Pharmaceutical Research, Vol. 26, No. 2, February 2009 (© 2008)
DOI: 10.1007/s11095-008-9748-3

Research Paper

R(+)-Methanandamide-Induced Apoptosis of Human Cervical Carcinoma Cells Involves A Cyclooxygenase-2-Dependent Pathway

Karin Eichele,[1] Robert Ramer,[1] and Burkhard Hinz[1,2]

Received July 9, 2008; accepted October 6, 2008; published online October 28, 2008

Purpose. Cannabinoids have received renewed interest due to their antitumorigenic effects. Using human cervical carcinoma cells (HeLa), this study investigates the role of cyclooxygenase-2 (COX-2) in apoptosis elicited by the endocannabinoid analog *R*(+)-methanandamide (MA).
Methods. COX-2 expression was assessed by RT-PCR and Western blotting. PGE_2/PGD_2 levels in cell culture supernatants and DNA fragmentation were measured by ELISA.
Results. MA led to an induction of COX-2 expression, PGD_2 and PGE_2 synthesis. Cells were significantly less sensitive to MA-induced apoptosis when COX-2 was suppressed by siRNA or the selective COX-2 inhibitor NS-398. COX-2 expression and apoptosis by MA was also prevented by the ceramide synthase inhibitor fumonisin B_1, but not by antagonists to cannabinoid receptors and TRPV1. In line with the established role of peroxisome proliferator-activated receptor γ (PPARγ) in the proapoptotic action of PGs of the D and J series, inhibition of MA-induced apoptosis was also achieved by siRNA targeting lipocalin-type PGD synthase (L-PGDS) or PPARγ. A role of COX-2 and PPARγ in MA-induced apoptosis was confirmed in another human cervical cancer cell line (C33A) and in human lung carcinoma cells (A549).
Conclusion. This study demonstrates COX-2 induction and synthesis of L-PGDS-derived, PPARγ-activating PGs as a possible mechanism of apoptosis by MA.

KEY WORDS: Apoptosis; cyclooxygenase-2; lipocalin-type prostaglandin D synthase; peroxisome proliferator-activated receptor γ; *R*(+)-methanandamide.

INTRODUCTION

In recent years, cannabinoids attracted a great deal of interest as promising novel anticancer agents, as they have been shown to inhibit the growth of tumor cells in culture and animal models by modulating key cell-signaling pathways (for review see (1,2)). By the beginning of the 1990s two cannabinoid-specific $G_{i/o}$ protein-coupled receptors, referred to as CB_1 and CB_2, have been cloned (3–5) and several endogenous ligands have been characterized. The first endocannabinoid to be isolated was anandamide (arachidonylethanolamine, AEA) (6), which also displays agonist activity at the transient receptor potential vanilloid-type 1 (TRPV1) (7), followed by 2-arachidonylglycerol (2-AG) (8). There is accumulating evidence, that endocannabinoids have the ability to modulate cell proliferation. AEA, for example, has been shown to possess anticancer properties in a number of different tumor-derived cell lines, including those derived from breast cancer (9), rat glioma (10), cervical carcinoma (11) and colorectal cancer (12). Moreover, upregulation of endocannabinoid levels in several cancer tissues and malignant cells has been suggested to be involved in the tonical control of tumor growth (12,13). Interestingly, however, AEA-mediated effects do not necessarily involve CB receptor or TRPV1 activation (14–17).

Over the past years, the mediators of cannabinoid effects have been intensively investigated. Thereby, a relationship between cannabinoids and prostaglandins (PGs) has been established by several lines of evidence. For instance, various actions of cannabinoids within the central nervous system, including dilation of cerebral arterioles (18), psychoactive and behavioural effects (19), or reduction of intraocular pressure (20,21) have been associated with an increased production of PGs. Consequently, Chan *et al.* (22) have shown that the hippocampal neuronal death induced

Karin Eichele and Robert Ramer contributed equally to this work.

[1] Institute for Toxicology and Pharmacology, University of Rostock, Schillingallee 70, D-18057, Rostock, Germany.
[2] To whom correspondence should be addressed. (e-mail: burkhard.hinz@med.uni-rostock.de)

ABBREVIATIONS: AEA, Anandamide; AM-251, *N*-(Piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide; AM-630, (6-Iodo-2-methyl-1-[2-(4-morpholinyl) ethyl]-1H-indol-3-yl) (4-methoxyphenyl) methanone; capsazepine, (*N*-[2-(4-Chlorophenyl)ethyl]-1,3,4,5-tetrahydro-7,8-dihydroxy-2H-2- benzazepine-2-carbothioamide; CB_1, Cannabinoid receptor 1; CB_2, Cannabinoid receptor 2; COX, Cyclooxygenase; L-PGDS, Lipocalin-type prostaglandin D synthase; MA, *R*(+)-methanandamide (*R*-(+)-arachidonyl-1′-hydroxy-2′-propylamide); NS-398, *N*-[2-(Cyclohexyloxy)-4-nitrophenyl]-methanesulfonamide; PG, Prostaglandin; PPARγ, Peroxisome proliferator-activated receptor γ; RT-PCR, Reverse transcriptase-polymerase chain reaction; siRNA, Small-interfering RNA; TRPV1, Transient receptor potential vanilloid-type 1; 2-AG, 2-Arachidonylglycerol; 15d-PGJ_2, 15-Deoxy-$\Delta^{12,14}$-PGJ_2.

by Δ^9-tetrahydrocannabinol was inhibited by non-steroidal anti-inflammatory drugs and inhibitors of de novo synthesis of mRNA and protein, implying the involvement of COX-2 in this process. Investigations from our group revealed upregulation of COX-2 by *R*(+)-methanandamide (MA), a stable synthetic analog of AEA, and a contribution of this enzyme to apoptotic death of human neuroglioma cells (23,24). In line with these findings, COX-2 has also been implicated in the proapoptotic and cytotoxic effects of diverse substances, including therapeutically used chemotherapeutics, in cancer cells (26–29), raising the hitherto unanswered question whether COX-2 inhibitors might interfere with the therapeutic benefit of COX-2-inducing anticarcinogenic agents.

Concerning the mechanisms underlying COX-2-mediated apoptosis, we were recently able to prove a significant contribution of lipocalin-type PGD synthase (L-PGDS), an isomerase that converts PGH_2 into PGD_2, to apoptosis of a cervical carcinoma cell line (HeLa) in response to several COX-2-inducing chemotherapeutics (29). PGD_2 and its biologically active metabolites, including J_2 series PGs such as PGJ_2, Δ^{12}-PGJ_2 and 15-deoxy-$\Delta^{12,14}$-PGJ_2 (15d-PGJ_2), are important regulators of cell proliferation and death (30) and have been shown to induce apoptotic death of human leukemia cells (25). Furthermore, evidence is accumulating to suggest, that PGD_2 and 15d-PGJ_2 induce apoptosis and cell growth inhibition in several types of cancer cells via their ability to activate the peroxisome proliferator-activated receptor γ (PPARγ) (30,31). In line with this assumption, COX-2-mediated apoptosis by chemotherapeutics was likewise shown to involve a PPARγ-dependent pathway (29).

Despite the well-known relationship between PGs and cannabinoids, an involvement of L-PGDS-derived PGs and PPARγ still remains to be established. To clarify this issue, the present study was designed to investigate apoptosis after MA treatment in HeLa cells with special emphasis on the role of COX-2, L-PGDS and PPARγ in this process. To exclude a cell line specific effect, we further performed key experiments with additional tumor cell lines.

