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Lipoxygenase: An Emerging Target for Stroke Therapy

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Abstract

Neuroprotection as approach to stroke therapy has recently seen a revival of sorts, fueled in part by the continuing necessity to improve acute stroke care, and in part by the identification of novel drug targets. 12/15-Lipoxygenase (12/15-LOX), one of the key enzymes of the arachidonic acid cascade, contributes to both neuronal cell death and vascular injury. Inhibition of 12/15-LOX may thus provide multifactorial protection against ischemic injury. Targeting 12/15-LOX and related eicosanoid pathways is the subject of this brief review.

Keywords

Ischemic stroke; neuroprotection; oxidative stress; eicosanoid; lipoxygenase; arachidonic acid cascade

Introduction

Neuroprotection in recent years has had a difficult standing in the stroke research field. Many failed clinical drug trials, notably the Saint II trial for the radical scavenger NXY-059[1], have contributed to a nihilistic view in both Industry and parts of Academia. With the identification of novel targets however, and given the continuing need for effective acute stroke treatment options, this stance is being re-evaluated. Several excellent recent reviews have covered various aspects of new approaches to neuroprotection, such as protection of the neurovascular unit, reduction of white matter injury, blockade of the inflammatory cascade, and others [2–6]. The approach of blocking the activity of 12/15-lipoxygenase (12/15-LOX) will be the major focus of this short review, and it touches upon several of these themes. In addition, we will very briefly highlight recent approaches to neuroprotection targeting other eicosanoid-producing pathways, including the cyclooxygenase and cytochrome P450 pathways.

The arachidonic acid cascade and lipoxygenase pathways

Free arachidonic acid is increased in the ischemic brain, mostly liberated from membrane phospholipids by phospholipases, especially phospholipase A2. The arachidonic acid cascade is a series of enzymatic reactions initiated by lipoxygenases, cyclooxygenases, and members of the cytochrome P450 family of proteins; collectively, the oxidation products are termed eicosanoids [7]. Lipoxygenases oxidize arachidonic acid at specific carbon atoms to generate a hydroperoxide (termed HPETE for hydroperoxyeicosatetraenoic acid), which is

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Conflict of Interest

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then reduced to the corresponding hydroxide (termed HETE). In this way, 5-LOX oxidizes arachidonic acid at C-5, generating 5-HPETE, which is then converted to 5-HETE and, in follow-up reactions by other enzymes, to pro-inflammatory leukotrienes. In a similar manner, 12/15-LOX generates a mixture of 12-HPETE and 15-HPETE, which again get reduced to the corresponding HETEs and undergo various follow-up reactions. In conjunction with other enzymes, this leads to production of both pro-inflammatory signaling molecules like the hepoxilins[8, 9], and anti-inflammatories like lipoxins[10]. Other polyunsaturated fatty acids besides arachidonic acid can serve as substrate; e.g., linoleic acid is oxidized to 9- and 13-HODE. Remarkably though, 12/15-LOX can also oxidize arachidonic acid and linoleic acid when they are esterified as phospholipids, meaning it can directly cause lipid peroxidation, and this may be its major contribution to ischemic brain damage (see below). In addition to 5- and 12/15-LOX, several other lipoxygenases exist, but little is known about their expression in the brain[11].

Another important class of eicosanoids are the prostaglandins, pro-inflammatory signaling molecules generated through the oxidation and cyclization of arachidonic acid by the cyclooxygenases COX-1 and COX-2. These enzymes are the target of non-steroidal anti-inflammatory drugs (NSAIDs), which have been extensively evaluated as stroke therapeutics. Finally, cytochrome P450 oxidizes arachidonic acid to 20-HETE as well as several epoxides (EETs), which are major vasoreactive molecules.

