0026-895X/06/6901-76–84$20.00
MOLECULAR PHARMACOLOGY
Copyright © 2006 The American Society for Pharmacology and Experimental Therapeutics
Mol Pharmacol 69:76–84, 2006

Vol. 69, No. 1
15206/3072198
Printed in U.S.A.

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

Yuyoung Joo, Hye-Sun Kim, Ran-Sook Woo, Cheol Hyoung Park, Ki-Young Shin, Jean-Pyo Lee, Keun-A Chang, Seonghan Kim, and Yoo-Hun Suh

Department of Pharmacology, College of Medicine, National Creative Research Initiative Center for Alzheimer's Dementia and Neuroscience Research Institute, Medical Research Council, Seoul National University, Seoul, South Korea (Y.J., H.-S.K., R.-S.W., C.H.P., K.-Y.S., K.-A.C., S.K., Y.-H.S.); and Department of Pediatrics, School of Medicine, University of California at San Diego, La Jolla, California (J.-P.L.)

Received June 2, 2005; accepted October 13, 2005

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 $(A\beta)_{1-42}$ treatment and the expression of a Swedish double mutation (KM595/596NL) 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 $A\beta_{1-42}$, Swe-APP, or APP-CTs in neuronal cells. In addition, mefenamic acid up-regulates expression of the antiapoptotic protein Bcl-X_L. Moreover, our study demonstrates for the first time that mefenamic acid improves learning and memory impairment in an $A\beta_{1-42}$-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 progress 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 progress of Alzheimer's disease, but this effect is independent of its typical anti-inflammatory mech-

This work was supported by a National Creative Research Initiative Grant (2003–2005) from the Ministry of Science and Technology, in part by the BK21 Human Life Sciences program, and by a grant from Seoul National University Bundang Hospital Research Fund.

Y.J., H.-S.K., and R.-S.W. contributed equally to this study.

Article, publication date, and citation information can be found at http://molpharm.aspetjournals.org.
doi:10.1124/mol.105.015206.

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; $\Delta\psi$m, mitochondrial membrane potential.

anism: inhibition of cyclooxygenase. Treatment with ibuprofen was shown to result in the selective reduction of SDS-soluble amyloid β peptide $(A\beta)_{1-42}$ in Tg2576 mice harboring mutant human amyloid precursor protein (Yan et al., 2003). In addition, in cell-based studies, it was found that NSAIDs such as sulindac sulfide, ibuprofen, indomethacin, and flurbiprofen efficiently reduce the intracellular pool of $A\beta_{1-42}$ and selectively decrease $A\beta_{1-42}$ production, as determined by cell-free γ-secretase activity assays (Weggen et al., 2003). NSAIDs have also been demonstrated to lower $A\beta_{1-42}$ by specifically affecting the proximity between APP and presenilin 1 and by altering the conformation of presenilin 1 (Lieo et al., 2004).

Mefenamic acid [(2-[2,3-dimethylphenyl]amino)benzoic acid], an anthranilic acid derivative, is a nonsteroidal anti-inflammatory, antipyretic, and analgesic agent that is used for relief of postoperative and traumatic inflammation and swelling, antiphlogistic analgesic treatment of rheumatoid arthritis, and antipyretic in acute respiratory tract infection (Winder et al., 1962; Fang et al., 2004). In this study, we examined the therapeutic potential of mefenamic acid in Alzheimer's disease and its mechanisms of action. Using an in vitro model of Alzheimer's disease, we demonstrated that mefenamic acid has neuroprotective effects. Moreover, our study demonstrates for the first time that mefenamic acid improves learning and memory impairment in an $A\beta_{1-42}$-infused Alzheimer's disease rat model.

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_L 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 polyethylenimine (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, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS), penicillin-streptomycin-amphotericin B mixture (Invitrogen) at 37°C and 5% CO_2. PC-12 cells were treated with nerve growth factor (50 ng/ml; Calbiochem, Darmstadt, Germany) to differentiate for 48 h. SH-SY5Y human neuroblastoma cells obtained from the American Type Cell Collection (Manassas, VA) were grown in DMEM containing 10% FBS, penicillin-streptomycin-amphotericin B mixture (Invitrogen). Nerve growth factor differentiated PC-12 cells and SH-SY5Y cells were transiently transfected with 1 μg of plasmid DNA (APP-CT59, APP-CT99, wt-APP, or Swe-APP) and 3 μl of FuGene 6 (Roche Molecular Biochemicals, Mannheim, Germany) in 1 ml of DMEM with 0.3% FBS 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.

Lactate Dehydrogenase Release Assay. Degrees of cell death were assessed by activity of lactate dehydrogenase (LDH) released into the culture medium using a Cytotox 96 nonradioactive cytotoxicity assay kit (Promega, Madison, WI) according to the manufacturer's instructions. The results are expressed as percentage of maximum LDH release obtained upon complete cell lysis by 0.9% Triton X-100.

Measurement of Reactive Oxygen Species Generation. Intracellular ROS in PC-12 cells were assayed using the dye 2′,7′-dichlorofluorescein diacetate (DCFH-DA; Invitrogen). Cells were washed 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_2O_2. 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_2^-), the oxidized product of nitric oxide, using the Griess reaction as described previously (Hirata et al., 1993). Nitric oxide is an electrochemically reactive species that can be oxidized. To measure the total nitric oxide oxidation products, NO_3^- is first reduced to NO_2^- 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-napthyl)-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 Pharmacia). 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).

