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Inhibition of 12/15-lipoxygenase as therapeutic strategy to treat stroke

Kazim Yigitkanli, MD¹, Anton Pekcec, DVM¹, Hulya Karatas, MD, PhD¹, Stefanie Pallast, PhD¹, Emiri Mandeville, MD¹, Netra Joshi², Natalya Smirnova, PhD³, Irina Gazaryan, PhD³, Rajiv R. Ratan, MD, PhD³, Joseph L. Witztum, MD⁴, Joan Montaner⁵, Theodore R. Holman, PhD², Eng H. Lo, PhD¹, and Klaus van Leyen, PhD¹

¹Neuroprotection Research Laboratory, Dept. of Radiology, Massachusetts General Hospital, Harvard Medical School, Charlestown, MA

²Department of Chemistry and Biochemistry, University of California, Santa Cruz, CA

³The Burke Medical Research Institute, Department of Neurology and Neuroscience, Weill Medical College of Cornell University, White Plains, NY

⁴Department of Medicine, University of California, San Diego, La Jolla, CA

⁵Neurovascular Research Laboratory, Vall d'Hebron University Hospital Research Institute, Barcelona, Spain

Abstract

Targeting newly identified damage pathways in the ischemic brain can help to circumvent the currently severe limitations of acute stroke therapy. Here we show that the activity of 12/15-lipoxygenase was increased in the ischemic mouse brain, and 12/15-lipoxygenase co-localized with a marker for oxidized lipids MDA2. This co-localization was also detected in the brain of two human stroke patients, where it also coincided with increased apoptosis-inducing factor, AIF. A novel inhibitor of 12/15-lipoxygenase, LOXBlock-1 protected neuronal HT22 cells against oxidative stress. In a mouse model of transient focal ischemia, the inhibitor reduced infarct sizes both 24 hours and 14 days post stroke, with improved behavioral parameters. Even when treatment was delayed until at least four hours after onset of ischemia, LOXBlock-1 was protective. Furthermore, it reduced tPA-associated hemorrhage in a clot model of ischemia/reperfusion. This study establishes inhibition of 12/15-lipoxygenase as a viable strategy for first line stroke treatment.

Introduction

Acute stroke drug therapy in the United States currently remains limited to tissue plasminogen activator (tPA), a protease with significant side effects including increased bleeding. Because of the need to rule out a hemorrhagic stroke, CT or MR imaging is required before tPA can be administered, narrowing the time window for tPA use. Currently only 1–5% of eligible patients are treated^{1, 2}.

Following experimental stroke, several oxidative stress-related pathways are activated, which contribute to enhanced ischemia/reperfusion injury^{3–6}. 12/15-lipoxygenase (12/15-LOX) is increased in both neurons and endothelial cells in the peri-infarct area^{7, 8}, contributing to delayed cell death in the penumbra, weakening of the blood – brain barrier,

*Corresponding Author: Klaus van Leyen, Ph. D., Massachusetts General Hospital, Neuroprotection Research Laboratory, 149 13th St., R. 2401, Charlestown, MA 02129, Tel.: (617) 724-6556, FAX: (617) 726-7830, klaus_vanleyen@hms.harvard.edu.

and edema formation. Previous studies using lipoxygenase inhibitors have demonstrated infarct size reduction in mice and rats^{9–14}, and improved behavioral deficits in rabbits¹⁵.

We have recently introduced novel lipoxygenase inhibitors, which were able to protect cultured neuronal and oligodendrocytic cells against oxidative stress¹⁶. In this study, we investigated 12/15-LOX activity in the ischemic brain, and we tested the inhibitor LOXBlock-1 in mouse models of cerebral ischemia and hemorrhage for its potential as acute phase therapy for ischemic stroke.

Materials and Methods

Detailed Materials and Methods are deposited in an online supplementary file. All animal experiments were performed following protocols approved by the Massachusetts General Hospital Institutional Animal Care and Use Committee in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Both the surgeon carrying out the operations, and the investigators evaluating data, were blinded as to treatment groups.

