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Effects of nimesulide on kainate-induced in vitro oxidative damage in rat brain homogenates

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Abstract

Background: The cyclooxygenase-2 inhibitor nimesulide is able to reduce kainate-induced oxidative stress in vivo. Here we investigate if this effect is mediated by the direct antioxidant properties of nimesulide using a well-characterized in vitro model of kainate toxicity.

Results: Exposure of rat brain homogenates to kainate (12 mM) caused a significant ($p < 0.01$) increase in the concentrations of malondialdehyde and 4-hydroxy-alkenals and a significant ($p < 0.01$) decrease in sulfhydryl levels. High concentrations of nimesulide (0.6–1.6 mM) reduced the extent of lipid peroxidation and the decline in both total and non-protein sulfhydryl levels induced by kainate in a concentration-dependent manner.

Conclusions: Our results suggest that the neuroprotective effects of nimesulide against kainate-induced oxidative stress in vivo are not mediated through its direct free radical scavenging ability because the concentrations at which nimesulide is able to reduce in vitro kainate excitotoxicity are excessively higher than those attained in plasma after therapeutic doses.

Background

Nimesulide (N-(4-nitro-2-phenoxy-phenyl)-methanesulfonamide) is a non-steroidal anti-inflammatory drug with potent anti-inflammatory, antipyretic and analgesic properties which is well tolerated gastrointestinally [1]. Nimesulide is considered a selective cyclooxygenase-2 (COX-2) inhibitor [2,3]. Although inhibition of prostanoids synthesis is a key effect of this drug, various non-prostaglandin mechanisms have been proposed to explain its mode of action: inhibition of 1) histamine release and activity [4], 2) cytokine release [5], 3) platelet-activating factor synthesis [6,7] and 4) phosphodiesterase type IV activity [9]. Moreover, nimesulide decreases the production of the superoxide anion (O_2•-) by polymorphonuclear leukocytes [8,10].

Maffei-Facino and co-workers [11] demonstrated the direct free radical scavenging activity of nimesulide. In two different cell free systems, nimesulide acts as a preventive antioxidant by specifically quenching the hydroxyl radical (HO•), the highly reactive species that, by promoting hydrogen abstraction from polyunsaturated fatty acids (PUFA), leads to cellular damage mediated by peroxidation of membrane phospholipids.

Kainic acid (2-carboxy-3-carboxymethyl-4-isopropenylpyrrolidine) is a non-degradable analog of glutamate isolated from the seaweed *Digenea simplex* with potent neuroexcitatory and neurotoxic properties [12]. Those effects seem to be mediated by a subclass of non-N-methyl-D-aspartate excitatory amino acid receptors [13]. It was found that free radical generation is associated with excitatory amino acid-induced brain injury [14]. Kainate has been shown to generate free radicals when added in vitro to rat cerebellar cell cultures [15] and in gerbil brain, following its systemic administration [16]. Also, reactive oxygen species were detected when kainate was added to isolated synaptoneurosomes derived from rat cerebral cortex [17]. Furthermore, it was demonstrated previously [18] that the addition of kainate to mouse disrupted brain cells caused a concentration-dependent increase in lipid peroxidation.

Recently, we have found that kainate-induced excitotoxicity, with the subsequent oxidative damage, is significantly reduced by the administration of nimesulide at a clinically relevant dose in the rat hippocampus [19]. In addition, we also found a marked neuroprotective effect of nimesulide against hippocampal neuronal damage following global cerebral ischemic brain damage in gerbils, a type of injury in which excitotoxicity plays a key role [20].

Given that the effects of nimesulide reducing oxidative damage in vivo might be attributed to its direct antioxidant properties, the aim of the present study was to determine whether nimesulide could attenuate the oxidative damage seen after the in vitro exposure of disrupted brain cell homogenates to kainate.

