Mefenamic Acid Shows Neuroprotective Effects and Improves Cognitive Impairment in in Vitro and in Vivo Alzheimer’s Disease Models

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ABSTRACT
Nonsteroidal anti-inflammatory drugs (NSAIDs) exert anti-inflammatory, analgesic, and antipyretic activities and suppress prostaglandin synthesis by inhibiting cyclooxygenase, an enzyme that catalyzes the formation of prostaglandin precursors from arachidonic acid. Epidemiological observations indicate that the long-term treatment of patients suffering from rheumatoid arthritis with NSAIDs results in reduced risk and delayed onset of Alzheimer’s disease. In this study, we investigated the therapeutic potential for Alzheimer’s disease of mefenamic acid, a commonly used NSAID that is a cyclooxygenase-1 and 2 inhibitor with only moderate anti-inflammatory properties. We found that mefenamic acid attenuates the neurotoxicities induced by amyloid β peptide (Aβ)1–42 treatment and the expression of a Swedish double mutation (KM595/K596NL) of amyloid precursor protein (Swe-APP) or the C-terminal fragments of APP (APP-CTs) in neuronal cells. We also show that mefenamic acid decreases the production of the free radical nitric oxide and reduces cytochrome c release from mitochondria induced by Aβ1–42. Swe-APP, or APP-CTs in neuronal cells. In addition, mefenamic acid up-regulates expression of the antiapoptotic protein Bcl-XL. Moreover, our study demonstrates for the first time that mefenamic acid improves learning and memory impairment in an Aβ1–42-infused Alzheimer’s disease rat model. Taking these in vitro and in vivo results together, our study suggests that mefenamic acid could be used as a therapeutic agent in Alzheimer’s disease.

Nonsteroidal anti-inflammatory drugs (NSAIDs) are widely-used therapeutic agents that have anti-inflammatory, analgesic, and antipyretic activities. NSAIDs are involved in the suppression of prostaglandin synthesis by inhibiting cyclooxygenases, enzymes that catalyze the formation of prostaglandin precursors from arachidonic acid. It has been reported that inflammatory processes are associated with the pathophysiology of Alzheimer’s disease and that treatment with NSAIDs reduces the risk of Alzheimer’s disease (Asanuma et al., 2001).

Inflammation is observed in numerous neurodegenerative disorders (e.g., Parkinson’s disease, stroke, and Alzheimer’s disease). In the case of Alzheimer’s disease, inflammatory evidence is observed near amyloid plaques, and such cytokines as interleukin-1, interleukin-6, tumor necrosis factor α, and transforming growth factor β have been implicated in this inflammatory process (Cacquevel et al., 2004). Thus, it has been postulated that the sustained use of NSAIDs could retard Alzheimer’s disease progression by reducing inflammatory response occurred in microglia (Sugaya et al., 2000; McGeer and McGeer, 2003; Yan et al., 2003).

Some recent reports have shown that certain NSAIDs have a direct effect on the progression of Alzheimer’s disease, but this effect is independent of its typical anti-inflammatory mech-

ABBREVIATIONS: NSAID, nonsteroidal anti-inflammatory drug; Aβ, amyloid β peptide; DMEM, Dulbecco’s modified Eagle’s medium; FBS, fetal bovine serum; GFP, green fluorescent protein; LDH, lactate dehydrogenase; ROS, reactive oxygen species; DCFH-DA, 2’,7’-dichlorofluorescein diacetate; TUNEL, terminal deoxynucleotidyl transferase biotin-DUTP nick-end labeling; APP, amyloid precursor protein; APP-CTs, C-terminal fragments of amyloid precursor protein; wt-APP, wild type of amyloid precursor protein; Swe-APP, Swedish mutant form of amyloid precursor protein; ANOVA, analysis of variance; ΔΨm, mitochondrial membrane potential.
Neuroprotective Effects of Mefenamic Acid

Materials and Methods

Reagents. Anti-cytochrome c and anti-β-tubulin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), and anti-active caspase-3 antibody was purchased from BD Transduction Laboratories (Lexington, KY). Anti-Bcl-X<sub>α</sub> and anti-cytochrome c oxidase IV antibodies were purchased from Cell Signaling Technology (Beverly, MA).

