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Research Paper

R(+)-Methanandamide Elicits a Cyclooxygenase-2-Dependent Mitochondrial Apoptosis Signaling Pathway in Human Neuroglioma Cells

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Purpose. Cannabinoids have been associated with tumor regression and apoptosis of cancer cells. Recently, we have shown that R(+)-methanandamide (R(+)-MA) induces apoptosis of H4 human neuroglioma cells via a mechanism involving *de novo* expression of the cyclooxygenase-2 (COX-2) enzyme. The present study investigated a possible involvement of a mitochondrial-driven pathway in this process.
Methods. Cell death was determined by the WST-1 cell viability test, and changes in apoptotic parameters [i.e., release of mitochondrial cytochrome *c*, activation of caspases, cleavage of poly(ADP-ribose) polymerase (PARP)] were detected by Western blotting.
Results. H4 cells treated with R(+)-MA showed typical signs of mitochondrial apoptosis, i.e., release of mitochondrial cytochrome *c* into the cytosol and activation of initiator caspase-9. Moreover, activation of the executor caspase-3 was observed following cannabinoid treatment. Cells were fully protected from apoptotic cell death by the caspase-3 inhibitor Ac-DEVD-CHO, indicating a crucial role for caspase-3 activation in R(+)-MA-elicited apoptosis. Furthermore, cleavage of the caspase-3 target protein PARP was registered. All of the aforementioned effects were substantially reduced by the selective COX-2 inhibitor celecoxib (1 μM) at a pharmacologically relevant, nonapoptotic concentration.
Conclusion. R(+)-MA-induced apoptosis is mediated via a mitochondrial-dependent pathway that becomes activated, at least in part, through up-regulation of the COX-2 enzyme.

KEY WORDS: cyclooxygenase-2; human neuroglioma cells; intrinsic apoptosis; mitochondrial apoptosis; R(+)-methanandamide.

INTRODUCTION

Cannabinoids, the active components of *Cannabis sativa* and their derivatives, exert a wide array of effects within the central nervous system as well as in the periphery such as immune, cardiovascular, digestive, reproductive, and ocular functions. Most of these effects are mediated via two cannabinoid-specific $G_{i/o}$ protein-coupled receptors, CB_1 and CB_2. The CB_1 receptor is particularly abundant in the central nervous system (1), whereas the CB_2 receptor is predominantly expressed by peripheral immune cells (2,3). The most important endogenous ligands on these receptors are anandamide (arachidonylethanolamine) and 2-arachidonylglycerol, which—together with their receptors and specific processes of synthesis, uptake, and degradation—constitute the endogenous cannabinoid system (4).

For decades, cannabinoids have been known to exert palliative effects in cancer patients. Δ^9-Tetrahydrocannabinol (THC), the most psychoactive phytocannabinoid, and its synthetic analog LY109514 (nabilone) are approved for treatment of chemotherapy-induced nausea and emesis. Other potential palliative properties of cannabinoids such as appetite stimulation and analgesia are presently tested in oncology (5). Apart from these actions, a number of plant-derived (e.g., THC and cannabidiol), synthetic (e.g., WIN-55,212-2), and endogenous cannabinoids (e.g., anandamide and 2-arachidonylglycerol) have been shown to block cancer cell proliferation and to induce apoptosis of cancer cells both *in vitro* (6,7) and *in vivo* (8,9). Several mechanisms have been proposed to be involved in these actions. In this context, cannabinoids have been reported to induce cell cycle arrest via mechanisms involving induction of the cyclin-dependent kinase inhibitor $p27^{kip1}$ (10), inhibition of the PI3K/Akt pathway (11), attenuation of epidermal growth factor receptor (EGFR) tyrosine kinase activity, or decrease in EGFR levels (9). On the other hand, direct induction of apoptotic death of tumor cells has been likewise documented to confer a significant portion of cannabinoids' anti-cancerogenic responses. In C6 glioma cells, for instance, cannabinoid-induced apoptosis involves sustained generation of ceramide and prolonged activation of the Raf-1/MEK/ERK signaling cascade (8). Sustained Akt inhibition has also been

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ABBREVIATIONS: COX, cyclooxygenase; PARP, poly(ADP-ribose) polymerase; PG, prostaglandin; R(+)-MA, R(+)-methanandamide [R-(+)-arachidonyl-1′-hydroxy-2′-propylamide)]; THC, Δ^9-Tetrahydrocannabinol.

associated with cannabinoid-induced apoptosis (11). Recent studies from our laboratory have shown that the apoptotic death of human neuroglioma cells induced by anandamide and its stable analog, R(+)-methanandamide [R(+)-MA], is mediated by up-regulation of the cyclooxygenase-2 (COX-2) enzyme (12). Enhanced COX-2 expression was found to occur via a cannabinoid receptor-independent pathway involving lipid rafts, increased synthesis of ceramide, and activation of p38 and p42/44 mitogen-activated protein kinases (13–15). However, the exact mechanisms underlying R(+)-MA-induced apoptotic cell death have not been established so far.