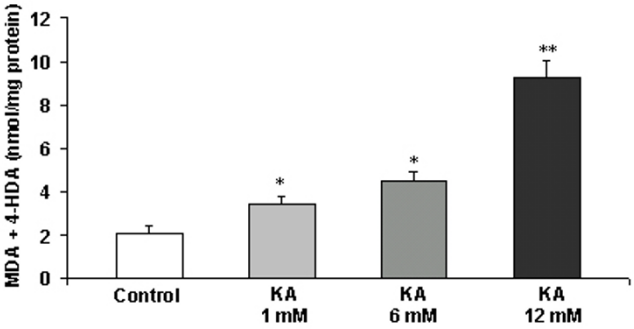

Figure 1
Effect of different concentrations (1, 6 and 12 mM) of kainic acid (KA) on malonaldehyde (MDA) and 4-hydroxyalkenals (4-HDA) concentrations in perfused rat brain homogenates following 20 min incubation. Values are means ± SEM of 3 experiments. Significant differences were determined by one-way ANOVA followed by Student-Newman-Keuls post-hoc test. *P < 0.05 and **P < 0.01 with respect to control.

Table 1: Effect of different concentrations (1, 6 and 12 mM) of kainic acid (KA) on total sulfhydryl groups (TSH) and non-protein sulfhydryl groups (NPSH) following 20 min incubation with rat brain homogenates.

	TSH (nmol/mg protein)	NPSH (μmol/g tissue)
Control	23.97 ± 0.25	1.85 ± 0.03
KA (1 mM)	22.97 ± 0.38	1.77 ± 0.05
KA (6 mM)	20.90 ± 0.27 *	1.65 ± 0.05 *
KA (12 mM)	19.25 ± 0.35 **	1.61 ± 0.04 **

Values are means ± SEM of 3 experiments. *P < 0.05 and **P < 0.01 with respect to control.

Results

The exposure of rat brain homogenates to different concentrations of kainate caused a concentration-dependent increase in the levels of MDA and 4-HDA compared to those in control samples (Fig. 1). On the other hand, kainate produced a significant decrease in TSH and NPSH levels, specially the highest concentrations (6 and 12 mM) as shown in Table 1. Because of the high oxidative damage induced by 12 mM kainate, this concentration was chosen for subsequent studies.

When kainate (12 mM) was added to the reaction mixture together with different concentrations of nimesulide (ranging from 0.6 to 1.6 mM) the increase in lipid peroxidation was reduced in a concentration-dependent manner (Fig. 2). In a similar way, nimesulide was able to protect sulfhydryl groups from kainate-induced oxidative damage (Table 2). The lowest concentrations of nimesulide tested (from 0.1 to 0.4 mM) were unable to confer protection against lipid peroxidation and sulfhydryl groups oxidation as shown in Fig. 2 and Table 2.

We also employed the widely used powerful antioxidant Trolox C (6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxilic acid; a water-soluble analogue of α-tocopherol) as a control and found that Trolox C at concentrations as low as 50–100 μM was able to significantly ($p < 0.05$) reduce oxidative damage induced by 12 mM kainate (data not shown).

In addition, in the absence of kainate, the exposure of brain homogenates to the different concentrations of nimesulide tested did not modify MDA and 4-HDA or sulfhydryl group levels, which were not significantly changed as compared to those in control samples (data not shown).

Discussion

Results from these studies show that kainate-induced oxidative damage as measured by the increase in the concentrations of MDA and 4-HDA and by the decrease in

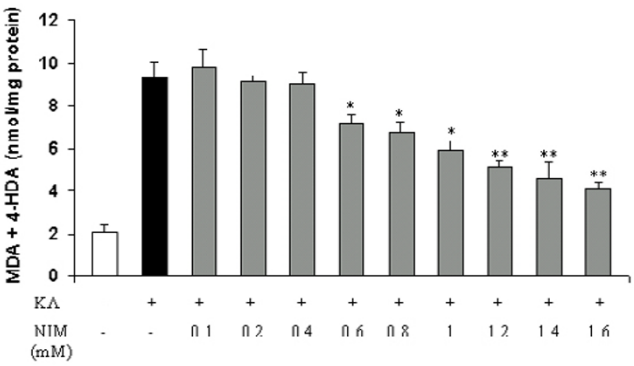

Figure 2
Effect of Nimesulide (NIM) on malonaldehyde (MDA) and 4-hydroxy-alkenals (4-HDA) concentrations in perfused rat brain homogenates following 20 min incubation with 12 mM kainic acid (KA). Values are means ± SEM of 3 experiments. Significant differences were determined by one-way ANOVA followed by Student-Newman-Keuls post-hoc test. *P < 0.05 and **P < 0.01 with respect to KA 12 mM.

