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The Anticancer Drug Ellipticine Induces Cytochromes P450 1A1, 1A2 and 3A, Cytochrome b_5 and NADPH:Cytochrome P450 Oxidoreductase in Rat Liver, Kidney and Lung

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The antineoplastic alkaloid ellipticine is a prodrug, the pharmacological efficiency of which is dependent on its cytochrome P450 (CYP)- and/or peroxidase-mediated activation in target tissues. Using the Western blotting, we found that this compound increases protein expression of cytochrome b_5, CYP1A1, 1A2, 3A and NADPH:CYP oxidoreductase (POR) in livers, lungs and kidneys of rats treated (i.p.) with ellipticine. The ellipticine-mediated induction of these enzymes resulted in an increase in their enzymatic activities and ellipticine oxidation to 7-hydroxy-, 9-hydroxy-, 12-hydroxy- and 13-hydroxyellipticine, the metabolites that are both detoxication products (7-hydroxy-, 9-hydroxyellipticine) and metabolites responsible for generation ellipticine-derived DNA adducts (12-hydroxy- and 13-hydroxyellipticine). The results demonstrate that by inducing CYP1A1/2, 3A, POR and cytochrome b_5, ellipticine increases its own metabolism in rats, thereby modulating its own pharmacological and/or genotoxic potential.

Keywords: Ellipticine; Cytochromes P450; Protein Expression; Western Blotting; Metabolism

1. INTRODUCTION

Ellipticine (5,11-dimethyl-6*H*-pyrido[4,3-*b*]carbazole, Fig. 1) and its derivatives are efficient anticancer compounds that function through multiple mechanisms participating in cell cycle arrest and

initiation of apoptosis (for a summary see [1-6]). Ellipticine was found (i) to arrest cell cycle progression due to modulation of levels of cyclinB1 and Cdc2, and phosphorylation of Cdc2 in human mammary adenocarcinoma MCF-7 cells, (ii) to initiate apoptosis due to formation of toxic free radicals, stimulation of the Fas/Fas ligand system and modulation of proteins of Bcl-2 family in several tumor cell lines, and (iii) to induce the mitochondria-dependent apoptotic processes (for a summary see [3,4]). The predominant mechanisms of ellipticine's biological effects were suggested to be (i) intercalation into DNA [5,6] and (ii) inhibition of topoisomerase II [3-6]. Further, we showed that this antitumor agent forms covalent DNA adducts after its enzymatic activation with cytochromes P450 (CYP) and peroxidases [1-4,7-11], suggesting an additional DNA-damaging effect of ellipticine.

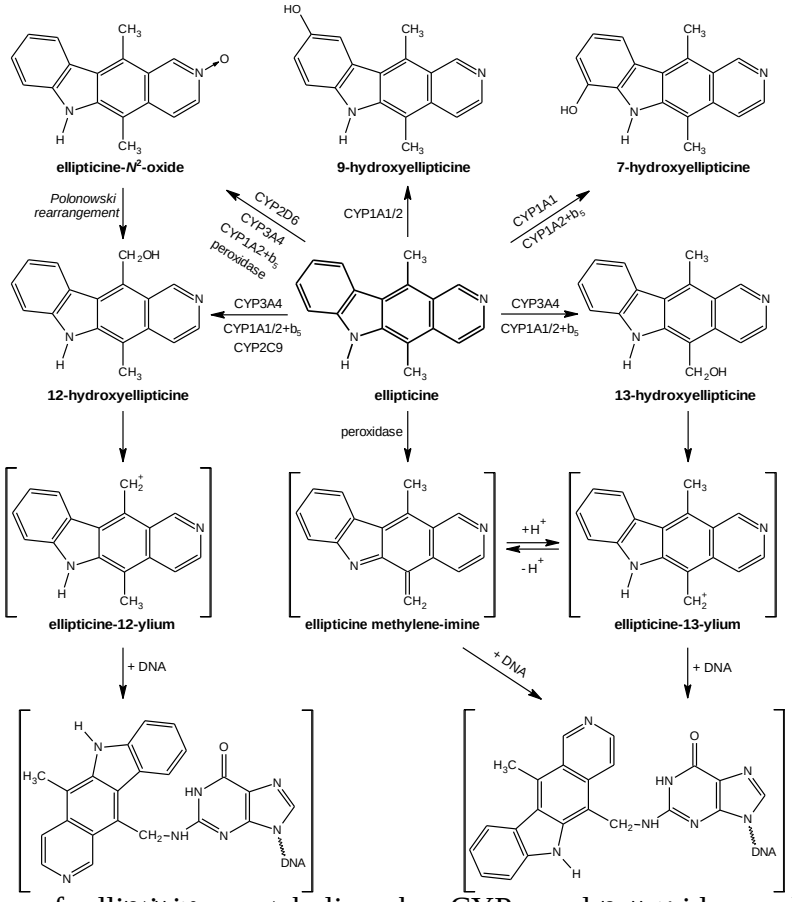

Figure 1. Scheme of ellipticine metabolism by CYPs and peroxidases showing the identified metabolites and those proposed to form DNA adducts. The compounds shown in brackets were not detected under the experimental conditions and/or not structurally characterized. The CYP enzymes predominantly oxidizing ellipticine shown in the figure were identified in our previous studies [7,10,11].

