Lipoxygenase inhibitors protect brain cortex macromolecules against oxidation evoked by nitrosative stress

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Abstract

Lipoxygenases (LOX) are a family of enzymes that are responsible for the metabolism of arachidonic and docosahexaenoic acid and for the formation of several eicosanoids and docosanoids, including leukotrienes, lipoxins and neuroprotectins. Depending on cells' redox state and other milieu conditions, these enzymes are engaged in oxidative stress and cell death mechanisms or in cell protection. In this study the antioxidative properties of several inhibitors of LOX isoforms were evaluated. We investigated the effect of a non-selective inhibitor of all LOXs and selective inhibitors of 5-LOX and 12-LOX on lipid and protein oxidation in brain cortex subjected to nitrosative stress, on dityrosine formation and on survival of an immortalized clonal mouse hippocampal cell line (HT22). The nitrosative stress was induced by nitric oxide (NO) donor, 0.5 mM sodium nitroprusside (SNP) and peroxynitrite (0.03 mM). Our data showed that nitrosative stress led to significant enhancement of lipid peroxidation and carbonyl group formation in brain cortex homogenate compared to control. Inhibitor of all LOXs nordihydroguaiaretic acid, 5-LOX inhibitors (zileuton, BWB70C), and inhibitors of 12/15-LOX baicalein and AA-861 (also an inhibitor of 5-LOX) significantly reduced, in a concentration dependent manner (1-10 μM), the level of lipid and protein oxidation. However, AA-861 and zileuton had no effect on carbonyl group formation. Moreover, we observed that LOX inhibitors protected a significant pool of HT22 cells against death evoked by 0.5 mM SNP. In summary, our results indicate that all LOX inhibitors in concentrations above 1-2.5 μM demonstrated antioxidative properties. These results should be taken into consideration during evaluation of experimental and clinical effects of LOX inhibitors.

Key words: lipoxygenases, nitrosative stress, brain cortex, HT22 cells.

Introduction

Lipoxygenases (LOXs) are a family of lipid peroxidizing enzymes that insert molecular oxygen into free form as well as esterified polyunsaturated fatty acids (PUFA). LOXs express a major function: they are involved in generation of several bioactive compounds (eicosanoids and docosanoids) that play an important role as mediators of inflammation and other signalling pathways. Moreover, these enzymes through lipid and protein oxidation influence membrane structure, properties and function [22,29]. The most abundant LOX isoform in the CNS is 12/15-LOX, while 12(S)-HETE and 15(S)-HETE are the major LOX metabolites in the brain. These metabolites play an important role as second messengers in synaptic transmission and are in-

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volved in learning and memory processes. 12/15-LOX has been described abundantly in neurons and in some glial cells throughout the cerebrum, hippocampus and basal ganglia [11,31]. Oxidative stress and inflammatory reactions may increase expression and activity of 12/15-LOX and other LOX isoforms. These enzymes initiate chemical reactions which modify the fatty acids and make them more susceptible to further oxidation [11,40]. The data demonstrated that reactive oxygen species-mediated neuronal lipids and protein oxidation were extensive in areas affected by Alzheimer's disease (AD). Oxidative imbalance was an important event in AD pathogenesis [6,32]. It was observed that 12/15-LOX activity was increased in pathologically affected areas of AD brains [40]. It was also shown that the metabolites of 12/15-LOX, 12(S)-HETE and 15(S)-HETE, significantly increased in the brains of AD patients, compared to control healthy brains. This increase correlated with brain lipid peroxidation and with alteration of tau protein. Genetic deletion of these enzymes reduces the cellular oxidative stress [32,40]. The last data also indicated the significant role of 5-LOX in AD pathology [18]. Our data indicated that amyloid beta peptides (Aβ)-mediated nitric oxide (NO) release was responsible for the enhancement of liberation of arachidonic acid (AA), the substrate for LOXs and cyclooxygenases (COXs) [10]. Nitric oxide plays an essential role as an extracellular and intracellular messenger, but excessive amounts of NO are toxic. NO interacts with superoxide radical, leading to formation of peroxynitrite anion [8,25,30, 34]. NO and peroxynitrite can induce dityrosine formation, nitrosylation and nitration of proteins [20, 30,39]. These alterations have been implicated in the pathology of multiple diseases, such as atherosclerosis, Alzheimer's and Parkinson's diseases, and in inflammatory neurodegeneration [7,15,39]. The activity of LOXs is connected with liberation of superoxide radicals that may accelerate oxidative stress through interaction with NO. Then oxidative stress and activation of ERK and PKC kinases lead to maximal cPLA$_2$ activation and AA release and then to further stimulation of LOXs, closing the vicious circle. Both LOX and COX isoforms play important roles not only in AD and other neurodegenerative disorders but also in brain ischaemia-reperfusion injury, in hypoxia and in glucose deprivation and glucose reload in diabetes [12,26,28,38,41]. LOXs through production of leukotrienes are involved in the pathomechanism of asthma [19,35,36]. The inhibitors of LOXs could offer a novel therapeutic strategy for these above-mentioned disorders. Several inhibitors of

LOXs are available and used in preclinical studies and some of them have been introduced to clinical practice. However, the specificity and mechanism of action of LOX inhibitors are still not well understood.

