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Contributions of 12/15-Lipoxygenase to bleeding in the brain following ischemic stroke

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Abstract

Ischemic strokes are caused by one or more blood clots that typically obstruct one of the major arteries in the brain, but frequently also result in leakage of the blood-brain barrier and subsequent hemorrhage. While it has long been known that the enzyme 12/15-lipoxygenase (12/15-LOX) is up-regulated following ischemic strokes and contributes to neuronal cell death, recent research has shown an additional major role for 12/15-LOX in causing this hemorrhagic transformation. These findings have important implications for the use of 12/15-LOX inhibitors in the treatment of stroke.

Keywords

lipoxygenase; eicosanoid; 12-HETE; 15-HETE; ischemic stroke; hemorrhage; hemorrhagic transformation; ischemia; blood-brain barrier; tissue plasminogen activator; tPA; neuroprotection; warfarin; STAT6

Strokes are typically classified into two major subtypes. Of these, ischemic strokes caused by blockage of a major artery account for around 85% of cases, while the remainder are caused by hemorrhage which can be either intracerebral or subarachnoid, depending on the location of the vessel rupture. However, even among the ischemic strokes a substantial number go on to include subsequent bleeding, leading to increased brain injury. This hemorrhagic transformation frequently occurs when tissue plasminogen activator (tPA) is used to lyse the obstructive blood clot, contributing to catastrophically low usage of this

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potentially lifesaving therapy - only a minor percentage of ischemic stroke patients receive thrombolytic treatment. Mechanisms involving the enzyme 12/15-lipoxygenase (12/15-LOX) contribute to the hemorrhagic transformation of ischemic strokes both in the presence and absence of tPA, as will be discussed in this mini-review.

The liberation of polyunsaturated fatty acids including arachidonic acid in the brain following a stroke has been recognized since the early 1970s[1]. These give rise to a dizzying spectrum of eicosanoids and related compounds produced by lipoxygenases, cyclooxygenases, and cytochromes P450, including prostaglandins, leukotrienes, and hydroxyeicosatetraenoic acids (HETEs). Increased levels of 12-HETE were found along with leukotrienes C4 and D4 in gerbil brains following an experimental stroke[2]. We have similarly seen massively increased levels of 12-HETE specifically on the infarcted side of the brain in mice both 12 and 24 hours after onset of ischemia (Figure 1). Therapeutically, initially 5-LOX was seen as the most promising target, mostly due to a much better understanding of leukotriene biology compared to the much less studied 12/15-LOX. However, two independent findings changed this perception: In 2004, a Japanese study found that *Alox5* knockout mice developed the same level of ischemic injury following experimental stroke as matched wild type mice[3]. Around the same time, the group of Chandan Sen at Ohio State University[4] and our group[5, 6] found that *Alox15* knockout mice were protected against the consequences of an experimental stroke, developing smaller infarct sizes (Sen group 90% reduction 72h after stroke onset, van Leyen group 40% infarct size reduction measured 24h after initiation of experimental stroke). Moreover, subsequent studies from our lab found that the mRNA encoding 12/15-LOX was up-regulated 2.2 fold in mice 24h after an experimental stroke[7]. Immunohistochemistry showed the increased 12/15-LOX signal both in neurons, and in endothelial cells[8]. Early work focused on injury to neurons, initially with the discovery that 12/15-LOX contributes to a form of cell death termed oxidative glutamate toxicity or oxytosis in neuronal cells[9]. More recently, a related redox pathway termed ferroptosis was introduced, which is characterized by the loss of glutathione peroxidase 4 (Gpx-4) activity[10] and in which 12/15-LOX is also involved. The commonalities and differences between these two pathways have yet to be clearly defined[10–12]. Glutathione as the major intracellular antioxidant in neurons is clearly important for both pathways, and glutathione levels also drop on the ischemic side of the brain following stroke, which presumably contributes to the activation of 12/15-LOX. The function of 12/15-LOX in this neuronal cell death pathway is to damage mitochondria and other organelles, for which the 12/15-LOX is uniquely qualified: in stress reticulocytes produced during severe anemia, as well as when incubated in vitro, the enzyme attacks mitochondrial membranes[13–16], priming the mitochondria for further degradation via the ubiquitin/proteasome system. This is one of three pathways by which reticulocytes lose their organelles during maturation to become functioning erythrocytes, the others being degradation via autophagic vacuoles and exosome formation[17, 18]. The redundancy of these pathways may be the reason why no outright defects in erythropoiesis were found in Alox15 knockout mice[19].

