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ORIGINAL ARTICLE

Involvement of heme oxygenase-1 expression in neuroprotection by piceatannol, a natural analog and a metabolite of resveratrol, against glutamate-mediated oxidative injury in HT22 neuronal cells

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Abstract Neuronal cell death caused by oxidative stress is common in a variety of neural diseases and can be investigated in detail in cultured HT22 neuronal cells, where the amino acid glutamate at high concentrations causes glutathione depletion by inhibition of the glutamate/cystine antiporter system, intracellular accumulation of reactive oxygen species (ROS) and eventually oxidative stress-induced neuronal cell death. Using this paradigm, we have previously reported that resveratrol (3,5,4′-*trans*-trihydroxystilbene) protects HT22 neuronal cells from glutamate-induced oxidative stress by inducing heme oxygenase (HO)-1 expression. Piceatannol (3,5,4′,3′-*trans*-trihydroxystilbene), which is a hydroxylated resveratrol analog and one of the resveratrol metabolites, is estimated to exert neuroprotective effect similar to that of resveratrol. The aim of this study, thus, is to determine whether piceatannol, similarly to resveratrol, would protect HT22 neuronal cells from glutamate-induced oxidative stress. Glutamate at high concentrations induced neuronal cell death and ROS formation. Piceatannol reduced glutamate-induced cell death and ROS formation. The observed cytoprotective effect was much higher when HT22 neuronal cells were pretreated with piceatannol for 6 or 12 h prior to glutamate treatment than when pretreated for 0.5 h. Piceatannol also increased HO-1 expression and HO activity via its activation of nuclear factor-E2-related factor 2 (Nrf2). Interestingly, neuroprotective effect of piceatannol was partly (but not completely) abolished by either down-regulation of HO-1 expression or blockage of HO-1 activity. Taken together, our results suggest that piceatannol, similar to resveratrol, is capable of protecting HT22 neuronal cells against glutamate-induced cell death, at least in part, by inducing Nrf2-dependent HO-1 expression.

Keywords Glutamate · Resveratrol · Piceatannol · Heme oxygenase-1 · Reactive oxygen species · Neuronal cells

Abbreviations

ARE	Antioxidant responsive element
DCF-DA	2′,7′-Dichlorofluorescein diacetate
DMEM	Dulbecco's modified Eagle's medium
ELISA	Enzyme-linked immunosorbent assay
Glu	Glutamate
HO-1	Heme oxygenase-1
MAPK	Mitogen-activated protein kinase
MTT	3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide
Nrf2	Nuclear transcription factor-E2-related factor 2
OD	Optical density
PBS	Phosphate-buffered saline
Pic	Piceatannol
PI3K	Phosphoinositide 3-kinase
PKC	Protein kinase C
ROS	Reactive oxygen species
siRNA	Small interfering RNA
SnPP	Tin protoporphyrin IX (SnPP)
RuCO	Tricarbonyldichlororuthenium-(II)-dimer

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Introduction

Glutamate (Glu) is an acidic amino acid with multiple roles in cell metabolism and physiology. When acting as an endogenous excitatory neurotransmitter, Glu is estimated to be used mainly in the central nervous system. At its high concentrations, Glu causes neuronal cell death, which contributes to the development of neurodegenerative diseases (Fukui et al. 2010). Glu-induced neuronal cell death is mediated by Glu receptor-mediated excitotoxicity and non-receptor-mediated oxidative toxicity (Fukui et al. 2010). Oxidative toxicity of Glu is initiated by high concentrations of extracellular Glu that can prevent cystine uptake into the cells via the cystine/Glu antiporter system, thereby resulting in depletion of intracellular cysteine and glutathione (Fukui et al. 2010). Depletion of the endogenous antioxidant glutathione induces excessive reactive oxygen species (ROS) accumulation, resulting in oxidative stress and subsequent neuronal cell death.

Although the production of ROS, including superoxide, hydroxyl radicals and peroxides such as hydrogen peroxide (H_2O_2), and their detoxification are normal physiological processes, an imbalance between ROS production and their removal leads to oxidative stress, which plays a crucial role in the development of the most common neurodegenerative diseases, namely Alzheimer's disease and Parkinson's disease (Jomova et al. 2010). Whether the oxidative stress is a cause or consequence of these neurodegenerative diseases, however, remains to be elucidated. A growing body of evidence suggests that oxidative stress directly initiates and progresses to neuronal cell death (Panickar and Anderson 2011; Kelsey et al. 2010; Esposito and Cuzzocrea 2010; Eghwrudjakpor and Allison 2010; Jellinger 2009). Therefore, a key therapeutic intervention would be used to reduce cellular levels of oxidative stress and/or to enhance cellular resistance to oxidative stress. Because naturally occurring antioxidants, such as phenolic compounds, can prevent neuronal cell death by scavenging of ROS and/or enhancing the cellular antioxidant system (Panickar and Anderson 2011; Kelsey et al. 2010), they may be thought to be potential neuroprotective agents.