The role of 12/15-LOX in stroke

Brain injury subsequent to an ischemic stroke typically follows a pattern with distinct temporal characteristics. The initial infarct core, where a sudden massive drop in the blood supply causes rapid cell death, is surrounded by a penumbra, where tissue is at risk for further injury, but not yet irreversibly affected. Blood flow to the penumbra region is reduced, and several additional factors contribute to the progression of the infarct over hours and days. One of these is the acidification of tissue, with pH drops to around pH 6.5, sometimes lower. In addition, depolarized neurons and dying cells from both the core and the penumbra release large amounts of glutamate, leading to excitotoxicity in neurons whose glutamate receptors become overloaded. The blood – brain barrier becomes leaky, leading to edema and massive secondary injury[12]. Oxidative stress-related injury underlies many of these pathologies, and 12/15-LOX (also known as 15-LOX-1, leukocyte-type 12-LOX, or 12/15-LO) may be a major contributor.

In the meantime, a fair body of evidence has accumulated to document the importance of 12/15-LOX in ischemic stroke. Early evidence of arachidonic acid metabolizing enzymes being involved in ischemic injury came from the work of Nic Bazan, who documented increased levels of arachidonic acid in the brain following ischemia [13]. This was followed by Pak Chan and Robert Fishman's demonstration that arachidonic acid injected into the brain can cause edema[14, 15]. Finally, Mike Moskowitz and colleagues showed in 1984 that lipoxygenase metabolites, including both 5-LOX derived leukotrienes and the 12/15-LOX metabolite 12-HETE, were increased in the gerbil forebrain following ischemia[16]. These early findings set the stage, but the next steps were not taken until much later. Fast forward to 1997, when Li, Maher, and Schubert showed an involvement of 12/15-LOX in oxidative stress-induced cell death in cultured neurons[17]. Then, in 2004/2005, the lab of Chandan Sen and ours independently demonstrated the specific involvement of 12/15-LOX in experimental stroke in rodents [18–20]. On the clinical side, a recent study showed elevated cerebrospinal fluid (CSF) levels of 12-HETE in patients with subarachnoid hemorrhage[21], and elevated levels of 12-HETE were also found in the CSF following traumatic brain injury[22]. Similar measurements in ischemic stroke patients have to our knowledge not been reported. Intriguingly, we have recently found 12/15-LOX increased in

the peri-infarct cortex of two stroke patients, suggesting 12/15-LOX may contribute to stroke injury in humans, as well[23].

To date, most studies of 12/15-LOX in stroke have been carried out in rodents. In mice, inducing an experimental stroke by transiently occluding the middle cerebral artery with a filament (transient MCAO) leads initially to a striatal infarct that expands into the cortex over time. This cortical region that is adjacent to the core infarct and goes on to die is seen as representative for the penumbra region in human stroke patients. 12/15-LOX was found by immunohistochemistry to be expressed in this peri-infarct region of the cortex[20]. 12/15-LOX expression progressively increases over 24 hours. In the same cells, but with a slight time delay, apoptosis-inducing factor (AIF) is also increased[24], linking 12/15-LOX to an apoptotic cell death mechanism. Importantly, 12/15-LOX is increased both in neurons and in endothelial cells, suggesting it may contribute to both neuronal cell death and vascular injury[25].

Cell death through organelle damage: the toxic mechanism of 12/15-LOX

The enzymology of 12/15-LOX is complex, but important for its mechanism of action[11, 26–28]. 12/15-LOX contains a non-heme iron, which shuttles between Fe^{3+} and Fe^{2+} during the oxidation reaction. An oxidative environment is thus required for enzyme activity. The enzyme inserts molecular oxygen into a C-H bond, which thus forms an unstable hydroperoxide that can undergo various follow-up reactions. As substrate, 12/15-LOX uses polyunsaturated fatty acids (PUFAs), mostly arachidonic and linoleic acid. In contrast to other lipoxygenases, cyclooxygenases, and cytochrome P450s, 12/15-LOX can oxidize PUFAs even when they are membrane-bound, as phospholipids. The important consequence of this is that 12/15-LOX can directly attack and modify membranes. It does so by binding to the membrane, generating pores that release soluble proteins from the luminal space of organelles, including mitochondria and endoplasmic reticulum (ER) membranes[29, 30].

