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Upregulation of 20-HETE synthetic cytochrome P450 isoforms by oxygen-glucose deprivation in cortical neurons

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Abstract

20-Hydroxyeicosatetraenoic acid (20-HETE), a potent vasoconstrictor, is a cytochrome P450 (CYP) 4A/4F-derived metabolite of arachidonic acid. Inhibition of 20-HETE synthesis protects brain from ischemic injury. However, that protection is not associated with changes in cerebral blood flow. The present study examined whether CYP4A isoforms are expressed in neurons, whether they produce 20-HETE in neurons, and whether neuronally derived 20-HETE exerts direct neurotoxicity after oxygen-glucose deprivation (OGD). The expression of Cyp4a10 and Cyp4a12a mRNA in cultured mouse cortical neurons increased significantly at 1 and 3 h after exposure to 1 h of OGD. Reoxygenation also markedly augmented the expression of CYP4A protein in neurons and increased 20-HETE levels in the culture medium. Cell viability after OGD increased after treatment with a 20-HETE synthesis inhibitor or an antagonist. That effect was reversed by co-administration of a 20-HETE agonist. These results indicate that neurons express Cyp4a10 and 4a12a, that expression of these isoforms is upregulated by OGD stress, and that neuronally derived 20-HETE directly contributes to neuronal death after reoxygenation.

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Contributions

H.Z., R.J.R., and Z.-J.Y. performed the experiments. R.C.K. and Z.-J.Y. conceived and designed the experiments and analyzed the data. J.R.F. provided 20-HETE agonist and antagonist. J.R.F., R.J.R., and D.R. H. provided oversight for the project. R.C.K. and Z.-J.Y. compiled the manuscript and figure preparation.

Conflict of interest

The authors declare that they have no conflicts of interest.

Keywords

20-HETE; cytochrome P450; eicosanoid; ischemia; oxygen-glucose deprivation

Introduction

Some cytochrome P450 (CYP) 4A and 4F isoforms catalyze the metabolism of arachidonic acid to produce 20-hydroxyeicosatetraenoic acid (20-HETE) (Williams et al. 2010). Cyp4a10, 4a12, and 4a14 catalyze ω -hydroxylation of fatty acids in mice, but only Cyp4a12 can catalyze the formation of 20-HETE from arachidonic acid (Muller et al. 2007). Currently two Cyp4a12 genes, Cyp4a12a and Cyp4a12b, have been identified (Nelson et al. 2004). Although CYP4A11, CYP4F2, and CYP4F3 are the predominant isoforms responsible for producing 20-HETE in most human tissues (Kroetz and Xu 2005; Hoopes et al. 2015; Edson and Rettie 2013; Wu et al. 2014), it remains to be determined whether the homologous CYP4F isoforms (4f13, 4f14, 4f15, 4f16, and 4f18) in mice produce 20-HETE (Stec et al. 2003). Thus, most work on 20-HETE synthesis has focused on CYP4A isoforms.

The CYP4A subfamily has been well studied in vascular smooth muscle, including cerebral arteries, where 20-HETE increases myogenic tone (Gebremedhin et al. 2000). Interestingly, inhibitors of 20-HETE synthesis have been reported to protect brains from focal (Omura et al. 2006; Poloyac et al. 2006) and global cerebral ischemia (Yang et al. 2012). However, the neuroprotective effects are not always attributable to changes in cerebral blood flow or pial artery diameter (Renic et al. 2009; Cao et al. 2009). In our effort to understand the effects of 20-HETE in neonatal hypoxic-ischemic (H-I) brains (Yang et al. 2012), we have found that post-ischemic administration of the 20-HETE synthesis inhibitor HET0016 reduces ischemic injury in piglet brains without altering post-ischemic cerebral blood flow. These results suggest that 20-HETE may directly promote neuronal damage after ischemia. Further evidence for direct effects of 20-HETE on brain parenchyma comes from work showing that an inhibitor of 20-HETE synthesis protects organotypic hippocampal slice cultures from oxygen-glucose deprivation (OGD) (Renic et al. 2012). However, no studies have identified the cellular sources of 20-HETE in ischemic brain tissues.