Table 2: Effect of nimesulide (NIM) on total sulfhydryl groups (TSH) and non-protein sulfhydryl groups (NPSH) following 20 min incubation with 12 mM kainic acid (KA).

	TSH (nmol/mg protein)	NPSH (μmol/g tissue)
Control	23.97 ± 0.25 **	1.85 ± 0.03 **
KA	19.25 ± 0.35	1.61 ± 0.04
KA + NIM (0.1 mM)	18.72 ± 0.38	1.57 ± 0.03
KA + NIM (0.2 mM)	20.21 ± 0.56	1.64 ± 0.03
KA + NIM (0.4 mM)	20.25 ± 0.35	1.71 ± 0.02
KA + NIM (0.6 mM)	21.96 ± 0.46 *	1.76 ± 0.03 *
KA + NIM (0.8 mM)	22.67 ± 0.30 **	1.76 ± 0.01 *
KA + NIM (1.0 mM)	23.14 ± 0.15 **	1.78 ± 0.02 *
KA + NIM (1.2 mM)	23.03 ± 0.30 **	1.81 ± 0.04 **
KA + NIM (1.4 mM)	23.68 ± 0.25 **	1.79 ± 0.03 **
KA + NIM (1.6 mM)	24.03 ± 0.31 **	1.81 ± 0.02 **

Values are means ± SEM of 3 experiments. Significant differences were determined by one-way ANOVA followed by Student-Newman-Keuls post-hoc test: *P < 0.05 and **P < 0.01 with respect to KA 12 mM.

sulfhydryl groups levels in disrupted cell homogenates from rat brain is markedly inhibited by high concentrations of nimesulide.

Brain cells are at particular risk from free radical damage because of their high content of iron and PUFA, the latter being a substrate for lipid peroxidation, and because of their relatively deficient antioxidant defense mechanisms [21].

Our results are consistent with previous reports which show that kainate-induced death of cerebellar neurons is prevented either by inhibiting the enzyme xanthine oxidase, a cellular source of superoxide anions, or by the addition of free radical scavengers to the culture medium [15]. The generation of free radicals by kainic acid and its correlation with excitotoxicity have been proposed by several groups [15,16,18,22]. The ability of kainate to induce lipid peroxidation was proven in brain by measuring the change in conjugated diene and lipid hydroperoxide concentrations after exposure of cerebellar granular cells to the excitotoxin [22]. Furthermore, the addition of kainate to mouse disrupted brain cells has been shown to cause a concentration-related increase in thiobarbituric acid-reactive substances generation [18].

It is generally accepted that neuronal degeneration after kainic acid administration is associated with a depletion of ATP levels and accumulation of $[Ca^{2+}]_i$. The increase in $[Ca^{2+}]_i$ may trigger Ca^{2+}-activated protease to convert xanthine dehydrogenase to xanthine oxidase with the simultaneous formation of free radicals [15]. In addition, xanthine dehydrogenase could be converted to xanthine oxidase by sulfhydryl oxidation [23]. On the other hand, the increase in $[Ca^{2+}]_i$ may activate enzymes such as phospholipase A_2 which hydrolyzes the membrane phospholipids to release PUFA leading to free radical generation [15].

