Endocannabinoids Prevent β-Amyloid-mediated Lysosomal Destabilization in Cultured Neurons*

Received for publication, July 7, 2010, and in revised form, October 4, 2010 Published, JBC Papers in Press, October 5, 2010, DOI 10.1074/jbc.M110.162040

Janis Noonan, Riffat Tanveer, Allan Klompas, Aoife Gowran, Joanne Mckierman, and Veronica A. Campbell

From the Department of Physiology, School of Medicine and Trinity College Institute of Neuroscience, Trinity College, Dublin 2, Ireland

Alzheimer disease (AD) is a debilitating illness of the brain defined by the progressive deterioration of cognition and memory as a result of selective neuronal loss in the hippocampus and surrounding areas of the cerebral cortex. There is substantial evidence to suggest that at least a subset of neurons in the AD brain die by apoptosis. The principal neuropathological hallmark of the disease, of neurons in the AD brain die by apoptosis (2). The principal

Recently, the lysosomal system has been implicated in AD pathogenesis (9, 10). Neurons of AD patients demonstrate alterations in the lysosomal system, including the cellular pathways that converge on it, namely endocytosis and autophagy (10, 11). Such alterations include an increase in the size and number of endosomes (10, 12), autophagosomes (13) and lysosomes (10) and an increase in the gene expression and synthesis of all classes of lysosomal hydrolases, including cathepsins (14). In addition to their role in the digestion of cellular waste, it has become clear that partial and selective lysosomal membrane permeabilization (LMP), followed by the release of lysosomal enzymes into the cytosol, can induce apoptotic cell death (15). Cathepsins D and L are among the lysosomal proteases that have been implicated in apoptosis by virtue of their ability to activate apoptotic effectors, such as mitochondrial uncoupling and caspases (16).

Among the agents that are capable of destabilizing lysosomes, Aβ has emerged as an inducer of LMP (17). Cultured primary neurons are able to internalize Aβ from the culture medium where it accumulates within lysosomes, resulting in the loss of lysosomal membrane integrity and activation of the apoptotic cascade (18). We have recently identified that Aβ destabilizes lysosomes early in the apoptotic cascade in a manner involving the tumor suppressor protein, p53, and its transcription target, Bax (9). However, the precise mechanism by which Aβ causes lysosomes to become vulnerable to LMP has not yet been resolved.

The endocannabinoid system has emerged as a promising new target for neuroprotective therapy in AD (19). This system comprises the G protein-coupled cannabinoid (CB) receptors, CB1 and CB2, their endogenous ligands, anandamide (AEA) and 2-arachidonoylglycerol (2-AG), and their degradative enzymes (20). Among their numerous functions, endocannabinoids are believed to play a role in the cell death/survival decision and thus govern cell fate (21). Numerous studies have identified neuroprotective roles of the endocannabinoid system against excitotoxicity (22), oxidative stress (23), and inflammation (24), all key pathological events in the AD brain. Moreover, endocannabinoids have been directly implicated in the protection of neurons against Aβ toxicity (25, 26). Recently, it was reported that CB1 receptors can be targeted to lysosomes via the adaptor protein, AP3, and are capable of mediating signal transduction while located at the lysosomal compartment (27). This spatial compartmentalization suggests a mechanism for diversity in CB1 receptor signaling and indicates a functional role for CB1 receptors at the lysosome. This has prompted us to investigate herein whether
Endocannabinoids Prevent Lysosomal Destabilization

the neuroprotective capacity of the endocannabinoid system is based on its ability to stabilize lysosomes and thus block the lysosomal branch of the apoptotic pathway.

The results from this study indicate that endocannabinoids have the ability to stabilize lysosomes against Aβ-induced LMP and thus may represent a novel target for therapeutic intervention in AD.

EXPERIMENTAL PROCEDURES

Culture of Cortical Neurons—Primary cortical neurons were established as described previously (28). Briefly, 1-day-old male Wistar rats were decapitated in accordance with institutional and national ethical guidelines, and their cerebral cortices were removed. The dissected cortices were incubated in phosphate-buffered saline (PBS) containing trypsin (0.3%) for 25 min at 37 °C. The tissue was then triturated (X5) in PBS containing soybean trypsin inhibitor (0.1%) and DNase (0.2 mg/ml) and gently filtered through a sterile mesh filter. Following centrifugation, 2000 x g for 3 min at 20 °C, the pellet was resuspended in neurobasal medium, supplemented with heat-inactivated horse serum (10%), penicillin (100 units/ml), streptomycin (100 units/ml), and GlutaMAX (2 mM). Suspended cells were plated out at a density of 0.25 – 10⁶ cells on circular 13 mm diameter coverslips, coated with poly-L-lysine (60 μg/ml), and incubated in a humidified chamber containing 5% CO₂, 95% air at 37 °C. After 48 h, 5 ng/ml cytosine-arabinoside was included in the culture medium to prevent proliferation of non-neuronal cells. Culture medium was exchanged every 3 days, and cells were grown in culture for 5 days prior to treatment.

