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SPIN STATE STUDIES ON CYTOCHROME P-450 IN LIVER MICROSOMES FROM OBESE AND DIABETIC ANIMALS

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The spin state of liver microsomal cytochrome P-450 from obese mice and streptozotocin-diabetic mice and rats has been studied both by the temperature and the type I substrates-induced spectral changes. The high spin cytochrome P-450 is significantly decreased in these animals. Moreover absolute spectra indicate that low spin cytochrome P-450 is stabilized in streptozotocin induced-diabetic animals. Thus the physiopathological state may modify the in vivo spin state of cytochrome P-450 and modifications of the microsomal fatty acid composition might contribute to these changes.

Both spontaneous diabetes (ob/ob mice) and streptozotocin (STZ)-induced diabetes modify the phospholipid composition of hepatic microsomal membranes (1, 2). Since Lu and Coon (3), phospholipids are considered as one of the components of the mixed-function oxidase system and, in fact, their presence is absolutly required to reconstitute the full activity of purified cytochrome P-450 with reductase (4). It was suggested that the lipid bilayer holds together the cytochrome P-450 and reductase, in a functional complex, facilitating the rate of electron transfer (5). Studies on LM2 cytochrome P-450 have shown that dilauryl-phosphatidylcholine induces a conformational change of the hemeprotein and a shift of the spin equilibrium towards the high spin state (6). These data prompted us to investigate the spin state of cytochrome P-450 from diabetic animals.

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MATERIAL AND METHODS

Normal and genetically diabetic (ob/ob) male mice of the C57BL/6 strain, 10 week-old, were purchased from the "Centre d'Elevage du CNRS, Orléans, France". STZ-diabetic mice were obtained by a single intraperitoneal injection of STZ (200 mg/kg, freshly dissolved in NaCl pH 4.5). Male Sprague-Dawley rats, 2 month-old, were made diabetic by a single intravenous injection of STZ (80 mg/kg). Diabetic rats and mice were killed 2 weeks later. Phenobarbital (PB) and 3-methylcholanthrene (MC) induced mice received intraperitoneal a daily dose (80 mg/kg) of either PB in saline for 4 days or MC in corn oil for 3 days. Liver microsomal membranes were prepared (7) then resuspended in 0.1 M phosphate buffer pH 7.4 and immediately used. Absolute spectra were obtained by the addition of linoleic acid hydroperoxide (7.5×10^{-4} M in final) to the reference cuvette as described by Nerland et al. (8). The temperature-induced spectral changes were recorded at 2°C intervals (reference cuvette maintened at 20° C) and absorbance (390-420 nm) analysed as indicating by Cinti et al. (9) to assess the spin state of cytochrome P-450. The magnitude of the shift towards the high spin state induced by increasing substrate concentration was inferred from the maximal amount of high spin induced at saturating substrate concentration, as compared with the total cytochrome P-450 assayed by the method of Omura and Sato (10). The former was calculated by the relation of Beer-Lambert using $\Delta\epsilon$(385-419 nm) = 126 mM^{-1} cm^{-1} (9) and ΔAmax (intersection with Y-axis in the Lineweaver-Burk plot of ΔA as a function of substrate concentration) (11).

Microsomal fatty acids were analysed by the modified technique described by Aubourg et al. (12) from 100 μl microsome samples with heptadecanoic acid (100 μM) as internal standard. After extraction and evaporation to dryness, the residue was dissolved in 100 μl of a mixture of N,O-bis-(trimethylsilyl)-trifluoroacetamide/pyridine (1:1), and heated at 60°C for 5 minutes. The following chromatographic conditions were used: 210° C, with immediate temperature program of 5° per minute. The mass spectrometric apparatus and conditions were the same as previously described (13). The fatty acids quantifications were run by selected ion monitoring of the [M - 15] fragments of the trimethylsilyl derivatives and area ratios compared to pre-established calibration curves.

Total phospholipids were estimated on a dried-down sample of chloroform/methanol (2:1 v/v) lipid extract. Mineralisation was performed with 0.2 ml of pure H_2SO_4 and 1 ml of 10% $HClO_4$ (w/v) at 250°C for 60 min, in pyrex tubes. The inorganic phosphate released was assayed by the Fiske-Subbarow method modified by Bartlett (14).

RESULTS

Calculated from the temperature-induced spectral changes (in absence of exogenous substrates), the high spin state of cytochrome P-450 is about 43% in normal mice and decreased to about 17% in both ob/ob and STZ-mice (table 1). By similar procedures of calculations we have verified that 3-methylcholanthrene treatment of mice led to a significant increase of the high spin state (78%), as expected. The STZ-induced diabetes in rats also produces a decrease of high spin state.

