Reactive Oxygen Species and Prostaglandin Production

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ABSTRACT. Resveratrol is a natural molecule with antioxidant action. Moreover, resveratrol is also considered to be a molecule with anti-inflammatory action, an effect attributed to suppression of prostaglandin (PG) biosynthesis. The aim of the present study was to investigate the effects of resveratrol, a polyphenol present in most red wines, on reactive oxygen species formation as well as on arachidonic acid (AA) release, cyclooxygenase expression, and PG synthesis in murine resident peritoneal macrophages. Results show that resveratrol exerted a strong inhibitory effect on superoxide radical (O_2^-) and hydrogen peroxide (H_2O_2) produced by macrophages stimulated by lipopolysaccharides (LPS) or phorbol esters (PMA). Resveratrol also significantly decreased [^{3}H]AA release induced by LPS and PMA or by exposure to O_2^- or H_2O_2. Resveratrol treatment caused a significant impairment of cyclooxygenase-2 (COX-2) induction stimulated by LPS and PMA or by O_2^- or H_2O_2 exposure. These effects of resveratrol on [^{3}H]AA release and COX-2 overexpression were correlated with a marked reduction of PG synthesis. Our results indicate that the antioxidant action of resveratrol affects AA mobilization and COX-2 induction. BIOCHEM PHARMACOL **59**;7:865–870, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. resveratrol; inflammation; arachidonic acid; superoxide; hydrogen peroxide; cyclooxygenase-2; prostaglandin

Resveratrol (3,4′,5-trihydroxystilbene) is a naturally occurring molecule present in high levels in grapes and wine. This phytoalexin, produced in response to environmental stress or pathogenic attack, is an antifungicide conferring disease resistance in the plant kingdom [1]. Recently, a large number of studies, carried out in cellular and animal models, have demonstrated that resveratrol regulates many biological activities. Thus, this molecule has been reported to protect against atherosclerosis by exerting antioxidant activity [2], modulating the synthesis of hepatic apolipoprotein and lipids [3], and inhibiting platelet aggregation and the production of proatherogenic eicosanoids by human platelets and neutrophils [4]. It also has beneficial effects on coronary degenerative disease(s). Moreover, resveratrol is considered to have anticancer and anti-inflammatory action [5] and is used for analgesic and therapeutic purposes in Oriental folk medicine [6].

The anti-inflammatory properties of resveratrol were demonstrated by suppression of carrageenan-induced paw edema [5], an effect attributed to the impairment of PG† synthesis via direct, selective inhibition of cyclooxygenase-1. However, the mechanisms by which resveratrol affects the AA cascade are unclear, although its antioxidant properties may be involved. In the current study, we extended prior observations on the effects of resveratrol on PG synthesis by investigating whether the antioxidant action of resveratrol could be modulated AA release and subsequent eicosanoid production. Previous data showed that ROS are involved in the pathways that result in [^{3}H]AA release by murine resident peritoneal macrophages. Thus, we suggested that phosphorylation and dephosphorylation processes stimulated by ROS could increase AA mobilization via a PKC-independent pathway [7]. Here, we show that resveratrol suppresses the production of O_2^- and H_2O_2 in murine peritoneal macrophages stimulated by LPS or PMA and correlate these results with an impairment of [^{3}H]AA mobilization, COX-2 overexpression, and the subsequent PGE_2 release induced by both agonists. These data provide an additional mechanistic basis for the anti-inflammatory properties of resveratrol.

MATERIALS AND METHODS

Materials

[5,6,8,9,11,12,14,15-^{3}H]AA (180–240 Ci/mmol) was obtained from DuPont–New England Nuclear. *trans*-Resveratrol (*trans*-3,4′,5-trihydroxystilbene), PMA, LPS from *Escherichia coli*, essential fatty acid-free BSA, H_2O_2, horseradish

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† *Abbreviations:* AA, arachidonic acid; COX-2, cyclooxygenase-2; DCPIP, 2,6-dichlorophenolindophenol; FBS, fetal bovine serum; H_2O_2, hydrogen peroxide; LPS, lipopolysaccharides; NBT, *p*-nitro blue tetrazolium; PMA, phorbol 12-myristate 13-acetate; PMSF, phenylmethylsulfonyl fluoride; PG, prostaglandin; PGE_2, prostaglandin E_2; PKC, protein kinase C; PLA_2, phospholipase A_2; ROS, reactive oxygen species; and O_2^-, superoxide radical.