Heme oxygenase-1 (HO-1) is a rate-limiting enzyme in heme degradation to produce free iron, carbon monoxide (CO) and biliverdin/bilirubin, thereby detoxifying the deleterious effects of the pro-oxidant heme (Kim et al. 2011; Pae et al. 2008, 2010; Jazwa and Cuadrado 2010). HO-1 expression is primarily regulated at the transcriptional level, and its induction by various inducers is related to the nuclear transcription factor-E2-related factor 2 (Nrf2) (Pae et al. 2008). Nrf2 is a basic leucine zipper transcription factor that resides in the cytoplasm bound to its inhibitor protein, Keap 1, and translocates to the nucleus after stimulation. It then forms heterodimers with small oncogene family proteins for the selective recognition of the antioxidant responsive element (ARE) on target genes, resulting in the regulation of gene expression of phase II detoxifying enzymes, including HO-1 (Pae et al. 2008). HO-1 expression can be induced by the HO substrate heme and various oxidative agents, strongly suggesting that HO-1 expression is an important cytoprotective/defense mechanism against oxidative injury (Pae et al. 2008). Although conflicting results have been published about the role of HO-1 in neuronal survival and protection, it has been increasingly clear that HO-1 expression is neuroprotective in various neuronal disease models associated with oxidative stress (Jazwa and Cuadrado 2010).

Piceatannol (Pic), which is first isolated from the seeds of *Euphorbia lagascae* (*Euphorbiaceae*), is a naturally occurring analog of resveratrol and this phenolic compound has been also identified as one of resveratrol metabolites (Piotrowska et al. 2012). In contrast to the extensive investigation on resveratrol, little is known about the biological effects of Pic. Because Pic is generated during phase I metabolism of resveratrol by cytochrome P450 enzymes, and represents one of its main phase I metabolites (Piotrowska et al. 2012), it was therefore hypothesized that Pic may have biological activities similar to those of resveratrol. Pic (3,5,4′,3′-*trans*-trihydroxystilbene; its chemical structure shown in Fig. 1a) possesses an additional hydroxyl group at the 3′ position of resveratrol (3,5,4′-*trans*-trihydroxystilbene). Although Pic, partly due to such a difference in the chemical structure, has a stronger antioxidant activity than resveratrol (Piotrowska et al. 2012), whether Pic would also exert other biological effects similar to those of resveratrol remains to be determined.

Previously, we have demonstrated that resveratrol can attenuate neuronal cell death caused by oxidative stress via its expression of the antioxidant/cytoprotective HO-1 (Kim et al. 2012). Whether Pic, similar to resveratrol, could also prevent neuronal cell death caused by oxidative stress by inducing HO-1 expression is currently unknown; and was therefore investigated in this study. Neuronal cell death caused by oxidative stress can be investigated in detail in cultured HT22 neuronal cells, where Glu at high concentrations causes glutathione depletion by inhibition of the cystine/Glu antiporter system, intracellular ROS accumulation and oxidative stress-induced neuronal cell death (Fukui et al. 2010). Using this paradigm, we investigated whether Pic could prevent neuronal cell death caused by Glu-mediated oxidative stress by inducing HO-1 expression.

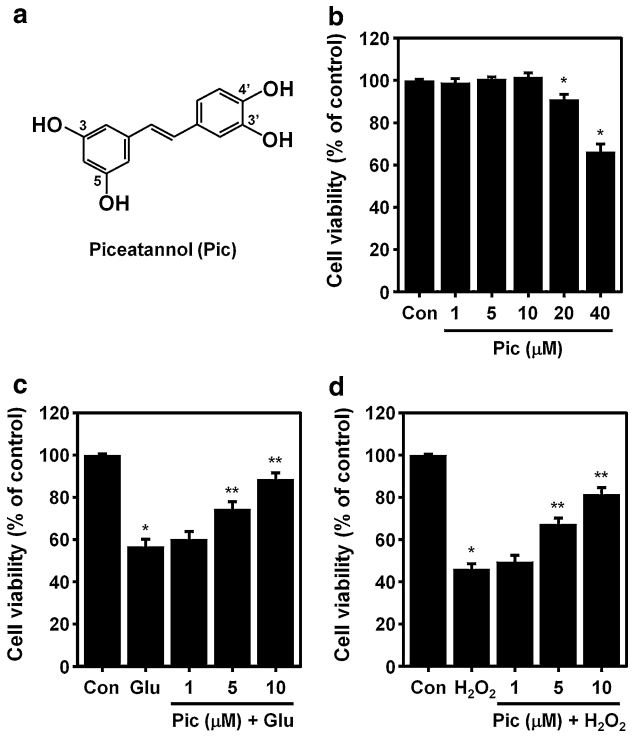

Fig. 1 Pic protects HT22 neuronal cells from Glu- and H_2O_2-mediated oxidative injury. **a** Chemical structure of Pic. **b** Cells were incubated for 24 h with indicated concentrations of Pic. **c** Cells were pretreated for 6 h with indicated concentrations of Pic, and then exposed to 2 mM Glu for 24 h. **d** Cells pretreated for 6 h with indicated concentrations of Pic were exposed to 100 μM H_2O_2 for 24 h. Cell viability was assessed by MTT assay. Values calculated as percentages of control (*con*) cells are presented as mean ± SEM ($n = 3$). *$P < 0.05$ vs. con and **$P < 0.05$ vs. Glu

Materials and methods

Reagents and antibodies

Pic, 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, 2′,7′-dichlorofluorescein diacetate (DCF-DA), tin protoporphyrin IX (SnPP), H_2O_2, bilirubin, tricarbonyldichlororuthenium-(II)-dimer (RuCO) and Glu were purchased from Sigma-Aldrich (St. Louis, MO, USA). Antibodies against HO-1, lamin B, Nrf2 and β-actin and small interfering RNA (siRNA) products against HO-1 and Nrf2 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). All other reagents used were of analytical grade.

