Kaempferol Attenuates 4-Hydroxynonenal-Induced Apoptosis in PC12 Cells by Directly Inhibiting NADPH Oxidase

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ABSTRACT

Kaempferol, a natural flavonoid isolated from various plant sources, has been identified as a potential neuroprotectant. In this study, we investigated the protective effect of kaempferol against 4-hydroxynonenal (HNE)-induced apoptosis in PC12 rat pheochromocytoma cells. Kaempferol inhibited 4-HNE-mediated apoptosis, characterized by nuclear condensation, down-regulation of antiapoptotic protein Bcl-2, and activation of proapoptotic caspase-3. Kaempferol inhibited 4-HNE-induced phosphorylation of c-Jun N-terminal protein kinase (JNK). More importantly, kaempferol directly bound p47phox, a cytosolic subunit of NADPH oxidase (NOX), and significantly inhibited 4-HNE-induced activation of NOX. The antiapoptotic effects of kaempferol were replicated by the NOX inhibitor apocynin, suggesting that NOX is an important enzyme in its effects. Our results suggest that kaempferol attenuates 4-HNE-induced activation of JNK and apoptosis by binding p47phox of NOX and potently inhibiting activation of the NOX-JNK signaling pathway in neuron-like cells. Altogether, these results suggest that kaempferol may be a potent prophylactic against NOX-mediated neurodegeneration.

Introduction

4-Hydroxynonenal (HNE) is a major aldehyde product generated by lipid peroxidation of cellular membranes (Zarkovic, 2003). Numerous studies have reported that 4-HNE has prooxidant effects, increasing intracellular reactive oxygen species (ROS) and eliciting apoptosis in cells (Uchida, 2003; Cho et al., 2009). 4-HNE is considered a possible cause of numerous diseases, including obesity, diabetes, and associated vascular and neurodegenerative disorders (Mattson, 2009). 4-HNE is highly elevated in the brains of patients with Alzheimer's disease, Parkinson's disease, and amyotrophic lateral sclerosis (Markesbery and Lovell, 1998; Shibata et al., 2001).

NADPH oxidase (NOX) is a transmembrane/cytosolic multi-subunit enzyme that transfers electrons from NADPH to molecular oxygen to produce superoxide. Superoxide produced by NOX can spontaneously form hydrogen peroxide, which undergoes further reactions to generate ROS (Brennan et al., 2009). Originally described in neutrophils, where it is the enzyme responsible for killing bacteria and fungi (DeLeo and Quinn, 1996), the essential function of NOX has been identified in many other cell types (Bedard and Krause, 2007). NOX is involved in a number of cellular signaling pathways, including kinase activation, regulation of ion channels, and Ca²⁺ signaling (Bedard and Krause, 2007); in the brain, it may modulate neuronal activity and play a role in cognitive function (Infanger et al., 2006). Overactivation of NOX generates excessive amounts of ROS and contributes to neurotoxicity and neurodegeneration (Infanger et al., 2006). Overactivated NOX has been observed in the brains of patients with Alzheimer’s disease and...
Kaempferol (3,5,7-trihydroxy-2-(4-hydroxyphenyl)-4H-1-benzopyran-4-one) (Fig. 1) is a natural flavonoid isolated from tea, mushrooms, kale, broccoli, and other plant sources (Hertog et al., 1994). Kaempferol has been reported to have several health-promoting effects. For example, kaempferol possesses antioxidative and anti-inflammatory properties (Burda and Oleszek, 2001; García-Lafuente et al., 2009) and exhibits antitumor activity (Leung et al., 2007). Kaempferol is neuroprotective against excitotoxic insults (Bedard and Krause, 2007). It has been reported that kaempferol could afford neuroprotection against 4-HNE-induced NOX activation and apoptosis (Yun et al., 2010).

In the present study, we sought to determine whether kaempferol prevents 4-HNE-induced NOX activation and apoptosis in PC12 rat pheochromocytoma cells. We confirmed that 4-HNE induces apoptosis through the activation of NOX in PC12 cells and showed that kaempferol protects the neuron-like cells against 4-HNE-induced c-Jun N-terminal protein kinase (JNK) activation and apoptosis by direct binding to p47^phox of NOX and blocking the activation of NOX.