Regulation of 12/15-LOX

In reticulocytes, the red blood cell precursors, organelle permeabilization is a desired effect, because it is the first step on the way to degrading the organelle. This is part of red blood cell maturation[31, 32], where mitochondria are absent in the mature erythrocyte, and it may also apply to lens cells of the eye[29, 33, 34]. But in almost all other cell types, organelle degradation is a detrimental process, and the cell thus limits 12/15-LOX activity through transcriptional, translational, and post-translational control. Depending on the cell type, transcriptional regulators of 12/15-LOX include STAT6[35], IL-4/IL-13[36], and GATA-6[37]. Translation of 12/15-LOX is regulated by proteins binding to the 3'-UTR of its mRNA, including hnRNP k and hnRNP E1[38, 39]. Nonetheless, the actual transcriptional or translational regulators leading to increased 12/15-LOX expression in the ischemic brain have not been reported to date. Post-translational control is exerted through a requirement for calcium to enable membrane binding[40], and through the intracellular antioxidant glutathione[17, 41]. Both of these become dysregulated in the ischemic brain, with glutathione levels dropping[42] and intracellular calcium levels rising[43]. This allows the 12/15-LOX to become activated and to start damaging organelles. Further activity increases are caused by the availability of free arachidonic acid, which is generated by phospholipase A2 under ischemic conditions[13]. To make matters worse, the enzyme is also activated by the hydroperoxides it produces, resulting in a destructive feed-forward loop[26]. These events are summarized in Fig. 1.

Inhibition of 12/15-LOX is protective in models of oxidative stress-related cell death in several types of brain cells

On a cellular level, much has been learned from a model of cell death through glutathione depletion, termed oxidative glutamate toxicity or oxytosis [44, 45]. Immature neurons, when challenged with high concentrations of glutamate, die despite the absence of functional glutamate receptors[44, 46]. The reason is a cystine/glutamate antiporter in the cell membrane termed Xc⁻, which supplies a building block for glutathione to the cells. Glutathione is the major endogenous antioxidant of neurons, and glutathione depletion causes oxidative stress and subsequently, cell death. This cell death model is replicated in the neuronal cell line HT22, which has been used to study its mechanisms. In both immature neurons and HT22 cells, oxytosis is dependent on 12/15-LOX activity[17]. Besides neuronal cells, oligodendrocytes are also subject to a similar mode of cell death, elicited by removal of cysteine from the culture medium. In both mature oligodendrocytes and their precursors, cell death is prevented by 12/15-LOX inhibition[47–49].

In HT22 cells, glutathione depletion occurs over a time period of several hours, after which the cells start experiencing oxidative stress. 12/15-LOX then becomes membrane-associated and activated[17]. Mitochondrial membranes are especially susceptible[24], and the mitochondrial membrane potential breaks down. This can be replicated in vitro by incubating 12/15-LOX with isolated mitochondria, which also leads to production of reactive oxygen species (ROS). Subsequently, cytochrome c is released to the cytosol[50], but without activation of caspase-3[51]. Instead, cell death proceeds through translocation of the apoptosis-inducing factor AIF from mitochondria to the nucleus. Pro-apoptotic Bid presumably contributes to the process[52]. This pathway may be highly relevant to neuronal death in the ischemic penumbra, where increased 12/15-LOX coincides with increased AIF in the peri-infarct region [24]. Further support comes from a study showing the importance of glutathione peroxidase-4 in preventing 12/15-LOX-and AIF-dependent cell death in cells and in vivo[53]. The link between GPX-4 and 12/15-LOX had previously been demonstrated in vitro[54]. Finally, proteasome inhibition blocks cell death in HT22 cells downstream of mitochondrial damage, but at present it is unclear which specific step of the cell death program requires proteasome activity[51].

Targeting 12/15-LOX in rodent models of stroke: gene knockout vs. pharmacological inhibition

Increased levels of 12/15-LOX in the ischemic peri-infarct area suggested that blocking its activity might be protective. Supporting this idea, 12/15-LOX knockout mice exhibit reduced infarct sizes[19, 20], and less edema than the corresponding wild-type mice[25]. Several direct inhibitors of 12/15-LOX have been successful in focal ischemia models in vivo.

a-tocotrienol

To block 12/15-LOX activity pharmacologically, Chandan Sen's group focused on a vitamin E compound, a-tocotrienol[55, 56]. Given by oral gavage for several weeks before the ischemic insult, a-tocotrienol reduced infarct size in rats[19]. It is not known what the time window would be for using a-tocotrienol in acute stroke, but it also reduced severity of stroke injury when dosed chronically in canines, suggesting it may have utility in secondary stroke prevention[57]. In addition to inhibiting 12/15-LOX and a general antioxidant effect, a-tocotrienol may also protect by targeting Multidrug Resistance-Associated Protein 1[58].