Beyond causing cell death in neurons, however, it has in recent years become clear that 12/15-LOX also contributes to vessel injury in rodent models of stroke. Alox15 knockout mice develop 51% less edema following experimental stroke than their wild type

counterparts, and 30% less immunoglobulin G (representative of blood proteins) extravasates into the brain parenchyma[8]. Several years later, we made the striking observation in a mouse model of thrombotic stroke that tPA infusion intended to lyse the occluding thrombus led to massive brain hemorrhage in these mice, which was reduced by 82% through simultaneous administration of a 12/15-LOX inhibitor, LOXBlock-1[20] (Figure 2). We have expanded on these results by investigating the effects of 12/15-LOX inhibition on both bleeding and infarct size in this thrombosis model, and found that LOXBlock-1 also improved behavioral scores in the mice[21].

This finding led us to systematically study mouse models of stroke where reperfusion following the ischemic event is associated with increased bleeding. In the classical filament model of transient focal ischemia, a filament is inserted into the internal carotid artery to partially block the middle cerebral artery on one side of the brain, which leads to a reduction of blood flow and ischemia in the striatum and cortex. The filament is then removed after a pre-specified time to allow for reperfusion. When the mouse is sacrificed after 24 hours an infarct is detected, the size of which is determined by the duration of the ischemia. Typically, this model does not lead to significant bleeding, but so called hemorrhagic transformation of the ischemic stroke can be induced, for example when mice are fed with the anticoagulant warfarin[22, 23]. Warfarin is a vitamin K inhibitor that is frequently given to patients with atrial fibrillation to reduce their risk of blood clot formation and subsequent stroke. While warfarin reduces the risk of stroke in these patients, this anticoagulant can cause excessive bleeding leading to increased injury when a stroke does occur. Moreover, thrombolysis with the clotbuster tissue plasminogen activator (tPA) is contraindicated in effectively anticoagulated patients on warfarin (international normalized ratio of coagulation time (INR) > 1.7) because tPA itself has bleeding as a side effect, thus eliminating the only drug currently approved by the FDA as a treatment option. Mice pretreated with warfarin via their drinking water for 24 hours prior to experimental stroke develop severe hemorrhage both when the mice receive a tPA infusion following removal of the filament, or in the absence of tPA when the stroke is severe enough (3 hours of filament occlusion; Figure 2)[23]. Along with the increased hemorrhage, we also found 25% higher levels of 12/15-LOX in the brains of the warfarin-treated mice. The increase was seen mostly in the vasculature, consistent with the idea that the increased vessel leakage following warfarin pretreatment is due to 12/15-LOX.

Consistent with the idea of 12/15-LOX as contributor to hemorrhage, 41% less bleeding was seen following warfarin pretreatment in Alox15 knockout mice[23]. In wild type mice, a similarly drastic reduction in hemorrhage by 38% was detected when the mice were treated with the second generation 12/15-LOX inhibitor ML351[24], administered intraperitoneally at the time of reperfusion, 3 hours after onset of ischemia. The reduction in bleeding remained significant even when the results were adjusted to account for the reduced infarct size in the ML351-treated mice, confirming that there is a specific effect on hemorrhage. When ML351 was administered along with tPA in warfarin pretreated mice, hemorrhage was similarly reduced by 59%. Taken together, these results demonstrated that increased 12/15-LOX in the brain vasculature can contribute to excessive bleeding in the brain, which is reduced by treatment with a 12/15-LOX inhibitor.

Much work remains to be done to elucidate the mechanism by which 12/15-LOX contributes to increased hemorrhage after an ischemic stroke. Important open questions include the selective up-regulation of 12/15-LOX in brain vascular endothelial cells following warfarin administration, both with and without subsequent tPA infusion. Is this a direct effect of warfarin and/or tPA, or are intermediate factors involved? Also, in neurons signal transducers and activators of transcription (STATs), specifically STAT1 and STAT6 are involved in up-regulating 12/15-LOX under ischemic conditions[7]. Are the same STATs active here, or is a different form of regulation relevant? Finally, what happens after 12/15-LOX is up-regulated in the endothelial cells and how does this lead to vessel rupture? In addition to destroying endothelial cells of the brain vasculature by damaging mitochondria, there may be a second injury mechanism induced by the signaling function of 12/15-LOX via metabolites of arachidonic acid. Both 12-HETE and its immediate precursor 12-HPETE are known as second messengers[25], activated along the semaphorin pathway[26, 27]. In neurons, this can lead to axon retraction[28, 29], but under some conditions also to cell death[30]. Semaphorin 3A has also been reported to increase vascular permeability in experimental stroke models[31]. Downstream of 12-HETE and 12-HPETE, secretion of destabilizing matrix metalloproteinases (MMPs) may play a role[32]. Both the molecular details of this signaling pathway, and the relative contributions of both pathways to vascular injury require further study.