Materials and Methods

Chemicals and Reagents. Kaempferol was purchased from Indofine Chemicals (Hillsborough, NJ). 4-HNE was obtained from Cayman Chemical (Ann Arbor, MI). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), trypsin blue solution (0.4%), and 4,6-diamidino-2-phenylindole (DAPI) were purchased from Sigma-Aldrich (St. Louis, MO). Dulbecco’s modified Eagle’s medium, fetal bovine serum, horse serum, and penicillin/streptomycin mixture were obtained from Invitrogen (Carlsbad, CA). Antibodies against poly(ADP-ribose) polymerase (PARP), Bcl-2, caspase-3, JNK, and p47^phox were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-phosphorylated-JNK and anti-β-actin antibody were purchased from Cell Signaling Technology (Beverly, MA) and Sigma-Aldrich, respectively. Lucigenin was obtained from Tokyo Chemical Industry (Tokyo, Japan). Hanks’ balanced salt solution (HBSS) was purchased from Mediatech (Herndon, VA). NADPH was obtained from AppliChem (Darmstadt, Germany). Cyanogen bromide-Sepharose 4B was purchased from GE Healthcare (Chalfont St. Giles, Buckinghamshire, UK). Apocynin (4-hydroxy-3-methoxyacetophenone) was obtained from Calbiochem (San Diego, CA), EDTA, dithiothreitol (DTT), phenylmethylsulfonyl fluoride (PMSF), and all other chemicals used were purchased from Sigma-Aldrich and were of analytical grade.

Cell Culture. Rat PC12 pheochromocytoma cells have been used widely to investigate oxidative stress-mediated neuronal damage (Behl et al., 1994; Kadowaki et al., 2005). PC12 cells were provided by Dr. Y.-J. Suh (Seoul National University, Seoul, Republic of Korea) and grown in Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated horse serum, 5% fetal bovine serum, and 0.1% penicillin/streptomycin at 37°C in a humidified 10% CO2 atmosphere. Treatment with 4-HNE, kaempferol, and apocynin was performed on cells in serum-free media.

MTT Assay. The MTT assay measures the reduction of the tetrazolium salt MTT to insoluble formazan as a result of mitochondrial activity and thus serves as a metabolic indicator of live cells. After PC12 cells (2 × 10^4 cells/well in 96-well plates) were incubated with 20 μM 4-HNE for 24 h, cell viability was determined using MTT solution.

Fig. 1. Chemical structure of kaempferol.

Fig. 2. Effects of kaempferol on 4-HNE-induced cell death. PC12 cells were pretreated with kaempferol (5 or 10 μM) for 30 min and then exposed to 20 μM 4-HNE for 24 h. Cell viability was determined using MTT assays (A) and trypan blue exclusion assays (B). Cell viabilities are presented as means ± S.E. (n = 3) expressed as a percentage of control values. Different letters on top of bars indicate a significant difference at p < 0.05 analyzed using ANOVA followed by Duncan’s multiple range test.
(final concentration, 1 mg/ml) was added, and cells were incubated for an additional 2 h. The dark-blue formazan crystals formed in viable cells were dissolved in dimethyl sulfoxide, and absorbance at 570 nm was measured with a microplate reader. Results are expressed as the percentage of MTT reduction, measured as absorbance values relative to those of control cells.

**Trypan Blue Exclusion Assay.** The trypan blue exclusion assay is based on the interaction of trypan blue dye with damaged membranes of dead cells. Trypan blue dye is excluded from viable cells. The trypan blue exclusion assay was performed on PC12 cells (10^5 cells/well in six-well plates) pretreated with 20 μM 4-HNE for 24 h with or without kaempferol. After centrifugation at 600g for 6 min, cells were resuspended in 200 μl of phosphate-buffered saline (PBS). Suspended cells then were mixed with 200 μl of trypan blue staining solution (0.4%) and incubated for 5 min at room temperature. The cells then were loaded onto a hemocytometer and stained. Dye-excluding cells were counted under a microscope, and the percentage of stained cells was determined by scoring 150 cells.