Drug Treatment—Aβ(1–40) (BioSource International Inc.) was made up as a 200 μM stock solution in PBS and double-deionized water and allowed to aggregate for 48 h at 37 °C. For treatment of cortical neurons, Aβ was diluted to a final concentration of 2 μM in prewarmed neurobasal medium. Control cells were exposed to the reverse peptide, Aβ(40–1) (2 μM), which we have previously demonstrated to lack toxicity (8). Cells were exposed to Aβ in the presence or absence of the caspase-3 inhibitor, Z-DEVD-FMK (10 μM) (29) (Tocris Bioscience), to assess whether the apoptotic pathway was activated in Aβ-induced cell death. Cells were then exposed to Aβ in the presence or absence of the endocannabinoids, AEA (10 nM; Sigma-Aldrich) or 2-AG (10 nM; Sigma-Aldrich) to assess their neuroprotective capacity. The dose of AEA and 2-AG used was based on dose response experiments using the TUNEL technique. 10 nM was subsequently chosen because that concentration had no effect on basal cell viability. In some experiments, the hydrolysis of AEA and 2-AG was prevented by incubation with an inhibitor of fatty acid amide hydrolase, URB 597 (1 μM) (30) (Cayman). The role of calpain in Aβ-induced toxicity was assessed using an inhibitor of its activity, MDL 28170 (10 μM) (6) (Merck). The involvement of CB1 receptors in endocannabinoid-mediated neuroprotection was investigated using various antagonists, SR 141716A (1 μM) (31) (gift from Dr. David Finn at The National University of Ireland, Galway. Original source: The National Institute of Mental Health’s Chemical Synthesis and Drug Supply Program), hemopressin (10 μM) (32, 33) (Tocris), and AM 251 (10 μM) (34) (Tocris). In all cases, the concentration of inhibitors used was in agreement with the literature or based on previous publications, as indicated.

The water-soluble CB1 receptor agonist, O-2545, was used as a positive control to demonstrate the ability of hemopressin to block surface CB1 receptor activity. Finally, a neutralizing cathepsin L antibody (1 μM) (Merck) was used to assess the toxicity of cathepsin L released into the culture medium.

Phospho-p53Ser-15/ERK(Tyr-205) Immunocytochemistry and Lysosomal Localization of Phospho-p53Ser-15—Following treatment, cells were fixed with paraformaldehyde (4%) for 30 min at 37 °C, permeabilized with Triton X-100 (0.2%), and refixed with 4% paraformaldehyde for 10 min. Cells were incubated overnight at 4 °C with a rabbit polyclonal antibody (p53; recognizes p53 phosphorylated on serine 15 (p-p53Ser-15)); 1:100 dilution in 10% serum; Cell Signaling Technologies) or with a mouse polyclonal antibody (ERK; recognizes ERK phosphorylated on tyrosine 205 (p-ERK Tyr-205); 1:200 dilution in 20% serum; Santa Cruz Biotechnology, Inc. (Santa Cruz, CA)). Immunoreactivity was detected using a biotinylated goat anti-rabbit IgG (p-p53Ser-15) and a biotinylated horse anti-mouse (p-ERK Tyr-205). Cells were then incubated with Alexa Fluor 488™ avidin conjugate for 1 h (1:2000 dilution in 10% serum; Invitrogen), and the nucleus was stained with Hoechst (1:1000 dilution in 5% serum; Invitrogen) for 15 min at room temperature. In order to assess colocalization of p-p53Ser-15 with lysosomes, the fluorescent probe, LysoTracker™ Red (Invitrogen), was used to visualize lysosomes in intact cells. Cells were exposed to prewarmed neurobasal medium containing LysoTracker™ Red (700 nm) for 25 min prior to exposure to cell treatments and p-p53Ser-15 immunocytochemistry.

Cells were viewed under X63 magnification using a confocal microscope (Zeiss LSM 510 META). The multitrack FITC/rhodamine channel configuration was selected (for Alexa 488, excitation at 488 nm and emission at 520 nm; for LysoTracker™ probe, excitation at 543 nm and emission at 599 nm).

Lysosomal Integrity Assay; Acridine Orange (AO) Relocation—The lysosomal integrity assay was carried out as described previously (9). Briefly, cells were exposed to prewarmed supplemented neurobasal medium containing acridine orange (5 μg/ml; Invitrogen) for 10 min at 37 °C. Cells were rinsed in neurobasal medium, exposed to cell treatments for 6 h, and viewed by confocal microscopy. Visualization of the fluorophore was achieved using the 488-nm argon laser in the λ mode, where emission over the 499–670 nm range was collected. The configuration parameters were as follows: 1) filters (Ch3-BP, 585–615 nm; Ch2-BP, 505–530 nm, and Ch51, 499.3–670.7 nm); 2) beam splitters (HFT 488 nm); 3) scan zoom 1. For each digital image, 512 x 512 pixels were used. The leakage of AO from the lysosome produces a decrease in the 633 nm emission, and this parameter was used. The leakage of AO from the lysosome produces a decrease in the 633 nm emission, and this parameter was used as an index of lysosomal integrity, as reported previously (35). The average cellular fluorescence at 633 nm was measured from at least 200 cells for each treatment, from at least four independent experiments.
Endocannabinoids Prevent Lysosomal Destabilization

Cathepsin D Localization—To assess the intracellular distribution of cathepsin D, neurons were incubated with BODIPY FL-pepstatin A (1 μM; Invitrogen) for 1 h at 37 °C. BODIPY FL-pepstatin A is pepstatin A (isovaleryl-l-valyl-l-4-amino-3-hydroxy-6-methylheptanoyl-l-alanyl-l-4-amino-3-hydroxy-6-methylheptanoic acid), covalently conjugated with the Boron dipyrromethene difluorophore (BODIPY). Following treatment, the incorporated fluorophore was examined with a confocal microscope (LSM-510 META, Zeiss), as described previously (36).

