The pseudokinase tribbles homologue-3 plays a crucial role in cannabinoid anticancer action

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ABSTRACT

Δ9-Tetrahydrocannabinol (THC), the major active ingredient of marijuana, and other cannabinoids inhibit tumor growth in animal models of cancer. This effect relies, at least in part, on the up-regulation of several endoplasmic reticulum stress-related proteins including the pseudokinase tribbles homologue-3 (TRIB3), which leads in turn to the inhibition of the AKT/mTORC1 axis and the subsequent stimulation of autophagy-mediated apoptosis in tumor cells. Here, we took advantage of the use of cells derived from Trib3-deficient mice to investigate the precise mechanisms by which TRIB3 regulates the anti-cancer action of THC. Our data show that RasV12/EIA-transformed embryonic fibroblasts derived from Trib3-deficient mice are resistant to THC-induced cell death. We also show that genetic inactivation of this protein abolishes the ability of THC to inhibit the phosphorylation of AKT and several of its downstream targets, including those involved in the regulation of the AKT/mammalian target of rapamycin complex 1 (mTORC1) axis. Our data support the idea that THC-induced TRIB3 up-regulation inhibits AKT phosphorylation by regulating the accessibility of AKT to its upstream activatory kinase (the mammalian target of rapamycin complex 2; mTORC2). Finally, we found that tumors generated by inoculation of Trib3-deficient cells in nude mice are resistant to THC anticancer action. Altogether, the observations presented here strongly support that TRIB3 plays a crucial role on THC anti-neoplastic activity. This article is part of a Special Issue entitled Dysregulated Lipid Metabolism in Cancer.

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1. Introduction

Today it is widely accepted that, out of the ~108 cannabinoids produced by Cannabis sativa, Δ9-tetrahydrocannabinol (THC) is the most relevant owing to its high potency and abundance in plant preparations [1,2]. THC exerts a wide variety of biological effects by mimicking endogenous substances – the endocannabinoids anandamide [3] and 2-arachidonoylglycerol (2-AC) [4,5] – that engage specific cell-surface cannabinoid receptors [6]. So far, two major cannabinoid-specific receptors – CB1 and CB2 – have been cloned and characterized from mammalian tissues [7,8]. In addition, other receptors such as the transient receptor potential cation channel subfamily V member 1 (TRPV1) and the orphan G protein-coupled receptor GPR55 have been proposed to act as endocannabinoid receptors [6]. Most of the effects produced by cannabinoids in the nervous system and in non-neural tissues rely on CB1 receptor activation. In contrast, the CB2 receptor was initially described to be present in the immune system [6], but more recently it has also been shown to be expressed in cells from other origins [9,10]. Of note, expression of CB1 and CB2 receptors has been found in many types of cancer cells, which does not necessarily correlate with the expression of these receptors in the tissue type of origin [9,11,12].

One of the most active areas of research in the cannabinoid field is the study of the potential application of cannabinoids as anti-tumoral agents [13,14]. Thus, cannabinoid administration has been shown to curb the growth of several models of tumor xenografts in rats and mice [13]. Based on this preclinical evidence, a pilot clinical study has been conducted to investigate the effect of THC on recurrent glioblastoma multiforme [11]. The mechanism of cannabinoid anti-tumoral action relies on the ability of these agents to inhibit tumor angiogenesis, inhibit cell cycle progression and stimulate cancer cell death [13]. We have...
recently found that cannabinoids activate an endoplasmic reticulum (ER) stress-related signaling route that leads to the up-regulation of the transcriptional co-activator nuclear protein 1 (Nupr1, also named p8) and its target the pseudo-kinase tribbles homolog 3 (TRIB3) [15,16]. The stimulation of this pathway promotes autophagia via TRIB3-mediated inhibition of the AKT/mammalian target of rapamycin complex 1 (mTORC1) axis and is indispensable for the pro-apoptotic and anti-tumoral action of THC [17]. However, the precise mechanisms by which TRIB3 inhibits AKT phosphorylation are only partially understood [18–20]. In this work we used RasV12/E1A-transformed embryonic fibroblasts derived from Trib3-deficient mice to investigate the role of this pseudokinase in the mechanism of THC-induced cancer cell death.

2. Materials and methods

2.1. Reagents

Δ9-tetrahydrocannabinol was obtained from THC-Pharm, (Frankfurt, Germany).