**DAPI Staining Assay.** The DNA-specific fluorescent dye DAPI was used to detect nuclear fragmentation characteristic of apoptotic cells. PC12 cells (5 × 10^4 cells/well in 24-well plates) were incubated with 20 μM 4-HNE for 24 h with or without kaempferol, washed with PBS, and fixed with 70% ethanol for 20 min. The fixed cells were washed with PBS again and incubated with DAPI (1 μg/ml) for 10 min. The cells then were washed with PBS and observed under a fluorescence microscope (Olympus, Tokyo, Japan). The degree of nuclear fragmentation was evaluated by counting the percentage of DAPI-stained cells in 100 to 120 randomly selected cells.

**Western Blot Analysis.** PC12 cells (4 × 10^5 cells/ml in a 6-cm dish) were incubated with 20 μM 4-HNE with or without kaempferol, washed, collected with ice-cold PBS, and centrifuged at 600g for 10 min. The cell pellet was resuspended in 100 μl of ice-cold lysis buffer (Cell Signaling Technology) and incubated on ice for 30 min. After centrifugation at 1000g for 15 min, the supernatant was separated and stored at −70°C. The protein concentration in lysates was determined using a protein assay kit (Bio-Rad Laboratories, Hercules, CA). Protein lysates were separated on an SDS-polyacrylamide gel and then transferred onto a polyvinylidene difluoride membrane. Membranes were blocked with a 5% skim milk solution containing 0.5 mM Tris-Cl (pH 7.5), 150 mM NaCl, and 0.05%Tween 20 for 2 h at room temperature and then incubated with primary antibody. After three washes with Tris-buffered saline containing 0.1% Tween 20 (TBST), the blots were incubated for 2 h at room temperature with horseradish peroxidase-conjugated secondary antibodies diluted 1:5000 in TBST/5% skim milk. The blots then were washed three times in TBST and developed using an enhanced chemiluminescence detection kit (GE Healthcare).

**NOX Activity Assay.** NOX activity was measured using superoxide-sensitive lucigenin chemiluminescence (Kim et al., 2007). Cells were grown on a 96-well plate (2 × 10^4 cells/well) for 24 h, and then the medium was removed and replaced with HBSS buffer. Cells were preincubated with the indicated drugs for 30 min before exposure to 20 μM 4-HNE for an additional 5 min. Cells then were loaded with HBSS buffer containing 25 μM lucigenin and 200 μM NADPH. Chemiluminescence was measured using Veritas microplate luminometer software (Tuner Biosystems, Sunnyvale, CA).

**Kaempferol-Sepharose 4B Pulldown of p47-phox.** A kaempferol-Sepharose 4B complex was generated by activating Sepharose 4B freeze-dried powder (0.3 g) in 30 ml of HCl (1 mM). Kaempferol (2 mg) was mixed with activated Sepharose 4B in coupling buffer (0.1 M HBSS buffer containing 25 μM lucigenin and 200 μM NADPH). After incubation for 2 min at room temperature and then incubation with primary antibody. After three washes with Tris-buffered saline containing 0.1% Tween 20 (TBST), the blots were incubated for 2 h at room temperature with horseradish peroxidase-conjugated secondary antibodies diluted 1:5000 in TBST/5% skim milk. The blots then were washed three times in TBST and developed using an enhanced chemiluminescence detection kit (GE Healthcare).

