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REGIO- AND STEREOSELECTIVE REGULATION OF MONOOXYGENASE ACTIVITIES BY ISOENZYME-SELECTIVE PHOSPHORYLATION OF CYTOCHROME P450

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The phosphorylation of the two major phenobarbital-inducible cytochrome P450 isoenzymes IIB1 and IIB2 was increased in hepatocytes by the action of the membrane permeating cAMP derivatives N^6-dibutyryl-cAMP and 8-thiomethyl-cAMP. Under these conditions the dealkylation of 7-pentoxyresorufin, a selective substrate of cytochrome P450IIB1 and P450IIB2 was markedly reduced. 16β-Hydroxylation of testosterone which is catalyzed specifically only by cytochrome P450IIB1 and IIB2 was strongly reduced; for 16α-hydroxylation which is also catalyzed by cytochrome P450IIB1 and IIB2 but additionally by 3 further cytochrome P450 isoenzymes, this reduction was less pronounced; for the oxidation of the 17β-hydroxyl group which besides cytochromes P450IIB1 and IIB2 is additionally catalyzed not only by other cytochromes P450 but also by 17β-hydroxysteroid dehydrogenase there was a clear tendency of reduction which, however, no longer reached statistical significance. Hydroxylation at other positions of testosterone which are catalyzed by other cytochrome P450 isoenzymes were not significantly changed. Hence isoenzyme-selective phosphorylation of cytochrome P450 leads to a corresponding isoenzyme-selective modulation of monooxygenase activity which holds promise to be especially important as a fast regulation of the control of genotoxic metabolites.

Cytochrome P450-dependent monooxygenases play a pivotal role in the control of genotoxic metabolites and hence in a very early crucial step of chemical carcinogenesis and mutagenesis (1-4). Obviously the relative enzyme activities are very important for this control. Moreover, the isoenzyme pattern governing alternative metabolic pathways is often crucial since the various isoenzymes may compete for the same pre-carcinogenic substrate and selective changes in their activities may therefore lead to drastic shifts between activating versus detoxifying pathways with consequent dramatic changes in the control of genotoxic metabolites (5,6). Such changes may be brought about by enzyme induction (5), a process studied in many laboratories since quite a long time (3,4). We have recently discovered a potentially faster process of such

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changes in the control of genotoxic metabolites by posttranslational modification of cytochrome P450 by phosphorylation. This process is both donor- and acceptor-selective in that specificity of the phosphorylation reaction was observed with respect to both the phosphoryl-donating protein kinase species as well as the phosphoryl-accepting cytochrome P450 isoenzymes (7-9). More recently we have demonstrated, that this phosphorylation does not only take place with the cell-free preparations of the purified protein kinase and cytochrome P450 enzymes but also in intact cells (hepatocytes), and is regulated by cAMP levels which, in turn, are under hormonal control (10). In the present study we show that the treatment of hepatocytes with the membrane permeating cAMP derivatives N^6-dibuturyl-cAMP and 8-thiomethyl-cAMP which leads to phosphorylation of the two major phenobarbital-inducible cytochromes P450IIB1[1] and P450IIB2 (P450b and e) (10) leads to marked and isoenzyme-selective changes in cytochrome P450-dependent monooxygenase activities. Thus, in addition to the well-studied control of cytochrome P450 dependent monooxygenase activities by enzyme induction there exists a further isoenzyme-selective control mechanism by posttranslational protein modification by phosphorylation which holds promise to be especially important since it works much faster than enzyme induction.

MATERIALS AND METHODS

Chemicals. (^{32}P) Orthophosphate (carrier free) was purchased from New England Nuclear. N^6-dibutyryl-cAMP and 8-thiomethyl-cAMP, 7-pentoxyresorufin, testosterone, 4-androstene-3,17-dione and corticosterone were supplied by Sigma (Deisenhofen, FRG). 6β-, 7α-, 16α- and 16β-hydroxytestosterone were purchased from Steraloids (Frankfurt, FRG). 2α-, 2β- and 6α-hydroxytestosterone were obtained from the Steroid Reference Collection (Prof. Dr. D.N. Kirk, Department of Chemistry, Queen Mary College, Mile End Road, London E1 4NS). Solvents for HPLC were from Baker (Gross-Gerau, FRG). Purified resorufin was kindly provided by Boehringer Ingelheim. All other chemicals used were of highest purity commercially available.

Preparation of cell fractions and incubation with cAMP derivatives. Male Sprague-Dawley rats (180-200 g; Interfauna Süddeutsche Versuchstierfarm,

[1] To facilitate it for the reader to follow the relatively complicated cytochrome P450 nomenclature, the individual isoenzymes are (as far as possible) designated by the recommended nomenclature for their (probable) genes (11) followed in brackets by a relatively simple and relatively widely used one letter nomenclature (12) and - at there first occurrence in the paper - very briefly characterized.

Tuttlingen, FRG) were treated intraperitoneally on two consecutive days with phenobarbital (75 mg/kg body wt). Intact hepatocytes were isolated by perfusion of rat liver in situ as described previously (13).