Immunohistochemical results have indicated that neurons express CYP4A protein early after brain ischemia or OGD. Granule and pyramidal cells in hippocampal slice culture (Renic et al. 2012) and cortical and striatal neurons in rat and piglet brain (Omura et al. 2006; Yang et al. 2012; Kawasaki et al. 2012) all exhibit positive CYP4A immuno-signals. Although 20-HETE levels in the brain parenchyma increase early after brain ischemia (Tanaka et al. 2007), neuronal CYP4A immuno-signals do not confirm that neurons are the source of 20-HETE production because only mouse Cyp4a12 can metabolize arachidonic acid into 20-HETE and CYP4A antibody cannot distinguish different CYP4A isoforms. In addition, glial cells could release 20-HETE under certain conditions. A recent study revealed that cultured rat astrocytes express Cyp4A protein and produce 20-HETE after mGluR agonist RS-3,5-dihydroxyphenylglycine treatment (Gebremedhin et al. 2016). Others have reported that microglia express CYP4A and may serve as the main cellular source of 20-HETE in rat brains 7 days after transient middle cerebral artery occlusion (Kawasaki et al. 2012).

Therefore, in the present study, we examined (1) which CYP4A isoforms are expressed in primary cultures of mouse cortical neurons; (2) whether CYP4A isoform expression is upregulated after OGD, and (3) whether synthesized 20-HETE can contribute to neuronal death after OGD.

Materials and Methods

Reagents

The ω -hydroxylase inhibitor *N*-hydroxy-*N'*-(4-*n*-butyl-2-methylphenyl)formamidine (HET0016) was purchased from Enzo (Farmingdale, NY). The 20-HETE stable agonist *N*-(20-hydroxyeicosa-5[Z],14[Z]-dienoyl)glycine (20-5,14-HEDGE) and the 20-HETE stable antagonist *N*-(20-hydroxyeicosa-6[Z],15[Z]-dienoyl)glycine (20-6,15-HEDGE) were synthesized in the laboratory of J.R. Falck (Regner et al. 2009).

Immunohistochemistry

The animal protocol was approved by the Animal Care and Use Committee of the Johns Hopkins University. In brief, anesthetized C57Bl/6 mice (7 days old, Charles River, Frederick, MD) were perfused with phosphate-buffered saline (PBS) and 4% paraformaldehyde. Brains were collected and cut into 40- μ m-thick frozen sections. The sections were blocked with 10% normal goat serum and incubated with rabbit anti-cytochrome P450 4A (CYP4A) antibody (1:300; ab3573, Abcam, Cambridge, MA) and mouse anti-NeuN (1:500, MAB377, EMD Millipore, Billerica, MA) overnight at 4°C, followed by 1 h incubation with anti-IgG antibodies conjugated with Alexa Fluor 488 or 594 (1:1000, Life Technologies, Grand Island, NY).

Cortical Neuron Culture

Culture conditions for cortical neurons were modified from the method described previously (Lesuisse and Martin 2002). In brief, neonatal pups were harvested on embryonic days 15-17 from anesthetized pregnant C57Bl/6 mice (Charles River). The cerebral cortex was collected, and cerebrocortical cells were isolated and plated (1000 cells/mm²) with Neurobasal medium supplemented with B27, glutamine, and streptomycin/penicillin (Life Technologies) onto poly-D-lysine (Sigma-Aldrich, St. Louis, MO)-coated 10-cm tissue culture dishes for CYP4A or 20-HETE measurement. Cells were also plated at a lower density (670 cells/mm²) onto poly-D-lysine-coated 96-well culture plates for neuronal cell viability experiments. Growth of glial cells was suppressed with 1 μ M cytarabine (Sigma-Aldrich), yielding cultured cells with approximately 90% neurons, as determined by NeuN and GFAP staining. Cells at 7-9 days *in vitro* were used for OGD or pharmacologic treatment.

OGD

OGD of mouse cortical neuron cultures was modified from methods described previously (Plesnila et al. 2001). Briefly, neurons were incubated with glucose-free Neurobasal medium with B27 minus AO (Life Technologies) in a chamber filled with 5% CO₂ and 95% N₂ at 37°C for 0.5, 1, or 2 h. Control cells were incubated in normal culture medium in a normoxic incubator for the same time period. OGD was terminated by switching back to

normal culture conditions with glucose. Cell viability was assessed 24 h after OGD by double staining with Hoechst 33342 (7 μ M, Life Technologies) and propidium iodide (PI; 2 μ M, Life Technologies), or at various times after OGD with the CellTiter Blue (CTB) cell viability assay (Promega, Madison, WI). We measured the effects of the 20-HETE synthesis inhibitor (HET0016; 1, 10, or 100 μ M), 20-HETE agonist (20-5, 14-HEDGE; 10 μ M), and 20-HETE antagonist (20-6,15-HEDGE; 1, 10 or 50 μ M) on cell survival by adding the compound or vehicle (0.1% DMSO) to the cell culture medium 15 min before and 1 h during exposure to OGD. Exposure was followed by a 24-h recovery period. The percent of dead cells was calculated from the ratio of the number of PI-positive-nuclei to the number of Hoescht 33342-positive nuclei in three fields per well and three to four wells per experiment. For CTB measurement, the value was averaged from fluorescent signals (using excitation and emission wavelengths of 560 nm and 590 nm, respectively) of seven wells per group. At least three independent experiments were performed for each intervention.