**Fig. 3.** Effects of kaempferol on 4-HNE-induced apoptosis. PC12 cells were pretreated with kaempferol (5 or 10 μM) for 30 min and then exposed to 20 μM 4-HNE for 24 h. A and B, apoptotic cells (condensed nuclei) were measured by DAPI staining assay. Nuclear morphologies were examined by fluorescence microscopy (A), and the numbers of apoptotic cells were calculated and presented as means ± S.E. (n = 3) expressed as a percentage of apoptotic cells in the 4-HNE-only group (B). C, the protein levels of Bcl-2, cleaved-caspase-3, and β-actin were determined by Western blot analysis. β-Actin was used as a loading control. D and E, the ratios of Bcl-2/β-actin (D) and procaspase-3/β-actin (E) were determined by densitometry. Data are presented as means ± S.E. of three independent experiments. Different letters on top of bars indicate a significant difference at p < 0.05 analyzed using ANOVA followed by Duncan’s multiple range test.
NaHCO₃ (pH 8.3) and 0.5 M NaCl and rotated at 4°C overnight. Coupling buffer was replaced with 0.1 M Tris-HCl buffer (pH 8.0), and the slurry was rotated again at 4°C overnight. The kaempferol-Sepharose 4B complex then was washed once with 0.1 M acetate buffer (pH 4.0) containing 0.5 M NaCl, followed by a second wash containing 0.5 M NaCl. Proteins from PC12 cells were incubated with kaempferol-Sepharose 4B beads or uncomplexed Sepharose 4B beads (100 µl, 50% slurry) in reaction buffer [50 mM Tris (pH 7.5), 5 mM EDTA, 150 mM NaCl, 1 mM DTT, 0.01% Nonidet P-40, 2 mg/ml bovine serum albumin, 0.02 mM PMSF, and 1 µg of protease inhibitor mixture] and rotated overnight at 4°C. The beads then were washed five times with washing buffer [50 mM Tris (pH 7.5), 5 mM EDTA, 150 mM NaCl, 1 mM DTT, 0.01% Nonidet P-40, and 0.02 mM PMSF].

**Replication and Statistical Analysis.** Experiments were repeated at least three to four times with consistent results. Means ± S.E. have been presented in figures. All of the statistical analyses were performed using SPSS software (SPSS 19.0 for Windows; SPSS Inc., Chicago, IL). Treatment effects were analyzed using one-way analysis of variance (ANOVA) followed by Duncan’s multiple range test. A probability cutoff of p < 0.05 was used as the criterion for statistical significance.

**Results**

**Kaempferol Inhibited 4-HNE-Induced Cell Death.** We first examined the cytoprotective effect of kaempferol against 4-HNE-induced cell death using MTT and trypan blue exclusion assays in PC12 cells. 4-HNE at a concentration of 10 to 20 µM has been widely used to investigate oxidative stress-mediated neuronal damage (Tang et al., 2008; Wang et al., 2010). Treatment of PC12 cells with 20 µM 4-HNE for 24 h reduced cell viability to 45.37 ± 6.65% that of untreated controls. Pretreatment with 5 or 10 µM kaempferol for 30 min increased the viability of 4-HNE-treated PC12 cells to 96.44 ± 18.71% and 117.07 ± 12.51%, respectively (Fig. 2A).

Similar results were obtained using the trypan blue exclusion assay, which showed that 20 µM 4-HNE reduced cell viability to 61.02 ± 3.24% of untreated control values, and pretreatment with 5 or 10 µM kaempferol for 30 min increased these values to 80.34 ± 4.58% and 86.18 ± 7.10%, respectively (Fig. 2B). These observations indicate that kaempferol inhibits 4-HNE-induced cell death in PC12 cells.

**Kaempferol Suppressed 4-HNE-Induced Apoptosis.** Apoptotic cells undergo nuclear condensation, as determined by DAPI staining. Treatment with 20 µM 4-HNE for 24 h resulted in an increase in the number of cells undergoing nuclear condensation (Fig. 3, Ab and B). Pretreatment with 5 or 10 µM kaempferol for 30 min significantly decreased the number of cells with condensed nuclei (Fig. 3, A, c and d, and B).

Bcl-2 is an antiapoptotic protein that stabilizes mitochondrial function and suppresses oxidative stress-mediated cellular damage (Mattson, 2000). In addition, it has been reported that Bcl-2 inhibits the activation of caspase-3, which is an important mediator of apoptosis (Cory and Adams, 2002). We thus investigated whether the protective effect of kaempferol against 4-HNE-induced apoptosis in PC12 cells involved changes in Bcl-2 levels and/or caspase-3 activation. Treatment of PC12 cells with 20 µM 4-HNE for 24 h decreased the amount of Bcl-2 and induced the cleavage of procaspase-3 (Fig. 3, C–E).

Pretreatment with 10 µM kaempferol for 30 min completely reversed the effects of 4-HNE on Bcl-2 (Fig. 3, C and D), and pretreatment with 5 or 10 µM kaempferol for 30 min blocked 4-HNE-induced procaspase-3 cleavage (Fig. 3, C and E). Collectively, these results indicate that kaempferol protects PC12 cells against 4-HNE-induced apoptosis.