The aim of this study was to evaluate the effect of selected LOX inhibitors on nitrosative stress induced by NO donor in a cell-free system of brain cortex. Moreover, the action of these inhibitors on hippocampal HT22 cell survival when subjected to SNP was investigated.

Material and methods

Chemicals

LOX inhibitors: *NDGA* – 1,4-bis (3,4-dihydroxyphenyl)-2,3-dimethylbutane, inhibitor of all LOX isoforms; *Baicalein* – 5,6,7-trihydroxyflavone, 12/15-LOX inhibitor; *BWB70C* – N-[3-[3-4 (-fluorophenoxy)phenyl]-1-methyl-2-propenyl]-N-hydroxyurea, dual 5-LOX and COX inhibitor; *AA-861* – 2-(12-hydroxydodeca-5,10-diynyl)-3,5,6-trimethyl-p-benzoquinone, 5-LOX and 12/15-LOX inhibitor. These compounds were purchased from Sigma (St. Louis, MO, USA). *Zileuton*, a 5-LOX inhibitor, was purchased from Cayman Chemical.

Others: Dimethyl sulfoxide (DMSO) and sodium nitroprusside (SNP) were purchased from Sigma (St. Louis, MO), 2-thiobarbituric acid from Merck KgaA (Darmstadt, Germany) and sodium peroxynitrite from Cayman Chemical (Ann Arbor, MI). DMSO was used as a solvent for tested compounds.

Animals

Male Wistar rats (250 g) were supplied by the Animal House of Mossakowski Medical Research Centre, Warsaw, Poland. All experiments on animals were approved by the Polish National Ethics Committee, and were carried out in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC).

For analysis of protein and lipid oxidation adult male Wistar rats were decapitated, their brains were removed, and brain cortices were isolated on ice and frozen at –80°C. Brain cortex was homogenized directly in ice-cold 50 mM Tris-HCl buffer, pH 7.4, to obtain 10% homogenate.

Cell culture

The studies were carried out using immortalized clonal mouse hippocampal cell line HT22. These cells were cultured in Dulbecco's modified Eagle's medium

supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1% penicillin/streptomycin (50 U/ml) and 2 mM glutamine at 37°C in a humidified incubator in 5% CO_2 atmosphere. Confluent cells were subcultured into 35 mm² dishes and used for experiments at 75-90% confluence.

Cell treatment protocols

Equal HT22 cell numbers were seeded into a 24-well 0.1% polyethyleneimine coated plate and after 24 h the growth medium was changed to low-serum (2% FBS) medium. Then HT22 cells were treated with SNP at 0.5 mM and incubated for 24 h with SNP alone or together with LOX inhibitors.

Determination of cell survival using MTT test

Mitochondrial function and cellular viability were evaluated by using 2-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). After treatment with investigated compounds, MTT (2.5 mg/ml) was added to all wells. The cells were incubated at 37°C for 2 h. Then cells were lysed and spectrophotometric measurement at 595 nm was performed.

Hoechst immunostaining

For morphological studies HT22 cells were subjected for 24 h to nitrosative stress evoked by NO donor 0.5 mM SNP together with LOX inhibitors. Nuclei were visualized with Hoechst 33342 (0.2 μg/ml, Riedel-de-Haën Germany) fluorescent staining. Plates containing HT22 cells were collected and washed in PBS. The cells were examined under a fluorescence microscope (Olympus BX51, Japan) and photographed with a digital camera (Olympus DP70, Japan).

Experimental conditions for dityrosine formation in protein-free system

Experiments were performed in the absence of protein using 0.5 mM tyrosine which was incubated with 30 μM peroxynitrite in the absence and presence of particular inhibitors of lipoxygenase at a concentration range of 1-10 μM in 200 mM Tris-HCl buffer pH 7.4. The fluorescence of formed dityrosine was determined spectrofluorimetrically at 325 nm excitation and 405 nm emission. The whole procedure was carried out according to [17].

It was found that with the exception of baicalein none of the tested compounds interfered with dityro-

sine fluorescence (data not shown). The effect of each investigated inhibitor at 1-10 μM in the presence of tyrosine and in the presence and absence of peroxynitrite was investigated.

