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Ibuprofen protects dopaminergic neurons against glutamate toxicity in vitro

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Abstract

Non-steroidal anti-inflammatory drugs (NSAIDs) reduce the risk of Alzheimer's disease, although the underlying mechanisms are unknown. Glutamate excitotoxicity has been implicated in Alzheimer's disease, Parkinson's disease, and others. We examined the effects of aspirin, acetaminophen, and ibuprofen on cultured primary rat embryonic neurons from mesencephalon, the area primarily affected in Parkinson's disease. We evaluated whether these drugs protect dopaminergic neurons against excitotoxicity. All three NSAIDs significantly attenuated the decrease in dopamine uptake caused by glutamate, indicating preservation of neuronal integrity. One hundred micro-moles ibuprofen protected both dopaminergic neurons and neurons overall against glutamate toxicity. In addition, ibuprofen alone increased the relative number of dopaminergic neurons by 47%. Thus, NSAIDs protected neurons against glutamate excitotoxicity in vitro, and deserve further consideration as neuroprotective agents in Parkinson's disease.

Keywords: Non-steroidal anti-inflammatory drugs; Aspirin; Acetaminophen; Ibuprofen; Neuroprotection; Neurodegeneration; Cell culture; Dopaminergic; Parkinson's disease

Several studies have demonstrated that chronic non-steroidal anti-inflammatory drug (NSAID) use reduced the incidence of Alzheimer's disease (AD), suggesting that NSAIDs may be neuroprotective (see [19] for review). NSAIDs are reported to oppose glutamate excitotoxicity [12], thought to underlie several neurodegenerative diseases [7]. Using cultures of rat mesencephalon, a brain region that includes dopaminergic neurons that are vulnerable in Parkinson's disease (PD) in humans, we found that NSAIDs protected neurons from glutamate toxicity.

Dissociated mescencephalic cell cultures were established from rat embryos at day 16 of gestation (E16) as described [6] and maintained at 37°C and 5% CO_2/95% air in equal volumes of minimal essential medium and Ham's F-12 supplemented with: 25 mM glucose, 25 mM $NaHCO_3$, 15 mM N-[2–hydroxyethyl]piperazine-N′-[2–ethanesulfonica-cid] (HEPES), 25 μg/ml insulin, 100 μg/ml transferrin, 60 μM putrescine, 20 nM progesterone, 30 nM sodium selenite (all from Sigma, St. Louis, MO) and 10% fetal calf serum (Life Technologies, Grand Island, NY). For experiments, growth medium was removed, and medium without serum containing NSAIDs and sodium glutamate (Sigma) was added.

Acetylsalicylate, acetaminophen, and ibuprofen (NSA IDs) were obtained from Sigma, dissolved in ethanol, due to their low water-solubility, and diluted 1:1000 in medium without serum, which was used in order to eliminate neuroprotection from factors therein. Six-day-old cultures were pre-incubated with NSAIDs for 48 h. Controls received ethanol (0.1%) alone, which had no significant effect on these cultures. Cultures were then treated with glutamate in the presence of NSAIDs for 24 h, fixed, and processed for immunocytochemistry as described [6]. Dopamine neurons were identified with an antibody to a key enzyme in dopamine synthesis, tyrosine hydroxylase (Boehringer–Mannheim, GmbH; diluted 1:1000). Neurons overall were identified with TG-2 [14] and PHF-1 [11] antibodies generously provided by Dr Peter Davies, (Albert Einstein College of Medicine; diluted 1:35). Neurons were visualized using

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Vectastain avidin-biotin peroxidase complex (ABC) and diaminobenzidine (DAB) (Vector Laboratories, Burlingame, CA). Neurons labeled with PHF-1 or TG-2 were quantified in a strip of five consecutive microscopic fields defined by a 1 × 1 cm reticule fitted into the eyepiece of the microscope. Dopaminergic neurons identified by tyrosine hydroxylase (TH) immunocytochemistry were counted in two perpendicular strips that spanned the diameter of the culture dishes and results expressed as mean counts of quadruplicate cultures. Statistical analysis was by analysis of variance (ANOVA) followed by the Fisher's protected least significant difference (PLSD) post-hoc test. In other experiments, toxicity was monitored by dopamine uptake assays, carried out as previously described [6]

Aspirin and acetaminophen did not affect the total number of neurons at 1 mM, while this concentration of ibuprofen reduced neuronal counts by 50% (Fig. 1a). Interestingly, all three compounds are toxic to glioma cells at this concentration; decreasing cell numbers by 39, 77 and 94%, respectively, [5]. This suggested that neurons are less sensitive to the toxic effects of NSAIDs.