MATERIALS AND METHODS

Materials

AM-251, AM-630, capsazepine and NS-398 were purchased from Alexis Deutschland GmbH (Grünberg, Germany). GW-9662 was obtained from Sigma-Aldrich Chemie (Steinheim, Germany). Fumonisin B_1 and MA 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 from Cambrex Bio Science Verviers S.p.r.l. (Verviers, Belgium). Fetal calf serum and penicillin-streptomycin were obtained from PAN Biotech (Aidenbach, Germany) and Invitrogen (Karlsruhe, Germany), respectively. Small-interfering RNA (siRNA) against COX-2 was synthesized by Qiagen (Qiagen GmbH, Hilden, Germany). The target sequence was 5′-aactgctcaacaccggaattt-3′ (bases 291–311 of NM000963.1) The siRNA sequence was controlled via BLAST search and did not show any homology to other known human genes. siRNA targeting L-PGDS (5′-cacaataaactccg gaagcaa-3′) and PPARγ (5′-gagggcgatcttgacaggaaa-3′) were commercially available (Qiagen GmbH, Hilden, Germany). A negative (non-silencing) control siRNA was purchased from Eurogentec (Seraing, Belgium; Cat. No. OR-0030-neg).

Cell culture and treatment protocols

The human cervical carcinoma cell lines HeLa and C33A, as well as the human lung carcinoma cell line A549 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.

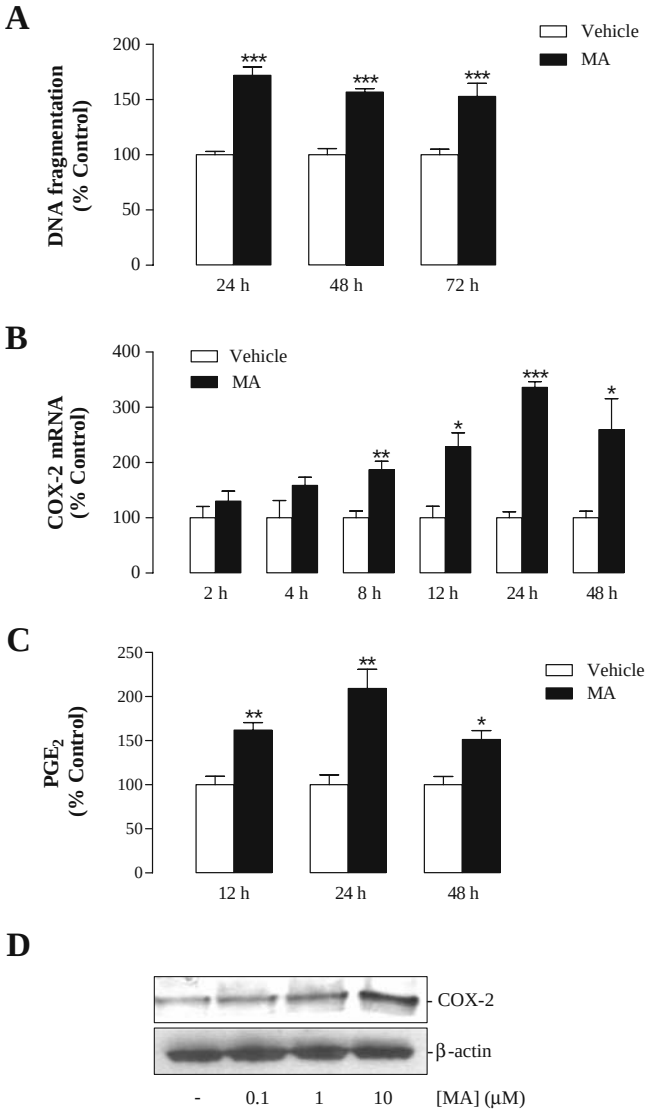

Fig. 1. Influence of MA on COX-2 expression, PGE_2 release and apoptosis by HeLa cells. **a** Effect of MA on DNA fragmentation. Effect of MA on COX-2 mRNA (**b**), PGE_2 release (**c**), and COX-2 protein expression (**d**). Cells were incubated with MA at 10 µM or its vehicle for the indicated times (**a**, **b**, **c**) and for 24 h (**d**). β-Actin was used as a loading control for Western Blot analysis. Percent control represents comparison with vehicle-treated cells (100%) in the absence of test substance. Values are means±SEM of n=4 experiments. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, *vs.* corresponding vehicle control (Student's *t* test).

AM-251, AM-630, capsazepine and NS-398 were used at a final concentration of 1 μM. GW-9662 was used at a 20-μM, and fumonisin B_1 at a 50-μM final concentration. Except concentration dependence experiments, MA was used at a final concentration of 10 μM. All inhibitors were added 1 hour prior to MA.

For transfection experiments, cells were seeded into 24-well plates (DNA fragmentation assays) or 6-well plates (Western blots) and grown to 50–80% confluence. Thereafter, cells were transfected with the respective silencing siRNA or non-silencing siRNA using RNAiFect® (Qiagen GmbH, Hilden, Germany) as described previously (23). The final concentration of siRNA or nonsilencing siRNA was 2.5 μg/ml (COX-2 siRNA) and 1.25 μg/ml (L-PGDS siRNA and PPARγ siRNA), respectively.

In experiments addressing the action of MA in COX-2-silenced cells, HeLa cells were first transfected with COX-2 siRNA or non-silencing siRNA for 24 h. Following medium change, cells were then treated with MA or vehicle in serum-free medium containing the same amounts of COX-2 siRNA or nonsilencing siRNA to provide constant transfection conditions.

The incubation times of the individual experiments are given in the Figure legends.

Quantitative RT-PCR analysis

HeLa cells were grown to confluence in 24-well plates. After incubation of cells with the respective test compounds or vehicle for 48 h (Fig. 4), or for the indicated times (2–48 h; Fig. 1), supernatants were removed and cells were lysed for subsequent RNA isolation. Total RNA was isolated using the RNeasy total RNA Kit (Qiagen, Hilden, Germany). β-Actin- (internal standard) and COX-2 mRNA levels were determined by quantitative real-time RT-PCR as described recently (23).

Western blot analysis

Western blot techniques were performed as described recently (23). In brief, cells were grown to confluence, and incubated with test substance or vehicle for the indicated times. Subsequently, cells were 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. Proteins derived from the supernatants were separated on a 10% sodium dodecyl sulfate-polyacrylamide gel. After transfer to

