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Novel Lipoxygenase Inhibitors as Neuroprotective Reagents

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Abstract

The lipid-metabolizing enzyme 12/15-lipoxygenase (12/15-LOX) mediates cell death resulting from oxidative stress in both neurons and oligodendrocytes. Specifically, it may contribute to the pathophysiology of stroke and Alzheimer's and Parkinson's diseases. We report here that two of three specific 12/15-LOX inhibitors, derived from a virtual screen by computer modeling and validated by inhibition of recombinant human 15-LOX in vitro, are able to rescue both neuronal as well as oligodendroglial cells from cell death induced by oxidative stress. Thus, in a fairly streamlined process, an initial virtual screen of 50,000 compounds in a library of drug-like molecules has led to the identification of two novel drug candidates for targeting LOX. Future studies of these novel neuroprotective inhibitors of 12/15-LOX may provide new therapeutic opportunities to combat stroke and other neurodegenerative diseases.

Keywords

neurodegeneration; stroke; Alzheimer's disease; Parkinson's disease; lipoxygenase; neuron; oligodendrocyte; HT22; oxidative stress; drug screening; neuroprotection

Oxidative stress is a major mechanism implicated in a variety of neurodegenerative diseases; including stroke, Alzheimer's disease, and Parkinson's disease (Lin and Beal, 2006). 12/15-LOX, also known as *leukocyte-type 12-LOX* in mice and *15-LOX-1* in humans (Brash, 1999; Yamamoto et al., 1999), may be one of the key mediators in neurodegenerative disease, because it is triggered by reactive oxygen species (ROS). Once activated, 12/15-LOX generates lipid hydroperoxides that serve to amplify oxidative stress further (Kühn et al., 1990; our unpublished results). Whereas lipoxygenases typically oxidize free polyunsaturated fatty acids such as arachidonic acid, 12/15-LOX can also directly oxidize and damage organelle membranes (Kühn et al., 1990; van Leyen et al., 1998). Elevated amounts of 12/15-LOX have been found in experimental stroke in mice (van Leyen et al., 2006) and in early phases of Alzheimer's in humans (Pratico et al., 2004). Cell culture studies have implicated 12/15-LOX in neuronal models of oxidative stress related to Alzheimer's disease (Lebeau et al., 2004; Zhang et al., 2004) and Parkinson's disease (Li et al., 1997; Mytilineou et al., 2002). Finally,

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12/15-LOX knockout mice are protected in middle cerebral artery occlusion (MCAO) models of stroke (Khanna et al., 2005; van Leyen et al., 2006). All of these studies suggest that finding novel inhibitors of 12/15-LOX may expand treatment options for these neurodegenerative diseases.

At present, drug discovery is still a tedious process, with multiple rounds of complicated experiments, both in vitro and in vivo, each of which can lead to failure for any given drug candidate. Any approach that reduces either the number of testing rounds, the complexity of the assays involved, or the number of compounds to be tested in vivo would constitute substantial progress in drug discovery. One approach that has recently come to the forefront is virtual screening of chemical libraries, based on known protein structures either derived from X-ray crystallographic studies or computer generated based on known structures (Jacobson and Sali, 2004). Compared with random screening of unknown compounds, this approach can increase the likelihood of finding specific inhibitors of a given enzyme, because modeling is based on an interaction of the drug candidate with the active site of the target enzyme. Here we have used the results of a virtual computer screen that yielded new inhibitors of human 15-LOX (Kenyon et al., 2006) to study their neuroprotective qualities in a cultured neuronal cell line, followed by verification in primary neuronal and oligodendroglial cells. With this strategy, we have identified two compounds that may be good candidates for further studies into the protective efficacy of LOX inhibition against neurons and oligodendroglia.