Oxidative glutamate toxicity in HT22 Cells

Glutathione depletion was induced in HT22 cells by glutamate treatment, and lactate dehydrogenase (LDH) release into the medium was measured to detect cell death as described¹⁶. Alternatively, 12-hydroxyeicosatetraenoic acid (12-HETE) was detected using a 12-HETE EIA kit (Assay Designs).

Transcription Factor Activation Assays

Cell-based reporter assays were used to measure activation of NRF2 and HIF1 α pathways^{17, 18}. Detailed descriptions given in Supplementary Methods file.

Human brain tissue samples

Patient 1 was a 59 year old male with a history of hypertension and diabetes mellitus type II. He suffered an acute ischemic stroke due to severe right carotid stenosis (atherothrombotic stroke)¹⁹. CT scan showed an infarct in the territory of the right Middle Cerebral Artery (MCA). The patient did not receive tPA and died 87 hours after stroke onset. It took 4 h from death until necropsy. On autopsy and during macroscopic examination, morphological features and last available neuroimages were used to guide brain tissue sampling from ischemic ipsilateral, or from contralateral hemisphere. Infarcted area was delineated by an experienced neuropathologist (mainly through the consistence and colour of the parenchyma) and 1 cm³ of the contiguous tissue was obtained as peri-infarct, which was confirmed by histopathological microscopic examination. Histology showed severe ischemic necrosis in the whole MCA territory, with extensive neuronal necrosis and no signs of neurodegenerative disease. The second patient was a 77 year old female with probable atrial fibrillation, presenting with ischemic stroke affecting the left MCA. This patient developed hemorrhagic transformation following thrombolytic treatment, and died 77 hours after stroke onset. Samples for immunohistochemistry were immediately fixed with 4% paraformaldehyde and kept at –80°C until use. This study was approved by the Ethics Committee of the Hospital Vall d'Hebron [PR(HG)85/04]. Informed consent was acquired from relatives prior to the autopsy.

Focal Cerebral Ischemia Model in Mice

The standard intraluminal middle cerebral artery occlusion method was used to induce transient focal cerebral ischemia in CD-1 mice⁸. 12 week old males were subjected to either 60 or 90 minutes of ischemia, followed by varying times of reperfusion. In all in vivo

experiments, LOXBlock-1 (50 mg/kg) was given by i.p. administration in a single dose, and investigators were blinded to the treatment groups. For the time of administration study, the procedure was slightly modified. To reduce variability, the common carotid artery was closed with 4-0 nylon suture during ischemia, which leads to more severe ischemia. After the filament was removed, the common carotid artery suture was also released to achieve reperfusion. Brain infarct sizes were assessed by staining with either 2,3,5-triphenyltetrazolium hydrochloride (TTC, for 24 hour samples), or hematoxylin&eosin staining (for animals sacrificed after 14 days). Immunohistochemistry was performed on formalin-fixed sections, according to an established protocol²⁰. The 12-HETE containing fraction was extracted from brain homogenates and measured with either the 12-HETE EIA kit, or by HPLC/MS. Behavioral tests and antibodies are described in the Supplementary Methods.

Intracerebral Hemorrhage Model

A standard intrastriatal collagenase injection model was used to induce hemorrhage in CD1 mice²¹. Hemorrhagic blood volume was measured photometrically, following sacrifice at 24 hours after collagenase injection. To assess functional outcome, we subjected the mice to a standardized hanging wire test before sacrifice, detecting both latency and a functional score^{21, 22}.

Distal MCAO Clot Model

A ferric chloride-induced clot model was used to occlude the distal MCA as described²³. Two hours later, 10 mg/kg tPA (Activase®, Genentech Inc.) was delivered intravenously over 20 minutes, and either LOXBlock-1 (50 mg/kg) or vehicle were injected intraperitoneally. After sacrifice at 24 hours, pictures were taken of the brains, and hemorrhage area was measured using NIH ImageJ in diaminobenzidine (DAB)-stained brain sections²⁴. Detailed descriptions are given in the Supplementary Methods.