$A\beta_{1-42}$-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 $A\beta_{1-42}$ 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 $A\beta_{1-42}$ (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

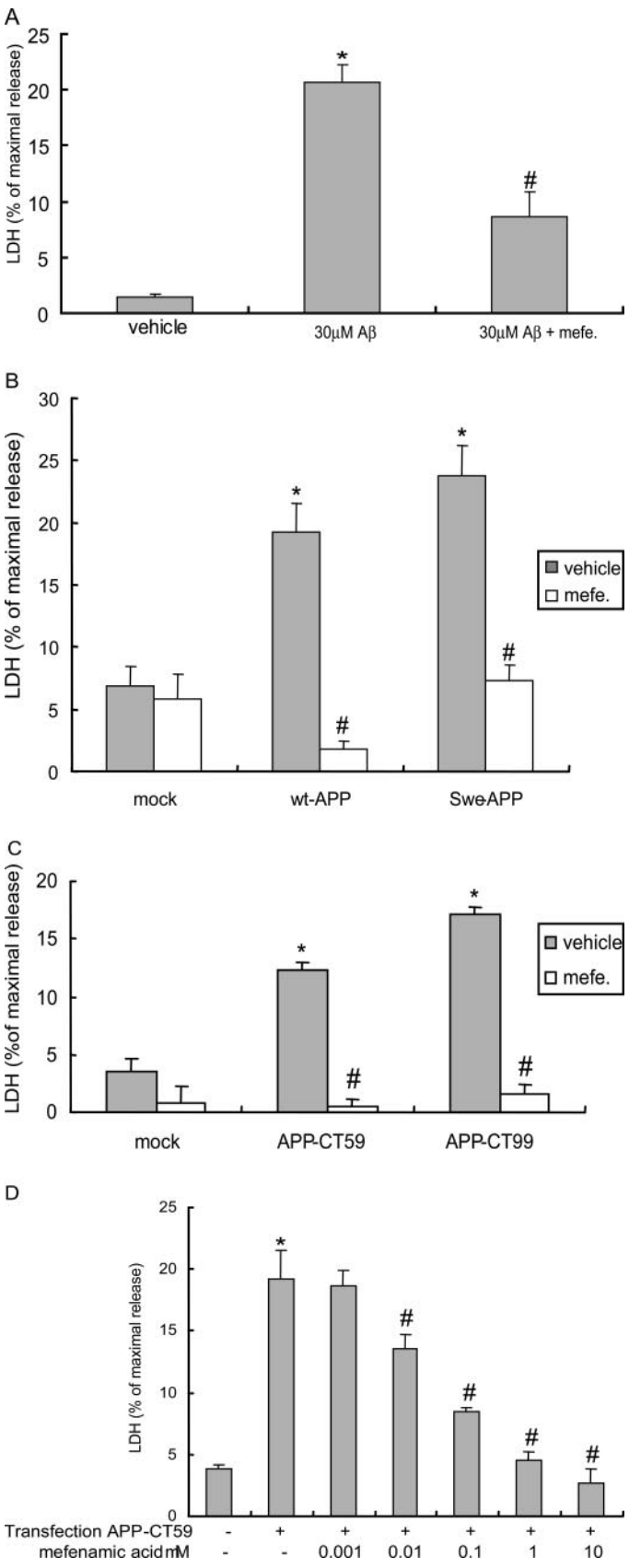


Fig. 1. Mefenamic acid attenuates the neuronal cell death induced by $A\beta_{1-42}$ treatment or Swedish APP expression in nerve growth factor-differentiated PC-12 cells. A, differentiated PC-12 cells were pretreated with vehicle or 5 μM mefenamic acid for 24 h and then treated with 30 μM $A\beta_{1-42}$. Twenty-four hours after $A\beta_{1-42}$ treatment, the degrees of cell death were assessed 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 $A\beta_{1-42}$-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 degrees of cell death were 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). C, nerve growth factor-differentiated PC-12 cells were transfected with mock, APP-CT59, 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 (by one-way ANOVA); #, $p < 0.05$ versus vehicle-treated APP-CT59-transfected cells (by one-way ANOVA).

4.0 ventral to the surface of the skull, according to the brain atlas of Paxinos and Watson (1986). Mefenamic acid (5 mg/kg/day) or PBS was then injected i.p. for 3 weeks.

Morris Water Maze Task. The experimental apparatus, a circular water tank (140 cm in diameter, 45 cm high) filled with opaque water made by adding dry milk powder to water at the temperature of 21~23°C, was located in a laboratory that contained prominent extra-maze cues. Animals were required to find a submerged platform (15 cm in diameter, 35 cm high) in the pool using the spatial cues. The two starting points were changed daily. Spatial training consisted of five sessions (two trials per session per day) during which the platform was left in the same position. In each training session, the latency to escape onto the hidden platform was recorded. After the final training session, a single probe trial was conducted. The escape platform was removed and each rat was allowed to swim for 90 s in the maze. The number of times the rat crossed the annulus where the platform had been located was recorded. Data collection was automated by a video image motion analyzer (Ethovision; Noldus Information Technology h.v., Wageningen, The Netherlands).