2.2. Cell culture

Mouse embryonic fibroblasts (MEFs) were extracted from Trib3+/+ and Trib3−/− mice and transformed as described below using a pBABE retroviral vector encoding the oncogenes RasV12 and E1A. Cells were cultured in DMEM containing 10% w/v FBS and penicillin/streptomycin (5 μg/ml), seeded at a density of 10,000 cells/cm² and transferred to medium containing 2% w/v FBS 18 h before performing the different treatments. RasV12/E1A-transformed Trib3+/+ and Trib3−/− MEFs correspond to a polyclonal mix of at least 20 different selected clones.

2.3. Generation of RasV12/E1A-transformed MEFs

2.3.1. Extraction of MEFs

Mouse embryonic fibroblasts (MEFs) were extracted from Trib3+/+ and Trib3−/− mice. Briefly, Trib3+/+ mice [B6;129S5-Erd215Flk/Lhr, Trib3+/+;Jax Model No:002869] were bred to Trib3−/− mice [B6;129S5-Trib3flOst324148Lek/Ieg, Trib3−/−;Jax Model No.002869]. The remaining tissue was minced, incubated with trypsin (1 ml; 0.2 mg/ml) at 37 °C for 5 min, pipetted up and down to eliminate all debris, and the embryos were dissected discarding head, limbs, and liver. The embryos were extracted at a total of 13–14.5 days post-coitus, the medium was removed, a 0.25% solution of collagenase was added to a concentration of 1% (v/v) and samples were subjected previously covalently-coupled to protein G-Sepharose using dimethyl pimelimidate (Sigma). Immunoprecipitations were carried out overnight on a rotating wheel. The immunoprecipitates were washed 4 times with lysis buffer, twice with Hepes buffer (25 mM Hepes pH 7.5 and 50 mM KCl), resuspended in 30 μl of sample buffer (without 2-mercaptoethanol) and filtered through a 0.22-μm pore Spin-X filter (Corning, Tokyo, Japan). Finally, 2-mercaptoethanol was added to a concentration of 1% (v/v) and samples were subjected to electrophoresis and immunoblot analysis following standard procedures. The following antibodies were used in Western blot analyses: anti-phospho-S6 ribosomal protein Ser235/236, anti-phospho-Akt Ser473, anti-phospho-p70 S6 kinase Thr389, anti-phospho-PRAS40 Thr246 (Cell Signaling Technology, Inc., Lake Placid, NY); anti-phospho-PRAS40 Thr246 antibodies (1:1000; kindly donated by Dr. Dario Alessi); anti-LC3 antibody (1:1000) and anti-alpha tubulin antibody (1:4000; Sigma).

2.3.2. Transduction of MEFs

MEFs were transduced with supernatants enriched in retroviral particles, obtained from Phoenix Ecotropic cells (packaging cells). Phoenix Ecotropic cells were plated in 10 cm diameter dishes with fresh medium (DMEM with 10% FBS). When cells had reached an 80 to 85% confluence the medium was removed, a retroviral-enriched supernatant was added and cells were incubated at 37 °C/5% CO2. Individual clones were evident after several weeks of incubation in the presence of medium containing gradually-increasing concentrations (1 to 10 μg/ml) of puromycin. Individual clones were collected and amplified and the genotype was verified.

2.4. Cell viability assays

Cell viability was determined by the MTT [(3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a yellow tetrazole)] test (Sigma Aldrich, St Louis, MO) following the manufacturer’s instructions.

2.5. Western blot and immunoprecipitation

Cells were lysed in a buffer containing 50 mM Tris HCl, pH 7.5, 1 mM phenylmethylsulfonyl fluoride, 50 mM NaF, 5 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 0.1% Triton X-100, 1 mg/ml leupeptin, 1 mM EDTA, 1 mM EGTA and 10 mM sodium β-glycerophosphate. For immunoprecipitation experiments, cells were lysed in a buffer containing 40 mM Hepes pH 7.5, 120 mM NaCl, 1 mM EDTA, 10 mM sodium pyrophosphate, 10 mM sodium glycero phosphosphate, 50 mM sodium fluoride, 0.5 mM sodium orthovanadate and 0.3% CHAPS. Briefly, lysates (1–4 mg protein) were pre-cleared by incubating with 5–20 μl of protein G-Sepharose coupled to pre-immune IgG. Lysate extracts were then incubated with 5–20 μl of protein G-Sepharose conjugated to 5–20 μg of the corresponding primary antibodies [anti-TRB3-aminoterminal end (ab50516; Abcam; Cambridge, UK); anti-SNI (kindly donated by Dr. Dario Alessi); Anti-total Akt (kindly donated by Dr. Dario Alessi)] or pre-immune IgG antibodies that had been previously covalently-coupled to protein G-Sepharose using dimethyl pimelimidate (Sigma). Immunoprecipitations were carried out overnight on a rotating wheel. The immunoprecipitates were washed 4 times with lysis buffer, twice with Hepes buffer (25 mM Hepes pH 7.5 and 50 mM KCl), resuspended in 30 μl of sample buffer (without 2-mercaptoethanol) and filtered through a 0.22-μm pore Spin-X filter (Corning, Tokyo, Japan). Finally, 2-mercaptoethanol was added to a concentration of 1% (v/v) and samples were subjected to electrophoresis and immunoblot analysis following standard procedures. The following antibodies were used in Western blot analyses: anti-phospho-S6 ribosomal protein Ser235/236, anti-phospho-Akt Ser473, anti-phospho-p70 S6 kinase Thr389, anti-phospho-TSC2 Thr1462; anti-phospho BAD Ser136 and anti-phospho GSK3β (Cell Signaling Technology, Inc., Lake Placid, NY); anti-phospho-PRAS40 Thr246 antibodies (1:1000; kindly donated by Dr. Dario Alessi); anti-LC3 antibody (1:1000) and anti-alpha tubulin antibody (1:4000; Sigma).