In order to examine the phosphorylation of cytochromes P450 in intact cells, $6x10^6$ hepatocytes were incubated at 37° C in Krebs-Henseleit buffer containing 25 mM 4(2-hydroxyethyl)-1-piperazineethane-sulfonic acid (HEPES), pH 7.4, 0,5% D-glucose, insulin (10 mg/Liter), and the amino acid mixture recommended by Seglen (14). Incubation was carried out in the presence and absence of the cAMP derivatives N^6-dibutyryl-cAMP (1.0 mM) and 8-thiomethyl-cAMP (0.5 mM) for 90 minutes. After incubation the hepatocytes were sedimented by centrifugation at 50 x g for 5 min. The supernatant was discarded, the pellets were resuspended in 10 mM sodium phosphate buffer pH 7.4 containing 110 mM KCl, 5 mM EDTA, 0.1 mM NaF, 50 mM sodium pyrophosphate, 0.5 mM phenylmethylsulfonylfluoride and Trasylol (30 U/ml), homogenized using an Ultraturrax followed by sonication (Branson Sonifier Cell Disruptor B15; microtip, output control: 20-40, 40 % duty cycle) for 30 sec. The homogenate was then centrifuged at 5000 x g for 10 min, the supernatant centrifuged again at 100.000 x g for 40 min. The sediment was resuspended in 0.1 M Tris-HCl, pH 7.4 containing 20 % glycerol, 5 mM EDTA, and 1 mM dithiothreitol. Protein concentration was determined by the biuret method with a BioRad protein assay kit (BioRad Laboratories, München, FRG) using bovine serum albumin as a standard. Enzyme Assays. The O-dealkylation of 7-pentoxyresorufin was measured essentially as described by Lubet et al. (15). A 2 ml reaction mixture consisted of 50 mM Tris-HCl, pH 7.5/25 mM $MgCl_2$/10 µl of substrate (10 µM), and about 10 µl of microsomes obtained from hepatocytes. The reaction was started by addition of 12.5 µM NADPH. The dealkylation of 7-pentoxyresorufin was monitored by recording the increase in the relative fluorescence of the product of this reaction (resorufin) as a function of time. Measurements were carried out on an Aminco-SPF 500 spectrophotofluorimeter with excitation at 522 nm and emission at 586 nm.

For assaying testosterone hydroxylation the incubations contained the 100.000 x g sediment of hepatocytes equivalent to 1.3 mg protein, 0.6 mM NADP, 8 mM glucose 6-phosphate, 1.4 units of glucose-6-phosphate dehydrogenase, 5 mM $MgCl_2$, 38 mM KCl in a final volume of 2 ml 50 mM HEPES buffer (pH 7.4). This mixture was preincubated for 2 min at 37 °C. The incubation was started by the addition of 1 mM testosterone dissolved in 25 µl methanol and performed with shaking at 37 °C. The incubation was stopped with 5 ml of ice-cold dichloromethane followed by the addition of 12 nmol corticosterone (internal standard) dissolved in 100 µl methanol. After vortexing for 1 min the phases were separated by centrifugation at 1000 x g for 10 min. The aqueous phase was once more extracted with 2 ml dichloromethane. The combined organic phases were extracted with 2 ml 0.02 N NaOH followed by thrice repeated washing with 2 ml water. Then the organic phase was brought to dryness with a stream of nitrogen and stored at -20 °C until HPLC separation for which the sample was dissolved in 40 µl of methanol.

High-performance liquid chromatography was carried out with a Waters 600 Multisolvent Delivery System, Model 2140 Rapid Spectral Detector LKB Broma and a 25 cm x 4.6 mm 5 µm C-18 reverse phase analytical column (Supelco Inc.). The metabolites of testosterone were separated according to earlier studies (16). The effluent was monitored at 240 nm and the area under the absorption peaks was integrated. The metabolites were quantitated by the internal standard technique.

RESULTS AND DISCUSSION

In the previous study (10) it had been shown that treatment of hepatocytes with the membrane permeating cAMP derivatives N^6-dibuturyl-cAMP and 8-thiomethyl-cAMP leads to phosphorylation of cytochrome P450IIB1 and P450IIB2 (P450b and

e). Since these two P450 forms occur only in very low amounts in the untreated rat liver and are highly inducible by phenobarbital, the effect was observable most clearly in heptocytes from livers of phenobarbital-pretreated rats. In the present study it was investigated whether the same treatment leads to changes in monooxygenase activities.

7-Pentoxyresorufin is a selective substrate of the two cytochrome P450 forms IIB1 and IIB2 (17) which were phosphorylated under the conditions described above. Table 1 shows, that after treatment of the hepatocytes with the membrane permeating cAMP derivatives the microsomal dealkylation of 7-pentoxyresorufin was substantially (35 %) lower compared with this enzyme activity determined from an aliquot of hepatocytes from the same phenobarbital-pretreated rats incubated for the same time in the same medium but not containing the cAMP derivatives.

