Endogenous Cannabinoid Anandamide Impairs Cell Growth and Induces Apoptosis in Chondrocytes

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ABSTRACT: Endocannabinoids has been described to be involved in articular degenerative disease by modulating nociception and immune system. However, the role of the endocannabinoid anandamide on chondrocyte cell viability is still unclear. Therefore, we decided to study anandamide's effects on chondrocytes viability and to evaluate its interactions with the catabolic factor TNF (tumor necrosis factor). Chondrocyte vitality was evaluated by MTT assay. We investigated LDH release, chromatin condensation, cleavage of focal adhesion kinase (FAK), and caspases-3, 8, and 9 activation. c-MYC mRNA levels were determined by RT-PCR. We studied by Western blot the activation patterns of AKT, AMPK, ERK, p38, and JNK kinases. Finally, we evaluate the effect of anandamide in TNF-induced caspase-3 cleavage. Anandamide decreased chondrocyte vitality independently of its receptors. It induced AMPK activation without LDH release. Anandamide induced chromatin condensation, activation of caspase-3, 8, and 9, and FAK cleavage. Surprisingly, despite anandamide inhibited cell proliferation, it increased c-MYC expression. Moreover anandamide inhibited AKT activation, whilst it induced a sustained activation of ERK, JNK, and p38. Finally, anandamide synergized with TNF-α in the cleavage of caspase-3. In conclusion, our findings suggest that anandamide, alone or in combination with TNF-α, may be a potential destructive agent in cartilage. © 2014 Orthopaedic Research Society. Published by Wiley Periodicals, Inc. J Orthop Res 32:1137–1146, 2014.

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The endocannabinoid system is constituted by the cannabinoid receptors (CB_1 and CB_2), by the endocannabinoids (including anandamide [AEA] and others) and the machinery for their biosynthesis and metabolism.[1] AEA, the prototype of the endocannabinoids, is the ethanol-amide of the arachidonic acid and it is synthesized by several cell types including: endothelial cells,[2] macrophages,[3] blood cells,[4] adipocytes,[5] as well as bone cells.[6] Cannabinoid receptors have seven transmembrane domains coupled to a pertussis toxin (PTX)-sensitive Gi/o protein. CB receptors (CBRs) differ in their amino acid sequence, signaling mechanisms, and tissue distribution. AEA binds to both cannabinoids receptors but also to vanilloid receptor 1 (VR1),[7] peroxisome proliferator–activated receptor (PPARγ)[8] and to GPR55, an orphan receptor that seems to be a novel cannabinoid binding site.[9] AEA exhibits different effects depending on the cell type. It induces death in liver cells, osteosarcoma cells, sertoli cells, and dendritic cells[10–13] whereas, it induces proliferation in other cell types such as lung carcinoma cells, glioblastoma cells (U37-MG), and hematopoietic growth factor-dependent blood cells (32D/EPO).[14,15]

Chondrocytes are the unique cells in the cartilage. They synthesize and maintain the homeostasis of their extracellular matrix. However, certain joint pathologies such as osteoarthritis (OA) are characterized by chondrocytes phenotype loss, apoptosis, cytokines production, and degeneration of extracellular matrix.

Cannabinoids has been considered interesting pharmacological tools in the management of chronic inflammatory diseases,[16] as arthritis, probably due to their activity as immune modulators and pain suppressors.[17,18] Actually, recent evidences, recently reviewed by La Porta et al.,[19] have demonstrated the antinociceptive effects of agonists of cannabinoid receptors in rodent models. However, despite recent behavioral and electrophysiological studies showed the antinociceptive effects of cannabinoids in experimental models of osteoarthritis, the administration of cannabinoids agonists in humans remained almost out of traditional clinical applications for their secondary side effects on central nervous system and the social prejudices conceived by "non medical" use of *Cannabis*.[19] On the other hand, it is known that AEA and 2-AG (2-arachidonyl glycerol) are elevated in the synovial fluid of OA and rheumatoid arthritis (RA) patients.[20] Also, cannabinoids exert a relevant role regulating the bone mass, which has a determinant impact in cartilage physiology.[6,21] Despite this, the role of endocannabinoids in the cellular compartment of joints is still

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unclear, including the effect of the cannabinoids in the metabolic activity and viability of chondrocytes.

In the present work, we have investigated whether AEA, alone or in combination with a classic chondrocyte-pro-apoptotic factor TNF, is able to modulate chondrocyte vitality and apoptosis rate. To gain further insights, we investigated the activation of caspases pathway and the presence of chromatin condensation upon AEA stimulation. Moreover we analyzed the effect of AEA on several signaling routes including AKT, AMPK, ERK1/2, P-38, JNK, and FAK. Finally, by means of specific pharmacological inhibitors, we explored the role of cannabinoid transmembrane receptors (CB_1, CB_2), and PTX sensitive G proteins.

METHODS

Reagents

Anandamide, TNF-α, MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide), pertussis toxin, Hoechst 33258 dye, and the antibody against actin were purchased from Sigma–Aldrich (St Louis, MO). Anti-caspase-3, anti-caspase-3 (Asp 175), anti-caspase 8, anti-AMPK-P (phosphorylated), anti-AMPK, anti-AKT, and anti AKT-P antibodies were purchased from Cell Signalling (Boston, MA). Anti-CB_1 and FAK antibodies were purchased to Santa Cruz Biotechnology, Inc., Santa Cruz, CA. Anti-ERK1/2, anti-ERK1/2-P, anti-p38, anti-p38-P, anti-JNK, and anti-JNK-P, antibodies were purchased to Upstate (Millipore), Virginia. CB_1 and CB_2 antagonists (SR141617A, SR144528) were a kind gift of Sanofi Aventis (Montpellier, France). Secondary antibodies, anti-mouse (NA9310V), anti-rabbit (NA9340V) were purchased to GE Healthcare (Freiburg, Germany). First strand kit, PCR master mix, and primers were purchased to SaBioSciences (Quiagen, Germantown, MD).

