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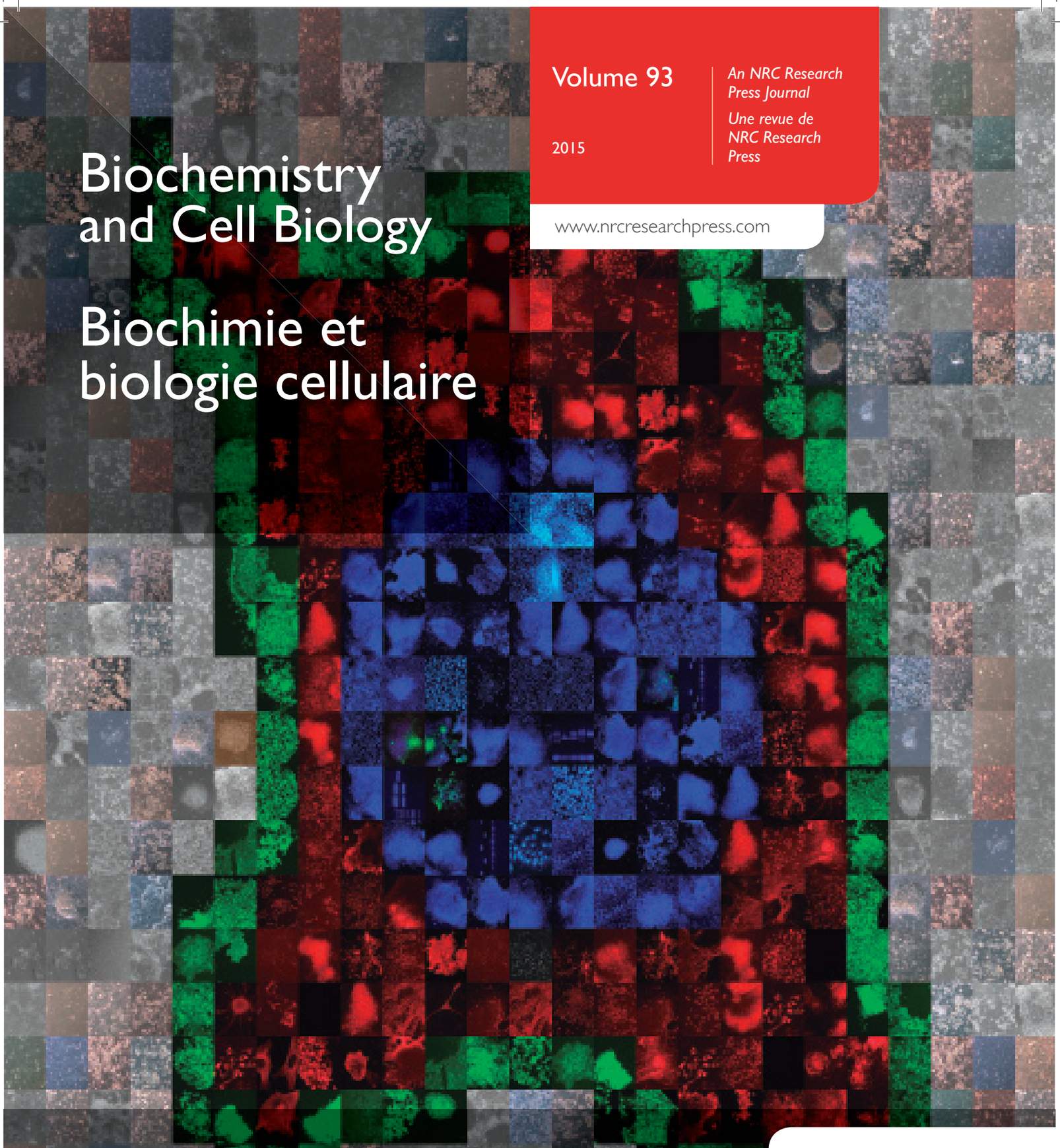

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NOTE

Cytochrome P450 expression in mouse brain: specific isoenzymes involved in Phase I metabolizing system of porphyrinogenic agents in both microsomes and mitochondria

Jimena Lavandera, Silvina Ruspini, Alcira Batlle, and Ana María Buzaleh

Abstract: Brain cytochrome P450 (CYP) metabolizes a variety of drugs to produce their pharmacological effects within the brain. We have previously observed that porphyrinogenic agents altered CYP levels in brain. The aim of this work was to further study the involvement of mice brain mitochondrial and microsomal Phase I drug metabolizing system when porphyrinogenic agents, such as Enflurane, Isoflurane, allylisopropylacetamide, veronal, ethanol, and Griseofulvin were administered. To this end, CYP2E1, CYP2B1, and CYP3A4 expression were measured. NADPH cytochrome P450 reductase (CPR) expression was also determined. Western Blots were performed in microsomes and mitochondria of whole brain. Some of the drugs studied altered expression mainly in microsomes. Chronic Isoflurane augmented mitochondrial isoform, although this anaesthetic diminished microsomal expression. Ethanol and topical Griseofulvin affected expression in microsomes but not in mitochondria. CYP2E1 mitochondrial activity was induced by acute Enflurane; while the activity of the microsomal protein was enhanced in alcoholised animals. Ethanol also induced CYP2E1 expression in microsomes, although Isoflurane provoked opposite effects in mitochondria and microsomes. Expression of CPR was also induced. Several reports support an emergent role of CYP enzymes in the pathogenesis of neurological disorders, so CYP response in brain could be one of the multiples factors influencing porphyria acute attacks.