In our previous study we found a significant neuroprotective effect of the cyclooxygenase-2 inhibitor nimesulide against the depletion of reduced glutathione and increase in lipid peroxidation at a therapeutically relevant dose (6 mg/kg; i.p) [19]. Similarly, in the present study nimesulide was able to protect against in vitro kainate-induced sulfhydryl group oxidation and lipid peroxidation, but this effect was observed only at high concentrations, which are unlikely to be attained in plasma after therapeutic doses. Even when the effects of nimesulide against kainate excitotoxicity in vivo and in vitro are similar, the mechanisms of nimesulide neuroprotection in vivo are quite different from the in vitro effects on measures of oxidative injury, because in the in vitro model (homogenates of rat brain), nimesulide might be acting directly as a scavenger of kainate-generated reactive oxygen species, producing a concomitant reduction in markers of oxidative stress. Unlike the in vitro situation, nimesulide treatment afforded a neuroprotective effect against kainate-induced in vivo oxidative stress in the rat hippocampus, probably acting through inhibition of cyclooxygenase-2, which is now considered a key cellular source of free radicals in the injured brain [24]. It is important to emphasize that several studies have found a marked expression of cerebral cyclooxygenase-2 mRNA and protein following the systemic administration of kai-

nate [25–27]. The induction of cyclooxygenase-2 expression parallels the appearance of neuronal apoptotic features in cell types affected by kainate, and excitotoxic neuronal death in vitro is accompanied by a selective elevation in cyclooxygenase-2 mRNA, indicating that cyclooxygenase-2 may be involved in pathways leading to neuronal death [28]. In support of this, results from our previous work indicated that the induction of cyclooxygenase-2 is involved in kainate-mediated free radicals formation [19]. Based on the pharmacological effects of nimesulide, additional mechanisms could account for the neuroprotection conferred by nimesulide against in vivo kainate excitotoxicity, such as inhibition of pro-inflammatory cytokines production, reduction in nitric oxide generation and blockade of apoptotic pathways, but these effects should be confirmed.

Several findings support the antioxidant activity of nimesulide [11,29,30]. Using the in vitro model of NADPH-supported lipid peroxidation in rat liver microsomes, nimesulide and its metabolites significantly inhibited MDA formation in a concentration-dependent manner [29]. Furthermore, using an in vitro assay based on the oxidation of phosphatidylcholine liposomes (exposed to a flux of hydroxyl radicals generated by water sonolysis), nimesulide exhibited a remarkable scavenging activity against free radicals at concentrations easily attainable in vivo (4.92 μM). Maffei-Facino and co-workers [11] investigated the superoxide anion (O_2•-) and hydroxyl radical (HO•) scavenging activities of nimesulide and its main metabolite 4-hydroxy-nimesulide, using the Electron Spin Resonance (ESR) spectroscopy with the spin trapping technique. According to their results, 4-hydroxy-nimesulide is a good scavenger of both oxygen free radicals and its rate constant of reaction with HO• is $8.9 \times 10^{10}\ M^{-1}\ s^{-1}$. Nimesulide, which has been shown by ESR to be inactive as a O_2•- quencher, has a rate constant of reaction with HO• slightly greater than that of its metabolite ($3.3 \times 10^{11}\ M^{-1}\ s^{-1}$). Taken together, all these studies indicate that antioxidant properties might account for the pharmacological effects of nimesulide.

In the present study, nimesulide conferred a concentration-dependent protection against kainate-induced peroxidation of membrane phospholipids when added together with kainate. The lowest concentrations of nimesulide (0.1 to 0.4 mM) were unable to reduce lipid peroxidation (Fig. 2). Similarly, only the highest concentrations of nimesulide (from 0.6 to 1.6 mM) were able to protect sulfhydryl groups from oxidation, suggesting that relatively high concentrations of nimesulide are able to protect against in vitro kainate toxicity.

Conclusions

In summary, since the concentrations of nimesulide in plasma are estimated to be around 10–20 μM after the administration of clinically used doses [3], results from the present study suggest that the in vivo neuroprotective effects of nimesulide against kainate excitotoxicity are not mediated by direct free radical scavenging ability of this compound because only excessively high concentrations (0.6–1.6 mM) were proven to reduce kainate-induced oxidative stress in our study. It is much more likely that the effects of nimesulide against kainate-induced oxidative damage [19] are mediated by the inhibition of cyclooxygenase-2, a key source of free radicals under damaging conditions to the brain [24].

