Effects of Resveratrol and trans-3,5,4′-Trimethoxystilbene on Glutamate-Induced Cytotoxicity, Heme Oxygenase-1, and Sirtuin 1 in HT22 Neuronal Cells

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Abstract
Resveratrol (trans-3,5,4′-trimethoxystilbene) has received considerable attention recently for the potential neuroprotective effects in neurodegenerative disorders where heme oxygenase-1 (HO-1) and sirtuin 1 (SIRT1) represent promising therapeutic targets. Resveratrol has been known to increase HO-1 expression and SIRT1 activity. In this study, the effects of resveratrol and trans-3,5,4′-trimethoxystilbene (TMS), a resveratrol derivative, on cytotoxicity caused by glutamate-induced oxidative stress, HO-1 expression, and SIRT1 activation have been investigated by using murine hippocampal HT22 cells, which have been widely used as an in vitro model for investigating glutamate-induced neurotoxicity. Resveratrol protected HT22 neuronal cells from glutamate-induced cytotoxicity and increased HO-1 expression as well as SIRT1 activity in a concentration-dependent manner. Cytoprotection afforded by resveratrol was partially reversed by the specific inhibition of HO-1 expression by HO-1 small interfering RNA and the nonspecific blockage of HO-1 activity by tin protoporphyrin IX, but not by SIRT1 inhibitors. Surprisingly, TMS, a resveratrol derivative with methoxyl groups in lieu of the hydroxyl groups, and trans-stilbene, a non-hydroxylated analog, failed to protect HT22 cells from glutamate-induced cytotoxicity and to increase HO-1 expression and SIRT1 activity. Taken together, our findings suggest that the cytoprotective effect of resveratrol was at least in part associated with HO-1 expression but not with SIRT1 activation and, importantly, that the presence of hydroxyl groups on the benzene rings of resveratrol appears to be necessary for cytoprotection against glutamate-induced oxidative stress, HO-1 expression, and SIRT1 activation in HT22 neuronal cells.

Key Words: Resveratrol, trans-3,5,4′-Trimethoxystilbene, Heme oxygenase-1, Sirtuin 1, Oxidative stress, Neuroprotection

INTRODUCTION
Although the production of reactive oxygen species and their detoxification are normal physiological processes, an imbalance between the production of reactive oxygen species and their removal may lead to oxidative stress (Steele et al., 2007). It is now well established that oxidative stress plays a crucial role in the development of the most common neurodegenerative diseases, namely Alzheimer’s disease and Parkinson’s disease (Floyd and Hensley, 2002). Although whether the oxidative stress is a cause or consequence of the neurodegenerative diseases remains to be elucidated, a growing body of evidence suggests that oxidative stress directly initiates and progresses to neuronal cell death (Pocernich et al., 2011). Therefore, a key therapeutic intervention would be to reduce cellular levels of oxidative stress and/or to enhance cellular resistance to oxidative stress. Because natural anti-oxidants, such as polyphenols, have been shown to prevent neuronal cell death via scavenging of reactive oxygen species and/or enhancing the cellular antioxidant system, they may be thought to be potential candidate neuroprotective agents (Kelsey et al., 2010). Among the natural polyphenols, resveratrol (trans-3,5,4′-trimethoxystilbene; RSV) that is present in a variety of plants is well known as one of neuroprotective antioxidants (Albani et al., 2010; Robb and Stuart, 2010; Sun et al., 2010; Richard et al., 2011; Li et al., 2012). Studies have shown that RSV is neuroprotective against oxidative stress (Darvesh et al., 2010; Hall et al., 2010). However, the mechanisms of actions involved in the antioxidant-related neuroprotective effects of RSV are not fully understood. RSV has been reported to have the capacity to chelate metal ions and to directly quench reactive oxygen species that contribute to oxidative damage (Martin et al., 2002; Ndiaye et al., 2005; Sun et al., 2008). While both of these physiochemical properties...
are known to contribute to the overall neuroprotective effects of RSV, RSV can also have the capacity to activate or inhibit various cellular signaling pathways and numerous additional regulatory molecules involved in cellular protection against oxidative stress (Sun et al., 2010). Interestingly, recent studies have shown that RSV can attenuate neuronal cell death caused by oxidative stress, indirectly through induction and/or activation of antioxidant/cytoprotective enzymes, including sirtuin 1 (SIRT1) and heme oxygenase-1 (HO-1) (Chen et al., 2005; Albani et al., 2009).