Cell culture

HT22 neuronal cells (a gift from Prof. Youn-Chul Kim at college of pharmacy, Wonkwang university) 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 mg/ml), and L-glutamine (2 mM) and were incubated at 37 °C in a humidified atmosphere containing 5 % CO_2 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 neuronal cells were cultured in a 96-well flat-bottom plate at concentration of 5×10^5 cells/ml. After 12 h of preconditioning, the cells were treated with various concentrations of Pic 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.

ROS measurement

For measurement of ROS, HT22 neuronal cells (2.5×10^4 cells/ml in 24-well plates) were pretreated for 6 h with Pic, and then exposed to 2 mM Glu for 6 h. After washing with phosphate-buffered saline (PBS), the cells were stained with 10 μM DCF-DA in Hanks' balanced salt solution for 30 min in the dark. The cells were then washed twice with PBS and extracted using 1 % Triton X-100 in PBS for 10 min at 37 °C. Fluorescence was recorded at an excitation wavelength of 490 nm and emission wavelength of 525 nm (Spectramax Gemini XS; Molecular Devices, Sunnyvale, CA, USA).

Western blot analysis

Proteins isolated from HT22 neuronal 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 (1:1000 dilution) or β-actin (1:1000 dilution) at 4 °C overnight. Afterward, membranes were incubated with horseradish peroxidase-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 measurement

HO activity was determined at the end of each treatment, as described previously (Kim et al. 2012). 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 ($\varepsilon = 40\ mM^{-1}\ cm^{-1}$). 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.

HO-1 and Nrf2 siRNA transfection

HT22 neuronal cells were transiently transfected with siRNA targeting to HO-1 or Nrf2 by Lipofectamine 2000™ (Invitrogen) according to the manufacturer's protocol. The knockdown efficiency of HO-1 and Nrf2 was determined by Western blot analysis using anti-HO-1 and Nrf2 antibodies. After 24 h of transfection, the cells were pretreated with 10 μM Pic for 6 h. Then, the cells were stimulated with culture medium supplemented with 2 mM Glu for 24 h.

Nuclear Nrf2-ARE binding assay

The amount of Nrf2 available in the nucleus to bind to AREs was determined using an enzyme-linked immunosorbent assay (ELISA)-based TransAM™ Nrf2 kit (Active Motif, Carlsbad, CA, USA). Briefly, nuclear extracts were added to wells containing the immobilized consensus ARE oligonucleotide. A primary antibody against Nrf2 was added to each well, and a secondary antibody conjugated to horseradish peroxidase that binds to the primary (Nrf2) antibody, then, was added to each well. The signal was detected at 450 nm, and Nrf2-ARE binding activity was reported as optical density (OD) units at 450 nm.

Statistical analysis

Data are expressed as mean ± SEM. 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 $P < 0.05$.

Results

Pic reduces Glu-induced cytotoxicity and ROS accumulation

HT22 neuronal cells were treated with different concentrations of Pic, and cell viability was performed after incubation for 24 h. As shown in Fig. 1b, no cytotoxic sign was observed up to 10 μM Pic. However, the cell viability was significantly reduced at 20 and 40 μM Pic. Therefore, maximum concentration was limited to 10 μM Pic in all subsequent experiments. As previously reported (Fukui et al. 2010), incubation of a high dose of Glu (2 mM) for 24 h markedly reduced the viability of HT22 neuronal cells (Fig. 1c). Interestingly, pretreatment with Pic for 6 h protected HT22 neuronal cells against Glu-induced cytotoxicity (Fig. 1c). Pic was also cytoprotective against H_2O_2-induced cytotoxicity (Fig. 1d). It should be noted that the cytoprotective effect of Pic was much higher when HT22 neuronal cells were pretreated with Pic for 6 or 12 h prior to Glu treatment than when pretreated for 0.5 h (Fig. 2a). In other experimental sets, Glu markedly increased intracellular ROS accumulation in HT22 neuronal cells, which was significantly reduced when the cells were pretreated with Pic for 6 h prior to Glu treatment (Fig. 2b).

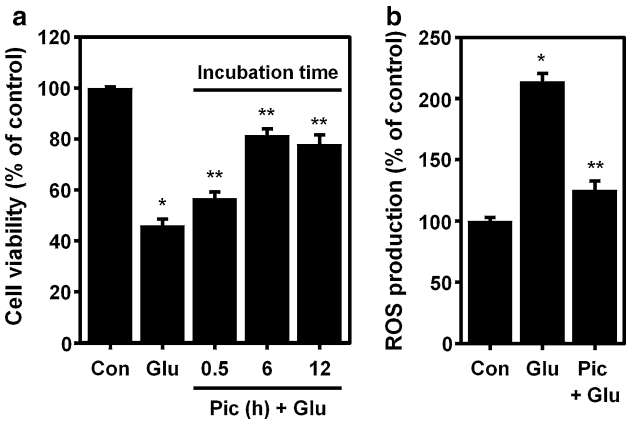

Fig. 2 Pretreatment of HT22 neuronal cells with Pic reduces more effectively Glu-mediated oxidative injury and ROS accumulation. **a** Cells were pretreated for indicated times with 10 μM Pic, and then exposed to 2 mM Glu. **b** Cells pretreated for 6 h with 10 μM Pic were exposed to 2 mM Glu for 12 h. Cell viability and ROS accumulation were assessed by MTT assay and DCF fluorescence measurement, respectively. Values calculated as percentages of control (*con*) cells are presented as mean ± SEM ($n = 3$). *$P < 0.05$ vs. con and ** $P < 0.05$ vs. Glu