**Kaempferol Blocked 4-HNE-Induced JNK Phosphorylation.** Because JNK is an important signaling molecule that leads to Bcl-2 down-regulation and caspase-3 activation (Mielke and Herdegen, 2000; Cho et al., 2009; Cao et al., 2010), we investigated the effect of kaempferol on JNK activation (phosphorylation). Treatment with 20 µM 4-HNE for 5 min significantly increased JNK phosphorylation (Fig. 4), an effect that was suppressed by pretreatment with 10 µM kaempferol for 30 min (Fig. 4). Collectively, these results indicate that kaempferol might inhibit 4-HNE-induced apoptosis, Bcl-2 down-regulation, and caspase-3 activation by inhibiting JNK phosphorylation.

**Kaempferol Attenuated 4-HNE-Induced Activation of NOX.** To investigate the effect of kaempferol on NOX activity, we performed NOX activity assays. Treatment with 20 µM 4-HNE for 5 min induced a 1.6-fold increase in NOX activity. This 4-HNE-induced increase in NOX activity was suppressed by pretreatment for 30 min with 5 or 10 µM kaempferol, which exerted an inhibitory effect comparable to that of apocynin, a well known pharmacological inhibitor of NOX (Fig. 5, A and B).

Because kaempferol inhibits NOX activity, we investigated...
whether kaempferol directly binds p47\textsuperscript{phox}, a cytosolic subunit of NOX, using kaempferol-Sepharose 4B pulldown assays. Cellular p47\textsuperscript{phox} did not bind unconjugated Sepharose 4B (Fig. 5C, lane 2) but did bind kaempferol-Sepharose 4B, suggesting that kaempferol interacts directly with p47\textsuperscript{phox} (Fig. 5C, lane 3). These observations indicate that kaempferol might inhibit 4-HNE-mediated activation of NOX by binding directly to p47\textsuperscript{phox} in PC12 cells.

**Apocynin, an Inhibitor of NOX, Inhibited 4-HNE-Induced Apoptosis.** To demonstrate that NOX plays a role in 4-HNE-induced apoptosis, we examined the effect of apocynin, a well-known inhibitor of NOX, on 4-HNE-induced apoptosis. Pretreatment of PC12 cells with apocynin (5 or 10 \mu M) for 30 min inhibited 4-HNE-induced cell death (Fig. 6A). We examined the levels of cleaved PARP, an important molecular indicator of the chromatin condensation process associated with apoptosis. Treatment with 20 \mu M 4-HNE for 24 h increased the level of cleaved PARP, and this increase was suppressed by pretreatment with 10 \mu M apocynin for 30 min (Fig. 6, B and C). Apocynin at a concentration of 5 or 10 \mu M attenuated 4-HNE-induced down-regulation of Bcl-2 and procaspase-3 cleavage (Fig. 6, B, D, and E). In addition, pretreatment with apocynin suppressed the 4-HNE-induced phosphorylation of JNK (Fig. 6, F and G). Thus, blocking the activity of NOX prevented the 4-HNE-induced increase in cell death and inhibited 4-HNE-mediated JNK phosphorylation, PARP cleavage, Bcl-2 down-regulation, and caspase-3 activation, indicating that NOX plays an important role in 4-HNE-induced apoptosis in PC12 cells.

**Discussion**

The consumption of flavonoid-rich foods and beverages has been suggested to limit the neurodegeneration associated with a variety of neurological disorders and to prevent or reverse deteriorations in brain (Spencer, 2010). Originally, it was thought that such actions were mediated by the antioxidant capacity of flavonoids (Spencer, 2010). However, the multiplicity of their effects appears to be underpinned by a number of routes, including interactions with important neuronal and glial signaling cascades in the brain (Spencer, 2010). In this study, we introduced the molecular interaction of kaempferol with p47\textsuperscript{phox} of NOX to explain its antiapoptotic and neuroprotective effects.

