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Potentiation of the inductive effect of phenobarbital on cytochrome P450 mRNAs by cannabidiol*

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Marijuana is known to prolong the sleep induced by some barbiturates in animals. The major cannabinoid responsible for this effect is cannabidiol (CBD) [1], a component of marijuana which is devoid of psycho-pharmacological activity, but which exhibits anticonvulsant activity [2]. In animals it has been demonstrated that administration of CBD inhibited the metabolism of barbiturates and other drugs, including tetrahydrocannabinol (THC), at the level of the mixed-function oxidase system [3–5]. The mechanism by which this occurs is not clear although it has been proposed that there may be inhibition of enzymatic activity or an actual reduction of hepatic microsomal cytochrome P450 [6, 7]. In human studies, prolonged administration of CBD also was found to inhibit the metabolism of other drugs (antipyrine and barbiturates) metabolized by liver mixed-function oxidase enzymes [8].

Cloning techniques have resulted in the synthesis of DNA complementary (cDNA) to cytochrome P450 mRNAs allowing quantitation of constitutive and inducible forms of the messages [9]. Using these reagents as probes in hybridization procedures, it has been shown previously that the induction of the cytochrome P450IIB1/2 (P450b and P450e) genes by phenobarbital is mediated by increased levels of mRNA from 25- to 100-fold [10, 11]. We report here that although cannabidiol by itself produced no effect on P450IIB1/2 mRNA levels, the combination of cannabidiol and phenobarbital resulted in the superinduction of these mRNAs.

It is also known that phenobarbital treatment induces cytochrome P450IIC7 enzyme levels in immature rats [12]. We report that phenobarbital treatment induced hepatic cytochrome P450IIC7 (P450f) mRNA in immature rats and that the combination of cannabidiol and phenobarbital potentiated this effect at a suboptimal inducing dose of phenobarbital. Although many studies have been undertaken on the cannabinoids and the cytochrome P450s at the enzymatic level, this is the first which focuses on the regulation of specific cytochrome P450 mRNAs.

* A preliminary report on part of this work was presented at the Third Annual International Cannabinoid Study Group Meeting, Richmond, VA, 1990, and at Marijuana '90: An International Conference on Cannabis and Cannabinoids, Crete, Greece, 1990.

Experimental Procedures

Animals and treatment. Male Sprague–Dawley rats, weighing approximately 140 g (6 weeks old), were injected intraperitoneally daily for 5 days with high (100 mg/kg) or low (30 mg/kg) dose phenobarbital. In the combined treatment the animals were co-administered, daily for 5 days, phenobarbital (high or low dose) and cannabidiol (25 mg/kg) dissolved in 50 μL ethanol, and killed approximately 20 hr after the last injection. Alternatively, animals were pre-injected with CBD (25 mg/kg) daily for 4 days and with phenobarbital (100 mg/kg) on day 5. Other animals received CBD (25 mg/kg) in ethanol daily for 5 days or just the ethanol vehicle (control) daily for 5 days.

Isolation of RNA. Livers were dissected and cut into small pieces which were immediately frozen in liquid nitrogen and subsequently ground to a fine powder, under nitrogen, in a large mortar and pestle. The powdered tissue was homogenized with a polytron in a guanidinium thiocyanate solution and total mRNA was purified by cessium chloride density gradient centrifugation using a modification [13] of the procedure of Chirgwin *et al.* [14] and Glisin *et al.* [15].