Cell Culture

The ATDC5 murine chondrogenic cell line was purchased from RIKEN Cell Bank (Japan). Cells were cultured as previously described.[22] Briefly, cells were cultured in DMEM/Hams'-F12 medium supplemented with 5% of FBS, 10 μg/ml human transferrin, 3×10^{-8} M sodium selenite, and antibiotics (50 U/ml penicillin and 50 μg/ml streptomycin).

The immortalized human juvenile costal chondrocytes cell lines T/C-28a2, C-20/A4, and C-28/I2 were cultured in DMEM/F12 supplemented with 10% FBS, L-glutamine, and antibiotics (50 U/ml penicillin and 50 μg/ml streptomycin).

RNA Isolation and Real Time Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

RNA was extracted with the NucleoSpin kit, according instructions and protocol provided by the purchaser Macherey-Nagel (Duren, Germany).

For relative quantification we performed a RT reaction with the first strand kit from SaBioSciences. Next, real time PCR reaction was performed with SaBioSciences master mix and specific primers for human and mouse mRNAs (SaBioSciences, sequences are not disclosed (human/mouse GAPDH (PPH00150E PPM02946E/A), human c-MYC (PPH00100A), human/mouse CB_2 (PPH02723A/PPM04826A), human/mouse FAAH (PPH23936B/PPM35386A)). Results of comparative real time PCR were analyzed with MxPro v4 software (Stratagene; Agilent Technologies, Inc., Santa Clara, CA). After the reaction, the PCR products were electrophoresed in a 2% agarose gel and stained with ethidium bromide for UV visualization.

Western Blot Assay

Protein lysates were extracted with NucleoSpin kit according to the protocol and reagents supplied by the provider (Macherey-Nagel). Lysates from control or stimulated cells were collected and separated by SDS/PAGE on a 10% or 12% polyacrylamide gel. Proteins were subsequently transferred to a polyvinylidene difluoride (PVDF) transfer membrane (Millipore, Spain). Blots were incubated with the appropriate antibodies. Immunoblots were visualized with the Immobilon Western detection Kit (Millipore, Spain) using horseradish peroxidase labeled secondary antibodies (GE Healthcare). To confirm equal load in each sample, after stripping in Glycine buffer at pH 3, membranes were re-blotted with the anti-actin antibody. The images were captured and analyzed with a EC3 imaging system (UVP, Inc., CA).

MTT Assay

Cell metabolic activity was examined using a colorimetric assay based on the MTT labeling reagent. Cells were seeded in 96-well plates (8,000 cells/well). Assays were performed according to the instructions and protocol provided by the manufacturer (Sigma–Aldrich). Spectrophotometrical absorbance was measured using a microtiter ELISA reader at 550 nm (Multiskan EX, Termo Labsystem, Barcelona, Spain).

LDH Determination

Assays were performed according to the instructions and protocol provided by the manufacturer. Briefly, cells were plated at an initial density of 12×10^4 cells/well in 24-well plates. Cells were cultured in the above-mentioned medium and after 8 h of starvation, cells were treated for 24 h. Culture medium was recovered for measure lactate dehydrogenase (LDH) activity. LDH catalyzes the conversion of pyruvate to L-lactate while NADH (nicotinamide adenine dinucleotide reduced) is oxidized. The rate of oxidation, which is directly proportional to LDH activity, was monitored by measuring the decrease in absorbance at 340 nm on an ADVIA 1650 Chemistry System.

Hoechst Dye Vital Staining

Cells were seeded at an initial density of 10,000 cells/well in 24-well plates. After 8 h of starvation the cells were treated with AEA during 12 h and then incubated for 45 min at 37°C in Hoechst 33258 dye at a final concentration of 5 mM. Next HEPES (pH 7.8) was added to a final concentration of 20 mM, and the cells were fixed with 0.4% paraformaldehyde for 30 min and examined by fluorescence microscopy.

Data Analysis

Data are reported as mean ± SEM of at least three independent experiments, each with at least three or more independent observations. Statistical analysis was performed by analysis of variance followed by the Bonferroni multiple comparison test or *t*-test analysis using the Prism computerized package (GraphPad Software). $p < 0.05$ was considered significant.

RESULTS

Expression of Endocannabinoid System in Human and Murine Chondrocytes

As shown in Figure 1A, CB_1 receptor, which exhibits the highest affinity for AEA, was expressed in human

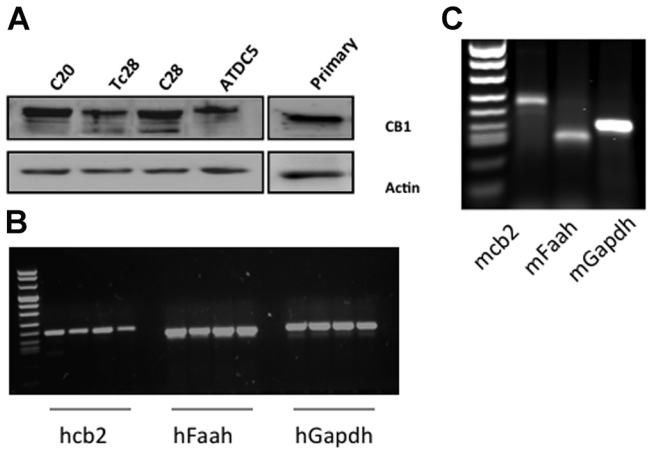

Figure 1. (A) Determination of CB_1 protein expression in C-20/A4, TC28, C28/I2, and ATDC5 cell lines and in cultured human primary chondrocytes. It was loaded 60 and 40 μg of total protein from chondrocytes cell lines and from human primary cultured chondrocytes respectively. (B) Determination of CB_2 mRNA and FAAH mRNA expression in human chondrocytes cultures: C-20/A4, TC28, C28/I2, and human primary cultured chondrocytes. Lanes: 1–4: CB_2. Lanes 6–9: FAAH. Lanes 11–14: GAPDH. (C) Determination of CB_2 mRNA and FAAH mRNA expression in ATDC5 cell line.