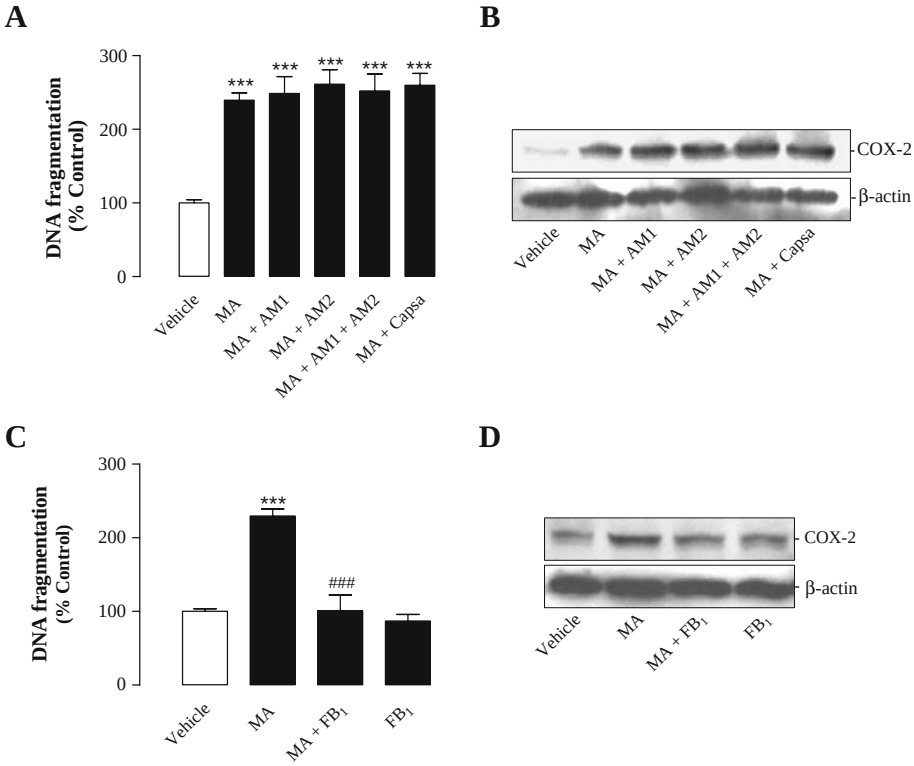

Fig. 2. Influence of the CB_1 antagonist AM-251 (AM1; 1 μM), the CB_2 antagonist AM-630 (AM2; 1 μM), the TRPV1 antagonist capsazepine (Capsa; 1 μM) and the ceramide synthase inhibitor fumonisin B_1 (FB_1; 50 μM) on MA-induced apoptosis (**a**, **c**) and COX-2 protein expression (**b**, **d**) by HeLa cells. Cells were incubated with MA at 10 μM or its vehicle for 24 h. All inhibitors were added to the cells 1 h prior to MA. β-actin was used as a loading control for Western Blot analysis. Percent control represents comparison with vehicle-treated cells (100%) in the absence of test substance. Values are means±SEM of n=4–8 experiments. $^{***}P<0.001$ *vs*. corresponding vehicle control; $^{\#\#\#}P<0.001$, *vs*. MA (one-way ANOVA plus Bonferroni test).

nitrocellulose and blocking of the membranes with 5% milk powder, blots were probed with specific antibodies raised to COX-2 (BD Biosciences, Heidelberg, Germany; 1:250 for detection in HeLa and A549, and 1:100 in C33A cells), PPARγ, L-PGDS (Cayman, Ann Arbor, MI, USA; 1:500 for PPARγ, and 1:250 for L-PGDS), and ß-Actin (Calbiochem, Bad Soden, Germany; 1:5,000). Thereafter, membranes were probed with horseradish peroxidase-conjugated Fab-specific anti-mouse IgG (New England BioLabs, Frankfurt, Germany) for detection of COX-2 and ß-actin, or anti-rabbit IgG (New England BioLabs, Frankfurt, Germany) for detection of L-PGDS and PPARγ, respectively. Antibody binding was visualized by enhanced chemiluminescence Western blotting detection reagents (Amersham Biosciences Inc., Freiburg, Germany).

Determination of prostaglandins

HeLa cells seeded in 24-well plates at a density of 5× 100,000 cells/well were grown to confluence. Thereafter, cells were incubated with the respective test compounds or its vehicles for 48 h (Fig. 4) or the indicated times (12–48 h; Fig. 1), in 300 µl cell culture medium without serum. PGE_2 and PGD_2 concentrations in cell culture supernatants were determined using commercially available enzyme-linked immuno assay (ELISA) kits (Cayman, Ann Arbor, MI, USA). In brief, the quantification of PGE_2 is based on the binding competition between PGE_2 from the cell culture supernatant, and a PGE_2-acetylcholinesterase conjugate serving as PGE_2 tracer to a limited amount of monoclonal antibody. Binding of the tracer-antibody-complex to the secondary antibody coated on the plate bottom is therefore inversely proportional to the amount of PGE_2 that is visualized by enzymatic reaction with Ellmann's reagent and can be calculated by a standard curve. The PGD_2 enzyme assay is based on the conversion of PGD_2 to a stable methoxylamine (MOX) derivative. Treatment of the sample with MOX hydrochloride (MOX-HCl) converts PGD_2 into PGD_2-MOX, thereby preventing its further chemical degradation following collection of supernatants.

Detection of DNA fragmentation

HeLa, C33A, and A549 cells were seeded in 24-well plates at a density of 1×100,000 cells/well and were grown to confluence. Thereafter, cells were incubated with the respective test compounds or its vehicles for the indicated times. Detection of DNA fragmentation in A549 cells was performed after 48 h, and in C33A cells after 24 h of incubation with the test substances or vehicles. For apoptosis detection, adherent cells were harvested by trypsinization and combined with the detached cells. Cytoplasmic histone-associated DNA fragments (mono and oligonucleosomes) were assessed using the cell death detection ELISA^{PLUS} kit (Roche Diagnostics, Mannheim, Germany).

Statistics

All data shown represent means±SEM referring to the respective vehicle control that is defined as 100%. Statistical analyses were undertaken using GraphPad Prism 4.01 (GraphPad Software, San Diego, CA, USA). Comparisons between two groups were performed with Student's t test. When the experimental set-up includes more than two groups, comparisons were performed with one-way ANOVA plus Bonferroni test.

RESULTS

MA-induced toxicity, apoptosis, COX-2 expression and PGE_2 synthesis

To investigate whether MA possesses antitumor properties in HeLa cells, we analysed induction of apoptosis by this compound using DNA fragmentation assays. As shown in Fig. 1A, MA led to a significantly increased apoptosis at different time points post-treatment.

In parallel, COX-2 mRNA expression was demonstrated to be induced in a time-dependent manner, with peak levels

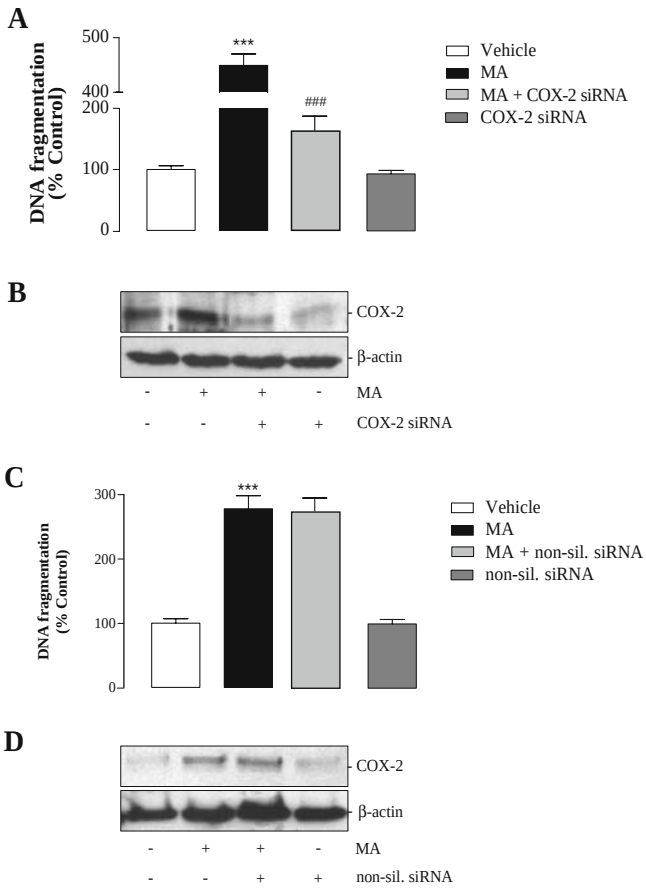

Fig. 3. Influence of COX-2 gene silencing on MA-induced apoptosis. **a** and **c**, Effect of COX-2 siRNA and non-silencing siRNA on DNA fragmentation. **b** and **d** Effect of COX-2 siRNA and non-silencing siRNA on COX-2 protein expression by HeLa cells. Cells were incubated with MA (10 µM) or its vehicle for 24 h. Transfection with COX-2 siRNA (2.5 µg/ml) or non-silencing siRNA (non-sil. siRNA; 2.5 µg/ml) was performed 24 h prior to addition of MA to the cells. β-actin was used as a loading control for Western Blot analysis. Percent control represents comparison with vehicle-treated cells (100%) in the absence of test substance. Values are means±SEM of n=3–4 experiments. $^{***}P<0.001$, *vs.* corresponding vehicle control; $^{###}P<0.001$, *vs.* MA (one-way ANOVA plus Bonferroni test).

occurring after a 24-h stimulation (Fig. 1B). MA also led to a concentration-dependent induction of COX-2 protein synthesis (Fig. 1D) accompanied by upregulation of COX-2 activity as measured by increased PGE_2 levels in supernatants (Fig. 1C).