Cell Culture and Transfection. PC-12 cells originating from rat pheochromocytoma were plated on a polyethyleneimine (0.2 mg/ml in sodium borate buffer, pH 8.3) coated six-well or 96-well plate and maintained in Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen) supplemented with 10%Bethesda, Maryland). Anti-adiponectin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), and anti-Bcl-2 and anti-cytochrome c oxidase IV antibodies were purchased from Cell Signaling Technology (Beverly, MA).

Cell Culture and Transfection. PC-12 cells originating from rat pheochromocytoma were plated on a polyethyleneimine (0.2 mg/ml in sodium borate buffer, pH 8.3) coated six-well or 96-well plate and maintained in Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen) supplemented with 10% fetal bovine serum (FBS), penicillin-streptomycin-amphotericin B mixture (Invitrogen) at 37°C and 5% CO<sub>2</sub>. PC-12 cells were treated with nerve growth factor (50 ng/ml; Calbiochem, Darmstadt, Germany) to differentiate at 37°C and 5% CO<sub>2</sub>. Cells were detached with trypsin-EDTA and washed with PBS. Mitochondrial and cytosolic fractions were isolated according to the manufacturer’s instruction. Transient transfection efficiencies using FuGene 6 was assessed with GFP vector in differentiated PC-12 cells and SH-SY5Y cells with HEPES-buffered saline and incubated in the dark for 1 h in HEPES-buffered saline containing 200 μM DCFH-DA. Upon incubation, DCFH-DA is taken up by cells, where intracellular esterase cleaves the molecule to 2',7'-dichlorofluorescein, which is oxidized to dichlorofluorescein in the presence of H<sub>2</sub>O<sub>2</sub>. The total fluorescence was measured using a spectrofluorometer (Shimadzu RF-5001PC) at an emission wavelength of 529 nm and an excitation wavelength of 504 nm (Wang and Zhu, 2003).

Measurement of Nitric Oxide Generation. Nitric oxide production was evaluated by measuring the level of nitrite (NO<sub>2</sub>·), the oxidized product of nitric oxide, using the Griess reaction as described previously (Hirata et al., 1993). Nitric oxide is an electrophilic reactive species that can be oxidized. To measure the total nitric oxide oxidation products, NO<sub>x</sub> is first reduced to NO<sub>2</sub> by NADH-dependent nitrate reductase. In brief, 50 μl of cell supernatant (exposed to the three experimental conditions described above) was mixed with 25.5 μl of 0.1 M potassium phosphate buffer, pH 7.4, 11 μl of 2 mM NADPH, 1.5 μl of 1 mM FAD and 12 μl (1.25 U/ml) of nitrate reductase. All samples were incubated for 2 h at room temperature in the dark and then 75 μl of 1% sulfanilamide and 75 μl of 1% N-(1-naphthyl)-ethylenediamine was added. Using a microplate reader, the nitrite production was determined by reading the absorbance at 550 nm. The amount of nitric oxide produced was normalized against the number of cells.

Assessment of Mitochondrial Membrane Potential. Cells were detached with trypsin-EDTA and washed with PBS. Mitochondrial membrane potential was measured by flow cytometry using the rhodamine-6G (Sigma, St. Louis, MO). Cells were resuspended in 0.5 ml of PBS containing 10 mg/ml of rhodamine-6G for 15 min at 37°C and then immediately submitted for flow analysis (BD Biosciences, San Jose, CA).

