INTRODUCTION

Human dental pulp inflammation is characterized by changes in blood flow (Olgart et al., 1991), immuno-competent cell function (Bergenholtz et al., 1991), and neuronal activity (Närhi and Hirvonen, 1983). Several mediators including histamine, prostaglandins, and neuropeptides are involved in one or more of these processes (Hirafuji et al., 1980; Grutzner et al., 1992), while all steps may involve nitric oxide (NO) and effects of NO synthase (NOS). NO acts as an intracellular messenger at physiological levels although it can be cytotoxic at higher concentrations. Epigallocatechin gallate (EGCG), a major green tea polyphenol, has diverse pharmacological activities in cell growth and death. This study is aimed to investigate the apoptotic mechanism by NO and effects of EGCG on NO-induced apoptosis in human dental pulp cells (HDPC). Sodium nitroprusside (SNP), an NO donor, decreased the cell viability of HDPC in a dose- and time-dependent manner. EGCG was administered for 1 hr before the SNP treatment, resulting in increased cell viability and reactive oxygen species (ROS) production inhibition. Expression of Bax, a pro-apoptotic Bcl-2 family, was upregulated, whereas expression of Bcl-2, an anti-apoptotic Bcl-2 family, was downregulated in SNP-treated HDPC. SNP augmented the release of cytochrome c from mitochondria into cytosol and enhanced caspase-9, -3 activities, a marker of the apoptotic executing stage. EGCG ameliorated caspase-9 and -3 activities and cytochrome c release increased by SNP. These results suggest that EGCG has a protective effect against NO-induced apoptosis in HDPC by scavenging ROS and modulating the Bcl-2 family.

Key words: Epigallocatechin gallate, Nitric oxide, Apoptosis, Human dental pulp cells

Original Article

Epigallocatechin gallate protects against nitric oxide-induced apoptosis via scavenging ROS and modulating the Bcl-2 family in human dental pulp cells

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ABSTRACT — Nitric oxide (NO) is produced by three different isoforms of the enzyme NO synthase (NOS). NOS isoforms are expressed in many cell types, including human dental pulp cells (HDPC). NO acts as an intracellular messenger at physiological levels although it can be cytotoxic at higher concentrations. Epigallocatechin gallate (EGCG), a major green tea polyphenol, has diverse pharmacological activities in cell growth and death. This study is aimed to investigate the apoptotic mechanism by NO and effects of EGCG on NO-induced apoptosis in HDPC. Sodium nitroprusside (SNP), an NO donor, decreased the cell viability of HDPC in a dose- and time-dependent manner. EGCG was administered for 1 hr before the SNP treatment, resulting in increased cell viability and reactive oxygen species (ROS) production inhibition. Expression of Bax, a pro-apoptotic Bcl-2 family, was upregulated, whereas expression of Bcl-2, an anti-apoptotic Bcl-2 family, was downregulated in SNP-treated HDPC. SNP augmented the release of cytochrome c from mitochondria into cytosol and enhanced caspase-9, -3 activities, a marker of the apoptotic executing stage. EGCG ameliorated caspase-9 and -3 activities and cytochrome c release increased by SNP. These results suggest that EGCG has a protective effect against NO-induced apoptosis in HDPC by scavenging ROS and modulating the Bcl-2 family.

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INTRODUCTION

Human dental pulp inflammation is characterized by changes in blood flow (Olgart et al., 1991), immuno-competent cell function (Bergenholtz et al., 1991), and neuronal activity (Närhi and Hirvonen, 1983). Several mediators including histamine, prostaglandins, and neuropeptides are involved in one or more of these processes (Hirafuji et al., 1980; Grutzner et al., 1992), while all steps may involve nitric oxide (NO). SNP, a NO donor, is pharmacologically active substance that releases NO in vivo and in vitro (Pirrone et al., 2006). NO is an intracellular messenger molecule with important cardiovascular, neurological, and immune functions (Nathan, 1992). NO is a short lived, highly reactive free radical gas that is synthesized from L-arginine in a reaction catalyzed by the nitric oxide synthase (NOS), of which three isoforms exist. NOS plays a significant role in the pathogenesis of pulpitis. Human dental pulp cells (HDPC) express eNOS and iNOS (Di Nardo Di Maio et al., 2004). The eNOS is constitutive isoforms that can rapidly synthesize small amounts of NO following receptor stimulation (Carmignani et al., 2000). The iNOS is mainly involved in the inflammatory processes. Pro-inflammatory stimuli triggers resident and immigrant inflammatory cell populations induce iNOS (Nussler and Billiar, 1993). NO has detrimental effects as well, because exaggerated NO production can be toxic against pulpal tissues (Lohinai and Szabó, 1998). Furthermore, the excessive vasodilation and increased vascular permeability elicited by locally high NO can raise the intrapulpal hydrostatic pressure, because the pulp is a low compliance system and is located in a closed and rigid dental chamber (Lohinai et al., 1995). Consequently, increased intrapulpal pressure may...
compress the pulpal venules and thus significantly impair the pulpal perfusion, potentially insulting the pulp tissue and even causing pulpal necrosis. However, the cellular mechanisms underlying the NO-induced cell death have not been fully described in HDPC.