Key words: cytochrome P450, brain, porphyrinogenic agents, volatile anaesthetics.

Résumé : Le cytochrome P450 (CYP) du cerveau métabolise une variété de médicaments afin de leur permettre d'exercer leurs effets pharmacologiques à l'intérieur du cerveau. Les auteurs ont observé précédemment que les agents porphyrinogènes affectaient les niveaux de CYP dans le cerveau. Le but de ce travail consistait à étudier plus en profondeur l'implication du système de métabolisme médicamenteux mitochondrial et microsomal de Phase I dans cerveau de la souris, lorsque des agents porphyrinogènes comme l'Enflurane, l'Isoflurane, l'allylisopropylacétamide, le véronal, l'éthanol et la Griséofulvine étaient administrés. À ces fins, l'expression de CYP2E1, CYP2B1 et CYP3A4 a été mesurée. L'expression de la NADPH cytochrome P450 réductase (CPR) a aussi été déterminée. Des buvardages Western ont été réalisés à partir des microsomes et des mitochondries du cerveau total. Certains des médicaments modifiaient l'expression principalement dans les microsomes. Un traitement chronique à l'Isoflurane accroissait l'isoforme mitochondriale, même si cet anesthésique diminuait l'expression microsomale. L'éthanol et la Griséofulvine topique affectaient l'expression dans les microsomes, mais pas dans les mitochondries. L'activité mitochondriale de CYP2E1 était induite par un traitement aigu à l'Enflurane, alors que l'activité de la protéine microsomale était accrue chez les animaux alcoolisés. L'éthanol induisait aussi l'expression de CYP2E1 dans les microsomes. L'Isoflurane provoquait des effets opposés dans les mitochondries et les microsomes. L'expression de CPR était aussi induite. Plusieurs rapports appuient un rôle émergent des CYP dans la pathogenèse de maladies neurologiques, ainsi, la réponse des CYP dans le cerveau pourrait constituer un des nombreux facteurs qui influencent les attaques porphyriques aigües. [Traduit par la Rédaction]

Mots-clés : cytochrome P450, cerveau, agents porphyrinogènes, anesthétiques volatils.

Introduction

Cytochrome P450 (CYP) isoenzymes are a superfamily of hemeproteins functioning as the terminal oxidases of the drug metabolizing enzyme system. In mammals, CYP isoforms are mainly localized in the endoplasmic reticulum, but some isoforms are bound to the internal membrane of mitochondria (Guengerich and Cheng 2011; Ortiz de Montellano and Nelson 2011).

The presence of drug metabolizing activity and the existence of some microsomal CYPs in mitochondria in several animal tissues including liver, brain, and lung have been reported since 1980 (Gervasini et al. 2004; Omura 2010). Mitochondrial CYP1A, CYP2A, CYP2B, CYP2C, CYP2E and CYP3A are usually found in a concentration lower than that detected in microsomes (Anandatheerthavarada et al. 1997; Genter et al. 2006; Avadhani et al 2011).

Both microsomal and mitochondrial CYP are coded to nuclear genes. Mitochondrial CYPs are synthesized by free polysomes in the cytoplasm, as precursor peptides with a cleavable presequence at

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J. Lavandera. Cátedra de Bromatología y Nutrición, Facultad de Bioquímica y Ciencias Biológicas, Universidad Nacional del Litoral, Santa Fe, Argentina.
S. Ruspini and A. Batlle. Centro de Investigaciones sobre Porfirinas y Porfirias (CIPYP), CONICET, Hospital de Clínicas José de San Martín, Universidad de Buenos Aires, Av. Córdoba 2351, 1120, Buenos Aires, Argentina.
A.M. Buzaleh. Centro de Investigaciones sobre Porfirinas y Porfirias (CIPYP), CONICET, Hospital de Clínicas José de San Martín, Universidad de Buenos Aires, Av. Córdoba 2351, 1120, Buenos Aires, Argentina; Departamento de Química Biológica, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Argentina.
Corresponding author: Ana María Buzaleh (e-mail: anamaria@qb.fcen.uba.ar).

the amino-terminus, then released into the cytoplasm and selectively targeted to the mitochondria (Ahn and Yun 2010).