Immunocytochemistry

Paraformaldehyde-fixed cells (on glass cover slips) were washed with phosphate-buffered saline (PBS), permeabilized with 0.1% Triton X-100 (Sigma-Aldrich), blocked for 30 min with 10% normal goat serum in PBS, and incubated with rabbit anti-CYP4A antibody and mouse anti-NeuN antibody overnight at 4°C, followed by 1 h incubation with anti-rabbit or anti-mouse IgG antibodies conjugated with Alexa Fluor 488 or 594.

Quantitative Reverse Transcriptase-PCR (qPCR)

Total RNA was extracted from cultured rat cortical neurons with an RNeasy Plus Mini Kit (Qiagen, Germany). RNA extracted from mouse kidney tissue was used as a positive control (Roman 2002; Zhang and Klaassen 2013). Complementary DNA was synthesized from 1 μ g of total RNA with a high-capacity RNA-to-cDNA kit (Life Technologies). The qPCR for total complementary DNA was performed in triplicate with power SYBR green PCR master mix (Life Technologies) in an ABI 7500 fast real-time PCR system (Applied Biosystems, Foster City, CA) according to standard protocols. Three independent experiments were used to analyze the expression of the target genes. We quantified the relative expression of CYP4a10, 4a12a, 4a12b, and 4a14 mRNA by quantification cycle (Cq) values using the 2^{-Cq} method relative to the expression of glyceraldehyde-3-phosphate dehydrogenase mRNA. Sequences of the forward (F) and reverse (R) primers used for real-time PCR were as follows (Zhang and Klaassen 2013): mouse CYP4a10 (Accession No. NM010011): F: 5'-TCCAGGTTTGCACCAGACTCT-3', R: 5'-TCCTGGCTCCTCCTGAGAAG-3'; mouse CYP4a12a (Accession No. NM177406): F: 5'-GCCTTATACGGAAATCATGGC-3', R: 5'-TGGAATCCTGGCCAACAATC G-3'; mouse CYP4a12b (Accession No. NM172306): F: 5'-CCTTCTACGGAAATCATGGCAGA-3', R: 5'-TGGAATCCTGGCCAACAATC-3'; mouse CYP4a14 (Accession No. NM007822): F: 5'-CAAGACCCTCCAGCATTTC-3', R: 5'-GAGCTCCTTGTCCTTCAGATGGT-3'; and mouse glyceraldehyde 3-phosphate dehydrogenase (Accession No. NM001001303): F: 5'-TGTGTCCGTCGTGGATCTGA-3', R: 5'-CCTGCTTCACCACCTTCTTGA-3'.

Western Blot Analysis

Western blot was performed as described previously (Yang et al. 2012). In brief, 20 µg of cell lysate protein (n = 4/group) was separated by polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane. The membranes were probed with antibodies against CYP4A (1:2000) and β-actin (1:5000, sc-47778, Santa Cruz Biotechnology, Inc., Dallas, TX) at 4°C overnight. Protein bands were visualized with the ECL kit (GE Healthcare, UK) and analyzed with NIH ImageJ software (V1.40).

20-HETE Measurement

Culture medium of OGD cells was collected at 1 and 3 h after OGD. Culture medium of control cells was collected at 3 h after 1-h control-operated duration. The medium was acidified to pH 3.5 and extracted with 3 volumes of ethyl acetate after the addition of 2 ng of 20-HETE-d6 internal standard. The organic phase was collected and dried under nitrogen. The samples were reconstituted in methanol, and 20-HETE was measured in an ABI-Sciex4000Q-Trap triple quad liquid chromatography/mass spectrometry/mass spectrometer as described previously (Williams et al. 2007).

Reactive Oxygen Species (ROS) Measurement

We assessed cellular ROS generation with the DCFDA (2',7'-dichlorofluorescein diacetate) cellular ROS detection assay kit (Abcam) according to the manufacturer's instructions. The cells were loaded with DCFDA at 37°C for 30 min and then underwent OGD in the presence and absence of HET0016, or treatment with 20-S,14-HEDGE. The fluorescence of the cells was measured with a microplate reader (SpectraMax M2, Molecular Devices, Sunnyvale, CA) at excitation and emission wavelengths of 485 and 535 nm. We included seven wells per group and completed at least three independent experiments for each intervention.