Our aim, therefore, was to examine the involvement of mitochondrial apoptosis in R(+)-MA-induced death of H4 human neuroglioma cells and the role of COX-2 in this process. To address this issue, we assessed the release of cytochrome *c*, which is associated with mitochondrial apoptosis, and the subsequent caspase activation cascade. Here we show that R(+)-MA induces apoptosis via COX-2-dependent cytochrome *c* release, caspase-9 and -3 activation, and cleavage of the caspase-3 substrate poly(ADP-ribose) polymerase (PARP).

MATERIALS AND METHODS

Materials

R(+)-MA and caspase-3 inhibitor Ac-DEVD-CHO were purchased from Calbiochem (Bad Soden, Germany). Dulbecco's modified Eagle's medium (DMEM) with 4 mM L-glutamine and 4.5 g/L glucose was obtained from Cambrex Bio Science Verviers (Verviers, Belgium). Fetal calf serum and penicillin–streptomycin were obtained from PAN Biotech (Aidenbach, Germany) and Invitrogen (Karlsruhe, Germany), respectively.

Cell Culture

H4 human neuroglioma cells were maintained in DMEM supplemented with 10% heat-inactivated fetal calf serum, 100 U/mL penicillin, and 100 μg/mL streptomycin. The cells were grown in a humidified incubator at 37°C and 5% CO_2. All incubations were performed in serum-free medium. Phosphate-buffered saline was used as a vehicle for the tested substances with a final concentration of 0.1% (v/v) ethanol [for R(+)-MA] or 0.1% (v/v) DMSO (for celecoxib), respectively.

Western Blot Analysis

Cells were grown to confluence in 10-cm dishes and incubated with test substances or vehicle for the indicated times.

For determination of cytochrome *c* release, cells were washed and lysed in ice-cold lysis buffer (20 mM HEPES, pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 1 mM EDTA, 1 mM DTT, 250 mM sucrose, 1 mM phenylmethylsulfonyl fluoride, 1 μg/mL leupeptin, and 10 μg/mL aprotinin) for 1 h and homogenized in a Dounce homogenizer (40 strokes). The homogenates were first centrifuged at 800 × *g* for 10 min at 4°C to remove nuclei and cell debris. Afterwards, supernatants were spun at 16,000 × *g* for 40 min at 4°C to separate membrane fractions from the cytosolic fraction. Supernatants (cytosolic fraction) were used for Western blot analysis.

For all other proteins, cells were washed, harvested, and pelleted by centrifugation. Cells were than lysed in solubilization buffer (50 mM HEPES, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% (v/v) Triton® X-100, 10% (v/v) glycerol, 1 mM phenylmethylsulfonyl fluoride, 1 μg/mL leupeptin, and 10 μg/mL aprotinin), homogenized by sonication, and centrifuged at 10,000 × *g* for 5 min. Supernatants were used for Western blot analysis. Proteins were separated on a 10% sodium dodecyl sulfate-polyacrylamide gel. Following transfer to nitrocellulose and blocking of the membranes with 5% milk powder, blots were probed with specific antibodies raised to the procaspase and cleaved forms of caspase-3, caspase-9, and caspase-7, PARP, or cleaved PARP (New England BioLabs, Frankfurt, Germany), cytochrome *c* (BD Biosciences, Heidelberg, Germany), or β-actin (Calbiochem), the latter being used as a loading control. Subsequently, membranes were probed with horseradish peroxidase (HRP)-conjugated antirabbit IgG (New England BioLabs) and with HRP-conjugated antimouse IgG (New England BioLabs) for cytochrome *c*, respectively. Antibody binding was visualized by enhanced chemiluminescence (ECL) Western blotting detection reagents (Amersham Biosciences, Freiburg, Germany). Quantification was achieved by scanning the films with an optical scanner (Gel Doc 2000; Bio-Rad Laboratories, Hercules, CA, USA) and then analyzing band intensities of the images with the Multi-Analyst program, version 1.1 (Bio-Rad Laboratories).