Almost all of the CYPs are inducible; the increase of expression could be related to gene transcription or post translating mechanisms such as RNA stabilization (Hewitt et al. 2007). In some cases, the administration of CYP inductors to animals increases total CYP in mitochondria (Genter et al. 2006; Avadhani et al. 2011).

NADPH cytochrome P450 reductase (CPR) is an essential component of a microsomal Phase I drug metabolizing system, which acts as electron donor to CYP (Riddick et al. 2013). The influence of CPR on CYP was demonstrated in transgenic mice by carrying out a deletion in hepatic CPR, leading to a diminished liver CYP activity (Wang et al. 2005). In mitochondria, the enzyme that functions as CPR is Adrenodoxin reductase (Kerstin et al. 2012).

Porphyrias are inherited disorders of heme metabolism. All acute hepatic Porphyrias display neurological symptoms (Batlle 1997; Parera et al. 2003; Piñeiro Pauwels et al. 2013). Enzyme inducing drugs are by far the most important triggering factors. δ-Aminolevulinic acid is responsible for the neuropsychiatric manifestations of several porphyric disorders. The administration of drugs that induce hepatic CYP and δ-Aminolevulinic acid synthetase can precipitate porphyric acute attacks (Hift et al. 2011). The importance of xenobiotics and CYP isoenzymes in the triggering of porphyrias have not been yet studied adequately in humans and animals.

Previously we have investigated the effects of some porphyrinogenic agents on brain mitochondrial and microsomal CYP levels and the activity of CPR, and our results revealed differential subcellular tissue responses and gave evidence on the participation of extrahepatic tissues in porphyrinogenic drug metabolization (Lavandera et al. 2007).

On the basis of the above findings, the aim of this work was to further study the involvement of mice brain mitochondrial and microsomal Phase I drug metabolizing enzyme system on the action of porphyrinogenic agents, such as the volatile anaesthetics Enflurane and Isoflurane, allylisopropylacetamide (AIA), veronal, ethanol, and Griseofulvin (Gris). Moreover, the effects of starvation were studied, because it is also an important factor in the triggering of acute attacts in the acute porphyrias (Batlle 1997). To this end, we have evaluated the response of the CYP isoforms that are mainly involved in the metabolism of the drugs mentioned above and that are present in the brain (Ferguson and Tyndale 2011). CYP2E1 expression was evaluated in a group of mice treated with volatile anaesthetics and ethanol and also under starvation conditions (Miksys and Tyndale, 2013). CYP2B1 expression was determined in mice receiving AIA and veronal (Wong et al. 1999; Upadhya et al. 2002). CYP3A4 expression was measured to evaluate the effect of Gris (Elewski and Tavakkol 2005). Moreover, the expression of CPR in microsomes was also determined.

Materials and methods

Enflurane and Isoflurane were from Abbott Laboratories S.A., Argentina. All other chemicals used were reagent grade obtained from Sigma-Aldrich Chem Co., St. Louis, Missouri, USA.

Animals

Albino male adult *CF1* mice (4–6 animals per group) weighing 25–30 g were maintained in controlled conditions and allowed free access to food (Purina 3, Asociación de Cooperativas Argentinas, San Nicolás, Buenos Aires, Argentina) and water. Animals received human care and were treated in accordance with guidelines established by the Animal Care and Use Committee of the Argentine Association of Specialists in Laboratory Animals (AADEALC). All treatments were performed at the same time of the day.

Treatments

The administration of drugs was performed as previously described (Lavandera et al. 2007). In brief, each group of mice received one (2 mL/kg, i.p.) or 10 doses (1 mL/kg, i.p.) of the anaesthetics Enflurane and Isoflurane; AIA (a single dose of 350 mg/kg, i.p); veronal (167 mg/kg, s.c., during 3 days), and ethanol (30%, *v*/*v*, in the drinking water for one week). Gris was administered in two different ways: topically (one dose of 50 mg/mL) and in the diet (2.5%, 15 days). Another group was starved 24 h prior to sacrifice. Control animals received the vehicle or were exposed to the same experimental conditions, and they were sacrificed at the times corresponding to each treatment.

Homogenate preparation

Whole brain previously perfused with saline solution was scissored and immediately processed. Brain tissue was homogenized (1:5, *w*/*v*) in 10 mmol/L TRIS-HCl pH 7.4, containing 20% glycerol (*v*/*v*), 1.14% KCl (*w*/*v*), 0.2 mmol/L EDTA, 0.1 mmol/L dithiothreitol, 0.1 mmol/L phenylmethylsulphonylfluoride, 10 μg/mL leupeptin and 1 μg/mL pepstatin A.