MATERIALS AND METHODS

Antioxidant Test

The inhibitors LOXBlock-1 (catalog No. 5680672), LOXBlock-2 (catalog No. 6635967), and LOXBlock-3 (catalog No. 6640337) were obtained from ChemBridge (San Diego, CA) and dissolved in dimethyl sulfoxide (DMSO) at 1–20 mM concentration (1,000-fold concentrated). The antioxidant activity of these compounds was assayed by monitoring the quenching of the stable free radical 1,1-diphenyl-2-picrylhydrazyl (DPPH) upon reaction with the testing compound (Wang et al., 2004). A known free radical scavenger, nordihydroguaiaretic acid (NDGA), was used as a positive control. Ten microliters of 1 mM testing reagents to achieve final concentrations of 5 μM was added to 2 ml 500 μM DPPH stirring in a cuvette. Optical absorbance was monitored and recorded at 25-sec intervals as described elsewhere (Wang et al., 2004). The decrease in optical absorbance at 517 nm was monitored using a spectrophotometer (Lambda 40; Perkin Elmer). The rate of reaction is proportional to the antioxidant potency of the test compounds.

HT22 Cell Culture

Maintenance and incubation of HT22 cells (obtained from David Schubert by way of Rajiv Ratan's laboratory) was carried out as described previously (van Leyen et al., 2005). Briefly, HT22 cells were cultured in DMEM containing 10% fetal bovine serum and penicillin/streptomycin (all media from Invitrogen). For viability experiments, cells were seeded at 5×10^5 cells/well in 24-well plates (Corning) and treated when approximately 70% confluent. Treatment consisted of exchanging the medium with fresh medium containing the inhibitor or DMSO (0.1% final concentration), then adding 5 mM glutamate 5 min later. After 24 hr of incubation in the presence or absence of inhibitor, lactate dehydrogenase (LDH) was measured in the medium and in cell lysates from each well using a Cytotoxicity Detection Kit (Roche). Percentage survival was calculated after subtraction of background values by the formula percentage survival = 100 × LDHcells/(LDHmedium + LDHcells). Data are presented as mean ± SEM, averaged from three separate experiments performed in duplicate. Statistical significance was determined by using the Tukey-Kramer HSD test. The results from these tests are generally similar to the outcome detected with the MTT assay in this model.

Rat Primary Cortical Neurons

Primary neurons were isolated from E17 rats and treated as described elsewhere (Ratan et al., 2002; van Leyen et al., 2005). This protocol typically results in a culture enriched to over 90% in neuronal cells at 18 hr after seeding. To induce oxidative stress, the medium was exchanged against fresh DMEM/10% fetal bovine serum (FBS). Inhibitors or DMSO (final concentration 0.1%) were added, followed after 5 min by glutamate (final concentration 5 mM) as indicated. After 24 hr of incubation, cells were lysed by removal of medium and addition of 0.5% Triton X-100. Because of the high background values in the medium, percentage survival was calculated from the intracellular LDH values compared with control-treated cells. Data are presented as mean ± SEM, averaged from three separate experiments performed in duplicate. Statistical significance was determined by using the Tukey-Kramer HSD test.

Rat Primary Oligodendroglial Cells

Primary oligodendroglial cells ($O4^+$ $O1^-$; preoligodendrocytes) were isolated, maintained, and treated as described by Wang et al. (2004). For treatment, the cells were washed twice with medium containing basic fibroblast growth factor and platelet-derived growth factor (both from Peprotech, Princeton, NJ) but lacking cystine. After these washing steps, the cells were incubated in fresh medium lacking cystine for 24 hr in the presence or absence of inhibitors. Percentage survival was determined by LDH measurement in medium and cell lysates. Data are presented as mean ± SEM, averaged from three separate experiments performed in duplicate. Statistical significance was determined by using the Tukey-Kramer HSD test.

RESULTS

Novel Lipoxygenase Inhibitors LOXBlock-1, -2, and -3 Have Low Antioxidant Activities

In preliminary experiments described elsewhere, a virtual screen of a library of 50,000 drug-like compounds was performed by computer modeling against the homology models of human 15-LOX-1 and human 12-LOX (Kenyon et al., 2006). The 20 hits derived from this screen were then tested in an enzymatic assay against the recombinant human 15-LOX-1, leading to three candidates with IC_{50}s in the low micromolar range. We have termed these three inhibitors *LOXBlock-1*, *LOXBlock-2*, and *LOXBlock-3* (Fig. 1A). Structurally, the compounds are clearly not related to one another, reinforcing the utility of the virtual screening/in vitro testing approach.