Methods

Chemicals

All reagents were of the highest quality available. Kainic acid and 5,5'-dithiobis-(2-nitrobenzoic acid) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Nimesulide was kindly provided by Gautier-Bagó Laboratories (Buenos Aires, Argentina). The Bioxytech LPO-586 kit for lipid peroxidation was obtained from Cayman Chemical Co. (Ann Arbor, MI, USA).

Animals

Adult male Sprague-Dawley rats (body weight 200–250 g; n = 12) were obtained from CENPALAB (Havana, Cuba) and housed in Plexiglass cages (4 per cage) and maintained in an air-filtered and automatic temperature controlled (20–22°C) room with a relative humidity of 50–52%. Rats were fed with standard laboratory chow and water ad libitum and were kept under artificial light-dark cycle of 12 h.

Solutions and range of concentrations

Nimesulide was dissolved in absolute ethanol (when added to the brain homogenate, the final alcohol concentration was 1%) and added as one of nine different concentrations (0.1, 0.2, 0.4, 0.6, 0.8, 1, 1.2, 1.4, and 1.6 mM) to the incubation mixture. The exposure of rat brain homogenates to 1% ethanol was proven not to produce any effect [31]. Kainic acid was dissolved in Tris-HCl buffer (20 mM, pH 7.4) and added in 3 different concentrations (1, 6 and 12 mM) to the homogenate.

Brain sample preparation

Rats were anesthetized with diethyl ether and subjected to intracardiac perfusion using a peristaltic pump (DIDT-CNIC Peristaltic, Cuba) with ice-cold saline in order to eliminate the excess of iron (bound to hemoglobin) that may artificially increase free radical generation. Brains were collected and immediately stored at -70°C.

Lipid peroxidation assay

This assay was conducted as previously reported [31]. Briefly, twelve brains were pooled and homogenized in ice-cold Tris-HCl buffer (20 mM, pH 7.4) with a motor driven homogenizer (Edmund Bühler, Germany) to produce a 1/10 homogenate. Aliquots of the homogenates were incubated in a shaking water bath for 20 min at 37°C with different concentrations of kainic acid and with or without nine different concentrations of nimesulide (from 0.1 to 1.6 mM). Moreover, nimesulide (at the concentrations tested) was added to the homogenate without kainate. After the incubation time, the reaction was stopped by cooling the samples in ice for 10 min. Thereafter, the samples were centrifuged at 12 000 × g for 5 min. The supernatant was collected and immediately tested for lipid peroxidation and sulfhydryl groups.

Lipid peroxidation was assessed by measuring the concentration of malonaldehyde (MDA) and 4-hydroxy-alkenals (4-HDA). These constituents are formed, for the most part, from the peroxidation of PUFA and are widely used as an index of lipid peroxidation [32]. The Bioxytech LPO-586 kit was used for these measurements; this kit takes advantage of a chromogenic reagent (N-methyl-2-phenylindole) which reacts with MDA and 4-HDA at 45°C yielding a stable chromophore with maximal absorbance at a wavelength of 586 nm. This wavelength and the low temperature of incubation used in this procedure minimize interferences and undesirable artifacts [32].

Sulfhydryl groups assay

Total (TSH) and non-protein (NPSH) sulfhydryl groups determinations were performed according to the method of Sedlak and Lindsay [33] using the Ellman's reagent.

Protein assay

Total protein concentration was determined using the Coomassie Blue method [34] with bovine serum albumin as standard.

Statistical analysis

Results are presented as mean ± SEM (standard error of means). All data were analyzed by one-way analysis of variance (ANOVA). If the F values were significant, the Student-Newman-Keuls post-hoc test was used to compare groups. The level of significance was accepted at $p<0.05$.

List of Abbreviations

O_2•-, superoxide anion; HO•, hydroxyl radical; PUFA, polyunsaturated fatty acids; MDA, malonaldehyde; 4-HDA, 4-hydroxy-alkenals; TSH, total sulfhydryl groups; NPSH, non-protein sulfhydryl groups; COX-2, cyclooxygenase-2.

Authors' contributions

Author 1 carried out the experimental procedures including the biochemical analyses, participated in the design of the study, performed the statistical analysis and drafted the manuscript. Author 2 participated in the design of the study and revised the manuscript.

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