SIRT1 is a class III protein deacetylase and there are seven members of the sirtuin class of enzymes in mammals; SIRT1 to SIRT7 (Srivastava and Haigis, 2011). Sirtuines regulate a number of intracellular pathways via the activation of transcription factors and enzymes responsive to nutrient availability (Zhang et al., 2011). SIRT1 is the major sirtuin activated through both calorie restriction and RSV (Borra et al., 2005). SIRT1 has been shown to promote cell survival by inhibiting apoptosis or cellular senescence induced by stresses, including DNA damage and oxidative stress (Hasegawa et al., 2008; Hasegawa and Yoshikawa, 2008; Albani et al., 2009). HO-1 is a rate-limiting enzyme in the degradation of pro-oxidant free heme to produce free iron, carbon monoxide and biliverdin, which is further converted to the antioxidant bilirubin by biliverdin reductase (Pae et al., 2010). HO-1 can be induced by various oxidative-inducing agents, including heavy metals, UV radiation, cytokines, and endotoxin (Pae et al., 2008). Recently, numerous in vitro and in vivo studies have shown that the induction of HO-1 is an important cytoprotective mechanism against oxidative injury (Jazwa and Cuadrado, 2010; Wu et al., 2011a).

Attempts to design the analogues of RSV with more potent biological activities have yielded many new stilbenes with different aryl substituents, of which trans-3,5,4′-trimethoxy-stilbene (TMS) is a primary candidate. It has been demonstrated that TMS is more potent than RSV as an antioxidant/cytoprotective agent (Kulawiak and Szewczyk, 2010). However, whether TMS, similar to RSV, would also exert antioxidant/cytoprotective effects remains to be investigated. Particularly, the effects of RSV on HO-1 expression and SIRT1 activation in neuronal cells are not compared with those of TMS. In the present study, we have investigated whether RSV and TMS would attenuate oxidative stress-induced cellular injury in neuronal cells and have further explored whether HO-1 expression and/or SIRT1 activation would be involved in these cytoprotective actions of RSV and TMS. For this end, we used HT22 neuronal cells (an immortalized mouse hippocampal cell line) which have been widely used as an in vitro model for elucidating the mechanism of oxidative stress-induced neurotoxicity (Behl, 1998). HT22 cells have been known to lack functional ionotropic glutamate receptor (Kulawiak and Szewczyk, 2012), thus excluding excitotoxicity as a cause for cell death induced by glutamate (Glut). In HT22 cells, Glut at high concentrations induces oxidative stress and subsequently cell death by inhibiting cysteine uptake, which results in the depletion of intracellular cysteine that is primarily associated to the cellular synthesis of the antioxidant glutathione (Behl, 1998; Kulawiak and Szewczyk, 2012).

### Materials and Methods

#### Reagents and antibodies

RSV, trans-stilbene (TS), Dulbecco’s modified Eagle’s medium (DMEM), hemin, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide (MTT), NADPH, glucose-6-phosphate dehydrogenase, glucose-6-phosphate, sirtolin, nicotinamide, tin protoporphyrin IX (SnPP), and glutamate were purchased from Sigma-Aldrich (St. Louis, MO, USA). TMS was obtained from Wako Pure Chemical Industries (Osaka, Japan). Antibodies against HO-1, SIRT1 and β-actin and small interfering RNA (siRNA) products against HO-1 and SIRT1 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). All other reagents used were of analytical grade.

#### Cell culture

Mouse hippocampal HT22 cells (kind gift of Prof. Youn-Chul Kim at Wonkwang University, Iksan, Korea) were maintained at 5 × 10^5 cells per 1 ml in DMEM medium supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen, Carlsbad, CA, USA), penicillin G (100 units/ml), streptomycin (100 μg/ml), and L-glutamine (2 mM) and were incubated at 37°C in a humidified atmosphere containing 5% CO₂ and 95% air.