Statistical Analysis

All data are expressed as mean ± standard deviation and analyzed by one-way ANOVA followed by the Student-Newman-Keuls multiple range test. A value of $p < 0.05$ was considered statistically significant.

Results

CYP4A immuno-signals were widely distributed in neurons from cerebral cortex of 7-day-old mice (Fig. 1). This finding is consistent with previous reports in newborn piglets (Yang et al. 2012), adult rats (Kawasaki et al. 2012), and cultured rat hippocampal slices (Renic et al. 2012). Moreover, expression of CYP4A protein was preserved in cultured cortical neurons (Fig. 1).

We first examined the effects of different OGD durations on cell viability. As expected, longer OGD duration produced more severe neuronal injury (Fig. 2a). At 24 h after 0.5, 1, and 2 h of OGD, neuronal viability was >90%, >60%, and <20%, respectively. Most of the neuronal injury occurred after 6 h after OGD (Fig. 2b). Cell death assessed by PI/Hoechst 33342 staining mirrored the results obtained with the CTB viability assay after 1-h OGD (Fig. 2c). Thus, we used a 1-h period of OGD in subsequent experiments because it was not

so severe to allow for detection of upregulation of certain genes and enabled us to identify the potential neuroprotective effects of HET0016.

Cyp4a10, 4a12a, 4a12b, and 4a14 mRNA were all expressed in mouse kidney tissue. However, only Cyp4a10 and 4a12a mRNA were detected in mouse cortical neurons (Fig. 3a). Even after the stress of OGD, Cyp4a12b and 4a14 mRNA remained undetectable. In contrast, the expression of Cyp4a10 and 4a12a mRNA increased at 1 and 3 h of reoxygenation after OGD.

The expression of CYP4A protein also increased significantly 6 h after OGD (Fig. 3b). Moreover, the concentration of 20-HETE that was released into the culture medium increased at 3 h after OGD (Fig. 3c).

HET0016 at 10 μ M attenuated neuronal death 24 h after 1-h OGD (Fig. 4a). The neuroprotective effect of HET0016 was reversed by 10 μ M of the 20-HETE agonist, 20-5,14-HEDGE. On the other hand, addition of the 20-HETE antagonist 20-6,15-HEDGE (50 μ M) afforded significant neuroprotection after OGD (Fig. 4b). In addition, HET0016 reduced OGD-induced ROS production after 1-h OGD and 3 h of reoxygenation (Fig. 5a). In contrast, addition of 20-5,14-HEDGE alone in cultured neurons. (Fig. 5b).

Discussion

In our study, (1) primary cultures of mouse cortical neurons expressed mRNA for Cyp4a10 and Cyp4a12a isoforms, which are known to metabolize arachidonic acid to 20-HETE; (2) the expression of both of these isoforms increased significantly after OGD; (3) the production of ROS in cultured neurons increased markedly after OGD, but the increase was attenuated by a 20-HETE synthesis inhibitor; and (4) inhibition of 20-HETE formation with a synthesis inhibitor or application of a 20-HETE antagonist attenuated neuronal injury after OGD and reoxygenation, but this protection could be reversed by administration of a 20-HETE agonist.

The expression of multiple CYP4A isoforms in neurons raises the possibility that they may serve different functions in different cell types in the brain or in different subpopulations of neurons. Because more than 90% of the cultured cells in our study were neurons, it is unlikely that the production of 20-HETE arose from contaminating glia or other cell types.

The 20-HETE levels in the medium increased at 3 h after neuronal exposure to OGD and reoxygenation. This increase is likely the result of increased arachidonic acid availability through the action of various lipases. In addition, the induction of the Cyp4a12a isoform, which is the primary enzyme responsible for formation of 20-HETE in the mouse, may contribute to the increase in 20-HETE production at this time. Previous studies have indicated that 20-HETE promotes increases in oxidative stress in ischemic brains (Zhu et al. 2015; Yang et al. 2012). Thus, the large increase in 20-HETE production at 3 h likely contributes to the ongoing neuronal cell death from that point forward.

