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Chronic treatment with the endocannabinoid anandamide increases cytochrome *P*450 metabolizing system in the rat

Barbara Costa [a], Daniela Parolaro [b], Mariapia Colleoni [c,*]

[a] *Dipartimento di Biotecnologie e Bioscienze, Facoltà di Scienze, Università Milano-Bicocca, Milan 20126, Italy*
[b] *Dipartimento di Biologia Strutturale e Funzionale, Sezione di Farmacologia, Università dell'Insubria-Varese, Busto Arsizio 21052, Italy*
[c] *Dipartimento di Farmacologia, Chemioterapia e Tossicologia Medica, Università di Milano, Milan 20129, Italy*

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Abstract

The aim of this work was to investigate the effects of single and repeated administration of the endogenous cannabinoid anandamide (20 mg/kg i.p.) on cytochrome *P*450-mediated biotransformation in the rat. In liver microsomes from chronically treated rats, an increase in cytochrome *P*450 content and in the activity and immunoreactivity of cytochrome *P*450 reductase was detected. Immunoblot analysis of the hepatic microsomal proteins revealed an increase in the relative level of cytochrome *P*450 2B1/2 and 3A2. The activity of monooxygenase enzymes linked to specific cytochrome *P*450 isoforms was significantly enhanced. This increase in the content and activity of the cytochrome *P*450 system was also seen in liver microsomes from acutely treated rats; however, these increases were smaller than those seen after prolonged treatment. After acute treatment, the brain cytochrome *P*450 and b_5 content was increased, whereas after chronic treatment, only that of b_5 was enhanced. Cytochrome *P*450 reductase activity and its relative abundance were increased only in the brains of chronically treated rats. The present findings demonstrate that anandamide administration increased the metabolic activity of the cytochrome *P*450 system in rat liver and brain.

Keywords: Cannabinoid; Anandamide; Tolerance; Cytochrome *P*450; Liver, rat; Brain, rat

1. Introduction

The first putative endogenous ligand for cannabinoid receptors, anandamide (*N*-arachidonoylethanolamine), was identified in porcine brain (Devane et al., 1992) and appears to have 30 times greater affinity for the cannabinoid CB_1 receptor in the brain than for that in the periphery (Munro et al., 1993). Anandamide is one of a family of arachidonic acid derivatives with cannabimimetic effects (see Di Marzo et al., 1999 for a review). Like Δ^9-tetrahydrocannabinol (THC) and other cannabinoids, anandamide produces antinociception, hypomotility, catalepsy and hypothermia effects, which are thought to be mediated by cannabinoid CB_1 receptor activation because they are reversed by pretreatment with SR141716A [*N*-piperidino-5-(4-chlorophenyl)-1-(2,4-dichlorophenhyl)-4-methylpyrazole-3-carboxamide], the specific cannabinoid CB_1 receptor antagonist (Costa et al., 1999). Like other cannabinoids, chronic intraperitoneally administered anandamide induces tolerance in mice and rats in various behavioral tests (Fride, 1995; Costa et al., 2000). Pharmacokinetics probably play a minor role in tolerance to the pharmacological effects of cannabinoids. Instead, tolerance development must be described as a pharmacodynamic event, such as receptor down-regulation or change in signal transduction system. Previously, we showed that chronic treatment of rats with CP-55,940 {(-)-*cis*-3-[2-hydroxy-4-(1,1-dimethylheptyl)-phenyl]-*trans*-4-(3hydroxypropyl)cyclohexanol)}, a potent bicyclic cannabinoid analog, increased the activity of the liver microsomal cytochrome *P*450 oxidative system, suggesting some pharmacokinetic tolerance (Costa et al., 1996), even though the same chronic treatment decreased cannabinoid receptor mRNA levels in the caudate-putamen area in the brains of rats tolerant to the cannabinoid-induced decrease in locomotor activity (Rubino et al., 1994). We have recently shown that rats chronically exposed to an active dose of anandamide for a period of 2 weeks developed behavioral tolerance (Costa et al., 2000), together with a widespread decrease in agonist-stimulated [^{35}S]GTPγS binding in all brain areas containing a high density of

* Corresponding author. Tel.: +39-2-5031-7038; fax: +39-2-5031-7036.
E-mail address: colleoni@mailserver.unimi.it (M. Colleoni).

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cannabinoid CB_1 receptors, without any change in cannabinoid CB_1 receptor binding and no alteration in cAMP levels or in protein kinase A activity (Rubino et al., 2000).

Furthermore, anandamide is an amide of arachidonic acid and ethanolamine, and in common with the arachidonic acid, it is a substrate in vitro for liver and brain microsomal *P*450-mediated processes. In fact, Bornheim et al. (1995) showed that incubation of mouse liver microsomes with anandamide resulted in the generation of at least 20 metabolites and that the pretreatment of mice with various cytochrome *P*450 inducers resulted in increased hepatic microsomal formation of several anandamide metabolites, with dexamethasone and phenobarbital being the most effective inducers, showing that cytochromes *P*450 3A and 2B are the major contributors to anandamide metabolism. The same authors showed that the in vitro metabolism of anandamide by brain microsomes resulted in the formation of two metabolites, and that cytochrome *P*450 3A antibody partially inhibited the formation of one of these metabolites. Furthermore, it has been reported that pretreatment with cannabidiol, a cannabinoid that inactivates cytochromes *P*450 2C and 3A in the mouse (Bornheim and Correia, 1991), decreases the formation of several anandamide metabolites (Bornheim et al., 1993).

On this basis, we examined whether anandamide administration in rats altered the hepatic and cerebral microsomal cytochrome *P*450(s).