Evaluation of Apoptosis with TUNEL Staining. TUNEL staining was performed according to the manufacturer’s protocol (in situ cell death detection kit, TMR red; Roche Diagnostics). TUNEL reaction was labeled with TMR red and analyzed by confocal microscopy with appropriate filters (LSM510; Carl Zeiss, Jena, Germany). 4',6-Diamidino-2-phenylindole (1 μM) staining was performed for nucleus staining. The number of TUNEL-positive cells in five random fields was counted in two sets of experiments, and the results were normalized as percentage ratios compared with the total cell numbers of mock transfected cells and those transfected with wt-APP, Swe-APP, or APP-Cts.

Western Blotting. Cells were lysed in a lysis buffer containing 50 mM Tris, pH 7.4, 150 mM NaCl, 1% Triton, 0.5% SDS, 0.5% sodium deoxycholate, and protease inhibitors. Protein was resolved in SDS polyacrylamide gel, electrophoresed at 30 to 50 μg of protein/lane, and transferred onto a nitrocellulose membrane (GE Healthcare, Little Chalfont, Buckinghamshire, UK). The protein blot was confirmed with appropriate antibodies and detected using horseradish peroxidase-conjugated secondary antibody (GE Healthcare Pharma- cia). Immunoreactive bands were visualized using an ECL enhanced chemiluminescence system (GE Healthcare). For detection of cytochrome c in mitochondrial and cytosolic fractions, after cell lysis, the mitochondrial and cytosolic fraction were obtained according to the method described previously (Kang et al., 1995).

Aβ<sub>1–42</sub>-Infused Alzheimer’s Disease Rat Model. Male Wistar rats weighing 200 to 250 g were housed in a specific pathogen-free room that was automatically maintained on a 12-h light/dark cycle at 25°C and proper humidity. Animals were handled in accordance with the Guidelines for Animal Experiments of Ethics Committee of Seoul National University. They had free access to food and water. The Aβ<sub>1–42</sub> peptides were dissolved in 35% acetonitrile/0.1% trifluoroacetic acid. The rats were anesthetized with sodium pentobarbital (40 mg/kg, i.p.). Continuous infusion of Aβ<sub>1–42</sub> (600 pmol/day) was maintained for a week by attachment of an infusion kit connected to an osmotic mini-pump (Alzet 1007D; ALZA Corporation, Mountain View, CA). The infusion kit was implanted into the right ventricle (1.2 mm posterior to the bregma, 1.5 mm lateral to the midline, and
Fig. 1. Mefenamic acid attenuates the neuronal cell death induced by Aβ1-42 treatment or Swedish APP expression in nerve growth factor-differentiated PC-12 cells and SH-SY5Y cells. In this study, we investigated the effects of mefenamic acid on wt-APP-, Swe-APP-, and APP-CTs–induced cytotoxicities, ROS production, nitrite accumulation, mitochondrial membrane potential loss, cytochrome c release, active caspase-3 and Bcl-XL in differentiated PC-12 cells and SH-SY5Y cells employing a transient transfection system. Under our experimental conditions, transient transfection efficiencies using FuGene 6 reagent was assessed with GFP vector in differentiated PC-12 cells and SH-SY5Y cells. The transfection efficiency was measured by LDH activity in the medium. The results are expressed as percentages of maximum LDH release obtained upon complete cell lysis (0.9% Triton X-100). Data represent mean ± S.E.M. obtained from 16 culture wells per experiment, determined in two independent experiments; *, p < 0.05 versus vehicle-treated cells, #, p < 0.05 versus 30 μM Aβ1-42-treated cells (by one-way ANOVA). B, nerve growth factor-differentiated PC-12 cells were mock-transfected or transfected with wt-APP or Swe-APP and then treated with vehicle or 5 μM mefenamic acid 6 h after transfection. After 48 h, the degree of cell death was assessed by LDH activity in the medium. The results are expressed as percentages of maximum LDH release obtained upon complete cell lysis. Data represent mean ± S.E.M. obtained from 16 culture wells per experiment, determined in two independent experiments; *, p < 0.05 versus vehicle-treated cells; #, p < 0.05 versus vehicle-treated mock-transfected vehicle-treated cells; & p < 0.05 versus vehicle-treated wt-APP- or Swe-APP-transfected cells (by one-way ANOVA). C, nerve growth factor-differentiated PC-12 cells were transfected with mock, APP-CT58, or APP-CT99, and then treated with vehicle or 5 μM mefenamic acid 6 h after transfection. Forty-eight hours after treatment, the degree of cell death was assessed by LDH activity in the medium. The results are expressed as percentages of maximum LDH release obtained upon complete cell lysis. Data represent mean ± S.E.M. obtained from 16 culture wells per experiment, determined in two independent experiments; *, p < 0.05 versus mock-transfected vehicle-treated cells; #, p < 0.05 versus vehicle-treated wt-APP- or Swe-APP-transfected cells (by one-way ANOVA). D, nerve growth factor-differentiated PC-12 cells were mock-transfected or transfected with C59 and then treated with vehicle or 0.001, 0.01, 0.1, 1, or 10 μM mefenamic acid 6 h after transfection. Forty-eight hours after treatment, the degree of cell death was assessed by LDH activity in the medium. The results are expressed as percentages of maximum LDH release obtained upon complete cell lysis. Data represent mean ± S.E.M. obtained from 16 culture wells per experiment, determined in two independent experiments; *, p < 0.05 versus mock-transfected vehicle-treated cells; #, p < 0.05 versus vehicle-treated APP-CT59-transfected cells (by one-way ANOVA).
ciencies were 40.0 ± 4.0% (n = 5) and 45.2 ± 8.7 (n = 4) in PC-12 cells and SH-SY5Y cells, respectively, as confirmed by the expression of GFP fluorescence. In addition, we confirmed the expression of wt-APP, Swe-APP, APP-CT59, and APP-CT 99 by Western blotting after transfection (data not shown).

