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INDUCTION OF HEPATIC AMINOLEVULINATE ACID SYNTHETASE ACTIVITY BY ISOFLURANE IN A GENETIC MODEL FOR ERYTHROPOIETIC PROTOPORPHYRIA

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Abstract – Erythropoietic Protoporphyria (EPP) is an inherited deficiency of ferrochelatase, the last enzyme of the heme pathway. Under general anaesthesia, some patients develop neurological dysfunction suggesting upregulation in heme biosynthesis similar to that described for acute porphyrias after xenobiotic administration. Our aim has been to evaluate whether Isoflurane induces alterations in the heme pathway in a mouse model for EPP. Administration of Isoflurane (a single dose of 2 ml/kg, i.p) to wild-type (+/+), heterozygous (+/Fech^{m1Pas}) and homozygous (Fech^{m1Pas}/Fech^{m1Pas}) mice, was evaluated by measuring the activity of δ-Aminolevulinic acid synthetase (ALA-S) and Porphobilinogen-deaminase (PBG-D) in different tissues, as well as Heme oxygenase (HO), cytochrome P-450, CYP2E1 and glutathione levels in liver. Porphyrin precursors were measured in 24h-urine samples. Fech^{m1Pas}/Fech^{m1Pas} mice receiving anaesthesia show enhanced ALA-S and CYP2E1 activities in the liver and increased urinary excretion of porphyrin precursors. No alterations were found in either PBG-D or HO activities. Diminished glutathione levels suggest that anaesthesia may produce oxidative stress in these animals. In conclusion, Isoflurane induces ALA-S activity and increased excretion of porphyrin precursors in EPP mice. These findings appear to confirm our previous hypothesis and indicate that Isoflurane may be an unsafe anaesthetic not only for patients with acute porphyrias but also for individuals with non acute porphyrias.

Key words: Erythropoietic Protoporphyria, Isoflurane, heme metabolism, cytochrome P-450, glutathione

INTRODUCTION

Anaesthesia is one of the factors triggering attacks of acute porphyrias. Isoflurane (Forane, 1-Cl-2,2,2-trifluoroethyldifluoromethylether) is an anaesthetic used to produce general anaesthesia (15, 28). It is metabolized through cytochrome P-450 (CYP), by the isoform CYP2E1 (10, 20, 21).We have previously demonstrated that when this xenobiotic was administered to mice, significant alterations in heme metabolism and its regulation occurred in a sex and strain dependent manner (8, 9).

Erythropoietic protoporphyria (EPP) is an inherited deficiency of the last enzyme in the heme pathway, ferrochelatase which is responsible for the insertion of a ferrous ion (Fe^{2+}) into protoporphyrin (PP). Reduced activity of ferrochelatase results in an increased PP concentration in erythrocytes, plasma, liver and feces (17, 27). The predominant clinical feature is cutaneous photosensitivity of childhood onset. Hepatic deposit of PP appears to be related to liver damage, including hepatitis, mild and severe forms of fibrosis and cirrhosis which may eventually lead to fulminant liver failure and need liver transplantation (5, 32). Although, no neurovisceral symptoms in EPP have been reported, some patients, with severe hepatic complications might develop motor neuropathy

Abreviations: ALA, δ-aminolevulinic acid; **ALA-D,** δ-Aminolevulinic acid dehydratase; **ALA-S,** δ-Aminolevulinic acid synthetase; **CYP,** cytochrome P-450; **EPP,** erythropoietic protoporphyria; **Fech,** Ferrochelatase; **GSH,** glutathione; **HO,** Heme oxygenase; **i.p. ,** intraperitoneal; **PBG,** porphobilinogen; **PBG-D,** Porphobilinogen-deaminase; **PP,** protoporphyrin; **S.D. ,** standard deviation

after liver transplantation, resembling the neurological crises of acute porphyrias (18, 22, 29). Thus, it was of interest to examine the effects of volatile anaesthetics on EPP to evaluate whether these drugs induce alterations in heme biosynthesis similar to those occurring in acute porphyria as was previously observed in CF1 mice (9).

To this end, we used a genetic model of EPP (Fech ^{m1pas}/Fech ^{m1pas}) (35). The activity of the heme enzymes, δ-Aminolevulinic acid synthetase (ALA-S), δ-Aminolevulinic acid dehydratase (ALA-D), Porphobilinogen-deaminase (PBG-D) and Ferrochelatase (Fech) was measured in liver, spleen and kidney from wild type and in the mouse model for EPP.

The effect of isoflurane administration to this genetic animal model of EPP on heme metabolism was evaluated by measuring the activity of the enzymes ALA-S, PBG-D and Heme oxygenase (HO). Moreover, total CYP levels and the activity of CYP2E1 isoform were determined in the liver. Taking into account that PP accumulation leads to oxidative stress in mice (1), the effect on hepatic glutathione (GSH) levels was also evaluated.