2. Materials and methods

2.1. Animals

Male Sprague–Dawley rats (weighing between 100 and 125 g upon arrival to the laboratory) were purchased from Charles River. The animals were housed in plastic cages and maintained on a 12-h light–dark cycle. Food and water were freely available. All procedures were carried out in accord-

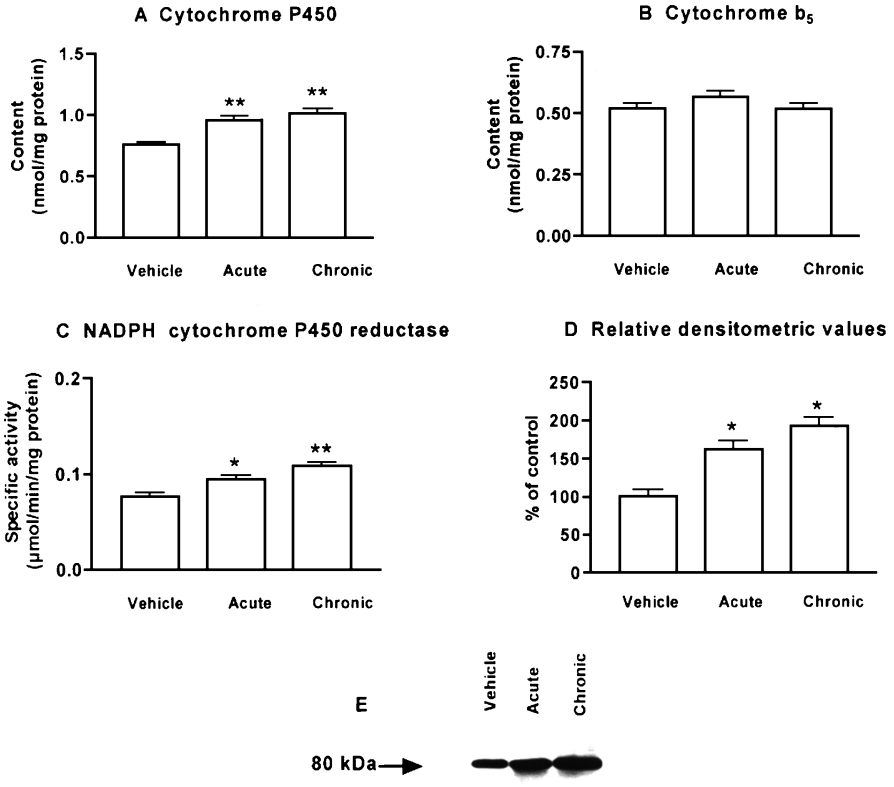

Fig. 1. Effect of acute and 15-day administration of anandamide (20 mg/kg i.p.) on total content of cytochromes *P*450 and b_5 and on NADPH cytochrome *P*450 reductase specific activity and levels in rat liver microsomes. The content of cytochromes *P*450 (A) and b_5 (B) and NADPH cytochrome *P*450 reductase activity (C) were spectrophotometrically determined as described in Section 2. Values are means ± S.E.M. for 13–20 rats. Statistical differences were assessed by one-way ANOVA followed by Tukey's test. $*P<0.05$, $**P<0.01$, significant as compared to vehicle-treated rats. (D) Immunoblot analysis of hepatic NADPH cytochrome *P*450 reductase protein was performed by electrophoresis on SDS-PAGE. Proteins were transferred to nitrocellulose membranes and probed with anti-NADPH cytochrome *P*450 reductase antibodies and visualized by using enhanced chemiluminescence, as described in Section 2. The representative immunoblot (E) shows levels of NADPH cytochrome *P*450 reductase protein in liver microsomes isolated from rats treated with vehicle or with anandamide acutely and chronically. Each lane was loaded with 15 µg of proteins. Data denote relative levels of expression determined by comparison with levels of expression in vehicle-injected rats, determined by computer-programmed densitometric analysis with NIH Image programs. Each value represents mean ± S.E.M. for three to five rats. Statistical differences were assessed by Kruskal–Wallis ANOVA applied on the medians followed by Rayan's test. $*P<0.05$, significant as compared to vehicle-treated rats.

ance with local ethical regulations for animal research (permission no. 94/2000-A).

2.2. Materials

Anandamide was a generous gift from the National Institute on Drug Abuse. Rabbit polyclonal antibodies raised against rat liver-specific isozymes of cytochrome *P*450 and NADPH cytochrome *P*450 reductase were purchased from Chemicon International. Enhanced chemiluminescence reagents were purchased from Roche Diagnostics; hyperfilm and secondary antibody (donkey anti-rabbit F(ab′)2 immunoglobulin conjugated to horseradish peroxidase) were purchased from Amersham Pharmacia Biotech. Nitrocellulose membrane was from Schleicher and Schuell. Acrylamide and protein molecular weight standards were purchased from Sigma Aldrich. Nonfat dry milk was from Marvel. All other reagents were purchased from commercial sources and were the highest quality available.

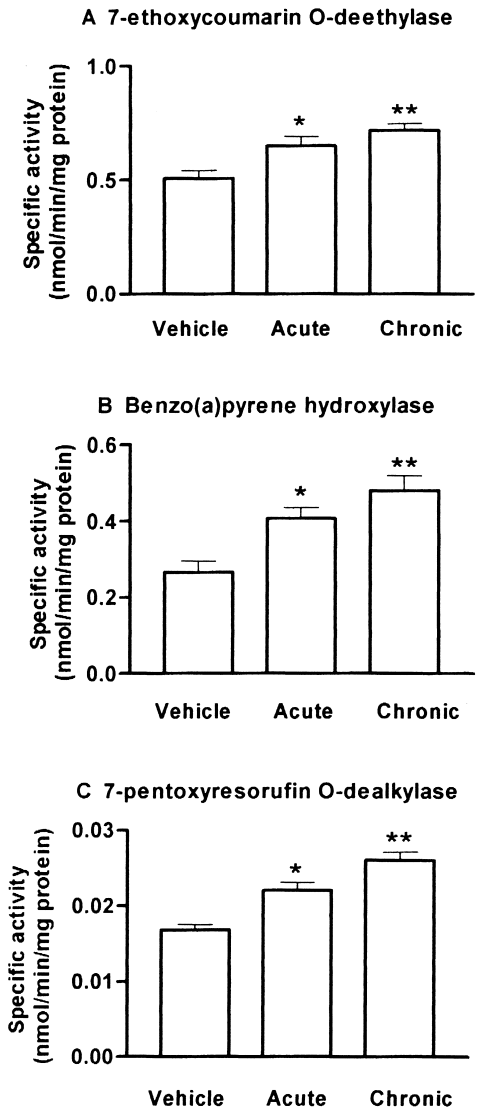

Fig. 2. Effect of acute and 15-day administration of anandamide (20 mg/kg i.p.) on liver cytochrome *P*450-linked monooxygenase enzyme activities. 7-Ethoxycoumarin *O*-deethylase (A), benzo(*a*)pyrene hydroxylase (B) and penthoxyresorufin *O*-dealkylase (C) activity was fluorimetrically assayed as described in Section 2. Values are means ± S.E.M. for 10–16 rats. Statistical differences were assessed by one-way ANOVA followed by Tukey's test. $*P<0.05$, $**P<0.01$, significant as compared to vehicle-treated rats.