Homogenates were centrifuged for 15 min at 1 500*g*. The supernatant obtained was then centrifuged for 15 min at 18 000*g*, and the pellet obtained was washed once with the same solution used for homogenization and again centrifuged for 15 min at 18 000*g* (mitochondrial fraction). The supernatants from the first and the second 18 000*g* centrifugations were pooled and centrifuged for 90 min at 105 000*g*; the pellet obtained was washed once with the buffer used for homogenization (microsomal fraction) (Lavandera et al. 2007).

Proteins were quantified according to Bradford's method (Bradford 1976).

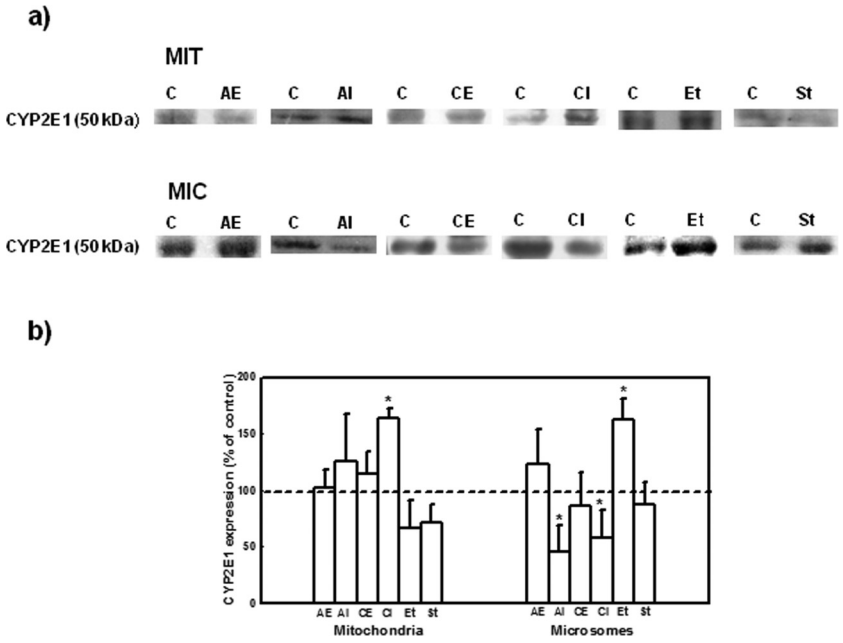
Immunoblotting of CYP isoforms and CPR

For the western Blot analysis of CYP isoenzymes, an equal amount of total protein was loaded in each lane: 3 μg of microsomal protein and 15 μg of mitochondrial protein. Proteins were separated on a 7.5% SDS-PAGE and transferred to nitrocellulose membranes. After blocking overnight with 5% bovine seroalbumine (BSA) in Tris-buffered saline containing 1% Tween 20 (TBS-T 1%), the blots were incubated for 1 h at room temperature with the specified primary antibody: anti-CYP2E1 (1:2.500 *v*/*v* in TBS-T 1% with 0.5% BSA, Stressgen, Canada), anti-CYP3A (1:500 *v*/*v* in TBS-T 1% with 0.5% BSA, Santa Cruz Biotechnology, Europe), anti-CYP2B1/2 (1:2.500 *v*/*v* in TBS-T 1% with 0.5% BSA, Chemicon, USA) for microsomal and mitochondrial fractions and anti-CPR (75 μg/mL in TBS-T 1% with 5% BSA, Stressgen, USA) to microsomal fraction. After at least 5 washings, blots were incubated for 1 h with a secondary goat anti-rabbit antibody conjugated with horseradish peroxidase (HRP) (1:8000 *v*/*v* in TBS-T 1%, Santa Cruz Biotechnology, USA). Blots were detected by chemiluminescence using ECL detection system (GE Healthcare, USA) and exposed to X-ray film. Quantification of bands was performed using the Scion Image software. Gel loadings were normalized according to total protein content and after immunoblotting the equivalence of sample loading was confirmed by Coomasie blue staining as was described by Welinder and Ekblad (2011).

CYP2E1 activity

CYP2E1 activity was measured spectrophotometricaly using p-nitrophenol as substrate according to Reinke and Moyer (1985). Microsomal or mitocondrial fractions (0.1 mL) were incubated in a solution containing 0.1 mol/L pH 7.4 phosphate potasium buffer; 1 mmol/L NADPH, and 0.2 mmol/L p-nitrophenol in a final volume of 1 mL. The mixture was incubated at 37 °C during 10 min and then the absorbance was register at 546 nm. One enzyme unit was expressed as the amount of enzyme that catalyze 1 nmol of 4-nitrochatecol (extinction coefficient at 546 nm = 12 $(mmol/L)^{-1}$ cm^{-1}). Specific activity was defined as nmol/mg protein.