Mefenamic Acid Attenuates Neuronal Cell Death Induced by Aβ1–42 Treatment or Swedish App Expression in Nerve Growth Factor-Differentiated PC-12 Cells. At the outset, we tested whether mefenamic acid affects the cytotoxicities observed in the nerve growth factor-differentiated PC-12 cell induced by Aβ1–42 treatment with the use of an LDH release assay. We found that pretreatment with mefenamic acid at 5 μM for 24 h significantly reduced the cytotoxicity induced by Aβ1–42 at 30 μM for 24 h in nerve growth factor-differentiated PC-12 cells versus vehicle-pre-treated cells (Fig. 1A). LDH release, as expressed by the percentage of maximal LDH release obtained upon complete cell lysis (0.9% Triton X-100), increased from 1.39% to 20.72% after treatment with Aβ1–42 at 30 μM for 24 h. In contrast, pretreatment with mefenamic acid at 5 μM for 24 h significantly reduced LDH release to 8.58% (Fig. 1A). In addition, mefenamic acid significantly reduced LDH release induced by the expression of wild type-APP, Swe-APP, or APP-CTs (CT59, CT99) in nerve growth factor-differentiated PC-12 cells (Fig. 1, B and C). It was reported previously that APP-CTs and Aβ play a role in inducing neuronal death in Alzheimer’s disease (Kim et al., 2000, 2003, 2004; Suh and Checler, 2002). In the present study, we found that mefenamic acid treatment reduced LDH release induced by APP-CT59 expression in a dose-dependent manner (Fig. 1D).