primary cultured chondrocytes as well as in all the human and murine chondrocyte cell lines tested so far (C-28/I2, T/C-28a2, C-20/A4, and ATDC5). Moreover, the mRNA of other members of the cannabinoid system, such as cannabinoid receptor CB_2 and fatty acid amide hydrolase (FAAH) were also expressed in these chondrocytes: Figure 1B (human primary chondrocytes and human cell lines), Figure 1C (murine cell line ATDC5).

Effect of AEA on Chondrocytes Vitality/Metabolic Activity

Upon AEA treatment, we observed early morphological alterations (data not shown). Considering that these changes in cellular shape are an early marker of vitality alteration, we decided to study by MTT assay the effect of AEA on human and murine chondrocytes (C28/I2, T/C-28a2, C-20/A4, and ATDC5) vitality. We determined that 24 h AEA treatment, at the dose of 5 and 15 μM, strongly decreased chondrocyte metabolic activity in all cell lines tested so far (Fig. 2A). To further confirm these data, we studied in C28/I2 chondrocytes the phosphorylation profile of the Adenosine 5′ monophosphate-activated protein kinase (AMPK), a regulator of cellular energy homeostasis usually activated upon mitochondrial failure.[23] As shown in Figure 2B, AEA stimulation elicited an early activation of AMPK that was sustained up to 120 min.

Study of the CBs Receptors Signaling Pathway

Once we determined the effect of AEA in chondrocytes vitality, we set up to study whether CBRs activation were involved in this process. To do this, we selected a suitable non-toxic concentration of CB_1 and CB_2 inhibitors, SR141716A (1 μM), and SR144528 (1 and 10 μM) respectively. Stimulation of C28/I2 chondrocytes during 24 h with AEA alone or in combination with SR141716A or SR144528 inhibitors revealed that neither CB_1 nor CB_2 inhibitors were able to revert the effect of AEA on chondrocytes vitality (Fig. 2C and D).

Despite CBRs are well conserved among species, in order to exclude a species-specific response of AEA on human C28/I2 chondrocytes, we also repeated these experiments in the murine cell line ATDC5. As shown in Figure 3A and B, similarly to human chondrocytes, CBR antagonists in ATDC-5 murine chondrocytes were unable to significantly block AEA effect. These results were further validated using PTX (100 ng/ml) pre-treatment, a classic inhibitor of $G_{i/o}$ proteins that are essential for CBRs signaling. Data obtained revealed that PTX treatment did not restore normal chondrocytes metabolic activity lost by AEA treatment (Fig. 3C), suggesting the presence of a non-conventional pathway for AEA effect.

Molecular Characterization of AEA Effect

Alteration of cellular metabolism is the previous step before the cell death. In this sense, data obtained pointed to a mitochondrial alteration in AEA treated cells, which is also a typical feature of several types of programmed cell death, such as apoptosis. Accordingly, we tried to elucidate which process was at play: apoptosis or cytotoxicity/necrosis. To test this, we determined after AEA stimulation the LDH levels in the culture medium of all the chondrocyte cell lines tested so far (ATDC5, C28/I2, T/C-28a2, and C-20/A4). Unlike apoptosis, necrosis, or cytotoxicity processes affect the cell membrane integrity enabling the release of LDH to the medium. Data obtained revealed that AEA did not induce LDH release from chondrocytes (Fig. 3D).

According to the LDH levels, AEA did not induce necrosis in these chondrocyte cell lines, thus we decided to figure out whether AEA induced apoptosis. Accordingly, we test a typical feature of apoptosis, such as chromatin condensation. As shown in Figure 4A, AEA was able to induce a strong staining of Hoechst vital dye in C28/I2 stimulated cells confirming the chromatin condensation and therefore the AEA mediated-apoptosis. To confirm these data, experiments were also carried on in all the other cell lines with similar results (Fig. 4A).

AEA-Induced Apoptosis

After determining that AEA was inducing apoptosis, irrespective of the chondrocyte cell line, we set out to obtain further insights into the molecular events associated to this process.

First, we studied the caspases pathway in human immortalized chondrocytes C28/I2. In agreement with data obtained previously, showing that AEA was able to induce a mitochondrial failure, Western-blot assays showed that AEA, in a dose dependent manner, was able to induce the cleavage of caspase-9 (that is classically involved in the mitochondrial apoptosis