Fig. 1. Effect of porphyrinogenic agents on mitochondrial and microsomal CYP2E1 expression. (*a*) Western blot of CYP2E1. An equal amount of total protein was loaded in each lane. (*b*) Columns represent normalized signals of control and treated animals that were quantified using an image analyzer. Gel loadings were normalized according to total protein content, and after immunoblotting, the equivalence of sample loading was confirmed by Coomasie blue staining. C: Control; AE: Acute Enflurane; AI: Acute Isoflurane; CE: Chronic Enflurane; CI: Chronic Isoflurane; Et: Ethanol; St: starvation; MIT: mitochondria; MIC: microsomes. Values are expressed as mean of at least 3 determinations run in duplicate and are expressed as a percentage, using the control group as 100% (dotted line). * $p < 0.05$, significance of differences between treated and control groups. Experimental details are described in the text.



Statistical analysis

Data were expressed as mean ± SD. Differences in mean values among the groups were analyzed using the analysis of variance (ANOVA) and $p < 0.05$ was considered statistically significant.

Results

Effect of porphyrinogenic agents on microsomal and mitochondrial CYP isoforms expression

CYP2E1

CYP2E1 expression (Fig. 1) was determined in brain mice microsomes and mitochondria treated with volatile anaesthetics, ethanol and under starvation.

In mitochondria, CYP2E1 expression was unchanged by the effect of acute anaesthetics, ethanol administration, and also under starvation. Instead, chronic Isoflurane anaesthesia induced 50% ($p < 0.05$) protein expression.

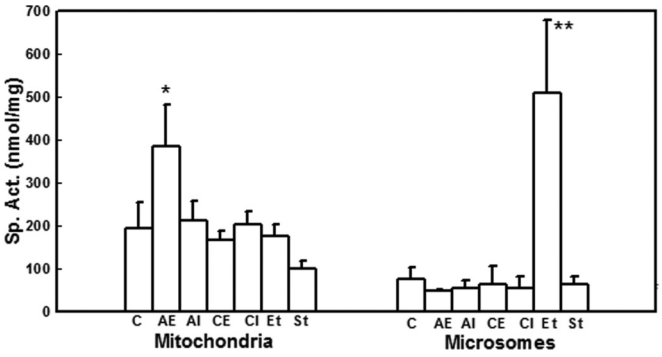
In microsomes, the administration of acute or chronic Isoflurane diminished around 50% ($p < 0.05$) protein expression. No alterations were observed by effect of Enflurane. Starvation produced no changes in CYP2E1 expression while ethanol caused a 50% ($p < 0.05$) induction.

Because of the alterations described above in CYP2E1 expression, it was of interest to evaluate if there were some correlation with CYP2E1 activity in mice brain microsomes and mitochondria (Fig. 2).

In mitochondria, CYP2E1 activity was 100% ($p < 0.05$) induced after acute Enflurane administration without any alteration when this anaesthetic was chronically administered or after Isoflurane anaesthesia. No effect was observed either by ethanol or under starvation status.

In microsomes, CYP2E1 activity was strongly induced (8-fold, $p < 0.01$) in the group receiving ethanol. No significant changes were observed due to any of the other porphyrinogenic agents assayed.

Fig. 2. Effect of porphyrinogenic agents on mitochondrial and microsomal CYP2E1 activity. C: Control; AE: Acute Enflurane; AI: Acute Isoflurane; CE: Chronic Enflurane; CI: Chronic Isoflurane; Et: Ethanol; St: starvation. *, $p < 0.05$, **, $p < 0.01$, significance of differences between treated and control groups. A unique control value is given, because no significant differences were obtained in any of the controls after administration of the corresponding vehicles. Other experimental details are described in the text.



CYP2B1

CYP2B1 expression was determined in brain mice microsomes and mitochondria treated with AIA and veronal (Fig. 3).

No changes were observed in CYP2B1 expression in either subcellular fraction.

CYP3A4

CYP3A4 expression was measured in brain mice microsomes and mitochondria treated with Gris (Fig. 4).

In mitochondria, protein expression was unchanged independently on the way the toxic was administered.

Fig. 3. Effect of porphyrinogenic agents on mitochondrial and microsomal CYP2B1 expression. (*a*) Western blot of CYP2B1. An equal amount of total protein was loaded in each lane. (*b*) Columns represent normalized signals in the control and treated animals that were quantified using an image analyzer. Gel loadings were normalized according to total protein content, and after immunoblotting the equivalence of sample loading was confirmed by Coomasie blue staining. C: Control; AIA: Allylisopropylacetamide; V: veronal; MIT: mitochondria; MIC: microsomes. Values are expressed as mean of at least 3 determinations run in duplicate and are expressed as a percentage, using the control group as 100% (dotted line). Other experimental details are described in the text.