Statistical Analysis. Data are expressed as mean ± S.E.M. values. One-way ANOVA tests were applied to study the relationship between the different variables. $p < 0.05$ was considered significant.

Results

Transient Transfection into 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-X_L 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 effi-

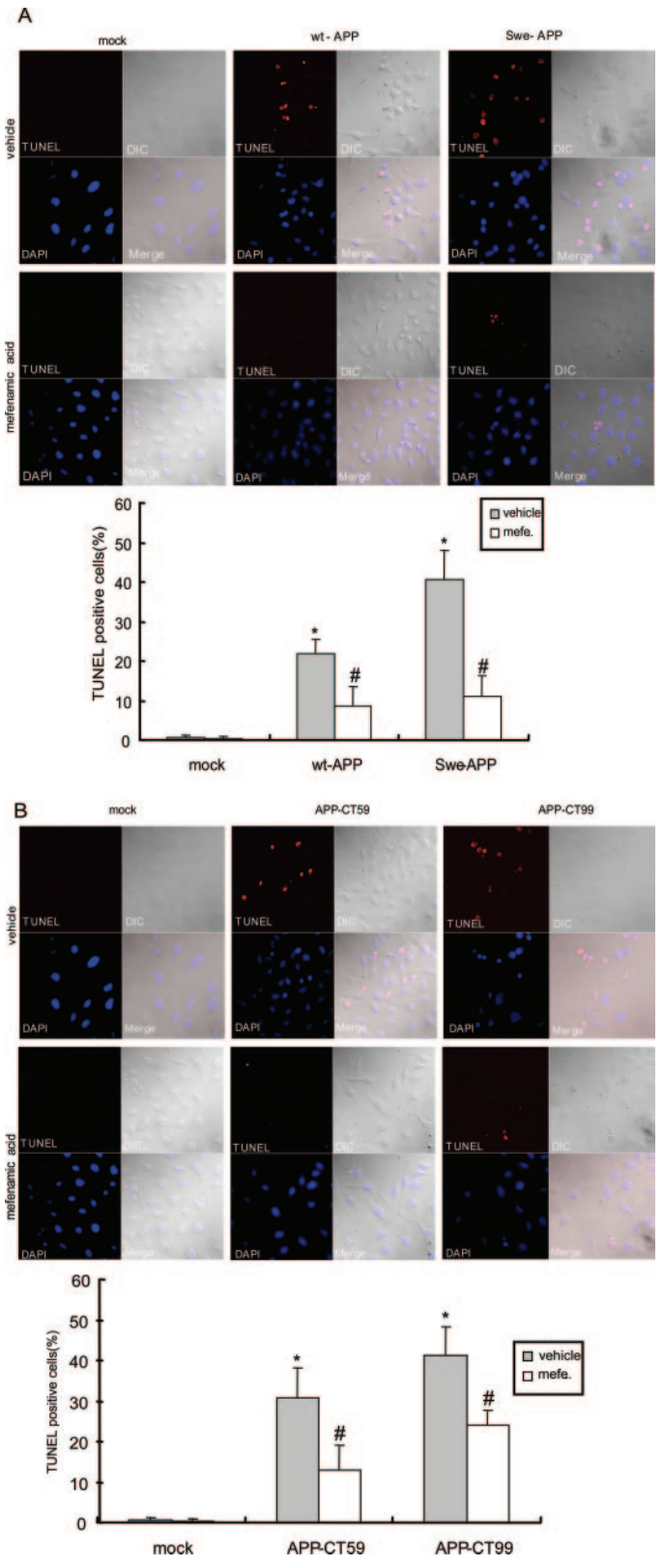


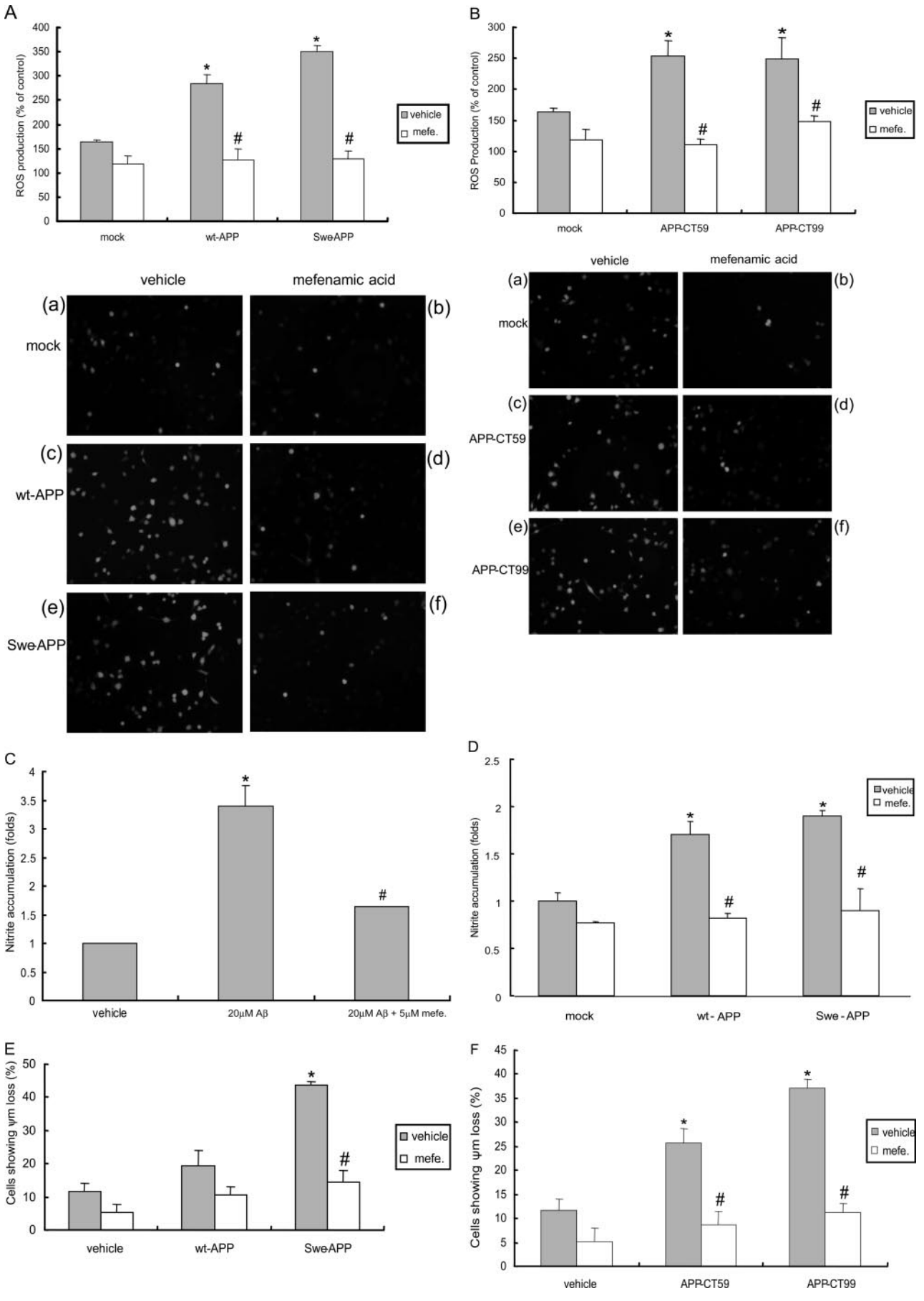
Fig. 2. Mefenamic acid attenuates apoptosis induced by Swedish APP or APP-CTs expression in nerve growth factor-differentiated PC-12 cells. A, apoptotic cells were detected using TUNEL staining among vehicle or 5 μM mefenamic acid-treated mock-, wt-APP-, or Swe-APP-transfected SH-SY5Y cells at 48 h after transfection. The percentage of TUNEL-positive cells 48 h after transfection is shown in the graph. 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. $*$, $p < 0.05$ versus vehicle-treated mock-transfected cells; #, $p < 0.05$ versus vehicle-treated wt-APP- or Swe-APP-transfected cells (by one-way ANOVA). DIC, differential interference contrast. B, apoptotic cells were detected using TUNEL staining among vehicle- or 5 μM mefenamic acid-treated mock-, APP-CT59-, or APP-CT99-transfected SH-SY5Y cells at 48 h after transfection. The percentage of TUNEL-positive cells 48 h after transfection is shown in the graph. $*$, $p < 0.05$ versus vehicle-treated mock-transfected cells; #, $p < 0.05$ versus vehicle-treated APP-CT59- or APP-CT99-transfected cells (by one-way ANOVA).