![Fig. 1. TRIB3 is required for THC-induced cell death. Effect of THC (24 h) on the viability of RasV12/E1A-transformed Trib3+/+ and Trib3−/− MEFs (n = 6). **P < 0.01 from THC-treated Trib3−/− RasV12/E1A MEFs. S.D. are omitted for clarity.](http://dx.doi.org/10.1016/j.bbalip.2013.03.014)
2.6. In vitro mTORC2 kinase assays

Cells were lysed in fresh Hepes lysis buffer. Lysates (1–4 mg protein) were pre-cleared by incubating with 5–20 μl of Protein G-Sepharose conjugated to pre-immune IgG and subsequently incubated with 5–20 μl of Protein G-Sepharose conjugated to 5–20 μg of anti-SIN1 (kindly donated by Dr Dario Alessi, Dundee University, UK) or pre-immune IgGs. Immunoprecipitations were carried out as described above. GST–AKT1 was isolated from serum-deprived HEK-293 cells incubated with PI-103 (1 μM for 1 h). mTOR reactions were initiated by adding 0.1 mM ATP and 10 mM MgCl2 in the presence of GST–AKT1 (0.5 μg). Reactions were carried out for 30 min at 30 °C on a vibrating platform and stopped by addition of SDS sample buffer. Reaction mixtures were then filtered through a 0.22-μm-pore Spin-X filter and samples were subjected to electrophoresis and immunoblot analysis.

2.7. Confocal microscopy and immunofluorescence of tumor samples

Samples from tumor xenografts were dissected, embedded in Tissue-Tek (Sakura; South Carolina, US) and, before the staining procedures were performed, fixed in acetone for 10 min at room temperature and frozen. After fixation, sections (5 μm) were permeabilized and blocked to avoid non-specific binding with 10% v/v goat antiserum and 0.25% v/v Triton X-100 in PBS for 45 min and subsequently incubated with the primary antibodies [anti-phospho-Akt (Ser 473; Cell Signaling Technology); anti-LC3 (1:100; Nanotools Antikörpertechnik GmbH & Co Antikörpertechnik GmbH & Co, Teningen, Germany; clone 5 F10) and polyclonal anti-cleaved caspase-3 Asp175 (1:100; Cell Signaling Technology)]. Then, samples were washed, incubated with the corresponding Alexa-488 or Alexa-594-conjugated secondary antibodies (Invitrogen; 90 min, room temperature) and nuclei were stained with Hoechst 33342 (Invitrogen; 10 min, room temperature) before mounting with Mowiol mounting medium (Merck, Darmstadt, Germany). Fluorescence images were acquired using Metamorph-Offline 6.2 software (Universal Imaging) and Zeiss Axioplan 2 Microscope.

2.8. In vivo generation of tumor xenografts

Tumors were induced by subcutaneous injection in nude mice of $10 \times 10^6$ RasV12/E1A-transformed Trib3+/+ or Trib3−/− MEFs cells
in PBS supplemented with 0.1% w/v glucose. When tumors had reached an average size of 250 mm³, animals were assigned randomly to an experimental group and injected peritumorally for 15 days with the corresponding treatment—THC (15 mg/kg day) or vehicle (100 μl of PBS supplemented with 5 mg/ml defatted and dialyzed BSA). Tumors were measured with external caliper, and volume was calculated as (4π/3) × (width/2)² × (length/2). At the end of the treatment, animals were killed, tumors excised and their weights determined. All procedures involving animals were performed with the approval of the Complutense University Animal Experimentation Committee according to Spanish and European official regulations.

