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Expression of a brain-type cannabinoid receptor (CB_1) in alveolar Type II cells in the lung: regulation by hydrocortisone

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Abstract

Using the polymerase chain reaction with degenerate primers to identify novel G-protein-coupled receptors of the rat alveolar Type II cell, we identified sequences expressed by the Type II cell identical to the sequence of the rat brain cannabinoid receptor (CB_1). The use of Northern blot analysis to examine expression of CB_1 mRNA in rat tissues revealed differences between the brain and lung. While rat brain expressed a 6.0 kb mRNA as previously described, rat lung expressed mRNA of 4.5 and 6.0 kb. Isolated lung alveolar Type II cells also expressed mRNA of 4.5 and 6.0 kb as determined by Northern analysis. However, only freshly isolated Type II cells contained cannabinoid receptor mRNA. Reverse transcriptase-polymerase chain reaction (RT-PCR) failed to detect CB_1 mRNA in Type II cells maintained in culture for 1 or 2 days. We next determined developmental changes in lung CB_1 mRNA expression using semi-quantitative RT-PCR. CB_1 expression was detected as early as gestational day 16 in rat lung and mRNA levels increased to fetal day 20 before birth, before declining to adult levels. Fetal rat lung explants were utilized to further examine the ontogeny and hormonal effects on CB_1 mRNA expression. Hydrocortisone induced a dose-dependent expression in 15-day and 18-day explants, similar to previous results for surfactant-associated proteins. Our results demonstrate expression of CB_1 mRNA in rat alveolar Type II cells and rat lung. This expression is ontogenically and hormonally regulated, with maximal expression noted just prior to birth in rat lung. Since CB_1 mRNA is only expressed in freshly isolated Type II cells, CB_1 may be useful as a Type II cell marker.

Keywords: Cannabinoid; Lung; Type II cell; Ontogeny; Development; Steroid

1. Introduction

Δ^9-Tetrahydrocannabinol is the major psychoactive component of marijuana which continues to be a widely abused recreational drug. While the existence of central nervous system receptors for this agent has been described for several years and a cannabinoid receptor (CB_1) has been cloned from rat brain (Matsuda et al., 1990), effects of Δ^9-tetrahydrocannabinol outside the central nervous system have been less well characterized. A cannabinoid receptor has also been identified on mouse spleen cells (CB_2), although the mRNA could not be identified in spleen tissue by Northern blot analysis (Munro et al., 1993). This receptor is assumed to be involved in cannabinoid-mediated immune effects. A cannabinoid receptor has also recently been described in testis (CB_1), where the expressed mRNA was noted to have the same size as brain cannabinoid receptor mRNA (Gérard et al., 1991). Utilizing more sensitive techniques, the brain-type cannabinoid receptor (CB_1) has also been identified in a majority of peripheral tissues, including the lung (Galieque et al., 1995).

Tetrahydrocannabinol use has been shown to affect pulmonary function (Graham, 1986). Increased airway conductance was noted among a group of 32 healthy, experienced male marijuana smokers following smoking or oral ingestion of tetrahydrocannabinol (Tashkin et al., 1983) while heavy marijuana smoking resulted in decreased airway conductance in 28 male subjects (Tashkin et al., 1976). Tetrahydrocannabinol also alters antibacterial defense in the lung (Huber et al., 1980). However, whether these effects are mediated by CB_1 receptors in the lung and the identity of cells in the lung expressing CB_1 receptors are unknown.

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The alveolar Type II cell of the lung synthesizes and secretes surfactant phospholipids and proteins in a highly regulated manner and this cell is responsible for successful adaptation to air-breathing at birth in the perinatal period (Rooney et al., 1994). Because of the key role this cell plays in regulation of surfactant secretion, G-protein-coupled receptors have been intensely studied in this cell type. To discern previously uncharacterized G-protein-coupled receptors of the Type II cell, we have utilized degenerate primers and the polymerase chain reaction with Type II cell cDNA prepared with reverse transcriptase from mRNA to identify and characterize novel G-protein-coupled receptors of the Type II cell. During these experiments, we identified sequences expressed in the Type II cell which were identical to sequence for the rat brain cannabinoid receptor (CB_1). We therefore undertook the present studies to further characterize the Type II cell cannabinoid receptor and determine whether the expression of the cannabinoid receptor in the lung is developmentally regulated.