ciencies were 40.0 $\pm$ 4.0% (n = 5) and 45.2 $\pm$ 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 Aβ_{1-42} 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 Aβ_{1-42} 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 Aβ_{1-42} at 30 μM for 24 h in nerve growth factor-differentiated PC-12 cells versus vehicle-pretreated 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 Aβ_{1-42} 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 $\pm$ 0.33, 21.99 $\pm$ 8.56, 40.75 $\pm$ 7.17, 30.90 $\pm$ 7.15, or 41.31 $\pm$ 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 $\pm$ 0.21, 8.55 $\pm$ 5.01, 11.02 $\pm$ 5.31, 12.88 $\pm$ 6.10, or 23.90 $\pm$ 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



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 $A\beta_{1-42}$ 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 $A\beta_{1-42}$ 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 ($\Delta\psi$m) 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 $\Delta\psi$m induced by Swe-APP or APP-CTs expression. Our results showed that mefenamic acid reduced the cells showing $\Delta\psi$m 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-X_L protein levels after mefenamic acid treatment. Mefenamic acid was found to up-regulate Bcl-X_L protein in mock-, 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 $A\beta_{1-42}$-Infused Alzheimer's Disease Rat Model. We assessed the neuroprotective effects of mefenamic acid in an in vivo system. $A\beta_{1-42}$ (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 ($n = 10$) $A\beta_{1-42}$-infused rat models 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 $A\beta_{1-42}$-infused rats was attenuated by mefenamic acid treatment, we performed a probe test and