Pic induces HO-1 expression via Nrf2 activation

Previously, we have shown that resveratrol is capable of inducing HO-1 expression in HT22 neuronal cells (Kim et al. 2012). Considering the close similarity between chemical structures of resveratrol and Pic, we examined whether Pic, similarly to resveratrol, could also induce HO-1 expression in HT22 neuronal cells. At its non-cytotoxic concentrations, Pic increased HO-1 expression in a time- and concentration-dependent manner (Fig. 3a, b), which was paralleled by increased HO activity (Fig. 3c, d). Moreover, treatment of HT22 neuronal cells with Pic resulted in a time-dependent increase in Nrf2 protein in the nucleus, along with a significant decrease in Nrf2 protein in the cytoplasm (Fig. 4a). To study Nrf2 activation, the nuclear extract of HT22 neuronal cells was isolated and the binding of Nrf2 to ARE was quantified using a TransAM assay. Pic increased Nrf2 activation in a concentration-dependent manner (Fig. 4b). Next, we conducted the experiments of Nrf2 knockdown by using Nrf2 siRNA to investigate whether Nrf2 activation would mediate HO-1 expression. HO-1 expression by Pic was prevented by siRNA against Nrf2 (Fig. 4c).

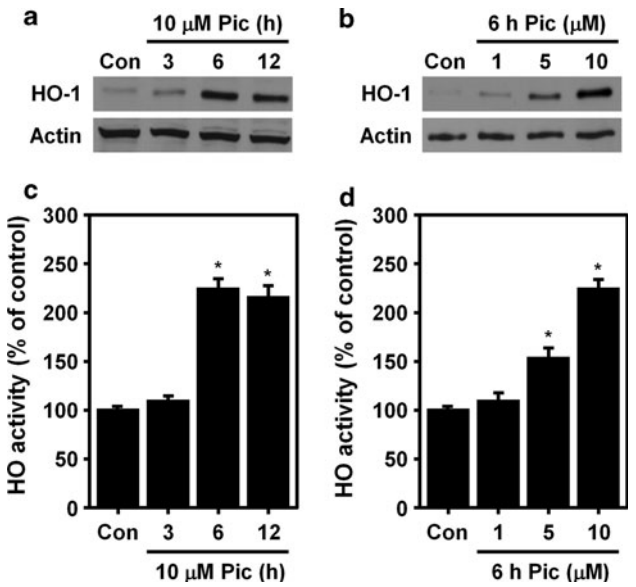

Fig. 3 Pic induces HO-1 expression in HT22 neuronal cells. **a** Proteins were isolated from cells incubated with 10 μM Pic for indicated times, and Western blot analysis for HO-1 was performed. **b** Proteins were isolated from cells incubated for 6 h with indicated concentrations of Pic, and Western blot analysis for HO-1 and HO enzyme activity measurement was performed. **c** HO enzymes were isolated from cells incubated with 10 μM Pic for indicated times, and HO enzyme activity measurement was performed. **d** HO enzymes were isolated from cells incubated for 6 h with indicated concentrations of Pic, and HO enzyme activity measurement was performed. Values calculated as percentages of control (*con*) cells are presented as mean ± SEM ($n = 3$). *$P < 0.05$ vs. con

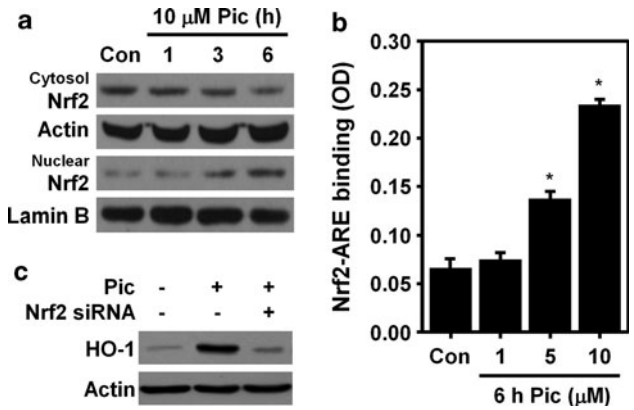

Fig. 4 Pic induces Nrf2 activation in HT22 neuronal cells. **a** Cytosol and nuclear proteins were isolated from cells incubated with 10 μM Pic for indicated times, and Western blot analysis for Nrf2 was performed. **b** Nuclear proteins were isolated from cells incubated with indicated concentration of Pic for 6 h, and Nrf2-ARE binding activity measurement was performed. **c** Proteins were isolated from normal or Nrf2 siRNA-transfected cells treated with 10 μM Pic for 6 h, and Western blot analysis for HO-1 was performed. Values calculated as percentages of control (*con*) cells are presented as mean ± SEM ($n = 3$). *$P < 0.05$ vs. con