#### MTT assay

Cell viability was determined by a modified MTT reduction assay. MTT is a pale yellow substance that is reduced by living cells to yield a dark blue formazan product. This process requires active mitochondria, and even fresh dead cells do not reduce significant amounts of MTT. HT22 cells were cultured in a 96-well flat-bottom plate at concentration of 5 × 10^5 cells/ml. After 12 h of pre-conditioning, the cells were treated with various concentrations of RSV, TMS, or TS for 24 h. Thereafter, culture medium was aspirated and 100 μl of MTT dye (1 mg/ml in phosphate-buffered saline) was added; the cultures were incubated for 4 h at 37°C. The formazan crystals produced through dye reduction by viable cells were dissolved using acidified isopropanol (0.1 N HCl). Index of cell viability was calculated by measuring the optical density of color produced by MTT dye reduction at 570 nm.

#### Western blot analysis

Proteins isolated from cells were separated by 10% SDS-PAGE denaturing gels and transferred to nitrocellulose membranes. After blocking, the membranes were incubated with antibodies against HO-1 (1:1,000 dilution), SIRT1 (1:1,000 dilution), or β-actin (1:1,000 dilution) at 4°C overnight. Afterward, membranes were incubated with HRP-conjugated secondary antibodies for 1 h at room temperature. Specific bands were detected using enhanced chemiluminescence detection system (Amersham Biosciences Inc., Piscataway, NJ, USA), and the membranes were exposed to X-ray film.

#### HO activity assessment

HO activity was determined at the end of each treatment as described previously (Son et al., 2010). Briefly, harvested cells were subjected to three cycles of freeze-thawing before addition to a reaction mixture consisting of phosphate buffer (1 ml final volume, pH 7.4) containing magnesium chloride (2 mM), NADPH (0.8 mM), glucose-6-phosphate (2 mM), glucose-6-phosphate dehydrogenase (0.2 Units), rat liver cytosol as a source of biliverdin reductase, and the substrate hemin (20
μM). The reaction mixture was incubated in the dark at 37°C for 1 h and was terminated by the addition of 1 ml of chloroform. After being vigorously vortexed and centrifuged, the extracted bilirubin in the chloroform layer was measured by the difference in absorbance between 464 and 530 nm (ε = 40 mM⁻¹ · cm⁻¹). The absorbance values recorded for each sample were normalized to μg of protein and the values were presented as the percentage of the control value.

**SIRT1 activity assessment**

SIRT1 activity was measured based on an enzymatic reaction using the fluorometric SIRT1 assay kit (Sigma-Aldrich) according to the manufacturer’s instructions. The substrate that is deacetylated by SIRT1 contains an acetylated lysine side chain, which reacts with the developing solution in the kit and leads to the release of a highly fluorescent substrate. The fluorescence intensity was measured at an excitation wavelength of 335 nm and emission wavelength of 444 nm. It is directly proportional to the deacetylation activity of the SIRT1 enzyme. The fluorescence values recorded for each sample were normalized to μg of protein and the values were presented as the percentage of the control value.

**HO-1 and SIRT1 siRNA transfection**

HT22 cells were transiently transfected with siRNA targeting to HO-1 or SIRT1 by Lipofectamine 2,000 (Invitrogen, Paisley, UK) according to the manufacturer’s protocol. The knockdown efficiency of HO-1 or SIRT1 was determined by Western blot using anti-HO-1 or anti-SIRT1. After 24 h of transfection, the cells were pretreated with 10 μM RSV for 12 h. Then, the cells were stimulated with culture medium supplemented with Glu (2 mM) for 24 h.

**Statistical analysis**

Data are expressed as means ± SE. One-way analysis of variance procedures were used to assess significant differences among treatment groups. For each treatment showing a statistically significant effect, the Newman-Keuls test was used for comparisons of multiple group means. The criterion for statistical significance was set at p<0.05.