Analytical procedures. For Northern blot analysis [16], RNA was separated on 1.4% agarose gels containing formaldehyde and transferred to nitrocellulose or nylon membranes for hybridization with the cDNA probes followed by autoradiography. The recombinant plasmids containing the cDNA inserts were radiolabeled by nick-translation with a ^{32}P nucleotide (New England Nuclear or Bethesda Research Laboratories). Quantitative analysis was performed directly on the membranes using the Ambis Radioanalytical Imaging System. This system has a detector composed of 896 elements that simultaneously detect multiple beta emissions. By moving the sample beneath the detector, counts are recorded from approximately 65,000 discrete locations on the nylon membrane used for hybridization. Sample patterns are recorded in a computer file which is then used for production of a composite picture and quantitation. Dot-blot hybridization experiments were conducted with purified RNA according to the procedure of White and Bancroft [17] using a Hybridot Manifold apparatus (Bethesda Research Laboratory). A semi-quantitative assessment of mRNA was made by visual inspection of the dot-blot autoradiogram: the relative

mRNA levels were estimated by determining the number of 2-fold serial dilutions of the experimental samples needed to yield comparable intensity to the control mRNA.

The following cDNA probes were employed in this study: (1) the cDNA probe R17 which is specific for the major forms of cytochrome P450 mRNA inducible by phenobarbital. This clone represents a cDNA containing exons 6–9 of P450IIB2 mRNA. It recognizes and measures sequences of both cytochrome P450IIB1 and P450IIB2 mRNA. Therefore, all results obtained using R17 reflect levels of both P450IIB1 and P450IIB2 mRNA [18]; (2) the cDNA clone pTF-1 which is specific for a constitutive mRNA [19] whose protein product is cytochrome P450IIC7 or a very closely (> 97%) related protein; and (3) the rat serum albumin cDNA clone (pRSA 13) [20], provided by Dr. C. Glackin (Phytogen, Pasadena, CA), which was employed as a control probe to test the integrity of the rat liver RNA preparations.

Results

Dot-blot analysis for P450IIB1/2 mRNA was performed on the isolated RNA from six groups of control and drug-treated 140 g rats using the radiolabeled R17 plasmid (Fig. 1A). As expected, both the high and low dose phenobarbital treatments (groups III and V, respectively) had dramatically higher levels of P450IIB1/2 mRNA than did the control animals group (I). The animals receiving CBD (II) showed no greater levels of mRNA than the controls did. It is striking, however, that the phenobarbital-mediated induction of cytochrome P450IIB1/2 mRNA, at both high (III) and low (V) dose, was potentiated by the co-administration of CBD (IV and VI, respectively). The potentiation appeared to be approximately 2-fold in each case. Using another injection regimen, similar results were obtained for animals preinjected daily for 4 days with CBD prior to a single phenobarbital injection on day 5. These

