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Review

Phosphorylation of cytochromes P450: First discovery of a posttranslational modification of a drug-metabolizing enzyme

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Abstract

Cytochromes P450 (CYP) are important components of xenobiotic-metabolizing monooxygenases (CYP-dependent monooxygenases). Their regulation by induction, most commonly by transcriptional activation, mediated by xenobiotics, normally substrates of the corresponding CYP, is well known and has been widely studied. Our team has discovered an additional important regulation of xenobiotic-metabolizing CYPs pertaining to posttranslational modification by phosphorylation. Individual CYPs are phosphorylated by different protein kinases, leading to CYP isoenzyme-selective changes in the metabolism of individual substrates and consequent drastic changes in the control of genotoxic metabolites. Best studied are the CYP phosphorylations by the cAMP-dependent protein kinase A. Most recently, we discovered that cAMP not only leads to drastic changes in the activity of individual CYPs, but also to drastic changes in the nuclear localization of the CYP-related transcription factor Ah receptor (AHR). The consequences are very different from those of AHR nuclear translocation mediated by the classical ligands (enzyme inducers such as dioxin) and are likely to represent the long-sought physiological function of the AHR, its persistent disturbance by long-lived ligands such as dioxin may well be the reason for its high toxicity.

Keywords: CYP phosphorylation; Protein kinase A; cAMP; Ah receptor nuclear translocation

Cytochromes P450 (CYP) are the substrate binding components of the CYP-dependent monooxygenases including a great number which metabolize xenobiotics. The regulation of many of them by enzyme induction is well known and has been widely studied: most commonly, it is due to transcriptional activation and mediated by inducers which at the same time are substrates of the corresponding CYP [1].

In the early 1980s, our team was the first to discover an additional important regulation of some xenobiotic-metabolizing CYPs, namely their posttranslational modification by phosphorylation [2]. The scope of this review was to summarize what is known about this fast regulation including our most recent findings on the influence of phosphorylating conditions on the best-studied CYP-related transcription factor Ah receptor (AHR).

Establishment of the biochemical actions by the use of purified components

Combining purified CYPs with purified protein kinases, the in vitro phosphorylation of essential components of the xenobiotic-metabolizing CYP-dependent monooxygenase system was investigated [2]. A cyclic AMP (cAMP)-independent phosvitin kinase (''kinase P'') (purified from the human cell line HeLa) was not able to phosphorylate any of the investigated purified CYPs. The catalytic subunit of the cAMP-dependent protein kinase A (PKA) (purified from rat muscle) catalyzed the phosphoryl transfer from [^{32}P]ATP to rabbit CYP2B4 and to rabbit CYP reductase (CYP red). CYP2B4 was phosphorylated when alone or when in a reconstituted system in which CYP red and phos-

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phatidylcholine, a membrane lipid component important for CYP activity of reconstituted CYP-dependent monooxygenase systems, were also present. The phosphorylation of CYP red was weak and was further considerably reduced when in the presence of CYP and phosphatidylcholine. The monooxygenase constituents themselves had no kinase activity to catalyze mutual phosphorylation or autophosphorylation [2].

Thus, purified CYP2B4 is phosphorylatable by purified PKA. Serine^{128} was reported as the sole phosphoryl acceptor amino acid in rabbit CYP2B4 [3] and in its homolog in rat, CYP2B1 [4], whilst site-directed mutation of the corresponding serine in rat CYP2E1 (Ser^{129}) showed in intact hepatocytes the existence of an additional phosphoryl acceptor site in either CYP2E1 itself or in a cooperating molecule [5]. With isolated, soluble CYP2B4 and with CYP2B4 incorporated into a model membrane, the K_m value was within the same order of magnitude (2–8 μM) [3], which was similar to or even smaller than the K_m values known for other phosphorylation reactions controlling diverse enzyme activities in vivo [6,7]. It therefore appears that CYP2B4 may well be a substrate of PKA also in the physiological environment of the complex system of the intact cell. Topological localization of CYP and PKA in the same subcellular endomembrane fraction (the microsomal fraction) gave further support for this possibility. It therefore appeared worthwhile to investigate whether in the more complex but more physiological system of whole cells and whole animals some CYPs are also phosphorylated.