Mefenamic Acid Attenuates Apoptosis Induced by Swedish App or APP-CTs Expression in SH-SY5Y Cells. We examined whether mefenamic acid affects apoptosis induced by wt-APP, Swe-APP, APP-CT59, or APP-CT99 in SH-SY5Y cells and found that mefenamic acid treatment reduced TUNEL staining in SH-SY5Y cells transfected with wt-APP, Swe-APP, APP-CT59, or APP-CT99 (Fig. 2, A and B). Forty-eight hours after transfection, TUNEL-positive cells were 0.66 ± 0.33, 21.99 ± 8.56, 40.75 ± 7.17, 30.90 ± 7.15, or 41.31 ± 6.92% versus all cells in mock-, wt-APP-, Swe-APP-, APP-CT59-, or APP-CT99-transfected cells, respectively (Fig. 2, A and B). Mefenamic acid treatment significantly reduced TUNEL-positive cells to 0.33 ± 0.21, 8.55 ± 5.01, 11.02 ± 5.31, 12.88 ± 6.10, or 23.90 ± 3.85% in mock-, wt-APP-, Swe-APP-, APP-CT59-, or APP-CT99-transfected cells, respectively (Fig. 2, A and B).

Mefenamic Acid Reduces ROS Accumulation and Attenuates Mitochondrial Membrane Potential Loss Induced by Swe-APP Expression. It has been reported that Swedish APP expression increases ROS in neuronal cells (Eckert et al., 2001). Swe-APP is associated with early-onset neuropathological changes in Alzheimer’s disease, including the accumulation of ROS and the loss of mitochondrial membrane potential. In this study, we found that mefenamic acid treatment significantly reduced ROS accumulation and attenuated mitochondrial membrane potential loss induced by Swe-APP expression in SH-SY5Y cells (Fig. 3, A and B).
familial Alzheimer's disease and results in a 3- to 6-fold increase in Aβ production. Thus, we examined whether mefenamic acid affects the ROS accumulation induced by Swe-APP or APP-CTs expression. Treatment with mefenamic acid significantly reduced the ROS generation induced by expressions of wt-APP, Swe-APP, or APP-CTs (CT59, CT99) (Fig. 3, A and B). ROS levels increased to 284% and 379% in wt-APP and in Swe-APP expressing differentiated PC-12 cells, respectively, versus nontreated control cells (as determined using DCFH-DA dye). We found that treatment with mefenamic acid at 5 μM for 24 h reduced ROS levels to 126% and 128% in wt-APP and Swe-APP expressing differentiated PC-12 cells, respectively, versus nontreated control cells (Fig. 3A). In addition, mefenamic acid also significantly reduced the ROS generation induced by APP-CTs (CT59 and CT99) (Fig. 3B).

Aβ treatment and Swe-APP expression have been shown to increase nitric oxide levels and lead to mitochondrial dysfunction (Keil et al., 2004). Here, we examined whether mefenamic acid affects the nitric oxide production induced by Aβ1–42 treatment or Swe-APP expression in differentiated PC-12 cells and found that mefenamic acid significantly reduces the nitric oxide generation induced by both. When Aβ1–42 was administered to PC-12 cells at 20 μM for 24 h, nitric oxide accumulation increased by 3.4-fold compared with vehicle-treated cells (Fig. 3C). Pretreatment with mefenamic acid significantly reduced nitric oxide accumulation (Fig. 3C), which is consistent with other reports (Asanuma et al., 2001) in which NSAIDs, including mefenamic acid, were found to exert neuroprotective effects by direct scavenging of nitric oxide radicals generated by nitric oxide radical donor NOC 18. In the present study, nitric oxide accumulation increased 1.7- or 1.7-fold in wt-APP- or Swe-APP-expressing differentiated PC-12 cells, respectively, versus vehicle-treated wt-APP- or Swe-APP-expressing cells (Fig. 3D).

Mitochondrial membrane potential (Δψm) has been reported to play a key role in apoptosis induction by regulating the mitochondrial permeability transition pore opening (Petronilli et al., 1993). We checked whether mefenamic acid affects changes in Δψm induced by Swe-APP or APP-CTs expression. Our results showed that mefenamic acid reduced the cells showing Δψm loss induced by both Swe-APP (Fig. 3E) and APP-CTs expression (CT59, CT99) (Fig. 3F). This may be because mefenamic acid significantly reduces the ROS levels generated by wt-APP, Swe-APP, or APP-CTs (CT59, CT99) as shown in Fig. 3, A and B.