Cell Viability Analysis

H4 cells were seeded in 96-well flat-bottom microplates and were grown to confluence. Thereafter, cells were incubated with the respective test compounds for 72 h in 100 μl medium without serum. New medium and test substances were added daily. Following the indicated incubation time, cell viability was measured by the colorimetric WST-1 test (Roche Diagnostics, Mannheim, Germany). This cell viability test is based on the cleavage of the tetrazolium salt WST-1 (4-[3-(4-Iodophenyl)-2-(4-nitrophenyl)-2*H*-5-tetrazolio]-1.6-benzene disulfonate) by mitochondrial dehydrogenases in metabolically active cells.

RESULTS

R(+)-MA Induces Cytochrome *c* Release and Activation of Caspase-9

During mitochondria-mediated apoptosis, a major apoptotic pathway induced by many chemotherapeutic agents, mitochondrial cytochrome *c* is released into the cytosol, where it promotes assembly of the apoptosome and activation of the initiator caspase-9. To investigate the contribution of cytochrome *c* release to R(+)-MA-induced apoptosis and the role of COX-2 in this process, H4 cells were treated with vehicle or R(+)-MA in the presence or absence of the selective COX-2 inhibitor celecoxib for 24 h. This incubation time was chosen based on kinetic experiments showing substantial induction of COX-2 at both mRNA and protein level at this time point (13,14). In Fig. 1A, R(+)-MA-treated cells showed an increase in cytosolic cytochrome *c* under

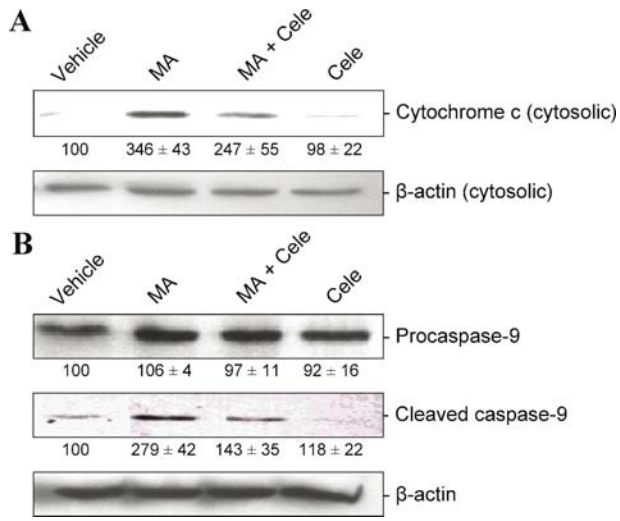

Fig. 1. (A) Effect of celecoxib on R(+)-MA-induced mitochondrial cytochrome *c* release (A) and activation of caspase-9 (B). Cells were incubated with R(+)-MA (10 μM) in the presence or absence of celecoxib (1 μM) or its vehicles for 24 h. Protein levels were determined by Western blotting and are shown by one representative Western Blot and a densitometric analysis. Values are means ± SEM of $n = 3$ observations and represent comparison with vehicle-treated cells (100%) in the absence of test substance. β-Actin was used as a loading control.

these experimental conditions. Coincubation with celecoxib substantially reduced cytochrome *c* release (Fig. 1A). As shown in Fig. 1B, the downstream target of cytochrome *c*, caspase-9, was activated by cleavage upon R(+)-MA treatment. Again, inhibition of COX-2 by celecoxib profoundly inhibited this response.

R(+)-MA Activates Caspase-3

Activated caspase-9 proteolytically activates other caspases, including effector caspases-3 and -7, thus initiating a process leading to apoptosis. To determine the involvement of these pathways in R(+)-MA-induced apoptosis, cleavage of caspase-3 and -7 was examined in further experiments. Western blot analysis clearly demonstrated the activation of caspase-3 upon R(+)-MA treatment, as indicated by increased levels of cleaved caspase-3 (Fig. 2A). Activation occurred within the same time frame as that examined for the upstream targets and could again be decreased by cotreatment with celecoxib. Even though the cleaved form of caspase-7 was observed in R(+)-MA-treated cells, there was no difference when compared to the vehicle control (Fig. 2C). Levels of both procaspase-3 and procaspase-7 remained unaltered. To determine the effect of caspase-3 activation on cell viability, cells were incubated with R(+)-MA in the presence or absence of the caspase-3 inhibitor Ac-DEVD-CHO. Loss of cell viability after R(+)-MA treatment was almost totally prevented by the caspase-3 inhibitor, whereas Ac-DEVD-CHO alone had no effect on cell viability at all (Fig. 2B).