Fig. 3. Mefenamic acid reduces ROS accumulation and attenuates the mitochondrial membrane potential loss induced by Swe-APP expression. A, ROS was detected using DCFH-DA dye in vehicle- or 5 μM mefenamic acid-treated nerve growth factor-differentiated PC-12 cells expressing mock, wt-APP, or Swe-APP by fluorescence microscopy (a–f). ROS production was also measured using DCFH-DA dye in vehicle- or mefenamic acid-treated nerve growth factor-differentiated PC-12 cells expressing mock, wt-APP, or Swe-APP. Fluorescence in cells in 96-well plates was read immediately at 504 nm (excitation) and 529 nm (emission) using a fluorescence plate reader (Molecular Devices, Sunnyvale, CA). *, $p < 0.05$ versus vehicle treated mock-transfected cells; #, $p < 0.05$ versus vehicle-treated wt-APP- or Swe-APP-transfected cells (by one-way ANOVA). B, ROS was detected using DCFH-DA dye in vehicle- or 5 μM mefenamic acid-treated nerve growth factor-differentiated PC-12 cells expressing mock, APP-CT59, or APP-CT99 by fluorescence microscopy (a–f). ROS production was also measured using DCFH-DA dye in vehicle- or mefenamic acid-treated nerve growth factor-differentiated PC-12 cells expressing mock, APP-CT59, or APP-CT99. Fluorescence in cells in 96-well plates was read immediately at wavelengths of 504 nm (excitation) and 529 nm (emission) using a fluorescence plate reader (Molecular Devices). *, $p < 0.05$ versus vehicle-treated, mock-transfected cells; #, $p < 0.05$ versus vehicle-treated APP-CT59- or APP-CT99-transfected cells (by one-way ANOVA). C, differentiated PC-12 cells were pretreated with PBS or 5 μM mefenamic acid for 24 h and then treated with 30 μM $A\beta_{1-42}$. Twenty-four hours after $A\beta_{1-42}$ treatment, nitric oxide production was evaluated by measuring the level of nitrite (NO_2^-, the oxidized product of nitric oxide) using the Griess reaction as described previously (13). The amount of nitric oxide produced was normalized against the number of cells. *, $p < 0.05$ versus vehicle-treated cells; #, $p < 0.05$ versus 20 μM $A\beta_{1-42}$-treated cells (by one-way ANOVA). D, nerve growth factor-differentiated PC-12 cells were mock-transfected or transfected with wt-APP or Swe-APP and then treated with PBS or 5 μM mefenamic acid 6 h after transfection. Forty-eight hours after treatment, nitric oxide production was evaluated as described in C. *, $p < 0.05$ versus vehicle treated mock-transfected cells; #, $p < 0.05$ versus vehicle-treated wt-APP- or Swe-APP-transfected cells (by one-way ANOVA). E and F, mitochondrial membrane potentials were measured by flow cytometry using rhodomine-6G (Sigma Chemical). The proportion of cells showing $\Delta\psi$m loss were plotted for vehicle- or 5 μM mefenamic acid-treated nerve growth factor-differentiated PC-12 cells expressing wt-APP or Swe-APP. *, $p < 0.05$ versus vehicle-treated mock transfected cells; #, $p < 0.05$ versus vehicle-treated wt-APP- or Swe-APP-transfected cells (by one-way ANOVA) (E). F, the proportion of cells showing $\Delta\psi$m loss are plotted for vehicle- or 5 μM mefenamic acid-treated nerve growth factor-differentiated PC-12 cells expressing APP-C59 or APP-C99. Each value represents a mean± S.E.M. ($n = 4$) expressed as a percentage of the corresponding vehicle-treated control culture. *, $p < 0.05$ versus vehicle-treated mock-transfected cells; #, $p < 0.05$ versus vehicle-treated APP-CT59- or APP-CT99-transfected cells (by one-way ANOVA).

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 Aβ_{1-42} treatment or expression of Swe-APP or APP-CTs (CT 59, CT 99) 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 $\Delta\psi$m induced by Aβ_{1-42} treatment and Swe-APP or APP-CTs expression. In addition, we found that mefenamic acid treatment up-regulated Bcl-X_L 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

