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The protective effect of astrocyte-derived 14,15-EET on H₂O₂-induced cell injury in Astrocyte-dopaminergic neuronal cell line co-culture

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Abstract

Astrocytes perform several functions that are essential for normal neuronal activity. They play a critical role in neuronal survival during ischemia and other degenerative injuries and also modulate neuronal recovery by influencing neurite outgrowth. In this study, we investigated the neuroprotective effects of astrocyte-derived 14,15-epoxyeicosatrienoic acid (14,15-EET), metabolite of arachidonic acid by Cytochrome P450 epoxygenases (CYP), against oxidative stress induced by hydrogen peroxide (H₂O₂). We found that dopaminergic neuronal cells (N27 cell line) stimulated with two different doses of H₂O₂ (0.1 and 1 mM) for 1h showed decreased cell viability compared to the control group, while astrocytes co-cultured with dopaminergic neuronal cell lines prevented cell during after stimulation with the same doses of H₂O₂ for 1h. Dopaminergic neuronal cells (N27 cell line) pretreated with different doses of 14, 15-EET (0.1–30 μM, 30 min) before H₂O₂ stimulation also showed increased cell viability. Furthermore, pre-treatment of the co-cultured cells with 12-(3-adamantan-1-yl-ureido)-dodecanoic acid (AUDA), an inhibitor of the EET metabolizing enzyme, soluble epoxide hydrolase (sEH), before H₂O₂ stimulation (1 mM, for 1h) increased cell viability. It also increased the endogenous level of 14,15-EET in the media compared to control group. However, pretreatment with the CYP epoxygenase inhibitor miconazole (1–20 μM, 1h) before H₂O₂ (1 mM, 1h) stimulation showed decreased cell viability. Our data suggest that 14,15-EET which is released from astrocytes, enhances cell viability against oxidant induced injury. Further understanding of the mechanism of 14,15-EET-mediated protection in dopaminergic neurons is imperative, as it could lead to novel therapeutic approaches for treating CNS neuropathologies, such as Parkinson's disease.

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Keywords

14,15-EET; H₂O₂; soluble epoxide hydrolase; AUDA; neuron-astrocyte co-culture; Parkinson's disease

Elevated production of hydrogen peroxide (H₂O₂) in the central nervous system has been implicated in the pathogenesis of several neurodegenerative diseases, including Parkinson's disease (PD). Superoxide dismutase (SOD) and catalase are important antioxidant enzymes that scavenge superoxide anion (O₂⁻) and H₂O₂ to protect cells from oxidative damage. If abnormal formation of O₂⁻ and H₂O₂ is over the capability of SOD/catalase defenses or the activities of SOD and catalase were to decrease abnormally, the production of reactive oxygen species (ROS) will induce cell death. However, the therapeutic strategy for neurodegenerative diseases using SOD/catalase has not been successful (Chen et al., 2008; Peng et al., 2005). Degeneration of dopaminergic neurons is thought to be the result of apoptosis induced by chronic oxidative stress. The source of increased oxidative stress is not completely known and therefore environmental factors, excitotoxins, dopamine homeostasis, and other factors have gained more attention (Sayre et al. 2008). Oxidative stress induces mitochondrial dysfunction, genetic mutation, and protein aggregation and ultimately causes cell death (Mattson et al. 2002).

Recognition of the importance of astrocytes in neurovascular regulation is increasing, specifically regarding the modulation of neural activity. Astrocytes actively participate in several aspects of neuronal growth and differentiation both by providing cell-cell interactions and by secreting neuronal growth-promoting factors. They take up and release several neurotransmitter substances and can modulate the concentration of a neurotransmitter substance at the synaptic cleft and thus monitor neuronal activity (Vernadakis A, 1988). Certain neurotrophic factors like glutathione released from astrocytes have been shown to protect neurons against oxidative damage induced by neurotoxins (Spina et al., 1992). More recently several reports show that epoxyeicosatrienoic acids (EETs), the metabolites of arachidonic acid (AA) by cytochrome P450 (CYP) epoxygenases, are released from neurons and astrocytes (Alkayed et al., 1996; Iliff et al., 2010). In the brain, EETs play an important role in cerebral blood flow regulation (Alkayed et al., 1996a) and neurovascular coupling (Koehler et al., 2006). Furthermore, the expression of CYP epoxygenase in brain is increased by ischemic preconditioning, which was associated with protection from ischemic stroke induced in the rat by middle cerebral artery occlusion (Alkayed et al., 2002). In addition, more recently the same group has demonstrated that EETs protect neurons (Koerner et al., 2007) and astrocytes (Liu and Alkayed, 2005) against ischemic cell death induced *in vitro* by oxygen-glucose deprivation, suggesting that EETs may exert a cytoprotective effect independent of its effect to dilate blood vessels and increase CBF. However the initial cellular mechanisms that mediate the action of EETs remain uncertain. One possibility is that EETs bind to a membrane receptor linked to an intracellular signal transduction pathway that initiates the functional response. The other is an intracellular mechanism in which EETs directly interact with and activate ion channels, signal transduction components, or transcription factors producing the functional response. It is likely that the actions of EETs are mediated by both mechanisms, thus accounting for