Cathepsin L Activity—Cathepsin L activity was measured using a commercially available fluorogenic assay under conditions that inactivate other thiol proteases, such as cathepsin B. Culture medium was used from cells treated with Aβ in the presence or absence of AEA and 2-AG and incubated with the cathepsin L substrate, Z-Phe-Arg, conjugated to aminofluorocoumarin (200 μM; Alexis Biochemicals) and the reaction buffer (20 mM NaOAc, pH 5.0, 4 mM EDTA, 8 mM DTT, and 0.4 M urea) for 1 h at 37 °C in a reaction volume of 100 μl. The amount of free aminofluorocoumarin produced was assessed by spectrofluorometry (excitation, 400 nm; emission, 505 nm).

Caspase-3 Analysis—Cleavage of the fluorogenic caspase-3 substrate (DEVD-aminofluorocoumarin; Alexis Corp.) to its fluorescent product was used to measure caspase-3 activity. Following treatment, cultured neurons were lysed in buffer (25 mM HEPES, 5 mM MgCl₂, 5 mM EDTA, 2 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml pepstatin, pH 7.4), sonicated for 2 s, and centrifuged at 10,000 rpm for 10 min at 4 °C. Samples of supernatant (90 μl) were incubated with the DEVD peptide (500 μM; 10 μl) for 1 h at 30 °C. Incubation buffer (900 μl; 50 mM HEPES, pH 7.4, containing 2 mM EDTA, 20% glycerol, 10 mM DTT) was added, and fluorescence was assessed (excitation, 400 nm; emission, 505 nm). Results are expressed as the fold change in caspase-3 activity induced by Aβ.

Calpain Activity—Calpain 1 and 2 activity was assessed as described previously (8). Briefly, cleavage of the fluorogenic calpain substrate, Suc-LLVY-AMC, was used to assess calpain activity in cell lysates. Samples were incubated with Suc-LLVY-AMC for 15 min at room temperature, and the amount of AMC released upon cleavage with calpain was measured fluorometrically at an excitation wavelength of 360 nm and an emission wavelength of 440 nm.

TUNEL—Apoptotic cell death was assessed using the Dead End™ colorimetric apoptosis detection system (Promega Corp.). Cells were treated with Aβ for 48 h in the presence or absence of Z-DEVD-FMK, AEA, 2-AG, or URB 597. Cells were then fixed with 4% paraformaldehyde and permeabilized with Triton X-100 (0.1%), and the biotinylated nucleotide was incorporated at 3'-OH DNA ends using the enzyme terminal deoxynucleotidyltransferase. Horseradish peroxidase-labeled streptavidin was then bound to the biotinylated nucleotide, and this was detected using the peroxidase substrate H₂O₂ and the chromogen diaminobenzidine. Cells were viewed under light microscopy at ×40 magnification, where the nuclei of TUNEL-positive cells stained brown. Apoptotic cells (TUNEL-positive) were counted and expressed as a percent-age of the total number of cells examined. To exclude the possibility that the number of living cells present on the coverslip had an affect on the TUNEL-positive ratio, the same number of cells (~500) were counted for each treatment.

Statistics—Data are reported as the mean ± S.E. of the number of experiments indicated in each case. Statistical analysis was carried out using one-way analysis of variance (ANOVA) followed by the post hoc Student-Newman-Keuls, test when significance (at the p < 0.05 level) was indicated. When comparisons were being made between two treatments, an unpaired Student’s t test was performed, and p < 0.05, p < 0.01, and p < 0.001 were considered significant.

RESULTS

Endocannabinoids Mediate Neuroprotection—Cultured cortical neurons were exposed to Aβ (2 μM; 48 h) with or without Z-DEVD-FMK (10 μM) and assessed for apoptosis (Fig. 1A). Aβ significantly increased the percentage of apoptotic cells from 2.7 ± 0.4% (mean ± S.E.) in control cells to 22.38 ± 2.5% (p < 0.001, ANOVA, n = 5), and this was prevented by the caspase-3 inhibitor, Z-DEVD-FMK (p < 0.001, n = 5, ANOVA). Neurons were also exposed to Aβ2-2-AG (10 nM), AEA (10 nM), or the fatty acid amide hydrolase inhibitor, URB 597 (1 μM). Fixed cells were assessed for apoptosis (Fig. 1B), and cell lysates were analyzed for caspase-3 activity (Fig. 1D). Aβ evoked a significant increase in the mean percentage of apoptotic cells from 7.8 ± 0.7% (mean ± S.E.) in control cells to 24.5 ± 0.9% (p < 0.001, n = 5, ANOVA), and this was significantly inhibited by 2-AG and AEA (p < 0.01, n = 5, ANOVA). URB 597 also significantly inhibited the Aβ-induced increase in DNA fragmentation (p < 0.05, ANOVA, n = 5). Sample TUNEL staining of control-treated (i), Aβ-treated (ii), Aβ- and 2-AG-treated (iii), Aβ- and AEA-treated (iv), and Aβ- and URB 597-treated (v) neurons is shown in Fig. 1C. In parallel, 2-AG, AEA, and URB 597 all significantly inhibited the stimulatory effect of Aβ on proapoptotic caspase-3 activity (Fig. 1D). Thus, in control cells, caspase-3 activity was 30 ± 5 pmol of p-nitroanilide produced/μg/min (mean ± S.E.), and this was significantly increased to 87 ± 24 pmol of p-nitroanilide produced/μg/min by Aβ (p < 0.01, n = 5, ANOVA), AEA, 2-AG, and URB 597 significantly inhibited the Aβ-mediated increase in caspase-3 activity (p < 0.01, ANOVA, n = 5).