Received 14 April 1999; accepted 21 September 1999.

peroxidase, NBT, DCPIP, catalase from human erythrocytes, superoxide dismutase from bovine erythrocytes, xanthine and xanthine oxidase, PMSF, aprotinin, leupeptin, dimethyldithiocarbamic acid, and α_2-macroglobulin were purchased from Sigma Chemical Co. Cell culture medium RPMI-1640, FBS, penicillin G, and streptomycin were supplied by GIBCO RBL. All other reagents were of analytical grade.

Isolation and Culture of Murine Resident Peritoneal Macrophages

Mouse resident peritoneal macrophages were collected from male CD-1 mice (Harlam) (20–25 g), which were killed by carbon dioxide asphyxiation, and the peritoneal cavity was lavaged with PBS containing 1% BSA, 20 U/mL of heparin, 100 U/mL of penicillin, and 100 μg/mL of streptomycin. Lavage fluids were pooled and centrifuged at 400 *g* for 10 min at 4° to pellet cells. Cells were re-suspended in RPMI-1640 medium supplemented with the antibiotics and 10% FBS, counted, and plated in 12-well plastic cluster dishes (Costar). Macrophages were allowed to adhere for 2 hr at 37° in an atmosphere of CO_2:air (1:19) and 100% humidity. The non-adherent cells were removed by washing the cell sheet in PBS. The adherent cells, which we had previously shown by morphological and functional criteria to be >95% macrophages, were maintained in RPMI-1640 medium supplemented with 10% FBS and antibiotics.

Incorporation and Release of [^{3}H]Arachidonic Acid

After a 20-hr incubation of macrophages, the medium was removed and replaced by 0.5 mL of RPMI containing 0.1 μCi of [^{3}H]AA, and the samples were incubated for an additional 6 hr at 37°. Cells were then washed three times in Ca^{2+}- and Mg^{2+}-free PBS containing 0.5% BSA to remove unincorporated [^{3}H]AA. Macrophages used in these studies incorporated 48 ± 3% of the [^{3}H]AA. At the end of each experiment, the cell monolayer was overlaid with 1% Triton X-100, and the cells were scraped off the dishes. Finally, radioactivity present in the medium and in the cell fraction was measured by scintillation counting, using a Packard Tri-Carb 1500 counter. The amount of [^{3}H]AA released into the medium as a result of the specified treatment was determined and expressed as a percentage of cell-incorporated [^{3}H]AA, which was determined in solubilized cells.

Assays for Superoxide and Hydrogen Peroxide Generation

O_2^- generation was determined by measuring the superoxide dismutase-inhibitable reduction of NBT [8]. Briefly, resident peritoneal macrophages were cultured (1×10^6 cells per well) in PBS supplemented with $CaCl_2$ (1 mM) and NBT (1 mM) and incubated with or without the specific reagents at 37°. The reaction was stopped by the removal of the supernatant and solubilization of formazan crystals in DMSO (100 μL). Reduction of NBT to formazan within the cells was assessed by measurement of optical density at 540 nm. H_2O_2 concentration was determined by the Mapson method [9]. Briefly, DCPIP (40 μM) was reduced by ascorbic acid, which decreased the blue color. H_2O_2 in samples with a few microliters of peroxidase induced an increase in absorbance (610 nm) due to reoxidation of DCPIP. Control reactions were performed by adding catalase.

Superoxide Generation System

In order to produce a flux of superoxide in culture cells, a system of xanthine oxidase and xanthine [10] was used. Generation of O_2^- was ascertained spectrophotometrically by monitoring the reduction of NBT at 560 nm.