their diverse effects. The mechanism involving a G protein-coupled receptor is provided by the observation that 11,12-EET induced activation of the BK_{Ca} channel and tissue plasminogen activator expression is mediated by the Gα_s component of a heterotrimeric GTP binding protein (Gebremedhin et al., 1992; Li and Campbell, 1997; Node et al., 2001). Angiogenesis initiated by 11,12-EET also involves a cAMP-PKA signaling pathway that induces cyclooxygenase-2 expression (Michaelis et al., 2005). In addition to the Gα_s-cAMP-PKA pathway, a number of other signal transduction mechanisms have been found to be active in EET functional responses under various conditions. Activation of tyrosine kinase cascade, Src kinase, mitogen-activated protein kinase (MAPK), and phosphatidylinositol 3-kinase (PI3K)/Akt pathways mediate actions of EETs in endothelial cells, arterial smooth muscle cells, glomerular mesangial cells, renal tubular epithelial cells, and myocardium (reviewed in Spector and Norris, 2007). In addition, the anti-inflammatory effect produced by 11,12-EET in the endothelium is due to inhibition of cytokine-activated nuclear factor-κB (NF-κB)-mediated transcription. This occurs by inhibition of IKK phosphorylation of IκBα (Spiecker and Liao, 2005). The fact that other agonists typically activate these pathways through membrane receptor mechanisms provides support for an EET receptor mechanism, but so far the putative EET receptor has not been conclusively identified.

There are 4 regioisomeric EETs: 5,6-, 8,9-, 11,12- and 14,15-EETs (Rifkind et al., 1995). Because the regioisomers have a number of similar metabolic and functional properties, EETs are generally considered as a single class of compound. But there are quantitative and qualitative differences in the actions of various regioisomers, such as 14,15-EET is the good substrate for sEH (Spector and Norris, 2007) and 11,12-EET is the only regioisomer that inhibits basolateral K⁺ channels in the renal cortical collecting duct (Wang et al., 2008).

Nevertheless, no information is available regarding the effect of EETs against the oxidant-induced neuronal damage one of the hallmarks of pathogenesis of PD. Therefore, this study was designed to evaluate the neuroprotective effects of 14,15-EET against H₂O₂-induced dopaminergic neuronal damage.

MATERIALS AND METHODS

Materials

Hydrogen peroxide (H₂O₂) and miconazole were obtained from Sigma (St. Louis, MO, USA). EETs were kindly donated by Dr. John R. Falck (Department of Biochemistry, University of Texas Southwestern Medical Center, Dallas, TX, USA). AUDA was also kindly donated by Dr. John D. Imig (Department of Pharmacology and Toxicology, Medical College of Wisconsin, Milwaukee, WI, USA). H₂O₂ was dissolved in a sterile saline solution (0.9% NaCl solution). Miconazole, EETs and AUDA were first dissolved in 100% alcohol and then diluted in a sterile saline solution containing 0.1 % alcohol. Vehicle for Miconazole, EETs and AUDA was a sterile saline solution containing 0.1 % alcohol.

Cell culture

Cell culture systems have provided powerful tools to delineate cellular and molecular events and for determining the neuroprotective effect of EETs. Experiments were performed on cell

culture using two different types of cells: astrocytes and N27 dopaminergic neuron cell line. It has been shown that astrocytes from the hippocampus and their conditioning media can support the survival of neurons from regions other than the hippocampus (Hewett, 2009). Based on this observation and our lab's long experience with successfully culturing astrocytes from this region of the brain (Alkayed et al., 1996) in this study we used hippocampal astrocytes. The N27 dopaminergic neuronal cell line was kindly donated by Dr. Balaraman Kalyanaraman, Department of Biophysics, Medical College of Wisconsin.

Primary astrocytes were cultured from 2–3 day old SD rat pups. The hippocampus was removed and digested in 20 U/ml papain (Worthington Biochemical, Freehold, NJ, USA) and L-Cystine (Sigma) for 45 min at 37°C. Digestion was stopped by washing the pellet three times with astrocyte growth media (10% FBS in DMEM with 1% Penicillin-Streptomycin, Invitrogen, Carlsband, CA, USA). The tissues were triturated and plated onto 10 cm dishes at a density of approximately 1×10^6 cells per dish. Like astrocytes, dopaminergic neurons were also incubated in the same type of growth media. The cells were incubated at 37°C in an atmosphere of 5% CO₂ in air. The cells were passaged every 7–10 days or when 80% confluent. In our experiments we have used cells from passages 2 to 4.

Co-cultures: For co-culture experiments, primary astrocytes were plated at a density of 300,000 cells per well in 6-well plates. Neurons were then plated at a density of 300,000 cells per well after 4 days on top of the mature primary astrocyte cultures. The medium was replaced 2 days later and experiments were performed on day 4 of the combined culture.

Determination of cell viability by MTT assay

The cell viability was assessed by the Vybrant MTT Cell Proliferation Assay Kit (V-13154, Molecular Probes, Inc. Eugene, OR, USA). Briefly, after treatment of cells with H₂O₂, 14,15-EET, CYP inhibitor-Miconazole or soluble epoxide hydrolase (sEH) inhibitor-AUDA, 50 μ l of MTT (5 mg/ml) was added to each well (six well plate) containing 500 μ l of phenol red free medium and incubated at 37°C for an additional 3 h. At the end of the incubation period, 500 μ l of the SDS-HCl solution was added to each well and incubated for 10 h. After the incubation, each well was mixed by a pipette and absorbance was read at 570 nm by using FLUOstar Omega plate reader (BMG Labtech GmbH, Allmendgruen, Ortenberg, Germany).