To demonstrate that general redox chemistry was not the mechanism of inhibition, we compared the antioxidant activity of these compounds, because many antioxidants are also known to inhibit lipoxygenase. NDGA, here used as positive control, is known as both a LOX inhibitor and a strong antioxidant (Whitman et al., 2002). Correspondingly, it led to rapid quenching of a stable radical, DPPH, in a well-established antioxidant assay (Wang et al., 2004). In contrast, the three compounds studied showed either no (LOXBlock-1 and LOXBlock-2) or little (LOXBlock-3) antioxidant activity in this assay (Fig. 1B).

LOXBlock-1 and -3 Protect Against Oxidative Glutamate Toxicity

We next tested these compounds in a simple cell culture-based screen, in which exogenously added glutamate leads to cell death through oxidative stress in a neuron-like mouse hippocampal cell line, HT22. This form of oxidative stress has previously been shown to depend on glutathione depletion and activation of 12/15-LOX (Li et al., 1997). Conversely, glutamate receptors do not appear to be responsible for the toxicity of glutamate in this model. In measuring leakage of intracellular LDH into the cell culture medium as an indicator of cell death, the compounds LOXBlock-1 and LOXBlock-3 showed strong neuroprotection at low micromolar concentration (Fig. 1C). In contrast, the third compound, LOXBlock-2, did not

show significant benefit. In separate experiments carried out in the absence of glutamate (data not shown), LOXBlock-2 showed some toxicity against these cells, suggesting that LOXBlock-2 might also affect other, unknown targets besides 12/15-LOX.

Rat Primary Cortical Neurons are Protected Against Oxidative Stress by LOXBlock-1 and -3

Although neuronal cell lines such as HT22 are excellent screening tools and very well suited for mechanistic studies, they do not always accurately reflect all characteristics of the primary cells from which they originate. To investigate further whether the protection through the novel LOX inhibitors also applies to brain-derived cells in primary culture, we subjected rat cortical primary neurons to oxidative glutamate toxicity. Increasing amounts of LOXBlock-1 and of LOXBlock-3 protected primary neurons in a dose-dependent manner (Fig. 2A,C, respectively). Again, LOXBlock-2 did not provide protection against glutamate treatment, and increasing concentrations of LOXBlock-2 led to increased cell death (Fig. 2B).

LOXBlock-1 and -3 Protect Oligodendroglial Cells

In addition to injury to neurons in the brain, recent studies have highlighted the importance of white matter deficiencies for neurological damage. We therefore investigated whether the novel LOX inhibitors might also protect oligodendrocytes against oxidative stress. Primary oligodendroglial cells ($O4^+$ $O1^-$) are subjected to oxidative stress when deprived of exogenous cystine, a building block needed for synthesis of the intracellular antioxidant glutathione. Similar to oxidative glutamate toxicity but in the absence of added glutamate, a depletion of intracellular glutathione follows. This typically leads to cell death after 24 hr. In a result strikingly similar to what was seen in neurons, both LOXBlock-1 and LOXBlock-3 were efficient protectors against oxidative stress (Fig. 2D–F). Interestingly, almost full protection was reached at 2 μM concentration for each compound, indicating that oligodendroglial cells are protected even more efficiently than primary neurons. Taken together, these results suggest that the novel LOX inhibitors LOXBlock-1 and LOXBlock-3 have the potential to protect against both gray matter and white matter injury.

DISCUSSION

We report here an efficient screening process for evaluating novel lipoxygenase inhibitors for their neuroprotective qualities. The path chosen to identify these candidate lipoxygenase inhibitors provides several advantages over more traditional methods. Because these compounds have been tested against the human enzyme, any positive results in animal models are likely to translate well to the human situation. With a relatively simple four-step process, two novel candidates have been filtered out of a library of 50,000 drug-like compounds that had previously not been evaluated for their neuroprotective qualities. The initial steps, identifying molecules that could bind the active site of human 15-lipoxygenase in a computer-based screen, followed by testing the in vitro efficacy in inhibiting the enzymatic activity of recombinant human 15-LOX, have been described in a previous publication (Kenyon et al., 2006). To this we have added a simple cellular assay to measure protection against oxidative stress in a neuronal cell line, HT22. These cells are easy to handle and could readily be adapted for high-throughput screening. Glutamate-induced oxidative stress leads to a lipoxygenase-dependent cell death in this cell line (Li et al., 1997). Among the three compounds that had been shown to inhibit human 15-LOX in vitro, two efficiently protected the HT22 cells against oxidative glutamate toxicity, LOXBlock-1 and LOXBlock-3. The third, LOXBlock-2, was not an effective neuroprotectant in this assay. Because of this somewhat surprising result, we repeated the experiment with both higher and lower concentrations of LOXBlock-2, ranging from 100 nM to 40 μM, to investigate whether protection could be achieved at these concentrations. Solubility in DMSO of LOXBlock-2 appears to be satisfactory, in that higher concentrations of the stock solution (40 mM) appeared clear, without a residual pellet. The