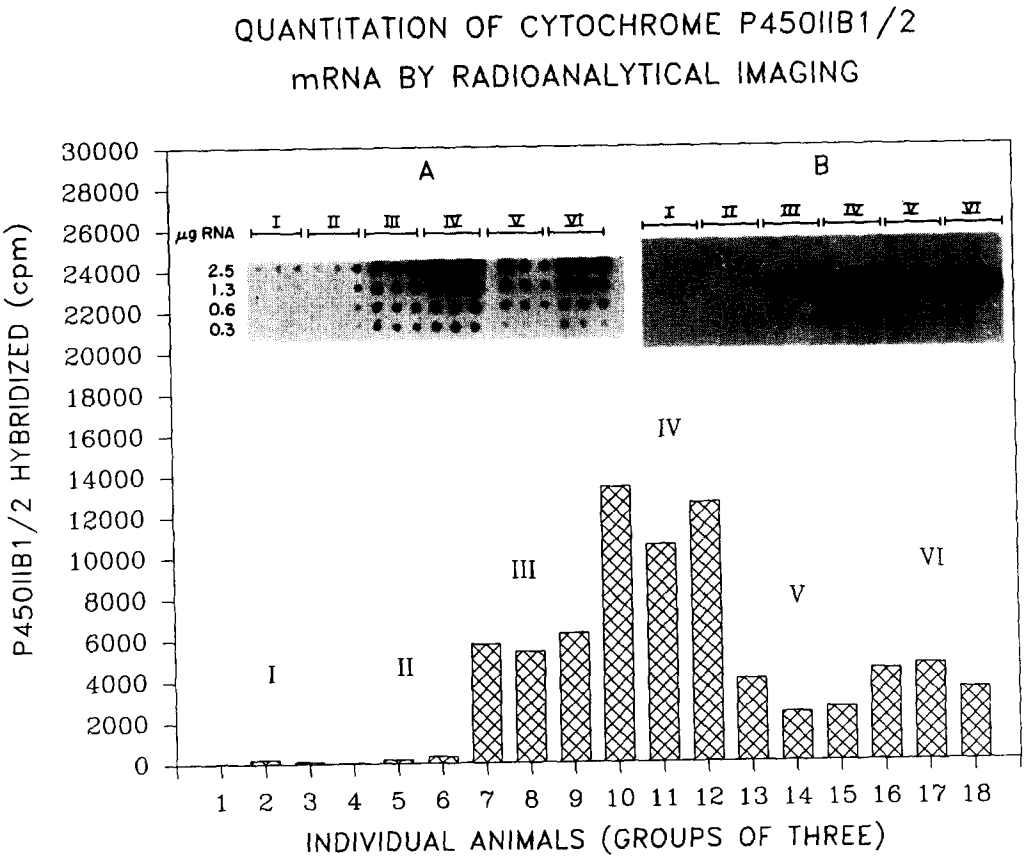

Fig. 1. Analysis of cytochrome P450IIB1/2 mRNA. Hybridization was with the radiolabeled R17 cDNA probe. Each treatment group had three animals. Group I (control) received the ethanol vehicle daily for 5 days. Group II (CBD) received CBD (25 mg/kg) in ethanol for 5 days. Group III (high dose phenobarbital) received phenobarbital (100 mg/kg) daily for 5 days. Rats in Group IV (CBD + high dose phenobarbital) were co-administered CBD (25 mg/kg) and phenobarbital (100 mg/kg) daily for 5 days. Group V (low dose phenobarbital) received phenobarbital (30 mg/kg) daily for 5 days. Rats in Group VI (CBD + low dose phenobarbital) were co-administered CBD (25 mg/kg) and phenobarbital (30 mg/kg) daily for 5 days. (A) Dot-blot hybridization. Sequential 2-fold serial dilutions of purified rat liver RNA (2.5 μg) were applied directly to nitrocellulose filters. Groups I–IV and groups V plus VI were blotted and hybridized on two separate membranes. The graph depicts radioanalytical imaging of the Northern-blot (B) for hepatic cytochrome P450IIIB1/2 mRNA. To obtain values that were within a linear range, results were derived from radioanalytical analyses of samples run on two separate Northern-blots.

animals, which received CBD before the PB treatment, showed approximately a 2-fold greater level of cytochrome P450IIB1/2 mRNA induction than did the animals treated with phenobarbital alone when examined by dot-blot analysis. It was also shown by dot-blot analysis that ethanol, the vehicle used for CBD injection, did not have any effect upon the levels of cytochrome P-450IIB1/2 mRNA (data not shown).

The CBD potentiation of the PB induction of P450IIB1/2 mRNA was confirmed using the Northern-blot procedure and quantitative analysis by radioanalytical imaging for the six groups of animals (Fig. 1B, Table 1). The control animals (I) and the animals receiving CBD (II) barely had sufficient levels of cytochrome P450IIB1/2 mRNA for the hybridization signal to be detected on the autoradiogram. Confirming the results of the dot-blots, treatment with 100 mg/kg of phenobarbital (III) dramatically induced the cytochrome P450IIB1/2 mRNA (approximately 60-fold). Induction also occurred with a dose of 30 mg/kg of phenobarbital (V), but to a lesser extent (approximately 30-fold). The novel observation seen on the dot-blots was confirmed on the Northern-blot; that is, co-administration of cannabidiol with either the 100 mg/kg (IV) or the 30 mg/kg (VI) dose of phenobarbital resulted in an even greater hybridization signal than observed with the corresponding dose of phenobarbital alone. That is, CBD potentiated the effect of phenobarbital by a factor of approximately 2.1 when co-injected with high dose PB and 1.4 with low dose PB (see Table 1). The combination of PB and CBD induced P450IIB1/2 mRNA approximately 42- and 120-fold for the low and high dose PB, respectively, relative to the control. CBD by itself exerted no significant effect relative to the control. Because of the low amounts of P450IIB1/2 mRNA of the uninduced animals, the calculated fold induction relative to these values are only approximations.