Of the CYP enzymes investigated, human CYP3A4 and rat CYP3A1 are the most active enzymes oxidizing ellipticine to 12-hydroxy- and 13-hydroxyellipticine, the reactive metabolites that dissociate to ellipticine-12-ylium and ellipticine-13-ylium which bind to DNA [3,7,8,10], while the CYP1A isoforms preferentially form the other ellipticine metabolites, 7-hydroxy- and 9-hydroxyellipticine, which are the detoxication products (Fig. 1). Recently we have found that

cytochrome b_5 alters the ratio of ellipticine metabolites formed by CYP1A1, 1A2 and 3A4. While the amounts of the detoxication metabolites (7-hydroxy- and 9-hydroxyellipticine) were either decreased or not changed with added cytochrome b_5, 12-hydroxy-, 13-hydroxyellipticine and ellipticine N^2-oxide increased considerably. The change in amounts of metabolites resulted in an increased formation of covalent ellipticine-DNA adducts, one of the DNA-damaging mechanisms of ellipticine antitumor action [10,11].

Because of the important role of cytochrome b_5 in CYP1A- and CYP3A-mediated ellipticine metabolism, expression levels of all these proteins are crucial for antitumor, cytostatic and/or genotoxic activities of this drug in individual tissues.

Recently we found that ellipticine as a ligand of aryl hydrocarbon receptor (AHR) [12] is a strong inducer of CYP1A1 and 1A2 in rat liver *in vivo* [13] and in several cancer cell lines *in vitro* [14,15]. In addition, levels of cytochrome b_5 are increased by ellipticine in liver of rats treated with this drug [11]. Moreover, ellipticine also induces expression of *CYP3A4* mRNA and CYP3A4 protein in several cancer cell lines [14,15]. However, in contrast to induction of CYP1A by ellipticine *in vivo* that has been partially studied previously [13], the effect of ellipticine on expression of CYP3A and cytochrome b_5 *in vivo* remains to be investigated.

Therefore, Western blotting analysis was used in this work to evaluate the effect of ellipticine on protein levels not only of CYP1A1/2, but also of CYP3A, POR and cytochrome b_5 proteins in rats *in vivo*.

2. EXPERIMENTAL PART

2.1 Animal experiments

The study was conducted in accordance with the Regulations for the Care and Use of Laboratory Animals (311/1997, Ministry of Agriculture, Czech Republic), which is in compliance with the Declaration of Helsinki. Male Wistar rats (~100 g) were treated with a single dose of 4 or 40 mg/kg body weight (n=3) of ellipticine by intraperitoneal (i.p.) injection as described previously [13]. Ellipticine was dissolved in 1% acetic acid at a concentration of 6 mg/ml. Three control animals received an equal volume of solvent only. Rats were placed in cages in temperature and humidity controlled rooms. Standardized diet and water were provided *ad libitum*. The animals were killed 48 hours after treatment by cervical dislocation. Livers, lungs and kidneys were removed immediately and directly used for the isolation of microsomal fractions. Microsomes were isolated from the livers, kidneys and lungs of rats as described [1].

2.2 Electrochemical determination of CYP, POR and cytochrome b_5 protein levels in microsomes of rat liver, kidney and lung

Immunoquantitation of rat liver, kidney and lung microsomal cytochrome b_5 , CYPs (CYP1A1, 1A2, and 3A) and POR was done by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-

PAGE). Samples containing 30-75 μg microsomal proteins were subjected to electrophoresis on 10% polyacrylamide gels (for CYPs and POR) and 15% gels (for cytochrome b_5) polyacrylamide gels [11,16,17]. After migration, proteins were transferred onto a polyvinylidene fluoride (PVDF) membrane and incubated with 5% non-fat milk to block non-specific binding. The membranes were then exposed to specific rabbit polyclonal anti-cytochrome b_5 (1:750, AbCam, MA, USA), anti-CYP1A1 (1:1000, Millipore, MA, USA), anti-CYP3A4 (1:5000, AbD Serotec, Oxford, UK) and anti-POR (1:1000, Millipore, MA, USA) antibodies overnight at 4 ^{o}C. The antigen-antibody complex was visualized with an alkaline phosphatase-conjugated goat anti-rabbit IgG antibody (1:1428, Sigma-Aldrich, USA) and 5-bromo-4-chloro-3-indolylphosphate/nitrobluetetrazolium as chromogenic substrate [16,17]. Antibody against glyceraldehyde phosphate dehydrogenase (GAPDH) (1:750, Millipore, MA, USA) was used as loading control.