Table 1

Spin state of liver microsomal cytochrome P-450 from diabetic animals estimated by the temperature-induced spectral changes

	mice	rats
Control animals	42.7 ± 10.2	31.6 ± 7.9
Ob/Ob mice	17.8 ± 5.4	/
STZ-diabetic animals	17.3 ± 2.2	22.9 ± 5.1

Results are expressed as percentage of high spin cytochrome P-450 at 20°C in absence of exogenous substrates. Mean ± SEM of 3 to 6 animals.

The extent of the spin equilibrium shifts towards the high spin state of cytochrome P-450 induced by type I substrates (benzphetamine and cyclohexane) is reported in table 2. Benzphetamine induces similar shifts in normal, ob/ob and STZ-mice (between 12 to 18%) and a slightly more important effect in phenobarbital-treated mice (23%). Cyclohexane produces shifts in normal and ob/ob mice identical to that of benzphetamine (about 15%) while in STZ-diabetic mice it is only of 8%.

Table 2

Substrate-induced spin state shifts of liver microsomal cytochrome P-450 from different mice

	benzphetamine	cyclohexane*
Control mice	11.4 ± 2.4	16.5
Ob/Ob mice	18.5 ± 4.9	13.9
STZ-diabetic mice	14.0 ± 0.5	7.9
PB-treated mice	23.4 ± 3.9	14.2

The range concentration for benzphetamine was from 1 to 10×10^{-5} M, excepted with microsomes from phenobarbital (PB)-treated animals where it was from 1 to 10×10^{-6}M. The range concentration for cyclohexane was from 1 to 10×10^{-4} M. Results are expressed as percentage of the substrate-induced high spin at 20° C. Mean ± SEM of 4 animals excepted *, mean of 2 animals.