**Fig. 1.** Effects of TS, RSV and TMS on cell viability and Glu-induced cytotoxicity. (A) Chemical structures of TS, RSV and TMS. (B) HT22 cells were incubated for 24 h with TS, RSV or TMS at indicated concentrations. (C) HT22 cells were pretreated for 12 h with TS, RSV or TMS at indicated concentrations. Then, the cells were stimulated with culture medium supplemented with 2 mM Glu for 24 h. MTT assay for cell viability was performed as described under Materials and Methods. Data are expressed as means ± SE from 3 and 4 experiments. *p<0.05 with respect to the untreated control group (Con) . **p<0.05 with respect to Glu-treated group.

**Fig. 2.** Effects of RSV on HO-1 expression and HO activity. (A, C) HT22 cells were incubated with or without 10 μM RSV for indicated time periods. (B, D) HT22 cells were incubated for 12 h with or without RSV at indicated concentrations. Western blot analysis for HO-1 expression (A, B) and HO activity assessment (C, D) were performed as described under Materials and Methods. Blots shown are representative of three independent experiments. Data are expressed as means ± SE from 3 and 4 experiments. *p<0.05 with respect to the untreated control group (Con).
RESULTS

Effects of pretreatment with RSV or TMS on Glu-induced cytotoxicity

HT22 neuronal cells were treated with different concentrations of each of three stilbene compounds shown in Fig. 1A, and cell viability was performed after 24 h incubation with TS, RSV, or TMS. As shown in Fig. 1B, no cytotoxic sign was observed up to 10 μM of each compound. However, the cell viability was significantly reduced at 20 μM of RSV and TMS. Therefore, maximum concentration was limited to 10 μM of each compound in all subsequent experiments. As previously reported (Jeong et al., 2008), incubation of a high dose of Glu (2 mM) for 24 h markedly reduced the viability of HT22 cells (Fig. 1C). Interestingly, pretreatment with RSV for 12 h protected HT22 cells against Glu-induced cytotoxicity in a concentration-dependent manner (Fig. 1C). However, no significant cytoprotection against Glu-induced cytotoxicity was observed when either TMS or TS was pretreated for 12 h, similarly to RSV (Fig. 1C). It should be noted that the cytoprotective effect of RSV was much higher when HT22 cells were pre-incubated with RSV for 12 h prior to Glu treatment than when RSV was added simultaneously with Glu (not shown).

Effects of RSV on HO-1 expression and SIRT1 activation

Incubation of HT22 neuronal cells with RSV at non-cytotoxic concentrations (2.5-10 μM) produced time- and concentration-dependent increases in HO-1 expression, with its maximum expression after exposure to 10 μM of RSV for 12 h (Fig. 2A and 2B). HO-1 expression by RSV in HT22 cells was associated with elevated HO activity levels (Fig. 2C, D). Similarly, stimulation of HT22 cells with increasing concentrations of RSV led to time- and concentration-dependent increases in SIRT1 expression and activity (Fig. 3). Obviously, a marked increase in SIRT1 expression and activity was observed after exposure to 10 μM of RSV for 12 h (Fig. 3).

Effects of TMS on HO-1 expression and SIRT1 activation

Because it has been demonstrated that TMS exhibits more potent anti-inflammatory and anticancer activities than RSV (Belleri et al., 2005; Pan et al., 2008; Yang et al., 2009; Alex et al., 2010; Deng et al., 2011; Hasiah et al., 2011), the effects of the three stilbene compounds shown in Fig. 1A on HO-1 expression and SIRT1 activation were compared in HT22 neuronal cells. Surprisingly, TS (10 μM) and TMS (10 μM) had no significant effect on HO-1 expression (Fig. 4A) and HO activity (Fig. 4B) under our experimental conditions. Also, TS (10 μM) and TMS (10 μM) failed to induce SIRT1 expression (Fig. 4A) and to increase SIRT1 activity (Fig. 4C).