CYP phosphorylation in whole cells and in whole animals

CYP phosphorylation was therefore investigated in unbroken cells. Hepatocytes were used for this purpose since hepatocytes are especially rich in xenobiotic-metabolizing CYPs. PKA transfers the γ-phosphoryl group from the ATP to substrate proteins. When in order to probe whether the γ-phosphoryl group of ATP is incorporated into a protein, cells are exposed to [γ-^{32}P]ATP in the medium, and the ATP molecule does not penetrate through the cellular plasma membrane. In order to investigate the possible phosphoryl transfer to CYP in the intact cell by following the incorporation of radioactivity into the CYP molecule, the cellular pool of ATP was therefore pre-labelled by exposing the hepatocytes to [^{32}P]*ortho*-phosphate which readily enters the cells and is intracellularly transformed into [^{32}P]ATP. Hepatocytes were isolated from the livers of adult male Sprague–Dawley rats pretreated with phenobarbital (PB) in order to enrich the cells with CYP2B1 and CYP2B2 (amongst 13 investigated CYPs, these two were the best substrates for PKA in the in vitro investigations using purified components [8]). Already in the absence of stimulation of the hepatocytes by PKA, an incorporation of [^{32}P]phosphate in CYP2B4 was observed, but this incorporation was very low. After stimulation of PKA by increased levels of cAMP mediated by exogenous addition of the hormone glucagon or of the membrane permeating cAMP derivative N^6,$O^{2'}$-dibutyryl-cAMP (dbcAMP) or 8-thiomethyl-cAMP, the incorporation was drastically increased. Four CYPs were selectively phosphorylated including CYP2B1 and CYP2B2 [9]. This was shown by the autoradiography of gel electrophoretically separated proteins from the solubilized microsomes of these hepatocytes and of purified CYPs as molecular standards followed by visualization of specific antibodies. Three further teams also reported subsequently, but in the same year, CYP phosphorylation in unbroken hepatocytes [4,10,11].

Koch and Waxman [11] showed in rat liver that in the situation of the whole organism phosphorylation of CYP2B1 clearly took place already without any exogenous stimulation and was increased by dbcAMP and a phosphodiesterase inhibitor. We [12] showed that treatment of rats with clinically used doses of glucagon and theophylline was sufficient to severely (by more than 50%) decrease the predominantly CYP2B1-mediated generation of genotoxic metabolites from cyclophosphamide (CPA) already at a time point of isolation of hepatocytes of only 15 min after the treatment of rats with glucagon and theophylline. This influence on the accumulation of genotoxic metabolites from CPA in the whole animals was at least as pronounced as it was when the same modulators had been given to the medium of hepatocytes. Thus, phosphorylation of CYP2B occurs in the whole animal and the hormonal status of an organism is an important determinant of it.

Consequence of CYP phosphorylation for CYP function and the control of genotoxic metabolites

Phosphorylation of CYP2B1/2B2 in hepatocytes led to a marked decrease of the monooxygenase activity associated with these CYPs [12]. Dealkylation of CYP2B1/2B2-selective substrate 7-pentylresorufin was markedly decreased [13]. The hydroxylations at various positions of testosterone were in a regio- and stereoselective manner reduced by varying degrees according to the degree of selectivity by which the individual hydroxylation reactions were catalyzed by CYP2B1/2B2 [13].

Incorporation of radioactive phosphate into the CYP2B1 molecule and loss of its ability to support monooxygenase activity occur very fast on introduction of conditions leading to an increase in its phosphorylation such as changes in the hormone (treatment with glucagon) or cAMP (treatment with dbcAMP) level. At the first experimentally monitored time point (4 min) both, phosphorylation and activity, were already markedly changed [12]. Also, the mutations induced in the *HPRT* gene of *CYP2E1*-transfected V79 cells took place to the maximal extent (numerically 89% of the maximal effect but not significantly different from it) at the first experimentally monitored time point of 2.5 min exposure to dbcAMP [5]. Moreover, the two time curves were superimposable, i.e., there was no observable lag time between phosphorylation and loss of monooxygenase activity. Thus, phosphorylation