2.3. Animal treatment

For the acute treatment, rats received the vehicle of anandamide (4% Tween 80 in saline solution) for 14 days, and on the 15th day, an i.p. injection of 20 mg/kg (0.1 ml/hg) anandamide. To induce behavioral tolerance, anandamide was injected i.p. once a day for 15 days at a dose of 20 mg/kg, as previously described (Costa et al., 2000). Control rats received equivalent volumes of the vehicle alone for 15 days. Rats were killed by rapid decapitation 30 min after the last injection of anandamide or vehicle. The livers and brains were quickly and carefully removed and washed with ice-cold saline solution. Tissues were homogenized in four volumes of ice-cold 0.15 M KCl. The microsomes were prepared according to Mitoma's procedure modified by Uemura et al. (1977). Microsomal pellets were stored at − 80 °C until analyzed.

2.4. Microsomal enzyme assays

The liver and brain microsomal content of cytochromes *P*450 and b_5 was measured spectrophotometrically according to Omura and Sato (1964) and Garfinkel (1958), respectively. NADPH cytochrome *P*450 reductase activity was measured spectrophotometrically by its NADPH cytochrome *c* reductase activity according to Omura and Takesue (1970). The following mixed-function oxidase cytochrome *P*450-mediated enzymes in the liver microsomes were determined fluorimetrically by referenced methods: benzo(*a*)pyrene hydroxylase according to Nebert and Gelboin (1968); 7-ethoxycoumarin *O*-deethylase according to Greenlee and Poland (1978); 7-pentoxyresorufin *O*-dealkylase according to Burke et al. (1985). Microsomal protein concentration was assayed by the method described by Lowry et al. (1951) with bovine serum albumin as standard.

2.5. Sodium dodecyl sulfate (SDS)-PAGE and immunoblotting

Microsomal proteins were diluted to obtain 15 μg of liver proteins and 80 μg of brain proteins in Laemmli buffer (0.3 M Tris–HCl, pH 6.8, containing 10% SDS; 50% glycerol; 5% dithiothreitol; 0.05% bromophenol blue) and heated at 100 °C for 4 min. Microsomal proteins were separated on 10/20-cm separating gels (mini gels or standard gels, respectively) containing 12.5% and 7.5% acrylamide, respectively. Proteins were electrophoretically stacked and separated at constant voltage, 120 V for 2 h for liver

samples and 100 V overnight at 4 °C for brain samples. Relative molecular weights were determined using prestained molecular weight standards. Proteins were transferred to nitrocellulose membranes by the semidry method for 20 and 45 min for liver and brain, respectively, at 15 V at room temperature. The membranes were incubated overnight at room temperature with 5% nonfat dry milk in phosphate buffer solution + Tween (PBST): Na_2HPO_4 16 mM, NaH_2PO_4 1.9 mM, NaCl 6.7 M, pH 7.5, 0.1% Tween 20 (blocking solution). Nitrocellulose membranes were then washed five times (5, 5, 15, 5 and 5 min) with PBST and incubated with primary antibodies (1:1000 in blocking solution) for 2 h at room temperature, with shaking. The primary antibodies were then removed and membranes were washed as described previously. Secondary antibody (1:1500 in 3% blocking solution) was then added and incubated for 1 h, with shaking; the secondary antibody was removed and membranes were washed with PBST, as above described. Membranes were then incubated for 20 s with enhanced chemiluminescence detection reagents and exposed to Hyperfilm for periods varying between 20 s and 1.5 min. Films were developed with Kodak D19 developer (25 °C, 4 min) and fixed in Kodak Unifix (8 min) and rinsed with water. The gray level of the bands was quantified by an image analysis system consisting of a video camera (Hamamatsu) connected to an Apple Macintosh II personal computer. Public domain Image 1.47 software was used (National Institute of Health). Each band was traced with the mouse cursor control and the light transmittance was determined as the gray level. The gray level of densitometric measurements was calculated after subtraction of the film background density. Gray levels obtained by densitometric analysis for treated rats are expressed as percentages of the levels for the respective vehicle-treated rats.

2.6. Statistical analysis

Data represent the means ± S.E.M. Statistical significance of the data was determined by either one-way analysis of variance (ANOVA) followed by comparison with Tukey's method for parametric results or Kruskal–Wallis ANOVA followed by Rayan's test for nonparametric results (the relative densitometric values). $P < 0.05$ was taken to indicate statistical significance.

3. Results

3.1. Effects of acute and chronic anandamide treatment on liver microsomal cytochrome P450 system

An increase in the content and activity of the liver microsomal oxidoreductive cytochrome *P*450 cycle was seen after both acute and prolonged administration of the endocannabinoid anandamide in rats (Fig. 1). In fact, the total *P*450 content significantly increased by about 26% after acute and 33% after chronic treatment (Fig. 1(A)). The cytochrome b_5 content did not change either after acute or chronic endocannabinoid treatment (Fig. 1(B)). The activity of an essential component of the monooxygenase system,