COX-2 Western Blot Assay

Macrophage cultures were washed twice with ice-cold PBS, scraped off in PBS containing 2 mM EDTA and pelleted, and protein concentration was measured by the Bradford method [11]. Cell pellets were sonicated in PBS containing 2 mM EDTA, 20 μg/mL PMSF, 20 μg/mL aprotinin, 20 μg/mL leupeptin, 200 μg/mL dimethyldithiocarbamic acid, and 0.2 mg/mL α_2-macroglobulin. Cell lysates (20 μg protein) were separated by a 10% SDS–PAGE gel [12] and blotted onto a nitrocellulose membrane. The membranes were blocked with 5% non-fat milk powder in PBS–0.1% Tween 20 for 1 hr, and COX-2 was immunodetected using a polyclonal antiserum directed against murine COX-2 (Cayman Chemical Co.). Finally, antibody binding was visualized by the enhanced chemiluminescence (ECL) technique from Amersham, following instructions from the supplier, using Kodak X-OMAT LS film. Levels of COX-2 were estimated by videodensitometry.

PGE_2 Levels in Culture Medium

An aliquot of culture supernatant medium (0.25 mL) was acidified with 1 mL of 1% formic acid. PGE_2 was extracted in ethyl acetate as previously described [13], and PGE_2 levels were determined using a PGE_2–monoclonal enzyme immunoassay kit (Cayman Chemical Co.), following the manufacturer's protocol.

Statistical Analysis

Data are expressed as the means ± SEM. All experiments were performed at least three times to ensure consistency of the observations. Significance of differences between data points and control was determined using a two-tailed Student's *t*-test with $P < 0.05$ confidence limits.

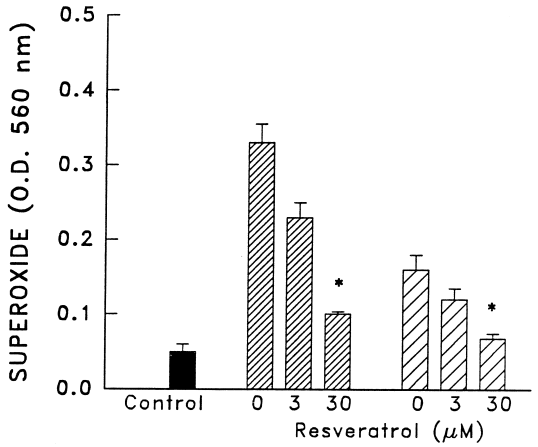

FIG. 1. Effect of resveratrol on superoxide anion produced by murine peritoneal macrophages stimulated with LPS (1 μg/mL, ▨) or PMA (1 μM, ▨) for 1 hr. Results are means ± SEM, N = 6. *P < 0.05, significantly different as compared to non-treated cells.

RESULTS

Preincubation of murine peritoneal macrophages with resveratrol strongly inhibited O_2^- and H_2O_2 production induced by LPS (1 μg/mL) or PMA (1 μM). Thus, at 30 μM, resveratrol significantly inhibited superoxide dismutase-inhibitable formazan production to about 30% of the non-treated value and to about 40% of the non-treated value of H_2O_2 (Fig. 1). Similar results were obtained when we determined the effect of the polyphenol on H_2O_2 production (about 60% inhibition at 30 μM) induced by LPS or PMA (Fig. 2).

The effect of resveratrol on AA release was examined by measuring the release of [^{3}H]AA from prelabeled macrophages. LPS (1 μg/mL) and PMA (1 μM) induced a marked [^{3}H]AA release (29.5% and 23.6%, respectively), and resveratrol (30 μM) significantly inhibited the [^{3}H]arachidonate release induced by both agonists (66% and 61%, respectively) (Table 1). Exposure to O_2^- or H_2O_2 also induced an enhancement of [^{3}H]AA release that was significant when O_2^- or H_2O_2 concentrations in the culture medium were similar to the concentration released during cell activation by LPS (1 μg/mL) or PMA (1 μM), which is consistent with previous data [7]. Here, our results showed that the mobilization of AA by macrophages after ROS exposure was decreased significantly by resveratrol (Table 2). Thus, resveratrol (30 μM) inhibited the [^{3}H]AA release induced by H_2O_2 (1 mM) and O_2^- (0.36, O.D. 560 nm) exposure by 51% and 67%, respectively.