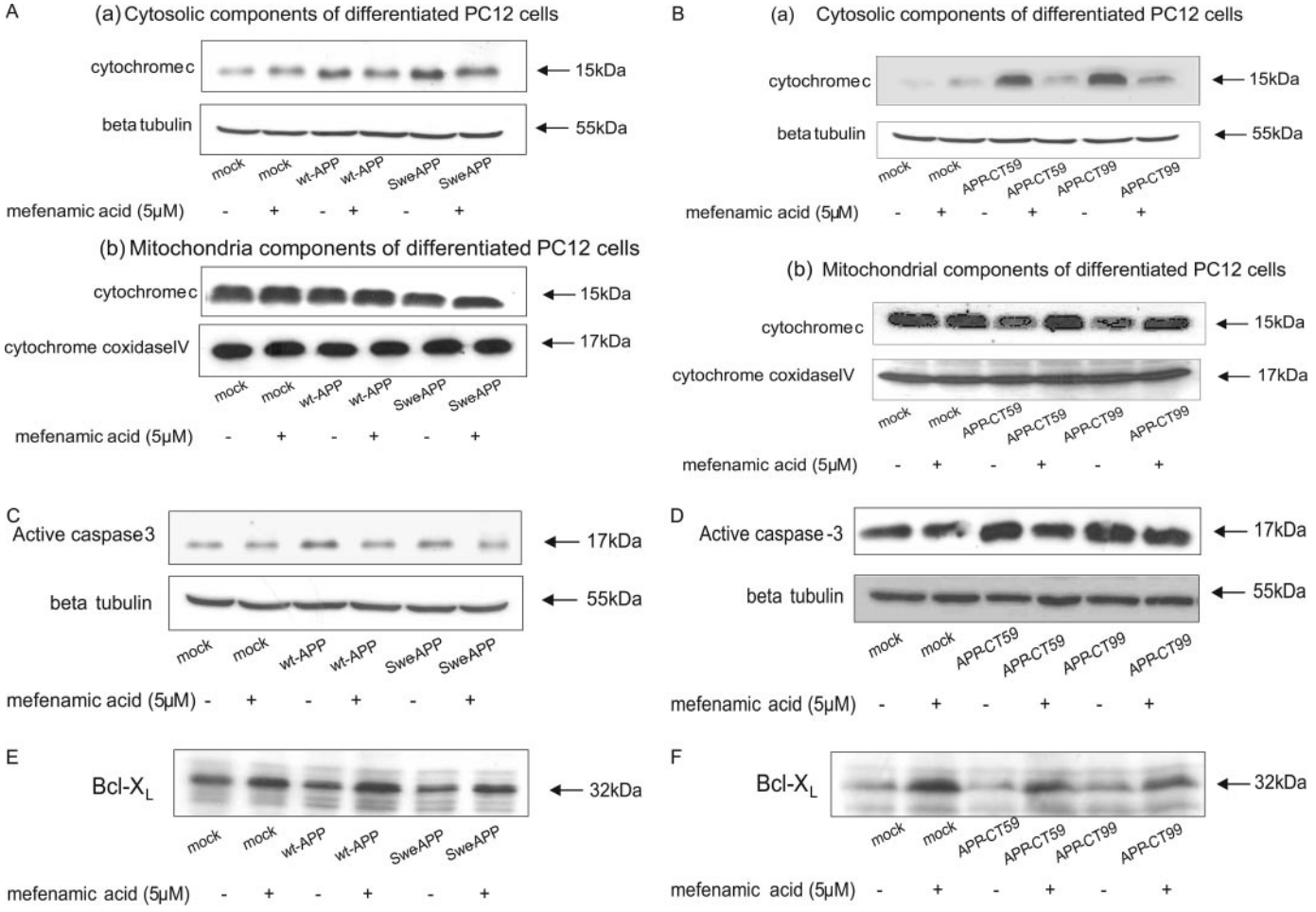


Fig. 4. Mefenamic acid inhibits cytochrome *c* release from mitochondria, and caspase-3 activation induced by Swe-APP expression. A, representative immunoblots for cytochrome *c*, cytochrome *c* oxidase IV, β-tubulin in mitochondrial or cytosolic fractions of vehicle or 5 μM mefenamic acid-treated wt-APP- or Swe-APP-transfected PC-12 cells are shown. The results are representative of three separate experiments performed with different samples. B, representative immunoblots for cytochrome *c*, cytochrome *c* oxidase IV, β-tubulin in mitochondrial or cytosolic fractions of vehicle- or 5 μM mefenamic acid-treated APP-CT59- or APP-CT99-transfected PC-12 cells are shown. The results are representative of three separate experiments performed with different samples. C, representative immunoblots for active caspase-3, β-tubulin in vehicle- or 5 μM mefenamic acid-treated wt-APP- or Swe-APP-transfected PC-12 cells. The results are representative of three separate experiments performed with different samples. D, representative immunoblots for active caspase-3, β-tubulin in vehicle- or 5 μM mefenamic acid-treated APP-CT59- or APP-CT99-transfected PC-12 cells. The results are representative of three separate experiments performed with different samples. E, Bcl-X_L was detected by immunoblotting in vehicle- or 5 μM mefenamic acid-treated wt-APP- or Swe-APP-transfected PC-12 cells. The results are representative of three separate experiments performed with different samples. F, Bcl-X_L was detected by immunoblotting in vehicle- or 5 μM mefenamic acid-treated APP-C59- or APP-C99-transfected PC-12 cells. The results are representative of three separate experiments performed with different samples.

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 $\Delta\psi m$, 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 $\Delta\psi m$ 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^+, Mg^{+2}, and Ca^{2+} ions) and water enter, thus swelling the mitochondrial matrix, rupturing the outer membrane (Ly et al., 2003). The accumulation of high levels of Ca^{2+} or the formation of ROS may overwhelm antioxidant defenses (i.e., induce oxidative stress) and destabilize mitochondria, thus reducing $\Delta\psi m$ (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 $\Delta\psi m$ 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-X_L 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-X_L 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 $A\beta_{1-42}$ 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 $\Delta\psi m$. In addition, mefenamic acid could promote cell survival by up-regulating the expression of the anti-apoptotic protein Bcl-X_L.

Furthermore, we found that mefenamic acid improves learning and memory impairment in an $A\beta_{1-42}$ 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.