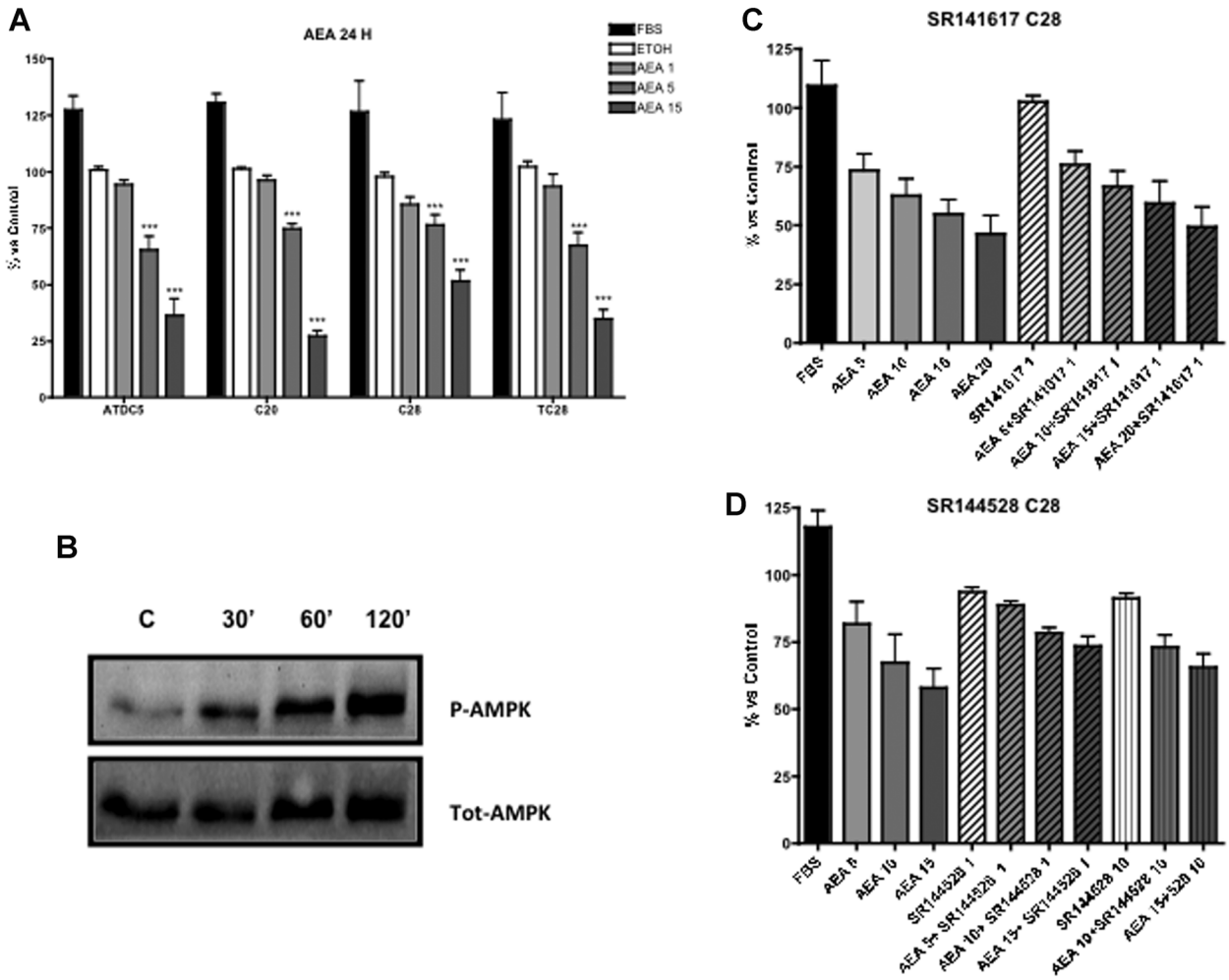

Figure 2. (A) MTT assays were performed in murine ATDC5 and in human immortalized C28/I2, T/C-28a2, and C-20/A4 cell lines. Results are expressed as percentage over control (***$p < 0.001$). Cells were treated with AEA (1, 5, and 15 μM) during 24 h. Experiments were performed in triplicate, with 16 independent observations for each treatment and experiment. (B) Western blots were performed with protein lysates coming from C28I2 cell line. Western blots are representative of at least three independent experiments. Cells were stimulated with AEA 15 μM during different times. (C and D) MTT assays were performed in human immortalized C28/I2. Results are expressed as percentage over control. Cells were treated with AEA (5, 10, 15, and 20 μM) and with SR141716A (1 μM) or SR144528 (1 and 10 μM) during 24 h. Experiments were performed in triplicate, with eight independent observations for each treatment and experiment.

pathway/intrinsic pathway) and caspase-3 (the main caspase that execute the cell death) (Fig. 4B and C). However, these experiments also revealed that AEA stimulation induced caspase-8 cleavage, which is involved in the death receptor apoptosis pathway/extrinsic pathway. Taken together, all these data suggested that AEA recruits both apoptotic pathways.

In order to obtain more information about the involvement of both apoptotic pathways in AEA-mediated apoptosis, we decided to study another early event in these processes, which is the cleavage of the focal adhesion kinase (FAK). This kinase exhibits a unique sensitivity to the activity of different caspases.[24] However, it also transduces to chondrocytes the surviving signals of the extracellular matrix.[25] As shown in Figure 4D, AEA treatment induced the cleavage of FAK (2 h) generating a 77 kDa fragment which is specific of caspase-6 activity.[24]

AEA Enhances TNF-α Mediated Caspase-3 Activation

After determine the involvement of both apoptotic pathways in AEA-mediated apoptosis, we set up to explore whether a low AEA concentration (2.5 μM), devoid of any effect on chondrocytes vitality (data not shown), was able to affect the caspase-3 activation mediated by a cell-death receptor, such as the TNF-α receptor (TNFR). Upon stimulation of C28/I2 cells with TNF-α and AEA we observed that, even at a low dose, AEA was able to synergize with TNF-α in the cleavage of caspase-3 (Fig. 5A).