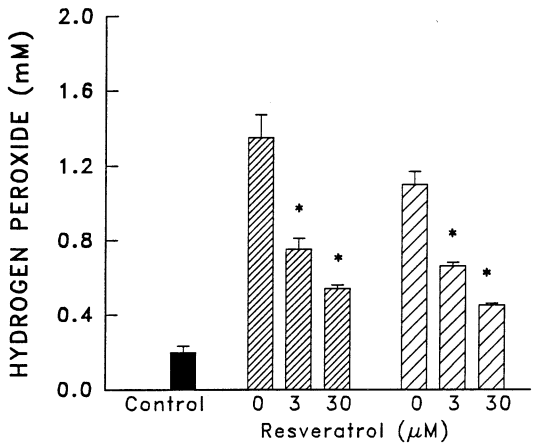

FIG. 2. Effect of resveratrol on hydrogen peroxide produced by murine peritoneal macrophages stimulated with LPS (1 μg/mL, ▨) or PMA (1 μM, ▨) for 1 hr. Results are means ± SEM, N = 6. *P < 0.05, significantly different as compared to non-treated cells.

TABLE 1. Effect of resveratrol on [^{3}H]AA mobilization induced by LPS and PMA

	%[^{3}H]AA released
Control	5.1 ± 0.2
LPS	34.6 ± 2.1
LPS + resveratrol (3 μM)	27.6 ± 1.3
LPS + resveratrol (30 μM)	15.1 ± 0.8*
PMA	28.7 ± 1.7
PMA + resveratrol (3 μM)	21.2 ± 1.1*
PMA + resveratrol (30 μM)	14.2 ± 0.9*

Resident peritoneal macrophages were incubated with resveratrol for 15 min. Cells were then stimulated with LPS (1 μg/mL) or PMA (1 μM) for an additional 60 min. Data represent the means ± SEM from 4–5 determinations performed in duplicate.
*$P < 0.05$ with respect to non-treated cells (Student's *t*-test).

We also studied whether resveratrol inhibited LPS- or PMA-mediated induction of PGE_2 synthesis as a consequence of the impairment of the AA release induced by the polyphenol. LPS and PMA caused an approximate 12- and 10-fold increase, respectively, in the synthesis of PGE_2. This effect was significantly suppressed by resveratrol in a dose-dependent manner (Fig. 3). Moreover, resveratrol (30 μM) completely reduced PGE_2 biosynthesis stimulated by LPS or PMA.

PGE_2, an active product of the COX pathway in murine resident peritoneal macrophages, is synthesized from AA by the action of both COX isoforms. To determine whether the above effect on PGE_2 production could be related to

TABLE 2. Effect of catalase, superoxide dismutase, and resveratrol on [^{3}H]AA mobilization induced by O_2^- and H_2O_2

	% [^{3}H]AA released
Control	4.3 ± 0.2
H_2O_2	45.2 ± 2.1
H_2O_2 + catalase	10.1 ± 0.3*
H_2O_2 + resveratrol (3 μM)	32.2 ± 0.4*
H_2O_2 + resveratrol (30 μM)	24.2 ± 0.3*
O_2^-	25.6 ± 0.9
O_2^- + SOD	13.9 ± 0.7*
O_2^- + resveratrol (3 μM)	18.2 ± 1.1*
O_2^- + resveratrol (30 μM)	11.3 ± 0.5*

Resident peritoneal macrophages were preincubated with catalase (1000 units), superoxide dismutase (SOD, 50 units), or resveratrol for 15 min. Cells were then incubated with O_2^- (0.36, O.D. 560 nm) generated by the xanthine/xanthine oxidase system or with H_2O_2 (1 mM) for an additional 30 min. Data represent the means ± SEM from 4–5 determinations performed in duplicate.
*$P < 0.05$ with respect to non-treated cells.