Parallel experiments to those performed for cytochrome P450IIB1/2 mRNA were undertaken using a cDNA probe (pTF-1) for a constitutive P450 mRNA (cytochrome P450IIC7 mRNA). The results of the dot-blot analysis for cytochrome P450IIC7 mRNA are shown in Fig. 2A. As expected for this constitutive cytochrome P450, the control animals (I) had appreciable levels of cytochrome P450IIC7 mRNA. The animals treated with CBD (II) appeared to have levels of P450IIC7 mRNA approximately equal to or slightly less than the control, while both high and low doses of PB induced this message (III and V, respectively). However, no enhanced induction was seen when CBD was co-administered with high doses of PB (IV). That is, unlike the case with P450IIB1/2, P450IIC7 did not exhibit potentiation at high doses of PB + CBD. At low doses of phenobarbital plus CBD (VI), on the other hand, there appeared to be some slight potentiation.

Quantitation of cytochrome P450IIC7 mRNA levels from the different groups of animals was also determined by radioanalytical imaging analysis of a Northern-blot using the radiolabeled pTF-1 plasmid (Fig. 2, Table 1). Confirming the dot-blots, it was found that the control animals (I) had appreciable levels of cytochrome P450IIC7 mRNA as did the animals treated with CBD (II). Also, as observed on the dot-blots, both high and low dose phenobarbital treatment resulted in a modest induction of cytochrome P450IIC7 mRNA. Co-administration of cannabidiol with high dose phenobarbital (IV) did not result in a greater hybridization signal than observed with the high dose of phenobarbital alone (III). Co-administration of cannabidiol with low dose phenobarbital (VI) did result in a slight superinduction of cytochrome P450IIC7 mRNA compared to low dose phenobarbital alone (V). The ethidium bromide staining of the gel indicated that the preparations were undegraded with distinct 18S and 28S bands (Fig. 2B) and that the message had the appropriate mobility for a 2kb fragment.

From Table 1, it can be calculated that CBD potentiated the effect of low dose phenobarbital by a factor of

Table 1. Cytochrome P450IIB1/2 and cytochrome P450IIC7 mRNA levels

		P450IIB1/2		P450IIC7	
Treatment group	Animal	cpm*	Ave.	cpm*	Ave.
I. Control	1	24	98	2,944	2,703
	2	185		2,772	
	3	84		2,392	
II. CBD (25 mg/kg)	4	25	175	2,244	2,011
	5	173		2,128	
	6	328		1,660	
III. PB (100 mg/kg)	7	5,762	5,810	5,065	6,995
	8	5,397		7,000	
	9	6,271		8,920	
IV. PB (100 mg/kg) CBD (25 mg/kg)	10	13,397	12,177	5,280	3,939
	11	10,549		3,020	
	12	12,586		3,516	
V. PB (30 mg/kg)	13	3,986	2,986	4,916	4,024
	14	2,367		3,480	
	15	2,605		3,676	
VI. PB (30 mg/kg) CBD (25 mg/kg)	16	4,437	4,204	4,608	5,788
	17	4,687		5,312	
	18	3,487		7,444	

* Counts per minute (cpm) were obtained from radioanalytical imaging data for cytochrome P450IIB1/2 (Fig. 1) and cytochrome P450IIC7 (Fig. 2).