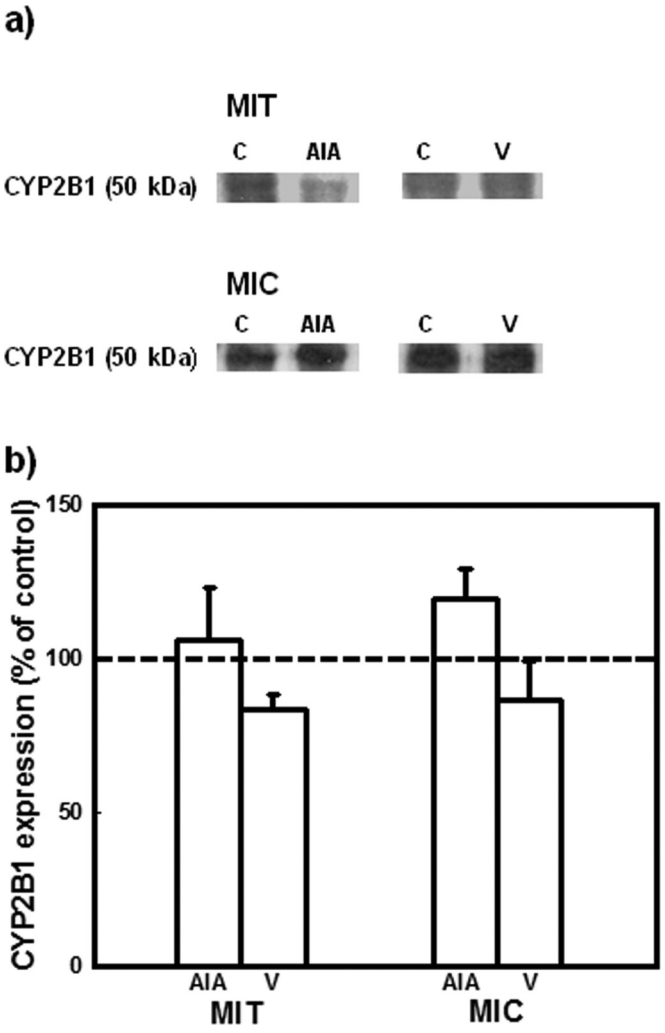
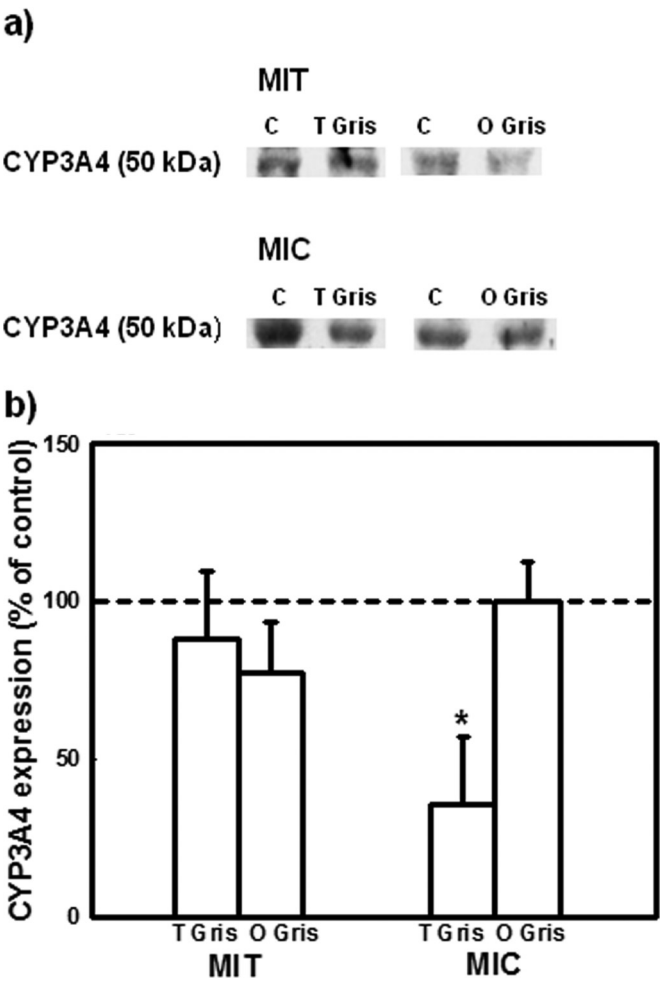


Fig. 4. Effect of porphyrinogenic agents on mitochondrial and microsomal CYP3A4 expression. (*a*) Western blot of CYP3A4. An equal amount of total protein was loaded in each lane. (*b*) Columns represent normalized signals in the control and treated animals that were quantified using an image analyzer. Gel loadings were normalized according to total protein content, and after immunoblotting the equivalence of sample loading was confirmed by Coomasie blue staining. C: Control; T Gris: Topical Griseofulvin; O Gris: Oral Griseofulvin; MIT: mitochondria; MIC: microsomes. Values are expressed as mean of at least 3 determinations run in duplicate and are expressed as a percentage, using the control group as 100% (dotted line). *, $p < 0.05$, significance of differences between treated and control groups. Other experimental details are described in the text.



In microsomes, CYP3A4 expression was 65% ($p < 0.05$) diminished after topical Gris administration, without any variation when this drug was given in the diet.