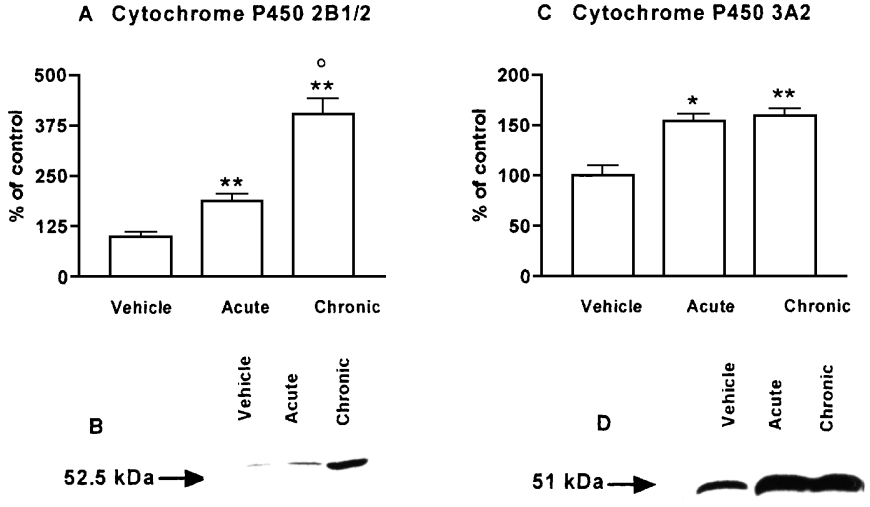

Fig. 3. Effect of acute and 15-day administration of anandamide (20 mg/kg i.p.) on cytochrome *P*450 isozyme levels in rat liver microsomes. Immunoblot analysis of cytochrome *P*450 2B1/2 (A) and 3A2 (C) proteins was performed by electrophoresis on SDS-PAGE. Proteins were transferred to nitrocellulose membranes and probed with anti-cytochrome *P*450 2B1/2 and anti-cytochrome *P*450 3A2 antibodies and visualized by using enhanced chemiluminescence, as described under Section 2. Typical autoradiograms of cytochrome *P*450 2B1/2 (B) and 3A2 (D) are shown. Each lane was loaded with 15 μg of proteins. Data denote relative levels of expression determined by comparison with levels in vehicle-injected rats, determined by computer-programmed densitometric with NIH Image programs. Each value represents mean ± S.E.M. for three to five rats. Statistical differences were assessed by Kruskal–Wallis ANOVA applied on the medians followed by Rayan's test. $*P < 0.05$, $**P < 0.01$, significant as compared to vehicle-treated rats; $°P < 0.05$, significant as compared to acute anandamide-treated rats.

NADPH cytochrome *P*450 reductase, increased by about 23% after acute and by about 40% after chronic treatment (Fig. 1(C)). In the same figure, the relative abundance of NADPH cytochrome *P*450 reductase is shown. The Western immunoblot analysis of liver microsomal proteins, using rabbit polyclonal antibody raised to liver rat NADPH cytochrome *P*450 reductase, showed a comparable increase in gray intensity of microsomal protein from acutely and chronically anandamide-treated rats (Fig. 1(E)). The statistical evaluation of data for all treated animals was done by using a nonparametric ANOVA applied to data obtained as arbitrary units of optical density and normalized as percentages over the mean of the corresponding values for the vehicle-injected groups. The relative abundance of protein increased by about 61% after single and by 92% after repeated injection, but again this difference in the percent increase between acute and chronic treatment did not reach statistical significance (Fig. 1(D)). As shown in Fig. 2, the activity of the cytochrome *P*450-associated enzymes was also increased by single and repeated treatment. The activity of 7-ethoxycoumarin *O*-deethylase (Fig. 2(A)) was increased by about 28% and 41%, respectively; that of the cytochrome *P*450 1A-mediated benzo(*a*)pyrene hydroxylase (Fig. 2(B)) by about 50% and 80%, respectively; and that of cytochrome *P*450 2B-mediated 7-pentoxyresorufin *O*-dealkylase (Fig. 2(C)) by about 28% and 40%, respectively. The increase in enzyme activity induced by chronic anandamide was higher than that induced by acute anandamide, even if this difference was not statistically significant. To evaluate whether this increase in total cytochrome *P*450 content was due to an increase in specific isozyme content, we studied particularly the relative abundance of the 2B, 1A and 3A cytochrome *P*450 isozymes (Fig. 3), because these isoforms are principally involved in anandamide metabolism in vitro (Bornheim et al., 1995). We showed that the level of cytochrome *P*450 1A1/2 immunoreactive protein, constitu-