Baicalein

In our experiments, we initially used baicalein, a flavonoid natural product from the root of *Scutellaria Baicalensis*[59]. It is a component of Chinese herbal medicine, and an inhibitor of both 12-LOX and 12/15-LOX[60]. In mice, it reduced infarct size and edema formation to about the same extent as the gene knockout[20, 25]. Beneficial effects have also been reported for baicalein in ischemia models in rats, and an embolic stroke model in rabbits[61–63]. It is however, not very specific, also blocking xanthine oxidase and having strong antioxidant activity[59]. In addition, we needed 300 mg/kg to provide neuroprotection in mice, although lower dosages have been reported to be protective in rats. The reason for the different protective dosages is not clear, but might relate to different sources of the compound.

Novel 12/15-LOX inhibitors

In order to develop new inhibitors of 12/15-LOX as drug candidates, we have collaborated with the group of Ted Holman at the University of California, Santa Cruz. Using an in vitro assay with recombinant human 15-LOX-1 and in silico modeling, they identified several candidate compounds that blocked 12/15-LOX activity[64]. We then used the oxidative glutamate toxicity model in HT-22 cells as secondary screen for novel neuroprotective inhibitors targeting 12/15-LOX. Two out of three compounds were protective in this model, and also successfully rescued primary neurons and oligodendrocytic cells against cell death through glutathione depletion[49]. These compounds, 2-amino-N-(3-chlorophenyl)-4,5,6,7-tetrahydro-1-benzothiophene-3-carboxamide and 4-ethyl-6-(4-phenoxy-1H-pyrazol-3-yl)-1,3-benzenediol (now termed LOXBlock-1 and -3, respectively) are currently being evaluated in several models of ischemic brain injury. Because of their identification through modeling to the active site of 12/15-LOX, and the finding that they have very little radical scavenging activity, they may show higher specificity than most previously established 12/15-LOX inhibitors, although cross-reactivity with other lipoxygenases cannot be ruled out. We have recently shown that LOXBlock-1 provides efficient neuroprotection in mice, even when given four hours after onset of ischemia. In addition, it does not worsen outcome in a hemorrhage model, suggesting it may be safe to give as first-line stroke treatment[23].

Direct and indirect 12/15-LOX inhibition: antioxidant inhibitors

In addition to the attempts to specifically inhibit 12/15-LOX activity to reduce stroke injury presented above, several other approaches to neuroprotection should also at least partially block 12/15-LOX activity. These neuroprotectants broadly fall into 2 categories: One, treatment with antioxidant drugs or chelators that either directly or indirectly reduce 12/15-LOX activation; two, increasing endogenous antioxidant defenses.

Drugs that block 12/15-LOX activity

Several antioxidants and some non-antioxidants that have been shown to reduce infarct size and/or edema following focal ischemia either directly or indirectly inhibit 12/15-LOX. Among these, edaravone stands out because it has clinical approval as stroke treatment in Japan[65, 66]. Known as a radical scavenger, it also inhibits 12/15-LOX activity in vitro[67]. Similarly, the glutathione peroxidase mimic ebselen is a lipoxygenase inhibitor. Ebselen has been investigated in clinical trials, where an average administration time of 25 hours post infarct presumably contributed to its lack of efficacy[68]. Other antioxidant 12/15-LOX inhibitors that have shown promise in animal studies include curcumin [69] and the green tea polyphenol epigallocatechin gallate[70]. AA-861, which is not an antioxidant, is an interesting case. Originally introduced as an inhibitor of 5-LOX, it has also been shown to block 12/15-LOX activity. Correspondingly, it protects neuronal cells against oxidative glutamate toxicity, and it also protects rodents against transient focal ischemia[71]. In all of

these cases, other protective mechanisms (radical scavenging, general antioxidant effects) are possible, but 12/15-LOX pathology should be considered as injury mechanism.