acts like a switch, in contrast to CYP induction, which depends on several time-consuming steps. Also, at least for the thoroughly studied case of CYP2B1, the loss of monooxygenase activity is not mediated by a phosphorylation-dependent degradation of CYP2B1. Whilst phosphorylation and inactivation were observable already at the earliest experimentally monitored time point of 4 min, the amount of CYP2B1 enzyme protein (monitored by densitometric scanning of Western blot signals obtained using specific antibodies against CYP2B1) remained unchanged for the entire monitored period of 3 h [12]. Also, during the entire time period of incubating intact hepatocytes with phosphorylation stimulators (dbcAMP) of 3 h, there was no measurable conversion of CYP2B1 from P450 to the spectrally altered (and enzymically inactive) P420 [12], in contrast to the situation when isolated microsomes were incubated with phosphorylation stimulators (ATP and catalytic subunit of PKA) [14].

With respect to CYP2E1, the reports on the relationship of phosphorylation, loss of monooxygenase activity, and protein degradation are somewhat controversial [5,15]. A report by Eliasson et al. [15] indicated that cAMP-dependent phosphorylation of CYP2E1 leads to its degradation. On the other hand, we [5] observed upon treatment with PKA-stimulating agents a marked decrease in CYP2E1-dependent monooxygenase (*p*-nitrophenol hydroxylase and *N*-nitrosodimethylamine *N*-demethylase) activities without an increased rate of CYP2E1 protein degradation. The different procedure of CYP2E1 induction—intragastric acetone and starvation for 2 days [15] versus 3 times 80 mg/kg isoniazid i.p. [5]—of the rats used as origin for the hepatocytes used may have contributed to the different results. With respect to CYP2B1, there is agreement that its phosphorylation leads to a loss of activity without increasing the rate of its degradation [12,15]. Consequently, the loss of activity is very fast, which is observed at the earliest time points investigated which in unbroken cells lasts 4 min for CYP2B1 [12] and 2.5 min for CYP2E1 [5].

CYP2B and 2E phosphorylation was catalyzed by protein kinase A (PKA) [5,8] and was dependent on the presence of a phosphorylatable serine (Ser) in the the PKA recognition sequence Arg Arg X Ser in the CYP (in the mouse CYP2E1 Ser^{129} [15]). After replacement of this serine by alanine or glycine, the mutant cDNAs produced catalytically active CYP2E1 having profoundly altered substrate specificities [5]. CYP2E1-Gly^{129} had a markedly higher specific activity for *p*-nitrophenol hydroxylation but a markedly lower specific activity for *N*-nitrosodimethylamine demethylation compared with CYP2E1-Ser^{129} [5]. Treatment of the cells with db-cAMP led to an expected decrease of the activity of the wild-type CYP2E1-Ser^{129} towards both substrates, but to a marked increase of these activities of the mutants CYP2E1-Gly^{129} and CYP2E1-Ala^{129} [5]. Thus, if the wild-type phosphoryl acceptor Ser^{129} in the CYP2E1 PKA recognition motif Arg-Arg-Phe-Ser^{129} is not available, PKA-mediated phosphorylation at another site of CYP2E1 or in another cooperating molecule leads to an increase of catalytic activity, i.e., to the opposite effect of PKA-mediated phosphorylation than its effect on wild-type CYP2E1-Ser^{129}. Thus, a binary phosphorylation-mediated control exists, directing CYP2E1 to opposite activity modulations. This may be important in potential human polymorphisms where the primary phosphorylatable serine may be mutated.