Effect of porphyrinogenic agents on microsomal CPR expression

CPR expression was evaluated in microsomal fraction of mice brain treated with all porphyrinogenic drugs described previously (Fig. 5).

CPR expression was around 50% and 80% ($p < 0.05$) enhanced after acute anaesthetics administration and starvation respectively, and it was 40% ($p < 0.05$) diminished after topical Gris. Chronic anaesthetics, AIA, veronal, and ethanol produced no changes in the expression of this protein.

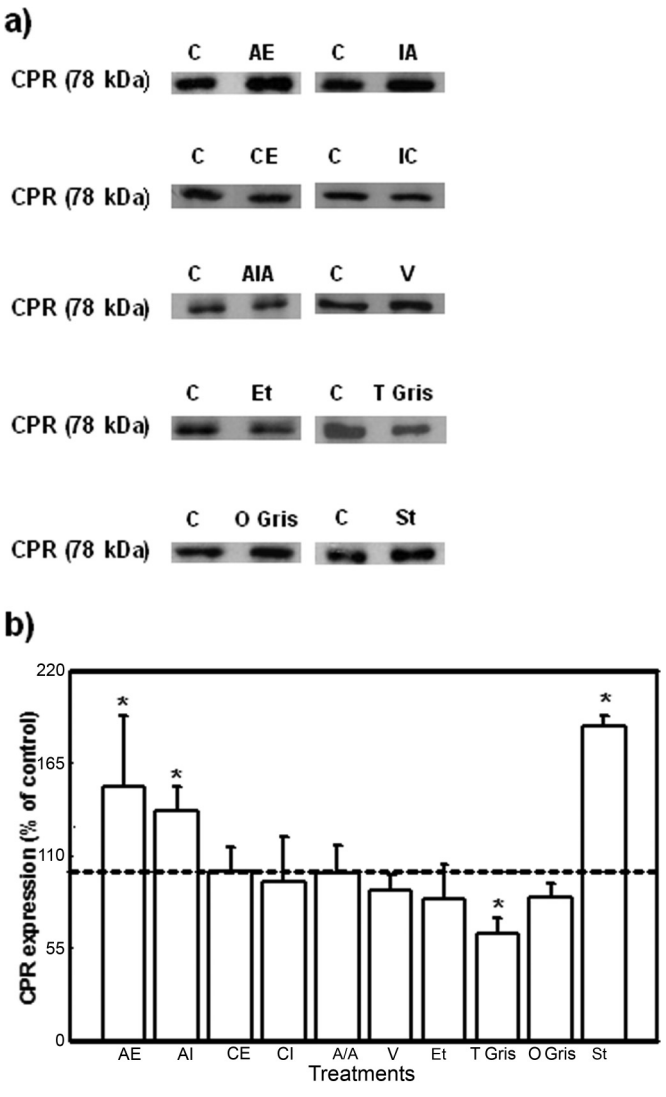
Discussion

An important factor in the alteration of hepatic heme biosynthesis induced by porphyrinogenic drugs is the interaction with one or several CYP isoenzymes (Correia et al. 2011). Depending on the delicate balance between detoxification and activation, the induction or inhibition of CYP could be a beneficial or harmful response. Inhibition is an immediate response, while induction is a slow regulatory process.

It was suggested that CYP induction in brain would have a severe impact on drugs metabolism in this organ. Classic inductors like phenobarbital, phenytoin, and ethanol induced the levels of several isoforms of CYP in brain, but this action did not occur in other tissues (Gervasini et al. 2004; Sanchez-Catalan et al. 2008).

In the brain, total CYP levels are 0.5%–2% of liver levels. Although, brain CYP can potentially have a considerable impact on

Fig. 5. Effect of porphyrinogenic agents on mitochondrial (MIT) and microsomal (MIC) CPR expression. (*a*) Western blot of CPR. An equal amount of total protein was loaded in each lane. (*b*) Columns represent normalized signals in the control, and treated animals that were quantified using an image analyzer. Quantification of bands was performed using the Scion Image software. Gel loadings were normalized according to total protein content, and after immunoblotting the equivalence of sample loading was confirmed by Coomasie blue staining. C: Control; AE: Acute Enflurane; AI: Acute Isoflurane; CE: Chronic Enflurane; CI: Chronic Isoflurane; Et: Ethanol; St: starvation; AIA: Allylisopropylacetamide: V: veronal; T Gris: Topical Griseofulvin; O Gris: Oral Griseofulvin. Values are expressed as mean of at least 3 determinations run in duplicate and are expressed as a percentage, using the control group as 100% (dotted line). *, $p < 0.05$, significance of differences between treated and control groups. Other experimental details are described in the text.



local metabolism and inducers, inhibitors or genetic variation may also play a role in the susceptibility to neurological diseases (Ferguson and Tyndale 2011).