Endocannabinoids Prevent Aβ-mediated Destabilization of Lysosomes and Cathepsin Release—Lysosomal destabilization has been reported as an upstream event in Aβ-mediated neurotoxicity (9). We tested the hypothesis that endocannabinoids could thwart the apoptotic pathway and confer neuroprotection by preventing lysosomal destabilization. Cells were loaded with AO prior to treatment with Aβ (2 μM; 6 h) with or without 2-AG (10 nM), AEA (10 nM), or URB 597 (1 μM) (i.e. prior to onset of DNA fragmentation), and emission at 633 nm was assessed as an index of lysosomal integrity (Fig. 2A). Aβ significantly reduced the fluorescence emission at 633 nm from 133 ± 18 fluorescence units (mean ± S.E.; arbitrary units) to 52 ± 10 (p < 0.05, ANOVA, n = 5), indicative of a loss in lysosomal integrity. Exposure to Aβ in the presence of AEA or 2-AG restored the fluorescence emission at...
Endocannabinoids Prevent Lysosomal Destabilization

633 nm to 124 ± 10 and 117 ± 10 fluorescence units, respectively (p < 0.05, ANOVA), demonstrating a stabilizing effect of both endocannabinoids on lysosomes. The fatty acid amide hydrolase inhibitor, URB 597, also restored lysosomal integrity because emission at 633 nm was comparable with control values in cells exposed to Aβ in the presence of URB 597 (130 ± 18 fluorescence units, n = 5). Thus, in control cells (Fig. 2B, i), the AO staining displayed a punctate pattern of fluorescence due to its accumulation within acidic lysosomal vesicles, indicative of intact lysosomal membranes. In contrast, the pattern of AO staining in Aβ-treated cells (Fig. 2B, ii) had shifted to a diffuse cytosolic green fluorescence, reflecting the release of AO from lysosomes into the cytosol. In cells exposed to Aβ in the presence of 2-AG (Fig. 2B, iii), AEA (Fig. 2B, iv), or URB 597 (Fig. 2B, v), the punctate pattern of AO staining was restored.

The stabilizing effect of endocannabinoids on lysosomes was confirmed with BODIPY FL-pepstatin A staining (Fig. 2C), which demonstrates that the punctate staining in control cells (Fig. 2C, i), indicative of intact lysosomes containing cathepsin D, becomes more diffuse in cells exposed to Aβ (Fig. 2C, ii), indicating a loss of lysosomal cathepsin D into the cytosol. In cells exposed to Aβ in the presence of 2-AG (Fig. 2C, iii), AEA (Fig. 2C, iv), or URB 597 (Fig. 2C, v), the punctate pattern of BODIPY FL-pepstatin A staining was restored. The frequency distributions of BODIPY FL-pepstatin A pixel intensity were compared for control and Aβ-treated cells. Four data sets per group were averaged and statistically compared with the Kolmogorov-Smirnov test. Exposure to Aβ was associated with a flattening of the high intensity frequency peak, and the curves were significantly different (p < 0.001), representing the Aβ-induced change in cathepsin D distribution (data not shown).

To further demonstrate a loss of lysosomal stability, we measured cathepsin L activity in the culture medium of treated cells (Fig. 2D) and thus examined its extracellular release. Aβ (2 μM, 6 h) significantly increased cathepsin L activity in the culture medium from 3.7 ± 0.9 relative fluorescence units (RFU; mean ± S.E.) to 7.6 ± 1.2 RFU (p < 0.05, ANOVA, n = 5), and this was inhibited when cells were treated with Aβ in the presence of AEA (p < 0.05, ANOVA, compared with cells treated with Aβ, n = 5) or 2-AG (p < 0.05, ANOVA, compared with cells treated with Aβ, n = 5).

To assess whether the cathepsin L release evoked by Aβ could be neurotoxic to neighboring neurons, the medium from Aβ-primed cells was incubated with naive cells, and apoptosis was assessed (Fig. 2E). The medium from Aβ-primed cells evoked a significant increase in the percentage of apoptotic cells from 7 ± 1% (mean ± S.E.) to 22 ± 2% (p < 0.001, ANOVA, n = 5), and this was significantly reduced to 14 ± 2% apoptotic cells (p < 0.05, ANOVA, n = 5) following co-treatment with the cathepsin L-neutralizing antibody (1 μM). This suggests that the cathepsin L released from the lysosomes by Aβ is still active and capable of inducing apoptosis in neighboring cells, thus highlighting the importance of blocking this lysosomal aspect of Aβ neurotoxicity.
Endocannabinoids Prevent Lysosomal Destabilization

Endocannabinoids Prevent the Aβ-mediated Up-regulation of p-p53Ser-15 and p-p53Ser-15-Lysosomal Interaction—We have recently reported that Aβ destabilizes lysosomes in a manner involving the interaction of p-p53Ser-15 with the lysosomal membrane. In Fig. 3, we demonstrate that the Aβ-mediated up-regulation of p-p53Ser-15 (Fig. 3A) and association of p-p53Ser-15 with the lysosomes (Fig. 3B) is prevented by the endocannabinoids, AEA and 2-AG. In Fig. 3A, the expression...
of p-p53Ser-15 is low in control cells (Fig. 3A, i), whereas p-p53Ser-15 immunoreactivity is increased in cells exposed to Aβ (2 μM, 6 h; Fig. 3A, ii). The Aβ-mediated increase in p-p53Ser-15 immunoreactivity is blocked by both 2-AG (Fig. 3A, iii) and AEA (Fig. 3A, iv). The Aβ-mediated increase in p-p53Ser-15 immunoreactivity is abolished by 2-AG and AEA in accordance with our previous findings (9). The co-localization of p-p53Ser-15 with lysosomes is abolished by 2-AG (Fig. 3B, iii) and AEA (Fig. 3B, iv). These results suggest that the Aβ-mediated up-regulation and association of p-p53Ser-15 with lysosomes are prevented by the endocannabinoid system.