2.9. Statistics

Unless otherwise specified, results shown represent mean ± S.D. Statistical analysis was performed by ANOVA with a post hoc analysis by the Student–Neuman–Keuls test.

3. Results

3.1. TRIB3-deficient cells are resistant to THC-induced autophagy-mediated cell death

To investigate the precise mechanism by which TRIB3 mediates cannabinoid-induced cancer cell death, we analyzed the response to THC treatment of cancer cells in which this pseudokinase had been genetically inactivated. To do this, we transformed embryonic fibroblasts derived from WT and Trib3-deficient mice with the oncogenes RasV12 and E1A. Characterization of these cells revealed that THC treatment reduces the viability of RasV12/E1A-transformed WT MEFs but does not affect that of their TRIB3-deficient counterparts (Fig. 1), thus strongly supporting that TRIB3 plays a crucial role in the mechanism of THC-induced cancer cell death.

We had previously found that THC promotes autophagy-mediated cell death via inhibition of the AKT/mTORC1 axis [17]. Therefore, we made use of the TRIB3-deficient RasV12/E1A-transformed MEFs to investigate the precise mechanisms by which this pseudokinase regulates these events.

As shown in Fig. 2, genetic inactivation of Trib3 abolished the ability of THC to reduce the phosphorylation of AKT as well as of several of its downstream targets including those (namely TSC-2 and PRAS40) directly involved in the regulation of mTORC1 activation [21]. Likewise, THC reduced the phosphorylation of the mTORC1 downstream target p70S6 kinase and of the ribosomal protein S6 – a well-established readout of the activity of the mTORC1 complex – in WT but not in TRIB3-deficient RasV12/E1A-transformed MEFs. Moreover, loss of Trib3 abrogated the ability of THC to induce autophagy (as evidenced by the increased levels of LC3-II — the lipidated and autophagosome-associated form of LC3 [21]). Taken together these observations suggest that RasV12/E1A-transformed Trib3-deficient MEFs are resistant to THC-induced inhibition of the AKT/mTORC1 axis and the subsequent stimulation of autophagy-mediated cell death.

3.2. THC regulates AKT via a TRIB3-dependent inhibition of its interaction with the mTORC2 complex

In order to be fully activated, AKT needs to be phosphorylated on Thr 308 and Ser473 [22,23]. Intriguingly, we found that treatment with THC decreases AKT phosphorylation on Ser 473 rather than on Thr 308 (Fig. 2 and data not shown). We therefore asked whether THC regulates the activity of the mTORC2 complex in a TRIB3-dependent manner, since this protein complex under many cellular settings is responsible for the phosphorylation of AKT on Ser473 [21]. Immunoprecipitation of SIN-1 – one of the protein components of the mTORC2 complex [21] – and further analysis of mTORC2 in vitro kinase activity showed that THC does not modify the phosphorylation of AKT on Ser 473 in either TRIB3-deficient or WT cells (Fig. 3A) indicating that the kinase activity of the complex is not affected upon treatment with THC either in the absence or in the presence of TRIB3. Next, we analyzed whether THC treatment affects the interaction between AKT and the mTORC2 complex and whether this event is regulated by TRIB3. As shown in Fig. 3B, treatment of RasV12/E1A-transformed WT cells with THC increased TRIB3 protein levels, enhanced the interaction of TRIB3 with AKT and decreased the interaction of AKT with SIN1. By contrast, treatment with THC did not modify the interaction of AKT with SIN1 in TRIB3-deficient cells (Fig. 3B). Taken together, these observations suggest that TRIB3 plays a role in the regulation of the accessibility of AKT to the mTORC2 complex.

3.3. TRIB3-deficient tumors are resistant to THC anticancer action

To investigate the in vivo relevance of our findings, we generated tumor xenografts by subcutaneous injection of RasV12/E1A-transformed WT and TRIB3-deficient MEFs in the flank of immune-deficient mice. As shown in Fig. 4, treatment with THC reduced the growth of tumors generated with WT but not with TRIB3-deficient cells, thus strongly supporting that TRIB3 plays a crucial role in THC anticancer action. Analysis of samples obtained from those tumors revealed that treatment with THC reduced the phosphorylation of AKT and enhanced autophagy and apoptosis (as determined by LC3 and active-caspase 3 immunostaining, respectively) in the tumors derived from WT cells (Fig. 5). By contrast AKT, autophagy and apoptosis were not modified in TRIB3-deficient tumors upon treatment with THC (Fig. 5). Taken together, these findings indicate that TRIB3 is required for THC-triggered...
inhibition of AKT and the subsequent stimulation of the autophagy-mediated cell death pathway in vivo.