2. Materials and methods

2.1. Animals

Pathogen-free Sprague-Dawley rats (200–250 g) were utilized for these experiments and obtained from Harlan (Indianapolis, IN, USA).

2.2. Chemicals

Materials for the Type II cell preparation were obtained from sources noted previously (Rice et al., 1990). Fetal calf serum, antibiotics and agarose were from Gibco-BRL. The AmpliTaq kit was purchased from Perkin-Elmer, Norwalk, CT, USA. Elastase was obtained from Worthington Biochemicals.

2.3. Preparation of alveolar Type II cells

Type II cells were isolated from rat lungs as previously described (Rice et al., 1990). The animals were anesthetized with sodium pentobarbital and the lungs perfused via the pulmonary artery, as previously noted. Elastase was used to digest the Type II cells from the basement membrane, and Type II cells were subsequently obtained by the panning method previously described by Dobbs et al. (1986).

2.4. Preparation of fetal lung tissue

Timed-pregnant Sprague-Dawley rats were obtained from Bantin and Kingman (Fremont, CA, USA). Mating was confirmed by the presence of a sperm-positive vaginal smear and was considered day 0 of gestation (day of birth = 22). Pregnant dams were killed with a lethal intraperitoneal dose of pentobarbital and transection of the aorta. After a rapid delivery through an abdominal hysterotomy, the fetuses were weighed, then killed by decapitation. Fetal age was determine by weight at delivery (Schellhase and Shannon, 1991). After removing the heart, trachea, and lungs en bloc from the chest cavity, individual lobes of the lung were dissected free from the major airways and maintained in sterile phosphate-buffered saline (PBS) on ice. Lungs from littermates were pooled and analyzed independently.

Fetal day 15 or 18 lung tissue prepared as above was cut into 1- to 2-mm^3 explants. Lung explants were cultured in 6-well tissue culture plates (Becton Dickinson, Lincoln Park, NJ, USA) on a semisolid medium consisting of 0.5% low gelling temperature agarose (Sigma, St. Louis, MO, USA) in Waymouth's 752/1 medium (Gibco/BRL, Grand Island, NY, USA) containing 100 U/ml penicillin G, 100 U/ml streptomycin, 2.5 μg/ml amphotericin (all from Gibco/BRL), and 10 μg/ml gentamicin sulfate (Sigma). Some media additionally contained hydrocortisone (HC, 10^{-9} to 10^{-6} M; Sigma), which was diluted in 100% ethanol; the final ethanol concentration used in explant cultures was less than 0.1%. Liquid Waymouth's (approximately 75–125 μl) containing no additions, or hydrocortisone in the same concentration as the semisolid medium was then added to maintain the explants at an air-liquid interface. Tissue culture plates were placed inside a modular incubator (Billups-Rothenberg, Del Mar, CA, USA), gassed with 95% air/5% CO_2, and rocked at 3 cycles/min inside a 37°C incubator. Explants were harvested after 3 days for RNA isolation. Uncultured fetal day 15, 18 and 21 and adult lungs were freshly isolated and used as age-matched controls.

2.5. Preparation of RNA

Total RNA was prepared from the tissues or cells noted using Trizol (Molecular Research Center, Cincinnati, OH, USA). Briefly, 1 ml of reagent was used per 100 mg of tissue or 10 cm^2 of culture dish, and the homogenate incubated 5–6 min at room temperature. A total of 0.2 ml of chloroform was added per 1 ml of Trizol, and the samples stored at room temperature for 15 min. Phases were separated by centrifugation, and the upper aqueous phase containing RNA was precipitated with isopropanol. The RNA precipitate was washed with ethanol and solubilized in water. Concentrations were determined spectrophotometrically.