Endocannabinoids Prevent Aβ-mediated Calpain Activation—In Fig. 4A, we demonstrate that the Aβ-mediated increase in p-p53Ser-15 expression is a calpain-dependent event. Cells were treated with the selective calpain inhibitor, MDL 28170 (0.1 μM) for 6 h in the presence or absence of 2-AG (10 nM) and AEA (10 nM) and assessed for p-p53Ser-15 expression. Aβ increased the expression of p-p53Ser-15 (i) compared with control (i), and this was prevented by 2-AG (ii) and AEA (iii). The arrows indicate p-p53Ser-15 immunoreactivity (scale bar, 10 μm). B, loading of cortical neurons with LysoTracker™ Red demonstrated the Aβ-induced co-localization of p-p53Ser-15 with lysosomes (i) compared with control (i). Inset, arrows indicate areas of colocalization (purple). This was also prevented by 2-AG (ii) and AEA (iv). Scale bar, 10 μm. n = 5. Error bars, S.E.
MDL 28170 (10 μM), for 1 h prior to exposure to Aβ (2 μM) for 6 h. Aβ significantly increased p-p53Ser-15 immunoreactivity from 48.1 ± 9.1 RFU in control cells (mean ± S.E.) to 155.3 ± 4.6 RFU (p < 0.001, ANOVA, n = 5), and this was prevented by the calpain inhibitor, MDL 28170 (47.05 ± 7.4 RFU; p < 0.001, ANOVA, n = 5).

In order to assess whether inhibition of calpain could also prevent the effects of Aβ on lysosomal membrane integrity, cultured cortical neurons were loaded with AO prior to treatment with Aβ (2 μM; 6 h) with or without MDL 28170 (10 μM). Fig. 4B demonstrates that inhibition of calpain prevents LMP evoked by Aβ. Thus, Aβ significantly reduced the fluorescence emission at 633 nm from 357.2 ± 31.2 in control cells to 81.2 ± 16.5 (p < 0.001, ANOVA, n = 5), indicative of a loss in lysosomal integrity. Exposure to Aβ in the presence of MDL 28170 restored the fluorescence emission at 633 nm to 414.1 ± 31.4 (p < 0.001, ANOVA, n = 5).

To demonstrate the importance of calpain activation in the apoptotic pathway evoked by Aβ, DNA fragmentation was also assessed in cells exposed to Aβ (2 μM; 48 h) in the presence or absence of MDL 28170. Fig. 4C demonstrates that the apoptotic pathway evoked by Aβ is dependent upon activation of calpain. Thus, in control cells, DNA fragmentation was 3.8 ± 0.5% (mean ± S.E.), and this was significantly increased to 24.1 ± 1.4% in cells exposed to Aβ (p < 0.001, ANOVA, n = 5). However, in the presence of MDL 28170, DNA fragmentation was significantly decreased to 5.5 ± 0.4%.

Finally, we investigated whether the neuroprotective pro-activity of endocannabinoids relied on their ability to prevent calpain activation. Calpain activity was assessed in cultured cortical neurons exposed to Aβ (2 μM; 6 h) in the presence or absence of 2-AG (10 nm), AEA (10 nm) or URB 597 (1 μM). Fig. 4D demonstrates that modulation of the endocan-nabinoid system can prevent the Aβ-induced increase in calpain activation. Thus, Aβ significantly increased calpain activity from 5.8 ± 0.3 RFU (AMC release/min/mg) in control cells to 8.1 ± 0.9 RFU (mean ± S.E.). This was significantly decreased to 4.8 ± 0.4, 4.7 ± 0.9, and 5.2 ± 0.2 RFU by co-treatment with 2-AG, AEA, and URB 597, respectively. These results demonstrate the role of calpain activation in Aβ-induced apoptosis and the ability of the endocannabinoid system to prevent this phenomenon.

Receptor Target for AEA—To investigate the involvement of the CB1 receptor in endocannabinoid-mediated neuroprotection, cortical neurons were exposed to Aβ (2 μM; 48 h) with or without 2-AG (10 nm) or AEA (10 nm) in the presence or absence of the CB1 receptor antagonist, AM 251 (10 μM). Fig. 5A demonstrates that AEA, but not 2-AG, provides neuroprotection against Aβ-induced DNA fragmentation in a CB1 receptor-dependent manner. Thus, when neurons were exposed to Aβ and 2-AG in the presence of AM 251, 2-AG maintained its ability to confer neuroprotection. However, AEA was unable to provide neuroprotection against Aβ while in the presence of AM 251. Subsequent experiments on the role of CB1 receptors in endocannabinoid-mediated neuroprotection thus focused solely on AEA. To determine whether surface or intracellular CB1 receptors were required for the neuroprotective effects of AEA on Aβ-induced lysosomal destabilization and apoptosis, we used the cell-permeable CB1 receptor antagonist, SR 141716A (1 μM), which can target both intracellular and plasma membrane CB1 receptors, and the cell-im-permeable CB1 receptor antagonist, hemopressin (10 μM), which can target only plasma membrane CB1 receptors (27).

Cortical neurons were exposed to Aβ (2 μM, 48 h) and AEA (10 nm) in the presence or absence of SR 141716A or hemopressin and assessed for DNA fragmentation. Fig. 5B demonstrates that the neuroprotective effect of AEA against Aβ-induced DNA fragmentation was blocked by SR 141716A. In contrast, AEA maintained the ability to confer neuroprotection against Aβ-induced toxicity while in the presence of hemopressin, thus providing evidence for the role of intracellular CB1 receptors in AEA-mediated neuroprotection. The water-soluble CB1 receptor agonist, O-2545, acted as a positive control to demonstrate the ability of hemopressin to block surface CB1 receptor activity. Thus, cortical neurons exposed to O-2545 expressed a higher level of p-ERK Tyr-205 phosphorylation compared with control cells, and this was prevented following co-treatment with hemopressin (Fig. 5B, inset).