Determination of the effect of lipoxygenase inhibitors on macromolecule oxidation

Protein oxidation

Protein oxidation was analysed by determination of the content of carbonyl groups. Brain cortex homogenate was incubated in a medium containing 50 mM Tris-HCl, pH 7.4, in the presence of 0.5 mM SNP. Incubation was carried out for 15 min at 37°C without and with the tested LOX inhibitor in a final volume of 0.2 ml. After incubation of the homogenate carbonyl group concentration was determined, as described previously [14,27].

Lipid peroxidation

To determine the effect of inhibitors on lipid peroxidation, the homogenates of brain cortex and hippocampus were incubated in the presence of 0.5 mM SNP. Incubation was carried out for 15 min at 37°C with the tested compound in a final volume of 0.2 ml. Thiobarbituric acid reactive substances (TBARS) were determined as an index of lipid peroxidation [1,14].

Statistical evaluation

All experiments were carried out at least three times in triplicate. The data are means ± SEM. Analysis was performed using one-way ANOVA and Newman-Keuls as a post-hoc test.

Results

To evaluate the action of LOX inhibitors, several experimental approaches were used in the studies. Our data indicated that nitrosative stress evoked by NO donor SNP significantly increased lipid peroxidation, as determined by the level of TBARS, the final product of free radical-dependent lipid oxidation. SNP activated lipid peroxidation in brain cortex homogenate in a concentration and time dependent manner (Fig. 1A,B). Significant enhancement of TBARS concentration was observed at 0.1 mM SNP in brain cortex homogenates after 1 h of incubation (Fig. 1A). The further enhancement of SNP concentration and time of incubation had a limited stimulatory effect (Fig. 1A,B). TBARS were increased 3-fold by
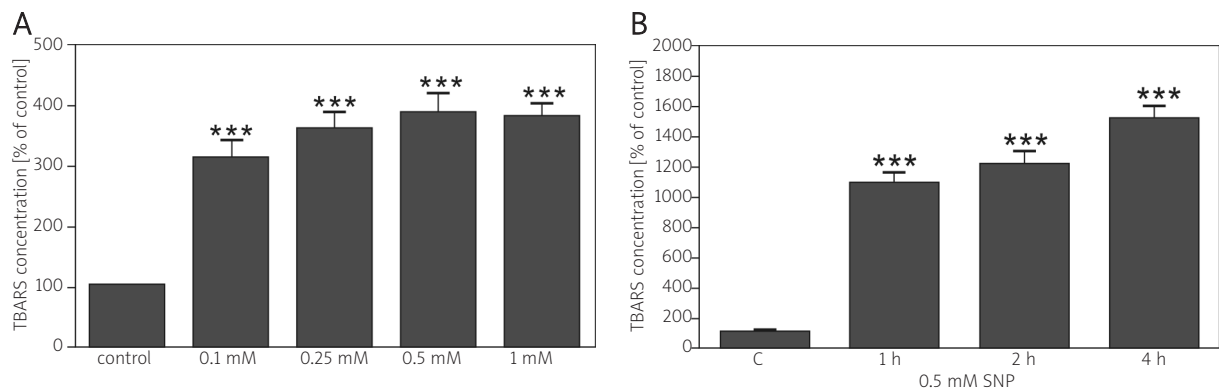
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Fig. 1. A) Effect of SNP concentration on lipid peroxidation of brain cortex. Brain cortex homogenates were incubated for 15 min at 37°C with different concentration of SNP. **B)** Effect of 0.5 mM SNP on brain cortex lipid peroxidation depending on time of incubation. Brain cortex homogenate was incubated for 1-4 h at 37°C. Lipid peroxidation was determined by measurement of TBARS. All values are means ± SEM from three experiments carried out in triplicate. Statistical significance compared to the control group: ***$P < 0.005$.
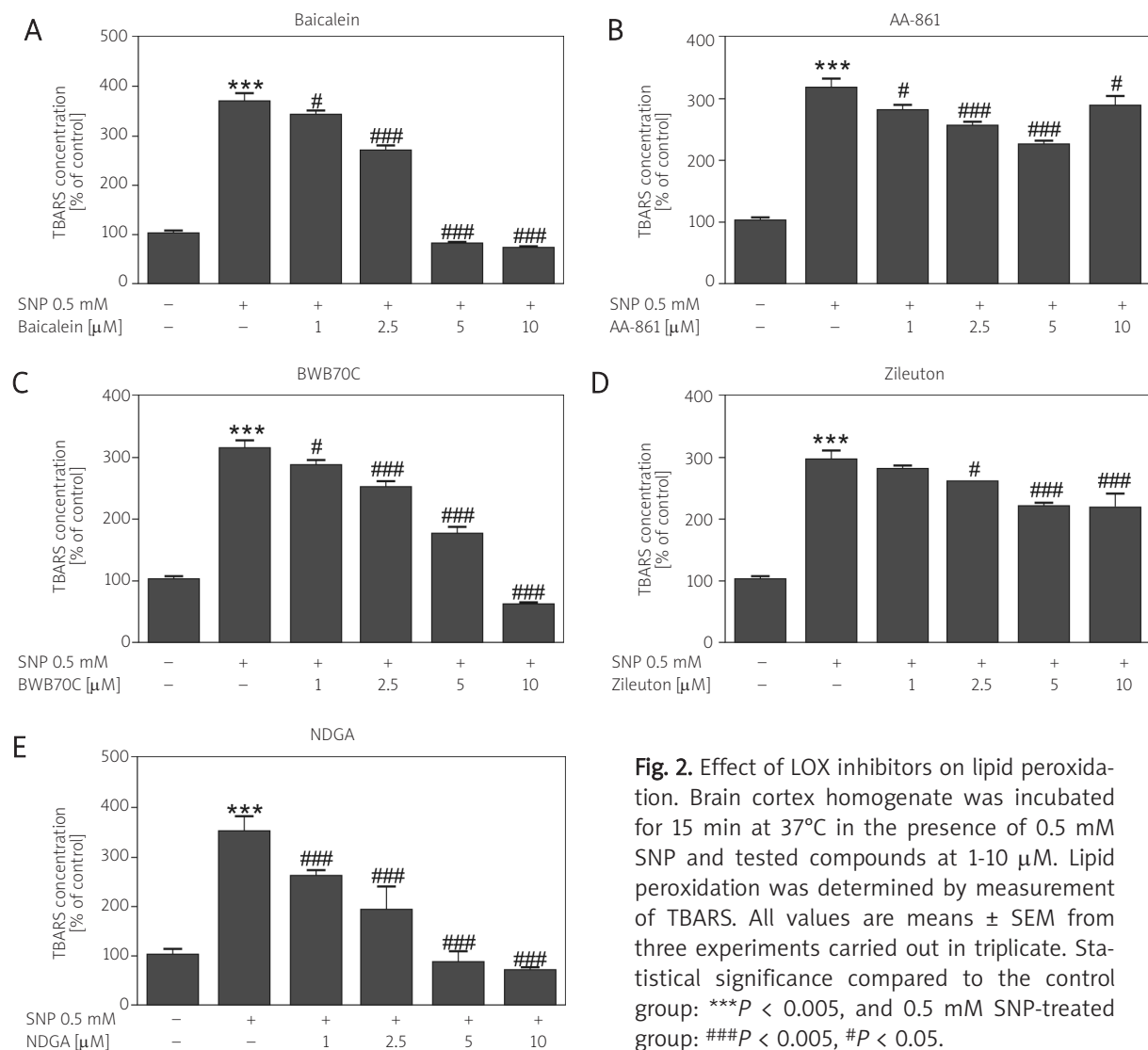