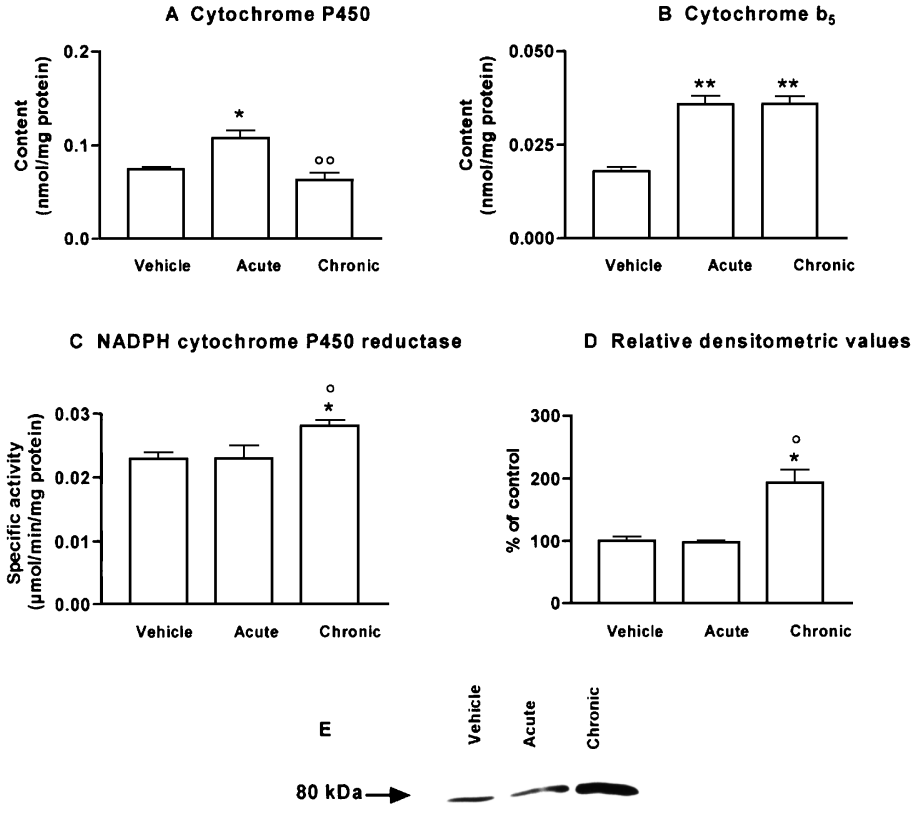

Fig. 4. Effect of acute and 15-day administration of anandamide (20 mg/kg i.p.) on total cytochrome *P*450 and b_5 content and on NADPH cytochrome *P*450 reductase specific activity in rat brain microsomes. The content of cytochromes *P*450 (A) and b_5 (B) and NADPH cytochrome *P*450 reductase activity (C) were spectrophotometrically determined as described in Section 2. Values are means ± S.E.M. for 6–10 rats. Statistical differences were assessed by one-way ANOVA followed by Tukey's test. $*P<0.05$, $**P<0.01$, significant as compared to vehicle-treated rats; $°P<0.05$, $°°P<0.01$, significant as compared to acute anandamide-treated rats. (D) Immunoblot analysis of brain NADPH cytochrome *P*450 reductase protein was performed by electrophoresis on SDS-PAGE. Proteins were transferred to nitrocellulose membranes and probed with anti-NADPH cytochrome *P*450 reductase antibodies and visualized by using enhanced chemiluminescence, as described in Section 2. The representative immunoblot (E) shows NADPH cytochrome *P*450 reductase protein levels in brain microsomes isolated from rats treated with vehicle or with anandamide acutely and chronically. Each lane was loaded with 80 µg of proteins. Data denote relative levels of expression determined by comparison with levels of expression in vehicle-injected rats, determined by computer-programmed densitometric analysis with NIH Image programs. Each value represents mean ± S.E.M. for four to eight rats. Statistical differences were assessed by Kruskal–Wallis ANOVA applied to the medians followed by Rayan's test. $*P<0.05$, significant as compared to vehicle-treated rats; $°P<0.05$, significant as compared to acute anandamide-treated rats.

tively present in liver, was not changed by either acute or chronic treatment with anandamide (data not shown). The acute and chronic treatment with endocannabinoid increased the level of cytochrome *P*450 2B1/2 immunoreactive protein (Fig. 3(B)). The relative densitometric values were significantly enhanced by about 90% and 300% versus vehicle after single and repeated doses, respectively (Fig. 3(A)). These increases were significantly different from each other. Also, the level of the constitutive cytochrome *P*450 3A2 isozyme was increased (Fig. 3(D)) by single and repeated treatment with cannabinoid by about 50% and 60%, respectively (Fig. 3(C)).

3.2. Effects of acute and chronic anandamide treatment on brain microsomal cytochrome P450 system

Only the acute treatment with endocannabinoid increased the total cytochrome *P*450 content (by about 45%) of brain microsomes, whereas the chronic treatment did not change it significantly (Fig. 4(A)). Both the acute and chronic treatments induced a large increase (100%) in the cytochrome b_5 content (Fig. 4(B)). Finally, only the repeated treatment with cannabinoid significantly increased the activity of NADPH cytochrome *P*450 reductase (22%) (Fig. 4(C)). This increase in activity could be explained by the increase (93%) in the relative abundance of the brain microsomal protein (Fig. 4(D) and (E)).

4. Discussion

Results from this study demonstrate for the first time that anandamide administration in rats increases the expression and the activity of liver cytochrome *P*450. Bornheim and Correia (1989) and Bornheim et al. (1994) reported that specific cytochrome *P*450 isozymes (2B, 3A and 2C) were induced in liver microsomes when cannabidiol, a major non-psychoactive constituent of marijuana (Hollister, 1973), was chronically administered to mice at 120 mg/kg i.p. for 4 days, and that the increased content of cytochrome *P*450 2B was consistent with the increase in cytochrome *P*450 2B mRNA and in cytochrome *P*450 2B-specific activity (7-pentoxyresorufin *O*-dealkylase activity). The effect of anandamide treatment to increase total liver cytochrome *P*450 content was associated with an increase in the level of immunoreactive proteins, namely the isozymes 2B and 3A of cytochrome *P*450, and in cytochrome *P*450-mediated enzyme activity, especially cytochrome *P*450 2B-mediated 7-pentoxyresorufin *O*-dealkylase activity. In addition, anandamide administration altered the liver activity and expression of NADPH cytochrome *P*450 reductase, an essential component of the monooxygenase system. The increases in *P*450 2B and NADPH cytochrome *P*450 reductase expression were much greater than the increases in the activity of 7-pentoxyresorufin *O*-dealkylase and NADPH cytochrome *P*450 reductase, probably because these proteins were not necessarily active and because other enzymes might depend on the cytochrome *P*450 2B isoform and on NADPH cytochrome *P*450 reductase protein. In our hands, treatment of rats with the classical cytochrome *P*450 2B inducer, phenobarbital (80 mg/kg, i.p., 4 days), increased the cytochrome *P*450 2B by 2000% but increased cytochrome *P*450 2B-mediated 7-pentoxyresorufin *O*-dealkylase activity by only 336% (data not shown). Bornheim et al. (1994) showed that the inducer cannabidiol increased cytochrome *P*450 2B content and activity by more than 50-fold over control values in mice, but in rats increased the amount of immunoquantified cytochrome *P*450 2B by only 9-fold and cytochrome *P*450 2B-catalyzed 7-pentoxyresorufin *O*-dealkylase activity by 7-fold (Bornheim et al., 1994). The same authors showed also that the 2-fold increase in cytochrome *P*450 3A mRNA detected after repeated cannabidiol administration was consistent with the increase in the amount of immunoquantified cytochrome *P*450 3A protein, but not with the increase in cytochrome *P*450 3A activity, suggesting inactivation of the induced cytochrome *P*450 3A. The repeated cannabidiol treatment increased the cytochrome *P*450 2C mRNA content 2-fold, but did not increase either the amount of cytochrome *P*450 2C or its activity.

The specific isoforms of cytochrome *P*450 induced by treatment with the endocannabinoid anandamide were those responsible for anandamide metabolism in vitro by mouse liver microsomes (Bornheim et al., 1993). In fact, in liver microsomes from mice pretreated with cannabidiol, which inactivates specific cytochrome *P*450s belonging to the 2C and 3A subfamilies (Bornheim and Correia, 1991), a decrease in anandamide metabolite production was shown (Bornheim et al., 1993). Bornheim et al. (1995) showed that mouse liver microsomal cytochrome *P*450 in vitro metabolized anandamide to 20 different metabolites, and that the cytochrome *P*450 isozymes involved in anandamide metabolism were principally 3A, 2B and 1A, even if the last isoform had a minor role.

