Δ⁹-Tetrahydrocannabinol disrupts mitochondrial function and cell energetics

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Sarafian, Theodore A., Shaghig Kouyoumjian, Farnaz Khoshaghidgeh, Donald P. Tashkin, and Michael D. Roth. Δ⁹-Tetrahydrocannabinol disrupts mitochondrial function and cell energetics. Am J Physiol Lung Cell Mol Physiol 284: L298–L306, 2003.—We have observed rapid and extensive depletion of cellular energy stores by Δ⁹-tetrahydrocannabinol (THC) in the pulmonary transformed cell line A549. ATP levels declined dose dependently with an IC₅₀ of 7.5 μg/ml of THC after 24-h exposure. Cell death was observed only at concentrations >10 μg/ml. Studies using JC-1, a fluorescent probe for mitochondrial membrane potential, revealed diminished mitochondrial function at THC concentrations as low as 0.5 μg/ml. At concentrations of 2.5 or 10 μg/ml of THC, a decrease in mitochondrial membrane potential was observed as early as 1 h after THC exposure. Mitochondrial function remained diminished for at least 30 h after THC exposure. Flow cytometry studies on cells exposed to particulate smoke extracts indicate that JC-1 red fluorescence was fivefold lower in cells exposed to marijuana smoke extract relative to cells exposed to tobacco smoke extract. Comparison with a variety of mitochondrial inhibitors demonstrates that THC produced effects similar to that of carbonyl cyanide p-trifluoromethoxyphenylhydrazone, suggesting uncoupling of electron transport. Loss of red JC-1 fluorescence by THC was suppressed by cyclosporin A, suggesting mediation by the mitochondrial permeability transition pore. This disruption of mitochondrial function was sustained for at least 24 h after removal of THC by extensive washing. These results suggest that exposure of the bronchopulmonary epithelium to THC may have important health and physiological consequences.

RECREATIONAL AND MEDICINAL USE of marijuana remains prevalent despite numerous reports of adverse and toxic consequences (1). The epithelial lining of the lung acts as the primary site exposed to the inhaled components of marijuana smoke, including the high concentrations of cannabinoids associated with the particulate tar fraction. Histopathological alterations in the lung resulting from marijuana smoking include proximal airway inflammation, reserve and goblet cell hyperplasia, squamous cell metaplasia, loss of epithelial microvilli, and cellular atypia (13, 35). These changes have been observed by bronchoscopic visualization and by microscopic inspection of human airway mucosal biopsy specimens. The mechanisms underlying these effects, however, are poorly understood and have not been investigated extensively.

We previously reported that exposure to marijuana smoke in vitro results in significant oxidative stress and suppression of fas-induced apoptosis (42, 43). We have also observed that agents that compromise cell energetics, including antioxidants, enhance the cytotoxic effects produced by Δ⁹-tetrahydrocannabinol (THC) (41). There have been several reports suggesting that THC alters mitochondrial function in brain and muscle tissue (4, 7, 8, 10). Effects of THC on glucose metabolism have also been described (38, 39) that may be a consequence of primary mitochondrial effects. Because direct effects of THC on pulmonary cell energetics have not been reported, we used the lung epithelial tumor cell line A549 to study in vitro responses to THC exposure. We observed numerous effects suggesting mitochondrial injury, which may help explain previously observed cytopathology.

MATERIALS AND METHODS

Materials. Carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP), cyclosporin A, propidium iodide, digitonin, and oligomycin were purchased from Sigma (St. Louis, MO). Reagents for ATP measurements include firefly lantern extract (FLE-50) and disodium ATP (Sigma). 5,5',6,6'-Tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) was purchased from Molecular Probes (Eugene, OR). FCS, BSA, PBS, RPMI 1640 medium, and trypsin-EDTA solution were purchased from Irvine Scientific (Irvine, CA). Marijuana cigarettes containing either 2.8% THC or 0% THC (placebo) were obtained from the National Institute on Drug Abuse (NIDA; Bethesda, MD). Placebo marijuana cigarettes were prepared at NIDA from marijuana leaves that had been extracted with ethanol to remove cannabinoids. Tobacco cigarettes were purchased commercially (Marlboro Red hard-pack filtered cigarettes, 10 cm, 1–1.05 g). Purified THC at a concentration of 7.5 μg/ml was prepared in dimethyl sulfoxide (DMSO).