Statistical analysis

For parametric and continuously variable measurements, we used ANOVA followed by Tukey-Kramer posthoc tests. For nonparametric ordinal data (e.g. functional outcomes), we used non-parametric Kruskal-Wallis followed by post-hoc Mann-Whitney tests. P values less than 0.05 were considered significant.

Results

Dose-ranging studies showed that 200 nM LOXBlock-1, which itself lacks antioxidant activity¹⁶, protected HT22 neuronal cells against oxidative glutamate toxicity (Figure 1A). Correspondingly, glutamate-induced elevation of the 12/15-LOX metabolite 12-hydroxyeicosatetraenoic acid (12-HETE) was also significantly suppressed by LOXBlock-1 (Figure 1B). Two major iron-dependent pathways counteracting oxidative stress are mediated by NRF2 and HIF1 α , and many traditional LOX inhibitors act by iron chelation. To investigate if LOXBlock-1 can activate these protective pathways, we used cell-based reporter assays^{17, 18}. Neither NRF2 nor HIF1 α were significantly activated (Figure 1C and D, respectively), suggesting that neuroprotection in our HT22 experiments was specific to the 12/15-LOX pathway.

Biochemical effects of 12/15-LOX were next examined *in vivo*. The brains of mice were analyzed at 12 or 24 hours after 60 minutes transient focal cerebral ischemia. 12/15-LOX activity was measured by assaying 12-HETE by enzyme immunoassay, which showed a marked increase in the ipsilateral brain at 12 and 24 hours post-ischemia (Figure 1E). The identity of 12-HETE was confirmed by HPLC and mass spectrometry (Figure 1F). At 24

hours, immunostaining with antibody MDA2^{25, 26} detected an increase in malondialdehyde-conjugated lysine in cells of the ischemic peri-infarct area. These cells were also 12/15-LOX positive (Figure 1G). Malondialdehyde is a breakdown product formed by oxidation of arachidonic acid, a substrate for 12/15-LOX.

To determine if these results are relevant to human stroke, we performed immunostaining with the 12/15-LOX and MDA2 antibodies in post mortem brain sections of two human stroke patients. Cell-specific staining for both antigens was detected in the penumbra surrounding the core infarct, but not on the contralateral side of the brain (Figure 2A and Supplementary Figure 1). Higher magnification of the ipsilateral staining pattern and overlay with a nuclear stain again showed colocalization of 12/15-LOX with the MDA2 epitope (Figure 2B). In a second patient, we combined 12/15-LOX staining with an antibody to AIF, which showed both antigens increased in the peri-infarct region of the brain, compared to the contralateral side (Figure 2C). The staining pattern closely resembles that found in our previously published mouse experiments, where both 12/15-LOX and AIF were also increased coincidentally in the peri-infarct region²⁰.

Consistent with a damaging role for increased 12/15-LOX activity, intraperitoneal administration of the 12/15-LOX inhibitor LOXBlock-1 significantly reduced infarct size 24 hours after focal cerebral ischemia (Figure 3A). Showing specificity, LOXBlock-1 also reduced the amount of 12-HETE in the ischemic hemisphere (Figure 3B). Neuroprotection was sustained long-term. Even 14 days post-ischemia, LOXBlock-1-treated mice still had significantly smaller infarct sizes compared to vehicle controls (Figure 3C). Behavioral testing supported this result, with LOXBlock-1 treated mice showing a reduced Neurological Severity Score in the acute phase, and returning towards pre-injury baseline in the Corner Test by Day 14, while vehicle-treated mice retained a significant deficit (Supplementary Figure 2).

The time window where acute phase stroke therapy provides a benefit is in many cases very limited. LOXBlock-1 proved to still protect when given 4 hours after induction of ischemia, and even at 6 hours still showed a trend towards protection, although this was not statistically significant (Figure 3D).

In a safety study, we tested the effects of LOXBlock-1 in a mouse model of collagenase-induced hemorrhage. LOXBlock-1 did not alter hematoma volumes (Figure 3E) or worsen neurological outcomes (Supplementary Figure 3), suggesting it does not increase hemorrhagic injury to the brain.