Samples of total RNA obtained in this fashion were utilized for preparation of poly(A)$^+$ RNA using a commercially available kit and the enclosed procedure (Promega, Madison, WI, USA). Biotinylated oligo dT was annealed to the total RNA in solution and hybrids captured with streptavidin-coated magnetic beads. Samples of poly(A)$^+$ RNA were evaporated to dryness, resuspended in diethyl

pyrocarbonate-treated water, and stored at −70°C until needed.

2.6. Northern blot analysis

RNA was isolated as noted above. Purified RNA was fractionated by electrophoresis through a denaturing 1% agarose gel and blotted onto Nytran (Schleicher & Schuell, Keene, NH, USA) by capillary action. Conditions for prehybridization, hybridization, and washing were identical to those described previously (Schellhase and Shannon, 1991). To determine equivalence of RNA loading of Northern blots, we analyzed the expression of β-actin mRNA using a 405-bp fragment from the 3′ untranslated region of human β-actin (Ponte et al., 1984), which has been shown to have >85% homology with the rat. Autoradiograms were generated using Kodak XAR-5 film (Eastman Kodak, Rochester, NY, USA). Northern blots were reprobed after removing previous ^{32}P label by washing them in 50% formamide and 6 × SSC (1 × SSC is 0.15 M sodium chloride and 15 mM trisodium citrate) at 65°C for 1.5 h and rinsing in 2 × SSC. The removal of cDNA probe was confirmed by an overnight autoradiogram. Probes were generated by random primer extension with a commercially available kit (Rediprime, Amersham, Chicago, IL, USA).

2.7. Polymerase chain reaction

The degenerate primers used to amplify Type II cell G-protein-coupled receptor cDNA were forward: 5′-TCT-AGACTGTG(C/T)G(C/T)(G/C) AT(T/C)GCIIT(T/G) GA(T/C)(C/A) G(G/C) TAC-3′ and reverse: 5′-AAG CTT A(T/G)G(A/T)AG(A/T)AG GGC AGC CAG CAG AI (C/G)(G/A)(T/C)AAA-3′. The oligonucleotide sequences were based on sequences of III and VI transmembrane domains of G-protein-coupled receptors and included *Xba*I and *Hin*dIII sites to facilitate cloning. The conditions of the PCR were as follows: 94°C × 30 s; 55°C × 30 s; 72°C × 1 min for 30 cycles. To amplify cannabinoid receptor (CB_1) sequences from Type II cell cDNA, the following primers based on the published rat brain cannabinoid sequence (Matsuda et al., 1990) were utilized: F-108-GGCATCTCTTTCTCAGTCAC; R-972-ATCAGGTAGGTCTCGTCAAT; F-191-CGTACCATC-ACCACAGACCT; R-1207-GCAGATGATCAACAC-CACCA and the same PCR conditions utilized. Other primers utilized were based on published sequences. SP-A (Sano et al., 1987): F-GGAGTCCTCAGCTTGCAAGGA and R-TTCACAAACAGCCAGCCGGTA, actin (Nudel et al., 1983): F-ACAGCTTCACCACCACAG and R-GCC-AGGGCAGTAATCTCC, β_2-microglobulin (Mauxion and Kress, 1987): F-CCGTGATCTTTCTGGTGCTTGTCT and R-CATCGGTCTCGGTGGGTGTGA. Semi-quantitative PCR was performed as previously described using actin or β_2-microglobulin cDNA as a control (Zhou et al., 1994; Murphy et al., 1990).

2.8. DNA sequencing

DNA was sequenced using the dideoxy chain termination method with a Sequenase sequencing kit (US Biochemical, Cleveland, OH, USA) and [α-^{32}P]ATP. Both strands were sequenced in their entirety.

3. Results

Utilizing degenerate primers to identify novel G-protein-coupled receptors in alveolar Type II cells, we generated a PCR product with sequence identical to the rat brain cannabinoid receptor (CB_1). CB_1-specific primers were then utilized to generate a 0.9-kb PCR product from alveolar Type II cell cDNA. The DNA sequence obtained was identical to CB_1, indicating expression of CB_1 mRNA by alveolar Type II cells.

