Baicalein and 12/15-Lipoxygenase in the Ischemic Brain

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Stroke is a multifactorial disease in which neuronal cell death proceeds through a mixture of mechanisms including oxidative stress, excitotoxicity, apoptosis, and inflammation. Infarction size is impacted both by transcriptional dysregulation, as well as by release of neurotoxic glutamate and other substances from dying cells. Genes aberrantly upregulated in neurons after ischemia and reperfusion thus represent possible targets for neuroprotective therapy. Oxidative stress-related gene expression is known to be susceptible to ischemic preconditioning. Especially 12/15-lipoxygenase (12/15-LOX, encoded by the ALOX15 gene), the dominant isoform in the brain, is known for its ability to damage neuronal cell death proceeds through a mixture of mechanisms including oxidative stress, excitotoxicity, apoptosis, and inflammation. Infarction size is impacted both by transcriptional dysregulation, as well as by release of neurotoxic glutamate and other substances from dying cells. Genes aberrantly upregulated in neurons after ischemia and reperfusion thus represent possible targets for neuroprotective therapy. Oxidative stress-related gene expression is known to be susceptible to ischemic preconditioning. Especially 12/15-lipoxygenase (12/15-LOX, encoded by the ALOX15 gene), the dominant isoform in the brain, is known for its ability to damage mitochondria. Both we and others have recently suggested that 12/15-LOX contributes to brain damage after middle cerebral artery occlusion (MCAO). It has previously not been studied if levels of 12/15-LOX are increased after stroke, but its gene expression is known to be susceptible to ischemic preconditioning, and large amounts of 12/15-LOX metabolites have been found in ischemic gerbil brains. It therefore seemed reasonable to study if inhibition of 12/15-LOX would provide a therapeutic benefit following experimental stroke. Baicalein is a specific lipoxygenase inhibitor, but as a flavonoid also has antioxidant activity. Originally published as an inhibitor of platelet-type 12-LOX, it is also highly inhibitory toward the human 15-LOX isoform, and thus has the potential to inhibit the 12/15-LOX pathway. In several settings, baicalein has been shown to be neuroprotective. In this study, we sought to address the question whether baicalein protects the ischemic brain by inhibiting the 12/15-LOX pathway.

Materials and Methods

Ischemia Protocol

All experiments were performed following an institutionally approved protocol in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. A standard intraluminal middle cerebral artery method was used. General anesthesia was maintained with 1 to 1.5% halothane via face mask. Laser Doppler flowmetry was used to measure cerebral cortical microperfusion (3 mm lateral to bregma). Body temperature was monitored and maintained at 36.5°C to 37.5°C with a feedback heating pad. After midline skin incision, the right external carotid artery was exposed, and its branches were electrocoagulated. A 7.0 nylon monofilament coated with silicon was introduced into internal carotid artery through the external carotid artery to occlude the origin of the middle cerebral artery. To allow reperfusion, blood flow was restored by withdrawal of the nylon suture. All animals were assessed with laser Doppler flowmetry to confirm adequate induction of focal ischemia and successful reperfusion between experimental

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groups. Blood gases and blood pressure were measured before induction of ischemia and again in ischemic mice after 1 hour of ischemia. Male mice \( \sim 12 \) weeks old were used in all experiments.

**Immunohistochemistry for LOX**

To assess the distribution and expression of LOX after middle cerebral ischemia, anesthetized C57Bl6J mice (Jackson Laboratories; Bar Harbor, Maine) were perfused transcardially with ice-cold phosphate-buffered saline (PBS), pH 7.4, followed with ice-cold 4% paraformaldehyde in PBS, pH 7.4. The brains were removed, immersed in the same fixative overnight at 4°C, and cryoprotected in 15% and 30% sucrose solutions in PBS at 4°C. Frozen coronal sections (20-μm-thick) were prepared using a cryostat. After blocking with PBS containing 0.2% Triton X-100 and 3% normal goat serum, sections were incubated with secondary antibody (anti-rabbit FITC; 1:100; Jackson Immunoresearch) for 30 minutes. Negative control sections from ALOX15 knockout mice (Jackson Laboratories; Bar Harbor, Maine) received identical treatment. As an additional control, wild-type mice received identical treatment except for the primary antibody (data not shown). Colocalization with neurons was analyzed by double staining with an antibody to NeuN (Chemicon).

**Infarction Sizes**

Male ALOX15 knockout mice and the genetically matched C57Bl6J wild-type controls were obtained from Jackson Laboratories. Treatment with baicalein was by intraperitoneal injection of 300 mg/kg baicalein, dissolved in DMSO, 5 minutes before ischemia. Control animals received an equal volume DMSO. In addition to the mice reported here, 2, 2, and 5 mice died in the studies depicted in Figure 3A, 3C, and 3D, respectively. In addition, 4, 0, and 1 mice, respectively, were excluded from the study because of either no infarction at all or infarction extending to the posterior cerebral artery territory. Ischemic brain damage was assessed by 2,3,5-triphenyltetrazolium chloride (TTC) staining on 1-mm brain slices after 90 minutes of ischemia and 22 hours 30 minutes of reperfusion and evaluation using the ImageJ program.

**Oxidative Stress in Primary Neurons**

Primary neurons were isolated from E17 rats as described, seeded at \( 3 \times 10^4 \) cells/well in 24-well plates and treated after 18 hours in culture. Treatment consisted of exchanging the medium to 1 mL fresh culturing medium and adding 5 mmol/L glutamate (stock solution 400 mmol/L in PBS) to induce oxidative stress in the presence or absence of either baicalein (Cayman Chemicals) or the solvent DMSO (maximum 0.1% final concentration) as control. After 24 hours of treatment, medium was collected and the cells were lysed in 1 mL 0.5% Triton X-100, by incubating for 30 minutes at 37°C. Lactate dehydrogenase (LDH) content was determined separately for the cell extracts and corresponding media using a Cytotoxicity Detection Kit (Roche), and the percentage of lactate dehydrogenase released to the medium calculated after subtracting the corresponding background value. Results are given as mean±SEM.