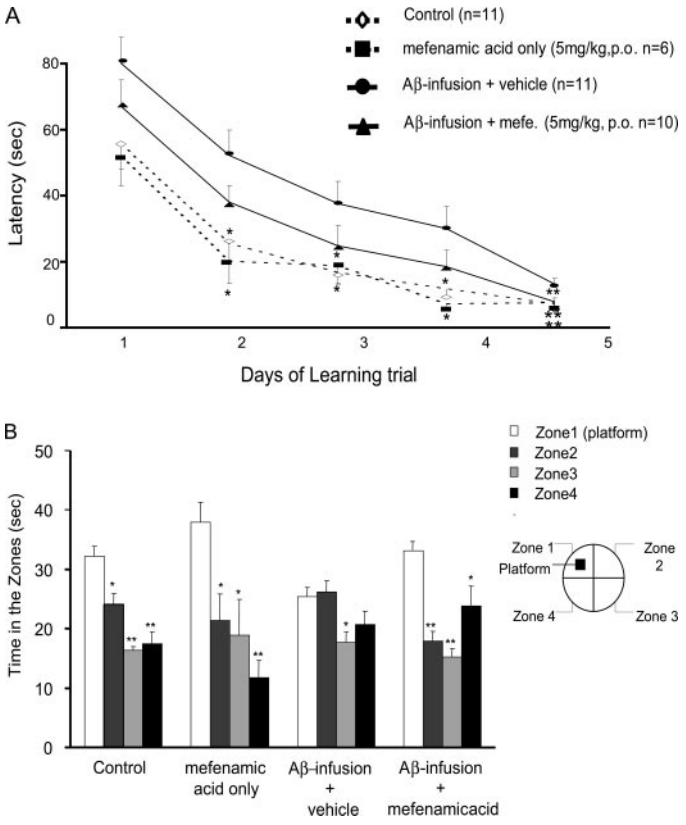


Fig. 5. Mefenamic acid attenuates learning and memory impairment in an $A\beta_{1-42}$-infused Alzheimer's disease animal model. A, $A\beta_{1-42}$ (600 pmol/day) was infused into lateral ventricles using an osmotic pump for 7 days. Mefenamic acid (5 mg/kg/day) was administered i.p. during the subsequent 3 weeks. After the water maze test training, testing was performed over five sessions after mefenamic acid or vehicle had been administered intraperitoneally for 3 weeks. Latency times for the animals in the mefenamic acid-treated group were compared with those of vehicle-treated animals (one-way ANOVA, $p < 0.05$ versus vehicle). B, the probe test was performed after the final training session. The times that rats of the mefenamic acid-injected group stayed in zones 1, 2, 3, and 4 were compared with those of the vehicle-injected group (one-way ANOVA, $p < 0.05$ versus vehicle).

Acknowledgments

We are grateful to Drs. Sangram S. Sisodia and Seoung-Hun Kim at the University of Chicago for providing the wt-APP and Swe-APP cDNA.

References

Ankarcrona M, Dypbukt JM, Bonfoco E, Zhivotovsky B, Orrenius S, Lipton SA, and Nicotera P (1995) Glutamate-induced neuronal death: a succession of necrosis or apoptosis depending on mitochondrial function. *Neuron* **15:**961–973.

Asanuma M, Nishibayashi-Asanuma S, Miyazaki I, Kohno M, and Ogawa N (2001) Neuroprotective effects of non-steroidal anti-inflammatory drugs by direct scavenging of nitric oxide radicals. *J Neurochem* **76:**1895–1904.

Cacquevel M, Lebeurrier N, Cheenne S, and Vivien D (2004) Cytokines in neuroinflammation and Alzheimer's disease. *Curr Drug Targets* **5:**529–534.

Dykens EM and Kasari C (1997) Maladaptive behavior in children with Prader-Willi syndrome, Down syndrome and nonspecific mental retardation. *Am J Ment Retard* **102:**228–237.