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stock concentration of 50 mg/ml in ethanol was obtained from NIDA.

**Tar extracts.** Tar-containing extracts from tobacco or marijuana smoke were prepared by passing whole smoke from a single cigarette through a Cambridge filter (Performance Systematic, Caledonia, MI), according to a standardized protocol (29). Filters were thoroughly dried and weighed, and the deposited tar was extracted with DMSO. Tar concentrations in these extracts were normalized based on standard curves for absorbance at 400 nm as described previously (43).

**Cell culture.** A549 cells, a human lung cancer cell line with alveolar epithelial characteristics (CCL-185; American Type Culture Collection, Bethesda, MD), were cultured in RPMI 1640 medium containing 10% FCS and 1% penicillin-streptomycin-fungizone (Irvine Scientific, Irvine, CA). Cells were passaged every 3–4 days and plated in 12-well plates at 1×10^4 per well or in 24-well plates at 5×10^4 per well, for assays.

**ATP and ADP assay.** A549 cells were grown in 24-well plates and exposed to various agents for 24 h, and an extract was prepared for measurement of the effects on cellular ATP.

**Mitochondrial membrane potential.** Mitochondrial membrane potential was measured by two methods using the fluorescent probe JC-1, which produces green fluorescence in the cytoplasm and red-orange fluorescence when concentrated in respiring mitochondria having a negative internal potential.

**Fluorescence microscopy.** Fluorescence microscopy was performed under a Zeiss Axioskop 2 fluorescence microscope equipped with rhodamine/fluoresceine dual filter set and a Spot 2 digital camera and imaging system (Diagnostic Instruments). Cells treated with THC, marijuana tar extract, or FCCP as above were treated with JC-1 for 30 min in a 37°C CO2 incubator. Cells were then washed twice with PBS and examined immediately under a ×40 aqueous immersible objective. We developed images using Spot 2 Software with the camera shutter set at 0.3 s for red light and 0.5 s for green light.

**Cell viability.** Cell viability was measured as described previously (44). After various exposures, 50 μM propidium iodide was added. After 15 min at room temperature, fluorescence measurements were taken with the Cytofluor 2300 plate reader at Ex = 546 and Em = 590 (sensitivity = 3). Background measurements (blank) were obtained from cell-free wells containing media and propidium iodide. Digitonin (160 μM) was then added, and, after 20 min at room temperature, fluorescence measurements were repeated to obtain F_max, a function of total cell number. Percentage viability was calculated as 100 – (F – blank/F_max – blank) × 100, where F is the measured propidium iodide fluorescence.

**Statistical methods.** Statistical analysis was performed by ANOVA and Fisher’s post hoc test using the Statview software program version 5.0 (SAS Institute).

**RESULTS**

We previously reported that a synergistic cytotoxicity occurred when butylated hydroxyanisole (BHA) and THC were added together to A549 cells, an effect that may have been mediated by their combined impact on cellular ATP levels (41). To characterize the capacity for THC to disturb energetics in these cells, we set up a dose-response study in which cellular ATP levels and viability were measured after 24-h exposure to various concentrations of THC (0–15 μg/ml). THC provided a dose-dependent effect on lowering ATP with an IC_{50} value of 7.5 μg/ml (Fig. 1).

