Resveratrol inhibits inducible nitric oxide synthase and cyclooxygenase-2 expression in β-amyloid-treated C6 glioma cells

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Abstract. Resveratrol has been reported to exert a variety of important pharmacological effects including anti-inflammatory, cardioprotective and cancer chemopreventive properties; however, its mechanisms of action are not completely understood. β-amyloid protein is considered to be responsible for the formation of senile plaques that accumulate in the brains of patients with Alzheimer disease. In the present study, we investigated the protective effect of resveratrol on β-amyloid-induced cytotoxicity in cultured rat astroglioma C6 cells. Preincubation of C6 cells with resveratrol concentration-dependently protected the cells from the growth inhibition induced by β-amyloid treatment. β-amyloid treatment led to increased nitric oxide (NO) synthesis and inducible nitric oxide synthase (iNOS) expression; however, cells pretreated with resveratrol showed a dose-dependent inhibition of NO production and iNOS expression following β-amyloid treatment. Resveratrol also attenuated β-amyloid-induced prostaglandin E2 (PGE2) release, which was associated with the inhibition of cyclooxygenase (COX)-2 expression. Furthermore, β-amyloid treatment induced nuclear translocation of NF-κB, which was suppressed by resveratrol pretreatment. Collectively, the present results indicate that modulation of nuclear factor-κB (NF-κB) activity is involved in the neuroprotective action of resveratrol against β-amyloid-induced toxicity.

Introduction

Alzheimer disease (AD) is a representative neurodegenerative disorder characterized by gradual degeneration and loss of neurons in the brain. The disease is characterized by three main pathogenic factors: senile plaques, neurofibrillary tangles and inflammation (1). One of the theories about the etiology of Alzheimer disease is the ‘β-amyloid protein toxicity’ hypothesis. β-amyloid is derived from proteolytic processing of a type I transmembrane glycoprotein called amyloid precursor protein, which is cleaved at two sites by two secretases, β- and α-secretase, and then yields short peptide β-amyloid (2). β-amyloid can mediate neurodegeneration through a complex interaction of neurodegenerative processes that involves increasing free radicals, raising intracellular Ca2+ concentrations, and triggering apoptosis (3). However, β-amyloid toxicity is eliminated by free radical scavengers, such as vitamin E, polyphenols and estrogens (4,5).

As shown by many epidemiological studies, diets with high contents of vegetables are cardioprotective. Some studies also showed a possible protective effect of vegetable rich diets on brain degenerative processes (6). The protective activities have been related to the high content of polyphenols and phytoestrogens in vegetables, fruits and beverages (for example, wine and tea). These compounds have an antioxidant activity, which is known as chelate transition metal ions, which interferes with transduction pathways involving protein phosphorylation and ion movements and are active on cell redox systems (6,7).

Resveratrol (3,5,4’-trihydroxy-trans-stilbene) is a naturally occurring phytoalexin found in grapes and wine, which is produced during environmental stresses such as ultraviolet light exposure and pathogen attacks (8,9). This polyphenol may play a role in the inverse correlation between red wine consumption and incidences of cardiovascular disease, and...
Table I. Gene-specific primers for RT-PCR.

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence of primers</th>
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<tbody>
<tr>
<td>iNOS</td>
<td>Sense: 5'-AGA-GAG-ATC-CGG-TTC-A 3'</td>
</tr>
<tr>
<td></td>
<td>Antisense: 5'-CAC-AGA-GCT-GAG-GGT-ACA 3'</td>
</tr>
<tr>
<td>COX-1</td>
<td>Sense: 5'-TGC-CCA-GCT-CCT-GGC-CGG-CCG-CTT-3'</td>
</tr>
<tr>
<td></td>
<td>Antisense: 5'-GTC-CA-CAT-AGG-CGC-CTC-TTC-3'</td>
</tr>
<tr>
<td>COX-2</td>
<td>Sense: 5'-TTC-AAA-TGA-GAT-TGT-GGG-AAA-AT-3'</td>
</tr>
<tr>
<td></td>
<td>Antisense: 5'-AGA-TC-AGA-AGG-GAG-TAT-CTT-3'</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Sense: 5'-CGG-AGT-CAA-CGG-ATT-TGG-TAT-3'</td>
</tr>
<tr>
<td></td>
<td>Antisense: 5'-AGC-CTT-CTC-CAT-GGT-GGA-GAC-3'</td>
</tr>
</tbody>
</table>

many studies, which may lead to the explanation of a health benefit of dietary resveratrol in humans, have concluded the same findings (10-12). A growing body of literature indicates that resveratrol is one of the most promising agents for prevention of heart disease, cancer and inflammatory disease (13). Resveratrol inhibits platelet aggregation, the production of pro-atherogenic eicosanoids, and the oxidation of low-density lipoproteins (14,15). Furthermore, this agent can inhibit, directly, several enzyme activities associated with cell proliferation and DNA replication such as ribonucleotide reductase, DNA polymerase, and soluble and membrane bound tyrosine kinases (16-18). However, the precise mechanisms of resveratrol remain largely unknown.