4. Discussion

In this work we took advantage of the genetic inactivation of Trib3 to investigate the participation of this pseudokinase in the mechanism of THC-induced cancer cell death. Several observations presented here strongly support that TRIB3 plays an essential role in the anticancer action of this agent: (i) genetic inhibition of Trib3 renders transformed-embryonic fibroblasts resistant to THC-induced cell death; (ii) tumors generated with RasV12/E1A-transformed Trib3-deficient MEFs are resistant to THC anticancer action; and (iii) THC does not trigger the inhibition of the AKT/mTORC1 axis nor produces the subsequent stimulation of autophagy-mediated cell death in Trib3-deficient cells or tumors.

Previous observations by our laboratory had shown that the mechanisms of THC-induced cancer cell death relies on the up-regulation of the stress-regulated protein NUPR1 (also named p8) and its downstream targets, the endoplasmic reticulum (ER) stress-related transcription factors ATF4 and CHOP, which lead in turn to the induction of Trib3 and the subsequent inhibition of the AKT/mTORC1 axis [13,15–17,24]. This cascade of events promotes the stimulation of autophagy, which is required for the induction of the apoptotic death of cancer cells that triggers THC [13,17]. Data presented in this report now indicate that the THC-induced Trib3-dependent inhibition of AKT relies on the regulation of mTORC2 complex. mTOR was initially shown to be associated with RAPTOR, PRAS40 and some other protein components to form the so-called mTORC1 complex — which, as mentioned above, plays a crucial role in the regulation of different cellular processes including protein translation and autophagy [21]. In addition, mTOR is also associated with a different set of proteins including RICTOR and SIN-1 to form the mTORC2 complex, which is responsible for the phosphorylation of AKT on Ser473, a crucial event for the full activation of this kinase [21,23]. Our data show that THC inhibits the interaction of mTORC2 and AKT in a Trib3-dependent manner. Moreover, the ability of mTORC2 to phosphorylate a recombinant form of AKT in vitro is not affected when the complex is immunoprecipitated from Trib3-deficient cells, suggesting that Trib3 does not directly regulate mTORC2 kinase activity. Although additional research is still necessary to fully elucidate the mechanism by which Trib3 and AKT interact with each other and with mTORC2, these observations suggest that the THC-triggered enhanced interaction of Trib3 and AKT inhibits the phosphorylation of AKT on Ser473 by regulating the accessibility of AKT to mTORC2.

Findings presented in this manuscript strongly support that TRIB3 is required for the cancer cell killing action of THC in vitro. Moreover, our observations indicate that this protein plays a crucial role in the stimulation of the autophagy-mediated cell death pathway by cannabinoids also in vivo. A potential consequence of these observations is that the loss of TRIB3 (or a decreased expression of this pseudokinase) could be a potential factor for resistance of tumor cells to the anticancer action of cannabinoids.

5. Conclusions

In summary, our findings support that TRIB3 plays a crucial role in the anti-cancer action of THC and suggest that this effect is based on the ability of this pseudokinase to interact with AKT and inhibit its full activation by mTORC2. These observations may contribute to set the basis for the design of novel anticancer strategies based on the up-regulation of TRIB3 and the resulting inhibition of the Akt/mTORC1 axis.

Acknowledgements

This work was supported by grants from the Spanish Ministry of Economy and Competitiveness (MINECO) (PS09/01401; PI12/02248, FR2009-0052 and IT2009-0053) to GV; Comunidad de Madrid (S2011/BMD-2308 to MG), GW Pharmaceuticals (to GV and MG) and Fundación Mutua Madrileña (AP101042012 to GV). Purchase of the Trib3-deficient mice (LEKKO-1947) line was funded by the Wellcome Trust. MS was recipient of a fellowship from the Spanish Ministry of Education and Science (MEC) and of a research formation contract from Comunidad de Madrid; ML was sequentially the recipient of a ‘Juan de la Cierva’ contract, a postdoctoral contract from the Spanish Ministry of Education and Science (MEC) and a postdoctoral contract from Comunidad de Madrid. We would like to thank Dr Dario Alesi (Dundee University, UK) for expert scientific and technical support and for kindly providing antibodies for the immunoprecipitation...
experiments. We also would like to thank other members of our laboratories for their continuous support.

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