Endogenous antioxidants

These include the peptide glutathione, as well as several important redox-active proteins. The best known of these is superoxide dismutase. Both the cytosolic (SOD1) and the mitochondrial (SOD2) version of superoxide dismutase protect against ischemic injury by scavenging ROS[72, 73], and in both cases this would be expected to reduce activation of 12/15-LOX. A direct connection between SOD and 12/15-LOX has to date not been made. In contrast, the glutathione peroxidase GPX-4 has been clearly linked to 12/15-LOX dependent cell death[53]; and overexpression of GPX-1 is protective in models of focal ischemia[74]. Another intracellular antioxidant in neurons is Peroxiredoxin-2, which when overexpressed protects against transient focal ischemia via a redox mechanism[75]. Finally, glutathione has long been a neuroprotection target. Because 12/15-LOX is activated when glutathione is depleted, raising glutathione levels should prevent activation of 12/15-LOX and thus, injury caused by 12/15-LOX. This cannot be achieved by glutathione itself, which cannot enter cells. Instead, either derivatives like glutathione methyl ester, or glutathione precursors may be beneficial[76].

The other side of the coin: anti-inflammatory lipoxins and protectins/ neuroprotectins

Paradoxically, 12/15-LOX can also generate protective molecules from either arachidonic acid, or the w3-fatty acids eicosapentaenoic and docosahexaenoic acid. In both cases, other enzymes – typically 5-LOX - are involved in the biosynthetic pathway as well. The arachidonic acid derivative lipoxin A4 is well known for its role in inflammation resolution[10, 77]. Lipoxin A4 may contribute to the neuroprotective effects of rosiglitazone through activation of PPAR γ (Sobrado et al., 2009). Its synthetic methyl ester derivative has been shown to reduce inflammation following transient focal ischemia[78]. Strikingly, an epi-lipoxin with equivalent activity is made when aspirin-treated COX-1 replaces 12/15-LOX.

The laboratories of Nicolas Bazan in New Orleans, and of Charles Serhan in Boston, have demonstrated strong neuroprotective effects of novel docosahexaenoic acid derivatives, termed protectins or neuroprotectins [79–83]. These can be either generated endogenously following infusion of docosahexaenoic acid, or made synthetically and used in a straight-up neuroprotectant approach.

This would pose the dilemma that 12/15-LOX can have both good and bad effects on stroke pathology. At least in experimental stroke studies in rodents to date, the bad clearly outweighs the good, because the net effect of 12/15-LOX inhibition or gene knockout is an improved outcome. Luckily, there may be a way to have the best of both worlds: in a fascinating twist to the story, low-dose aspirin converts COX-2 into a pseudo-lipoxygenase, which generates the R-enantiomers of 12/15-LOX products, which are usually in S-configuration. These R-enantiomers are also active in resolving inflammation[10, 79, 80]. Importantly, the modified COX-2 would not be expected to be able to damage organelles in the way 12/15-LOX does. Therefore, combining low-dose aspirin with a 12/15-LOX inhibitor could provide dual protection, blocking intracellular organelle damage while allowing for the production of anti-inflammatory aspirin-triggered (AT) epi-lipoxin A4 and AT-neuroprotectin D1.

Do other lipoxygenases contribute to stroke injury?

As outlined above, the case for involvement of 12/15-LOX in stroke pathology is strong. But the lipoxygenases are a large family of proteins, with varying tissue distributions and activities[11]. So do other lipoxygenases contribute to ischemic brain injury as well? Presumably not via the same mechanism, because only 12/15-LOX can directly oxidize membrane-bound phospholipids. For example, 5-LOX needs Five-lipoxygenase activating protein FLAP to associate with membranes, but even then a phospholipase A2 is required to liberate polyunsaturated fatty acids from the membrane before they can be oxidized. This may relate to differences in the binding pocket between 5- and 12/15-LOX, which favor head-on insertion for 5-LOX, but tail first engagement for 12/15-LOX. While the attack on mitochondria and other organelles inside the neural cell appears to be restricted to 12/15-LOX, other mechanisms of injury may be elicited by lipoxygenases.