In the present study, using the rat astroglioma cell line (C6), we investigated the effects of resveratrol on nitric oxide (NO) production and prostaglandin E2 (PGE2) synthesis induced by b-amyloid. Our results indicate that resveratrol clearly inhibited the overexpression of cyclooxygenase (COX)-2 and inducible nitric oxide synthase (iNOS) by b-amyloid, which were connected with inhibition of NO generation and PGE2 release, and suppressed nuclear transition factor nuclear factor-kB (NF-kB) signaling.

Materials and methods

Cell culture, a-amyloid, resveratrol and growth inhibition study. Rat C6 glioma cells were obtained from the American Type Culture Collection and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum and antibiotics (100 μg/ml streptomycin, 100 U/ml penicillin and 0.25 μg/ml amphotericin B) at 37˚C in a humidified incubator under an atmosphere of 5% CO2 and 95% air. Amyloid b-protein fragment 31-35 (b-amyloid) and resveratrol were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). b-amyloid was dissolved in sterile water at a concentration of 2.5 mM and was stored frozen at -20˚C for at least 1 week prior to use. Resveratrol was dissolved in ethanol as a stock solution at 200 mM concentration, and stored in aliquots at -20˚C. For cell growth analysis, cells were treated with b-amyloid in the presence or absence of resveratrol for 6 h. The cells were trypsinized and washed with phosphate-buffered saline (PBS) and the viable cells were scored using the trypan blue method.

RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was isolated using TRIzol reagent (Invitrogen Co., Carlsbad, CA) following the manufacturer's recommendations. Total RNA was digested with RNase-free DNase (Boehringer Mannheim, Indianapolis, IN) for 15 min at 37˚C and repurified using an RNeasy kit according to the manufacturer's protocol (Qiagen, La Jolla, CA). cDNA was synthesized from 2 μg total RNA by incubation at 37˚C for 1 h with AMV reverse transcriptase (Amersham Corp., Arlington Heights, IL) with random hexanucleotide according to the manufacturer's instructions. The reaction mixture was subjected to polymerase chain reaction (PCR) to amplify sequences to desired primers (Table I). Amplification was performed in a mastercycler (Eppendorf, Hamburg, Germany) with cycles of denaturation at 94˚C, annealing at 58˚C, and extension at 72˚C for 30 sec, respectively. The amplified PCR products were run in 1.5% agarose gels and visualized by ethidium bromide (EtBr, Sigma).

Protein extraction, gel electrophoresis and Western blot analysis. Total cell lysates were lysed in an extraction buffer as previously described (19). Protein concentration was determined using a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA). For Western blot analysis, proteins were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and then electrotransferred to a nitrocellulose membrane (Schleicher & Schuell, Keene, NH). The membranes were subjected to immunoblot analysis with relevant antibodies and proteins were visualized using the enhanced chemiluminescence (ECL) method (Amersham). The primary antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Peroxidase-labeled donkey anti-rabbit immunoglobulin and peroxidase-labeled sheep anti-mouse immunoglobulin were purchased from Amersham.

Assay for NO synthesis. Cells were plated in DMEM and allowed to adhere overnight. The cells were treated with b-amyloid in the presence or absence of resveratrol for 6 h. Synthesis of NO was determined by assay of culture supernatants for nitrite, a stable reaction product of NO with molecular oxygen. Briefly, 100 μl of culture supernatant was
allowed to react with 100 μl of Griess reagent [0.1% N-(1-naphthyl) ethylenediamine, 1% sulfanilamide, 2.5% H₃PO₄], and then incubated at room temperature for 5 min. The optical density of the assay samples was measured at 550 nm using a microplate reader (Molecular Devices). Fresh culture media served as the blank in all experiments. Nitrite concentrations were calculated from a standard curve derived from the reaction of NaNO₂ in the assay.

Prostaglandin E₂ (PGE₂) accumulation assay. Cells were treated with β-amyloid in the presence or absence of resveratrol for 6 h. The medium was removed, and PGE₂ release by cells was measured. To measure the PGE₂ accumulation, enzyme immunoassay was performed using a commercial kit (Cayman Chemicals, Ann Arbor, MI) according to the manufacturer's protocol. The levels of total cellular PGE₂ production from endogenous arachidonic acids were measured using an ELISA reader at 630 nm. PGE₂ production was normalized with respect to the number of viable cells present in the particular culture.