AEA Induces c-Myc Expression

All the previous described facts including: the mitochondrial alteration, morphological changes, FAK cleavage, the recruitment of both apoptotic pathways and the sensitization to the apoptotic effect of a cell-death receptor, prompted us to study the expression

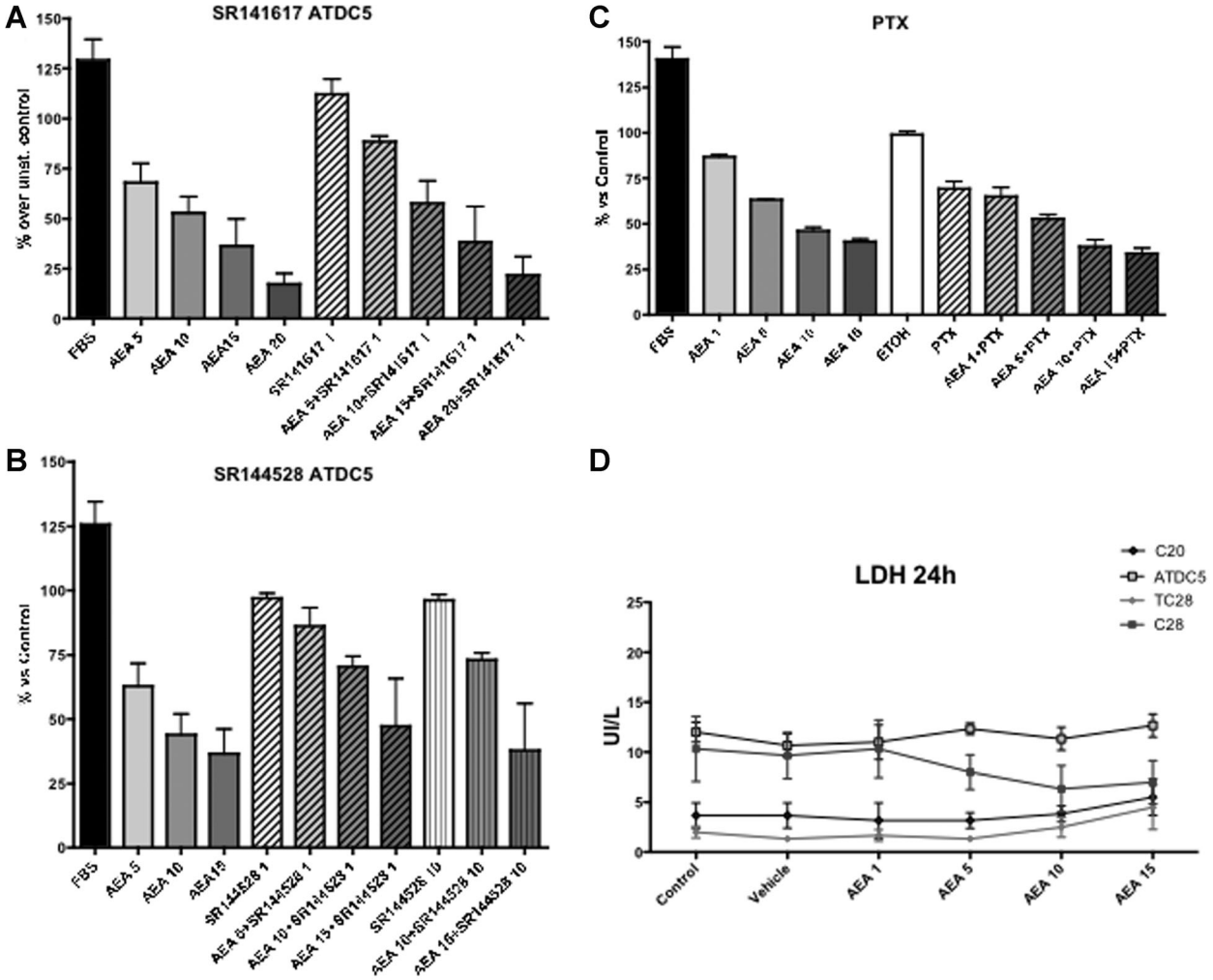

Figure 3. (A and B) MTT assays were performed in murine ATDC5 cells. Results are expressed as percentage over control. Cells were treated with AEA (5, 10, 15, and 20 μM) and with SR141716A (1 μM) or SR144528 (1 and 10 μM) during 24 h. Experiments were performed in triplicate, with eight independent observations for each treatment and experiment. (C) MTT assays were performed in murine ATDC5 cells. Results are expressed as percentage over control. Cells were treated with AEA (1, 5, 10, and 15 μM) during 24 h and pre-treated with PTX 16 h before AEA stimulation. Experiments were performed in triplicate, with eight independent observations for each treatment and experiment. (D) LDH determinations in cell supernatants were performed in ATDC5, C28/I2, T/C-28a2, and C-20/A4 cell lines. Experiments were performed in triplicate. Cells were treated during 24 h with AEA. Results are expressed in UI/L.

levels of *c*-MYC.[26,27] *c*-MYC is a classic transcription factor positively correlated to cellular proliferation. However, in certain circumstances, it may be also involved in apoptosis induction.[26] Hence, we determined the kinetic profile expression of c-MYC in AEA treated C28/I2 chondrocytes. As shown in Figure 5B, AEA (15 μM) was able to induce a significant increase of c-MYC expression in a time dependent fashion. Although, the precise mechanism of c-MYC induced-apoptosis is not well defined, it is known that certain pathways might underpin its apoptotic activities by inducing its expression or enhancing its stability.[28,29] At this point, we decided to study two major pathways ERK1/2 and AKT that modulate the activity of this transcription factor.[28] As shown in Figure 5C and D, AEA (15 μM) treatment induced a rapid and sustained phosphorylation of ERK1/2. In addition, AEA produced a rapid (after 5 min) and transient AKT phosphorylation followed by a sustained de-phosphorylation prolonged for all experimental time set (Fig. 5D). Moreover, according to the reported role of p-38 and JNK in the c-Myc mediated apoptosis,[30] we tested whether AEA was also able to activate both stress activated kinases. As shown in Figure 5E, Western-blot analyses revealed that AEA (15 μM) treatment increased, in a time dependent manner, the phosphorylation of p38 and JNK kinases.