Measurement of mitochondrial membrane potential by JC-1 assay

Mitochondrial membrane potential was assessed by the MitoProbe 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazole-carbocyanide iodine (JC-1) assay kit (Molecular Probes, Inc). JC-1 labels mitochondria with high membrane potential orange and mitochondria with low membrane potential green. Following the incubation with AUDA pretreatment and/or H₂O₂, 5 μ g/ml JC-1 was added to each well (6 well plates) into the medium for 30 min at 37°C and washed twice with PBS. The mitochondrial membrane potentials were analyzed by Flow Cytometer core on a FACSCalibur Flow cytometer (Becton Dickinson, San Jose, CA, USA). The carbonyl cyanide 3-chlorophenylhydrazone (CCCP), an uncoupler of cell respiration and oxidative phosphorylation, was used as a control to show the dissipation of mitochondrial membrane potential.

Measurement of endogenous level of 14,15-EET in media by Liquid Chromatography-Mass Spectrometry (LC/MS) technique

Co-cultured cells were pretreated with AUDA or vehicle for 1 h followed by 1 mM of H₂O₂ stimulation for 1 h. After H₂O₂ stimulation media from each group was collected and transferred to glass extraction tubes. To each sample 10 µl of internal standard (d6-20-HETE, 1ng/µl) was added and the samples were extracted with diethyl ether. The ether layer containing the extracted lipids were dried under nitrogen and stored at -80° C until measurement of 14,15-EET by LC/MS.

LC/MS Analysis of Eicosanoids

Samples were reconstituted in 50 µL of 50% solution of methanol and the metabolites of AA were separated in the Biochemical core by high-pressure liquid chromatography on a Betabasic C18 column (150-2.1mm, 3 mm; Thermo Hypersil-Keystone, Bellefonte, PA, USA) at a flow rate of 0.2 mL/min using an isocratic elution with a 51:9:40:0.01 mixture of acetonitrile/methanol/water/acetic acid for 30 min followed by a step gradient to 68:13:19:01 acetonitrile/methanol/water/acetic acid and water for 15 min. The effluent was ionized using a negative ion electrospray (4501C, 4,500 V) with the collisional activated dissociation gas set at 7 L/min. All transitions had a scan time of 0.2 s and a unit resolution in both Q1 and Q3 set at 0.7±0.1 full width at half maximum. The peaks eluting with a mass/charge ratio (m/z) of 319 > 301 (HETEs and EETs), 337 > 319 (DiHETEs), 319 > 245 (20-HETE) and 325 > 251 (d6-20-HETE) were monitored in the multiple reaction monitoring mode using a triple quadrupole mass spectrometer (ABI 3000; Applied Biosystems, Foster City, CA, USA). The ratio of ion abundances in the peaks of interest versus that seen in the internal standard was determined and compared with four-point individual standard curves generated for each product analyzed in each sample run over a range from 0.2 to 10 ng.

Statistical Analysis

Mean values ± S.E.M. are presented. One-way analysis of variance (ANOVA) followed by Dunnett's post-test or Student's *t*-test was used to determine the difference between groups. The GraphPad Prism software was used to perform the statistics (version 4.03; GraphPad Software, Inc., San Diego, CA, USA). *P* < 0.05 was considered to be significant.

RESULTS

Toxic effect of hydrogen peroxide (H₂O₂) on co-cultured cells

Increased intracellular levels of reactive oxygen species (ROS) causes oxidative stress by activating a common pathway leading to neuronal cell death (Griendling and FitzGerald, 2003). In our study the effect of H₂O₂ on astrocytes, N27 cell lines were studied. Astrocytes, N27 cells were stimulated with two different doses of H₂O₂ for 1h and then cell viability was measured by MTT assay. We found that neuronal cells stimulated with different doses of H₂O₂ (0.1 and 1 mM) for 1 h decreased cell viability to about 33 to 52 % (respectively) compared to control group (Fig 1A). However, in astrocytes alone stimulated with the same dose of H₂O₂ the loss of cell viability was much lower (3 to 31% respectively) (Fig. 1B)

compared to that in the N27 cell lines. The resistance of astrocytes to oxidative injury led us to investigate the neuromodulator released from astrocytes, which can prevent the cells from free radical damage. It has been previously reported that in the brain, four regio-isomeric epoxyeicosatrienoic acids (EETs) are released from astrocytes and neurons (Alkayed et al., 1996, Iliff et al., 2010) which lead us to determine the effect of exogenously applied 14,15-EET against H₂O₂ induced cell death.

The effect of pretreatment with 14,15-EET on H₂O₂-induced neuronal cell death

Experiments were then performed to determine if exogenously applied 14,15-EET will protect neuronal cells from free radical damage. Groups of neuronal cells were pretreated with different concentrations (0.1, 1, 10 or 30 μ M) of 14,15-EET or vehicle for 30 min before H₂O₂ (1 mM) stimulation (Fig. 2) and the cell viability was measured 1 h thereafter. We found that 14,15-EET dose dependently increased cell viability compared to vehicle pretreatment group (Fig. 2).

The effect of AUDA, a soluble epoxide hydrolase inhibitor, on H₂O₂-induced cell death

In support of our finding that exogenously applied 14,15-EET prevents cells from free radical damage, we used AUDA, an inhibitor for the EETs metabolizing enzyme, soluble epoxide hydrolase (sEH), to increase the endogenous level of 14,15-EET which in turn may prevent cell death from free radical injury. Groups of co-cultured cells were pretreated with different concentrations (8, 40, 80 or 800 nM) of AUDA or vehicle for 1 h before H₂O₂ (1 mM) stimulation and the cell viability was measured with MTT assay 1h thereafter. We found that AUDA dose dependently increased the cell viability compared to vehicle pretreatment group (Fig. 3).