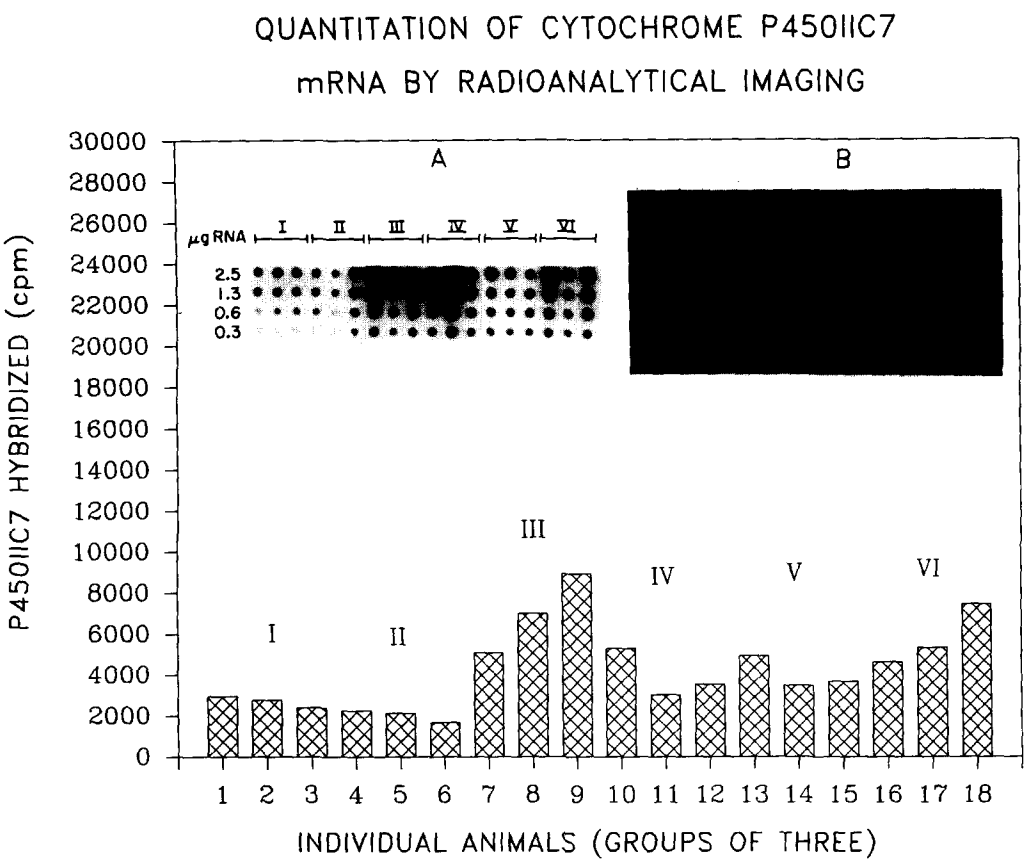

Fig. 2. Analysis of cytochrome P450IIC7 mRNA. (A) Dot-blot hybridization with the pTF-1 cDNA probe. Sequential 2-fold serial dilutions of purified rat liver RNA (2.5 μg) were applied directly to the nitrocellulose filters. Groups I–IV and groups V and VI were blotted and hybridized on two separate membranes. The graph depicts radioanalytical imaging of a Northern-blot for cytochrome P450IIC7 mRNA employing the radiolabeled plasmid pTF-1. Samples within a group were normalized relative to albumin mRNA to adjust for sample loading as follows: average albumin values (for each group of three animals) were divided by individual values to obtain normalization factors which were then in turn multiplied by the P450IIC7f readings to obtain normalized values for the eighteen animals. (B) an ethidium bromide stained gel of the individual RNA preparations from eighteen animals (six groups of three) used for quantitative analysis. See legend of Fig. 1 for a description of each of the treatment groups.

approximately 1.4. The induction of P450IIC7 by PB relative to the control animals was found to be approximately 1.5- and 2.6-fold at low and high dose, respectively.