This PCR product was used to probe a rat tissue Northern blot (Fig. 1). A 6.0-kb mRNA was detected in rat brain, kidney and lung, as previously reported for rat brain (Matsuda et al., 1990). However, a 4.5-kb mRNA species was also detected in lung tissue and was also noted on a Northern blot of Type II cell mRNA (Fig. 2). We also utilized RT-PCR to examine tissue expression of CB_1 mRNA. The predicted 0.9-kb product was noted in the same three rat tissues, and additionally in spleen and testis (Fig. 3).

RT-PCR was also utilized to determine expression of CB_1 in alveolar Type II cells during culture (Fig. 4). Expression of CB_1 mRNA was only noted in freshly isolated Type II cells (day 0) and could not be detected by RT-PCR at later time points.

To determine whether CB_1 is differentially expressed during rat lung development, mRNA was obtained from

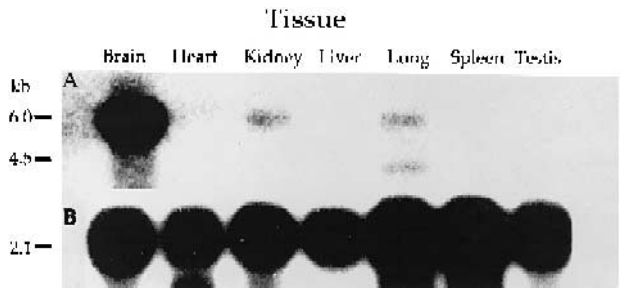

Fig. 1. Northern analysis of rat tissue CB_1 expression. This blot is representative of three independent experiments. Total tissue RNA was extracted as noted in Section 2, fractionated through a 1% denaturing gel, transferred to Hybond-N, probed with a radiolabeled cDNA probe for CB_1, and an autoradiogram performed (A). 20 μg of total RNA was loaded in each lane; equivalent mRNA loads were determined by reprobing the same blot with a radiolabeled cDNA probe for actin (B) as noted in Section 2.

fetal rat lungs, and semi-quantitative RT-PCR performed. Expression of the surfactant-associated protein, SP-A and β_2-microglobulin mRNA were used for comparison. CB_1 mRNA expression in rat lung increased throughout gestation, reaching a peak prior to birth on gestational day 20. Levels of CB_1 mRNA expression then fell in the adult lung. In contrast, SP-A mRNA levels rose throughout gestation and did not drop as precipitously in adult lung (Fig. 5).

Since steroids regulate expression of surfactant-associated proteins in the lung, we next examined regulation of CB_1 mRNA expression by hydrocortisone in developing lung. While expression of CB_1 mRNA was undetectable in fetal lung from gestational day 15 or day 18, hydrocortisone induced dose-dependent expression of the 4.5-kb CB_1 mRNA in day 15 and day 18 fetal lung explants (Figs. 6 and 7). Expression of the 6.0-kb CB_1 mRNA was not

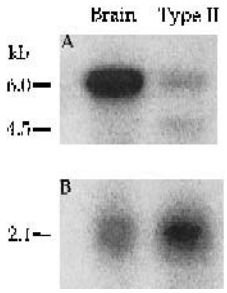

Fig. 2. Northern analysis of Type II cell CB_1 expression. This blot is representative of five independent experiments. 2 μg of poly(A)$^+$ RNA was extracted and Northern analysis performed as noted for Fig. 1. Radiolabeled cDNA for CB_1 (A) or actin (B) were utilized as noted.

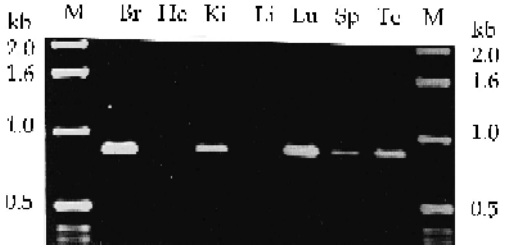

Fig. 3. Expression of CB_1 mRNA in rat tissues determined by RT-PCR. RT-PCR was used to detect expression of CB_1 mRNA in rat tissues as detailed in Section 2. Results are representative of at least three separate preparations for each tissue. The CB_1 primers used were F-108 and R-972. M, size markers; Br, brain; He, heart; Ki, kidney; Li, liver; Lu, lung; Sp, spleen; Te, testis.