lower concentrations were at best marginally protective against glutamate, but 20 μM and 40 μM LOXBlock-2 led to reduced survival of HT22 cells, suggesting some toxicity of the compound (data not shown).

After this screening process, we tested whether rat-derived primary brain cells are also protected against this form of lipoxygenase-mediated cell death. It has recently become clear that, to protect against ischemia/reperfusion injury, it is not sufficient to salvage gray matter, but white matter must also be rescued (Dewar et al., 1999). It is therefore useful to investigate the effect of a neuroprotective drug not just on neurons, but also on the oligodendrocytes that provide the supportive myelin sheaths for neuronal axons. Both primary neurons and cells of the oligodendrocyte lineage were protected with similar efficiency by LOXBlock-1 and by LOXBlock-3, whereas LOXBlock-2 again provided no protection. Possibly, LOXBlock-2 also affects another target besides 12/15-LOX, but in any case this compound does not seem to be a good candidate for further studies.

Surprisingly, both LOXBlock-1 and LOXBlock-3 were protective at lower concentrations than their inhibitory concentrations against the human 15-LOX in vitro. Several factors may account for this discrepancy. The efficacy against mouse and rat 12/15-LOX may be somewhat higher than that against the human enzyme. Also, the experimental conditions used to measure the inhibition of human 15-LOX in vitro may not be optimal; indeed, the addition of detergent can have a major impact on the inhibitory concentration measured. In this context, it is interesting to note that the lipoxygenase-dependent cell death in neural cells may depend on the oxidation of intracellular membranes, not of free arachidonic acid, as measured in the in vitro screen. In any case, the fairly good protection afforded by both LOXBlock-1 and LOXBlock-3 even at 2 μM concentration suggests that these may be efficient neuroprotectants. Importantly, these compounds were identified in a computer screen by their potential to fit into the active site of human 12-LOX or human 15-LOX. This, together with the finding that LOXBlock-3 exhibits only mildly antioxidant properties and LOXBlock-1 is clearly not an antioxidant, suggests that these inhibitors function through direct inhibition of 12/15-LOX.

The results of this study may be applicable to neuroprotection in a variety of neurodegenerative diseases where oxidative stress is a major cause of injury. The best-studied example of this may be stroke, one of the deadliest and most debilitating diseases. Much has been learned in recent years about mechanisms leading to brain damage following stroke, but as yet this knowledge has not translated into successful drug compounds. At the same time, there is a dire need for treatment options, given that tissue plasminogen activator (tPA) is currently the only drug with FDA approval. Similarly, although there are therapies that relieve symptoms in Alzheimer's and Parkinson's diseases, no effective neuroprotectants exist for these chronic neurodegenerative disorders either. Furthermore, LOX inhibitors might be suitable as preventive agents in premature infants and sick term infants, whose developing brains seem to be very susceptible to oxidative injury. Oxidative stress to preoligodendrocytes in the developing white matter has been implicated in the pathogenesis of periventricular leukomalacia, the lesion underlying most cases of cerebral palsy in premature infants (Wang et al., 2004; Gerstner et al., 2006). Therefore, the LOX inhibitors identified by our stepwise screening process here may provide new opportunities for targeting a wide range of disorders. The two novel 12/15-LOX inhibitors, LOXBlock-1 and LOXBlock-3, may now be evaluated for their pharmacological properties in vivo.