DISCUSSION

In elderly population, OA is the most frequent chronic disease, which is characterized by progressive cartilage degradation, which induces pain, disability, and loss of joint architecture. This degenerative process has been associated to an alteration in the metabolic rate and surviving rate of articular chondrocytes. It has been determined the presence and activity of

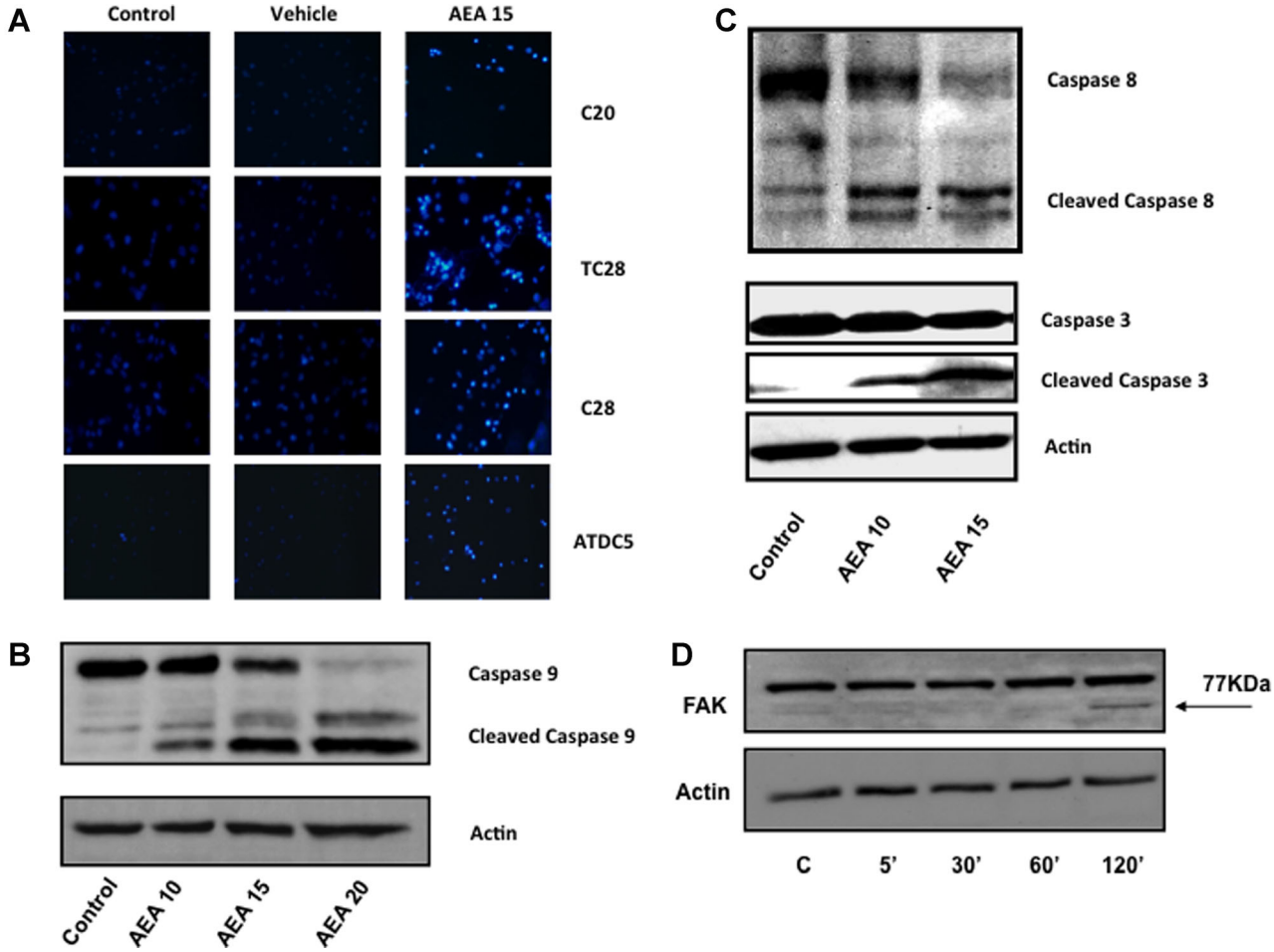

Figure 4. (A) Hoechst dye vital stainings were performed in ATDC5, C28/I2, T/C-28a2, and C-20/A4 cell lines. Cells were treated with AEA (15 μM) and vehicle during 12 h. Experiments were performed in triplicate. (B and C) Western blots were performed with protein lysates coming from C28I2 cell line. Western blots are representative of at least three independent experiments. Cells were stimulated with AEA at 10, 15, and 20 μM during 24 h. (D) Western blots were performed with protein lysates coming from C28I2 cell line. Western blots are representative of at least three independent experiments. Cells were stimulated with AEA 15 μM during different times.

different neurotransmitters in joint environment.[31,32] However, little is know about others neurotransmitters like endocannabinoids.

The cannabinoid system has been involved in innumerable physiological and pathophysiological processes. Many works in animal models have confirmed the immunosuppressive and anti-nociceptive activities of cannabinoids in chronic inflammatory diseases, such as rheumatoid arthritis.[16–18] However, these compounds also have a dark side since they are able to activate peripheral nociceptors in normal and arthritic rat joints.[33] In fact, the CB_1 inhibitor (SR141617A) is able to suppress the sensorial hypersensitivity associated to CFA (complete Freund's adjuvant induced arthritis).[34] In agreement with these data, it has been reported that AEA and 2-AG (2-arachidonyl glycerol) are elevated in the synovial fluid of osteoarthritis (OA) and rheumatoid arthritis (AR) patients.[20] Nonetheless, despite the source of these molecules are not clear, certain studies pointed to infiltrated macrophages or osteoblasts and osteoclasts as a potential source.[3,6] Among cannabinoids AEA has been associated to the control of cell proliferation and apoptosis in many different cell types. However, nothing is known about the effect of AEA on chondrocyte metabolism and vitality. Therefore, the main goal of this work was to determine the expression of the cannabinoid system in cultured chondrocytes, as well as, to characterize the effect of AEA on the proliferation and apoptotic rate of normal and/or apoptosis-stimulated chondrocytes.