Fig. 2. Effect of LOX inhibitors on lipid peroxidation. Brain cortex homogenate was incubated for 15 min at 37°C in the presence of 0.5 mM SNP and tested compounds at 1-10 μM. Lipid peroxidation was determined by measurement of TBARS. All values are means ± SEM from three experiments carried out in triplicate. Statistical significance compared to the control group: ***$P < 0.005$, and 0.5 mM SNP-treated group: ###$P < 0.005$, #$P < 0.05$.
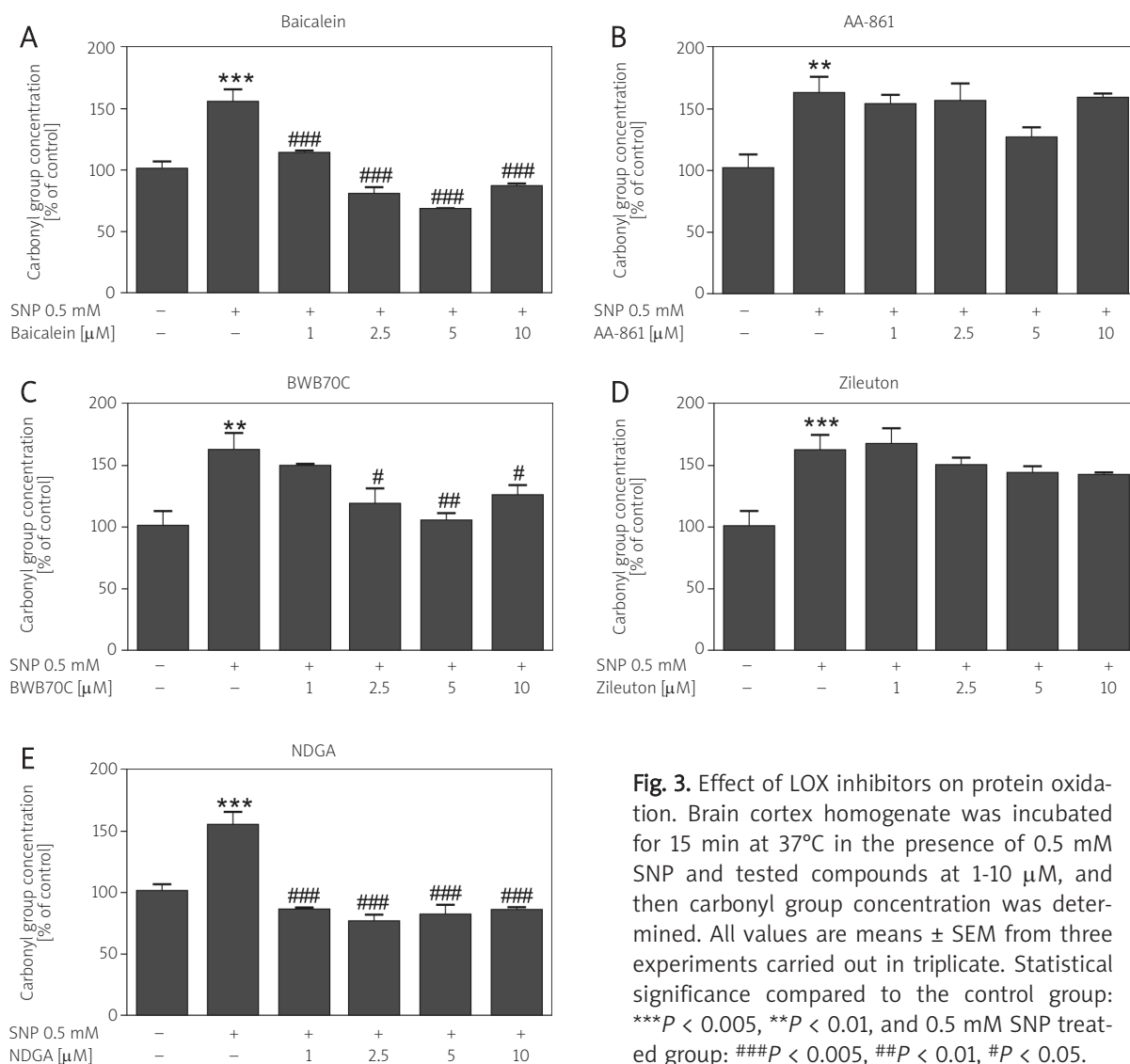
Fig. 3. Effect of LOX inhibitors on protein oxidation. Brain cortex homogenate was incubated for 15 min at 37°C in the presence of 0.5 mM SNP and tested compounds at 1-10 μM, and then carbonyl group concentration was determined. All values are means ± SEM from three experiments carried out in triplicate. Statistical significance compared to the control group: ***$P < 0.005$, **$P < 0.01$, and 0.5 mM SNP treated group: ###$P < 0.005$, ##$P < 0.01$, #$P < 0.05$.