Immunochemistry for NF-κB translocation. Cells were fixed with 3.7% paraformaldehyde for 30 min at 4°C and washed with PBS, and permeabilized with 0.2% Triton X-100 for 30 min at 4°C. The cells were washed with PBS, blocked with 2% bovine serum albumin (BSA, Pierce, Rockford, IL) for 1 h and treated with anti-NF-κB antibody for 2 h at 4°C. The anti-NF-κB stained cells were washed with PBS, incubated with FITC-conjugated anti-rabbit IgG (Sigma) for 1 h at 4°C and analyzed via a fluorescence microscope (Carl Zeiss, Germany).

Results

Resveratrol prevented β-amyloid-induced loss of C6 cell growth. To evaluate the effects of β-amyloid on the growth of C6 cells, hemocytometer counts were performed after treatment for 6 h. The results showed that β-amyloid exhibited a time-dependent decrease of cell growth (Fig. 1A). After 6 h, the growth of C6 cells decreased approximately 20% by 10 μM β-amyloid. However, preincubation of C6 cells with resveratrol for 40 min concentration-dependently protected the cells from the toxicity of β-amyloid (Fig. 1B). Resveratrol alone (up to 20 μM) had no cytotoxic effect in C6 cells (data not shown).

Effects of resveratrol on β-amyloid-induced NO production and iNOS expression. To investigate whether resveratrol can inhibit β-amyloid-induced NO synthesis and iNOS expression, C6 cells were treated with β-amyloid alone or together with resveratrol. When cells were stimulated in 10 μM β-amyloid, nitrites were time-dependently increased in culture supernatants (~3.5-fold after 6 h, Fig. 2A). However, after pretreatment with 20 μM resveratrol for 40 min, NO products decreased up to 30% in β-amyloid-treated C6 cells (Fig. 2B). To examine...
whether an inhibitory effect of resveratrol on ß-amyloid-induced NO production was attributable to its influence on iNOS synthesis, RT-PCR and Western immunoblot analyses were carried out. As shown in Fig. 3, iNOS mRNA and protein expression were hardly detectable in untreated control C6 cells, but markedly increased after ß-amyloid treatment. However, cells pretreated with resveratrol showed a dose-dependent inhibition of iNOS mRNA and protein expression following ß-amyloid treatment. These results clearly demonstrated that resveratrol produced a concentration-dependent inhibition of NO production and iNOS expression in response to ß-amyloid.

Effects of resveratrol on PGE2 secretion and COX-2 expression in ß-amyloid-treated C6 cells. The effects of resveratrol on the production of PGE2 and expression of COX-2 were examined in C6 cells activated with ß-amyloid. As shown in Fig. 4, ß-amyloid alone secreted large amounts of PGE2 in C6 cells. Within the range of doses used, pretreatment with various concentrations of resveratrol markedly inhibited ß-amyloid-induced PGE2 secretion in a dose-dependent manner. Furthermore, COX-2 mRNA and protein expression were barely detectable in untreated control C6 cells but markedly increased after ß-amyloid treatment without alteration of COX-1 expression (Fig. 5). However, cells pre-treated with resveratrol showed a dose-dependent inhibition of COX-2 mRNA and protein expression following ß-amyloid treatment. The data suggested that the inhibitory effect of resveratrol on the accumulation of PGE2 by amyloid-ß was associated with the down-regulation of COX-2 in C6 cells.
Repression of β-amyloid-induced NF-κB nuclear translocation by resveratrol. To investigate whether the induction of iNOS and COX-2 by β-amyloid treatment was connected with the activation of NF-κB through nuclear translocation, we examined the localization of NF-κB p65 by immunohistochemical analysis. As presented in Fig. 6, the immunostaining results indicated that NF-κB was seen in the cytosol of untreated control and resveratrol-alone-treated C6 cells and, in cells treated with β-amyloid, NF-κB was translocated from the cytosol into the nucleus. However, the data showed no basic difference between control cells and resveratrol-pretreated cells before treatment with β-amyloid, suggesting that the translocation of NK-κB by β-amyloid was prevented by pretreatment of resveratrol.

Discussion

Several studies within the last few years have shown that resveratrol has many biological and pharmacological effects. This agent inhibits platelet aggregation, the production of pro-atherogenic eicosanoids and the oxidation of low-density lipoproteins (14,15). Resveratrol can directly inhibit several enzyme activities associated with cell proliferation and DNA replication, such as ribonucleotide reductase, DNA polymerase and soluble and membrane bound tyrosine kinases (16-18). Furthermore, it has been suggested that resveratrol is one of the most potent chemopreventive agents able to block all three phases of tumor development, i.e. initiation, promotion, and progression (10), which was partly associated with the suppression of COX-2 expression and the activity of NF-κB (12,20,21). Recently, this compound also has been shown to exhibit neuroprotective effects against β-amyloid-induced neurotoxicity in several experimental models (22,23); however, its mechanisms of action are not completely understood.