The effect of astrocyte-conditioned media on H₂O₂-induced neuronal cell death

The results of the experiments described above indicate that pretreatment of cells with different concentrations of AUDA may modulate the cell viability by increasing the endogenous level of 14,15-EET. To confirm our hypothesis we separated the cells, stimulated astrocytes with different concentrations (8, 80 or 800 nM) of AUDA or vehicle; after 1 h astrocyte-conditioned media was transferred to neuronal cells and thereafter the neuronal cells were stimulated with H₂O₂ (1 mM) (Fig. 4). MTT assay was performed after 1 h. We found that astrocyte-conditioned media with AUDA treatment concentration-dependently prevented neuronal cells from H₂O₂-induced cells death compared to vehicle treated group (Fig. 4).

The effect of AUDA on the modulation of endogenous level of 14,15-EET

To prove that pretreatment of cells with different doses of AUDA may modulate the cell viability by increasing the endogenous level of 14,15-EET, groups of co-cultured cells were pretreated with different concentrations (8, 40, 80 or 800 nM) of AUDA or vehicle for 1 h before H₂O₂ (1 mM, for 1 h) (Fig. 5). In addition we also measured endogenous level of 14,15-EET in a separate cell co-culture (neurons and astrocytes) after stimulation of cells with different doses (8 or 80 nM, for 1 h) of AUDA (Fig. 6). The media from each group was collected and the endogenous level of all four regioisomeric EETs: 5,6-, 8,9-, 11,12-

and 14,15-EETs were measured by LC/MS technique. We found that in co-cultured cells after pretreatment of cells with AUDA increased the endogenous level of 14,15-EET (Fig. 5) but not 5,6-, 8,9- or 11,12-EETs (data not shown) compared to vehicle pretreatment group. Also we found that after AUDA treatment or in control groups endogenous level of 14,15-EET was much higher in astrocytes than in neurons as measured by LC/MS technique (Fig. 6).

The effect of AUDA on mitochondrial dysfunction in co-cultured cells

Reactive oxygen species, such as H_2O_2 , play an important role in neurodegenerative disease by causing oxidative damage and mitochondrial dysfunction (Bowling and Beal, 1995). In our experiments we pretreated cells with two different concentrations (8 and 800 nM) of AUDA or vehicle 1 h before H_2O_2 stimulation and measured the changes of mitochondrial membrane potential by the uptake of the 5,5',6,6'-tetrachloro1,1',3,3'-tetraethylbenzimidazolcarbocyanine iodide (JC-1) into the mitochondria 1 h thereafter. We found that the group of co-cultured cells pretreated with vehicle for 1 h before application of different concentrations (0.1 and 1) of H_2O_2 concentration-dependently depolarized the mitochondrial membrane (Fig. 7), however, pretreated cells with AUDA (80 or 800 nM, for 1 h) before H_2O_2 (1 mM, for 1 h) almost completely protected the cells against H_2O_2 -induced membrane depolarization (Fig. 7).

The effect of miconazole, a cytochrome P450 inhibitor, on H_2O_2 -induced cell death

Since increased endogenous level of 14,15-EET by inhibiting sEH enzyme prevents the cells from free radical damage, in following experiments we wanted to know if decreasing synthesis of endogenous level of 14,15-EET by CYP enzyme inhibitor miconazole will increase H_2O_2 -induced cell death. Group of co-cultured cells were pretreated with different concentrations (1, 10 or 20 μM) of miconazole for 1 h before H_2O_2 (1 mM) treatment and cell viability was measured 1 h thereafter (Fig. 8). We found that miconazole pretreatment dose dependently decreased cell viability compared to H_2O_2 treated group, while miconazole alone (20 μM , 2 h) had no effect on cell viability as compared to the control group (Fig. 8).

DISCUSSION

Oxidative stress has been considered as one of the major risk factors exacerbating neuronal damage in degenerative disorders of the central nervous system via different molecular pathways. (Chan, 2001; Jenner, 2003). Several components of ROS generated during neurodegeneration can cause damage to cardinal cellular components, such as lipids, proteins and DNA, initiating subsequent cell death via necrosis or apoptosis (Gorman et al., 1996). Based on these findings, there is increased interest in research for neuroprotective drugs of natural origin against ROS-induced neuronal death. Also recognition of the importance of astrocytes in neurovascular regulation is increasing, specifically regarding the modulation of neural activity. Numerous studies demonstrated that astrocytes play a significant role in neurodegenerative disorders (Salmina 2009) and exert a fundamental protective function against oxidative stress because of their effects on the metabolism of the antioxidant glutathione (GSH) and the defense against ROS (Schulz et al., 2000). Recent

attention has been focused on CYP epoxygenase-dependent metabolites of arachidonic acid – epoxyeicosatrienoic acids (EETs). It has been shown that main sources for synthesis of EETs in the brain are astrocytes (Alkayed et al., 1996, Iliff et al., 2010). In our study, we observed that the toxic effect of H₂O₂ was more prominent in dopaminergic neuronal cell lines than in astrocytes and this could be due to the release of EETs from astrocytes. To support these findings, we further attempted to study the neuroprotective effects of exogenously applied EETs against H₂O₂-induced dopaminergic neuronal cell damage. We found that only 14,15-EET but not 5,6-; 8,9- or 11,12-EETs (data not shown) protected dopaminergic cells against H₂O₂-induced cell damage. EETs have also been demonstrated to promote capillary angiogenesis and reduce platelet aggregation, inflammation, and hyperemia in the brain. In addition, inhibition of EETs formation in brain tissue attenuates the protection acquired after transient ischemic attacks, and inhibition of EETs breakdown by preventing their hydrolysis via the epoxide hydrolase is neuroprotective, which most likely occurs via the phosphoinositide 3-kinase (PI3-K)/AKT cell survival pathway (Alkayed et al., 2002). Furthermore, EETs exhibit direct cytoprotective properties, as evidenced by their ability to reduce ischemic cell death induced in primary cultured astrocytes by oxygen-glucose deprivation (OGD) (Liu and Alkayed, 2005).