**Results**

We first studied the relevance of 12/15-LOX to stroke pathology by determining its expression in a mouse model of transient MCAO by immunohistochemistry. No significant differences in physiological parameters were detected between the ALOX15 knockout mice and corresponding wild-type C57Bl6J mice when subjected to MCAO (Table), suggesting that differences in infarct size are not caused by differences in the vascular response. In healthy mice, LOX protein is only weakly detected using an antibody raised against the rabbit lipoygenase, 15-LOX-1 (data not shown). However, after 2 hours of transient focal cerebral ischemia and 4 hours of reperfusion, LOX levels were increased primarily in the peri-infarct region of the ischemic cortex (compare Figure 1A to Figure 1B, 1C, 1E, 1F). This region, adjacent to the core infarct, is taken to be at risk for subsequent damage. The specificity of these data were confirmed by the lack of LOX staining in ALOX15 knockout mice, in which the gene encoding 12/15-LOX has been deleted (Figure 1D). To confirm which cells exhibit the increased amounts of 12/15-LOX, we performed double labeling with an antibody directed against the neuronal marker NeuN. Most of the staining was associated with neurons (Figure 2A to 2C), although endothelial cell staining was also detected in some cases (data not shown).

These increased levels of 12/15-LOX in neurons of the peri-infarct region suggested to us that inhibiting neuronal LOX activity might be beneficial. Correspondingly, we tested baicalein, an inhibitor of the 12- and 15-lipoygenases, in primary cortical neurons subjected to oxidative stress (Figure 2D). At 10 μmol/L, baicalein efficiently reduced cell death by glutamate treatment.

In the next step, we treated mice subjected to MCAO with baicalein, applied by intraperitoneal injection 5 minutes before ischemia. After 90 minutes of ischemia and 22 hours 30 minutes of reperfusion, infarct sizes were significantly reduced compared with vehicle-treated mice (Figure 3A, 3B; n=6 per group). To investigate whether or not the beneficial effect of baicalein treatment is caused by lipoygenase inhibition, we next compared brain infarct sizes in ALOX15 knockout mice with corresponding wild-type mice after 90 minutes ischemia and 22 hours 30 minutes of reperfusion. Infarction sizes in the ALOX15 knockouts were significantly smaller than those in wild-type mice, similar to the effect of baicalein treatment in wild-type mice (Figure 3C; n=7 per group). If baicalein protected the ischemic mice through lipoygenase inhibition, then 12/15-LOX knockout mice should not derive an additional benefit from baicalein treatment. Indeed, baicalein treatment of the knockout mice did not provide significant additional protection (Figure 3D; n=4 per group). This result suggests that baicalein protects the ischemic brain mostly through inhibition of 12/15-LOX.
ase contributes to brain damage after stroke, and that 12/15-LOX inhibition by baicalein is neuroprotective.

**Discussion**

Lipoxygenases are a family of lipid-oxidizing enzymes that generate a wide array of pro- and anti-inflammatory mediators. The well-known 5-lipoxygenase and its activating protein, encoded by the gene ALOX5AP, have recently been intensely studied because of a genetic linkage to stroke risk.19–22 This must be separated from stroke severity, however, and deletion of the ALOX5 gene did not prove beneficial in a mouse model of transient focal ischemia.23 In contrast, the 12/15-lipoxygenase...
knockout mice $ALOX15^{−/−}$ have been shown by us and others to be protected. In addition, $12/15$-LOX may contribute to oxidative stress in mouse brains in vivo.

For the first time, the results presented here document an increase of $12/15$-LOX on the ischemic side of the infarcted brain, in the peri-infarct region of the cortex. This increase is detected predominantly in neurons, consistent with previous studies showing $12/15$-LOX involvement in neuronal cell death in vitro. In addition, we also found increased levels of $12/15$-LOX in endothelial cells in some cases, the significance of which we are currently investigating (data not shown). The strongest increases in $12/15$-LOX expression were thus detected in neurons and sometimes endothelial cells, but $12/15$-LOX in other cell types of the brain may also contribute to the effects on stroke pathophysiology shown here.

In contrast, the $ALOX15^{−/−}$ mice were not further protected by baicalein. This suggests that the protection afforded by baicalein may be mediated in large part through its inhibition of $12/15$-LOX. This inhibition is likely to be direct, because baicalein is known to inhibit the $12/15$-lipooxygenases in vitro with an IC50 in the low micromolar range, and baicalein has been modeled to interact directly with the iron located in the active site of the enzyme. Alternately, baicalein could also be decreasing oxidative stress, which would prevent activation of $12/15$-LOX. Either way, the decisive factor appears to be the activity of $12/15$-lipooxygenase, because its removal abolishes the protective effect of baicalein. In this initial study, infarct size reductions were apparent at 24 hours, indicating a critical role for $12/15$-LOX in acute ischemic injury. The effect of LOX inhibition on delayed infarct maturation and long-term outcome remains to be studied.

In summary, this is the first study to show that baicalein protects specifically through inhibition of the $12/15$-LOX pathway. There are 2 consequences of our findings: (1) we confirm that $12/15$-LOX is a valid drug target for stroke neuroprotective therapy, because its increased neuronal expression appears to be detrimental; and (2) baicalein should...
be further investigated for its therapeutic potential to combat stroke.

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Disclosures

None.

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