Similarly, SR 141716A, but not hemopressin, abolished the ability of AEA to conserve the integrity of the lysosomal membrane (arbitrary units; Fig. 5C). Representative images of AO-stained neurons treated with hemopressin control (i), hemopressin, Aβ and AEA (ii), SR 141716A control (iii), and SR 141716A, Aβ and AEA (iv) are shown in Fig. 5D.

DISCUSSION

Endocannabinoids Prevent Aβ-induced Apoptosis—In this study, we demonstrate that endocannabinoids, both directly and through pharmacological enhancement, are able to protect primary cerebral cortical neurons from Aβ-induced apoptosis in vitro. Thus, 2-AG, AEA, and URB 597 prevented the Aβ-induced increase in DNA fragmentation and caspase-3 activation, both of which are hallmarks of apoptosis. Whether apoptosis is responsible for all of the neuronal cell loss observed in AD is a subject of some controversy. The findings of our study would suggest that at least a subset of neurons in the AD brain are vulnerable to cell death by apoptosis. This is in agreement with a number of studies that have reported an increase in DNA fragmentation (37, 38) and caspase-3 activation (39) in the AD brain as well as the proclivity of Aβ to induce apoptosis in neuronal cells in vivo (3).

The findings of our study also suggest that modulation of the endocannabinoid system could be therapeutically advantageous in preventing the neuronal cell loss associated with AD. We demonstrate that both direct applications of 2-AG and AEA and their pharmacological enhancement using URB 597 are able to provide protection against Aβ toxicity. The relevance of the endocannabinoid system to AD treatment has been contemplated for many years. Several studies have reported on its potential neuroprotective capabilities in various models of AD (25, 26, 40) as well as highlighting disease-related alterations that occur over time (40 – 42). However, certain discrepancies also exist regarding the benefits of endocannabinoid therapeutics to AD research, with a number of...
studies reporting that it has no effect on AD neuropathology (43, 44). Many of these inconsistencies can be explained by the use of different AD models, application of a diverse range of endocannabinoid system-modulating drugs (e.g., phytocannabinoids, endogenous cannabinoids, and synthetic cannabinoids), and different drug concentrations used. Contradictions in results may also be due to variable timing of drug application, as was suggested by van der Stelt et al. in 2006 (26). Thus, our findings are specific to an in vitro model of AD consisting of primary cerebral cortical neurons exposed to toxic concentrations of Aβ and the neuroprotective proclivity of co-applied low concentrations of endocannabinoids. The concentration of AEA and 2-AG used in this study was based on a dose-response experiment using the TUNEL technique, whereby 10 nM had no effect on cell viability. However, in this experiment, higher concentrations (2-AG 10 μM and AEA 10 nM) induced a significant increase in DNA fragmentation. However, in the presence of hemopressin, AEA maintained the proclivity to prevent Aβ-induced DNA fragmentation (***, p < 0.001 versus control). In the presence of SR 141716A, the neuroprotective effect of AEA was abolished, and a significant increase in DNA fragmentation was observed (§§§, p < 0.001 versus AEA). Inset, the water-soluble CB1 receptor agonist, O-2545, was used as a positive control to demonstrate the inhibitory effect of hemopressin on p-ERK Tyr-205 expression. Representative images for p-ERK Tyr-205 immunoreactivity are displayed in cells treated with control (i), hemopressin (ii), O-2545 (iii), and hemopressin and O-2545 (iv). Emission at 633 nm was significantly decreased in cells exposed to Aβ, indicative of lysosomal destabilization (***, p < 0.001 versus control), and this was prevented by AEA (###, p < 0.001 versus Aβ). In the presence of hemopressin, AEA maintained the proclivity to prevent Aβ-induced lysosomal destabilization (888, p < 0.001 versus control). However, in the presence of hemopressin and AEA, the stabilizing effect of AEA on lysosomes was abolished (#, p < 0.05 versus Aβ and AEA).
increase in DNA fragmentation compared with control cells, thus representing the neuroprotection/neurotoxicity paradox of endocannabinoid modulation, which has been the focus of a recent review (45).

Consistent with our finding, other studies have reported on the neuroprotective properties of the endocannabinoid system in hippocampal and cortical neurons against a number of toxic insults, including excitotoxicity (22, 46), ischemia (47), and oxidative damage (23), all of which are known to induce cell death by apoptosis. This endocannabinoid-mediated neuroprotection has been reported to occur through a variety of mechanisms, including activation of prosurvival signaling pathways like inositol triphosphate (48), PI3K (49), focal adhesion kinase (50), and ERK (49–50) as well as through inhibition of calcium currents and opening of potassium channels (51–53). The non-psychoactive component of cannabis, cannabidiol, has a number of additional characteristics that highlight the benefits of using cannabinoid-based therapeutics for the treatment of AD, such as its ability to scavenger reactive oxygen species (23), reverse Tau hyperphosphorylation (54), and reduce activation of the inflammatory transcription target, nuclear factor-κB (54). This study demonstrates for the first time that endocannabinoid-mediated neuroprotection can also occur through stabilization of the lysosomal membrane.