We have determined that human primary cultured chondrocytes, as well as human and murine chondrocytes cell lines, express both cannabinoid receptors (CB1 y CB2) and the main enzyme involved in AEA metabolism (FAAH). These results showed that chondrocytes are equipped with the complete biochemical machinery necessary to process endocannabinoids signaling. Accordingly, we assayed the potential functional relevance of these findings by designing a set of experiments aimed to study the effect of AEA on chondrocyte vitality. Our results revealed that AEA inhibited chondrocytes vitality and their mitochondrial

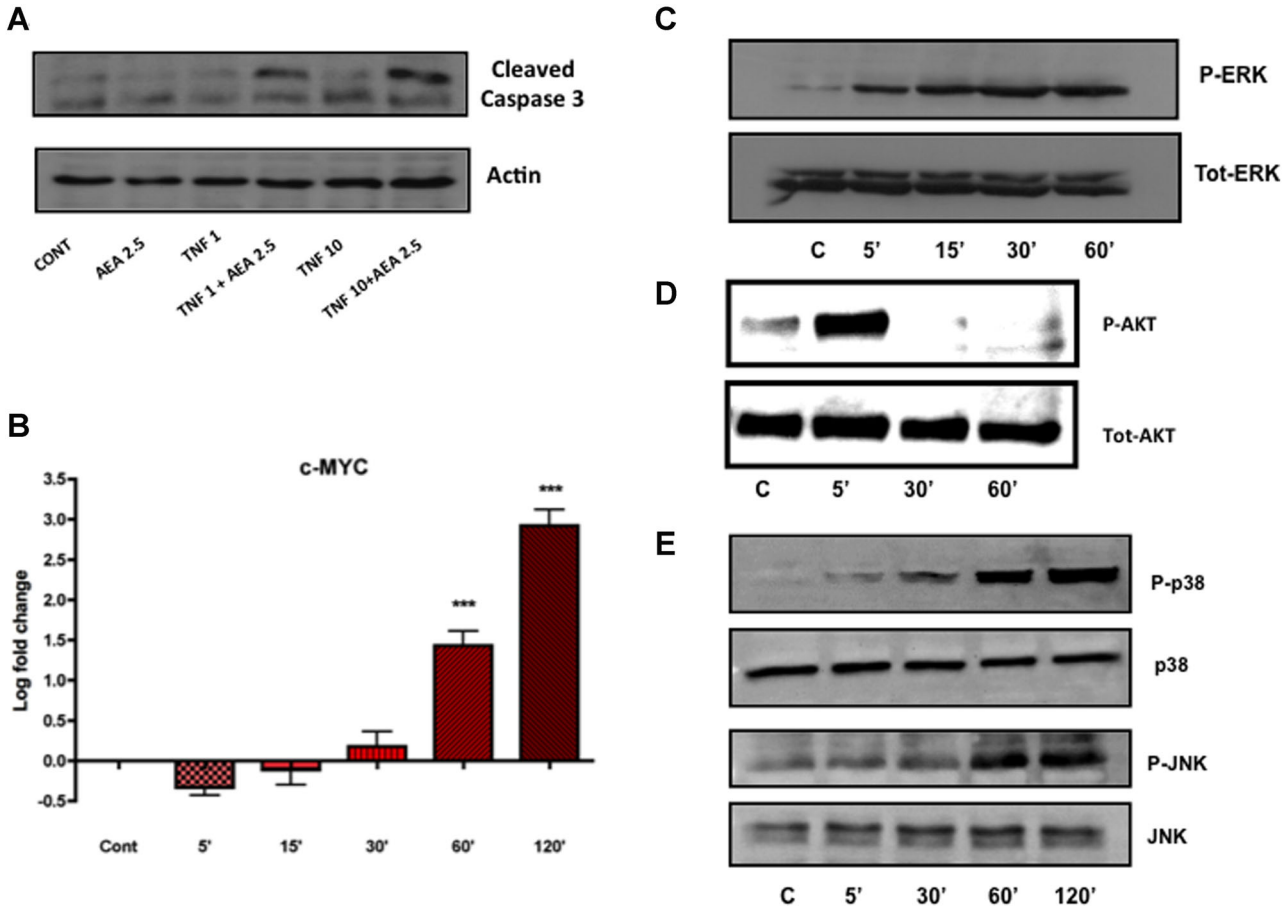

Figure 5. (A) Western blots were performed with protein lysates coming from C28I2 cell line. Western blots were representative of at least three independent experiments. Cells were stimulated with AEA at 2.5 μM and TNF (1, 10 μM) during 24 h. (B) Relative quantification of c-MYC mRNA expression. C28I2 cell line was stimulated with AEA 15 μM during different times. Real-time PCR results were expressed as $\log_2$ fold versus control. Experiments were performed at least in triplicate ($^{***}p < 0.001$). (C and E) Western blots were performed with protein lysates coming from C28I2 cell line. Western blots are representative of at least three independent experiments. Cells were stimulated with AEA 15 μM during different times.

activity, which is in agreement with the already reported activities of cannabinoids in other cell types.[35–39] Surprisingly, although these activities suggested that cannabinoid system was at play in the effect of AEA, our experimental set using CBR inhibitors showed that the effect of AEA was not mediated by binding to its cognate receptors. Likewise, the effect of AEA was also independent of its potential binding to GPR55 receptor, since this receptor is also sensible to the inhibition by SR141617A.[40] In agreement with this, we could also verify that different CBRs expression across the studied chondrocytes cell lines did not involve different responses upon AEA stimulation. Altogether, these data supported the fact that cannabinoid receptors were not involved in AEA effect, pointing to a non-conventional mechanism for AEA action.