0.1 mM SNP, compared to control conditions without SNP. The significant lipid oxidation induced by nitrosative stress was suppressed by the 5-LOX inhibitor BWB70C, zileuton and by the 12/15-LOX inhibitor baicalein, and also by NDGA, the inhibitor of all LOX isoforms. The other 12/15-LOX and 5-LOX inhibitor, AA-861, also exerted a protective effect against nitrosative stress induced by 0.5 mM SNP. All LOX inhibitors protected brain lipid against oxidation evoked by SNP (Fig. 2A-E). Nitrosative stress enhanced protein oxidation evaluated by the estimation of carbonyl group level. A significant pronounced antioxidative effect was observed in the presence of NDGA, baicalein and BWB70C. The other inhibitors,

zileuton and AA-861, had no effect on carbonyl group formation, but, as described above, significantly reduced lipid oxidation (Fig. 3A-E). The antioxidative effect of all LOX inhibitors was further confirmed in the protein-free system, where they exerted a protective effect against dityrosine formation in the presence of tyrosine and peroxynitrite (Fig. 4A-D). All LOX inhibitors with the single exception of baicalein had a significant concentration dependent inhibitory effect on dityrosine formation. Baicalein itself exerted endogenous fluorescence and was excluded from this study (data not shown).

In the following experiments immortalized hippocampal neuronal HT22 cells were subjected to
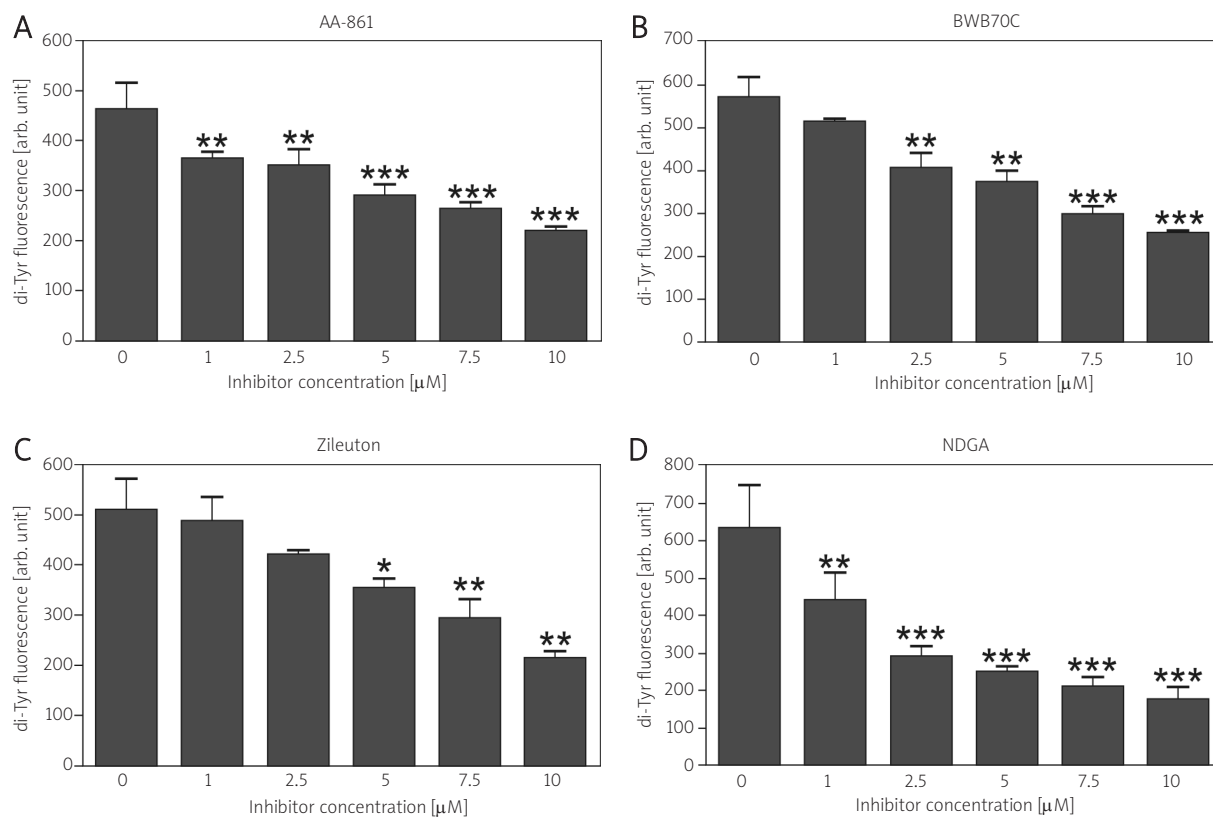
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Fig. 4. Effect of LOX inhibitors on dityrosine formation. L-tyrosine (0.5 mM) was incubated in 200 mM Tris-HCl buffer pH 7.4 for 10 min at 37°C in the presence of 30 µM ONOO⁻ and tested compounds at 1-10 µM. All values are means ± SEM from three experiments carried out in triplicate. Statistical significance compared to the control group: ***P < 0.005, **P < 0.01, *P < 0.05.

nitrosative stress for 24 h by using 0.5 mM SNP. Under these conditions about 60% of cells died. LOX inhibitors had a protective effect on cell death evoked by 0.5 mM SNP (Fig. 5A-D). Inhibitors of LOXs (zileuton, BWB70C, AA-861, baicalein and NDGA) also reversed morphological changes evoked by 0.5 mM SNP (cytoplasm shrinkage and degeneration of neuritic processes, apoptotic cell death) that were observed in the fluorescence microscopic study (Fig. 6A-F).

Discussion

Our data demonstrated that the inhibitor of all lipoxygenase isoforms (NDGA), inhibitors of 12/15-LOX baicalein and AA-861 and 5-LOX inhibitors protected brain cortex lipids and proteins against oxidation evoked by NO donor SNP. In concentrations over 1-2.5 µM all investigated compounds exerted an anti-