**Endocannabinoids Prevent Aβ-induced Lysosomal Membrane Permeabilization**—Our finding that endocannabinoids stabilize lysosomes against Aβ toxicity is novel and represents an early event in the apoptotic pathway that may be targeted by the endocannabinoid system to confer neuroprotection. LMP, as a distinct event in the apoptotic pathway, is now widely accepted (16, 55, 56) and is believed by many to occur upstream of mitochondrial outer membrane permeabilization and caspase-3 activation (57, 58). This is consistent with our findings, given that LMP was evoked after 6 h of exposure to Aβ, whereas DNA fragmentation and caspase-3 activation typically do not occur at a significant level until 48 h post-Aβ treatment.

Although it is possible that Aβ increased the luminal pH of lysosomes, leading to the redistribution of AO into the cytosol, our finding that Aβ promotes the translocation of the lysosomal enzymes cathepsin D to the cytosol and cathepsin L to the extracellular matrix supports our proposal that Aβ evokes permeabilization of the lysosomes. Both of these enzymes have been implicated in the initiation and execution of cell death pathways (15). For example, cathepsin D has the proclivity to activate the proapoptotic protein, Bax, in a Bid-independent (59) and Bid-dependent (60) manner, resulting ultimately in mitochondrial outer membrane permeabilization, whereas cathepsin L is capable of activating caspase-dependent apoptosis through the cleavage of the proapoptotic protein, Bid, and the antiapoptotic proteins Bcl-2 and Bcl-xL (61).

The diffuse appearance of BODIPY FL-pepsinatin A in Fig. 2C (ii) demonstrates the ability of Aβ to translocate cathepsin D from the lysosomal lumen into the cytosol. It is still unknown whether all lysosomes within a cell are uniformly susceptible to LMP; however, there is some evidence to suggest that larger lysosomes may be more vulnerable than smaller ones (62). This discrepancy may account for the small amount of punctuate staining that is visible in the Aβ-treated neurons. Another possibility is that alterations in the amount of Aβ loading into lysosomes may in turn affect the extent of LMP that occurs. However, it should be noted that a study by Roberg et al. (63) demonstrated that microinjection of cathepsin D enzyme into the cytosol is sufficient to trigger mitochondrial outer membrane permeabilization and thus highlights the impact of Aβ toxicity on a single lysosome.

We also report in this study that cathepsin L activity was significantly increased in the cell culture medium concomitant with LMP. Furthermore, the Aβ-primed cells, rich in cathepsin L, were sufficient to induce apoptosis in fresh neurons, thus suggesting that the secreted cathepsin L is active and capable of initiating apoptosis in neighboring cells. This could be of relevance to AD, a disease characterized by neuronal cell loss, because it implies that loss of lysosomal integrity in one cell could be enough to trigger cell death in a whole population of adjacent neurons. Most cells secrete small amounts of newly synthesized procathepsin L, but under certain physiological conditions, mature cathepsin L has also been detected in cell culture medium (64). The mechanism of this secretion remains to be determined, but there is some evidence to suggest that endosomes can become enriched with cathepsin L. Under certain stimuli, cathepsin L is then activated and secreted via a distinctive targeting pathway (64). Of interest, when cathepsin L is up-regulated, targeting to lysosomes continues at a constant level, whereas the additional enzyme produced enters a different targeting pathway (e.g. endosomes) (64, 65). We have previously demonstrated that Aβ can promote a time- and dose-dependent release of cathepsin L into the cytosol, with cathepsin L release being induced 6 h after Aβ treatment (8). Thus, it is possible that the cytosolic release of cathepsin L is the result of Aβ-induced LMP, whereas the cathepsin L detected in our cell culture medium is a result of its up-regulation and subsequent targeting to secretory endosomes. Using a cathepsin L-neutralizing antibody, we determined that cathepsin L in our cell culture medium was inducing apoptosis in neighboring cells. However, the effect of the neutralizing antibody was only partial and not sufficient to return DNA fragmentation to control levels. This suggests that other lysosomal enzymes may be released alongside cathepsin L following exposure to Aβ (e.g. the cysteine protease cathepsin B).

Precisely how Aβ induces LMP remains to be clarified. Findings from our laboratory would suggest that it may evoke structural alterations in lysosome-associated membrane proteins, which in turn cause lysosomes to become vulnerable to LMP (data not shown). In this study, we report that this harmful effect of Aβ on lysosomal integrity is dependent upon the phosphorylation of p53Ser-15.

Our finding that endocannabinoids have the proclivity to stabilize lysosomes is novel and may represent a vital mechanism by which these lipid modulators confer neuroprotection. Our results suggest that this stabilizing effect of endocannabinoids on lysosomal membranes is based upon their ability to prevent Aβ-induced alterations in p53Ser-15. Given that the
Endocannabinoids Prevent Lysosomal Destabilization

A well-established neuroprotective property of endocannabinoids is their ability to reduce Ca\(^{2+}\) influx through the CB\(_1\) receptor by blocking the activity of voltage-dependent N-, P/Q-, and L-type Ca\(^{2+}\) channels and through the presynaptic inhibition of glutamate release (22). Because a small change in intracellular Ca\(^{2+}\) concentration is sufficient to activate calpains, it is a possibility that the capacity of endocannabinoids to confer neuroprotection is through their inhibition of Ca\(^{2+}\) influx. Indeed, we have demonstrated that endocannabinoids prevent the Aβ-induced increase in calpain activity. Given that endocannabinoids are produced in response to an increase in intracellular Ca\(^{2+}\) concentration, it is possible that under normal circumstances, this leads to the inhibition of calpain and its downstream affects on p53\(^{\text{Ser-15}}\) and lysosomal integrity. However, this regulatory effect may be lost in the AD brain. Thus, therapies that modulate endocannabinoid tone may be advantageous based on their ability to prevent a rise in intracellular Ca\(^{2+}\). In accordance with this theory, we have demonstrated that URB 597 can prevent the Aβ-induced increase in calpain activation, destabilization of the lysosomal membrane, and ensuing cell death.