Consistent with previous studies (41, 43), 24-h exposure to 10 μg/ml of THC resulted in <15% cell death, indicating that reductions in ATP levels did not result in immediate cytotoxicity and were not simply due to cell death. At 15 μg/ml, ATP levels were ≤5% those of control, and cell viability declined by 20%. Cellular ADP levels were also found to decline by 28 ± 2.3%, 55 ± 1.8%, and 68 ± 3% (SE) after 24-h exposure to 5, 10, and 15 μg/ml of THC, respectively (data not shown), indicating further decrement in cellular energy charge.

To examine mitochondrial function, we incubated cells treated with THC for 24 h with the fluorescent probes.
probe JC-1. Examination of ethanol vehicle control cells by fluorescent microscopy revealed bright orange-red punctate staining in cytoplasmic regions in virtually all cells, consistent with formation of JC-1 “J-aggregates” within actively functioning mitochondria (Fig. 2). Nuclei were unstained as expected. Variable and faint green staining was observed in most cells. THC produced a concentration-dependent decrease in number and intensity of orange spots, similar to results produced by FCCP, an electron transport chain uncoupler. Analysis of cells by flow cytometry revealed a concentration-dependent decrease in red fluorescence detectable at 0.5 μg/ml of THC with an IC50 between 1 and 2.5 μg/ml of THC (Fig. 3). This decrease in red J-aggregate fluorescence is indicative of loss of mitochondrial membrane potential. Green fluorescence, reflecting cytoplasmic staining, increased only at THC concentrations >5 μg/ml. The THC-induced effects on JC-1 fluorescence provided a more sensitive measure of the mitochondrial function than did measurements of changes in ATP levels (Fig. 4). The fluorescent changes in JC-1 produced by THC exposure closely resembled that of the uncoupling agent FCCP, with decreased red fluorescence and enhanced green fluorescence (Fig. 5). In contrast, the ATP synthase inhibitor oligomycin increased the percentage of cells with high red fluorescence (upper quadrants) from 66 to 94%. Cyclosporin A, which stabilizes membrane pore transition, increased red fluorescence when added to cells alone and attenuated the loss of red fluorescence produced by THC. In response to 4 μM cyclosporin A, the percentage of gated cells in the upper quadrants increased from 12% for cells treated with 5 μg/ml of THC alone to 37% from cells cotreated with THC and cyclosporin A.

We evaluated the ability of THC to affect mitochondrial membrane potential in the presence of cigarette smoke components by comparing the effects of particulate tar extracts from different cigarettes (Fig. 6). Extracts from Marlboro Red cigarettes added to culture medium for 24 h at 50 μg/ml of tar caused an increase in the mean red fluorescence intensity in the upper quadrants from 732 in solvent-treated control to 1,662. Exposure to the same concentration of marijuana tar extract, however, reduced red fluorescence to only 293. To further characterize the specific role of THC in the decrease in red fluorescence, we examined the effect of placebo marijuana tar extract lacking THC. A 24-h exposure to 50 μg/ml of tar produced a red fluorescence intensity of 1,578, similar to that of tobacco extract. When 5 μg/ml of THC was added to the placebo tar extract, Y-mean red fluorescence declined to 299. No increase in green fluorescence was observed.

JC-1 fluorescence was also examined in situ using a Cytofluor 2300 plate reader. Time course studies on the effect of THC revealed a rapid decrease in mitochondrial membrane potential measured as the ratio of red/green fluorescence (Fig. 7). Within 1 h of exposure to 2.5 μg/ml or 10 μg/ml of THC, the red/green ratio declined by 24 and 69%, respectively. At 2.5 μg/ml of THC, this value declined to 49% of control by 3 h and remained low for up to 30 h without detectable loss of cell viability. At 10 μg/ml of THC, the red/green fluorescence ratio remained at ~20–25% of control for up to 30 h with ~25% loss of cell viability observed at 30 h.