We have previously observed that some of the porphyrinogenic agents studied altered mitochondrial brain total CYP but not the microsomal levels. That was the case of chronic anaesthesia with Isoflurane and starvation that diminished total CYP levels (Lavandera et al. 2007).

In the present work, the effect of porphyrinogenic agents such as volatile anaesthetics, ethanol, Gris, and starvation on some CYP isoforms expression was studied. In particular we were interested in evaluating whether mitochondrial and microsomal CYPs were or not responding both in the same manner.

Some of the porphyrinogenic agents studied altered expression mainly in microsomes. Chronic Isoflurane increased the mitochondrial isoform, however this same anaesthetic inhibited the microsomal expression. Ethanol and topical Gris affected specific isoforms in microsomes but not in mitochondria.

CYP2E1 is one of the most studied CYP enzymes in animals and humans, due to its role in ethanol metabolism and its participation in metabolic activation of some procarcinogens (Knockaert et al. 2011). Moreover, CYP2E1 has particular relevance in several pathologies, because high amounts of reactive oxygen species are producing during the detoxification process (Knockaert et al. 2011). Hepatic and renal toxicity of volatile anaesthetics is due to their biotransformation to toxic metabolites mediated by CYP, being the isoform CYP2E1, responsible for the metabolism of these compounds in humans (Miksys and Tyndale 2013). We have previously observed that this isoform is always induced in liver after administration of Enflurane or Isoflurane (Buzaleh et al. 1997; Martínez et al. 2000).

When the effect of anaesthetics, ethanol, and starvation on CYP2E1 activity was evaluated, induction of enzymatic activity in microsomes was only observed after ethanol administration. The same action was produced by acute Enflurane in mitochondria. CYP2E1 expression was enhanced or diminished depending on the agent, and the response was also differential in the mitochondria with respect to the microsomes. In all instances, Isoflurane produced more alterations than Enflurane.

Veronal, a phenobarbital derivative, induces specific CYP synthesis of 2B and 3A family in several tissues (Handschin and Meyer 2003). Upadhya et al. (2002) reported CYP2B1 induction in cortex after Phenobarbital administration in rats but not in other brain areas.

AIA and veronal did not affect the expression of CYP2B1 either in mitochondria or in microsomes. It would be likely that there were other isoforms mainly involved in the metabolization of these xenobiotics, as suggested by other authors when they evaluated the effects of these xenobiotics in rat brain and they described the induction of CYP3A based on N-alkylporphyrins formation (Wong et al. 1999), or mRNA expression (Schilter et al. 2000), or that their response might be different in the brain. Further studies are needed to support this hypothesis.

Schilter et al. (2000) reported that animals treated i.p. with four daily doses of Phenobarbital (80 mg/kg) demonstrated markedly induced levels of CYP2B1 mRNA in the striatum and cerebellum, with no or slight alterations after 1 or 2 days of Phenobarbital treatment, although the latter treatments produced marked induction of the corresponding genes in the liver. The differences with our results could be attributed to the fact that we are evaluating protein expression in whole brain.

There are some cases where the effect is different in liver and brain; for example, ethanol induces CYP2E1 in liver rat but not in encephalon (Schoedel et al. 2001); nicotine also, induces CYP2B1 in rat encephalon but not in liver (Miksys et al. 2000).

CYP3A4 was altered in brain mice depending on the way that Gris was administered.

Some hormones and drugs that are known to enhance CYP isoform expression, showed only a weak effect on CPR levels, so that CPR and CYP ratios in hepatic microsomes can vary from 1:15 to 1:100 under different treatments (Riddick et al. 2013).

In this work, we have observed an enhanced expression of CPR concomitant with the increased enzymatic activity previously reported (Lavandera et al. 2007), when the anaesthetics Enflurane and Isoflurane were administered in an acute way and when ani-

mals were starved. Topical Gris also affected protein expression. In the other groups, expression was not altered.

In conclusion, these results revealed a differential tissue response after administration of various xenobiotics. Although, the liver is the principal organ involved in the exogenous drugs metabolization, we have observed that other hepatic tissues can also participate in this process.

Developments occurring in a multitude of disciplines support the fact of an emergent role of CYP enzymes, their metabolic substrates and final products, in the pathogenesis and treatment of central nervous system disorders. Therefore CYP response in brain could be one of the multiple factors having a great influence in neurological disorders found in the acute porphyrias. The roles and regulation of brain CYPs may be useful for the development of novel strategies to better predict, prevent, and treat diseases (Ferguson and Tyndale 2011).

Although the physiological functions and toxicological implications for the mitochondrial forms of CYPs are poorly understood (Genter et al. 2006; Avadhani et al. 2011), this work provides a new insight for this organelle in brain, suggesting important roles in the metabolization of xenobiotics, as proposed earlier by Anandatheerthavarada et al. (1997) for liver mitochondria.

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