2.3 *CYP1A, 3A and POR enzyme activity assays*

The rat liver, kidney and lung microsomal samples were characterized for CYP1A activity using 7-ethoxyresorufin *O*-deethylation (EROD) [18], for 6-β-hydroxylation of testosterone (a marker for CYP3A) [19] and for POR activity using reduction of cytochrome *c* [10,13].

2.4 CYP1A mRNA contents in rat liver, kidney and lung

The mRNA contents of CYP1A in rat liver, kidney and lung were carried out as described previously [13].

2.5 *Determination of cytochrome b_5 content*

The concentration of cytochrome b_5 was determined spectrophotometrically (the absolute absorbance spectrum) using the molar extinction coefficient ε_{413} = 117 $mM^{-1}.cm^{-1}$ [20,21], or from the difference spectrum of reduced minus oxidized form, using molar extinction coefficient $\varepsilon_{424\text{-}409}$ = 185 $mM^{-1}.cm^{-1}$, respectively [21].

2.6 Oxidation of ellipticine by rat hepatic, renal and pulmonary microsomes

Incubation mixtures used to study ellipticine oxidation to its metabolites contained 50 mM potassium phosphate buffer (pH 7.4), 1 mM $NADP^{+}$, 10 mM D-glucose 6-phosphate, 1 U/ml D-glucose 6-phosphate dehydrogenase (NADPH-generation system), 0.2 mg protein of pooled hepatic, renal and pulmonary microsomal fraction from 3 male rats, either untreated or treated with 40 mg/kg body weight ellipticine and 10 μM ellipticine (dissolved in 5 μl methanol) in a final volume of 500 μl. The reaction was initiated by adding the substrate. In the control incubation, ellipticine was omitted from the incubation mixture. After incubation in open glass tubes (37 ^{o}C, 20 min) the reaction was stopped by adding 100 μl of 2 M NaOH. The oxidation of ellipticine is linear up to 30 min of

incubation [7]. After incubation, 5 µl of 1 mM phenacetine in methanol was added as an internal standard and the ellipticine metabolites were extracted twice with ethyl acetate (2 x 1 ml). Analyzes of ellipticine metabolites were performed by HPLC as described [7,10,11]. Recoveries of ellipticine metabolites were around 95%.

3. RESULTS AND DISCUSSION

Using Western blot analysis with antibodies raised against CYP1A1, 3A4, POR and cytochrome b_5, the expression levels of these proteins were analyzed in livers, kidneys and lungs of rats exposed to ellipticine. Rats treated i.p. with 4 and 40 mg/kg body weight were used as a model. Microsomes isolated from livers, kidneys and lungs of untreated (control) and ellipticine-treated rats were used for the analyses.