This increase in the liver cytochrome *P*450 system, observed in anandamide-treated rats, could be ascribed to anandamide per se, but also to arachidonic acid, which results from the breakdown of anandamide (Deutsch and Chin, 1993) and which is a known substrate of the cytochrome *P*450 system (Capdevila et al., 1992). Recent and preliminary results exclude this hypothesis, because intraperitoneal treatment of rats with an equimolar dose of arachidonic acid did not change significantly the cytochrome *P*450 system and particularly the content of 2B isozyme (data not shown). The small increase in the *P*450 system after the acute treatment with anandamide in as little as 30 min after endocannabinoid treatment suggests that this increase may not be due to enzyme induction, which typically requires 12–24 h, but may be related to some other phenomena, such as altered degradation or higher yield of enzyme in the microsomal preparation. There were some differences in the increase in the cytochrome *P*450 system after acute and repeated anandamide: the enhance-

ment induced by acute anandamide was smaller than that after prolonged treatment, particularly regarding the increase in cytochrome *P*450 2B1/2 content. On this basis we cannot exclude that the increase in the liver cytochrome *P*450 system after prolonged exposure to anandamide could be related to the development of tolerance, with the particular involvement of the cytochrome *P*450 2B1/2 isozyme.

Since anandamide has been identified as a lipid constituent of the brain and since the physiological effects of cannabinoids are believed to be mediated through binding to brain cannabinoid CB_1 receptors, anandamide metabolism at the brain level may be of relevance to the physiological actions of the endocannabinoid (as well as in tolerance development). Furthermore, enhancement of the cytochrome *P*450 system in the brain could have profound effects not only on the pharmacological responses to cannabinoids but also on the metabolism of exogenous (psychoactive drugs, nicotine, ethanol) and endogenous (neural steroids, neurotransmitters) compounds. Bornheim et al. (1995) demonstrated that anandamide is metabolized by mouse brain microsomes in vitro into two principal metabolites, hydroxylated or epoxidized. The antibody against the hepatic cytochrome *P*450 3A isoform partially inhibited the formation of only one of these metabolites, thus cytochrome *P*450 3A seems to have a minor role in anandamide metabolism in the brain. We showed that a single administration of anandamide increased both the function and the content of the cytochrome *P*450 system in brain microsomes. In fact, the content of cytochromes *P*450 and b_5 and the activity and relative abundance of NADPH cytochrome *P*450 reductase increased. We hypothesize that this increase in cytochrome *P*450 could represent an early and regulatory local protective response against the sudden increase in the cerebral concentration of the endocannabinoid. To explain the results obtained at a brain level after chronic anandamide treatment, namely that was no change in the content of cytochrome *P*450 and an increase in the activity and relative abundance of NADPH cytochrome *P*450 reductase, we hypothesized that the cytochrome *P*450-mediated increased degradation of anandamide in the liver after chronic treatment lowered the plasma concentration of anandamide, and consequently the amount of anandamide which reaches the brain. Surprisingly, the cytochrome b_5 content significantly increased after acute and chronic anandamide administration. Two homologues of cytochrome b_5 were recently identified in pig brain microsomes (Yoo, 1997); one of these was present only in brain and differed from the hepatic cytochrome b_5. The brain cytochrome b_5 had a maximum absorbance at wavelength 425.42 nm, whereas that of liver cytochrome b_5 was at 426.48 nm. Thus, cerebral cytochrome b_5 may have a different amino acid sequence and, hence, different functions. Cytochrome *P*450-catalyzed oxidation of endogenous as well as exogenous substrates has been reported to occur in the mammalian brain (Warner et al., 1989; Miyairi et al., 1988; Perrin et al., 1990), so brain cytochrome *P*450 may play a role in the metabolism of centrally active compounds (Ravindranath et al., 1989). These include the biotransformation of diverse drugs, such as morphine, codeine, benzamphetamine (Minn et al., 1991). It is unclear whether the expression and activity of cytochrome *P*450 in the brain are subject to regulation, as has been demonstrated for liver cytochrome *P*450. Studies indicate that the cytochrome *P*450 in the brain may be induced following chronic administration of phenobarbital (Strobel et al., 1989; Anandatheerthavarada et al., 1990) and nicotine in rats (Anandatheerthavarada et al., 1993). Since the total cytochrome *P*450 content was not changed after chronic treatment with anandamide, but the activity of NADPH cytochrome *P*450 reductase was significantly increased, we think that it is possible that repeated treatment with anandamide results in the induction of a specific cytochrome *P*450 isozyme at the expense of others, resulting in no net increase in total cytochrome *P*450 content. Our future aim will be to measure the content of different cytochromes *P*450 in distinct brain regions by using semi-quantitative methods, such as RT-PCR, because of the low level of the various isoforms.

In conclusion, this work shows that the cytochrome *P*450 system in both liver and brain was increased by single and chronic administration of anandamide. A certain physiological relevance needs to be mentioned, even if the observed increase in the cytochrome *P*450 system was small, because the levels of pharmacologically active endocannabinoid in tissues may change during certain physiological or pathological responses and other drugs may interfere with the biosynthesis or degradation of endogenous anandamide. In the future, anandamide or other analogs may be administered therapeutically in single and repeated doses. Leweke et al. (1999) measured elevated levels of anandamide in schizophrenic patients, and Di Marzo et al. (2000a) found increased levels in the external layer of the globus pallidus in an animal model of Parkinson's disease. Anandamide accumulates in cultured immature neurons following neuronal damage due to glutamate excitoxicity. In vivo, in neonatal rat models characterized by widespread neurodegeneration as a consequence of altered glutamatergic neurotransmission, cortical anandamide concentration increased 13-fold 24 h after intracerebral NMDA injection, and less severe insults triggered by mild concussive head trauma or NMDA receptor blockade produced a less pronounced anandamide accumulation (Hansen et al., 2001). Many authors have found a striking increase in the anandamide content both in vivo and in vitro and even in patients (Kondo et al., 1998; Varga et al., 1998; Wagner et al., 1997; Wang et al., 2001). Bisogno et al. (1998) reported high levels of anandamide in human breast cancer and rat pheocromocytoma cells, and more recently Pagotto et al. (2001) showed that human pituitary adenomas had a higher content of anandamide than did the normal hypophysis.