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REFERENCES

Brash AR. Lipoxygenases: occurrence, functions, catalysis, and acquisition of substrate. J Biol Chem 1999;274:23679–23682. [PubMed: 10446122]

Dewar D, Yam P, McCulloch J. Drug development for stroke: importance of protecting cerebral white matter. Eur J Pharmacol 1999;375:41–50. [PubMed: 10443563]

Gerstner B, Buhrer C, Rheinlander C, Polley O, Schuller A, Berns M, Obladen M, Felderhoff-Mueser U. Maturation-dependent oligodendrocyte apoptosis caused by hyperoxia. J Neurosci Res 2006;84:306–315. [PubMed: 16673399]

Jacobson MP, Sali A. Comparative protein structure modeling and its applications to drug discovery. Annu Rep Med Chem 2004;39:259–276.

Kenyon V, Chorny I, Carvajal WJ, Holman TR, Jacobson MP. Novel human lipoxygenase inhibitors discovered using virtual screening with homology models. J Med Chem 2006;49:1356–1363. [PubMed: 16480270]

Khanna S, Roy S, Slivka A, Craft TK, Chaki S, Rink C, Notestine MA, DeVries AC, Parinandi NL, Sen CK. Neuroprotective properties of the natural vitamin E alpha-tocotrienol. Stroke 2005;36:2258–2264. [PubMed: 16166580]

Kühn H, Belkner J, Wiesner R, Brash AR. Oxygenation of biological membranes by the pure reticulocyte lipoxygenase. J Biol Chem 1990;265:18351–18361. [PubMed: 2120232]

Lebeau A, Terro F, Rostene W, Pelaprat D. Blockade of 12-lipoxygenase expression protects cortical neurons from apoptosis induced by beta-amyloid peptide. Cell Death Differ 2004;11:875–884. [PubMed: 15105833]

Li Y, Maher P, Schubert D. A role for 12-lipoxygenase in nerve cell death caused by glutathione depletion. Neuron 1997;19:453–463. [PubMed: 9292733]

Lin MT, Beal MF. Mitochondrial dysfunction and oxidative stress in neurodegenerative diseases. Nature 2006;443:787–795. [PubMed: 17051205]

Mytilineou C, Kramer BC, Yabut JA. Glutathione depletion and oxidative stress. Parkinsonism Rel Disord 2002;8:385–387.

Pratico D, Zhukareva V, Yao Y, Uryu K, Funk CD, Lawson JA, Trojanowski JQ, Lee VM. 12/15-Lipoxygenase is increased in Alzheimer's disease: possible involvement in brain oxidative stress. Am J Pathol 2004;164:1655–1662. [PubMed: 15111312]

Ratan RR, Ryu H, Lee J, Mwidau A, Neve RL. In vitro model of oxidative stress in cortical neurons. Methods Enzymol 2002;352:183–190. [PubMed: 12125346]

van Leyen K, Duvoisin RM, Engelhardt H, Wiedmann M. A function for lipoxygenase in programmed organelle degradation. Nature 1998;395:392–395. [PubMed: 9759730]

van Leyen K, Siddiq A, Ratan RR, Lo EH. Proteasome inhibition protects HT22 neuronal cells from oxidative glutamate toxicity. J Neurochem 2005;92:824–830. [PubMed: 15686484]

van Leyen K, Kim HY, Lee SR, Jin G, Arai K, Lo EH. Baicalein and 12/15-lipoxygenase in the ischemic brain. Stroke 2006;37:3014–3018. [PubMed: 17053180]

Wang H, Li J, Follett PL, Zhang Y, Cotanche DA, Jensen FE, Volpe JJ, Rosenberg PA. 12-Lipoxygenase plays a key role in cell death caused by glutathione depletion and arachidonic acid in rat oligodendrocytes. Eur J Neurosci 2004;20:2049–2058. [PubMed: 15450084]

Whitman S, Gezginci M, Timmermann BN, Holman TR. Structure-activity relationship studies of nordihydroguaiaretic acid inhibitors toward soybean, 12-human, and 15-human lipoxygenase. J Med Chem 2002;45:2659–2661. [PubMed: 12036375]

Yamamoto S, Suzuki H, Nakamura M, Ishimura K. Arachidonate 12-lipoxygenase isozymes. Adv Exp Med Biol 1999;447:37–44. [PubMed: 10086181]

Zhang Y, Wang H, Li J, Jimenez DA, Levitan ES, Aizenman E, Rosenberg PA. Peroxynitrite-induced neuronal apoptosis is mediated by intracellular zinc release and 12-lipoxygenase activation. J Neurosci 2004;24:10616–10627. [PubMed: 15564577]