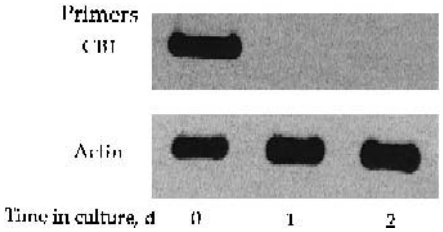

Fig. 4. Expression of CB_1 mRNA in cultured alveolar Type II cells. Semi-quantitative RT-PCR was used to detect expression of CB_1 mRNA in freshly isolated alveolar Type II cells (day 0) or alveolar Type II cells cultured for the times noted. Primers for CB_1 or actin were used where noted. Data are representative of five independent experiments.

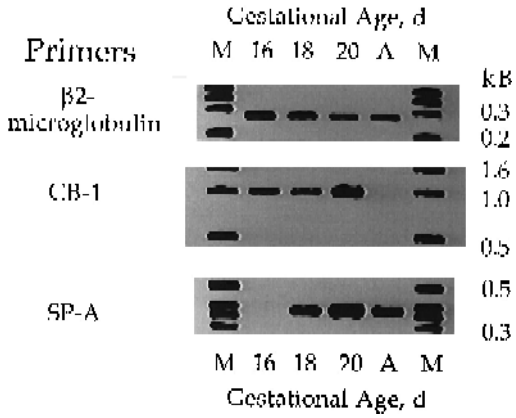

Fig. 5. Ontogeny of CB_1 and SP-A mRNA expression in developing lung. Total cellular RNA was harvested from fetal rat lungs at the gestations noted and semi-quantitative RT-PCR performed as detailed in Section 2. Primers for CB_1, SP-A or β_2-microglobulin were utilized as noted. The CB_1 primers used were F-191 and R-1207. Gels are representative of three independent experiments. d, day of gestation; M, size markers; A, adult.

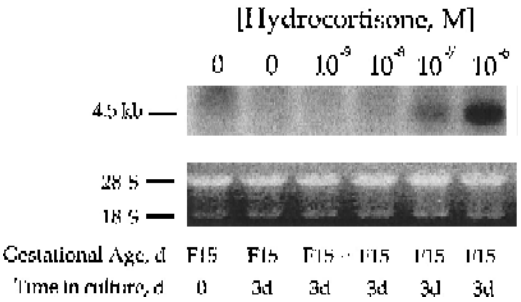

Fig. 6. Representative Northern analysis of fetal day 15 rat lung explant cultures. Lung explants from fetal day 15 rats were grown for 3 days in culture medium without hormone (0) or in the presence of the indicated concentrations of hydrocortisone. Total RNA was isolated, fractionated, transferred to Nytran, and probed with ^{32}P-labelled cDNA for CB_1. This blot is representative of three independent experiments. 15 μg of RNA was loaded in each lane; equivalent mRNA loads were determined by ethidium bromide staining. d, day.

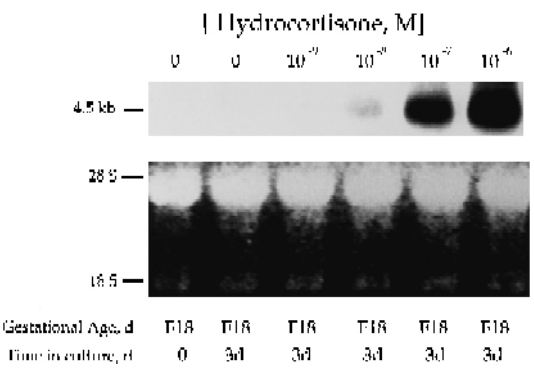

Fig. 7. Representative Northern analysis of fetal day 18 rat lung explant cultures. Lung explants from fetal day 18 rats were analyzed as noted for Fig. 6. d, day.

detected at either age in the presence or absence of hydrocortisone.