Studies on the reversibility of THC-induced mitochondrial injury demonstrated that the rate and degree of recovery varied as a function of THC concentration (Fig. 8). After 3 h of exposure to 5 μg/ml of THC, the red/green fluorescence ratio decreased by 74% compared with ethanol-treated control cells. Upon removal of THC by extensive washing with PBS containing 2% fetal calf serum, the fluorescence ratio increased significantly (P < 0.01) yet remained 30–60% lower than similarly washed control cells for up to 24 h. THC at 10 μg/ml initially decreased fluorescence ratio by 95%, and values following THC removal were never higher than 50% of control. Similar results were obtained following 24-h exposure to THC (data not shown). However, exposure to THC for only 1 h produced lower initial decrements in JC-1 fluorescence ratio, and complete recovery to control levels was observed 4 h after washing (data not shown). However, exposure to 5 μg/ml of THC for only 1 h produced lower initial decrements in JC-1 fluorescence ratio and complete recovery to control levels by 2 h after washing (Fig. 8B).

DISCUSSION

Prior studies in this laboratory characterized oxidative stress associated with exposure of cultured cells to marijuana smoke (42). This oxidative stress was found to increase as a function of THC content of the cigarette, suggesting a direct role for THC. Removal of the THC-containing particulate smoke fraction exacerbated the oxidative stress, however, indicating that
THC was not acting as a primary oxidant. This conclusion is consistent with voltametry studies revealing a weak reducing potential for THC. Nonetheless, studies with A549 cells revealed that THC exerts a cytotoxic effect and that this effect was potentiated by the antioxidant food additive BHA (41). Because BHA is known to be a mitochondrial toxicant, a possible mechanism for the observed synergistic toxicity of THC and BHA was suggested on the basis of aggravated mitochondrial injury.

To investigate this hypothesis, we have examined the effects of THC on cell energetics. THC was found to decrease A549 ATP levels in a time- and dose-dependent manner with effects observed at concentrations <1 μg/ml. A decline in ATP >80% was observed without significant loss of cell viability, indicating that ATP loss was not a secondary consequence of death-associated cell permeability. This decline in ATP would be expected to affect numerous cellular functions, including membrane ion transport, macromolecule synthesis, and cell signaling.

When marijuana cigarettes are smoked, milligram quantities of THC are deposited directly onto the respiratory epithelium in the form of THC-laden smoke particulates (36, 54). Although local pulmonary concentrations of THC have not been measured, they likely exceed the range of 5–250 ng/ml that has been observed in the peripheral blood of subjects smoking one or more marijuana cigarettes (5, 30, 33, 47). Exposing mice to marijuana smoke similarly produced peak blood levels of 400 ng/ml (25), whereas intravenous administration of 10 mg/kg THC resulted in a blood level of 720 ng/ml after 20 min (25). These systemic THC concentrations in human studies and animal models are near the range that begin to disrupt mitochondrial membrane potential and ATP levels. The effects on respiratory epithelial cells, exposed to even higher local con-