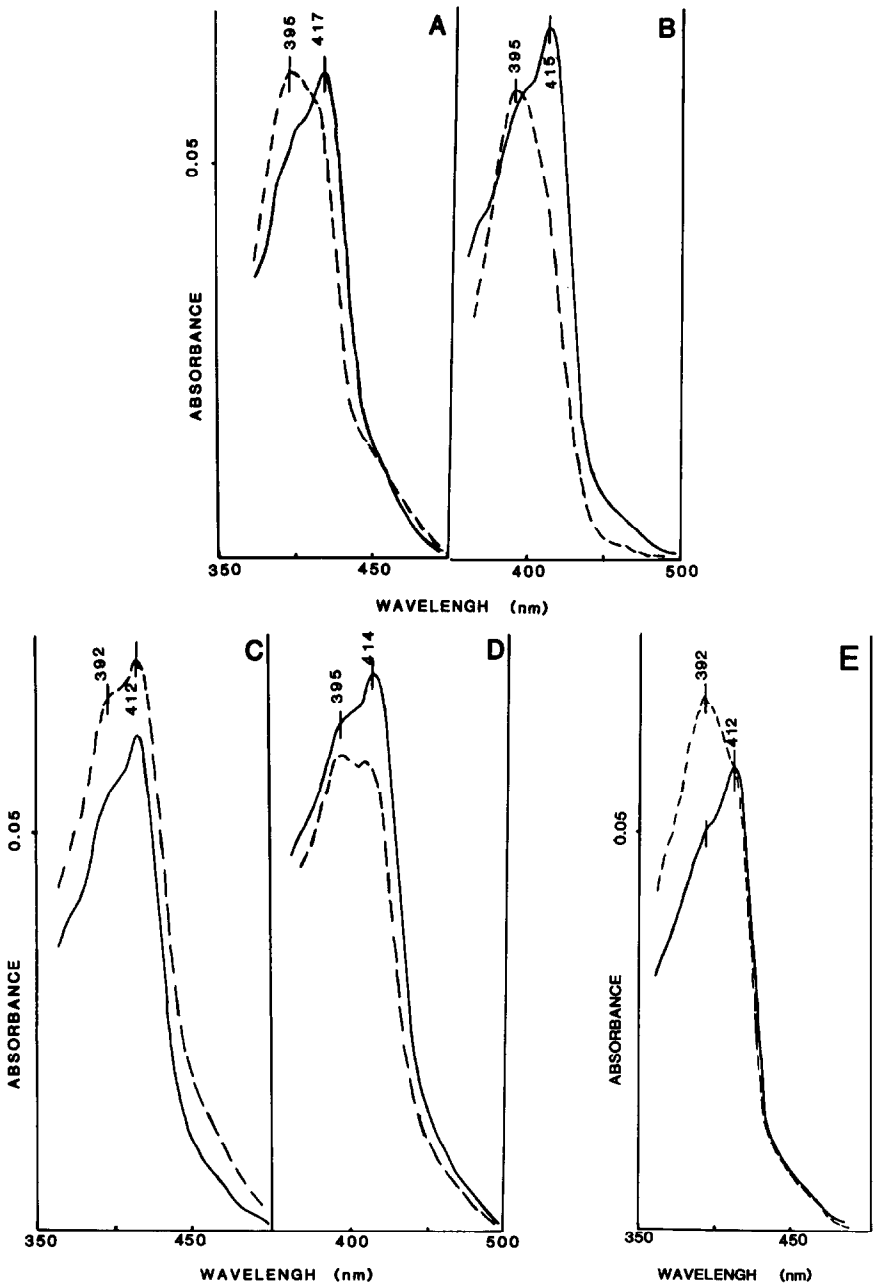

Fig. 1: Absolute (—) and type I substrate-induced (- - -) spectra of liver microsomal cytochrome P-450 from normal mice (A), PB-treated (B), STZ-diabetic mice (C and D) and ob/ob mice (E). The spectral changes were obtained in A, B, C and E by cyclohexane (2.5×10^{-3}M) and benzphetamine (2.5×10^{-3}M) in D.

All absolute spectra of liver microsomal cytochrome P-450 exhibit a peak at 415-417 nm and a shoulder at 395 nm (figure 1). When cyclohexane is added (2.5×10^{-3} M) to microsomes from phenobarbital-treated mice

(figure 1B), a unique absorbance peak is observed at 395 nm; with microsomes from normal or ob/ob mice, the increase of the 395 nm peak is obvious but a shoulder remains at 415 nm (figure 1A and 1D); with microsomes from STZ-diabetic mice, in spite of an increase of the 395 nm peak, the 415 nm peak remains the more important one, even with subsequent additions of cyclohexane (figure 1C). Benzphetamine (2.5×10^{-3} M) added to microsomes from STZ-diabetic mice induces a more important shift since the absorbance at 395 nm is identical to that at 415 nm (figure 1D).

The fatty acid composition of microsomal lipids is strongly modified in ob/ob mice, with important decreases of C16:1 and C18:2 while the other fatty acids increase 2 to 5-fold. In STZ-diabetic mice the main result is the decrease of C16:1 level. Total phospholipids content remains unchanged (Table 3).

Table 3
Fatty acid composition of total microsomal phospholipids from liver of normal and different diabetic mice

	Control mice	Ob/Ob mice	STZ-diabetic mice
Phospholipids *	325 ± 37	360 ± 12	371 ± 42
Fatty acids **			
C16:0	1991 ± 369	2196 ± 241	2131 ± 532
C16:1	124 ± 38	20 ± 2	24 ± 9
C18:0	495 ± 135	880 ± 147	539 ± 20
C18:1	226 ± 30	750 ± 83	175 ± 50
C18:2	188 ± 15	26.1 ± 0.4	115 ± 28
C20:3	73 ± 11	347 ± 43	66 ± 14
C20:4	128 ± 33	316 ± 35	166 ± 36

* : μg/mg protein, mean ± SEM of 6 animals
** : nmole/mg protein, mean ± SEM of 3 animals

DISCUSSION

The in vivo high spin cytochrome P-450 is decreased in diabetic animal (STZ-mice or rats and ob/ob mice) as judged by the temperature-induced spectral changes. As a consequence, the type I substrate-induced shift towards the high spin form is increased in diabetic mice by addition of benzphetamine. This effect is peculiarly obvious with microsomes from phenobarbital-treated mice, since pretreatment by phenobarbital is known to induce low spin cytochromes P-450 (15).

Thus, this study shows that diabetes, associated with either insulin resistance (ob/ob mice) or insulinopenia (STZ-diabetic mice), modify the in vivo spin state of cytochrome P-450. This study extends to pathological states the previously reported modifications of the spin state in some peculiar physiological conditions: starvation (16) and pregnancy (17).

As the high spin of cytochrome P-450 possesses a higher redox potential than the low spin state form (18), its reduction by NADPH cytochrome P-450 reductase is easier, and biotransformation of substrates starts owing to the initial amount of high spin cytochrome P-450. Hence a change in this level might explain, at least partially, some of the alterations previously reported (19) of the drug metabolism by liver microsomes from ob/ob mice. In STZ-diabetic animals this effect might occur besides the synthesis of peculiar forms of cytochrome P-450 as reported for mice (20) and rats (21).

Absolute spectra indicate that low spin cytochrome P-450 is stabilized in STZ-diabetic animals in this state (but not in ob/ob mice) upon addition of exogenous substrates and confirm that benzphetamine is a much more efficient type I substrate to induce the equilibrium shift than cyclohexane. In spite of a similar total amount of phospholipids, modifications of the fatty acid composition may be responsible for these spin state stabilisations.

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