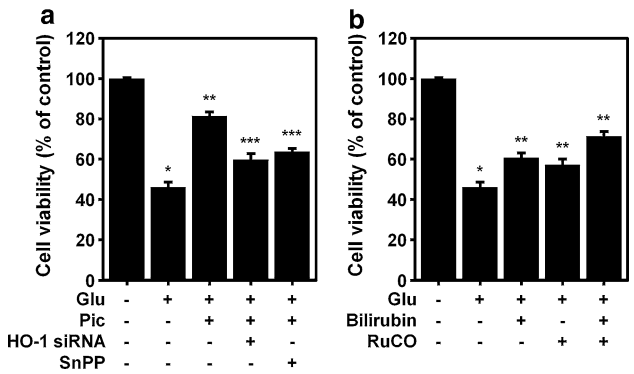

Fig. 5 HO-1 expression mediates cytoprotective effect of Pic against Glu-mediated oxidative injury in HT22 neuronal cells. **a** Cells treated with 10 μM Pic for 6 h in the presence or absence of HO-1 siRNA or 50 μM SnPP were exposed to 2 mM Glu for 24 h. **b** Cells were exposed to 2 mM Glu for 24 h in the presence or absence of 20 μM bilirubin, 20 μM RuCO, or bilirubin plus RuCO. Values calculated as percentages of control (*con*) cells are presented as mean ± SEM ($n = 3$). *$P < 0.05$ vs. con, **$P < 0.05$ vs. Glu and ***$P < 0.05$ vs. Glu + Pic

Cytoprotection by Pic correlates with HO-1 expression

In order to assess the potential role of HO-1 in Pic-induced cytoprotection, HT22 neuronal cells were pretreated with 10 μM Pic for 6 h in the presence or absence of HO-1 siRNA, a specific inhibitor of HO-1 expression, or SnPP, a competitive inhibitor of HO-1 activity, followed by Glu treatment. As shown in Fig. 5a, cytoprotective effect of Pic was partly (but not completely) abolished by either HO-1 siRNA or SnPP. In addition, we examined whether HO-1

reaction products, such as the antioxidant/cytoprotective bilirubin and the cytoprotective/anti-inflammatory CO, could be also protective against Glu-induced cytotoxicity. As shown in Fig. 5b, both bilirubin and RuCO, a CO donor, protected HT22 neuronal cells against Glu-induced cytotoxicity. Interestingly, the combined addition of bilirubin and RuCO was more effective in protecting Glu-induced neuronal cell death than either bilirubin or RuCO alone (Fig. 5b). HO-1 siRNA, SnPP, bilirubin, and RuCO alone had no effect on cell viability (not shown).

Discussion

There is considerable current interest in the neuroprotective effects of natural antioxidants against oxidative stress and the different defense mechanisms involved. Although the neuroprotective effects of resveratrol have been extensively studied (Li et al. 2012; Kelsey et al. 2010; Albani et al. 2010; Sun et al. 2010; Robb and Stuart 2010), whether the naturally occurring resveratrol analog Pic would exert a neuroprotective effect was not yet investigated. This study demonstrates that Pic is capable of protecting HT22 neuronal cells, which were derived from the immortalized mouse hippocampal neurons, from oxidative toxicity caused by high concentrations of the neurotransmitter Glu. Other studies have reported the direct cytotoxic effects of Pic in several types of tumor cells, although at micromolar concentrations (Billack et al. 2008; Chowdhury et al. 2005). In line with these findings, Pic reduced cell viability only at the concentrations above 20 μM under our experimental conditions. However, Pic appeared to be predominantly neuroprotective at its lower concentrations. We also demonstrate that Pic seems to exert a neuroprotective effect, at least in part, by inducing Nrf2-dependent HO-1 expression.

The experiments were carried out in HT22 neuronal cells, which were previously introduced as an excellent model system to study mechanisms of Glu-mediated neurotoxicity and its prevention (Fukui et al. 2010; Yang et al. 2011). Our experimental protocol follows procedures outlined in those earlier publications. HT22 neuronal cells are phenotypically most similar to neuronal precursor cells and express neuron-specific enolase and neurofilament proteins (Morimoto and Koshland 1990). HT22 neuronal cells lack ionotropic Glu receptors, and this, therefore, can exclude the receptor-mediated excitotoxicity as a cause for Glu-mediated cell death. Because HT22 neuronal cells divide rapidly in culture and lack ionotropic Glu receptors, they have been widely used to examine the in vitro effects of Glu-induced intracellular oxidative stress on neuronal cells. Indeed, a number of studies have shown that Glu at high concentrations could induce intracellular oxidative stress and subsequent cell death in HT22 neuronal cells by inhibiting cystine uptake, which results in decreased intracellular glutathione levels and ultimately in oxidative stress and cell death (Fukui et al. 2010). PC12 cells, which were developed from rat adrenal pheochromocytoma cells, have also been used to study the mechanisms of Glu-mediated neurotoxicity (Tan et al. 2013). However, in these cells the cytotoxicity caused by Glu may be attributed to both the presence of ionotropic Glu receptors and the blockade of cystine uptake into the cell via its inhibition of the Glu/cystine antiporter.

Oxidative neuronal cell death is thought to contribute importantly to neuronal injury and degeneration in many brain disorders (Jomova et al. 2010; Jellinger 2009). Thus, agents that can prevent neuronal ROS formation and/or oxidative neuronal cell death may have therapeutic potentials in neurodegenerative diseases associated with oxidative stress (Kelsey et al. 2010). Using cultured HT22 neuronal cells, we showed the neuroprotective effect of Pic against Glu-induced oxidative toxicity. Interestingly, Pic was also neuroprotective against oxidative toxicity caused by direct addition of the well-known oxidant hydrogen peroxide, a relatively stable form of ROS. Thus, it is most likely that Pic may attenuate neuronal cell death caused by various types of oxidant, not restricted only to Glu.