3.1 CYP1A1, 1A2 and 3A protein levels and enzymatic activities in rat liver, kidney and lung

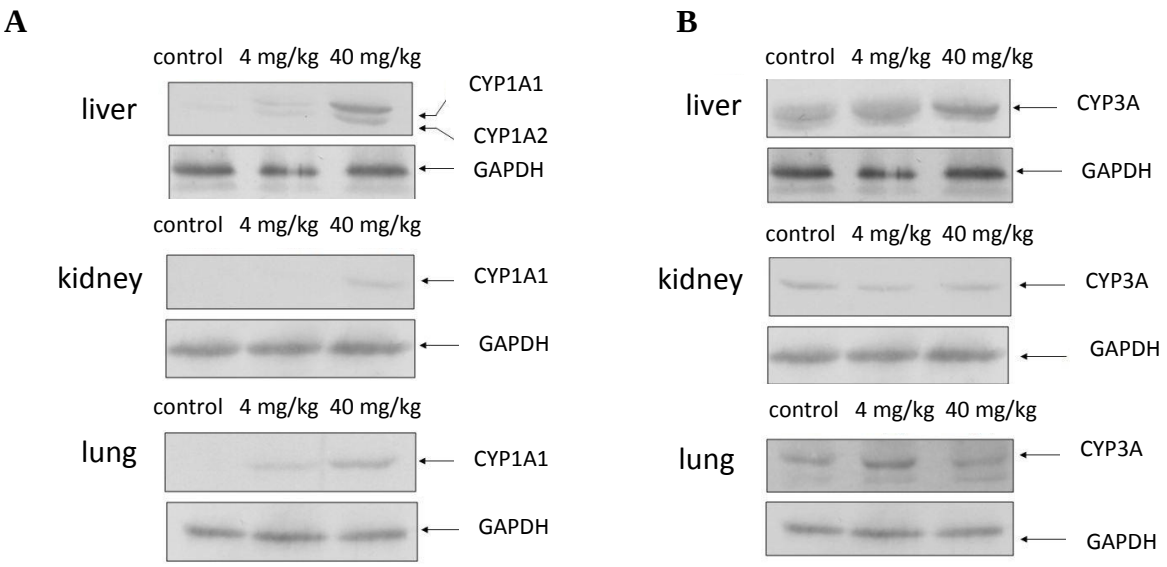

Figure 2. Immunoblots of microsomal CYP1A1, 1A2 (**A**) and 3A (**B**) from livers, lungs, and kidneys of untreated and ellipticine-treated (4 and 40 mg/kg) rats stained with antibody against rat CYP1A1 (**A**) and human CYP3A4 (**B**). Microsomes isolated from rat organs were subjected to SDS-polyacrylamide gel electrophoresis, and proteins were transferred to PVDF membranes and probed with antibodies. Glyceraldehyde phosphate dehydrogenase (GAPDH) was used as loading control.

As shown in Figure 2A, ellipticine is capable of inducing expression levels of CYP1A1 and 1A2 proteins in liver, kidney and lung of rats treated i.p. with this compound. The increase in CYP1A1 and 1A2 protein levels in rat organs is dose-dependent; the highest expression levels of these enzymes were found in the liver of rats treated with 40 mg/kg body weight of ellipticine (Fig. 2A). A dose-dependent increase in CYP1A1 and 1A2 protein levels due to ellipticine correlated with their

enzymatic activities (EROD for CYP1A1/2) in all organs studied (Fig. 3A). Moreover, as we found in our former work [13], this increase in CYP1A1 protein levels corresponded also to higher expression of *CYP1A1* mRNAs in liver, kidney and lung (see [13] and Table 1). mRNA levels of *CYP1A2* showed no significant difference between ellipticine treated and control rats in all three organs. Interestingly the highest *CYP1A2* mRNA levels were observed in lung and kidney, while protein expression determined by Western blot was only seen in livers of ellipticine treated rats.

Table 1. Expression of mRNA of CYP1A1 and 1A2

	CYP1A1		CYP1A2	
	Δc_T[a]	Fold Change	Δc_T	Fold Change
control rats				
liver	6.37 ± 0.07		-3.9 ± 0.12	
kidney	4.45 ± 0.36		14.07 ± 0.54	
lung	10.48 ± 0.16		13.97 ± 1.06	
ellipticine-treated rats				
liver	3.87 ± 1.73	5.66*	-4.04 ± 0.22	1.1
kidney	1.12 ± 0.13	10.08**	13.8 ± 0.76	1.21
lung	5.89 ± 0.59	24.2**	15.37 ± 0.03	0.38

[a]Results shown are mean ± standard deviation from data found for three male rats (control and treated with 40 mg/kg body weight) and are taken from our previous work [13].
Significantly different from controls: $^{*}p<0.05$; $^{**}p<0.001$ (Student's t-test).