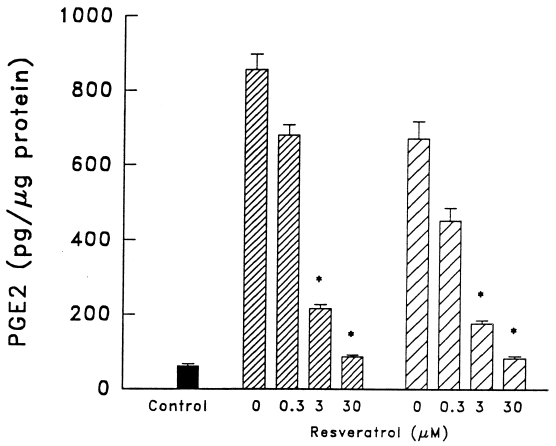

FIG. 3. Effect of resveratrol on PGE_2 produced by murine peritoneal macrophages stimulated with LPS (1 μg/mL, ▨) or PMA (1 μM, ▨) for 1 hr. Results are means ± SEM, N = 6. *P < 0.05, significantly different as compared to non-treated cells.

differences in the levels of COX, Western blotting of cell lysate protein was carried out. Figure 4 shows that LPS and PMA induced COX-2 induction. Interestingly, the treatment with resveratrol (30 μM) caused a significant decrease in COX-2 induction. However, neither LPS/PMA nor resveratrol modified the amounts of COX-1 (data not shown). In a similar way, O_2^- and H_2O_2 exposure produced COX-2 induction. Resveratrol treatment significantly decreased the enhancement of COX-2 levels induced by both ROS (Fig. 4).

DISCUSSION

Resveratrol has been reported to protect against atherosclerosis [2] and coronary degenerative disease(s) [14]. The down-regulation of PG and prostacyclin and proatherogenic eicosanoid synthesis is probably due to the inhibition of cyclooxygenase and hydroperoxide activities by this polyphenol [5]. Recently, the anti-inflammatory properties of resveratrol were demonstrated by suppression of carrageenan-induced paw edema [5], an effect attributed to suppression of PG synthesis via direct, selective inhibition of COX-1. In the current study, we have extended prior observations on the effects of resveratrol on PG synthesis by determining whether the antioxidant action of resveratrol modulates AA mobilization and subsequent metabolism by the COX-2 pathway.

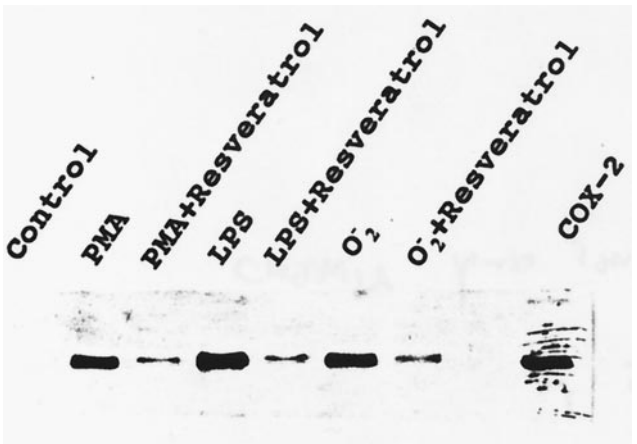

FIG. 4. COX-2 overexpression was significantly induced by incubation with LPS (1 μg/mL) or PMA (1 μM) for 2 hr and was inhibited by resveratrol (30 μM). O_2^- exposure (0.36, O.D. 560 nm) for 2 hr also induced an enhancement of COX-2 levels that was reduced by resveratrol (30 μM). Ovine COX-2 (100 ng) was used as standard. The Western blot shown is representative of four experiments with similar results.