Roles of HO-1 expression and SIRT1 activation by RSV in cytoprotection

In order to explore a potential involvement of HO-1 activation in the observed cytoprotection mediated by RSV, we used HO-1 siRNA to down-regulate endogenous HO-1 synthesis and SnPP to inhibit HO-1 activity. The cytoprotective effect of RSV on Glu-induced cytotoxicity was partially (but not completely) abolished in HT22 cells either transfected transiently with HO-1 siRNA or treated with SnPP (Fig. 5). We next tested whether SIRT1 activation could be also involved in cytoprotection afforded by RSV against Glu-induced cytotoxicity, and
found that three different SIRT1 inhibitors (i.e., SIRT1 siRNA for down-regulation of endogenous SIRT1 synthesis, sirtinol for specific inhibition of SIRT1 activity, and nicotinamide for nonspecific inhibition of activities of sirtuins) all failed to reverse RSV-mediated cytoprotection (Fig. 5). Inhibitors alone had no significant effect on cell viability (data not shown).

**DISCUSSION**

The present study demonstrates that the natural polyphenol RSV protected HT22 neuronal cells from Glu-induced cytotoxicity. Other authors have reported the direct cytotoxic effect of RSV, although at micromolar concentrations (Delmas et al., 2011; Huang and Zhu, 2011). In line with these findings, RSV reduced the viability of HT22 cells only at concentrations above 20 μM under our experimental conditions. However, at lower concentrations, RSV appeared to be predominantly cytoprotective according to our results. This study also demonstrates that RSV seems to exert a cytoprotective effect, at least under our experimental conditions, by inducing HO-1 expression but not by activating SIRT1 pathway. We have also assessed whether TMS, a derivative of RSV with methoxyl groups in lieu of the hydroxyl groups, and TS, a non-hydroxylated analog, could be also cytoprotective against Glu-induced cytotoxicity, similarly to RSV. According to our results, neither TMS nor TS reduced Glu-induced cytotoxicity in HT22 cells, thus implicating that the presence of hydroxyl groups on the benzene rings of the RSV structure appears to be associated with its cytoprotective activity.

It has been previously reported that oxidative stress plays a crucial role in induction of cell death by Glu treatment in HT22 neuronal cells (Behl, 1998; Kulawiak and Szewczyk, 2012). It is therefore conceivable that the known antioxidant effects of RSV may contribute to the increased resistance of HT22 cells against Glu-induced cytotoxicity. However, the observed effects might not be solely due to the antioxidant properties of RSV itself, because pretreatment of the cells with RSV significantly attenuated Glu-induced cytotoxicity. Alternatively, it is most likely that RSV might up-regulate and/or activate antioxidant/cytoprotective proteins, including SIRT1 and HO-1 (Chen et al., 2005; Albani et al., 2009), which could contribute to the attenuation of Glu-induced cytotoxicity.

The physiological mechanism(s) by which RSV exerts antioxidant/cytoprotective actions is still the subject of debate. Several studies suggest that the beneficial effects of RSV directly or indirectly depend on its roles in activating SIRT1, a key regulator of cell defense and survival under various stress conditions, such as oxidative stress (Wang et al., 2011a; Chong et al., 2012). Indeed, RSV has been shown to prevent cytotoxicity triggered by H2O2 or 6-hydroxydopamine, two well-known oxidative stress inducers, in SK-N-BE neuronal cells, and this protective pathway involved SIRT1 activation (Albani et al., 2009). Moreover, RSV has been reported to have SIRT1-dependent neuroprotective effects both in vitro and in vivo, mainly against oxidative stress inducers (Kim et al., 2007; Ho et al., 2010; Shindler et al., 2010; Wang et al., 2011b; Wu et al., 2011b). In HT22 neuronal cells, RSV significantly increased SIRT1 expression and activity, as the ability of RSV to activate SIRT1 has been examined in other cell lines (Kao et al., 2010; Xia et al., 2011), and we therefore examined whether the cytoprotection afforded by RSV could account for its activation of SIRT1. Surprisingly, the cytoprotective effect of RSV against Glu-induced cytotoxicity in HT22 cells was independent of SIRT1 activation, because specific and nonspecific SIRT1 inhibitors failed to reverse the protective effect of RSV. Our results suggest that the cytoprotective effect of RSV might not be due to SIRT1 activation but could rather be attributed to other antioxidant/cytoprotective proteins.