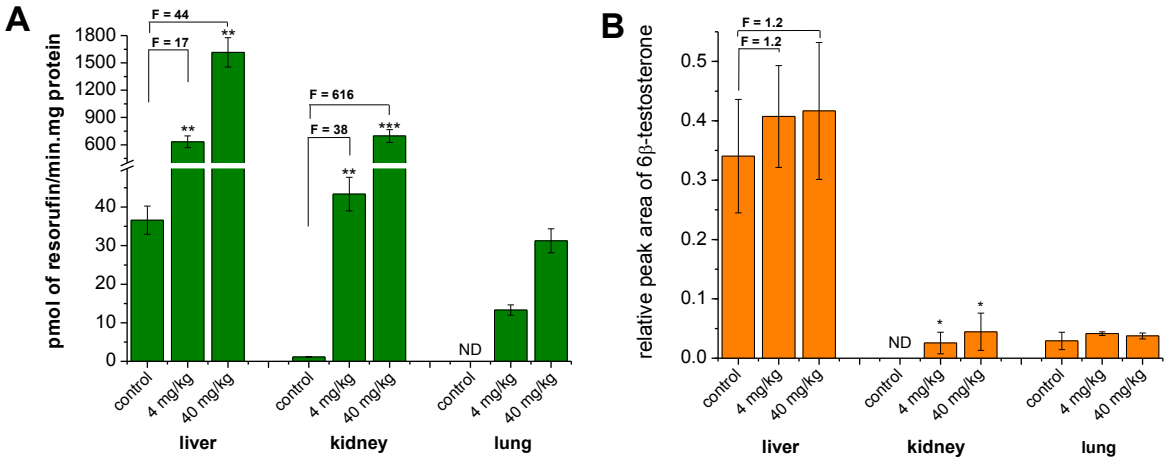

Figure 3. CYP1A enzymatic activity measured by 7-ethoxyresorufin *O*-deethylation (EROD) (**A**) and CYP3A enzymatic activity measured by testosterone 6-β-hydroxylation (**B**) in microsomes of control and ellipticine-treated rats (ND – not detected). F, fold of increase in CYP1A and 3A enzymatic activities in rats treated with ellipticine compared with those of control (uninduced) rats. Values significantly different from control: **p*<0.05, ***p*<0.01, ****p*<0.001 (Student's t-test).

Besides CYP1A1 and 1A2, expression levels of CY3A protein were also increased in livers of rats treated with ellipticine (Fig. 2B). In contrast, lower CYP3A protein levels were found in kidney of rats treated with both doses of ellipticine and in lung of rats exposed to the higher dose of ellipticine (40 mg/kg). Dose-dependent increases in marker activity of the CYP3A enzymes, testosterone 6-β-hydroxylation, were produced in all studied organs of rats treated with ellipticine (Fig. 3B). Nevertheless, an increase in this enzymatic activity in liver and lung was not statistically significant.

3.2 Cytochrome b_5 protein levels and its specific content in rat liver, kidney and lung

Similarly to CYP1A and 3A enzymes, expression of additional protein of the mixed-function oxidase system located in the membrane of endoplasmic reticulum, cytochrome b_5, was increased in liver and kidney of rats treated with ellipticine (Fig. 4). Using only 30 μg microsomal proteins were sufficient to detect expression of this protein in rat livers by Western blotting (Fig. 4A), but higher levels of microsomal proteins (75 μg) were utilized to detect expression of this protein in rat kidney (Fig. 4B). An increase in expression levels of cytochrome b_5 by treating rats with ellipticine was dose-dependent. No cytochrome b_5 expression was detectable in lung microsomes.

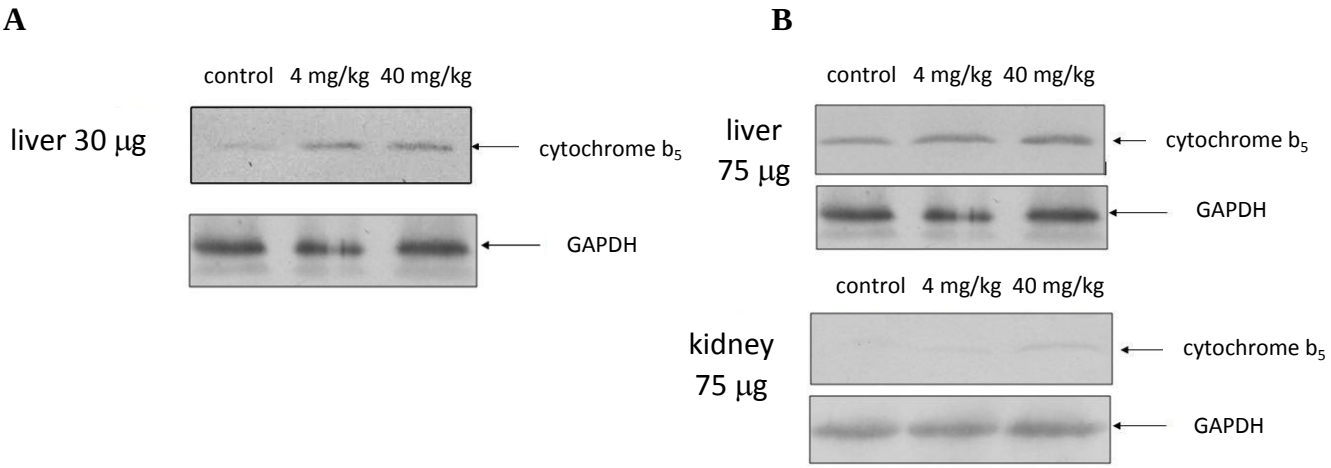

Figure 4. Immunoblots of microsomal cytochrome b_5 from livers (30 μg microsomal proteins) (A), and livers and kidneys (75 μg microsomal proteins) (B) of untreated and ellipticine-treated (4 and 40 mg/kg) rats stained with antibody against rat cytochrome b_5. Microsomes isolated from rat organs were subjected to SDS-polyacrylamide gel electrophoresis, and proteins were transferred to PVDF membranes and probed with antibody. Glyceraldehyde phosphate dehydrogenase (GAPDH) was used as loading control.