Next we examined the involvement of CB receptors and TRPV1 in MA responses, using the selective CB_1 receptor antagonist AM-251, the selective CB_2 receptor antagonist AM-630, a combination of AM-251 and AM-630 as well as the TRPV1 antagonist capsazepine. However, all preincubations with receptor antagonists neither significantly altered DNA fragmentation (Fig. 2A) nor COX-2 expression induced by MA (Fig. 2B).

On the other hand, treatment of cells with the ceramide synthase inhibitor fumonisin B_1 completely abolished DNA fragmentation by MA (Fig. 2C). Likewise, a suppression of MA-induced COX-2 protein was observed (Fig. 2D).

Influence of COX-2 siRNA on MA-induced apoptotic cell death

One main emphasis of the present study was the question whether the proapoptotic action of MA on HeLa cells is dependent on COX-2 activity. To address this issue, a set of experiments using siRNA targeting COX-2 was performed. As shown in Fig. 3A, transfection of cells with COX-2 siRNA significantly inhibited DNA fragmentation by the endocannabinoid analog. At the concentration tested, COX-2 siRNA completely inhibited MA-induced COX-2 protein expression (Fig. 3B). Control experiments revealed no significant effect of non-silencing siRNA on all these parameters investigated (Fig. 3C and D)

Influence of NS-398 on MA-induced apoptotic cell death

In case of a key role of COX-2 activity in apoptosis induction, selective inhibition of COX-2 activity is supposed to mimic the protective effect of COX-2 knock-down. Therefore, further experiments using the selective COX-2 inhibitor NS-398 were performed. Coincubation of MA with NS-398 (1 μM) abolished MA-induced PGE_2 and PGD_2 formation (Fig. 4A, middle panels) and significantly protected HeLa cells against DNA fragmentation (Fig. 4A, left). Incubation of cells with NS-398 (1 μM) alone had no significant effect on basal DNA fragmentation (Fig. 4A). Interestingly, NS-398 at 1 μM also tended to suppress induction of COX-2 mRNA expression by MA (Fig. 4A, right). However, a statistical significance could not be calculated in this respect.

Role of L-PGDS in MA-induced apoptotic cell death

As COX-2-dependent PGs generated by the enzymatic activity of L-PGDS are known to induce apoptosis (28), we next analysed, whether this enzyme is involved in HeLa cell apo-

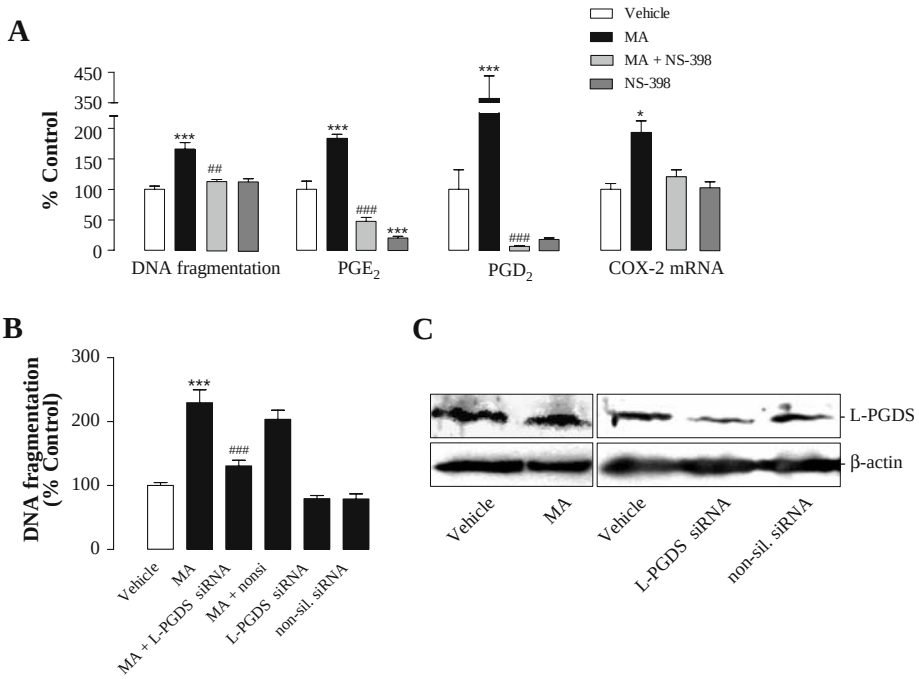

Fig. 4. Involvement of COX-2 in MA-induced apoptosis in HeLa cells. **a** Effect of NS-398 on DNA fragmentation, levels of PGs (PGE_2, PGD_2) and COX-2 mRNA. **b** Effect of L-PGDS siRNA or non-silencing siRNA on DNA fragmentation. **c** Effect of L-PGDS siRNA or non-silencing siRNA on L-PGDS protein expression. Cells were incubated with MA (10 μM), NS-398 (1 μM) or its vehicles for 48 h (**a**, **b**) and 24 h (**c**), respectively. Transfection with L-PGDS siRNA (1.25 μg/ml) or non-silencing siRNA (non-sil. siRNA; 1.25 μg/ml) was performed 24 h prior to addition of test compounds to the cells. β-Actin was used as a loading control for Western Blot analysis. Percent control represents comparison with vehicle-treated cells (100%) in the absence of test substance. Values are means±SEM of n=3–10 experiments. $^{*}P<0.05$, $^{***}P<0.001$, *vs.* corresponding vehicle control; $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$, *vs.* MA (one-way ANOVA plus Bonferroni test).

ptosis. As shown in Fig. 4B, transfection of cells with L-PGDS siRNA significantly inhibited apoptosis elicited by the cannabinoid. At the concentration tested, L-PGDS siRNA was shown to inhibit basal L-PGDS protein expression (Fig. 4C), which was not altered by the investigated compound. L-PGDS siRNA alone had no effect on DNA fragmentation (Fig. 4B). Moreover, non-silencing siRNA which was used as a negative control, left all investigated parameters virtually unaltered (Fig. 4B and C).

Role of PPARγ in MA-induced apoptosis

Previously, PGD_2 was reported to mediate its effects as a ligand on the PPARγ receptor (31). This led us to hypothesize that MA-induced apoptosis might involve PPARγ activation. As shown in Fig. 5A, knock-down of PPARγ by siRNA transfection significantly inhibited MA-induced apoptosis. Again, siRNA at the concentration tested was shown to inhibit basal PPARγ protein expression (Fig. 5B), which was not altered by the investigated cannabinoid. PPARγ siRNA did not affect DNA fragmentation, as well as a non-silencing siRNA which was used as a negative control had no effect on all parameters investigated (Fig. 5A and B). Additionally, coincubation of cells with the selective PPARγ antagonist GW-9662 attenuated MA-induced apoptosis (Fig. 5C). In line with these findings, MA-induced apoptosis was mimicked by the synthetic PPARγ agonist rosiglitazone (Fig. 5D).

Involvement of COX-2 and PPARγ in MA-induced apoptosis in A549 and C33A cells

To exclude a restriction to one single cell line, we repeated the key experiments in another cervical carcinoma cell line (C33A) as well as in a cell line derived from human lung cancer (A549). As shown in Fig. 6A, COX-2 expression was increased in both cell lines after treatment with 10 μM MA. In line with the experiments addressing the role of COX-2 and PPARγ in HeLa cells, induction of DNA fragmentation after MA treatment again proved to be sensitive to NS-398 and GW-9662 in both C33A and A549 cells (Fig. 6B and C).