oxidative effect. NDGA, baicalein and BWB70C significantly reduced endogenous lipid oxidation also in the absence of SNP (data not shown). Moreover, it was found that LOX inhibitors protected tyrosine against peroxynitrite oxidation in the absence of protein. However, inhibitors of COXs remained without effect on lipid and protein oxidation evoked by nitrosative stress, which indicates their specific action (data not shown). In our study we have evaluated the concentration dependent effect of most available LOX inhibitors on lipid and protein oxidation evoked by NO donor. SNP was selected for this study because NO and nitrosative stress play a significant role in pathogenesis of most neurodegenerative disorders. Moreover, NO plays an important role in brain ischemia-reperfusion pathology and in inflammation [4,8]. It was found that NO inhibited cytochrome oxidase by competition with oxygen [21]. NO led to
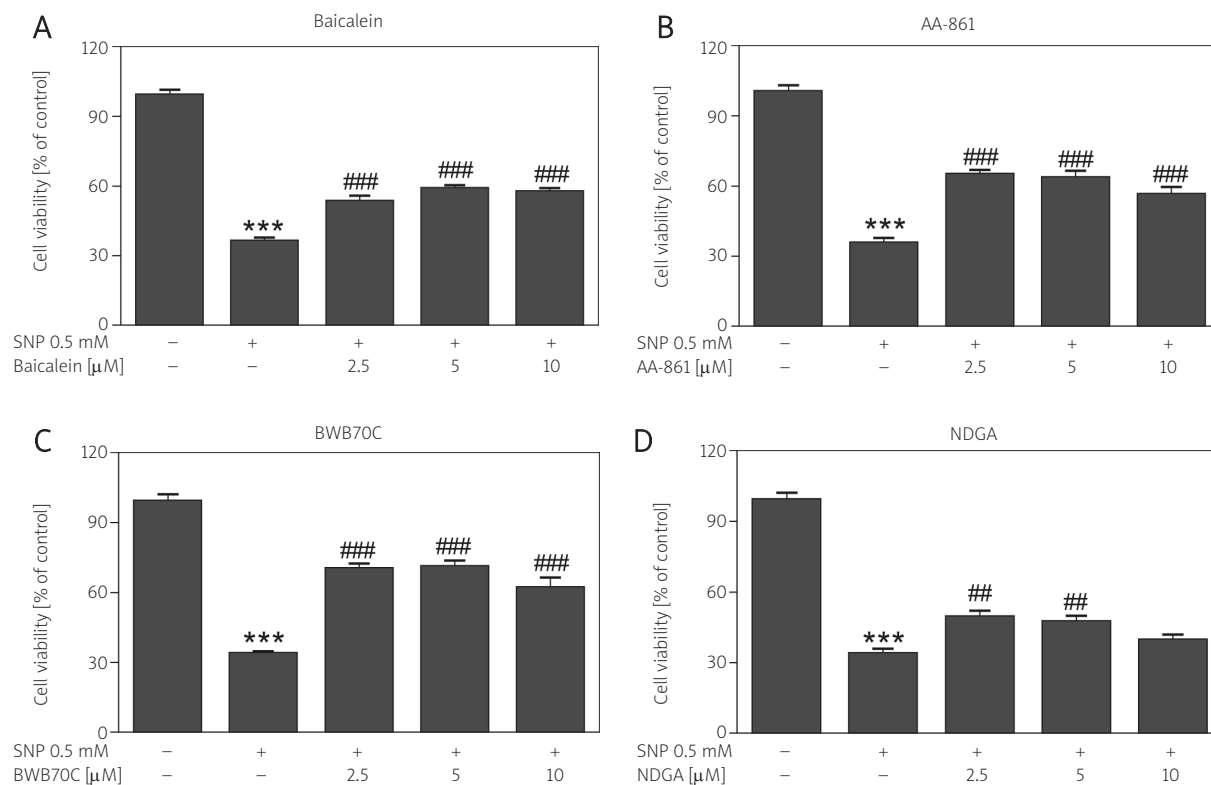
Fig. 5. Effect of LOX inhibitors on HT22 cell survival when subjected for 24 h to nitrosative stress evoked by NO donor 0.5 mM SNP. All values are means ± SEM from three experiments carried out in triplicate. Statistical significance compared to the control group: ***$P < 0.005$ and 0.5 mM SNP treated group: ###$P < 0.005$, ##$P < 0.01$.

glutamate release, excitotoxicity and mitochondria failure [5,16,24,37]. NO and superoxide radicals were also main players in inflammatory neurodegeneration [3,7,23]. LOXs may be an important source of superoxide radicals, and during interaction with NO by enhancement of nitrosative stress these enzymes could be responsible for cell death, as was also demonstrated in this study using hippocampal neuronal HT22 cells in culture. NO donor SNP at 0.5 mM after 24 h induced death of about 60% of HT22 cells. All the inhibitors of LOXs used in this study at 2.5-10 μM significantly protected these cells against death evoked by SNP. In the majority of recently published studies NADPH oxidase is considered as an important source of peroxy radicals, and it is possible that this enzyme may also be involved in generation of nitrosative stress [2,23]. Our previous data showed that inhibitors of LOXs protected PC12 cells against SNP evoked death [33]. This NO donor induced signi-