An increase in expression of liver and kidney cytochrome b_5 in rats after treatment with ellipticine was paralleled by the specific content of this protein determined spectrophotometrically (Fig. 5).

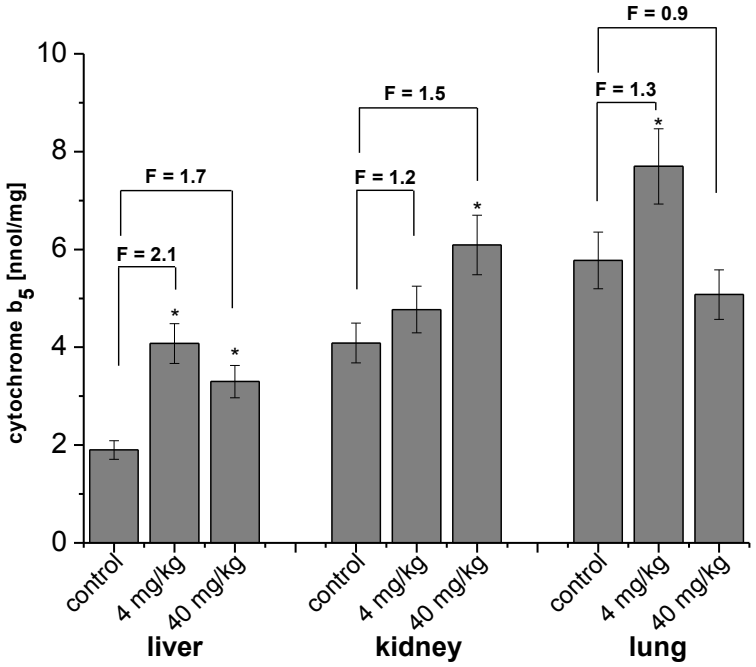

Figure 5. Specific contents of cytochrome b_5 in microsomes of control and ellipticine-treated rats. F, fold of increase in cytochrome b_5 in rats treated with ellipticine compared with those of control (uninduced) rats. Values significantly different from control: *p<0.05 (Student's t-test).

In contrast to the cytochrome b_5 protein detected by Western blot, spectrophotometric analyses revealed higher cytochrome b_5 contents in lung (approximately three fold over liver) than liver or kidney (Fig. 5). Low interaction of antibody with cytochrome b_5 caused by worse availabilities of epitopes of this protein in lung microsomes or the presence of certain inhibitor(s) in these microsomes that decrease interaction of the protein with its antibody might the reasons of this observation. Nevertheless, these suggestions remain to be investigated.

3.3 POR protein levels and enzymatic activity in rat liver, kidney and lung

Another enzyme that is a component of the mixed-function oxidase system, POR, was also detected by Western blotting in the liver, kidney and lung microsomes of rats treated with ellipticine (Fig. 6), whereas almost no expression of this enzyme was detected in kidney of untreated (control) rats. An increase in levels of rat liver POR protein by ellipticine was paralleled by an increase in its enzymatic activity measured as reduction of cytochrome *c* (Fig. 7).

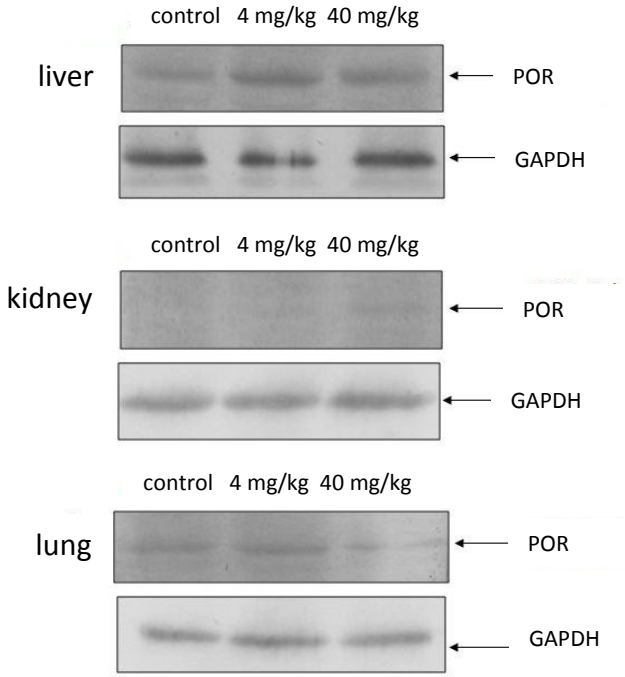

Figure 6. Immunoblots of microsomal POR from livers, kidneys and lungs of untreated and ellipticine-treated (4 and 40 mg/kg) rats stained with antibody against rat POR. Microsomes isolated from rat organs were subjected to SDS-polyacrylamide gel electrophoresis, and proteins were transferred to a PVDF membrane and probed with antibody. Glyceraldehyde phosphate dehydrogenase (GAPDH) was used as loading control.