The ω -hydroxylase inhibitor HET0016 attenuated neuronal cell death after OGD. Although substrates other than arachidonic acid can undergo ω -hydroxylation by these CYPs, reversal

of the protective effect of HET0016 by 20-5,14-HEDGE suggests that 20-HETE is the primary metabolite responsible for neuronal injury. On the other hand, ROS is an important cause of cell death. Studies have indicated that many free radical scavengers attenuate cell death after OGD (Zhang et al. 2008; Song et al. 2006; Wu et al. 2006). Direct 20-5,14-HEDGE-induced ROS generation and HET0016-attenuated ROS production after OGD in cultured neurons, and the similar neuroprotection provided by HET0016 and 20-HETE antagonist 20-6,15-HEDGE support the view that 20-HETE contributes to the adverse effects after OGD. Comparable neuroprotective effects of HET0016 and other 20-HETE analogs have been reported in rat hippocampal slices after OGD (Renic et al. 2012) and *in vivo* after ischemic stroke (Renic et al. 2009). These findings further support the view that 20-HETE contributes to neuronal cell death after ischemic stress. Collectively, our findings indicate that upregulation of Cyp4a12a in injured neurons contributes to the increased production of 20-HETE after OGD and that blockade of the synthesis or actions of 20-HETE has a protective effect against OGD-induced neuronal injury. These findings further indicate that endogenous 20-HETE production within neurons amplifies ischemic-like neuronal cell death by, at least in part, increasing oxidative stress.

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Fig. 1. Double-immunofluorescence staining indicates that CYP4A (red) and NeuN (green), a neuronal marker, are colocalized in the cerebral cortex of 7-day-old mice (a) and in cultured mouse cortical neurons (b). DAPI is used to display nuclei of cultured cells. Scale bar = 40 μm

Fig. 2.

CellTiter Blue (CTB) assay results indicate reduced neuronal viability 24 h after exposure to 0.5-, 1-, and 2-h OGD (a) and at various times after 1-h OGD (b). (c) Propidium iodide/Hoechst 33342 staining was used to determine the percentage of injured neurons 24 h after exposure to 1-h OGD. The percent of viable neurons was similar to that obtained with the CTB assay 24 h after 1-h OGD in panel b. Data are expressed as mean $\pm$ standard deviation and analyzed by one-way ANOVA followed by the Student-Newman-Keuls multiple range test. * $p < 0.05$ vs. control. Scale bar = 250 μ m. The percent of dead cells was calculated from the ratio of the number of PI-positive nuclei to Hoescht 33342-positive nuclei in three fields per well and three or four wells per experiment. Values represent the mean of fluorescent signals (Ex/Em 560 nm/590 nm) of seven wells per group. At least three independent experiments were performed for each intervention.

Fig. 3.

Expression of CYP4A and 20-HETE after OGD. (a) Mouse CYP4A mRNA expression in control and OGD neurons; M, base pair (bp) marker; K, kidney; N, neuron; ND, no detection. (b) Western blot analysis comparing the expression of CYP4A protein at various times after exposure to 1-h OGD. (c) Liquid chromatography/mass spectrometry analysis showed that the concentration of 20-HETE increased significantly in the culture medium 3 h after OGD. $n = 3-4$ per group. Data are expressed as mean $\pm$ standard deviation and analyzed by one-way ANOVA followed by the Student-Newman-Keuls multiple range test. $*p < 0.05$ vs. control.

Fig. 4.

20-HETE exacerbates neuronal injury from OGD. (a) HET0016 dose-dependently protected neurons from OGD injury, but this protection was attenuated by administration of the 20-HETE agonist, 20-5,14-HEDGE. (b) At a concentration of 50 μ M, the 20-HETE antagonist 20-6,15-HEDGE protected neurons from OGD. Data are expressed as mean $\pm$ standard deviation and analyzed by one-way ANOVA followed by the Student-Newman-Keuls multiple range test. * $p < 0.05$ vs. control; # $P < 0.05$ vs. OGD+vehicle. At least three independent experiments were performed for each intervention

Fig. 5.

20-HETE exacerbates ROS production after OGD. (a) HET0016 attenuated OGD-induced ROS generation in cultured neurons 3 h after exposure to 1-h OGD. (b) ROS production increased 3 h after administration of the 20-HETE agonist 20-5,14-HEDGE (10 μ M) to cultured neurons. Data are expressed as mean $\pm$ standard deviation and analyzed by one-way ANOVA followed by the Student-Newman-Keuls multiple range test. * p < 0.05 vs. control; # p < 0.05 vs. vehicle (Veh). The value was averaged from fluorescent signals (Ex/Em 485 nm/535 nm) of seven wells per group. At least three independent experiments were performed for each intervention. DCFDA, 2',7'-dichlorofluorescein diacetate