AEA Targets Intracellular CB\(_1\) Receptors—Our findings also suggest that AEA, but not 2-AG, mediates its neuroprotection in a CB\(_1\) receptor-dependent manner. Furthermore, the neuroprotective and lysosome stabilizing effects of AEA were dependent upon intracellular and not plasma membrane CB\(_1\) receptors. Thus, when cortical neurons were exposed to hemopressin, a CB\(_1\) receptor antagonist that cannot cross the plasma membrane and therefore can only target plasma membrane CB\(_1\) receptors, AEA still had the proclivity to protect against the Aβ-induced increase in DNA fragmentation and LMP. In contrast, in the presence of SR 141716A, a cell-permeable CB\(_1\) receptor antagonist, AEA could no longer confer neuroprotection. Hemopressin is a naturally occurring peptide that is capable of functionally antagonizing the activity of CB\(_1\) receptors. Recent studies have reported that hemopressin’s mechanism of action reflects those of the well-characterized CB\(_1\) inverse agonists, AM 251 and SR 141716A (32, 33).

In this study, we demonstrated the ability of hemopressin to prevent p-ERK\(^{\text{Tyr-205}}\) signaling by the water-soluble CB\(_1\) agonist O-2545 and thus exhibit the proclivity of hemopressin to antagonize this receptor. Further evidence that hemopressin selectively antagonizes CB\(_1\) receptors comes from the recent finding that it can prevent agonist-induced receptor internalization into endosomes (32). However, it should be noted that Gomes et al. (77) have identified that N-terminally extended forms of hemopressin can function as CB\(_1\) receptor agonists, activating a signal transduction pathway distinct from that activated by endocannabinoids.

It has recently been reported that the intracellular pools of CB\(_1\) receptors can directly associate with lysosomes and moreover that they retain their ability to couple to Go\(_i\) to activate ERK (27). Given our finding that intracellular CB\(_1\) receptors are required for AEA-mediated neuroprotection, it is...
possible that direct signaling of CB₁ receptors at the lysosome can be attributed to this neuroprotective effect. Furthermore, the finding that CB₁ receptors are trafficked to lysosomes via AP-3 (27), the same adaptor protein responsible for trafficking lysosome-associated membrane proteins 1 and 2 to lysosomes, suggests that a far more intimate relationship may exist between the endocannabinoid and lysosomal systems. Moreover, given that lysosomes are stores for Ca²⁺ (78), it is an intriguing possibility that endocannabinoids govern lysosomal integrity by preventing Aβ-induced lysosomal calcium release, a small amount of which would be sufficient to activate calpain.

In 2005, Ramirez et al. (40) reported that CB₁ receptor expression was decreased on neurons remote from amyloid plaques in post-mortem AD brains and suggested that they might be extremely vulnerable to the toxic species generated by microglia. Similarly, autoradiographic studies with [³H]CP55.940 in patients with AD demonstrated that CB₁ receptor density was reduced in the entorhinal cortex, hippocampus, and caudate of the latter (41). However, in this study, reductions in CB₁ receptor density were not associated with the characteristic neuropathological hallmarks of AD. Recent findings from Cudaback et al. (79) suggest that cannabinoid receptor expression may determine their efficacy for activating proapoptotic and prosurvival signals, whereby low levels of cannabinoid receptor expression in astrocytoma sub-clones couple to apoptotic pathways, whereas high levels of expression also couple to prosurvival pathways. Thus, it is a possibility that endocannabinoids have a direct modulating effect on the expression of cannabinoid receptors, which in turn couple to prosurvival signaling pathways to confer neuroprotection. Moreover, given that endocytic processes are required for the axonal targeting of CB₁ receptors (80), which in turn depend upon an efficient lysosomal system, endocannabinoid-mediated stabilization of lysosomal membranes may in turn affect the expression level of cannabinoid receptors within a given cell.

CB₂ receptor expression is increased on microglia within amyloid plaques (40, 42), and evidence is emerging in support of the neuroprotective actions of CB₂ receptor agonists for the treatment of neurodegenerative diseases (81–83). Although the results generated from these experiments were obtained with primary cultured neurons, we cannot exclude a small contributing role for glia. However, in this study, the use of AM630, a selective CB₂ receptor antagonist, had no effect on the proclivity of 2-AG to confer neuroprotection (data not shown).

In contrast to AEA, the mechanism of 2-AG-mediated neuroprotection was not CB₁ receptor-dependent. Over the last decade, other receptor targets and oxygenation pathways for endocannabinoids have been identified. For example, numerous studies have now reported on the ability of 2-AG and AEA to activate peroxisome proliferator-activated receptors (84–86) and putative cannabinoid receptors, such as GPR55 (87). In addition, 2-AG has been demonstrated to be a natural substrate for cyclooxygenase-2 (COX-2) (88, 89); thus, the possibility cannot be ruled out that downstream metabolites of 2-AG oxygenation account for the neuroprotective effects of 2-AG in our model of AD. Further experiments are required to decipher precisely how 2-AG confers neuroprotection. In light of these results, we propose that stabilization of lysosomes by endocannabinoids may represent a key mechanism by which these lipid molecules can confer neuroprotection.

Acknowledgments—We acknowledge the assistance of Dr. J. F. X. Jones with BODIPY FL-pepstatin A analysis and Dr. A. J. Irving in providing comments on the manuscript.