Fig. 2. THC alters the pattern of 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetramethyl-2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) staining in A549 cells. Cells were cultured in 35-mm dishes and exposed for 24 h to 0.1% ethanol (control, A), 2 μg/ml of THC (B), 4 μg/ml of THC (C), 6 μg/ml of THC (D), 50 μg/ml marijuana tar extract (E), or 50 μM carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP, F). Photos reveal punctate nonnuclear red-orange staining characteristic of JC-1 accumulation in respiring mitochondria. THC, marijuana tar extract, and FCCP caused a decrease in number and intensity of red-stained mitochondria while slightly increasing the green intensity of cytoplasmic JC-1. Magnification = ×1,200.
centrations during smoking, are likely to be significant. Such severe loss in cellular ATP suggested impairment of mitochondrial function. To investigate this possibility, we used the fluorescent probe JC-1 (2, 32, 37, 52). JC-1 is a lipophilic cationic molecule that is readily taken up by cells and produces a green fluorescent signal when present in the cytosolic fraction. In actively respiring mitochondria, the electronegative inner membrane potential of these mitochondria promote accumulation of JC-1 and formation of the J-aggregate form that fluoresces, producing a red-orange color. Control A549 cultures displayed >90% cells with bright orange punctate staining within a faint green cytosolic background. Exposure of cells to THC caused a dose-dependent decrease in red fluorescence, indicating loss of mitochondrial membrane potential. Both uncoupling agents, such as FCCP, and inhibitors of electron transport, such as antimycin A, are known to produce similar effects (46). At higher concentrations of THC, green cytosolic fluorescence increased, suggesting release of JC-1 monomer from mitochondria to cytoplasm. Decreased red fluorescence induced by THC was not due to direct interaction of THC with JC-1, since cell-free incubation with these compounds resulted in no decrease in red fluorescence (data not shown). In fact, red fluorescence was enhanced at [THC] >10 μg/ml under these conditions. THC-induced loss of mitochondrial membrane potential and cellular ATP was partially prevented by cyclosporin A, suggesting that mitochondrial injury was mediated by the permeability transition pore complex. Cyclosporin A acts by binding to the mitochondrial matrix protein cyclophilin D and subsequently preventing interaction between adenine nucleotide translocator and the voltage-dependent anion channel (11, 20). This channel is regulated by vicinal thiol groups and damaged by oxidative stress, ganglioside GD3, and FCCP (9, 26, 31). It should be noted that the binding of cyclosporin A to cyclophilin D also results in alterations in Ca2+ -mediated cell signaling pathways (21) that could, in turn, be responsible for the partial attenuation of the observed THC-induced mitochondrial effects. Nevertheless, the mitochondrial permeability transition is an important regulator for both apoptotic and necrotic cell death (19).

Fig. 3. Flow cytometric analysis of THC effects on mitochondrial membrane potential as detected by JC-1 fluorescence. A549 cells were treated with THC for 24 h, washed, and stained with 2 μg/ml of JC-1 for 30 min at 37°C. Cells were trypsinized to provide a single cell suspension and analyzed for green (FL-1) and red (FL-2) fluorescence by flow cytometry. A: flow cytometry data were analyzed using Cell Quest software and dot-plot generated to track fluorescence in response to treatment. Con, control. B: bar graphs indicating the percentage of gated cells falling in upper quadrants (Upper Quads) or lower quadrants (Lower Quads) as shown in A. This experiment was repeated twice with similar results.

Fig. 4. Simultaneous analysis of the effects of THC on A549 mitochondrial membrane potential, cellular ATP, and cell viability. JC-1 fluorescence was measured both with a flow cytometer (FACS JC-1) and with a Cytofluor 2300 fluorescence plate reader measuring the ratio of red/green fluorescence (Cyto JC-1). ATP levels expressed as pmol/μg protein were assayed using a luminescence firefly luciferase assay, and % viability was assayed with propidium iodide staining. All values are expressed as a percentage of vehicle-treated controls and represent means of duplicate assays. The experiment was repeated twice with similar results.
We reported previously that anti-fas-induced apoptosis in A549 cells was suppressed by THC, as shown by a THC-induced decrease in caspase-3 activity, phosphatidyl serine externalization, and nuclear chromatin condensation assays (43). The inhibitory action of THC on A549 apoptosis could occur by one of several mechanisms, including alteration of fas receptor activity or death-inducing signaling complex protein assembly. The present findings suggest an alternative mechanism for apoptosis inhibition, since apoptotic cell death is known to require a minimal level of ATP or dATP estimated to be ~25% of control levels (12, 29, 34, 48). ATP is required as a critical component of the apoptosome in which caspase-9 complexes with cytochrome c and Apaf-1 to activate caspase-3 (19, 50). This pathway can be influenced by the fas receptor via activation of truncated BH3-interacting domain, which mediates release of cytochrome c from mitochondria (55). Although some mitochondrial inhibitors are known to induce apoptosis (28, 53), inhibitors that deplete ATP levels severely do not allow expression of the apoptotic pathway. Such may be the case with THC. The observed increase in necrotic cell death caused by THC with or without fas-receptor activation may result from an inability to maintain energy-dependent membrane transport functions.