Tea polyphenols are natural plant flavonoids found in the leaves and stem of tea plant. The green tea polyphenols have a variety of pharmacological properties such as anti-inflammatory, anticarcinogenic, and antioxidant effects (Stoner and Mukhtar, 1995; Gensler et al., 1996; Shi et al., 2000). Green tea contains an abundance of flavonoids called catechins, specifically epigallocatechin-3-gallate (EGCG), epigallocatechin (EGC), epicatechin-3-gallate (ECG), and epicatechin (EC) (Nanjo et al., 1996; Shankar et al., 2007). Among these polyphenols, EGCG is a major component responsible for biological effects. EGCG exhibits potent antioxidant property because it possesses two triphenolic groups in its structure (Jin et al., 2000).

There is an increasing body of evidence that NO may give rise to cytotoxicity on HDPC (Yasuhara et al., 2007). Nevertheless, the underlying mechanisms of NO-induced cytotoxicity have not been studied in HDPC. This study was designed to investigate the mechanism of NO-induced apoptosis and the effects of EGCG on NO-induced apoptosis in HDPC.

**MATERIALS AND METHODS**

**Cell culture and treatment with SNP and EGCG**

Human dental pulp cells (HDPC) were obtained from healthy tooth of two patients in Chonnam National Hospital according to a protocol approved by the Ethics Committee. Informed consent had been obtained from each patient. The human dental pulp tissues obtained from sectioned teeth were removed aseptically, rinsed with Hanks’ buffered saline solution, and placed in a 60 mm Petri dish. The dental pulp tissues were then minced with a blade into small fragments and cultured in Dulbecco modified Eagle medium containing 10% fetal bovine serum (Gibco, Carlsbad, CA, USA) along with 100 U/ml penicillin and 100 U/ml streptomycin (Life Technologies, Gaithersburg, MD, USA). Cultures were maintained at 37°C in a humidified atmosphere of 5% CO₂ and 95% air. Sodium nitroprusside (SNP, Sigma, St. Louis, MO, USA) was dissolved in distilled water and sterilized through 0.2 μm filter. Cells were treated with each concentration of SNP for needed time in the same medium and EGCG (Sigma) was pretreated for 1 hr before SNP treatment.

**Cell viability assay by MTT assay**

The MTT assay relies on the observation that viable cells with active mitochondria reduce 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma) into a visible dark-blue formazan reaction product and providing an indirect measurement of cell viability. HDPC were plated onto 96 well plates (5 × 10⁴ cells/well) and exposed to SNP alone or pretreated with different concentration of EGCG for 1 hr. After treatment, MTT was added to the culture medium at a final concentration of 0.1 mg/ml and incubated at 37°C for 3 hr. The absorbance of MTT was extracted in dimethyl sulfoxide (DMSO, Sigma) and optical density (OD) was spectrophotometrically measured at 570 nm with DMSO as a blank using a absorbance microplate reader (ELx800uv, BioTek, St. Winooski, USA).

**Annexin V-positive cell assay**

Annexin V assay was performed using FITC Annexin V Apoptosis Detection kit II (Pharmingen, San Jose, CA, USA). After treatment with various concentrations of SNP for 24 hr, cells were collected and washed twice with cold PBS and then resuspended in 1xbinding buffer at a density of 10⁶ cells/ml. An aliquot of 100 μl was transferred to a 5 ml test tube, 5 μl of Annexin V-FITC and 5 μl of PI were added, gently vortexed and incubated for 15 min at RT in the dark. Then 400 μl of 1X binding buffer was added to each tube and samples were analyzed by flow cytometry (Beckman, Krefeld, Germany).