A study by Di Marzo et al. (2000b) showed that chronic treatment of rats with THC resulted in higher amounts of anandamide in the limbic forebrain than in vehicle-treated

rats, so the authors (Di Marzo et al., 2000b) suggested that it was possible that dopamine released in the nucleus accumbens following chronic administration of THC or more potent drugs of abuse, such as morphine and alcohol (Tanda et al., 1997), stimulated the formation of anandamide in this region, thus opening the way to the possibility that anandamide or drugs derived from anandamide could be used in the treatment of depression and related nervous disturbances.

Recently, Quistad et al. (2001) showed that organophosphorus pesticides induced the accumulation of anandamide in the mouse brain, because they inhibit fatty acid amide hydrolase activity.

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References

Anandatheerthavarada, H.K., Shankar, S.K., Ravindranath, V., 1990. Rat brain cytochrome *P*450: catalytic, immunochemical properties and inducibility of multiple forms. Brain Res. 536, 339–343.

Anandatheerthavarada, H.K., Williams, J.F., Wecker, L., 1993. The chronic administration of nicotine induces cytochrome *P*450 in rat brain. J. Neurochem. 60, 1941–1944.

Bisogno, T., Katayama, K., Melck, D., Ueda, N., De Petrocellis, L., Yamamoto, S., Di Marzo, V., 1998. Biosynthesis and degradation of bioactive fatty acid amides in human breast cancer and rat pheochromocytoma cells—implications for cell proliferation and differentiation. Eur. J. Biochem. 254, 634–642.

Bornheim, L.M., Correia, M.A., 1989. Purification and characterization of a mouse liver cytochrome *P*-450 induced by cannabidiol. Mol. Pharmacol. 36, 377–383.

Bornheim, L.M., Correia, M.A., 1991. Purification and characterization of the major hepatic cannabinoid hydroxylase in the mouse: a possible member of the cytochrome *P*-450IIC subfamily. Mol. Pharmacol. 40, 228–234.

Bornheim, L.M., Kim, K.Y., Chen, B., Correia, M.A., 1993. The effect of cannabidiol on mouse hepatic microsomal cytochrome *P*450-dependent anandamide metabolism. Biochem. Biophys. Res. Commun. 197, 740–746.

Bornheim, L.M., Everhart, E.T., Li, J., Correia, M.A., 1994. Induction and genetic regulation of mouse hepatic cytochrome *P*450 by cannabidiol. Biochem. Pharmacol. 48, 161–171.

Bornheim, L.M., Kim, K.Y., Chen, B., Correia, M.A., 1995. Microsomal cytochrome *P*450-mediated liver and brain anandamide metabolism. Biochem. Pharmacol. 50, 677–686.

Burke, M.D., Thompson, S., Elcombe, C.R., Halpert, J., Haaparanta, T., Mayer, R.T., 1985. Ethoxy-, pentoxy- and benzyloxyphenoxazones and homologues: a series of substrates to distinguish between different induced cytochromes *P*-450. Biochem. Pharmacol. 34, 3337–3345.

Capdevila, J.H., Falck, J.R., Estabrook, R.W., 1992. Cytochrome *P*450 and the arachidonate cascade. FASEB J. 6, 731–736.

Costa, B., Parolaro, D., Colleoni, M., 1996. Chronic cannabinoid, CP-55,940, administration alters biotransformation in the rat. Eur. J. Pharmacol. 313, 17–24.

Costa, B., Vailati, S., Colleoni, M., 1999. SR141716A, a cannabinoid receptor antagonist, reverses the behavioural effects of anandamide-treated rats. Behav. Pharmacol. 10, 327–331.

Costa, B., Giagnoni, G., Colleoni, M., 2000. Precipitated and spontaneous withdrawal in rats tolerant to anandamide. Psychopharmacology 149, 121–128.

Deutsch, D.G., Chin, S.A., 1993. Enzymatic synthesis and degradation of anandamide, a cannabinoid receptor agonist. Biochem. Pharmacol. 46, 791–796.

Devane, W.A., Hanus, L., Breuer, A., Pertwee, R.G., Stevenson, L.A., Griffin, G., Gibson, D., Mandelbaum, A., Etinger, A., Mechoulam, R., 1992. Isolation and structure of a brain constituent that binds to the cannabinoid receptor. Science 258, 1946–1949.

Di Marzo, V., Bisogno, T., De Petrocellis, L., Melck, D., Martin, B.R., 1999. Cannabimimetic fatty acid derivatives: the anandamide family and other "endocannabinoids". Curr. Med. Chem. 6, 721–744.

Di Marzo, V., Hill, M.P., Bisogno, T., Crossman, A.R., Brotchie, J.M., 2000a. Enhanced levels of endogenous cannabinoids in the globus pallidus are associated with a reduction in movement in an animal model of Parkinson's disease. FASEB J. 14, 1432–1438.

Di Marzo, V., Berrendero, F., Bisogno, T., Gonzalez, S., Cavaliere, P., Romero, J., Cebeira, M., Ramos, J.A., Fernandez-Ruiz, J.J., 2000b. Enhancement of anandamide formation in the limbic forebrain and reduction of endocannabinoid contents in the striatum of Δ^9-tetrahydrocannabinol-tolerant rats. J. Neurochem. 74, 1627–1635.

Fride, E., 1995. Anandamides: tolerance and cross-tolerance to Δ^9-tetrahydrocannabinol. Brain Res. 697, 83–90.

Garfinkel, D., 1958. Studies on pig liver microsomes: I. Enzymic and pigment composition of different microsomal fractions. Arch. Biochem. Biophys. 77, 493–509.

Greenlee, W.F., Poland, A., 1978. An improved assay of 7-ethoxycoumarin *O*-deethylase activity: induction of hepatic enzyme activity in C57BL/6J and DBA/2J mice by phenobarbital, 3-methylcholantrene and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. J. Pharmacol. Exp. Ther. 205, 596–605.