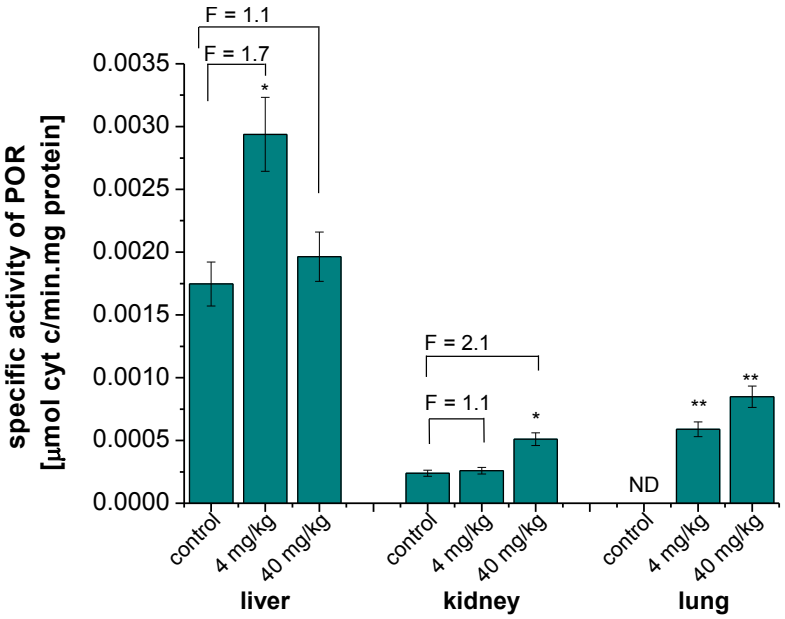

Figure 7. POR enzymatic activity in microsomes of control and ellipticine-treated rats (ND – not detected). F, fold of increase in POR enzymatic activities in rats treated with ellipticine compared with those of control (uninduced) rats. Values significantly different from control: *p<0.05, **p<0.01, ***p<0.001 (Student's t-test).

The mechanisms responsible for induction of CYP1A have already been investigated [12,22,,23]. CYP1A1 and 1A2 induction might be a consequence of the binding of ellipticine to AHR [12]. Binding of ellipticine allows cytosolic AHR to translocate into the nucleus and to dimerize with AHR nuclear translocator (ARNT). The AHR-ARNT complex functions as a transcriptional activator by binding to the aryl hydrocarbon responsive element in the regulatory domains of *CYP1A1* and *CYP1A2* genes [12], thus stimulating their transcription as we previously found in livers [13] and in kidneys and lungs in the present study. Another mechanism of CYP1A1 induction might result from inhibitory potential of ellipticine to this enzyme [22]. As shown previously [23], a decrease in levels of CYP1A1 enzymatic activity by ellipticine results in an increase in constitutive activation of AHR-ARNT transcriptional complexes. Moreover, low levels of ellipticine antagonize AHR activation by inducing ligands such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin or certain azo dyes.

Effects of ellipticine upon CYP3A, POR and cytochrome b_5 protein expression and enzymatic activities were much lower than upon CYP1A. For these proteins the induction mechanisms are not yet entirely elucidated and await further investigation. Because the pregnane X receptor (PXR) and the constitutive active receptor (CAR) are known to coordinate multifaceted responses to inducers of CYPs of a 3A subfamily and POR [24, 25], we plan to investigate whether ellipticine or its metabolites function as ligands of either of these receptors, thereby inducing expression of these enzymes.

3.3 Ellipticine as an inducer of cytochrome b_5, CYP1A and 3A increases efficiencies of rat hepatic, pulmonary and renal microsomes to oxidize ellipticine to its hydroxylated metabolites

Ellipticine is oxidized by hepatic, pulmonary and renal microsomes to four metabolites, 7-hydroxy-, 9-hydroxy-, 12-hydroxy and 13-hydroxyellipticine (Fig. 8). Ellipticine N^2-oxide (Fig. 1) was also produced, but this metabolite was not quantitated in this study because of its spontaneous rearrangement to 12-hydroxyellipticine [7,8].