5-LOX

Initially, 5-LOX was a preferred target for stroke therapy, based on its role as generator of the pro-inflammatory leukotrienes. Inhibitors that target 5-LOX directly have been reported to reduce ischemic injury in several models. Nonetheless, 5-LOX knockout mice developed the same infarct size in both transient and permanent focal ischemia as the corresponding wild-type mice [84]. While this result has put a damper on targeting 5-LOX directly, its activating protein FLAP is still being investigated. In rats, the FLAP inhibitor MK-886 decreased leukotriene levels and reduced infarct size[85] 24h after onset of permanent ischemia. It is somewhat unclear how to reconcile these results with one another. In addition, polymorphisms in ALOX5AP (the gene encoding FLAP) have been implicated in stroke risk[86, 87], although this has recently been drawn into question[88]. Similarly, downstream enzymes of the leukotriene pathway have been identified as conferring an increased risk of stroke[89, 90].

12-LOX and other lipoxygenases

Besides 12/15-LOX, there is also a separate 12-LOX enzyme, termed platelet-type 12-LOX[91, 92]. While its distribution in the brain has not been thoroughly studied, it is being investigated as a possible target for anti-platelet therapy[93–95], and this could be beneficial for ischemic stroke. A variety of other lipoxygenases exist, including a 12R-LOX, epidermal LOX-3, and 15-LOX-2, but not much is known about their expression and possible functions in the brain[91].

Other enzymes of the arachidonic acid cascade: phospholipase A2, cyclooxygenase, and cytochrome P450

Besides the lipoxygenase family, other enzymes of the arachidonic acid cascade have also long been implicated in stroke-related brain injury. Phospholipases that liberate arachidonic acid from membrane phospholipids, cyclooxygenases which produce pro-inflammatory prostaglandins, and cytochrome P450 which generates vasoactive 20-HETE, all may contribute to ischemic neurotoxicity. A variety of excellent reviews is available for these subjects, so we will here just very briefly highlight some of the concepts involved.

Phospholipases

because phospholipases, especially of the extended A2 family (PLA2), increase the pool of free poly unsaturated fatty acids following ischemia, their importance was recognized very early[13]. Cytosolic phospholipase A2 (cPLA2) is a type IV phospholipase, and the major arachidonic acid-releasing enzyme in the neuronal cell. It can thus be seen as a master regulator of the arachidonic acid cascade. Besides supplying the arachidonic acid substrate

when activated by calcium influx, it is also literally a regulator for the cyclooxygenase COX-2 following ischemia: COX-2 protein is increased two-fold in wild-type, but not in cPLA2 knockout mice following cerebral ischemia[96]. Correspondingly, cPLA2 knockout mice are protected against transient focal ischemia[97]. Either directly or indirectly, cPLA2 also contributes to disruption of the blood – brain barrier[98]. The drawback of being at the initiation step of the arachidonic acid cascade is that it may not be a good drug target. Because cPLA2 modulates early events, the time window for phospholipase inhibition after a stroke may be fairly short[96]. Another phospholipase that has received much attention for its role in atherosclerosis is the lipoprotein-associated phospholipase, Lp-PLA2. In the stroke field, it is being studied mostly for its contributions to stroke risk[99–101]. Isoforms of secretory PLA2 (sPLA2) may contribute to neuronal death[102, 103]. Conversely, another phospholipase PAF-acetylhydrolase II appears to be neuroprotective[104].

Cyclooxygenases

Similar to phospholipases, the cyclooxygenases COX-1 and COX-2 have long been studied for their involvement in stroke. Especially COX-2 is known to be increased, and contribute to injury, in animal models of stroke[105, 106], while COX-1 deficiency actually leads to increased infarct size[107], although recently the case has been made for reinvestigating COX-1 as drug target[108]. In part, COX-2 is modulated by NMDA receptor activity, at the same time it contributes to excitotoxicity[109]. However, because of the finding that COX-2 inhibitors like Vioxx and Bextra increase the risk of cardiovascular events[110, 111], recent research has mostly focused on downstream elements of the COX-2 pathway[112]. Here, especially the Prostaglandin E2 receptors EP1 – EP4 are seen as promising targets, as well as the microsomal Prostaglandin E synthase[113]. Receptors of the D and F series have also been targeted[114, 115].