**Detection of ROS production and caspase activity**

Reactive oxygen species (ROS) production was monitored by a fluorescence spectrometer (Hitachi F-4500, Tokyo, Japan) using 2', 7'-dichlorofluor- bescin diacetate (DCF-DA). Cells were plated on 96-well plates (5 × 10⁴ cells/well) and treated with N-acetyl-cysteine (NAC, Sigma) and SNP. DCF-DA (25 μM) was added into the media for further 10 min at 37°C. Emission was measured at 530 nm. Caspase activities were assayed by spectrometer using the caspase-9 and caspase-3 activity assay kit (Calbiochem, Gibbstown, NJ, USA) according to the manufacturer’s instructions.

**Subcellular fractionation**

Cells were harvested by centrifugation at 600 x g for 10 min at 4°C. The cell pellets were washed and suspended in ice-cold cytosol extraction buffer (250 mM sucrose, 70 mM KCl, 250 μg/ml digitonin, 10 μg/μl aprotinin, 10 μg/μl leupeptin, 2 μg/μl pepstatin and 1 mM PMSF in PBS). After a 15 min incubation on ice, samples were centrifuged at 1,000 × g for 5 min and supernatant
was collected (cytosolic fraction). Cytosolic fraction were resolved on a 15% SDS-PAGE and analyzed by Western blot using anti-human cytochrome c antibody (1:1,000).

Western blotting
Cells were washed twice with PBS and proteins were solubilized in the lysis buffer (500 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1 mM Benzenoiden, 1 μg/ml Trypsin inhibitor) containing a cocktail of protease inhibitor (Roche, Mannheim, Germany). Lysates were incubated for 30 min at 4°C, centrifuged at 12,000 x g for 20 min and protein concentrations were determined by BCA protein assay (Pierce, Rockford, IL, USA). The protein extracts (100 μg) were boiled for 5 min with an SDS-sample buffer and then subjected to electrophoresis on 12% polyacrylamide gel. Proteins were electroblotted onto a nitrocellulose membrane (Santa Cruz, Finnell Street Dallas, TX, USA) and blocked with 5% skim milk (BD, Franklin Lakes, NJ, USA) in Tris-buffered saline-0.1% Tween 20 (TBS-T) for 1 hr and incubated with the respective primary antibody. Rat monocloned antibodies against cytochrome c, Bcl-2, Bax, and Actin (Santa Cruz) were applied. The blots were subsequently washed three times in TBS-T for 5 min and incubated with specific peroxidase-coupled secondary antibodies (Sigma). The bound antibodies were visualized using an enhanced chemiluminescent detection system (GE Healthcare, Pittsburgh, PA, USA).

Statistical analysis
Results are expressed as mean ± standard deviations (S.D.) and analyzed by Student’s t-test. Level of p < 0.05 was regarded as statistically significant.

RESULTS

Effect of EGCG on cells viability and apoptosis in SNP-treated cells
To evaluate whether HDPC express eNOS and iNOS, HDPC was analyzed by Western blot. As a result, the HDPC endogenously expressed eNOS and iNOS, which are critical for the synthesis of NO (Fig. 1A). To assess cell viability for the time period, cell viability was measured by MTT assay. The cell viability was greatly reduced in dose- and time-dependent manners when HDPC were exposed to SNP (Figs. 1B and 1C). The survival rate of the HDPC treated with 4 mM SNP for 24 hr was approximately 30%, whereas the EGCG pretreatment at a concentration from 5 to 50 μM for 1 hr prevented the loss of viability (Fig. 1D). To examine whether EGCG blocks SNP-induced apoptosis, the HDPC were incubated with 50 μM EGCG for 1 hr and treated with 4 mM SNP for 24 hr. Annexin V-positive cell assay was used to show the rate of apoptosis induced by SNP in HDPC. The rate of Annexin V-positive cells was 0.56% in the control cells and 42.65% in SNP-treated cells. However, the rate of apoptosis was significantly reduced (approximately 5.45%) when the cells were pretreated with EGCG before SNP exposure (Fig. 1E). Thus, these results suggest that EGCG suppresses SNP-induced apoptosis in HDPC.