Because 12/15-LOX contributes to both neuronal cell death and to vessel leakage following a stroke, 12/15-LOX inhibition appears to be a particularly promising approach to stroke therapy by targeting two separate modes of injury, killing two birds with one stone. Both our group[20, 23, 24, 33, 34] and others[35, 36] have in recent years focused on developing improved inhibitors of 12/15-LOX, and it will be exciting to see if these novel molecules can turn the tide in the seemingly endless war to combat stroke. The finding that we can reduce bleeding subsequent to an ischemic stroke in the rodent model broadens the spectrum of patients that could be treated with a 12/15-LOX inhibitor. In addition to its use as a stand-alone neuroprotective treatment that could already be given in the ambulance on the way to the hospital, 12/15-LOX inhibition could also be combined with tPA thrombolysis to make the use of tPA safer. By removing the most serious side effect of tPA, this approach may lead to significantly more patients receiving tPA treatment. Furthermore, the recently introduced endovascular treatment in which a stent retriever is used to remove the obstructing blood clot could also be rendered more effective by adding a 12/15-LOX inhibitor, because even after thrombus removal cognitive deficits are seen in many patients who could benefit from the added neuroprotection[37]. Finally, besides the more common ischemic strokes there may also be a place for 12/15-LOX inhibition in the treatment of hemorrhagic strokes. We have recently completed a study to investigate the function of 12/15-LOX in subarachnoid hemorrhage, where we found increased 12/15-LOX in the brains of mice 24 hours after hemorrhage induction (92 ± 60 12/15-LOX positive cells/field vs. 2 ± 2 in sham-operated controls, $p<0.05$)[38]. In this case, 12/15-LOX expression was detected mostly in macrophages however, rather than in neurons and endothelial cells; the injury mechanism may thus differ from that in ischemic stroke. Regardless, Alox15 knockout mice developed 72% less injury than wild type mice, and 12/15-LOX inhibition also reduced injury by 55% compared to vehicle-treated mice in this model of hemorrhagic stroke.

In conclusion, despite different triggers - in the presence or absence of anticoagulant, with or without tPA treatment - 12/15-LOX is activated in various models of stroke-related hemorrhage. In addition to its benefits in infarct size reduction, 12/15-LOX inhibition may thus independently reduce hemorrhagic conversion of ischemic strokes by protecting the vasculature.

Acknowledgment:

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A
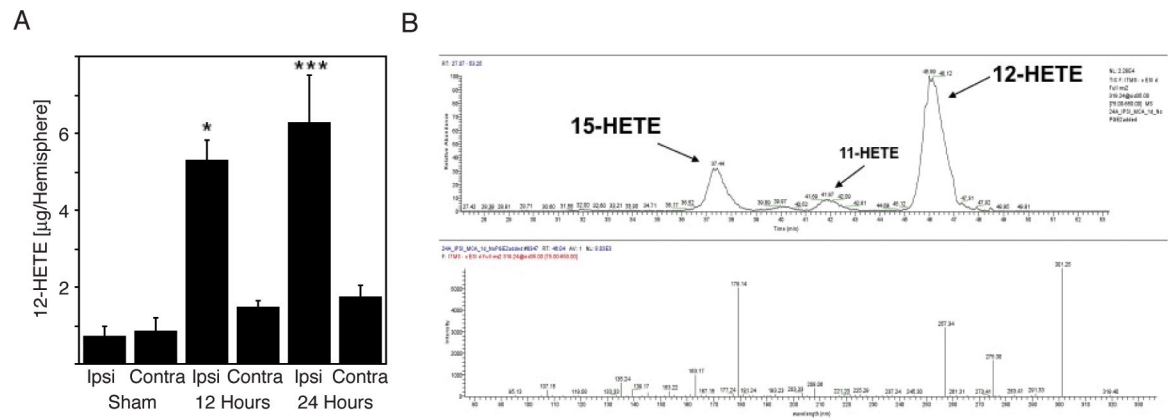