β-amyloid, the major component of senile plaques, is considered to have a causal role in the development and progress of Alzheimer disease (24). Previous studies indicate that β-amyloid affects a wide array of neuronal and glial functions, thereby leading to neuronal cell death and dys-regulation of the normal inflammatory processes which have been implicated in the pathophysiology of Alzheimer disease (25,26). Since activated glia-derived pro-inflammatory cytokines are important pathological factors in the progression of Alzheimer disease, neuropathological changes, and inflammation in the central nervous system (27), the anti-inflammatory drug can reduce Alzheimer disease risk and delay some forms of Alzheimer disease pathology.

One of the principal enzymes that play a crucial role in mediating inflammatory response is COX-2. Although both isoforms (COX-1 and COX-2) are involved in the formation of PG endoperoxides, they are likely to have fundamentally different biological roles. COX-1 is a housekeeping enzyme which is constitutively expressed in most mammalian tissues and is thought to be involved in maintaining physiological functions. In contrast, COX-2 is barely detectable under normal physiological conditions, but can be induced rapidly and transiently by proinflammatory mediators and mitogenic stimuli, thereby mediating deleterious effects in the neurodegenerative disorders (28). In an Alzheimer brain, COX-2 is notably up-regulated, which seems to be associated with β-amyloid plaque formation (28,29). COX-2 upregulation is thought to mediate neuronal damage presumably by producing excessive amounts of harmful prostanoids and free radicals. An expanding body of data suggests that PGs are involved in the pathogenesis of Alzheimer disease. Elevated production of PGs, especially PGE2, has been found in the brains of patients with Alzheimer disease (30). Based on these findings, it is conceivable that COX-2 represents a therapeutic target for anti-inflammatory treatment of Alzheimer disease dementia. In the present results, resveratrol clearly inhibited the secretion of PGE2, and COX-2 expression induced by β-amyloid (Figs. 4 and 5). Therefore, these results suggest that resveratrol might have a beneficial effect in the treatment of COX-2-mediated Alzheimer disease.

The intercellular messenger, NO, is a short-lived free radical that participates in the physiology and pathophysiology of many systems including Alzheimer disease (31,32).
Production of NO is formed by NOS, which converts L-arginine to L-citrulline (33). Three isoforms of NOS have been identified, including endothelial NOS (eNOS), iNOS, and neural NOS (nNOS). The small amount of NO produced by constitutive NOS, including eNOS and nNOS, is an important regulator of physiological homeostasis, whereas the large amount of NO produced by iNOS has been closely correlated with the pathophysiology in a variety of diseases and inflammation (33-35). As shown in Fig. 2, resveratrol was discovered to have a dose-dependent inhibitory effect on β-amyloid-induced NO production in C6 cells. Furthermore, resveratrol attenuated β-amyloid-induced synthesis of both mRNA and protein of iNOS suggesting that resveratrol down-regulates β-amyloid-induced iNOS expression at both transcriptional and translational levels (Fig. 3).

On the other hand, NF-κB, which is a redox-sensitive transcription factor, regulates the expression of genes that are involved in cellular differentiation, proliferation, apoptosis, oxidative response, inflammation, and immune responses (36). The COX-2 promoter harbors at least two NF-kB response elements (37) and activated NF-kB plays a crucial role in COX-2 gene expression in response to proinflammatory signals or cellular stress. The iNOS promoter also has a number of binding sites for transcription factors, including NF-kB, and NF-kB sites which are essential for NO production (38). In the cytosol, NF-kB is constitutively present as homo- or heterodimers and is linked to inhibitory IκB proteins. Activation of NF-kB results in phosphorylation, ubiquitination, and proteasome-mediated degradation of the IκB proteins, followed by translocation of NF-kB to the nucleus and induction of gene transcription through its binding to the cis-acting NF-kB element (39,40). NF-kB activation was evident in postmortem Alzheimer disease brains (41), and β-amyloid treatment resulted in NF-kB activation in cultured cells (42,43). Therefore, inhibition of NF-kB activity promises to be an effective approach in the prevention and treatment of β-amyloid toxicity. In the present study, as shown in Fig. 6, resveratrol markedly inhibited β-amyloid-mediated nuclear translocation of NF-κB, indicating that resveratrol can suppress β-amyloid-mediated NF-κB activation, specific to the nuclear translocation step of NF-κB.

In conclusion, resveratrol was discovered to inhibit NO production and iNOS expression, and PGE_2 secretion and COX-2 expression in β-amyloid-stimulated C6 glial cells, which was attributable to its suppression of nuclear translocation events of NF-κB. Resveratrol could provide an invaluable tool for investigating NF-κB-dependent iNOS and COX-2 expression, in addition to its therapeutic potential in inflammatory diseases.

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References