HO-1 has been reported to be one of other key mediators of antioxidant/cytoprotective actions of RSV (Bastianetto and Quirion, 2010; Ren et al., 2011). In our study, we found that RSV induced HO-1 expression and increased the ensuring HO activity in HT22 neuronal cells. Importantly, we have shown here that the specific down-regulation of HO-1 cellular synthesis by using HO-1 siRNA partially (but not completely) reduced the ability of RSV to prevent Glu-induced cytotoxicity, indicating that HO-1 expression may be at least partly responsible for cytoprotective actions of RSV in HT22 cells. This result was further confirmed by using the nonspecific HO inhibitor SnPP; SnPP, similarly to HO-1 siRNA, partially abrogated the cytoprotective effect of RSV. It should be noted that RSV, together with HO-1 pathway, might also activate other antioxidant/cytoprotective pathways, and overall cytoprotection afforded by RSV could be achieved by virtue of the concerted actions of the multiple pathways being activated. In this regard, it has been previously demonstrated that RSV protected HT22 cells from Glu-induced cytotoxicity by inducing the expression of the mitochondrial antioxidant superoxide dismutase 2 (Fukui et al., 2010). The importance of the HO-1 pathway in physiology and pathophysiology has been confirmed by many experimental studies. It has been reported that the human HO-1-deficient case described in the literature and HO-1-deficient mice exhibit increased susceptibility to oxidative stress (Pae et al., 2008). The beneficial effects of HO-1 expression have been attributed to several factors, including the degradation of pro-oxidant heme, formation of biliverdin and/or bilirubin with their antioxidant properties, as well as the release of carbon monoxide, which has cytoprotective and anti-inflammatory effects.
(Pae et al., 2008). Although the exact mechanisms involved in cytoprotective actions of the HO-1 system have not been fully elucidated, one or more of the HO-1 reaction products may mediate the cytoprotective actions of RSV under our experimental conditions. Interestingly, it has been demonstrated that pretreatment of rats with RSV had neuroprotective effect on cerebral ischemia/reperfusion injury, which is likely exerted by up-regulated HO-1 expression to ameliorate oxidative damage, reduce neuronal apoptosis, protect ischemic penumbra, reduce infarct size, reduce reperfusion damage of brain tissue (Ren et al., 2011). Our results, together with previous reports (Bastianetto and Quirion, 2010; Ren et al., 2011), suggest that HO-1 may play an important role in neuroprotection of RSV against the damage from oxidative stress.

In our study, the biological properties of RSV have been compared with those of TMS, one of the well-known analogues of RSV. In particular, the cytoprotective activity was investigated in vitro by measuring the ability to increase HO-1 expression and SIRT1 activity. In HT22 cells, TMS, unlike RSV, failed to increase HO-1 expression as well as SIRT1 activation. Moreover, TMS also failed to reduce Glu-induced cytotoxicity; this is, presumably, because it was inactive in increasing HO-1 expression. Similarly, T5, of which chemical structure lacks three hydroxyl groups, had no significant effects on HO-1 expression and SIRT1 activation. These findings suggest that the presence of hydroxyl groups in the benzene rings of RSV may be required at least in part for HO-1 expression and SIRT1 activation.

In summary, the results of the present study demonstrate that: (i) pretreatment of HT22 neuronal cells with RSV can confer a marked protection against Glu-induced cytotoxicity, which appears to be associated at least in part with HO-1 expression; (ii) the cytoprotective activity of RSV was independent of its activation of SIRT1; and (iii) TMS failed to protect HT22 cells from Glu-induced cytotoxicity and to increase HO-1 expression and SIRT1 activation, suggesting that the hydroxyl groups on the benzene rings of RSV was necessary for cytoprotective activity, HO-1 expression, and SIRT1 activation. Although we are not concluding that HO-1 is the only pathway by which RSV can be cytoprotective, we believe that HO-1 may be a unique candidate by which RSV can induce an endogenous cellular pathway that leads to building cellular resistance to oxidative stress. Further studies will be required in order to understand the exact mechanisms of overall cytoprotective actions of RSV.

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