The neuroprotective effects of the naturally occurring phenolic compounds are generally thought to be due to their direct antioxidant and free radical scavenging properties. It is, therefore, conceivable that the observed antioxidant effect of Pic, as this phenolic compound reduced Glu-induced intracellular ROS accumulation, may contribute to the increased resistance of HT22 neuronal cells against Glu-induced oxidative toxicity. However, the observed effects might not be solely due to the antioxidant properties of Pic itself, because pretreatment of the cells with Pic for 6 or 12 h augmented its ability to attenuate Glu-induced oxidative toxicity. Alternatively, it is most likely that Pic might up-regulate and/or activate antioxidant/cytoprotective proteins that could contribute to the attenuation of Glu-induced oxidative toxicity. Previously, we have demonstrated that resveratrol, of which chemical structure is similar to Pic, can reduce Glu-induced oxidative toxicity by inducing HO-1 expression in HT22 neuronal cells (Kim et al. 2012). In this study, we found that Pic also induced HO-1 expression and increased HO activity in HT22 neuronal cells, presumably by inducing Nrf2 activation. Importantly, we have shown here that the specific down-regulation of HO-1 cellular synthesis using HO-1 siRNA partly (but not completely) reduced the ability of Pic to prevent Glu-induced oxidative toxicity, indicating that HO-1 expression may be, at least in part, responsible for neuroprotective actions of Pic in HT22 neuronal cells. This result was further confirmed using the

non-specific HO inhibitor SnPP; SnPP, similar to HO-1 siRNA, partly abrogated the neuroprotective effect of Pic. It should be noted that Pic, together with HO-1 pathway, might also activate other cytoprotective pathways, including modulation of excitotoxicity, stimulation of nitric oxide synthase (Frombaum et al. 2011), regulation of mitochondrial functions and activation of sirtuins (Frombaum et al. 2011), and overall cytoprotection afforded by Pic could be achieved by virtue of the concerted actions of the multiple pathways being activated. It is worth pointing out that activation of sirtuin 1 by Pic is most likely to contribute to additional cytoprotective effects of Pic, because resveratrol prevented cytotoxicity triggered by H_2O_2 or 6-hydroxydopamine via its activation of sirtuin 1 in SK-N-BE neuronal cells (Albani et al. 2009).

The transcription factor Nrf2 plays a key role in the ARE-mediated expression of phase II detoxifying and antioxidant enzymes and stress-inducible proteins (Pae et al. 2008). In HT22 neuronal cells, Pic increased the nuclear translocation of Nrf2 and its ARE binding activity, thereby stimulating the transcriptional activity of Nrf2. Inhibition of Nrf2 synthesis by the use of siRNA abolished Pic-induced HO-1 expression, corroborating the role for Nrf2 as an essential regulator in the Pic-induced HO-1 expression. This is largely consistent with previous findings showing that Pic is capable of inducing HO-1 expression via Nrf2 activation in MCF10A epithelial cells (Lee et al. 2010) and bovine aortic endothelial cells (Wung et al. 2006). The upstream signaling events that lead to nuclear localization of Nrf2 and subsequent expression of HO-1 include activation of several kinases, such as mitogen-activated protein kinases (MAPKs), protein kinase C (PKC) and phosphoinositide 3-kinase (PI3 K)/Akt (Lee et al. 2010; Wung et al. 2006). However, activation of these kinases by Pic may be different in cell type used. In MCF10A epithelial cells, Pic-induced HO-1 expression was effectively inhibited by a selective inhibitor of PI3 K/Akt, but the HO-1 expression was not influenced by inhibitors of MAPKs (Lee et al. 2010). In bovine aortic endothelial cells, Pic-induced HO-1 expression was blocked by PKC inhibitors, but inhibitors of PI3 K/Akt or MAPKs had no significant effect on Pic-induced HO-1 expression (Wung et al. 2006). In HT22 cells, none of inhibitors of PI3 K/Akt, PKC or MAPKs influenced Pic-induced HO-1 expression (data not shown). Activation of other putative kinases that may be involved in Pic-induced HO-1 expression in HT22 cells is currently under investigation.

The importance of the HO-1 pathway in physiology and pathophysiology has been confirmed by many experimental studies (Pae et al. 2008; Jazwa and Cuadrado 2010). 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 CO, 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 neuroprotective actions of Pic under our experimental conditions. This hypothesis was strongly supported by our data showing the neuroprotective effects of bilirubin and CO against Glu-induced oxidative toxicity in HT22 neuronal cells. Overall, our work shows that Pic can attenuate neuronal cell death caused by oxidative stress via HO-1 expression in HT22 neuronal cells in vitro and suggests that pharmacological up-regulation of HO-1 expression may represent a viable clinical strategy for the prevention of oxidative neuronal damage. It should be noted that we examined the neuroprotective effect of Pic against Glu-induced cell death only in in vitro culture system. Further studies are needed to clarify whether this effect can be reproduced in an in vivo model of oxidative neuronal damage. Interestingly, an in vivo study using mice has demonstrated that resveratrol can induce HO-1 expression in the brain to provide resistance against oxidative stress and the cascade of events that leads to infarct brain damage after ischemia–reperfusion injury (Sakata et al. 2010). Considering that Pic is one of resveratrol metabolites, we speculate that Pic, similarly to resveratrol, may be also neuroprotective in vivo.