Treatment of rats with 40 mg/kg of ellipticine resulted in changes of patterns of metabolites formed by microsomes isolated from all three organs of these rats. In all organs 9-hydroxyellipticine was formed at significantly higher amounts by "ellipticine microsomes" than by those of control rats, while 7-hydroxyellipticine formation was induced in kidney and liver only. The influence of ellipticine exposure on the levels of these two metabolites was expected, because they are predominantly formed by CYP1A1/2 [7,11] and CYP1A1 was induced in all three organs. An up to 2-fold increase in formation of 13-hydroxy- and 12-hydroxyellipticine, the metabolites generating DNA adducts 1 and 2 (see Fig. 1), was induced in hepatic microsomes (Fig. 8A). This phenomenon is caused not only by a strong induction of CYP1A1/2, but also by induction of CYP3A (Fig. 2B) and cytochrome b_5 (Fig. 4) in livers of rats treated with ellipticine. Ellipticine treatment causing induction of cytochrome b_5 seems to play a crucial role for these metabolic changes. Cytochrome b_5 influences the enzymatic activity of CYP1A1/2 [11] and CYP3A [10], mediating the increased formation of 12-hydroxy- and 13-hydroxyellipticine *in vitro*. Hence, higher levels of cytochrome b_5 protein in liver (Fig. 4) results in the elevated formation of 12-hydroxy- and 13-hydroxyellipticine in rat liver microsomes shown in this study (Fig. 8A). In order to prove that elevated formation of these ellipticine metabolites leads to

higher levels of ellipticine-derived DNA adducts will be analyzed in our laboratory in future. The ^{32}P-postlabeling assay [1-3,4-11] or electrochemical techniques [26,27] will be used for such a study. In contrast to liver microsomes, no increase in formation of 12-hydroxy- and 13-hydroxyellipticine was found during oxidation of ellipticine catalyzed by lung and kidney nicrosomes of rats treated with ellipticine (Fig. 8B,C). Only an increase in oxidation of ellipticine to 7-hydroxyellipticine and/or 9-hydroxyellipticine was found in lung and kidney microsomes. These results are consequences of both the effective ellipticine-mediated induction of CYP1A1 in these organs and a lower induction effect of ellipticine on expression levels of CYP3A and cytochrome b_5 in these tissues (compare Figs. 2 and 4).

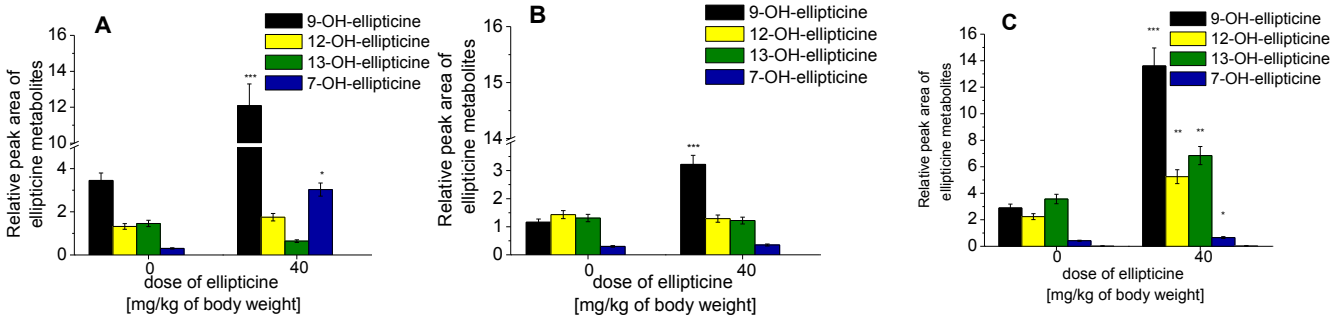

Figure 8. Ellipticine metabolism in rat liver (A), lung (B) and kidney microsomes (C) of control animals and those treated with 40 mg/kg ellipticine. Microsomes containing 0.2 mg microsomal protein, and 10 μM ellipticine were used in all experiments. Levels of ellipticine metabolites are averages ± standard deviations of triplicate incubations. Values significantly different from control: *p<0.05, **p<0.01, ***p<0.001 (Student's t-test).

4. CONCLUSIONS

Utilizing the Western blotting, protein levels of CYP1A, 3A, POR and cytochrome b_5 were found to be increased by treating rats with ellipticine. The most prominent change observed was the induction of CYP1A expression in all organs analyzed, namely liver, kidney and lung. This induction of expression resulted in an increase in CYP1A, 3A and POR marker activities as well as in an enhanced oxidation of ellipticine itself. By inducing these proteins, ellipticine modulates its own metabolism in rats, thereby dictating its own pharmacological and/or genotoxic potential. Therefore, further studies that would shad more light on the mechanism(s) of this induction are needed to be carried out. Studies investigating whether ellipticine or its metabolites activates receptors such as AHR, CAR and PXR which consequently leads to induction of CYP1A, 3A, and POR expression is planned to be performed in our laboratory in future.

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