EGCG reduces the generation of ROS induced by SNP in HDPC
The involvement of ROS in the SNP-induced apoptosis of HDPC was examined by measuring the level of ROS production using DCF-DA. The fluorescence images were obtained under confocal microscopy. An increase in fluorescence observed in cells exposed to 4 mM SNP for 1 hr was significantly reduced in the presence with 50 μM EGCG or 5 mM NAC (Fig. 2A). In addition, DCF fluorescence results evaluated by fluorescent spectrometer demonstrated that SNP induced the production of ROS in a dose-dependent manner, whereas EGCG ameliorated the SNP-induced ROS production (Fig. 2B).

EGCG ameliorates the increase of the Bax to Bcl-2 ratio and the release of mitochondrial cytochrome c to cytosol induced by SNP in HDPC
The Bcl-2 family is an important regulator in various apoptosis pathways. To determine whether EGCG protects SNP-induced apoptosis of HDPC by modulating the Bcl-2 family, expression of Bax and Bcl-2 were observed using Western blot analysis. In the SNP-treated cells, the expression of Bcl-2 was downregulated significantly. Therefore, the ratio of Bax to Bcl-2 was high, which indicates a low apoptotic threshold. However, the pretreatment with EGCG modulated the Bcl-2 and Bax expression altered by SNP, resulting in a decrease of the Bax to Bcl-2 ratio (Fig. 3A).

To evaluate whether EGCG inhibits the loss of mitochondrial integrity, cytochrome c released from the mitochondria into the cytosol in the SNP-treated cells was analyzed by Western blot. As shown as Fig. 3B, SNP caused the accumulation of cytochrome c in the cytosol. When the cells were pretreated with EGCG prior to SNP treatment, cytochrome c in the cytosol was significantly reduced compared with the SNP-treated group. These results suggest that SNP induces the release of cytochrome c from mitochondria into cytosol during the apoptotic process induced by SNP and EGCG blocks the release of cytochrome c into the cytosol.
EGCG attenuates caspase-9 and -3 activities induced by SNP in HDPC

Caspases have been known to be important triggers for the execution of apoptosis. To determine whether EGCG protects against SNP-induced apoptosis via the inhibition of caspases, the activities of caspase-9 and -3 were measured. In HDPC treated with 4 mM SNP, caspase-9 and -3 activation were evident at 8 hr but declined to normal by 24 hr. In addition, pretreatment of HDPC with 50 μM EGCG for 1 hr prior to 4 mM SNP completely inhibited the SNP-induced increase in caspase-9 and -3 activities at 8 hr after SNP treatment (Figs. 4A and 4B).

DISCUSSION

Green tea extract has been demonstrated to protect brain, liver, and kidney from lipid peroxidation injury. Besides, EGCG exhibits potent antioxidant property and these effects have been extensively studied (Cheng et al., 1996; Guo et al., 1996; Kondo et al., 1999; Nagai et al., 2002). The antioxidant and free-radical scavenger properties of EGCG are responsible for the protective effect against cardiovascular and metabolic disease (Chung et al., 2001; Pianetti et al., 2002; Bose et al., 2008). Variations in cell signaling and cell cycle regulation, oxidative stress environment, antioxidant metabolism, induction and/or suppression of apoptosis are considered involved in discriminating effects of tea polyphenols depending on different cell types (Raza and John, 2005). Recent evidence reported that EGCG inhibits the expression of pro-inflammatory cytokines and adhesion molecules in HDPC (Nakanishi et al., 2010). However, there are no reports of the effects of EGCG against NO-induced toxicity of HDPC.
Protection of EGCG against NO-induced apoptosis in HDPC

Fig. 2. Effect of EGCG on ROS produced by SNP in HDPC. Intracellular levels of ROS in 4 mM SNP-treated cells with or without 50 μM EGCG, or 5 mM NAC for 1 hr were imaged on confocal laser microscope (A). Bar, 50 μm. HDPC loaded with DCF-DA were incubated with different concentration of SNP in the presence or absence of 50 μM EGCG for 1 hr. The intracellular levels of ROS were determined by measuring the DCF fluorescence (B). The data is reported as the mean ± S.D. from triplicate independent experiments. *p < 0.05 vs. the SNP-treated group.