Because Pic is rapidly metabolized in the liver and is converted mainly to a glucuronide conjugate (Piotrowska et al. 2012), the concentrations of Pic used in our in vitro study may not be attained in vivo. It has been demonstrated that a single intravenous dose of 10 mg/kg Pic in rats resulted in a decline of plasma concentrations of Pic from 41 to 1 μM in 6 h (Roupe et al. 2006). Similarly, a single intravenous administration of resveratrol (20 mg/kg) to rats showed maximum plasma concentrations of resveratrol and its metabolites in the range from 2 to 13 μM (Yu et al. 2002). However, it has been reported that the poor bioavailability of resveratrol can be enhanced by various methods, such as encapsulation with cyclodextrin, formulation with piperine, and incorporation of resveratrol into microparticles, liposomes, nanocapsules, and emulsion (Amri et al. 2012). Considering that the chemical structure of Pic is similar to that of resveratrol, it is most likely that the poor bioavailability of Pic may be enhanced by similar methods applied to resveratrol.

In vivo studies have shown that resveratrol is neuroprotective in both acute neuronal injuries as well as chronic neurodegenerative diseases. Resveratrol protects spinal cord neurons from ischemic reperfusion injury (Kaplan et al. 2005), and mimics ischemic preconditioning in hippocampal slices (Raval et al. 2006). The benefits of

resveratrol in animal models of Alzheimer disease have been also investigated (Marambaud et al. 2005). To our best knowledge, no direct evidence that Pic, similar to resveratrol, would be neuroprotective in in vivo models of neuronal diseases is reported in the scientific literature. It is now clear that Pic, the less-known congener of the famous resveratrol, is a similarly interesting natural compound possessing a similarly broad spectrum of biological activity. Indeed, both Pic and resveratrol protected 6-day-old primary mixed (glial/neuronal) hippocampal cells against amyloid β-peptide-mediated cytotoxicity (Bastianetto et al. 2009). In many, although not all, of the experimental models in which resveratrol exerts a neuroprotective effect, there was accompanying evidence that resveratrol attenuated neuronal injuries via activation of antioxidant pathways (Richard et al. 2011). Because Pic, similar to resveratrol, activates antioxidant pathways in our in vitro study and differs from resveratrol only by possessing an additional hydroxyl group, it is likely that Pic may have an in vivo neuroprotective effect.

In conclusion, the results of the present study demonstrate that pretreatment of HT22 neuronal cells with non-cytotoxic concentrations of Pic can confer a marked protection against glutamate-induced oxidative toxicity, which appears to be associated at least in part with Nrf2-dependent HO-1 expression. Although we are not concluding that HO-1 is the only pathway by which Pic can be neuroprotective, we believe that HO-1 may be a unique candidate by which Pic can induce an endogenous cellular pathway that leads to building cellular resistance to oxidative stress. Further studies will be required to understand the exact mechanisms of overall cytoprotective actions of Pic. Although Pic showed strong neuroprotective potential using in vitro model systems, it is not clear whether it exerts the same effect in vivo, partly because most flavonoids are usually extensively metabolized in the body and their physiological activities could be altered significantly. Even so, there are a number of reports that antioxidant phytochemicals contribute to benefits of human health.

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Conflict of interest The authors declare that they have no conflict of interest.

References

Albani D, Polito L, Batelli S, De Mauro S, Fracasso C, Martelli G, Colombo L, Manzoni C, Salmona M, Caccia S, Negro A, Forloni G (2009) The SIRT1 activator resveratrol protects SK-N-BE cells from oxidative stress and against toxicity caused by alpha-synuclein or amyloid-beta (1–42) peptide. J Neurochem 110:1445–1456

Albani D, Polito L, Signorini A, Forloni G (2010) Neuroprotective properties of resveratrol in different neurodegenerative disorders. BioFactors 36:370–376

Amri A, Chaumeil JC, Sfar S, Charrueau C (2012) Administration of resveratrol: what formulation solutions to bioavailability limitations? J Control Release 158:182–193

Bastianetto S, Dumont Y, Han Y, Quirion R (2009) Comparative neuroprotective properties of stilbene and catechin analogs: action via a plasma membrane receptor site? CNS Neurosci Ther 15:76–83

Billack B, Radkar V, Adiabouah C (2008) In vitro evaluation of the cytotoxic and anti-proliferative properties of resveratrol and several of its analogs. Cell Mol Biol Lett 13:553–569

Chowdhury SA, Kishino K, Satoh R, Hashimoto K, Kikuchi H, Nishikawa H, Shirataki Y, Sakagami H (2005) Tumor-specificity and apoptosis-inducing activity of stilbenes and flavonoids. Anticancer Res 25:2055–2063

Eghwrudjakpor PO, Allison AB (2010) Oxidative stress following traumatic brain injury: enhancement of endogenous antioxidant defense systems and the promise of improved outcome. Niger J Med 19:14–21

Esposito E, Cuzzocrea S (2010) New therapeutic strategy for Parkinson's and Alzheimer's disease. Curr Med Chem 17:2764–2774

Frombaum M, Therond P, Djelidi R, Beaudeux JL, Bonnefont-Rousselot D, Borderie D (2011) Piceatannol is more effective than resveratrol in restoring endothelial cell dimethylarginine dimethylaminohydrolase expression and activity after high-glucose oxidative stress. Free Radic Res 45:293–302