Nevertheless, EETs cytoprotective effects are limited by their conversion to diol derivatives via the enzyme, soluble epoxide hydrolase (sEH). Epoxide hydrolases are localized in the cytosol (soluble form) as well as in microsomes and peroxisomes (Newman et al., 2005). Koerner et al. (2007) reported that sEH overexpression exacerbates primary cortical neuronal cell death by increasing the metabolism of EETs. EETs are preferred substrates for sEH, but 14,15-EET is the regioisomer that is hydrolyzed with the highest $V_{\max}$ and lowest K_m (Revermann, 2010). An alternative strategy that has been used to increase EETs systemically is sEH inhibition (Imig, 2006). Previously it has been shown that the sEH inhibitor 12-(3-adamantan-1-yl-ureido)-dodecanoic acid (AUDA) protects cells against cerebral ischemia in spontaneously hypertensive stroke-prone (SHRSP) rats (Dorrance et al., 2005). Interestingly, chronic AUDA treatment in SHRSP rats effectively decreased infarct size induced by middle cerebral artery occlusion without decreasing blood pressure (Dorrance et al., 2005). In our study we found that baseline-endogenous level of 14,15-EET is much higher in astrocytes than in neurons. In addition, after stimulation of cells with AUDA the increase in endogenous level of 14,15-EET was found to be much higher in astrocytes than in neurons. However, after AUDA treatment we were able to measure 14,15-EET but not other EETs. It is possible that in the time frame when we measured 14,15-EET, other EETs were already metabolized to DiHETE. Furthermore, recently our laboratory has shown that epoxygenase activity and production of EETs regioisomers is much higher in cultured neonatal hippocampal astrocytes than in neurons from the same region (Sarkar et al., 2011), which re-enforces the presently observed capacity of astrocytes as neuroprotective cell types.

Moreover, Zhang et al (2007) suggested that administration of AUDA-butyl ester protects cells against ischemia reperfusion injury in brains of normotensive mice by mechanisms that may involve neural protection rather than vascular protection (Zhang, 2007). In this study

we found that the sEH inhibition by AUDA provide protection against H₂O₂-induced cell death by increasing the half-life of endogenous 14,15-EET.

The loss of mitochondrial membrane potential has been identified as the first step in the apoptotic process (Zamzami et al., 1995). Our results were consistent with previous reports that ROS can disturb mitochondrial membrane potential (Mao et. al., 2007), however AUDA pretreatment showed protective effect against H₂O₂-induced depolarization of mitochondrial membrane potential. Therefore, we suggest that AUDA mediates its protective effect against H₂O₂ induced cell death through increasing the release of 14,15-EET from astrocytes, which in turn prevents the disruption of mitochondrial membrane potential.

It has been shown before that H₂O₂ inhibits P450 epoxygenases and this interaction modulates EET bioavailability (Larsen et al., 2008) and a nonspecific inhibitor of CYP enzyme-Miconazole blocks formation of EETs in the brain tissue (Alkayed et. al., 1996; Peng et. al., 2002). Based on these investigators' results, we tested the possibility that decreased endogenous level of 14,15-EET will aggravate H₂O₂-induced cell death. We found that miconazole pretreatment decreased the cell viability compared to vehicle pretreated group, while miconazole alone did not have any effect on cell viability compared to control group. This result supports our finding that EETs, especially 14,15-EET as an important neuroprotectant against free radical induced neuronal damage. However, the cellular mechanism of 14,15-EET neuroprotection is unclear and these observations make it essential to understand the role of 14,15-EET on cell protection against oxidative stress and future studies are in progress to address such issues.

As regards the actions of miconazole, this agent has pleiotropic actions- besides inhibiting P450 epoxygenases it also increases the endogenous level of free radicals (Kobayashi et al., 2002). At this point it is not clear if miconazole-reduced cell viability is through increased endogenous level of free radicals or P450 epoxygenase inhibition. An important limitation of the present study is the inability to verify whether redox potential was actually changed or cytochrome P450 was inhibited by miconazole in these cells and, therefore, the mechanism of miconazole remains to be investigated.

In summary, this study demonstrates that 14,15-EET released from astrocytes shows neuroprotective effects against H₂O₂-induced cell injury and it is essential to understand the mechanism of action of 14,15-EET on neuroprotection of dopaminergic neurons from oxidative stress, as they could become novel therapeutics for the treatment of neuronal pathologies such as Parkinson's disease.

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Abbreviations

14,15-EET	14,15-Epoxyeicosatrienoic Acid
AA	arachidonic acid
ROS	Reactive Oxygen Species
EETs	Epoxyeicosatrienoic Acids
H₂O₂	hydrogen peroxide
sEH	soluble epoxide hydrolase
AUDA	12-(3-adamantan-1-yl-ureido)-dodecanoic acid
CYP	Cytochrome P450 epoxygenases
PD	Parkinson's disease