4. Discussion

While the presence of cannabinoid receptors (CB_1) in the central nervous system has been well-established, the presence of such receptors in sites outside the central nervous system has only recently been appreciated, although expression of a related cannabinoid receptor (CB_2) in cells of lymphoid origin has been noted (Munro et al., 1993). From the present work, the lung, and specifically the alveolar Type II cell, can now be added to the list of sites of cannabinoid receptor (CB_1) expression outside the central nervous system.

In contrast to the brain (Matsuda et al., 1990), the cannabinoid receptor mRNA expressed in alveolar Type II cells is 4.5 kb as well as 6.0 kb in size. The reason for this size discrepancy is presently unclear. Differential splicing of the lung mRNA may lead to the smaller sized message. cDNA from the IM-9 cell line contains an alternatively spliced form of the CB_1 receptor (Shire et al., 1995). Expression of this alternatively spliced mRNA generates an amino-terminal variant of the CB_1 receptor (Shire et al., 1995). It is also possible that a different gene exists for production of the lung cannabinoid receptor. However, only one product was obtained from lung mRNA by RT-PCR, arguing against this possibility. Polyadenylation may also differ in the lung. In support of this possibility, two possible polyadenylation signals have been identified in the human CB_1 3′ untranslated region (Shire et al., 1995).

A cDNA for the human brain cannabinoid receptor has also recently been described which hybridized with dog testis by Northern blot analysis (Gérard et al., 1991). We were unable to observe similar expression in rat testis by Northern blotting. Whether this represents a true species difference is unclear. Gérard and colleagues utilized 10 μg of poly$(A)^+$ RNA and observed multiple hybridizing bands, while we examined 2 μg of poly$(A)^+$ RNA and only observed one predominant band in brain, as previously described. These technical differences may be responsible for the observed experimental differences, since we did detect CB_1 expression in rat testis by RT-PCR.

Unclear at the present time is the function of the Type II cell cannabinoid receptor and the nature of the endogenous ligand which binds to this receptor. While CB_1 ligands inhibit cAMP production (Condie et al., 1996; Howlett et al., 1986) and cAMP is known to regulate surfactant secretion from alveolar Type II cells (Rice et al., 1984), whether CB_1 agonists inhibit surfactant secretion in vivo is not known. Given the rapid disappearance of CB_1 mRNA in Type II cells, we were not able to directly test this hypothesis utilizing current methodology. It is also interesting to note that production of the surfactant-associated protein, SP-A, is regulated by cyclic AMP and inhibition of cyclic AMP production in the Type II cell would also inhibit production of SP-A (Boggaram et al., 1988). Since tetrahydrocannabinol is typically inhaled, alveolar Type II cells may be exposed to much higher concentrations of cannabinoids than other sites in the body, such as the central nervous system. Such exposure could potentially interfere with normal regulation of surfactant secretion and other Type II cell functions.

Devane and colleagues have recently identified anandamide, an arachidonic acid metabolite, as an endogenous agonist for the cannabinoid receptor in the brain (Devane et al., 1992; Felder et al., 1993). The lung has also been noted as a site of anandamide synthesis (Pertwee, 1995), as well as the kidney, noted to express CB_1 mRNA in the present work. It is therefore, possible that anandamide is the endogenous ligand for the lung cannabinoid receptor. Further data are necessary to test this hypothesis.

The expression pattern of CB_1 in developing lung is also of interest. Prior work in developing rat brain failed to demonstrate any change in CB_1 mRNA expression in rats from postnatal day 3 to adulthood by Northern blotting (McLaughlin et al., 1994). Utilizing RT-PCR and focusing on prenatal development, we were able to demonstrate a peak in CB_1 mRNA expression shortly before birth and a dramatic drop to lower adult levels. This expression pattern may indicate a role for CB_1 in developing lung and is distinct from the expression pattern noted for the surfactant-associated protein, SP-A.

In summary, we have described the presence of cannabinoid receptor mRNA in alveolar Type II cells. Two species of mRNA are noted in the lung, in contrast to other tissues, and expression of the 4.5 kb mRNA is ontogenically and hormonally regulated in the lung. Whether other lung cell types also express CB_1 and the role of CB_1 in regulation of normal lung function remain to be clarified.

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