Fig. 3. Effect of EGCG on the expression of the Bcl-2 family by SNP in HDPC. The cytosolic proteins were prepared from the cells with or without 50 μM EGCG pretreatment for 1 hr in the presence of 4 mM SNP for 24 hr. Western blot analysis was performed for Bax and Bcl-2 protein levels (A) and cytosolic cytochrome c (Cyt c) levels (B). The Bax to Bcl-2 ratio determined by densitometer was plotted. The data is reported as the mean ± S.D. from triplicate independent experiments.
To investigate the mechanism mediating the protective effect by EGCG in NO-induced toxicity, the SNP-treated HDPC culture system was used. EGCG remarkably reduced cell death of HDPC by NO. SNP-induced apoptotic cell death of HDPC was prevented by pretreatment with EGCG before SNP exposure. This result suggests that EGCG has a protective effect on NO-induced apoptosis. It was reported that NO-induced apoptosis is driven by the production of ROS, resulting in the formation of peroxynitrite (Bolaños et al., 1997; Peuchen et al., 1997). However, lipid peroxidation as a result of ROS production can be attenuated by the addition of EGCG (Guo et al., 1996). It provides one of the possible protective mechanisms of EGCG which might be its direct scavenging of ROS production by NO. In the present study, the SNP-treated HDPC enhanced the intracellular ROS levels, which were attenuated by EGCG, similarly to NAC, a ROS scavenger. This result demonstrates that NO-induced apoptosis may be driven by oxidative stress and ROS scavenging effects of EGCG may be an important factor in reducing the level of the cell death induced by NO.

Another anti-apoptotic mechanism of EGCG may be driven by the regulation of the signaling molecules that participate in the apoptotic process induced by NO. Anti-apoptotic proteins (e.g., Bcl-2 and Bcl-xL) and pro-apoptotic proteins (e.g., Bax and Bid) of Bcl-2 family are important modulators in regulating cell death (Boise et al., 1993; Oltvai et al., 1993). The altered ratio of pro- to anti-apoptotic Bcl-2 family proteins is significant in determining if apoptosis occurs. In the present study, the Bax/Bcl-2 ratio was increased significantly by the treatment with SNP and was attenuated by the pretreatment with EGCG. This result suggests that the anti-apoptotic action of EGCG may occur by regulating the expression of Bcl-2 family. Previous studies have shown that EGCG directly regulates the expression of the Bcl-2 family such as Bax, Bad, Mdm2, Bcl-2 and Bcl-X.<sub>L</sub> (Mandel et al., 2004). Bcl-2 family plays an important role in regulating the release of cytochrome c from the mitochondria into the cytosol and caspase activation (Gross et al., 1999). Therefore, the modulation of the Bcl-2 family by EGCG might block the downstream apoptosis pathway. The Bcl-2 family controls the release of mitochondrial cytochrome c by regulating the mitochondrial permeability transition (PT) pore composed of the voltage-dependent anion channel (VDAC) in the outer membrane, the adenosine nucleotide translocated (ANT) in the inner membrane, and cyclophilin-D (Cyp-D) in the matrix assemblies (Crompton, 2000; Gross et al., 1999). Once cytochrome c is released into the cytosol during apoptosis, it binds to Apaf-1, which recruits and activates pro-caspase-9, an initiator caspase.

**Fig. 4.** Effect of EGCG on caspases in SNP-treated HDPC. The caspases activities were determined in the 4 mM SNP treated HDPC for 24 hr with or without 50 μM EGCG for 1 hr. The total protein (50 μg) with the LEHD-pNA substrate (200 μM), the IETD-pNA substrate (200 μM) and the DEVD-pNA substrate (200 μM), respectively, for the enzymatic activity of caspase-9 protease (A) and caspase-3 protease (B) were assayed. The data is reported as the mean ± S.D. from triplicate independent experiments. *p < 0.05 vs. the SNP-treated group.
Caspases transduce the apoptotic cell death signals in a cascade manner, where the initiator caspases cleave and activate the effector caspases, which then degrade other cellular targets leading to cell death (Wolf and Green, 1999). Caspase-3 is one of the effector caspases found in apoptotic cells, which is activated by action of upstream signaling, such as caspase-9 (Masumura et al., 2000). In the present study, we demonstrated that the induction of apoptosis by NO was accompanied by a significant increase in caspase-9 and -3 activities. These effects were attenuated by the pretreatment with EGCG, before SNP treatment. These results suggest that EGCG inhibits NO-induced apoptosis by suppressing the release of cytochrome c from the mitochondria into the cytosol, which in turn blocks the activation of caspase-9 and -3.

Taken together, these results suggest that EGCG prevents NO-induced apoptosis by scavenging ROS and regulating the expression of the Bcl-2 family and the activation of caspases which play a key role in NO-induced apoptosis in HDPC.

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