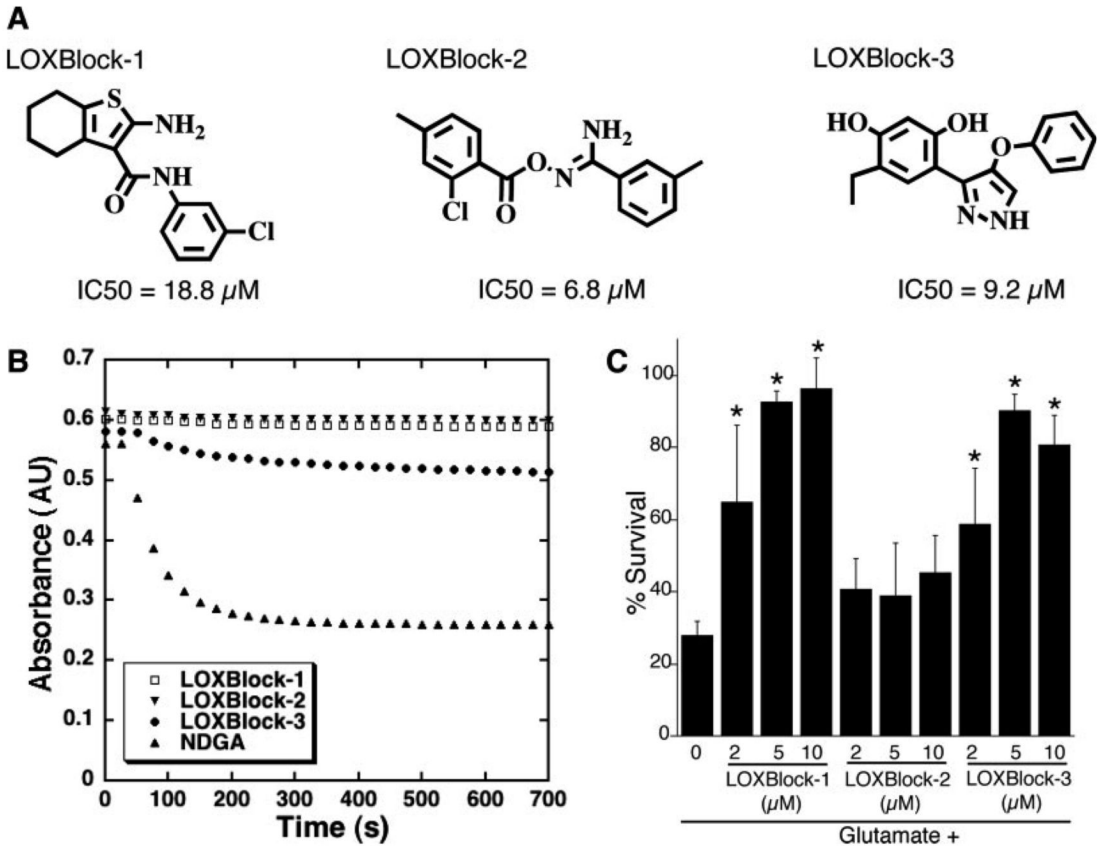

Fig. 1.

A: Chemical structures of the LOX inhibitors LOXBlock-1, LOXBlock-2, and LOXBlock-3. **B:** Antioxidant activity assay of LOXBlock-1, LOXBlock-2, and LOXBlock-3. Antioxidant activity was assessed by monitoring quenching of the stable free radical DPPH upon reaction with the testing compounds. NDGA (5 μM) was used as a positive control. LOXBlock-1, LOXBlock-2, and LOXBlock-3 (all 5 μM) were tested in the assay. LOXBlock-1 and LOXBlock-2 do not quench DPPH, whereas minor quenching is observed with LOXBlock-3. **C:** Protection of HT22 cells from oxidative glutamate toxicity. Survival of glutamate-treated cells was enhanced by coincubation with either LOXBlock-1 or LOXBlock-3, but LOXBlock-2 did not significantly protect the cells. ⋆$P < 0.05$ compared with glutamate alone.