NOX requires p47\textsuperscript{phox}, a key cytosolic subunit, to be activated (Taura et al., 2009). Antisense oligonucleotides against
p47\textsubscript{phox} have been shown to suppress NOX-mediated apoptosis (Yu et al., 2005), suggesting that p47\textsubscript{phox} plays an important role in NOX-induced apoptosis. N-Methyl-D-aspartate-induced superoxide production and cell death are attenuated in p47\textsubscript{phox}(/H11002(/H11002/) neurons compared with those in wild-type neurons (Brennan et al., 2009), indicating that p47\textsubscript{phox} is required for N-methyl-D-aspartate-mediated NOX activation and neuronal death. Therefore, attenuation of excessive activation of NOX through blocking p47\textsubscript{phox} might be a useful therapeutic strategy for preventing neuronal injury.

In this study, we report that kaempferol could be a potent prophylactic against NOX-mediated neurodegeneration through direct binding to p47\textsubscript{phox}. The interaction may be responsible for its inhibition of 4-HNE-mediated NOX activation and apoptosis. Kaempferol might bind functional regions in p47\textsubscript{phox}: the PX domain, which interferes with membrane binding, or the SH3 domain, which interacts with the catalytic subunit p22\textsubscript{phox} (Sumimoto, 2008). However, the results of kaempferol-Sepharose 4B pulldown assays showed that kaempferol did not interact with p67\textsubscript{phox}, the other cytosolic regulatory component of NOX (data not shown). More precise structure-activity relationship studies such as virtual computer modeling or X-ray cocrystallography might define the exact molecular interaction of kaempferol with p47\textsubscript{phox}.

JNK is an important signaling molecule in pathways that lead to 4-HNE-induced apoptosis (Song et al., 2001) and has been shown to be involved in caspase-3 activation (Cho et al., 2009). Our observations demonstrate that apocynin, a specific inhibitor of NOX, blocks 4-HNE-induced JNK phosphorylation, caspase-3 activation, and apoptosis, suggesting that
JNK is involved in the NOX-mediated signaling pathway leading to apoptosis. We also found that apocynin did not decrease the phosphorylation of mitogen-activated protein kinase kinase 4, an upstream kinase of JNK, indicating that mitogen-activated protein kinase kinase 4 is not regulated by NOX (data not shown). Therefore, our results indicate that kaempferol might suppress NOX activation through direct binding to p47phox and subsequently attenuate JNK-caspase-3 activation. Previous reports have similarly demonstrated that kaempferol inhibits neuronal apoptosis by regulating the c-Jun and caspase cascade (Schroeter et al., 2001).

The pharmacokinetic parameters after intravenous administration of 25 mg/kg kaempferol in male rats showed that the concentration of kaempferol reaches up to approximately 30 μM in plasma (Barve et al., 2009). Kaempferol can be transported into the neurons in a concentration-dependent manner when the neurons were incubated with the culture medium containing kaempferol (Liu et al., 2006). Because of its permeability in neurons, kaempferol at concentrations of 5 and 10 μM might directly bind cytosolic p47phox and interfere with 4-HNE-induced NOX activity in PC12 cells. Consistent with our result, NOX has been suggested as a molecular target that might interact with kaempferol (Tauber et al., 1984; Steffen et al., 2008). Kaempferol inhibits NOX activity at an IC50 value of 92 ± 18.3 μM in human neurophil (Tauber et al., 1984). Kaempferol has an IC50 value of 10.0 ± 3.6 μM against NOX activity in human endothelial cells, making it more potent than the known NOX inhibitor apocynin (IC50 = 50.0 ± 9.1 μM) in these cells (Steffen et al., 2008). Even though there is a lack of direct pharmacokinetic data in brain, kaempferol has been shown to be neuroprotective under pathological conditions in animal models (López-Sánchez et al., 2007; Lagoa et al., 2009). These observations suggest that kaempferol might be able to cross the blood-brain barrier and reach the brain tissue.

In conclusion, 4-HNE-induced apoptosis, characterized by the induction of nuclear condensation, down-regulation of antiapoptotic protein Bcl-2, and activation of proapoptotic caspase-3, was attenuated by kaempferol treatment. This inhibition was associated with the binding of kaempferol to p47phox and suppression of the NOX-JNK pathway. Taken together, these results indicate that kaempferol may act through direct binding to p47phox and inhibit 4-HNE-induced NOX activation and JNK-mediated apoptosis in neuron-like PC12 cells.


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