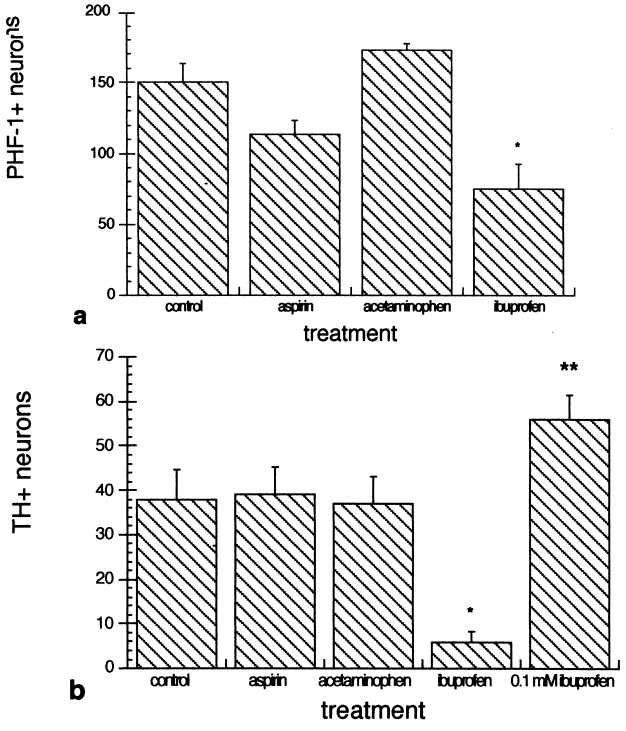

Fig. 1. Effects of aspirin, acetaminophen, and ibuprofen on total neurons and dopaminergic neurons. Cultures established from embryonic rat mesencephalon were treated with 1 mM aspirin, acetaminophen, and ibuprofen for 2 days. Cells were then fixed and processed for PHF-1 or TH immunocytochemistry to identify neurons and dopaminergic neurons, respectively. Bars represent mean counts ± SEM) for total neurons (panel a), or dopaminergic neurons (panel b). The additional bar in (panel b) represents cell numbers after treatment with 0.1 mM ibuprofen. Asterisks denote cell numbers in treatment groups which significantly lower (*) or higher (**) from those in untreated cultures ($P < 0.05$).

NSAIDs affected the dopaminergic subpopulation in a similar fashion to neurons in general, as illustrated in Fig. 1b. While incubation with 1 mM aspirin and acetaminophen for 2 days had no effect on dopaminergic neurons, 85% of these neurons were lost with 1 mM ibuprofen after 2 days of treatment. In contrast to its toxicity at 1 mM, 0.1 mM ibuprofen increased the number of dopaminergic neurons by an average of 47% in four replicate experiments. Glutamate excitotoxicity is a suspected mechanism in many neurodegenerative diseases [7]. Conversely, clinical studies indicate that NSAIDs may protect against neurodegeneration [19]. Therefore, we tested whether NSAIDs could protect dopaminergic neurons against glutamate excitotoxicity in our in vitro model of Parkinsonian neurodegeneration. Mesencephalic cultures (above) were pre-treated with 1 mM aspirin and acetaminophen, or 0.1 mM ibuprofen for 2 days, and then exposed to 150 μM glutamate, previously determined to be neurotoxic in this system [4]. After 24 h, the integrity of dopaminergic neurons was assessed as high-affinity dopamine uptake as described [6]. Glutamate (150 μM) reduced dopamine uptake by 55% compared to control (Fig. 2). Pretreatment with either 1 mM aspirin, 1 mM acetaminophen or 0.1 mM ibuprofen preserved dopamine uptake, indicating significant reductions of glutamate excitotoxicity by approximately 55, 36 and 45%, respectively, (Fig. 2). None of the NSAIDs studied were toxic to dopaminergic neurons judged by specific dopamine uptake; both acetaminophen and ibuprofen by themselves actually