Eckert A, Schindowski K, Leutner S, Luckhaus C, Touchet N, Czech C, and Muller

WE (2001) Alzheimer's disease-like alterations in peripheral cells from presenilin-1 transgenic mice. *Neurobiol Dis* **8:**331–342.
Fang L, Numajiri S, Kobayashi D, Ueda H, Nakayama K, Miyamae H, and Morimoto Y (2004) Physicochemical and crystallographic characterization of mefenamic acid complexes with alkanolamines. *J Pharm Sci* **93:**144–154.
Hirata Y, Emori T, Eguchi S, Kanno K, Imai T, Ohta K, and Marumo F (1993) Endothelin receptor subtype B mediates synthesis of nitric oxide by cultured bovine endothelial cells. *J Clin Investig* **91:**1367–1373.
Hou Q, Cymbalyuk E, Hsu SC, Xu M, and Hsu YT (2003) Apoptosis modulatory activities of transiently expressed Bcl-2: roles in cytochrome C release and Bax regulation. *Apoptosis* **8:**617–629.
Kang D, Nishida J, Iyama A, Nakabeppu Y, Furuichi M, Fujiwara T, Sekiguchi M, and Takeshige K (1995) Intracellular localization of 8-oxo-dGTPase in human cells, with special reference to the role of the enzyme in mitochondria. *J Biol Chem* **270:**14659–14665.
Keil U, Bonert A, Marques CA, Scherping I, Weyermann J, Strosznajder JB, Muller-Spahn F, Haass C, Czech C, Pradier L, et al. (2004) Amyloid β-induced changes in nitric oxide production and mitochondrial activity lead to apoptosis. *J Biol Chem* **279:**50310–50320.
Kim HS, Kim EM, Kim NJ, Chang KA, Choi Y, Ahn KW, Lee JH, Kim S, Park CH, and Suh YH (2004) Inhibition of histone deacetylation enhances the neurotoxicity induced by the C-terminal fragments of amyloid precursor protein. *J Neurosci Res* **75:**117–124.
Kim HS, Kim EM, Lee JP, Park CH, Kim S, Seo JH, Chang KA, Yu E, Jeong SJ, Chong YH, et al. (2003) C-Terminal fragments of amyloid precursor protein exert neurotoxicity by inducing glycogen synthase kinase-3beta expression. *FASEB J* **13:**1951–1953.
Kim HS, Park CH, Cha SH, Lee JH, Lee S, Kim Y, Rah JC, Jeong SJ, and Suh YH (2000) Carboxyl-terminal fragment of Alzheimer's APP destabilizes calcium homeostasis and renders neuronal cells vulnerable to excitotoxicity. *FASEB J* **11:**1508–1517.
Lieo A, Berezovska O, Herl L, Raju S, Deng A, Bacskai BJ, Frosch MP, Irizarry M, and Hyman BT (2004) Nonsteroidal anti-inflammatory drugs lower Abeta42 and change presenilin 1 conformation. *Nat Med* **10:**1065–1066.
Ly JD, Grubb DR, and Lawen A (2003) The mitochondrial membrane potential (deltapsi(m)) in apoptosis; an update. *Apoptosis* **8:**115–128.
McGeer EG and McGeer PL (2003) Inflammatory processes in Alzheimer's disease. *Prog Neuropsychopharmacol Biol Psychiatry* **5:**741–749.
McGeer PL and McGeer EG (1996) Anti-inflammatory drugs in the fight against Alzheimer's disease. *Ann NY Acad Sci* **777:**213–220.
Menze MA, Hutchinson K, Laborde SM, and Hand SC (2005). Mitochondrial permeability transition in the crustacean *Artemia franciscana*: absence of a Ca^{2+}-regulated pore in the face of profound calcium storage. *Am J Physiol* **289:**R68–R76.
Orrenius S (2004) Mitochondrial regulation of apoptotic cell death. *Toxicol Lett* **149:**19–23.
Paxinos G and Watson C (1986) *The Rat Brain in Stereotaxic Coordinates.* Academic Press, San Diego.
Petronilli V, Cola C, and Bernardi P (1993) Modulation of the mitochondrial cyclosporin A-sensitive permeability transition pore. II. The minimal requirements for pore induction underscore a key role for transmembrane electrical potential, matrix pH and matrix Ca^{2+}. *J Biol Chem* **268:**1011–1016.
Sugaya K, Uz T, Kumar V, and Manev H (2000) New anti-inflammatory treatment strategy in Alzheimer's disease. *Jpn J Pharmacol* **82:**85–94.
Suh YH and Checler F (2002) Amyloid precursor protein, presenilins, and α-synuclein: molecular pathogenesis and pharmacological applications in Alzheimer's disease. *Pharmacol Rev* **54:**469–525.
Tsujimoto Y and Shimizu S (2000) Bcl-2 family: life-or-death switch. *FEBS Lett* **466:**6–10.
Wang RG and Zhu XZ (2003) Subtoxic concentration of manganese synergistically potentiates 1-14 methyl-4-phenylpyridinium-induced neurotoxicity in PC12 cells. *Brain Res* **961:**131–138.
Weggen S, Eriksen JL, Sagi SA, Pietrzik CU, Ozols V, Fauq A, Golde TE, and Koo EH (2003) Evidence that nonsteroidal anti-inflammatory drugs decrease amyloid β42 production by direct modulation of gamma-secretase activity. *J Biol Chem* **278:**31831–31837.
Winder CV, Wax J, Scotti L, Scherre RA, Jones EM, and Short FW (1962) Anti-inflammatory, antipyretic and antinociceptive properties of *N*-(2,3-xylyl) anthranilic acid (mefenamic acid). *J Pharmacol Exp Ther* **38:**405–413.
Yan Q, Zhang J, Liu H, Babu-Khan S, Vassar R, Biere AL, Citron M, and Landreth G (2003) Anti-inflammatory drug therapy alters beta-amyloid processing and deposition in an animal model of Alzheimer's disease. *J Neurosci* **23:**7504–7509.
Zou H, Henzel WJ, Liu X, Lutschg A, and Wang X (1997) Apaf-1, a human protein homologous to C. elegans CED-4, participates in cytochrome *c*-dependent activation of caspase-3. *Cell* **90:**405–413.

Address correspondence to: Yoo-Hun Suh, Department of Pharmacology, College of Medicine National Creative Research Initiative Center for Alzheimer's Dementia Seoul National University Seoul, 110-799, South Korea. E-mail: yhsuh@plaza.snu.ac.kr