Mefenamic Acid Inhibits Cytochrome c Release from Mitochondria and Inhibits the Caspase-3 Activation Induced by Swe-APP Expression. Cytochrome c release from mitochondria is the central event in the apoptotic process (Zou et al., 1997). We found that mefenamic acid treatment significantly inhibited cytochrome c release from mitochondria as induced by wt-APP, Swe-APP, APP-CT59, or APP-CT99 expression (Fig. 4, A and B). We also checked the effects of mefenamic acid on the caspase-3 activation induced by wt-APP, Swe-APP, or APP-CTs expression. Figure 4, C and D, shows that mefenamic acid was found to inhibit the caspase-3 activation induced by wt-APP, Swe-APP, or APP-CTs expression.

Cytochrome c release is known to be governed by Bcl-2 family proteins. We investigated changes in Bcl-XL protein levels after mefenamic acid treatment. Mefenamic acid was found to up-regulate Bcl-XL protein in mock- or wt-APP-, Swe-APP-, APP-CT59-, and APP-CT99-expressing differentiated PC-12 cells (Fig. 4, E and F). However, mefenamic acid showed no significant effects in Bax expression, one of the proapoptotic Bcl-2 family members (data not shown).

Mefenamic Acid Attenuated Learning and Memory Impairment in an Aβ1–42-Infused Alzheimer's Disease Rat Model. We assessed the neuroprotective effects of mefenamic acid in an in vivo system. Aβ1–42 (600 pmol/day) was continuously infused into the lateral ventricle of rats for 1 week. Mefenamic acid (5 mg/kg/day) or PBS was then injected i.p. for 3 weeks. After that time, we tested the vehicle-treated (n = 11) or mefenamic acid-treated (n = 10) Aβ1–42-infused rats for spatial learning and memory impairment using the Morris Water Maze test. From the second learning session day, we observed that the mefenamic acid-treated group showed a shorter latency time than the vehicle-treated group (p < 0.05; Fig. 5A). We also confirmed that the mefenamic acid treatment alone did not affect latency time (n = 6, Fig. 5A). To confirm whether memory impairment shown in the Aβ1–42-infused rats was attenuated by mefenamic acid treatment, we performed a probe test and...
recorded average latencies in zone 1 without platform. Mefenamic acid-treated rats stayed significantly longer in zone 1 than at the other zones (zones 2, 3, and 4) (p < 0.05; Fig. 5B). However, no differences were observed for stays in zones 1, 2, 3, and 4 in the vehicle-treated group (Fig. 5B). We confirmed that mefenamic acid treatment alone did not affect stays in zones 1, 2, 3, and 4 (n = 6; Fig. 5B).

**Discussion**

Alzheimer’s disease is characterized by the deposition of fibrillar forms of Aβ in the brain and by the compaction of these Aβ fibrils into senile plaques (Suh and Checler, 2002). Compelling epidemiological evidence indicates that long-term NSAID therapy has a marked effect on the incidence of Alzheimer’s disease (McGeer and McGeer, 1996). Mefenamic acid, an anthranilic acid derivative, is a well known NSAID, although its anti-inflammatory properties are considered to be only moderate (Winder et al., 1962). In this study, we investigated the therapeutic potential of mefenamic acid in Alzheimer’s disease and its possible action mechanisms. We found that mefenamic acid reduces cell death induced by Aβ1-42 treatment or expression of Swe-APP or APP-CTs (CT59, CT99) in differentiated PC-12 cells; in addition, mefenamic acid reduces ROS and nitric oxide accumulation, cytochrome c release from mitochondria, caspase-3 activation, and the loss of Δψm induced by Aβ1-42 treatment and Swe-APP or APP-CTs expression. In addition, we found that mefenamic acid treatment up-regulated Bcl-XL protein levels in wt-APP-, Swe-APP-, or APP-CTs-transfected differentiated PC-12 cells (Fig. 4, E and F).