References

- Alkayed NJ, Narayanan J, Gebremedhin D, Medhora M, Roman RJ, Harder DR. Molecular characterization of an arachidonic acid epoxygenase in rat brain astrocytes. *Stroke*. 1996; 27:971–979. [PubMed: 8623121]
- Alkayed NJ, Birks EK, Hudetz AG, Roman RJ, Henderson L, Harder DR. Inhibition of brain P-450 arachidonic acid epoxygenase decreases baseline cerebral blood flow. *Am J Physiol*. 1996a; 271:H1541–H1546. [PubMed: 8897950]
- Alkayed NJ, Goyagi T, Joh HD, Klaus J, Harder DR, Traystman RJ, Hurn PD. Neuroprotection and P450C11 upregulation after experimental transient ischemic attack. *Stroke*. 2002; 33:1677–1684. [PubMed: 12053011]
- Alkayed, NJ.; Goyagi, T.; Peng, X.; Harder, DR.; Hurn, PD.; Traystman, RJ.; Koehler, RC. Role of P450 arachidonic acid epoxygenase in ischemic preconditioning in brain. In: Kriegelstein, J., editor. *Pharmacology of Cerebral Ischemia*. Stuttgart, Germany: Medpharm Scientific Publishers; 2002. p. 303-310.
- Bowling AC, Beal MF. Bioenergetic and oxidative stress in neurodegenerative diseases. *Life Sci*. 1995; 56:1151–1171. [PubMed: 7475893]
- Chan PH. Reactive oxygen radicals in signaling and damage in the ischemic brain. *J Cereb Blood Flow Metab*. 2001; 21:2–14. [PubMed: 11149664]
- Chen P, Li A, Zhang M, He M, Chen Z, Wu X, Zhao C, Wang S, Liang L. Protective effects of a new metalloporphyrin on paraquat-induced oxidative stress and apoptosis in N27 cells. *Acta Biochim Biophys Sin*. 2008; 40:125–132. [PubMed: 18235974]
- Dorrance AM, Rupp N, Pollock DM, Newman JW, Hammock BD, Imig JD. An epoxide hydrolase inhibitor, 12-(3-adamantan-1-yl-ureido)dodecanoic acid (AUDA), reduces ischemic cerebral infarct size in stroke-prone spontaneously hypertensive rats. *J Cardiovasc Pharmacol*. 2005; 46:842–848. [PubMed: 16306811]
- Gorman AM, McGowan A, O'Neill C, et al. Oxidative stress and apoptosis in neurodegeneration. *J Neurol Sci*. 1996; 139:45–52. [PubMed: 8899658]
- Griendling KK, FitzGerald GA. Oxidative stress and cardiovascular injury: Part I: basic mechanisms and in vivo monitoring of ROS. *Circulation*. 2003; 108:1912–1916. [PubMed: 14568884]
- Gebremedhin D, Ma YH, Falck JR, Roman RJ, VanRollins M, Harder DR. Mechanism of action of cerebral epoxyeicosatrienoic acids on cerebral arterial smooth muscle. *Am J Physiol*. 1992; 263:H519–25. [PubMed: 1510149]
- Hewett JA. Determinants of regional and local diversity within the astroglial lineage of the normal central nervous system. *J Neurochem*. 2009; 110:1717–1736. [PubMed: 19627442]