R(+)-MA Causes Proteolytic Cleavage of PARP

To confirm the role of caspase-3 in R(+)-MA-induced apoptosis, cleavage of the DNA repair enzyme and caspase-3 substrate, poly(ADP-ribose) polymerase (PARP), was tested via further Western Blot analysis. As shown in Fig. 3, levels of cleaved PARP increased, whereas levels of native PARP slightly decreased after exposure to R(+)-MA. In line with the results described above, alterations of cleaved as well as native PARP were profoundly reduced by celecoxib cotreatment.

DISCUSSION

Recent investigations from our group have shown that R(+)-MA causes the apoptotic death of H4 human neuroglioma cells via a mechanism involving *de novo* expression of COX-2 (12). However, the molecular mechanisms underlying R(+)-MA-induced apoptosis downstream to COX-2 expression remained to be established. The present study therefore addressed this issue and investigated the apoptotic process activated upon R(+)-MA treatment.

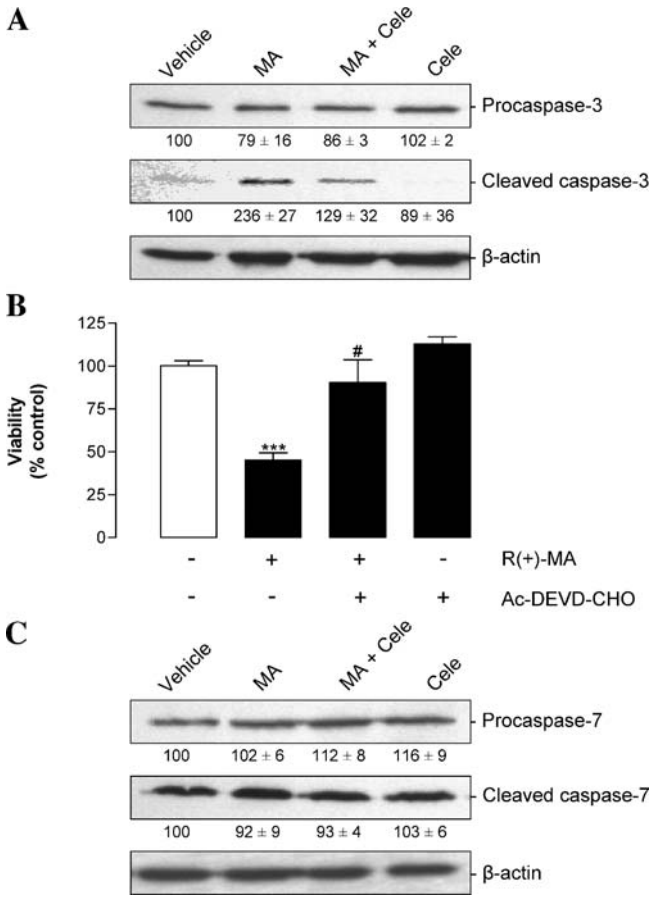

Fig. 2. Effect of R(+)-MA (10 μM) in the presence or absence of celecoxib (1 μM) on the activation of caspase-3 (A) and caspase-7 (C). Effect of a caspase-3 inhibitor (Ac-DEVD-CHO, 100 μM) on loss of cell viability induced by R(+)-MA (10 μM) (B). Cells were incubated with the indicated substances or its vehicles for 24 h (A, C) or 72 h (B). Protein levels were determined by Western blotting and are shown by a representative Western Blot and a densitometric analysis. β-Actin was used as a loading control. Values are means ± SEM of $n = 3$ (A, C) or $n = 4$ (B) observations and represent comparison with vehicle-treated cells (100%) in the absence of test substance. $^{***}p < 0.001$ *vs.* corresponding vehicle control; $^{\#}p < 0.05$ *vs.* sole R(+)-MA treatment (Student's *t*-test).

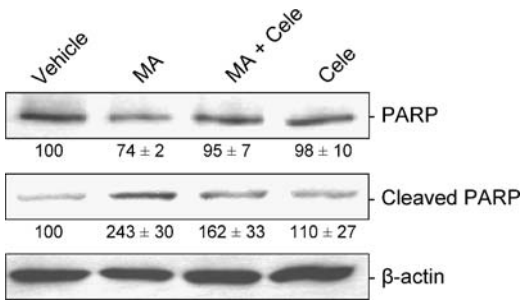

Fig. 3. Effect of celecoxib on R(+)-MA-induced PARP cleavage. Cells were incubated with R(+)-MA (10 μM) in the presence or absence of celecoxib (1 μM) or its vehicles for 24 h. Protein levels were determined by Western blotting and are shown by a representative Western Blot and a densitometric analysis. Values are means ± SEM of $n = 3$ observations and represent comparison with vehicle-treated cells (100%) in the absence of test substance. β-Actin was used as a loading control.