DISCUSSION

In recent years, evidences for an antitumorigenic action of cannabinoids and their endogenous analogs was mainly derived from studies demonstrating their proapoptotic and antiproliferative properties (for review see (1,2)). Moreover, several cellular investigations revealed a contribution of

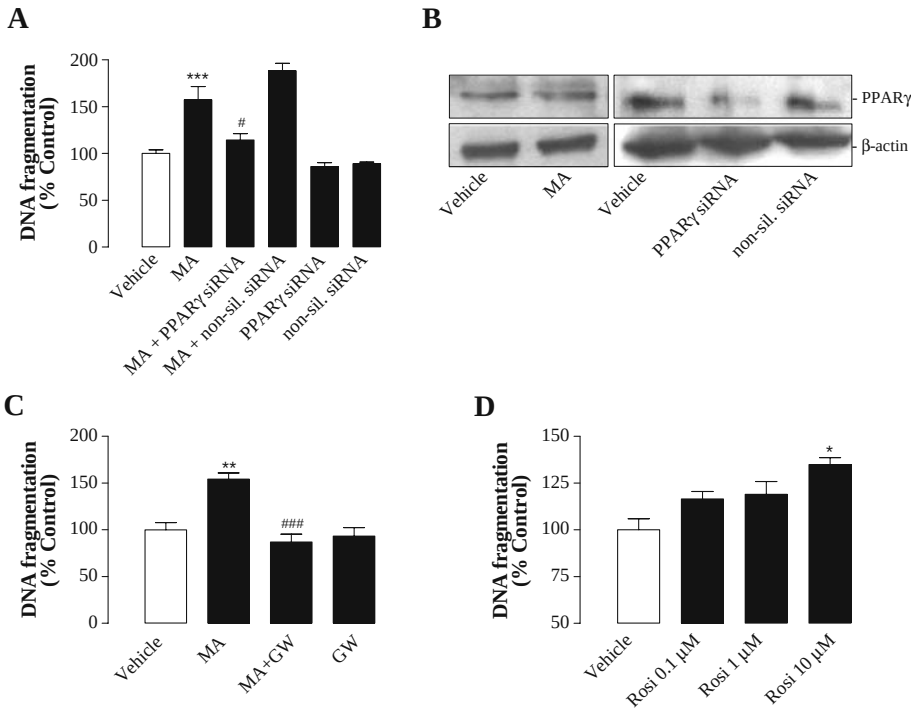

Fig. 5. Role of PPARγ in MA-induced apoptosis. **a** Effect of PPARγ siRNA and non-silencing siRNA on DNA fragmentation. **b** Effect of PPARγ siRNA and non-silencing siRNA on PPARγ protein expression. **c** Effect of GW-9662 on DNA fragmentation. **d** Effect of rosiglitazone on DNA fragmentation. Cells were incubated with MA (10 μM), GW-9662 (20 μM), rosiglitazone (Rosi; 0.1–10 μM) or its vehicles for 48 h (**a**, **c**, **d**) and 24 h (**b**), respectively. Transfection with PPARγ siRNA (1.25 μg/ml) or non-silencing siRNA (non-sil. siRNA; 1.25 μg/ml) was performed 24 h prior to addition of test compounds to the cells. β-Actin was used as a loading control for Western blot analysis. Percent control represents comparison with vehicle-treated cells (100%) in the absence of test substance. Values are means±SEM of $n=3$–4 experiments. $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$, *vs.* corresponding vehicle control; $^{\#}P<0.05$, $^{\#\#\#}P<0.001$, *vs.* MA (one-way ANOVA plus Bonferroni test).

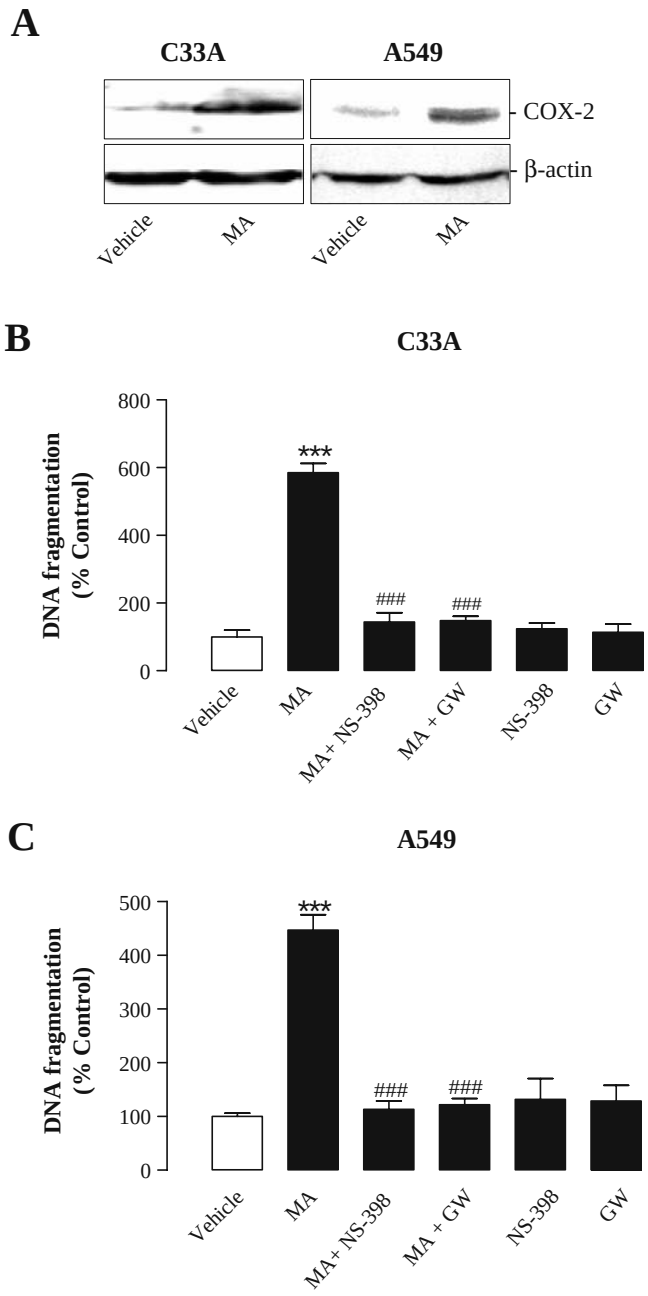

Fig. 6. Influence of NS-398 and GW-9662 on MA-induced DNA fragmentation in C33A and A549 cells. **a** Western Blot analysis of COX-2 protein expression after a 24-h exposure to MA (10 μM). **b**, **c** Effect of NS-398 or GW-9662 on DNA fragmentation in C33A (**b**) and A549 (**c**) and cells. Cells were incubated with MA (10 μM), NS-398 (1 μM), GW-9662 (20 μM) or the respective vehicle controls for 24 (**b**) or 48 (**c**) h. Percent control represents comparison with vehicle-treated cells (100%) in the absence of test substances. Values are means±SEM of n=4 experiments. $^{***}P<0.001$ *vs.* corresponding vehicle control; $^{###}P<0.001$, *vs.* MA (one-way ANOVA plus Bonferroni test).

endocannabinoids to the tonical control of cancer growth (12,13). However, despite the fact that many signalling pathways contributing to the antitumorigenic properties have been clarified, several phenomena reported after cannabinoid treatment have not been analysed yet regarding their potential as key mediators of cannabinoid-induced apoptosis. In this respect we were particularly interested in the modulation of COX-2 expression and PG production which has been reported to be involved in several biological effects of cannabinoids (18–21,32,33). Recent investigations of our group have shown that MA causes apoptotic death of H4 neuroglioma cells by a mechanism involving de novo expression of COX-2 (23,24).