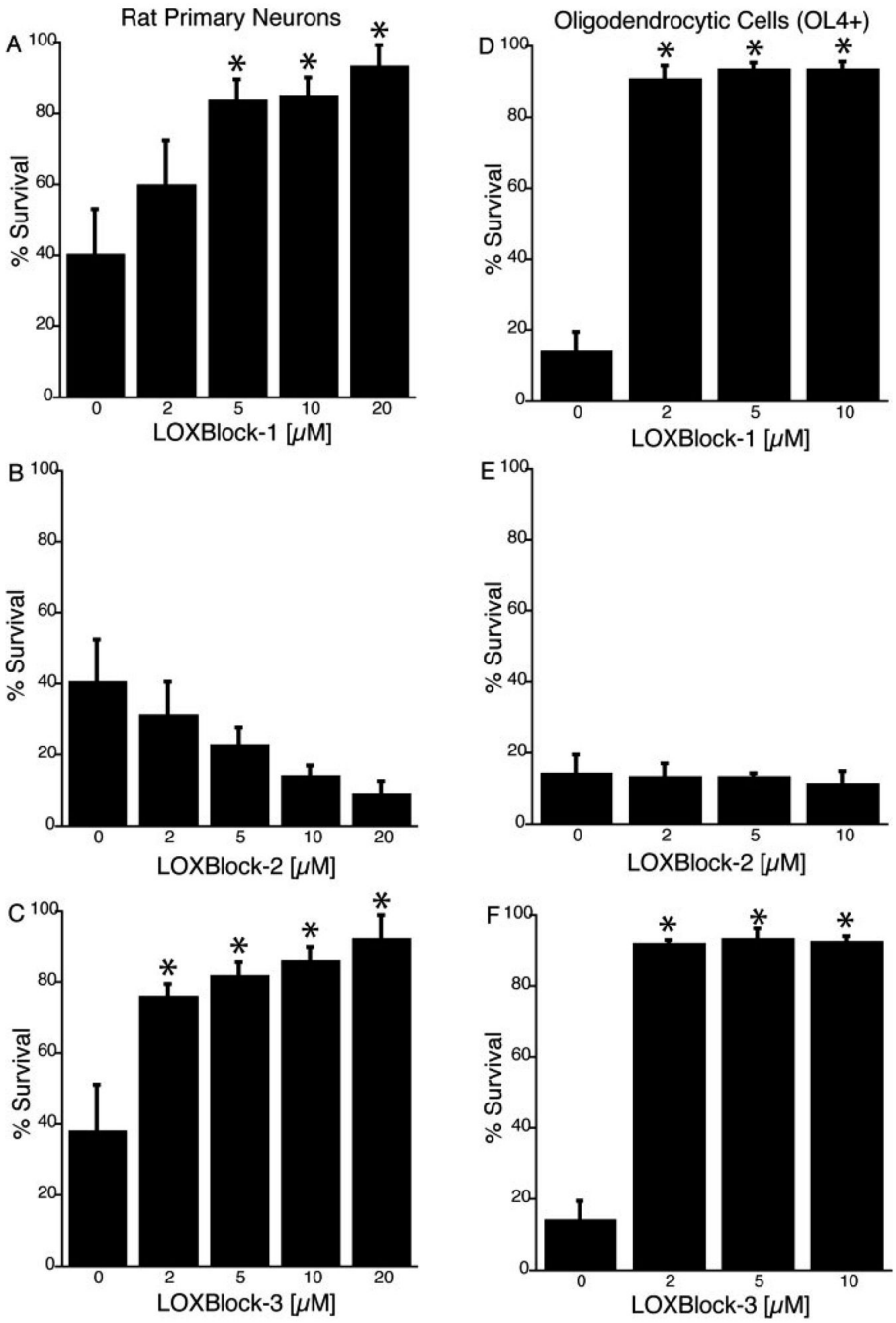

Fig. 2.

Protection of brain-derived primary cells from LOX-mediated cell death. **A–C:** Rat primary neurons subjected to oxidative glutamate toxicity were protected in a dose-dependent fashion by coincubation with LOXBlock-1 and LOXBlock-3, but not by LOXBlock-2. **D–F:** Likewise, oligodendrocyte survival following oxidative stress was greatly improved in the presence of LOXBlock-1 and LOXBlock-3. $^{\star}P < 0.05$ compared with glutamate alone.