ficant increase of expression of the gene for 12/15 LOX in PC12 cells [13]. Our study showed that NO was involved in the regulation/activation of PLA$_2$ and release of AA [9,10]. Moreover, oxidative stress-regulated kinases may further phosphorylate and activate PLA$_2$, leading to increased release of AA, the substrate for LOX/COX. Using available LOX inhibitors we showed that they exerted a significant antioxidant effect at very low 1-2.5 μM concentration. The well-known free radical scavenger NDGA at 1 μM concentration significantly reduced the level of TBARS after NO donor treatment. However, baicalein, BWB70C and AA-861 at 1 μM concentration exerted a similar effect. Moreover, zileuton, which is widely used in clinical practice, exerted a significant antioxidative effect at 2.5 μM concentration. We believe that the results of this study may be applicable in evaluation of LOX inhibitors in experimental and clinical medicine.
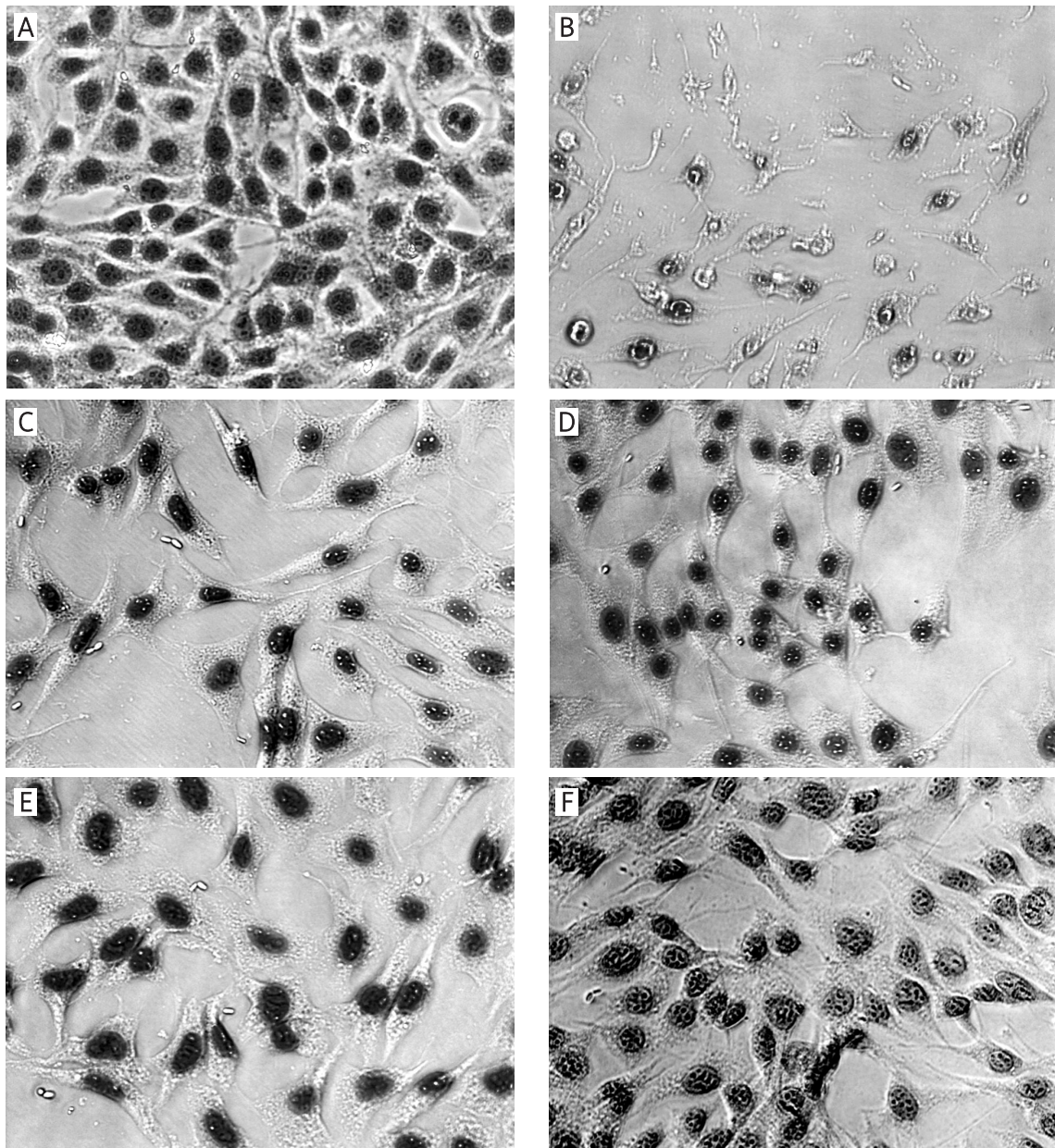
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Fig. 6. Effect of LOX inhibitors on HT22 cell morphology when subjected for 24 h to nitrosative stress evoked by NO donor 0.5 mM SNP. Nuclei were visualized with Hoechst staining. The cells were cultured in the presence of 0.5 mM SNP (**B**) or together with one of the LOX inhibitors (**C** – 10 μM BWB70C, **D** – 10 μM NDGA, **E** – 10 μM AA-861, **F** – 10 μM Baicalein). The control cells (**A**) were not treated.

Acknowledgments

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