We here demonstrate an induction of COX-2 expression and subsequent PG synthesis by MA in HeLa cells. Additional analysis provided evidence that MA-induced COX-2 expression and apoptosis were not mediated by either CB receptors or TRPV1. Instead, MA-induced COX-2 expression was shown to involve a ceramide-dependent pathway (for the complete proposed mechanism see Fig. 7). Ceramide is an ubiquitous sphingolipid second messenger that plays an important role in the control of cell fate at different sites (34) and has previously been reported to induce COX-2 expression and to activate MAPKs (35), which have been implicated as upstream targets regulating COX-2 expression (36). Moreover, ceramide induction by cannabinoids has been shown to mediate apoptosis of glioma cells (23,37). Ceramide can be generated by hydrolysis of sphingomyelin by sphingomyelinases, de novo, or via reacylation of free sphingoid bases (38). The data presented here suggest that MA-induced apoptosis is mediated via de novo ceramide formation by the enzyme ceramide synthase. Accordingly, induction of DNA fragmentation by MA was prevented by the mycotoxin fumonisin B_1, a structural sphingolipid analog that inhibits the acylation step in ceramide synthesis by binding competition with sphinganine and sphingosine (38). This mechanism is in line with our recent data showing a contribution of de novo ceramide synthesis to COX-2 induction and subsequent apoptosis in H4 neuroglioma cells (39,23).

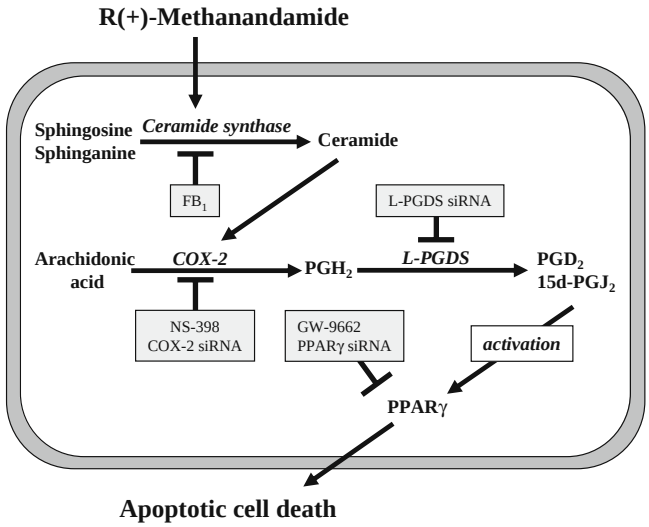

Fig. 7. Scheme illustrating the proposed mechanism by which MA enhances apoptotic cell death of HeLa, C33A and A549 cells. MA increases synthesis of ceramide via the enzyme ceramide synthase, which in turn leads to COX-2 expression, and subsequent synthesis of PGD_2 and 15d-PGJ_2 by the L-PGDS. Finally, activation of PPARγ by the indicated PGs is proposed to elicit apoptotic cell death.

The main emphasis of this study, however, was to establish a COX-2-dependent mechanism of apoptosis induction by the investigated endocannabinoid analog. In fact, we confirmed a direct correlation between COX-2 and apoptosis induction by means of COX-2 siRNA transfection. Additionally, NS-398 significantly interfered with the proapoptotic and cytotoxic action of MA at a concentration of 1 μM, which itself did not induce apoptosis, but which completely inhibited MA-induced PGE_2 and PGD_2 synthesis. Interestingly, NS-398 also inhibited, albeit not significantly, COX-2 mRNA expression suggesting a positive feedback regulation of COX-2 expression by COX-2-derived PGs, a phenomenon reported by us and others previously (40–42).

Looking for the targets conferring COX-2-induced apoptosis, we have recently focussed on diverse PGs synthesized upon COX-2 stimulation. In this context, PGD_2 and its dehydration product 15d-PGJ_2 were both shown to elicit apoptosis in HeLa cells (29). On the other hand, PGE_2, which was proven to cause apoptosis of H4 neuroglioma cells (23) and primary cultures of glioblastoma multiforme (43), failed to induce DNA fragmentation in HeLa cells (29). Based on these findings, the contribution of PGD_2 to cannabinoid-induced apoptosis was assessed using siRNA targeting its biosynthesizing enzyme L-PGDS. L-PGDS is an *N*-glycosylated, monomeric protein, which catalyzes the conversion of PGH_2 to PGD_2 (44) and is known to induce apoptosis (45,46). In our hands, L-PGDS siRNA significantly interfered with cannabinoid-induced apoptosis, suggesting an involvement of COX-2-dependent L-PGDS-derived PGs in the apoptotic response to MA. These results are in line with findings by Kim *et al.* (31) showing that L-PGDS and PGD_2 metabolites produced by normal prostate stromal cells inhibit tumor cell growth.

Several natural and synthetic PPARγ activators, including COX-2-dependent PGD_2 and 15d-PGJ_2, have been found to inhibit cancer cell growth *in vitro* and in animal models (30,31,47). Accordingly, we hypothesized that PPARγ might be involved in MA-induced COX-2-mediated apoptosis. With respect to cannabinoids, PPARγ is predominantly associated with preadipocyte differentiation, insulin resistance and vasorelaxation (48–50). Furthermore, 2-AG was recently shown to mediate interleukin-2 suppression via PPARγ activation, independently of CB receptor activation (51). However, to our knowledge, this is the first study to assess the involvement of PPARγ activation in the apoptotic response of tumor cells to cannabinoids. Accordingly, MA-induced apoptosis was abolished by siRNA targeting PPARγ, and by coincubation with the PPARγ antagonist GW-9662. Finally, the synthetic PPARγ agonist rosiglitazone mimicked the apoptotic response to MA. Referring to the fact that PGD_2 activates PPARγ (31), PPARγ activation by COX-2-dependent PGD_2 is supposed to be involved in MA-induced apoptosis in human cervical carcinoma cells.

Finally, we confirmed a COX-2- and PPARγ-dependent proapoptotic effect of MA in another human cervical cancer cell line (C33A) as well as in human lung carcinoma cells (A549), suggesting both proteins as key mediators of MA-elicited apoptosis. Future studies to be performed *in vivo* are needed to further shed light on the role of COX-2 in tumor progression and to verify a potential interaction of a concomitant treatment of MA and COX-2 inhibitors.

Collectively, this study supports the general understanding of cannabinoids as antitumorigenic substances, provides additional evidence for cannabinoid-induced apoptosis, and suggests a novel COX-2-mediated mechanism for apoptosis induction by MA. In this context, our results suggest a relationship between PG synthesis, PPARγ activation and endocannabinoid-induced apoptosis. Although PGs as well as PPARγ are known mediators of cannabinoid-dependent effects, this is the first study to show a combined involvement of these parameters in cannabinoid-induced apoptosis.

ACKNOWLEDGMENT

This study was supported by grants from the Deutsche Krebshilfe e.V. (Bonn, Germany), Deutsche Forschungsgemeinschaft (SFB 539 TP BI.6) and Johannes und Frieda Marohn Stiftung (Erlangen, Germany).