Cytochrome P450

The Cytochrome P450 family of proteins generates a variety of blood flow regulators, including 20-HETE and EETs. These have been investigated as opportunities to increase blood flow following ischemia[116]. In addition, increasing the levels of EETs by inhibiting soluble epoxide hydrolase (sEH) is being investigated[117].

Summary and Outlook

Several important questions have been raised concerning the feasibility of neuroprotection to combat stroke[118–123]. While not all of these can be answered with theoretical arguments, the basic principle still applies: the injury that can be prevented, does not need to be healed later. Some of the novel approaches presented here, alone or in combination, may provide us with treatments that have proven elusive so far.

So can inhibition of 12/15-LOX be turned into a promising drug approach to treat stroke? Several findings suggest this may be the case. One, low-level expression of the enzyme in the non-ischemic mouse brain, and the relatively normal brain histology of 12/15-LOX knockout mice, both suggest blocking 12/15-LOX activity may not be too disruptive, and thus not saddled with overly severe side effects. Two, the increased expression in both neurons and endothelial cells of the peri-infarct zone, together with the reduction in both infarct size and edema formation through 12/15-LOX inhibition or knockout, show that despite hitting only one target, a suitable 12/15-LOX inhibitor may disrupt several injury pathways in the ischemic brain. Three, the finding of increased 12/15-LOX protein, as well as increased plasma levels of 12-HETE in human stroke patients, indicate that this injury mechanism may also apply to humans. Four, the time window for inhibitor administration appears to be large enough to make 12/15-LOX inhibition clinically relevant, with

LOXBlock-1 providing significant neuroprotection when given four hours after onset of ischemia in mice. Although initial studies look promising, effects of 12/15-LOX inhibition on hemorrhage (both in intracerebral hemorrhage models, and in conjunction with tPA) need to be evaluated in more detail. This will help to define the settings in which a 12/15-LOX inhibitor can be employed. If hemorrhagic complications are not worsened, this would make the strategy more widely applicable. Furthermore, the relationship with possible beneficial effects of 12/15-LOX through protectins and similar anti-inflammatory mechanisms needs to be clarified. Finally, studies should be expanded to include aged animals[124], and additional models of stroke and cerebral ischemia in other organisms. Nonetheless, the promising results with 12/15-LOX inhibitors thus far establish that this is a strategy that should be pursued, and hopefully, the current impasse on neuroprotection as stroke treatment strategy can be broken.

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List of Abbreviations

LOX	lipoxygenase (e.g., 12/15-LOX)
H(P)ETE	hydro(pero)xyeicosatetraenoic acid
COX	cyclooxygenase
NSAIDs	non-steroidal anti-inflammatory drugs
EET	epoxyeicosatrienoic acid
CSF	cerebrospinal fluid
AIF	apoptosis-inducing factor
PUFA	polyunsaturated fatty acid
ER	endoplasmic reticulum
ROS	reactive oxygen species
SOD	superoxide dismutase
GPX	glutathione peroxidase
FLAP	five-lipoxygenase activating protein
cPLA2	cytosolic phospholipase A2
lp-PLA2	lipoprotein-associated phospholipase A2

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Figure 1. Model for activation of 12/15-LOX in the ischemic brain

Under ischemic conditions, cPLA₂ is activated to release arachidonic acid from membrane phospholipids. This free arachidonic acid, together with a calcium influx from the extracellular milieu and the depletion of glutathione, leads to activation of 12/15-LOX, which starts binding to and oxidizing mitochondrial and ER membranes. This permeabilizes the organelles, leading to release of calcium from ER stores and generation of ROS by mitochondria, which further activates 12/15-LOX. Mitochondria release the pro-apoptotic proteins cytochrome c and AIF, which then translocates to the nucleus. Abbreviations: AA, arachidonic acid; Cyt., cytochrome; AIF, apoptosis-inducing factor; ROS, reactive oxygen species.