Discussion

It has been demonstrated (for reviews see Adesnik and Atchison [9] and Okey [21]) that treatment of rats with phenobarbital markedly induced the hepatic cytochrome P450IIB1/2 mRNA. In this study, the combination of phenobarbital and cannabidiol were evaluated for their ability to modulate the levels of the two liver cytochrome P450 mRNA subfamilies P450IIB1 + IIB2 and P450IIC7. We report here the novel observation that although administration of cannabidiol alone did not lead to enhanced P450IIB1/2 mRNA levels, pretreatment with cannabidiol or the co-administration of cannabidiol with phenobarbital (high or low dose) resulted in the superinduction of cytochrome P450IIB1/2 mRNA. The probe employed in this study, R17, hybridizes with the two inducible cytochrome P450 mRNAs P450IIB1 and P450IIB2 which have the same enzymatic specificity and differ in only 13 of 491 total amino acids [22].

Although this is the first report regarding the effect of CBD upon cytochrome P450IIB1/2 mRNA, other studies have noted the effect of CBD upon cytochrome P450 isozymes. As mentioned previously, CBD treatment has been shown to inhibit the mixed-function oxidase system and to decrease cytochrome P450 content. In contrast to our results in rats, it was shown in mice that with repetitive CBD treatment, an isozyme is induced which is immunologically similar to the phenobarbital-inducible isozyme found in rats [23, 24].

We also observed that injection of a high or low dose of phenobarbital resulted in a modest increase in the level of the "constitutive" cytochrome P450IIC7 mRNA. Although it was shown previously that with pTF-1 as a probe, cytochrome P450IIC7 mRNA levels do not change significantly in concentration after phenobarbital treatment in mature rats [25], our results are consistent with those showing that phenobarbital treatment causes a modest induction of cytochrome P450IIC7 enzyme levels in immature rats, which were used in this study, but not in adults [12]. In this regard, it is noteworthy that Barroso *et al.* [26] cloned a cDNA that displays 98.4% sequence

similarity to P450IIC7 cDNA. Northern-blot analysis of liver RNA utilizing this cDNA clone, which clearly must hybridize to P450IIC7 mRNA, revealed a significant induction of complementary RNA in immature rats. Taken together, these results indicate that the ability of cytochrome P450IIC7 to be induced by xenobiotics is age-related. It may very well be that the loss of phenobarbital inducibility of P450IIC7 mRNA as rats mature is a consequence of the age-dependent increase in basal mRNA levels [12, 19]. In the case of cytochrome P450IIC7 mRNA, co-administration of cannabidiol and high dose phenobarbital had no greater effect than phenobarbital alone. However, the combination of cannabidiol and low dose phenobarbital did result in a small increase of cytochrome P450IIC7 mRNA levels compared to treatment with only low dose phenobarbital. These results for the inducible and this constitutive mRNA indicate that a component of marijuana, cannabidiol, interacts with the mixed-function oxidase system to affect the levels of cytochrome P450 messenger ribonucleic acids.

We have no information on the mechanism by which CBD potentiates the inductive effect of phenobarbital on cytochrome P450 mRNA. A hypothetical mechanism to explain these results resides at the level of metabolism and gene regulation. It is known that CBD inhibits cytochrome P450 enzymatic activity and may actually reduce the hepatic microsomal cytochrome P450 levels [6, 7]. Furthermore, CBD has been shown to inhibit metabolism of other drugs by the liver mixed-function oxidase enzymes [3–5]. Therefore, a straight-forward hypothesis would be that CBD causes a decrease (or inhibition) in the cytochrome P450 enzymes and this results in slower metabolism of phenobarbital and, thus, the resulting higher level of phenobarbital (compared to the animals only treated with PB) could result in enhanced transcriptional activation.

It is known that the mechanism for induction by phenobarbital is the increased rate of P450IIB1 and P450IIB2 gene transcription [27, 28]. Therefore, another hypothetical mechanism is that CBD may interact with some transcription factors or accessory proteins in such a way that it enhances transcription in the presence of phenobarbital. Alternatively, CBD could post-transcriptionally act to stabilize mRNA or to enhance the processing efficiency of P450IIB1/2 nuclear transcripts without affecting gene transcription. Indeed, numerous instances of post-transcriptional regulation of P450 mRNA accumulation have been documented [reviewed in Ref. 29]. Moreover, dexamethasone enhancement of P450IIB1/2 induction by phenobarbital has been attributed to a glucocorticoid-mediated stabilization of nuclear transcripts [30].