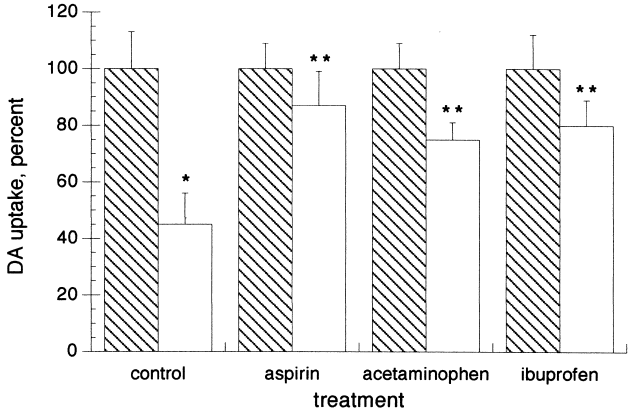

Fig. 2. Aspirin, acetaminophen and ibuprofen protect dopaminergic neurons from glutamate toxicity. Cultures were established from embryonic rat mesencephalon as described, and treated with 1 mM aspirin, 1 mM acetaminophen, and 0.1 mM ibuprofen for 2 days, followed by 1 day of incubation with 150 μM glutamate. Dopamine uptake was determined after incubation with ^{3}H-dopamine for 10 min. Bars represent normalized percent uptake of four replicates for each treatment group for drug alone (striped bars) and drug followed by glutamate (open bars) ±SEM. A single asterisk (*) denotes significant differences in uptake between glutamate treated and control cultures in the absence of drugs. Double asterisks (**) denote significant differences in glutamate treated groups with and without drug treatments ($P < 0.05$).

increased dopamine uptake, although this did not achieve statistical significance (data not shown).

Since ibuprofen protected dopamine uptake, an important functional property of dopaminergic neurons, we examined whether ibuprofen protected all neurons in general and/or dopaminergic neurons in particular, from glutamate toxicity (Fig. 3). Cultures were pretreated with 0.1 mM ibuprofen, exposed to 150 μM glutamate as above, and then fixed and processed for TG-2 immunocytochemistry. Like PHF-1, TG-2 stains rat neurons [14], but the site at which the TG-2 antibody binds is not altered by glutamate treatment, as is the site for PHF-1 binding (D. Casper, unpublished data). Treatment with glutamate alone resulted in a significant reduction in the number of neurons (28%), but ibuprofen completely protected the overall neuronal population from glutamate toxicity (Fig. 3a). Moreover, the number of neurons treated with ibuprofen and glutamate increased consistently, but not significantly, with respect to control cultures.

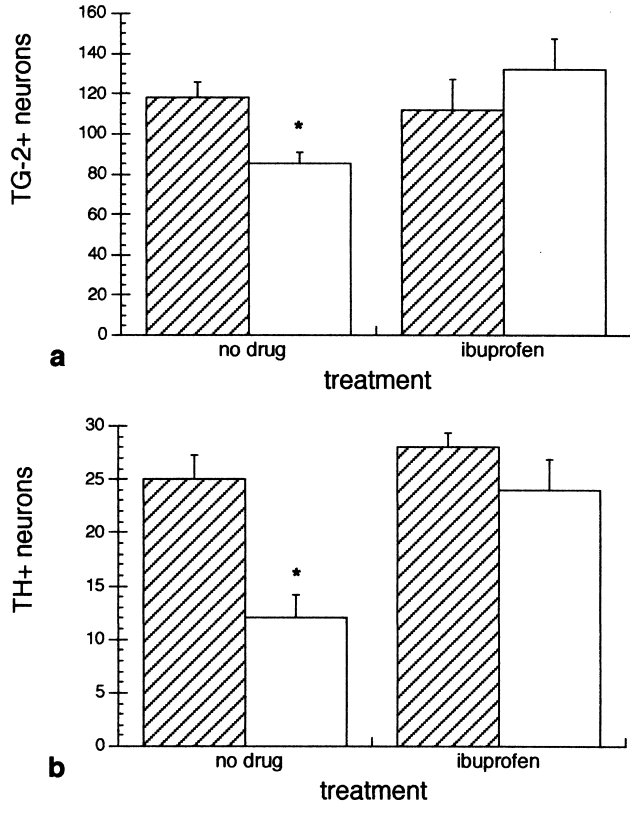

Fig. 3. Ibuprofen protects total neurons and dopaminergic neurons from glutamate toxicity. Cultures were established from embryonic rat mesencephalon as described, and treated with 0.1 mM ibuprofen for 2 days followed by 1 day incubation with 150 μM glutamate. Cells were fixed and processed for immunocytochemistry with TG-2 and TH antibodies to identify neurons and dopaminergic neurons, respectively. Immunoreactive neurons were counted in representative microscopic fields. (a) TG-2+ neurons; (b) TH + dopaminergic neurons. Bars represent mean counts of four cultures for each treatment group (±SEM). Striped bars, without glutamate treatment; open bars, with glutamate treatment. Asterisks (*) denote groups in treated with glutamate in which cell numbers differed significantly from those in cultures without glutamate treatment ($P < 0.05$).