REFERENCES

1. M. Guzman. Cannabinoids: potential anticancer agents. *Nat. Rev. Cancer.* **3**:745–755 (2003) doi:10.1038/nrc1188.
2. M. Bifulco, C. Laezza, S. Pisanti, and P. Gazzerro. Cannabinoids and cancer: pros and cons of an antitumour strategy. *Br. J. Pharmacol.* **148**:123–135 (2006) doi:10.1038/sj.bjp.0706632.
3. L. Matsuda, S. Lolait, M. Brownstein, A. Young, and T. Bonner. Structure of a cannabinoid receptor and functional expression of the cloned cDNA. *Nature*. **346**:561–564 (1990) doi:10.1038/346561a0.
4. S. Munro, K. Thomas, and M. Abu-Shaar. Molecular characterization of a peripheral receptor for cannabinoids. *Nature*. **365**:61–65 (1993) doi:10.1038/365061a0.
5. S. Galiegue, S. Mary, J. Marchand, D. Dussossoy, D. Carriere, P. Carayon, M. Bouaboula, D. Shire, G. L. Fur, and P. Casellas. Expression of central and peripheral cannabinoid receptors in human immune tissues and leukocyte subpopulations. *Eur. J. Biochem.* **232**:54–61 (1995) doi:10.1111/j.1432-1033.1995.tb20780.x.
6. W. A. Devane, L. Hanus, A. Breuer, R. G. Pertwee, L. A. Sevenson, G. Griffin, D. Gibson, A. Mandelbaum, A. Etinger, and R. Mechoulam. Isolation and structure of a brain constituent that binds to the cannabinoid receptor. *Science*. **258**:1946–1949 (1992) doi:10.1126/science.1470919.
7. D. Smart, M. Gunthorpe, J. Jerman, S. Nasir, J. Gray, A. Muir, J. Chambers, A. Randall, and J. Davis. The endogenous lipid anandamide is a full agonist at the human vanilloid receptor (hVR1). *Br. J. Pharmacol.* **129**:227–230 (2000) doi:10.1038/sj.bjp.0703050.
8. R. Mechoulam, S. Ben-Shabat, L. Hanus, M. Ligunsky, N. E. Kaninski, A. R. Schatz, A. Gopher, S. Almog, B. R. Martin, D. R. Compton, R. G. Pertwee, G. Griffin, M. Bayewitch, J. Barg, and Z. Vogel. Identification of an endogenous 2-monoglyceride, present in canine-gut, that binds to cannabinoid receptors. *Biochem. Pharmacol.* **50**:83–90 (1995) doi:10.1016/0006-2952(95)00109-D.
9. L. D. De Petrocellis, D. Melck, A. Palmisano, T. Bisogno, C. Laezza, M. Bifulco, and V. D. Marzo. The endogenous cannabinoid anandamide inhibits human breast cancer cell proliferation. *Proc. Natl. Acad. Sci. USA*. **95**:8375–8380 (1998) doi:10.1073/pnas.95.14.8375.
10. S. Jacobsson, T. Wallin, and C. Fowler. Inhibition of rat C6 glioma cell proliferation by endogenous and synthetic cannabinoids. Relative involvement of cannabinoid and vanilloid receptors. *J. Pharmacol. Exp. Ther.* **299**:951–959 (2001).
11. E. Contassot, M. Tenan, V. Schnuriger, M. F. Pelte, and P. Y. Dietrich. Arachidonyl ethanolamide induces apoptosis of uterine cervix cancer cells via aberrantly expressed vanilloid receptor-1. *Gynecol. Oncol.* **93**:182–188 (2004) doi:10.1016/j.ygyno.2003.12.040.