In summary, the combination of phenobarbital and cannabidiol was evaluated for their ability to modulate the levels of the two liver cytochrome P450 mRNA subfamilies P450IIB1/2 and P450IIC7. Treatment of rats with either a low or a high dose of phenobarbital induced the hepatic cytochrome P450IIB1/2 mRNA. We report here the novel observation that co-administration of cannabidiol with phenobarbital resulted in the superinduction of cytochrome P450IIB1/2 mRNA. Cannabidiol by itself, unlike the treatment with phenobarbital or phenobarbital plus cannabidiol, did not have an effect upon the levels of cytochrome P450IIB1/2 mRNA.

Injection with phenobarbital also resulted in a modest increase in the level of cytochrome P450IIC7 mRNA. Co-administration of cannabidiol with a high dose of phenobarbital had no greater effect than phenobarbital alone. However, the combination of cannabidiol and a low dose of phenobarbital did potentiate slightly the effect of phenobarbital on cytochrome P450IIC7 mRNA levels. These results, taken as a whole, indicate that a component of marijuana, cannabidiol, interacts with the mixed-function oxidase system to affect the levels of two subfamilies of cytochrome P450 messenger ribonucleic acids.

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* *Department of Biochemistry and Cell Biology*
State University of New York at Stony Brook
Stony Brook, NY 11794, U.S.A.
‡ *Department of Cell Biology*
New York University School of Medicine
New York, NY, U.S.A.

DALE G. DEUTSCH*†
EUGENE R. TOMBLER*
JAMES E. MARCH*
STEPHEN H. C. LO*
MILTON ADESNIK‡

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† Correspondence: Dale G. Deutsch, Ph.D., Department of Biochemistry and Cell Biology, Life Sciences Building, SUNY at Stony Brook, Stony Brook, NY 11794–5215.

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Effect of cyclosporin A *in vivo* on taurocholate uptake by rat hepatocytes*

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Cyclosporin A (CsA†) is a fungal metabolite that has proved to be a potent immunosuppressant, treatment with which has resulted in the significantly improved survival of kidney, liver and heart allograft patients [1, 2]. However CsA therapy is associated with various side effects, mainly affecting the kidney [3] and liver [4, 5]. The most common abnormalities related to hepatotoxicity both in humans and experimental animals are elevations of bilirubin and bile acid in blood [6, 7]. These occur without evidence of liver cell damage as necrosis and increases in serum liver enzymes are essentially absent [7–10]. The ability of CsA to cause elevation in SBA level has been linked to its ability to interfere with bile acid transport by hepatocytes [5, 11, 12] and hepatocyte membrane vesicles [13]. However, most of the data that support this mechanism are from *in vitro* studies [5, 11, 12]. Furthermore the reports that show rises in SBA in rats treated with CsA used high doses and long duration protocols [7, 14, 15]. While the nature of inhibition of CsA *in vitro* seems to be direct it is possible that there may be an indirect effect *in vivo*. To determine whether small, therapeutically relevant doses of CsA as well as large doses administered for a short duration can produce changes in the levels of serum bilirubin and SBA, it was decided to investigate the effects of different concentrations of CsA on these parameters. In addition, the uptake pattern of taurocholate by hepatocytes isolated from CsA-treated rats was also determined to see if this correlated with elevated SBA levels in similarly treated rats.

* Disclaimer: The conclusions reached and scientific views expressed in this paper are solely those of the authors. They do not necessarily reflect the views and policies of the organization in which they work.

† Abbreviations: CsA, Cyclosporin A; SBA, serum bile acids; HEPES, 4-(2-hydroxymethyl)-1-piperazineethanesulfonic acid; AST, aspartate aminotransferase; ALT, alanine aminotransferase; AP, alkaline phosphatase.