- Iliff JJ, Jia J, Nelson J, Goyagi T, Klaus J, Alkayed NJ. Epoxyeicosanoid signaling in CNS function and disease. *Prostaglandins and Other Lipid Mediators*. 2010; 91:68–84. [PubMed: 19545642]
- Imig JD. Cardiovascular therapeutic aspects of soluble epoxide hydrolase inhibitors. *Cardiovasc Drug Rev*. 2006; 24:169–188. [PubMed: 16961727]
- Jenner P. Oxidative stress in Parkinson's disease. *Ann Neurol*. 2003; 53:S26–S36. [PubMed: 12666096]
- Kobayashi D, Kondo K, Uehara N, Otokozawa S, Tsuji N, Yagihashi A, Watanabe N. Endogenous Reactive Oxygen Species Is an Important Mediator of Miconazole Antifungal Effect. *Antimicrobial Agents and Chemotherapy*. 2002; 46(10):3113–3117. [PubMed: 12234832]
- Koehler RC, Gebremedhin D, Harder DR. Role of astrocytes in cerebrovascular regulation. *J Appl Physiol*. 2006; 100:307–317. [PubMed: 16357084]
- Koerner IP, Jacks R, DeBarber AE, Koop D, Mao P, Grant DF, Alkayed NJ. Polymorphisms in the human soluble epoxide hydrolase gene EPHX2 linked to neuronal survival after ischemic injury. *J Neurosci*. 2007; 27:4642–4649. [PubMed: 17460077]
- Larsen BT, Gutterman DD, Sato A, Toyama K, Campbell WB, Zeldin DC, Manthathi VL, Falck JR, Miura H. Hydrogen peroxide inhibits cytochrome p450 epoxygenases: interaction between two endothelium-derived hyperpolarizing factors. *Circ Res*. 2008; 102(1):59–67. [PubMed: 17975109]
- Li PL, Campbell WB. Epoxyeicosatrienoic acids activate K⁺ channels in coronary smooth muscle through a guanine nucleotide binding protein. *Circ Res*. 1997; 80:877–884. [PubMed: 9168791]
- Liu M, Alkayed NJ. Hypoxic preconditioning and tolerance via hypoxia inducible factor (HIF) 1alpha-linked induction of P450 2C11 epoxygenase in astrocytes. *J Cereb Blood Flow Metab*. 2005; 25:939–948. [PubMed: 15729289]
- Mao Y-R, Jiang L, Duan Y-L, et al. Efficacy of catalpol as protectant against oxidative stress and mitochondrial dysfunction on rotenone-induced toxicity in mice brain. *Environ Toxicol Pharmacol*. 2007; 23:314–318. [PubMed: 21783774]
- Mattson MP, Duan W, Chan SL, Cheng A, Haughey N, Gary DS, Gau Z, Lee J, Furukawa K. Neuroprotective and neurorestorative signal transduction mechanisms in brain aging: modification by genes, diet and behavior. *Neurobiol Aging*. 2002; 23:695–705. [PubMed: 12392775]
- Michaelis UR, Fisslthaler B, Barbosa-Sicard E, Falck JR, Fleming I, Busse R. Cytochrome P450 epoxygenases 2C8/9-derived are implicated in hypoxia-induced endothelial cell migration and angiogenesis. *J Cell Sci*. 2005; 118:5489–5498. [PubMed: 16291720]
- Newman JW, Morisseau C, Hammock BD. Epoxide hydrolases: their roles and interactions with lipid metabolism. *Prog Lipid Res*. 2005; 44:1–51. [PubMed: 15748653]
- Node K, Ruan XL, Dai J, Yang SX, Graham L, Zeldin DC, Liao JK. Activation of Gα_s mediates induction of tissue-type plasminogen activator gene transcription by epoxyeicosatrienoic acids. *J Biol Chem*. 2001; 276:15983–15989. [PubMed: 11279071]
- Peng J, Stevenson FF, Doctrow SR, Andersen JK. Superoxide dismutase/catalase mimetics are neuroprotective against selective paraquat-mediated dopaminergic neuron death in the substantia nigra. *J Biol Chem*. 2005; 280:29194–29198. [PubMed: 15946937]
- Peng X, Carhuapoma JR, Bhardwaj A, Alkayed NJ, Falck JR, Harder DR, Traystman RJ, Koehler RC. Suppression of cortical functional hyperemia to vibrissal stimulation in the rat by epoxygenase inhibitors. *Am J Physiol Heart Circ Physiol*. 2002; 283:H2029–H2037. [PubMed: 12384482]
- Revermann M. Pharmacological inhibition of the soluble epoxide hydrolase-from mouse to man. *Current Opinion in Pharmacology*. 2010; 10:173–178. [PubMed: 20079692]
- Rifkind AB, Lee C, Chang TK, Waxman DJ. Arachidonic acid metabolism by human cytochrome P450s 2C8, 2C9, 2E1, and 1A2: regioselective oxygenation and evidence for a role for CYP2C enzymes in arachidonic acid epoxyoxygenation in human liver microsomes. *Arch Biochem Biophys*. 1995; 320:380–389. [PubMed: 7625847]
- Salmina AB. Neuron-glia interactions as therapeutic targets in neurodegeneration. *J Alzheimers Dis*. 2009; 16:485–502. [PubMed: 19276541]
- Sarkar P, Narayanan J, Harder DR. Differential effect of amyloid beta on the cytochrome P450 epoxygenase activity in rat brain. *Neuroscience*. 2011; 194:241–9. [PubMed: 21843605]
- Sayre LM, Perry G, Smith MA. Oxidative stress and neurotoxicity. *Chem Res Toxicol*. 2008; 21:172–188. [PubMed: 18052107]

- Schulz JB, Lindenau J, Seyfried J, Dichgans J. Glutathione, oxidative stress and neurodegeneration. *Eur J Biochem.* 2000; 267:4904–4911. [PubMed: 10931172]
- Spector AA, Norris AW. Action of epoxyeicosatrienoic acids on cellular function. *Am J Physiol Cell Physiol.* 2007; 292:C996–C1012. [PubMed: 16987999]
- Spina MB, Hyman C, Squinto S, Lindsay RM. Brain-derived neurotrophic factor protects dopaminergic cells from 6-hydroxydopamine toxicity. *Ann NY Acad Sci.* 1992; 648:348–350. [PubMed: 1637069]
- Vernadakis A. Neuron-glia interrelations. *Int Rev Neurobiol.* 1988; 30:149–224. [PubMed: 3061968]
- Wang Z, Wei Y, Falck JR, Atcha KR, Wang WH. Arachidonic acid inhibits K channels in the cortical collecting duct via cytochrome P-450 epoxygenase-dependent metabolic pathways. *Am J Physiol Renal Physiol.* 2008; 294:F1441–F1447. [PubMed: 18417544]
- Zamzami N, Marchetti P, Castedo M, et al. Sequential reduction of mitochondrial transmembrane potential and generation of reactive oxygen species in early programmed cell death. *J Exp Med.* 1995; 182:367–377. [PubMed: 7629499]
- Zhang W, Koerner IP, Noppens R, Grafe M, Tsai HJ, Morisseau C, Luria A, Hammock BD, Falck JR, Alkayed NJ. Soluble epoxide hydrolase: a novel therapeutic target in stroke. *J Cereb Blood Flow Metab.* 2007; 27:1931–1940. [PubMed: 17440491]

Highlights

- 14,15-EET protects dopaminergic neurons from H₂O₂-induced cell death.
- Co-culture of these neurons with astrocytes and astrocyte-conditioned media also showed this protective effect.
- Increasing bioavailability of endogenous EETs confers cytoprotection.
- Blocking astrocytic EETs production obliterates the protective effect.

Figure 1.

Toxic effect of H₂O₂ on dopaminergic neurons (A), Astrocytes (B). Groups of cells were treated with 0.1 or 1 mM of H₂O₂ or vehicle for 1h. The cell viability was measured by MTT assay. After stimulation of cells with the same doses of H₂O₂ the cell viability in astrocytes (A) was much higher than in dopaminergic neurons (B). Data are represented as mean $\pm$ SEM, n = 5–6 groups/each experiment. One-way ANOVA followed by Dunnett's post-test was used to determine the difference between groups. * P < 0.05, ** P < 0.01 compared to control groups.