In parallel experiments, we studied the protective effect of ibuprofen on glutamate excitotoxicity by counting dopaminergic (TH-positive) neurons. Incubation with 150 μM glutamate killed over 50% of the dopaminergic neurons (Fig. 3b). Thus, compared to the 28% loss in the general neuronal population (Fig. 3a), dopaminergic neurons were selectively vulnerable to glutamate toxicity. Pretreatment with ibuprofen also protected them from glutamate toxicity, largely attenuating the loss by excitotoxicity by over 80%. Therefore, ibuprofen protected both neurons in general and dopaminergic neurons in particular against glutamate excitotoxicity.

Although structurally distinct, all three NSAIDs tested inhibit cyclooxygenases, enzymes that convert arachidonic acid to prostaglandins. Recent studies showed strong evidence that chronic NSAID use reduced the risk of AD ([19] for review). However, these studies could not address the protective mechanism, nor was it possible to identify the primary cellular target being protected. Known systemic targets of NSAIDs include the vasculature and the immune system. However, little is known about the direct effects of NSAIDs on central nervous system (CNS) cells. Here, we found that all three NSAIDs protected dopamine neurons from glutamate excitotoxicity in our mixed neuronal and glial cultures, which reflect the cellular composition of the brain. Moreover, ibuprofen treatment alone increased the number of dopaminergic neurons by almost half (Fig. 1b); most likely protecting them from the known excitotoxicity associated with culture medium change [9]. In contrast, aspirin and acetaminophen killed glioma cells at concentrations now found to be non-toxic and/or neuroprotective to dopamine neurons [5], which supports a favorable therapeutic ratio for potential NSAID treatment of glioma.

Proposed mechanisms for glutamate excitotoxicity include: (1) loss of ion gradients and metabolic rundown; (2) calcium-dependent activation of apoptotic cascades; and (3) oxidative stress. Likewise, several pathways have been proposed for the neuroprotective actions of NSAIDs: (1) decreasing prostaglandin synthesis by inhibition of cyclooxygenase [21], (2) inhibition of nuclear factor kappa B (NF-κB), a transcription factor associated with inflammation [12], and (3) inhibition of peroxisome proliferator-activated receptor gamma (PPAR γ), a retinoic acid receptor-like molecule that mediates inflammation- and beta-amyloid-induced neurotoxicity [8].

Glutamate has been shown to induce neuronal cyclooxygenase-2 (COX-2) [20], which may mediate glutamate toxicity. COX-2 is inhibited by NSAIDs [21] and prostaglandins can modulate synaptic transmission, including glutamate release [2]. Glutamate and oxidative stress both induce NF-κB [18], a transcription factor found in neurons [17], which is elevated in neuronal nuclei in Parkinson's disease, indicating activation [16]. NSAIDs may modulate neuronal NF-κB

activity, and this has been implicated in the neuroprotection by aspirin against glutamate neurotoxicity in cerebellar granule cell cultures [12]. Recent studies have also demonstrated that both aspirin and salicylate abolished the neurodegeneration caused by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) in mice [1,10]. However, this protection was not solely due to inhibition of NF-κB, since dexamethazone, a potent NF-κB inhibitor, had no neuroprotective effect, while some data point suggest a free radical scavenging effect of these NSAIDs.

NSAIDs have also been shown to activate the PPAR, which inhibits microglia- and monocyte-mediated neurotoxicity [8]. Since our cultures may contain microglia, it is possible that NSAIDs also inhibit the inflammatory response in vitro. Finally, NSAIDs or their metabolites could modify glutamate metabolism by binding to both glutamate dehydrogenase and glutamine synthetase [3,13], which modulate brain glutamate levels.

In conclusion, our results demonstrate that NSAIDs can protect neurons against glutamate toxicity. This is especially significant for dopaminergic neurons, which are particularly vulnerable to glutamate toxicity in vitro, and are the main cellular targets in PD [15]. The study of the neuroprotection afforded by NSAIDs against glutamate excitotoxicity could provide new information about the molecular pathways of neurodegeneration and neuroprotection. NSAIDs may be a promising new therapeutic avenue for the treatment of neurodegenerative diseases.

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