B

Figure 1:

A. 12-HETE was significantly increased in the infarcted ipsilateral hemisphere of mice both 12 and 24 hours after transient focal ischemia, compared to sham-operated mice (*p < 0.05, ***p < 0.001; sham, n = 6 brains; 12 hours, n = 3 brains; 24 hours, n = 5 brains). B. The identity of 12-HETE was confirmed by high-performance liquid chromatography (HPLC)/ mass spectrometry analysis. The smaller peak for 15-HETE in the HPLC profile (top panel) is also a 12/15-LOX product. Reprinted with permission from reference 20.
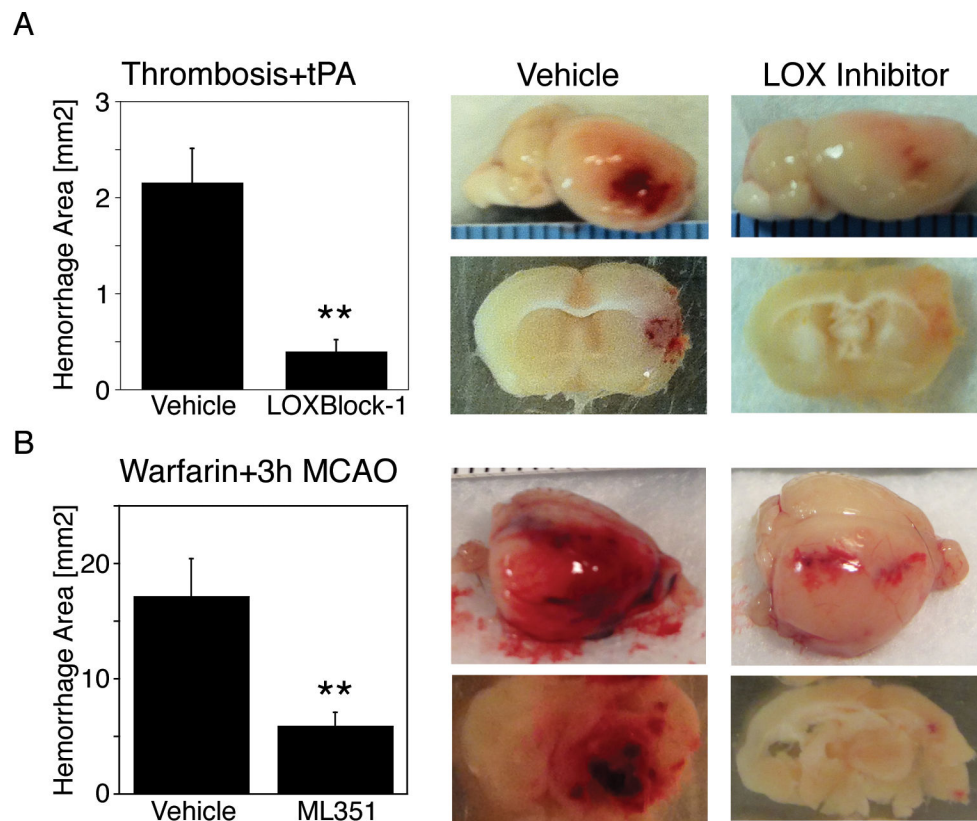
A

B

Figure 2:

Examples of stroke models associated with increased hemorrhage. A. Thrombosis was induced when 10% ferric chloride solution was topically applied to the brain. An infusion of tissue plasminogen activator (tPA) two hours later led to distinct hemorrhage in vehicle-treated mice after 24 hours, visible both on the surface of the brain (top) and in sections (below). In contrast, when mice were intraperitoneally injected with the 12/15-LOX inhibitor LOXBlock-1 (50 mg/kg), significantly less hemorrhage was detected in the brain. B. Pretreatment of mice for 24 hours with the anticoagulant warfarin added to the drinking water causes massive hemorrhage following a severe form of experimental ischemic stroke with 3 hours of occlusion of the middle cerebral artery. This is again visible both on the surface, as well as in brain sections. Mice treated with the 12/15-LOX inhibitor ML351 (50 mg/kg) develop far less hemorrhage (bottom right). Quantitation graphs represent hemorrhage area measured in brain sections and are reprinted with permission from references 20 (top) and 23 (bottom).