Figure 2AB.

Effect of pretreatment with different doses of 14,15-EET against H₂O₂-produced cell death (A) and the chemical structure of 14,15-EET (B). Groups of cells were pretreated with different doses of 14,15-EET (0.1, 1, 10 and 30 M) or vehicle 1h before H₂O₂ (1 mM) treatment and the cell viability was measured 1h thereafter by MTT assay. Pretreatment of 14,15-EET increased the cell viability compared to vehicle-pretreated group. Data are represented as mean ± SEM, n=5–6 groups/each experiment. One-way ANOVA followed by Dunnett's post-test was used to determine the difference between groups. #*P* < 0.001 compare to control group, **P* < 0.001 compared to vehicle-H₂O₂ group.

Figure 3.

Effect of pretreatment with different doses of AUDA against H₂O₂-induced cell death. Groups of cells were pretreated with different doses of AUDA (8, 40, 80 and 800 nM) or vehicle 1h before H₂O₂ (1 mM) treatment and the cell viability was measured 1h thereafter by MTT assay. Pretreatment of AUDA increased the cell viability compared to vehicle-pretreated group. Data are represented as mean $\pm$ SEM, n=5–6 groups/each experiment. One-way ANOVA followed by Dunnett's post-test was used to determine the difference between groups. * $P < 0.001$ compared to vehicle-H₂O₂ group.

Figure 4.

The effect of astrocyte-conditioned media on H₂O₂-induced neuronal cell death. The groups of astrocytes were stimulated with different doses (8, 80 or 800 nM) of AUDA or vehicle; after 1 h astrocyte-conditioned media was transferred to neuronal cells and thereafter the neuronal cells were stimulated with H₂O₂ (1 mM). MTT assay was performed after 1 h of H₂O₂ stimulation.

Data are represented as mean $\pm$ SEM, n=5–6 groups/each experiment. Student's *t*-test and One-way ANOVA followed by Dunnett's post-test was used to determine the difference between groups. #*P* < 0.01 compared to control group; **P* < 0.001 compared to vehicle-H₂O₂ treated group.

Figure 5AB.

Effect of pretreatment with different doses of AUDA on endogenous level of 14,15-EET (**A**) and the representative LC/MS chromatograms of each AUDA treated groups (**B**) in co-culture cells. Groups of co-cultured cells were pretreated with different doses of AUDA (8, 40, 80 and 800 nM) or vehicle 1h before H₂O₂ (1 mM) treatment and the endogenous level was measured 1h thereafter. Pretreatment of AUDA before H₂O₂ (1 mM, 1h) treatment increased the endogenous level of 14,15-EET compared to vehicle-pretreated group measured by LC/MS technique. Data are represented as mean $\pm$ SEM, n=5 groups/each experiment (A). One-way ANOVA followed by Dunnett's post-test was used to determine the difference between groups. * $P < 0.01$ compared to vehicle-H₂O₂ treated group.

Figure 6.

Effect of pretreatment with different doses of AUDA on endogenous level of 14,15-EET in neurons and astrocytes. Groups of neuronal cells and astrocytes were pretreated with different doses of AUDA (8 and 800 nM) 1h before H₂O₂ (1 mM) treatment and the endogenous level was measured 1h thereafter. Pretreatment of AUDA before H₂O₂ (1 mM, 1h) treatment increased the endogenous level of 14,15-EET compared to vehicle-pretreated group measured by LC/MS technique. Data are represented as mean $\pm$ SEM, n=5 groups/each experiment. One-way ANOVA followed by Dunnett's post-test was used to determine the difference between groups. * P < 0.01 compared to control-H₂O₂ treated group, # P < 0.001 compared to control-H₂O₂ treated group and ♣ P < 0.001 compared to control-H₂O₂ treated neuronal group.

Figure 7.

Effect of pretreatment with different doses of AUDA on the mitochondrial membrane potential. Groups of cells were pretreated with different doses of AUDA (80 and 800 nM) or vehicle 1h before H₂O₂ (1 mM) treatment and mitochondrial membrane potential was measured 1h thereafter by JC-1 assay. Different doses (0.1 and 1) of H₂O₂ treatment for 1h after vehicle (1h) pretreatment dose-dependently decreased the mitochondrial membrane potential while, pretreatment with different doses of AUDA 1 h before H₂O₂ (1 mM, 1h) treatment increased the mitochondrial membrane potential compared to vehicle-pretreated group measured by JC-1 assay. Data are represented as mean $\pm$ SEM, n=4 groups/each experiment. One-way ANOVA followed by Dunnett's post-test was used to determine the difference between groups. * P < 0.01, ** P < 0.001 compared to control group; # P < 0.01 compared to vehicle-H₂O₂ (1 mM) group.

Figure 8.

Effect of pretreatment with different doses of Miconazole against H_2O_2 -produced cell death. Groups of cells were pretreated with different doses of Miconazole (1, 10 and 20 M) or vehicle 1h before H_2O_2 (1 mM) treatment and the cell viability was measured 1h thereafter by MTT assay. Pretreatment of Miconazole decreased the cell viability compared to vehicle-pretreated group. However, high dose of Miconazole alone for 2 h did not change the cell viability compared to control. Data are represented as mean $\pm$ SEM, $n=5-6$ groups/each experiment. One-way ANOVA followed by Dunnett's post-test and student's t -test was used to determine the difference between groups. # $P < 0.01$ compared to control; * $P < 0.001$ compared to vehicle- H_2O_2 group.