Finally, we investigated the effects of LOXBlock-1 when combined with tPA in a model of tPA-associated hemorrhagic transformation. Intravenous administration of tPA two hours after onset of clotting allowed for efficient reperfusion (details in Supplementary Information), but led to hemorrhage in C57Bl6J mice. This was significantly reduced by intraperitoneal injection of LOXBlock-1 (Figures 3F and G), demonstrating that 12/15-LOX inhibition may provide benefits when co-administered with tPA.

Discussion

Our main findings are that 12/15-LOX inhibition by LOXBlock-1 efficiently protected against oxidative stress-related cell death, both in neuronal HT22 cells, and in mouse models of transient focal ischemia. In an established mouse model of stroke, 12/15-LOX activity was increased. 50 mg/kg LOXBlock-1 was sufficient to reduce elevated 12-HETE in the ischemic brain, and to provide long-lasting protection against ischemic stroke. Furthermore, LOXBlock-1 still protected when administered 4 hours, possibly even six hours after onset of ischemia. Importantly, LOXBlock-1 did not have adverse effects in a mouse hemorrhage

model, and significantly reduced hemorrhage associated with tPA administration. Because increased 12/15-LOX was also detected in the peri-infarct region of two human stroke patients, these results may translate well to human stroke.

Glutathione depletion in HT22 cells is an established cell culture model of oxidative stress-related toxicity. Cell death in these cells, as in immature primary cortical neurons and in primary oligodendrocytic cells, depends on 12/15-LOX^{15, 16, 27–30}. The effective concentration for half-maximal rescue of HT22 cells by LOXBlock-1 (EC₅₀) was around 300 nM, suggesting reasonable efficacy for an enzyme inhibitor. Release of 12-HETE into the culture medium is presumably not part of the death mechanism; addition of exogenous 12-HETE does not affect viability of HT22 cells³¹. Instead, an intracellular mechanism of lipid peroxidation in endoplasmic reticulum and mitochondria, followed by translocation of apoptosis-inducing factor AIF to the nucleus likely kills the cell²⁰. Nevertheless, the detection of 12-HETE provides a good readout for 12/15-LOX inhibition both in cell culture, and in the ischemic brain. Here, the decrease of 12-HETE in the ischemic hemisphere of LOXBlock-1 treated mice suggests that the inhibitor reaches its target in the brain. 12-HETE was still elevated 24 hours after MCAO, and a recent biomarker study found increased HETE levels up until 7 days in the plasma of stroke patients³², suggesting that lipoxygenase inhibition may be beneficial even at relatively late times of administration. From the initial studies identifying LOXBlock-1^{16, 33}, we know that it also inhibits the human version of 12/15-LOX, termed 15-lipoxygenase-1. Our case study shows increased 12/15-LOX after stroke in two human stroke cases as well (Figure 2). LOXBlock-1 may thus also protect against 12/15-LOX related brain damage in humans.

Because of its propensity to cause bleeding, tPA administration currently requires time-consuming imaging to rule out a hemorrhagic stroke. A drug that does not worsen hemorrhage could be given earlier, before advanced imaging by CT or MRI. While no current hemorrhage model can completely reflect the complexities of both intracerebral (ICH) and subarachnoid (SAH) hemorrhagic strokes, it is significant that LOXBlock-1 does not have adverse effects in the collagenase-induced hemorrhage model. Should this be confirmed, or LOXBlock-1 even provide a benefit to victims of ICH or SAH, this would raise the intriguing possibility of giving a neuroprotective drug already in the ambulance, or directly upon arrival in the hospital and initial stroke diagnosis.