In order to investigate the isoenzyme selectivity of this modulation with respect to a broader range of cytochrome P450 isoenzymes, the influence of the treatment of the hepatocytes with the cAMP derivatives on the regio- and stereoselective hydroxylation of testosterone was studied. Testosterone is exquisitely suitable for such studies, since it had been demonstrated by other investigators (18,19) that the regio- and stereoselectivity of the various testosterone hydroxylation reactions is much more sharply cytochrome P450 isoenzyme selective (and in several instances apparently fully isoenzyme specific) than with more commonly used "diagnostic" but largely overlapping substrates. Thus, of all 10 purified cytochrome P450 isoenzymes investigated, only the two isoenzymes P450IIB1 and P450IIB2 which were phosphorylated in the hepatocytes treated with the cAMP derivatives catalyze the testosterone hydroxylation in position 16β (18,19). Table 1 shows that this reaction is markedly (44 %) decreased by the treatment with the cAMP derivatives. Hydroxylation of testosterone in position 16α is also catalyzed by cytochromes P450IIB1 and IIB2 (18,19), but less specifically. This reaction is also catalyzed by cytochromes P450IIC7 (P450f, constitutive form immunologically cross-reactive with P450IIB1), P450g (male specific, strain-dependent) and

Table 1

Isoenzyme-selective repression of monooxygenase activities in hepatocytes treated with cAMP derivatives

Treatment of hepatocytes	7-Pentoxyresorufin dealkylation (pmol resorufin/mg protein/min)	Testosterone metabolites[#] (nmol hydroxytestosterone/mg protein/min)						
		16β-OH	16α-OH	7α-OH	6β-OH	2β-OH	2α-OH	A
None (control)	2565 ± 81	0.81±0.10	2.27±0.20	0.49±0.07	1.10±0.21	∿ 0.1[§]	0.51±0.05	2.45±0.25
cAMP derivatives[+]	1680 ± 52* (65)*	0.45±0.07* (56)*	1.56±0.14* (68)*	0.36±0.04 (73)	0.94±0.25 (85)	∿ 0.1[§]	0.51±0.10 (100)	1.83±0.25 (74)

Microsomes were prepared from hepatocytes of phenobarbital-pretreated adult male Sprague-Dawley rats and incubated with 7-pentoxyresorufin or testosterone as described under Materials and Methods. The dealkylation of 7-pentoxyresorufin was monitored by spectrophotofluorimetry. Testosterone metabolites were resolved and quantitated by HPLC. Values represent means ± S.E.M., numbers in brackets represent percent of control.

No further testosterone metabolite peaks unequivocally above background noise were noted in the HPLC chromatogram. The abbreviations denote the hydroxylated testosterone metabolites formed, "A" represents androst-4-ene-3,17-dione, the oxidation product of the 17β-hydroxy group of testosterone.

§ Clearly identifyable metabolite, but too close to background for accurate quantification.

+ 1 mM N^6-dibutyryl-cAMP and 0.5 mM 8-thiomethyl-cAMP.

* $p < 0.05$ compared with corresponding control.

P450IIC11 (P450h, male-specific testosterone 2α/16α-hydroxylase) (18,19). The depression of the hydroxylation at this site by treatment of the hepatocytes with the cAMP derivatives is therefore "diluted" by the contribution of these other cytochrome P450 isoenzymes and hence is of a smaller magnitude (32 %) (Table 1). The third reaction of cytochromes P450IIB1 and IIB2 catalyzed on the teststerone molecule is the oxidation of the 17β-hydroxyl group leading to androst-4-ene-3,17-dione (18,19). This reaction is still less specific, in that it is not only catalyzed by a further cytochrome P450 isoenzyme (the male-specific P450IIC11, also called P450h) but also by a 17β-hydroxysteroid dehydrogenase (18,19). Hence here the decrease in activity due to the treatment of the hepatocytes with the cAMP derivatives was even more "diluted" and did not reach statistical significance at the $P < 0.05$ level (Table 1). Also at other positions no statistically significant changes in the hydroxylation of testosterone were observed (Table 1). This includes hydroxylation in 2α which is specifically catalyzed by cytochrome P450IIC11 (P450h); in 2β which is selectively catalyzed by the synthetic steroid-inducible P450IIIA1 (P450p); in 6β which is catalyzed by several cytochromes P450 (P450IIIA1, the major 3-methylcholanthrene-inducible P450IA1 also called P450c, the major isosafrol-inducible P450IA2 also called P450d and by P450g); and in 7α specifically catalyzed by the constitutive P450IIA1 (P450a) (18,19).

Hence this study shows, that those cytochrome P450-dependent monooxygenase reactions are markedly and selectively reduced by treatment of hepatocytes with membrane permeating cAMP derivatives, which are selectively catalyzed by those isoenzymes which are phosphorylated under these conditions. Short-term regulation of cytochrome P450-dependent monooxygenase activities by post-translational protein modification by phosphorylation is therefore highly promising to exert a fast and important control on genotoxic metabolites, especially in those cases in which the modulated isoenzyme competes with another isoenzyme for the same substrate and where the isoenzymes in question govern metabolic pathways of opposite (activating versus detoxifying) biological significance.

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