Fukui M, Choi HJ, Zhu BT (2010) Mechanism for the protective effect of resveratrol against oxidative stress-induced neuronal death. Free Radic Biol Med 49:800–813

Jazwa A, Cuadrado A (2010) Targeting heme oxygenase-1 for neuroprotection and neuroinflammation in neurodegenerative diseases. Curr Drug Targets 11:1517–1531

Jellinger KA (2009) Recent advances in our understanding of neurodegeneration. J Neural Transm 116:1111–1162

Jomova K, Vondrakova D, Lawson M, Valko M (2010) Metals, oxidative stress and neurodegenerative disorders. Mol Cell Biochem 345:91–104

Kaplan S, Bisleri G, Morgan JA, Cheema FH, Oz MC (2005) Resveratrol, a natural red wine polyphenol, reduces ischemia–reperfusion-induced spinal cord injury. Ann Thorac Surg 80:2242–2249

Kelsey NA, Wilkins HM, Linseman DA (2010) Nutraceutical antioxidants as novel neuroprotective agents. Molecules 15:7792–7814

Kim HP, Pae HO, Back SH, Chung SW, Woo JM, Son Y, Chung HT (2011) Heme oxygenase-1 comes back to endoplasmic reticulum. Biochem Biophys Res Commun 404:1–5

Kim DW, Kim YM, Kang SD, Han YM, Pae HO (2012) Effects of resveratrol and trans-3,5,4'-trimethoxystilbene on glutamate-induced cytotoxicity, heme oxygenase-1, and sirtuin 1 in HT22 neuronal cells. Biomol Ther 20:306–312

Lee HH, Park SA, Almazari I, Kim EH, Na HK, Surh YJ (2010) Piceatannol induces heme oxygenase-1 expression in human mammary epithelial cells through activation of ARE-driven Nrf2 signaling. Arch Biochem Biophys 501:142–150

Li F, Gong Q, Dong H, Shi J (2012) Resveratrol, a neuroprotective supplement for Alzheimer's disease. Curr Pharm Des 18:27–33

Marambaud P, Zhao H, Davies P (2005) Resveratrol promotes clearance of Alzheimer's disease amyloid-beta peptides. J Biol Chem 280:37377–37382

Morimoto BH, Koshland DE Jr (1990) Induction and expression of long- and short-term neurosecretory potentiation in a neural cell line. Neuron 5:875–880

Pae HO, Kim EC, Chung HT (2008) Integrative survival response evoked by heme oxygenase-1 and heme metabolites. J Clin Biochem Nutr 42:197–203

Pae HO, Son Y, Kim NH, Jeong HJ, Chang KC, Chung HT (2010) Role of heme oxygenase in preserving vascular bioactive NO. Nitric Oxide 23:251–257

Panickar KS, Anderson RA (2011) Effect of polyphenols on oxidative stress and mitochondrial dysfunction in neuronal death and brain edema in cerebral ischemia. Int J Mol Sci 12:8181–8207

Piotrowska H, Kucinska M, Murias M (2012) Biological activity of piceatannol: leaving the shadow of resveratrol. Mutat Res 750:60–82

Raval AP, Dave KR, Pérez-Pinzón MA (2006) Resveratrol mimics ischemic preconditioning in the brain. J Cereb Blood Flow Metab 26:1141–1147

Richard T, Pawlus AD, Iglésias ML, Pedrot E, Waffo-Teguo P, Mérillon JM, Monti JP (2011) Neuroprotective properties of resveratrol and derivatives. Ann N Y Acad Sci 1215:103–108

Robb EL, Stuart JA (2010) *Trans*-resveratrol as a neuroprotectant. Molecules 15:1196–1212

Roupe KA, Yáñez JA, Teng XW, Davies NM (2006) Pharmacokinetics of selected stilbenes. Rhapontigenin, piceatannol, and pinosylvin in rats. J Pharm Pharmacol 58:1443–1450

Sakata Y, Zhuang H, Kwansa H, Koehler RC, Doré S (2010) Resveratrol protects against experimental stroke: putative neuroprotective role of heme oxygenase 1. Exp Neurol 224:325–329

Sun AY, Wang Q, Simonyi A, Sun GY (2010) Resveratrol as a therapeutic agent for neurodegenerative diseases. Mol Neurobiol 41:375–383

Tan JW, Tham CL, Israf DA, Lee SH, Kim MK (2013) Neuroprotective effects of biochanin a against glutamate-induced cytotoxicity in PC12 cells via apoptosis inhibition. Neurochem Res 38:512–518

Wung BS, Hsu MC, Wu CC, Hsieh CW (2006) Piceatannol upregulates endothelial heme oxygenase-1 expression via novel protein kinase C and tyrosine kinase pathways. Pharmacol Res 53:113–122

Yang H, Sung SH, Kim J, Kim YC (2011) Neuroprotective diarylheptanoids from the leaves and twigs of *Juglans sinensis* against glutamate-induced toxicity in HT22 cells. Planta Med 77:841–845

Yu C, Shin YG, Chow A, Li Y, Kosmeder JW, Lee YS, Hirschelman WH, Pezzuto JM, Mehta RG, van Breemen RB (2002) Human, rat, and mouse metabolism of resveratrol. Pharm Res 19:1907–1914