The effects of THC on cell energetics appear to occur both when THC is applied as a purified synthetic compound and in preparations of marijuana smoke extract, both of which strongly diminished JC-1 red fluorescence. Neither tobacco smoke extract nor 0% THC placebo marijuana extract produced this effect. These results indicate that THC exerts its mitochon-

![Fig. 5. Mitochondrial inhibitors alter JC-1 fluorescence and alter the response to THC. A549 cells were pretreated for 24 h with 0.2% ethanol [control (Con)], 5 μg/ml of THC, 25 μM FCCP, 5 μM cyclosporin A (Cys A), 50 μM oligomycin (Oligo), or 5 μg/ml of THC and 5 μM Cys A and analyzed for the impact on JC-1 by flow cytometry. Data are presented as dot plots (A) and as bar graphs representing the percentage of gated cells in upper or lower quadrants (B). This experiment was repeated twice with similar results.

![Fig. 6. Marijuana tar extract produces effects similar to THC. A549 cells were pretreated 24 h with a 50 μg/ml concentration of cigarette smoke extracts prepared from the particulate phase of tobacco (Tob Extr), marijuana (MJ Extr), or placebo [Plac (0% THC)] marijuana smoke. A: FL-1 (green) fluorescence intensity is plotted on the x-axis and FL-2 (red) fluorescence on the y-axis. B: bar graphs depicting the mean red fluorescence intensity (y-axis) for cells in upper quadrants or lower quadrants under the conditions shown. Exposure of A549 cells to cigarette tar extract resulted in a nonspecific general increase in red fluorescence compared with control cells (see Fig. 3). This experiment was repeated 3 times with similar results.](http://ajplung.physiology.org/)
drial action in the presence of the numerous components of particulate cigarette smoke. This complex mixture by itself in the absence of THC does not cause loss of mitochondrial membrane potential, nor does it interfere with the action of THC. Both flow cytometry and fluorescent microscopy studies reveal that marijuana smoke extract stored at 15°C for up to 6 mo retains the ability to damage A549 mitochondria (data not shown).

Further studies will be necessary to identify the mechanism underlying THC-induced mitochondrial dysfunction. Because aromatic and phenolic compounds are known to interfere with mitochondrial electron transport (15, 51), this mechanism represents one possible site of interaction for THC consistent with previous studies. Another possibility that could account for impaired mitochondrial membrane potential and ATP depletion would be a decline in NADH supply, either through inhibition of Krebs cycle activity or through cytoplasmic depletion. Such depletion is known to occur via activation of poly(ADP-ribose) polymerase (PARP) resulting from DNA damage (27). PARP activation would be promoted in cells deficient in caspase-3 activity, a condition previously demonstrated in THC-treated A549 cells (43). The inactivation of PARP is one of the primary actions of caspase-3 associated with apoptosis (24).

Marijuana smoking is associated with increased risk of pulmonary infection (3, 6, 45, 49) and bronchial mucosa inflammation and injury (13, 35). Disruption of cell energetic pathways, particularly when sustained long after initial exposure to THC, might help explain these health effects in several ways. First, immune cell-mediated defense mechanisms could be compromised due to impairment in macromolecule synthesis.
by immune cells (16, 48). Second, disruption of apoptotic mechanisms in epithelial cells has been shown to increase risk of infection in gut lining due to the inability to eliminate and process infected cells (18, 22). Third, pathogenic infectious agents are routinely removed from the pulmonary epithelium by mucociliary transport, which is driven by the coordinated action of epithelial microvilli powered by cellular ATP. One of us (D. P. Tashkin) has observed a dramatic loss of microvilli in the tracheobronchial lining of marijuana smokers (14, 17). Further studies will be required to characterize the role of THC-mediated mitochondrial injury in pulmonary pathophysiology.

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