Results from this study show that R(+)-MA-induced apoptosis of glioma cells is mediated via the mitochondrial pathway. There are several lines of evidence supporting this notion. First, release of mitochondrial cytochrome *c* into the cytosol was detected, which is a necessary step for the formation of the apoptosome complex (16). Second, induction of caspase-9, a substrate of the apoptosome complex (16), was observed. Third, activation of effector caspase-3 followed by cleavage of PARP, a DNA repair enzyme and substrate of caspase-3, was shown to be involved in R(+)-MA-induced apoptosis. The decisive role of caspase-3 in R(+)-MA-induced cytotoxicity was corroborated by experiments using the caspase-3 inhibitor Ac-DEVD-CHO, which abolished cannabinoid-elicited apoptotic cell death. Caspase-7, an executioner caspase with an almost indistinguishable enzymatic activity from that of caspase-3 (17), is usually activated concurrently with caspase-3. Surprisingly, in the present study caspase-7 was not altered during R(+)-MA-induced apoptosis. The reasons for this phenomenon are unclear and deserve further investigation. However, comparable data have been reported for doxorubicin-induced apoptosis in human lymphoma cells (18).

A crucial role of COX-2 in mediating up-regulation of mitochondrial apoptosis by R(+)-MA was confirmed in further experiments with the selective COX-2 inhibitor celecoxib. In a recent study from this laboratory, suppression of COX-2 activity by pharmacological concentrations of this substance was accompanied by a substantial inhibition of the proapoptotic action of R(+)-MA (12). In line with these data, celecoxib was demonstrated to interfere with all of the aforementioned effects. Accordingly, cotreatment with celecoxib suppressed cytochrome *c* release and cleavage of caspase-9. Likewise, celecoxib substantially inhibited caspase-3 cleavage and proteolysis of PARP.

Our data are in contrast to the current view implying a contribution of COX-2 to carcinogenesis (19–21) and resistance to apoptosis (22). However, a major part of this evidence is based on *in vitro* experiments showing growth-inhibitory and proapoptotic actions of COX-2 inhibitors on various cancer cell lines. Noteworthy, most of these *in vitro* studies focused on the single effect of nonsteroidal anti-inflammatory drugs (NSAIDs) and COX-2 inhibitors, using suprapharmacological concentrations of these drugs that confer its proapoptotic action, at least in part, via effects independent of prostaglandin synthesis inhibition (23–26). In support of this notion, higher concentrations of COX-2 inhibitors were recently shown to stimulate angiogenesis of both COX-2 positive and COX-2 negative pancreatic cancer cells, and to mediate opposite effects on growth and angiogenesis in pancreatic cancer *in vivo*, depending on COX-2 expression (27).

In the present study, celecoxib inhibited R(+)-MA-induced apoptotic signaling at a nonapoptotic concentration that was well within the pharmacological range of plasma concentrations in patients receiving celecoxib (28). These data are consistent with other reports focusing on interactions between chemotherapeutic agents and COX-2 inhibitors. Therefore, the selective COX-2 inhibitor NS-398 was recently shown to inhibit the proapoptotic and antiproliferative effect of paclitaxel, an inducer of COX-2 (29,30), on epithelial ovarian cancer cells (31). In another study, the apoptotic death of human mammary epithelial cells elicited by the chemotherapeutic edelfosine was attenuated by celecoxib (32). Likewise, the proapoptotic action of the endocannabinoid-like compound stearoylethanolamide on C6 glioma cells was reported to be associated with activation of prostaglandin formation (33). More recently, different COX inhibitors (i.e., NS-398, aspirin) were shown to attenuate reduced viability of human leukemia cells treated with the COX-2 inducer 12-*o*-tetradecanoylphorbol-13-acetate (TPA) and arachidonic acid (34). In the same study, prostaglandin (PG) D_2 and PGJ_2 products were demonstrated to confer apoptosis of leukemia cells via activation of caspase-3, thus providing a feasible mode of action for COX-2-induced apoptosis. Collectively, these data strongly imply a need for further studies that should address a possible attenuation of the therapeutic efficacy of COX-2-inducing anticancer compounds by COX-2 inhibitors.

As a whole, the present study demonstrated a COX-2-dependent mitochondrial pathway for R(+)-MA-elicited apoptosis in human neuroglioma cells and thus proposes a mechanism by which certain cannabinoids and inhibitors of endocannabinoid inactivation might exert their antitumorigenic properties.

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