Both vitality assays and morphological changes appreciated in AEA treated cells suggested that AEA exerted a lethal effect on chondrocytes. However, to objectively characterize this effect we performed a set of experiments to study the cellular membrane integrity, nuclear chromatin status, and caspases activation in AEA treated cells. Data obtained revealed that plasmatic membrane of AEA treated cells remains unaltered, suggesting that AEA was inducing a programmed cell death instead of a cytotoxic/necrotic process. Confirming this, it was observed that, after AEA stimulation, chondrocytes' chromatin became condensed and fragmented. Moreover, caspases activation after AEA treatment underpinned with solid proofs the apoptotic process. Overall, these data provided clear evidence that AEA is able to induce apoptosis in chondrocytes, which suggests for the first time that AEA could be a harmful agent for chondrocytes.

Considering that previous experiments suggested that AEA effect involved a mitochondrial-mediated apoptosis we set up to uncover how AEA could recruit the apoptotic extrinsic pathway. To achieve this goal we studied FAK integrity, a kinase with unique sensitivity to the activity of different caspases that is also upstream of chondrocytes key surviving signals.[24,25,41] Study of FAK integrity after AEA stimulation revealed that AEA induced a rapid

caspase-6-mediated proteolysis. Therefore, since caspase-6 is the only caspase from the mitochondrial pathway able to activate caspase-8,[42] these results put forward a mechanism whereby AEA may recruit the extrinsic apoptosis pathway. First, by direct activation of the caspase-8 by caspase-6 and second by avoiding the negative control that FAK exerts over the extrinsic apoptotic pathway.[43]

In RA excessive production of TNF-α can drive synovial inflammation and cartilage degradation.[44] However, OA has also an inflammatory component. In fact, TNF-α levels have been associated with OA knee cartilage loss.[45] Therefore, taken into account that TNFR is as cell death receptor, upstream of the extrinsic apoptosis pathway, and that we observed the recruitment of both apoptotic pathways in AEA-mediated apoptosis, we decide to test whether AEA could prime chondrocytes to the apoptotic effect of a cell-death receptor such as the TNFR. According to previous reports,[46] the apoptotic effect of TNF-α alone in chondrocytes was limited. However, a low dose of AEA, without any activity on cell vitality, amplified TNF-α-induced caspase-3 cleavage. Accordingly, this fact suggests that AEA could work as a susceptibility factor in death receptors mediated apoptosis.

c-MYC is a classic transcription factor positively correlated to cellular proliferation.[26,47]. In agreement with this, the fact that AEA stimulation induced cell growth inhibition and at the same time a strong and sustained increase of c-MYC suggested that this transcription factor might be involved in AEA-mediated apoptosis. Supporting this idea, it has been reported the involvement of c-MYC in the recruitment of both apoptotic pathways (intrinsic and extrinsic) as well as in the sensitization to the apoptotic effect of TNF-α.[27] In addition, FAK cleavage is considered a typical sign of c-MYC induced apoptosis.[48]

In line with the potential involvement of c-MYC in the AEA-mediated apoptotic effect, we also found that AEA induced a strong and sustained ERK½ activation. Unlike normal ERK½ activation (transient) that is associated to cell proliferation, prolonged activation of ERK½ has been associated to an aberrant increase and stabilization of c-MYC,[28,49,50] which has been related to cell proliferation inhibition and apoptosis.[37,49,51] This activity has already been reported for cannabinoids in prostate cells, where ERK sustained phosphorylation lead to an increase in the apoptotic rate.[37]

Loss of surviving signals has a key role in programmed cell death processes. In fact, inhibition of surviving signals has been associated to an enhanced c-MYC mediated apoptosis.[26,47,52,53] In agreement to this, in AEA stimulated cells we observed a strong and sustained AKT inhibition, which has been considered a death and anti-proliferation signal.[54] Actually, it is also known that AKT activation is coupled to caspase-9 inhibition. Therefore, observed AKT inhibition was consistent with the detected high caspase-9 activity.[55] Accordingly, this sustained AKT inactivation together with FAK proteolysis, which transduces the surviving signals from chondrocytes' extracellular matrix,[25] put forward a cellular environment optimum for the c-MYC apoptotic activities. This pro-apoptotic scenario, induced by the suppression of the surviving signals, was further increased by AEA's ability to activate the stress-activated kinases (p38 and JNK) since these kinases have been associated to c-MYC activation, stabilization, and expression.[29,56,57] To note, the activation of both kinases has already been associated to cannabinoids actions.[11,58–60]

In agreement to the pro-apoptotic role of c-MYC suggested here, it is noteworthy that in OA cartilage lesions exists an association between chondrocytes apoptosis and high levels of c-MYC.[61] In OA chondrocytes, apoptosis induced by an increased hydrostatic pressure has been related to an increased expression of c-MYC and TNF-α.[62] Moreover, it is known that constitutive overexpression of this transcription factor inhibits their differentiation.[63]

In conclusion, our data describe a different facet of AEA that goes beyond the previously described in pain modulation of articular degenerative diseases.

Our results, together with those obtained by other authors in synovial tissues,[20] suggest that AEA and the endocannabinoid system might be of potential therapeutic interest to target joint degenerative inflammatory diseases such as RA and OA.

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