A further possible use for 12/15-LOX inhibition may be as an adjuvant to tPA thrombolysis. As a first step to investigate this possibility, we used a mouse model of clot-induced distal MCAO²³. We found surprisingly high levels of hemorrhagic transformation when we reperused after two hours using tPA, which in humans typically occurs when tPA is given at later time points. We used tPA at 10 mg/kg. This is the standard dose for tPA in rodents, but tenfold higher than in humans, possibly explaining the difference in timing. Nevertheless, the striking finding here is that LOXBlock-1 administered at the same time as tPA can significantly reduce hemorrhage (Figure 3F–G), suggesting that combining tPA with LOXBlock-1 may be a useful therapeutic strategy. Further studies will be required to determine whether administration at the same time is required, and to find a suitable time window for this combination treatment.

In summary, we found increased 12/15-LOX activity following ischemia, and have tested a novel inhibitor of 12/15-LOX for its utility as a neuroprotectant in vitro, as well as in mouse models of transient focal ischemia. The robust protection shown by LOXBlock-1 suggests it is a strong candidate for a successful acute phase stroke treatment.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

LOXBlock-1 targets increased 12/15-LOX activity in oxidatively stressed neuronal cells, and 12/15-LOX activity is increased in the ischemic brain. A) Neuronal HT22 cells are protected against oxidative glutamate toxicity by nanomolar levels of LOXBlock-1 (** $p < 0.001$). B) 5 μM LOXBlock-1 brings increased levels of 12-HETE in the medium back down to baseline (** $p < 0.01$ against control, or glutamate plus LOXBlock-1 treated cells). C) LOXBlock-1 (■) does not activate NRF2 in the Neh2-luc reporter assay (with tertiary butylhydroquinone (●) as positive control). D) Likewise, LOXBlock-1 (■) does not activate HIF1 α in a HIF1 ODD-luc reporter assay (ciclopirox (○) as positive control). E) 12-HETE was significantly increased in the ipsilateral hemisphere both 12 and 24 hours after transient focal ischemia (* $p < 0.05$, *** $p < 0.001$; sham $n = 6$ brains, 12hours $n = 3$ brains, 24hours $n = 5$ brains). F) The identity of 12-HETE was confirmed by HPLC/Mass Spectrometry analysis. The smaller peak for 15-HETE in the HPLC profile (top panel) is also a 12/15-LOX product. G) 24h after transient focal ischemia, LOX co-localizes with a

marker for malondialdehyde-modified proteins, MDA2 in the peri-infarct cortex. Nuclei appear as blue stain (To-Pro-3) in the merged picture; scale bar = 20 μm .

Figure 2.

12/15-LOX co-localizes with the oxidative stress marker MDA2 and pro-apoptotic AIF in the infarcted human brain. A) 12/15-LOX is increased in the peri-infarct region of a human brain 87 hours after an ischemic stroke, compared to the contralateral side (scale bar = 50 nm). B) Co-localization of 12/15-LOX in the peri-infarct area with MDA2, merged with nuclear stain To-Pro-3 (scale bar = 20 nm). C) In a second human brain, 12/15-LOX co-localizes with AIF, and both are clearly increased on the infarcted vs the contralateral side (scale bar = 20 μm).

Figure 3.

LOXBlock-1 efficiently protects against transient focal ischemia in mice, and does not worsen outcome after hemorrhage. A) Treatment with 50 mg/kg LOXBlock-1 significantly reduced infarct size 24 hours after 90 minutes of MCAO (**p<0.01; n=12 per group). B) Levels of 12-HETE were lower in the ischemic brain hemisphere of LOXBlock-1 treated mice (n=11 for vehicle, n=10 for LOXBlock-1). C) Infarct sizes remained smaller 14 days after MCAO (**p<0.01; n=12 per group). D) Delayed administration of LOXBlock-1 retains efficacy against MCAO-induced brain injury (**p<0.01 at 2 hours with n=8 per group; *p<0.05 at 4 hours). E) Hemorrhage volume after collagenase administration was not increased by LOXBlock-1 (n=7 per group). F) Following tPA thrombolysis in a clot model, vehicle-treated mice exhibit brain hemorrhage, which is reduced by LOXBlock-1 treatment (n=4 per group). G) Quantitative evaluation of DAB-stained brain sections confirms a significant reduction in hemorrhage by treatment with LOXBlock-1 (**p<0.01).