The phosphorylation-dependent control of toxicity/genotoxicity becomes especially important when it refers to phosphorylatable CYPs which control the intended therapeutic activity of drugs which, because of their high unwanted toxicity, possess a narrow therapeutic window, as is the case for the widely used cytostatic oxazaphosphorine cancer drugs cyclophosphamide (CPA), ifosfamide, and their relatives. Drastic changes in the control of cytotoxic and mutagenic metabolites of CPA upon changes of CYP2B phosphorylation have been shown treating hepatocytes [16] or whole animals [12]. As an illustration, the latter will be briefly described: PB-induced rats were treated with clinically used doses of glucagon for activation of adenylate cyclase and theophylline for inhibition of phosphodiesterase, both leading to an increase in cAMP and therefore to PKA activation. Fifteen minutes after i.p. application of these compounds, hepatocytes were isolated and used as metabolic system for activation of CPA in the Ames test. The hepatocytes from the glucagon/theophylline-treated rats had a severely (by more than 50%) impaired ability for accumulation of mutagenic CPA metabolites [12]. This shows that these modulations also occur in the whole organism and that the hormonal status of the organism and its perturbation by clinically used doses of drugs are sufficient to affect the accumulation of mutagenic CPA metabolites. Since the actual activity of CYP2B undergoes a fast regulation by PKA-mediated phosphorylation, controlled by cAMP and this, in turn, controlled by many factors including hormones and clinically used drugs (such as glucagon, adrenaline, follicle-stimulating hormone, isoproterenol, dopamine or methylxanthines), these influences should not be underestimated. Their consideration will help to more precisely hit the narrow window of sufficient effectivity combined with acceptable systemic toxicity during oxazaphosphorine cancer therapy.

The most drastic influence of the phosphorylation status on the accumulation of mutagenic metabolites was observed by the use of phosphatase inhibitors with aromatic amines or aromatic amides as pro-mutagens [17]. The treatment of rat hepatocytes or rat liver homogenate or fractions from it with okadaic acid (an inhibitor of the serine/threonine protein phosphatases PP1 and PP2A) and with *ortho*-vanadate (an inhibitor of tyrosine phosphatases) reduced the mutagenicity of all three aromatic amines/amides investigated, 2-aminoanthracene, 2-aminofluorene, and 2-acetylaminofluorene by more than 80%. The mutagenicity of the CYP1A1/1A2-formed metabolite 2-*N*-hydroxyacetylaminofluorene in the presence of hepatocytes or liver homogenate fractions as metabolizing system was not influenced by *ortho*-vanadate. Thus

in this case, the drastic reduction of mutagenicity must have occurred prior to the presence of the metabolically formed *N*-hydroxyacetylaminofluorene. Major contributors to its formation are CYP1A1 and CYP1A2. However, no incorporation of ^{32}P from the pre-labelled intracellular [^{32}P]ATP pool into CYP1A1 or CYP1A2 was observed. This led to the question whether transcription factors involved in the control of the expression of these CYPs, such as the Ah receptor (AHR), may undergo changes upon PKA activation. Treatment of the mouse hepatoma cell line, Hepa1c1c7 cells (Hepa1) with cAMP, activated the AHR, translocating the receptor to the nucleus [18]. The consequences were fundamentally different from AHR activation by classical AHR ligands such as dioxin. This cAMP-mediated activation may reflect the physiological function of AHR. Disruption of the cAMP-mediated activation by dioxin that is present for binding to the AHR for an extremely long time might be fundamental to the mechanism of dioxin toxicity. Understanding this endogenous activation of the AHR by cAMP may help in developing methods to counteract the toxicity caused by many AHR binding environmental and food-borne toxic chemicals.

References

[1] A.H. Conney, Induction of drug-metabolizing enzymes: a path to the discovery of multiple cytochromes P450, Annu. Rev. Pharmacol. Toxicol. 43 (2003) 1–30.

[2] W. Pyerin, C.R. Wolf, V. Kinzel, D. Kübler, F. Oesch, Phosphorylation of cytochrome P-450-dependent monooxygenase components, Carcinogenesis 4 (1983) 573–576.

[3] W. Pyerin, H. Taniguchi, A. Stier, F. Oesch, C.R. Wolf, Phosphorylation of rabbit liver cytochrome P-450 LM_2 and its effect on monooxygenase activity, Biochem. Biophys. Res. Commun. 122 (1984) 620–626.

[4] W. Pyerin, H. Taniguchi, Phosphorylation of hepatic phenobarbital-inducible cytochrome P-450, EMBO J. 8 (1989) 3003–3010.