Hansen, H.H., Schmid, P.C., Bittigau, P., Lastres-Becker, I., Berrendero, F., Manzanares, J., Ikonomidou, C., Schmid, H.H., Fernandez-Ruiz, J.J., Hansen, H.S., 2001. Anandamide, but not 2-arachidonoylglycerol, accumulates during in vivo neurodegeneration. J. Neurochem. 78, 1415–1427.

Hollister, L.E., 1973. Cannabidiol and cannabinol in man. Experientia 29, 825–826.

Kondo, S., Sugiura, T., Kodaka, T., Kudo, N., Waku, K., Tokumura, A., 1998. Accumulation of various *N*-acylethanolamines including *N*-arachidonoylethanolamine (anandamide) in cadmium chloride-administered rat testis. Arch. Biochem. Biophys. 354, 303–310.

Leweke, F.M., Giuffrida, A., Wurster, U., Emrich, H.M., Piomelli, D., 1999. Elevated endogenous cannabinoids in schizophrenia. NeuroReport 10, 1665–1669.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 265–275.

Minn, A., Ghersi-Egea, J.F., Perrin, R., Leininger, B., Siest, G., 1991. Drug metabolizing enzymes in the brain and cerebral microvessels. Brain Res. Rev. 16, 65–82.

Miyairi, S., Sugita, O., Sassa, S., Fishman, J., 1988. Aromatization and 19-hydroxylation of androgens by rat brain cytochrome *P*450. Biochem. Biophys. Res. Commun. 150, 311–315.

Munro, S., Thomas, K.L., Abu-Shaar, M., 1993. Molecular characterization of a peripheral receptor for cannabinoids. Nature 365, 61–65.

Nebert, D.W., Gelboin, H.V., 1968. Substrate-inducible microsomal aryl hydroxylase in mammalian cell culture. J. Biol. Chem. 243, 6250–6261.

Omura, T., Sato, R., 1964. The carbon monoxide-binding pigment of liver microsomes: I. Evidence for its hemoprotein nature. J. Biol. Chem. 239, 2370–2378.

Omura, T., Takesue, S., 1970. A new model for simultaneous purification of cytochrome b_5 and NADPH-cytochrome *c* reductase from liver microsomes. J. Biochem. 67, 249–257.

Pagotto, U., Marsicano, G., Fezza, F., Theodoropoulou, M., Grubler, Y., Stalla, J., Arzberger, T., Milone, A., Losa, M., Di Marzo, V., Lutz, B., Stalla, G.K., 2001. Normal human pituitary gland and pituitary adeno-

mas express cannabinoid receptor type 1 and synthesize endogenous cannabinoids: first evidence for a direct role of cannabinoids on hormone modulation at the human pituitary level. J. Clin. Endocrinol. Metab. 86, 2687–2696.

Perrin, R., Minn, A., Ghersi-Egea, J.F., Grassiot, M.C., Siest, G., 1990. Distribution of cytochrome *P*450 activities towards alkoxyresorufin derivatives in rat brain regions, subcellular fractions and isolated cerebral microvessels. Biochem. Pharmacol. 40, 2145–2151.

Quistad, G.B., Sparks, S.E., Casida, J.E., 2001. Fatty acid amide hydrolase inhibition by neurotoxic organophosphorus pesticudes. Toxicol. Appl. Pharmacol. 173, 48–55.

Ravindranath, V., Anandatheerthavarada, H.K., Shankar, S.K., 1989. Xenobiotic metabolism in human brain—presence of cytochrome *P*-450 and associated mono-oxygenases. Brain Res. 496, 331–335.

Rubino, T., Massi, P., Patrini, G., Venier, I., Giagnoni, G., Parolaro, D., 1994. Chronic CP-55,940 alters cannabinoid receptor mRNA in the rat brain: an in situ hybridization study. NeuroReport 5, 2493–2496.

Rubino, T., Viganò, D., Costa, B., Colleoni, M., Parolaro, D., 2000. Loss of cannabinoid-stimulated guanosine 5′-*O*-(3-[^{35}S]thiotriphosphate binding without receptor down-regulation in brain regions of anandamide-tolerant rats. J. Neurochem. 75, 2478–2484.

Strobel, H.W., Cattaneo, E., Adesnik, M., Maggi, A., 1989. Brain cytochromes *P*-450 are responsive to phenobarbital and tricyclic amines. Pharmacol. Res. 21, 169–175.

Tanda, G., Pontieri, F.E., Di Chiara, G., 1997. Cannabinoid and heroin activation of mesolimbic dopamine transmission by a common mu 1 opioid receptor mechanism. Science 276, 2048–2050.

Uemura, T., Chiesara, E., Cova, D., 1977. Interaction of epinephrine metabolites with the liver microsomal electron transport system. Mol. Pharmacol. 13, 196–215.

Varga, K., Wagner, J.A., Bridgen, D.T., Kunos, G., 1998. Platelet- and macrophage-derived endogenous cannabinoids are involved in endotoxin-induced hypotension. FASEB J. 12, 1035–1044.

Wagner, J.A., Varga, K., Ellis, E.F., Rzigalinski, B.A., Martin, B.R., Kunos, G., 1997. Activation of peripheral CB1 cannabinoid receptors in haemorrhagic shock. Nature 390, 518–521.

Wang, Y., Liu, Y., Ito, Y., Hashiguchi, T., Kitajima, I., Yamakuchi, M., Shimizu, H., Matsuo, S., Imaizumi, H., Maruyama, I., 2001. Simultaneous measurement of anandamide and 2-arachidonoylglycerol by polymixyn B-selective adsorption and subsequent high-performance liquid chromatography analysis: increase in endogenous cannabinoids in the sera of patients with endotoxic shock. Anal. Biochem. 294, 73–82.

Warner, M., Stromstedt, M., Moller, L., Gustafsson, J.A., 1989. Distribution and regulation of 5*a*-androstane-2*b*,17*b*-diol hydroxylase in the rat central nervous system. Endocrinology 125, 2699–2706.

Yoo, M., 1997. Identification of two homologous cytochrome b_5s in rat brain. Biochem. Biophys. Res. Commun. 236, 641–642.