The antioxidant properties of resveratrol have already been described [3]. Here, we found that resveratrol exerted a powerful antioxidant effect on multiple ROS such as O_2^- and H_2O_2 produced by LPS- or PMA-stimulated macrophages. The system responsible for O_2^- production in phagocytic cells is the multicomponent enzyme NADPH oxidase. This complex includes membrane-bound cytochrome b_{558} and cytosolic proteins $p47^{phox}$, $p67^{phox}$, Rac 1/2, and $p40^{phox}$ [15] that translocate to the membrane during stimulation to form a catalytically active oxidase [16]. Recently, Cachia *et al.* [17] reported that α-tocopherol, another natural antioxidant, inhibits O_2^- production by impairing the assembly of the NADPH oxidase. Thus, a direct radical scavenger effect or an effect on NADPH oxidase activation are mechanisms that could be involved in the effect of resveratrol on ROS production. On the other hand, these toxic oxygen metabolites, considered to be mediators in cellular injury, have been shown to induce AA release [7] and the subsequent PGE_2 synthesis by the participation of COX-2 in murine peritoneal macrophages [18].

The signaling pathways activated by exogenous or endogenous oxidative stress have been the subject of intense research. Thus, the redox state of the cell may act as a molecular switch to regulate the activity of many enzymes and genes in concert. The release of AA from cellular phospholipids by PLA_2 occurs as a result of a variety of physiological stimuli and is the rate-limiting step in the subsequent metabolism by the cyclooxygenase pathway. Recently, we reported that ROS produced by resident peritoneal macrophages could be involved in the regulation by phosphorylation of PLA_2 activity and the subsequent [^{3}H]AA release [7]. Here, we found evidence showing that the antioxidant effect of resveratrol on O_2^- and H_2O_2 generation could be involved in the effect of the polyphenol on [^{3}H]AA release.

Following AA release from membranes by phospholipase activity, free intracellular AA can be metabolized to prostanoids. There are two isoforms of cyclooxygenase (COX) that catalyze the formation of PGs from AA: COX-1 is a housekeeping gene that is expressed constituently [19], while COX-2 is an immediate, early response gene that is highly inducible by mitogenic and inflammatory stimuli [12,20,21]. This led to the hypothesis that a highly expressed COX-2 is responsible for the high levels of prosta-

noids present in inflamed tissues. Moreover, many transcription factors are regulated by oxygen radicals [22], and ROS generation in LPS- or PMA-stimulated macrophages is involved in COX-2 induction [18]. Our results showed that resveratrol, at concentrations that inhibit O_2^- and H_2O_2 production, was able to reduce COX-2 levels induced by LPS or PMA. Thus, these findings suggest that the effect of resveratrol on PG biosynthesis may be explained not only by the action on COX-1 or COX-2 activity, as proposed previously Jang *et al.* [5], but also by the impairment of COX-2 induction.

With regard to the mechanism by which resveratrol modulates gene expression, Subbaramaiah *et al.* [23] reported recently that this polyphenol inhibits PKC translocation and the enhancement of COX-2 promoter activity mediated by PKC, by extracellular signal-regulated kinase-1 (ERK-1), and by c-Jun. Thus, this inhibitory effect of resveratrol could be explained by the antioxidant properties of resveratrol and other phenolic antioxidants that inhibit the action of PKC [24].

Bertelli *et al.* showed that resveratrol is quickly absorbed [25, 26], reaching its peak concentration of 25 ng/mL 60 min after wine ingestion (100 μg resveratrol/kg). The *in vivo* effects of resveratrol observed by Jang *et al.* [5] occur after an administration of 3 mg/kg of resveratrol, and the concentrations used in our *in vitro* study were 3–30 μM. Taken together, these data suggest that the effects observed in the present *in vitro* experiments could be correlated with the effects of resveratrol *in vivo*.

Finally, we must consider that endogenous fatty acids such as AA and lysophospholipids liberated by PLA_2 action on membrane phospholipids might be in a compartment that effectively activates the NADPH oxidase responsible for the respiratory burst, as reported recently Robinson *et al.* [27]. Thus, the effect of resveratrol on the AA cascade could also modulate ROS production.

To conclude, our results provide evidence that the antioxidant effect of resveratrol could be involved in the inhibitory effect of the polyphenol on AA mobilization, COX-2 overexpression, and the subsequent PGE_2 release induced by LPS and PMA in murine resident peritoneal macrophages.

This study was supported by grants from the Spanish Ministry of Education (DGES PM97-0110) and (DGES PM98-0191). We are very grateful to Robin Rycroft for his valuable assistance in preparation of the manuscript.

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