[5] B. Oesch-Bartlomowicz, P.R. Padma, R. Becker, B. Richter, J.G. Hengstler, J.E. Freeman, C.R. Wolf, F. Oesch, Differential modulation of CYP2E1 activity by cAMP-dependent protein kinase upon Ser129 replacement, Exp. Cell Res. 242 (1998) 294–302.

[6] E.G. Krebs, J.A. Beavo, Phosphorylation-dephosphorylation of enzymes, Ann. Rev. Biochem. 48 (1979) 923–959.

[7] D.A. Flockhart, J.D. Corbin, Regulatory mechanisms in the control of protein kinases, CRC Crit. Rev. Biochem. 12 (1982) 133–186.

[8] W. Pyerin, H. Taniguchi, F. Horn, F. Oesch, Z. Amelizad, T. Friedberg, C.R. Wolf, Isoenzyme-specific phosphorylation of cytochromes P-450 and other drug metabolizing enzymes, Biochem. Biophys. Res. Commun. 142 (1987) 885–892.

[9] B. Bartlomowicz, D.J. Waxman, D. Utesch, F. Oesch, T. Friedberg, Phosphorylation of carcinogen metabolizing enzymes: regulation of the phosphorylation status of the major phenobarbital inducible cytochromes P-450 in hepatocytes, Carcinogenesis 10 (1989) 225–228.

[10] I. Johansson, E. Eliasson, A. Johansson, A.L. Hagbjörk, K. Lindros, M. Ingelman-Sundberg, Mechanism of ethanol and acetone-dependent induction of cytochromes P-450, in: I. Schuster (Ed.), Cytochrome P-450: Biochemistry and Biophysics. Proceedings of 6th International Conference, Taylor and Francis, London/New York/Philadelphia, 1989, pp. 592–595.

[11] J.A. Koch, D.J. Waxman, Posttranslational modification of hepatic cytochrome P-450. Phosphorylation of Phenobarbital-inducible P-450 forms PB-4 (IIB1) and PB-5 (IIB2) in isolated rat hepatocytes and in vivo, Biochemistry 28 (1989) 3145–3152.

[12] B. Oesch-Bartlomowicz, B. Richter, R. Becker, S. Vogel, P.R. Padma, J.-G. Hengstler, F. Oesch, cAMP-dependent phosphorylation of CYP2B1 as a functional switch for cyclophosphamide activation and its hormonal control in vitro and in vivo, Int. J. Cancer 94 (2001) 733–742.

[13] B. Bartlomowicz, T. Friedberg, D. Utesch, E. Molitor, K. Platt, F. Oesch, Regio- and stereoselective regulation of monooxygenase activities by isoenzyme-selective phosphorylation of cytochrome P450, Biochem. Biophys. Res. Commun. 160 (1989) 46–52.

[14] H. Taniguchi, W. Pyerin, A. Stier, Conversion of hepatic microsomal cytochrome P-450 to P-420 upon phosphorylation by cyclic AMP-dependent protein kinase, Biochem. Pharmacol. 34 (1985) 1835–1837.

[15] E. Eliasson, S. Mkrtchian, M. Ingelman-Sundberg, Hormone- and substrate-regulated intracellular degradation of cytochrome P-450 (2E1) involving MgATP-activated rapid proteolysis in the endoplasmic reticulum membranes, J. Biol. Chem. 267 (1992) 15765–15769.

[16] B. Oesch-Bartlomowicz, S. Vogel, H.-J. Arens, F. Oesch, Modulation of the control of mutagenic metabolites derived from cyclophosphamide and ifosfamide by stimulation of protein kinase A, Mutat. Res. 232 (1990) 305–312.

[17] B. Oesch-Bartlomowicz, H.-J. Arens, B. Richter, J.G. Hengstler, F. Oesch, Control of the mutagenicity of aromatic amines by protein kinases and phosphatases, Arch. Toxicol. 71 (1997) 601–611.

[18] B. Oesch-Bartlomowicz, A. Huelster, O. Wiss, P. Antoniou-Lipfert, C. Dietrich, M. Arand, C. Weiss, E. Bockamp, F. Oesch, Aryl hydrocarbon receptor activation by cAMP vs. dioxin: divergent signaling pathways, Proc. Natl. Acad. Sci. USA 102 (2005) 9218–9223.