12. A. Ligresti, T. Bisogno, I. Matias, L. D. Petrocellis, M. Cascio, V. Cosenza, G. D'argenio, G. Scaglione, M. Bifulco, I. Sorrentini, and V. D. Marzo. Possible endocannabinoid control of colorectal cancer growth. *Gastroenterology*. **125**:677–687 (2003) doi:10.1016/S0016-5085(03)00881-3.
13. M. Bifulco, C. Laezza, M. Valenti, A. Ligresti, G. Portella, and V. DiMarzo. A new strategy to block tumor growth by inhibiting endocannabinoid inactivation. *FASEB J.* **18**:1606–1608 (2004).
14. V. DiMarzo, T. Bisogno, L. De Petrocellis, D. Melck, and B. R. Martin. Cannabimimetic fatty acid derivatives: the anandamide family and other endocannabinoids. *Curr. Med. Chem.* **6**:721–744 (1999).
15. V. DiMarzo, C. S. Breivogel, Q. Tao, D. T. Bridgen, R. K. Razdan, A. M. Zimmer, A. Zimmer, and B. R. Martin. Levels, metabolism, and pharmacological activity of anandamide in CB_1 cannabinoid receptor knockout mice: evidence for non-CB_1, non-CB_2 receptor-mediated actions of anandamide in mouse brain. *J. Neurochem.* **75**:2434–2444 (2000) doi:10.1046/j.1471-4159.2000.0752434.x.
16. E. Berdyshev, P. Schmid, R. Krebsbach, C. Hillard, C. Huang, N. Chen, Z. Dong, and H. Schmid. Cannabinoid-receptor-independent cell signalling by *N*-acylethanolamines. *Biochem. J.* **360**:67–75 (2001) doi:10.1042/0264-6021:3600067.
17. K. Sarker, and I. Maruyama. Anandamide induces cell death independently of cannabinoid receptors or vanilloid receptor 1: possible involvement of lipid rafts. *Cell. Mol. Life Sci.* **60**:1200–1208 (2003).
18. E. Ellis, S. Moore, and K. Willoughby. Anandamide and Δ^9-THC dilation of cerebral arterioles is blocked by indomethacin. *Am. J. Physiol.* **269**:H1859–1864 (1995).
19. S. Burstein, K. Hull, S. Hunter, and J. Shilstone. Immunization against prostaglandins reduces Δ^1-tetrahydrocannabinol-induced catalepsy in mice. *Mol. Pharmacol.* **35**:6–9 (1989).
20. D. Pate, K. Jarvinen, A. Urtti, P. Jarho, M. Fich, V. Mahadevan, and T. Jarvinen. Effects of topical anandamides on intraocular pressure in normotensive rabbits. *Life Sci.* **58**:1849–1860 (1996) doi:10.1016/0024-3205(96)00169-5.
21. K. Green, E. Kearse, and O. McIntyre. Interaction between Δ^9-tetrahydrocannabinol and indomethacin. *Ophthalmic Res.* **33**:217–220 (2001) doi:10.1159/000055673.
22. G. Chan, T. Hinds, S. Impey, and D. Storm. Hippocampal neurotoxicity of Δ^9- tetrahydrocannabinol. *J. Neurosci.* **18**:5322–5332 (1998).
23. B. Hinz, R. Ramer, K. Eichele, U. Weinzierl, and K. Brune. Upregulation of cyclooxygenase-2 expression is involved in R(+)-methanandamide-induced apoptotic death of human neuroglioma cells. *Mol. Pharmacol.* **66**:1643–1651 (2004) doi:10.1124/mol.104.002618.
24. K. Eichele, U. Weinzierl, R. Ramer, K. Brune, and B. Hinz. R(+)-methanandamide elicits a cyclooxygenase-2-dependent mitochondrial apoptosis signaling pathway in human neuroglioma cells. *Pharm. Res.* **23**:90–94 (2006) doi:10.1007/s11095-005-8815-2.
25. Y. C. Chen, S. C. Shen, and S. H. Tsai. Prostaglandin D_2 and J_2 induce apoptosis in human leukemia cells via activation of the caspase 3 cascade and production of reactive oxygen species. *Biochim. Biophys. Acta*. **1743**:291–304 (2005) doi:10.1016/j.bbamcr.2004.10.016.
26. M. Maccarrone, R. Pauselli, M. DiRienzo, and A. Finazzi-Agro. Binding, degradation and apoptotic activity of stearoylethanolamide in rat C6 glioma cells. *Biochem. J.* **366**:137–144 (2002).
27. Y. E. Dommels, M. M. Haring, N. G. Keestra, G. M. Alink, P. J. van Bladeren, and B. van Ommen. The role of cyclooxygenase in n-6 and n-3 polyunsaturated fatty acid mediated effects on cell proliferation, PGE_2 synthesis and cytotoxicity in human colorectal carcinoma cell lines. *Carcinogenesis*. **24**:385–392 (2003) doi:10.1093/carcin/24.3.385.
28. H. K. Na, H. Inoue, and Y. J. Surh. ET-18-O-CH_3-induced apoptosis is causally linked to COX-2 upregulation in H-ras transformed human breast epithelial cells. *FEBS Lett.* **579**:6279–6287 (2005) doi:10.1016/j.febslet.2005.09.094.
29. K. Eichele, R. Ramer, and B. Hinz. Decisive role of cyclooxygenase-2 and lipocalin-type prostaglandin D synthase in chemotherapeutics-induced apoptosis of human cervical carcinoma cells. *Oncogene*. **27**:3032–3044 (2008) doi:10.1038/sj.onc.1210962.
30. H. K. Na, and Y. J. Surh. Peroxisome proliferator-activated receptor γ (PPARγ) ligands as bifunctional regulators of cell proliferation. *Biochem. Pharmacol.* **66**:1381–1391 (2003) doi:10.1016/S0006-2952(03)00488-X.
31. J. Kim, P. Yang, M. Suraokar, A. L. Sabichi, N. D. Llansa, G. Mendoza, V. Subbarayan, C. J. Logothetis, R. A. Newman, S. M. Lippman, and D. G. Menter. Suppression of prostate tumor cell growth by stromal cell prostaglandin D synthase-derived products. *Cancer Res.* **65**:6189–6198 (2005) doi:10.1158/0008-5472.CAN-04-4439.
32. B. Gardner, L. X. Zhu, S. Sharma, D. P. Tashkin, and S. M. Dubinett. Methanandamide increases COX-2 expression and tumor growth in murine lung cancer. *FASEB J.* **17**:2157–2159 (2003).
33. L. Mestre, F. Correa, F. Docagne, D. Clemente, and C. Guaza. The synthetic cannabinoid WIN 55,212–2 increases COX-2 expression and PGE_2 release in murine brain-derived endothelial cells following Theiler's virus infection. *Biochem. Pharmacol.* **72**:869–880 (2006) doi:10.1016/j.bcp.2006.06.037.
34. Y. Hannun, and L. Obeid. The ceramide-centric universe of lipid-mediated cell regulation: stress encounters of the lipid kind. *J. Biol. Chem.* **277**:25847–25850 (2002) doi:10.1074/jbc.R200008200.
35. K. Subbaramaiah, W. Chung, and A. Dannenberg. Ceramide regulates the transcription of cyclooxygenase-2. evidence for involvement of extracellular signal-regulated kinase/c-Jun N-terminal kinase and p38 mitogen-activated protein kinase pathways. *J. Biol. Chem.* **273**:32943–32949 (1998) doi:10.1074/jbc.273.49.32943.
36. R. Ramer, K. Brune, A. Pahl, and B. Hinz. R(+)-methanandamide induces cyclooxygenase-2 expression in human neuroglioma cells via a non-cannabinoid receptor-mediated mechanism. *Biochem. Biophys. Res. Commun.* **286**:1144–1152 (2001) doi:10.1006/bbrc.2001.5518.
37. G. Velasco, I. Galve-Roperh, C. Sanchez, C. Blazquez, A. Haro, and M. Guzman. Cannabinoids and ceramide: two lipids acting hand-by-hand. *Life Sci.* **77**:1723–1731 (2005) doi:10.1016/j.lfs.2005.05.015.
38. E. Wang, W. P. Norred, C. W. Bacon, R. T. Riley, and A. H. Jr Merrill. Inhibition of sphingolipid biosynthesis by fumonisins. Implications for diseases associated with *Fusarium moniliforme*. *J. Biol. Chem.* **266**:14486–14490 (1991).
39. R. Ramer, U. Weinzierl, B. Schwind, K. Brune, and B. Hinz. Ceramide is involved in R(+)-methanandamide-induced cyclooxygenase-2 expression in human neuroglioma cells. *Mol. Pharmacol.* **64**:1189–1198 (2003) doi:10.1124/mol.64.5.1189.
40. B. Hinz, K. Brune, and A. Pahl. Cyclooxygenase-2 expression in lipopolysaccharide-stimulated human monocytes is modulated by cyclic AMP, prostaglandin E_2, and nonsteroidal anti-inflammatory drugs. *Biochem. Biophys. Res. Commun.* **278**:790–6 (2000) doi:10.1006/bbrc.2000.3885.
41. S. Debey, J. Meyer-Kirchrath, and K. Schror. Regulation of cyclooxygenase-2 expression by iloprost in human vascular smooth muscle cells. Role of transcription factors CREB and ICER. *Biochem. Pharmacol.* **65**:979–988 (2003) doi:10.1016/S0006-2952(02)01661-1.
42. S. Rosch, R. Ramer, K. Brune, and B. Hinz. Prostaglandin E_2 induces cyclooxygenase-2 expression in human non-pigmented ciliary epithelial cells through activation of p38 and p42/44 mitogen-activated protein kinases. *Biochem. Biophys. Res. Commun.* **338**:1171–1178 (2005) doi:10.1016/j.bbrc.2005.10.051.
43. L. Lalier, P. F. Cartron, F. Pedelaborde, C. Olivier, D. Loussouarn, S. A. Martin, K. Meflah, J. Menanteau, and F. M. Vallette. Increase in PGE_2 biosynthesis induces a Bax dependent apoptosis correlated to patients' survival in glioblastoma multiforme. *Oncogene*. **26**:4999–5009 (2007) doi:10.1038/sj.onc.1210303.
44. J. L. Herlong, and T. R. Scott. Positioning prostanoids of the D and J series in the immunopathogenic scheme. *Immunol. Lett.* **102**:121–131 (2006) doi:10.1016/j.imlet.2005.10.004.
45. J. K. Maesaka, T. Palaia, L. Frese, S. Fishbane, and L. Ragolia. Prostaglandin D_2 synthase induces apoptosis in pig kidney LLC-PK1 cells. *Kidney Int.* **60**:1692–1698 (2001) doi:10.1046/j.1523-1755.2001.00989.x.
46. L. Ragolia, T. Palaia, L. Frese, S. Fishbane, and J. K. Maesaka. Prostaglandin D_2 synthase induces apoptosis in PC12 neuronal cells. *Neuroreport*. **12**:2623–2628 (2001) doi:10.1097/00001756-200108280-00008.

47. S. Han, and J. Roman. Peroxisome proliferator-activated receptor γ: a novel target for cancer therapeutics. *Anticancer Drugs*. **18**:237–244 (2007) doi:10.1097/CAD.0b013e328011e67d.
48. M. Bouaboula, S. Hilairet, J. Marchand, L. Fajas, G. Le Fur, and P. Casellas. Anandamide induced PPARγ transcriptional activation and 3T3-L1 preadipocyte differentiation. *Eur. J. Pharmacol.* **517**:174–181 (2005) doi:10.1016/j.ejphar.2005.05.032.
49. S. Burstein. PPAR-γ: a nuclear receptor with affinity for cannabinoids. *Life Sci.* **77**:1674–1684 (2005) doi:10.1016/j.lfs.2005.05.039.
50. S. E. O'Sullivan, E. J. Tarling, A. J. Bennett, D. A. Kendall, and M. D. Randall. Novel time-dependent vascular actions of Δ^9-tetrahydrocannabinol mediated by peroxisome proliferator-activated receptor gamma. *Biochem. Biophys. Res. Commun.* **337**:824–831 (2005).
51. C. E. Rockwell, N. T. Snider, J. T. Thompson, J. P. Vanden Heuvel, and N. E. Kaminski. Interleukin-2 suppression by 2-arachidonyl glycerol is mediated through peroxisome proliferator-activated receptor γ independently of cannabinoid receptors 1 and 2. *Mol. Pharmacol.* **70**:101–111 (2006).