In eukaryotic cells, mitochondria are contributors to both the energetic processes necessary for life and signaling events leading to death. The mitochondrial pathway is a major road to apoptosis in vertebrate cells and is defined by a pivotal event, mitochondrial permeability transition (Menze et al., 2005). Mitochondrial permeability transition...
pores, consisting of a mitochondrial outer-membrane, voltage-dependent anion channel, inner-membrane adenine nucleotide translocase, and benzodiazepine receptor, are known to exert a central role in the sequence of events leading to apoptosis (Ly et al., 2003). The mitochondrial permeability transition pore opening is regulated by both Δψm, the predominant result of a proton gradient between the matrix and intermembrane space, and by the pH of the mitochondrial matrix (Petronilli et al., 1993). We found that mefenamic acid attenuated ROS accumulation induced by wt-APP, Swe-APP, or APP-CTs expression in differentiated PC-12 cells (Fig. 3, A and B), although further study remains to clarify the mechanisms of how mefenamic acid prevents ROS generation or accumulation.

Our results also showed that mefenamic acid reduced cells showing Δψm loss induced by Swe-APP (Fig. 3E), APP-CT59, or APP-CT99 expression (CT59, CT99) (Fig. 3F). These results are believed to be due to the action of mefenamic acid in significantly reducing the ROS levels generated by wt-APP, Swe-APP, or APP-CTs (CT59, CT99), as shown in Fig. 3, A and B.

Mitochondrial permeability transition pore opening allows the leakage of mitochondrial proteins, such as cytochrome c, apoptosis inducing factor, and Smac/Diablo by making solutes (K+, Mg2+, and Ca2+ ions) and water enter, thus swelling the mitochondrial matrix, rupturing the outer membrane (Ly et al., 2003). The accumulation of high levels of Ca2+ or the formation of ROS may overwhelm antioxidant defenses (i.e., induce oxidative stress) and destabilize mitochondria, thus reducing Δψm (Dykens and Kasari, 1997), which, depending on its severity, can lead to either apoptotic or necrotic cell death (Ankarcrona et al., 1995). Figure 4, A and B, shows that mefenamic acid treatment significantly inhibits cytochrome c release from mitochondria induced by wt-APP, Swe-APP, APP-CT59, or APP-CT99 expression. This result is consistent with mefenamic acid attenuating ROS accumulation and Δψm loss induced by wt-APP, Swe-APP, APP-CT59, or APP-CT99 expression.

Cytochrome c release is also known to be governed by Bcl-2 family proteins. The release of the solubilized mitochondrial pool of cytochrome c into the cytosol may follow the permeabilization of the outer mitochondrial membrane mediated by proapoptotic Bcl-2 family proteins, notably Bax and Bak, and/or by mitochondrial permeability transition (Orrenius, 2004).

Bcl-Xl is a pro-survival member of the Bcl-2 family and has been shown to antagonize the proapoptotic activity of Bax and promote cell survival by blocking Bax translocation from the cytosol to mitochondria, which prevents cytochrome c release (Tsujimoto and Shimizu, 2000; Hou et al., 2003). It is noteworthy that in the present study, Bcl-Xl protein expression was found to be up-regulated by mefenamic acid treatment; however, the detailed mechanism involved remains to be clarified.

In summary, our in vitro results suggest that mefenamic acid exerts a neuroprotective effect against Aβ1–42 treatment or Swe-APP or APP-CTs expression by inhibiting cytochrome c release from mitochondria and caspase-3 activation. This effect may be derived from the inhibitory effects of mefenamic acid on ROS and nitric oxide accumulation and from the loss of Δψm. In addition, mefenamic acid could promote cell survival by up-regulating the expression of the anti-apoptotic protein Bcl-Xl.

Furthermore, we found that mefenamic acid improves learning and memory impairment in an Aβ1–42 infused animal model, as determined by the Morris water maze test, suggesting that mefenamic acid has therapeutic potential for the treatment of Alzheimer's disease.

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