MATERIALS AND METHODS

Animals

The Fech^{m1Pas}/Fech^{m1Pas} mutant mice with a BALB/cJ background were obtained from the Jackson Laboratory (Bar Harbor, ME). Subsequently, a colony was established in the animal facility of the Hospital Doce de Octubre, Madrid, Spain. Mice were kept with unlimited water supply and standard laboratory chow under 12 hours light/dark cycles. Three groups of 4-6 animals (8-12 weeks of age) were used: wild-type mice (+/+), heterozygous (+/Fech^{m1Pas}) and homozygous (Fech^{m1Pas}/Fech^{m1Pas}). Homozygous pups were distinguished from their heterozygous littermates by genomic DNA PCR followed by restriction analysis as previously described (6). All animal experimentation was conducted in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals.

Experimental design

Animals received a single dose of 2 ml/kg (0.6:3, v/v. in oil) i.p, of Isoflurane. The dose of 2 ml/kg was chosen as the optimum anaesthetic dose producing more alterations in the heme pathway, as previously described (9). Control animals received the vehicle used for anaesthetic solubilisation (oil) and were sacrificed at the same time as treated mice. All the experiments were performed at the same time of the day. To overload the effect of Isoflurane administration on heme precursor excretion, ALA and porphobilinogen (PBG), five EPP mice were used. On the first day the aninals received saline serum. On the second day, mice were i.p. injected with 3 doses of oil (vehicle) every 4 hours, and on the last day animals received fresh prepared isoflurane oil mixture in the same protocol as vehicle.

Sample collection

Blood was collected in heparinised tubes through cardiac puncture. After perfusion, liver, spleen and kidney were excised and were immediately homogenized with buffers adequate for each enzyme determination. EPP mice were housed in metabolic cages (reference 3600M021 BIOSIS S.L. Biologic Systems) to collect 24-h. urine samples.

Biochemical assays

The activity of ALA-S was determined in liver and kidney according to Marver et al. (24). ALA-D activity was measured in liver, spleen, kidney and blood following Berlin et al. (4). PBG-D activity was measured in liver, kidney and blood by the method described by Anderson et al. (2). Ferrochelatase activity was measured in the kidney, spleen and liver (16). HO was determined in liver according to Tenhunen et al. (33) with minor modifications. Total CYP levels and CYP2E1 activity were measured in liver microsomes as previously described (26, 30) respectively. GSH levels were quantified in liver homogenates according to Rossi et al. (31). Protein concentration was measured by Bradford assay (7) (Bio-Rad Protein Assay, Bio-Rad Laboratories, München, Germany). ALA and PBG were quantified in urine using a quantitative ion exchange column method (BioSystems SA, Barcelona) and measured by spectrophotometry (Ultrospc 3000, Pharmacia Biotech, Buckinghamshire, UK) at 555 nm.

Statistical analysis

The mean and Standard deviation of all parameters for each group were calculated. The significance of differences between groups was analyzed using the analysis of the variance (ANOVA) test and $p < 0.05$ was regarded as significant.

RESULTS

Enzyme activity of the critical steps in the heme synthesis pathway in the mouse model of EPP

In Fech^{m1Pas}/Fech^{m1Pas} mice, the activity of the first two enzymes of heme synthesis, ALA-S and ALA-D was significantly diminished in liver, with no alterations in kidney and spleen (Table I). The activity of PBG-D was not modified in the liver or kidney of the EPP mice when compared with the wild-type animals. However, the enzyme was induced in blood (Table I**).** In the Fech^{m1Pas} mice the genetic defect is transmitted in an autosomal recessive fashion resulting in a Ferrochelatase activity 45 to 65 % of normal in the heterozygous mice. Heterozygous animals did not accumulate PP, had normal liver function and were not sensitive to light exposure. Instead, homozygous mice showed a marked reduction of the catalytic activity of the encoded enzyme (up to 10 % of normal in various tissues) (Table I).

Table 1. Heme synthesis parameters in wild type and **Fech $^{ml\ Pas}$/Fech $^{ml\ Pas}$** mice

Enzyme	Tissue	Specific Activity		p
		+/+	**Fech $^{ml\ Pas}$/Fech $^{ml\ Pas}$**	
ALA-S (pmol/mg protein)	Liver	126.30 ± 22,50	68.60 ± 22.9	P<0.01
	Spleen	107.90 ± 22.60	92.70 ± 16.5	NS
	Kidney	122.40 ± 12.60	134.90 ± 30.7	NS
ALA-D (nmol/mg protein)	Liver	30.60 ± 5.80	22.00 ± 2.90	P<0,05
	Spleen	62.50 ± 10.60	57.00 ± 9.50	NS
	Kidney	18.70 ± 1.50	21.60 ± 2.10	NS
	Blood	19.10 ± 4.60	29.50 ± 4.50	P<0.05
PBG-D (pmol/mg protein)	Liver	3.40 ± 0.90	2.60 ± 0.31	NS
	Spleen	7.30 ± 2.70	6.30 ± 1.34	NS
	Kidney	3.10 ± 0.96	2.30 ± 0.87	NS
	Blood	0.13 ± 0.16	0.23 ± 0.12	P<0,05
Fech (nmol/mg protein)	Liver	2.80 ± 0.69	0.42 ± 0.06	p<0.001
	Spleen	1.40 ± 0.44	0.36 ± 0.13	p<0.001
	Kidney	4.80 ± 1.64	0.47 ± 0.29	p<0.001

Data represent mean value ± S.D. N.S.: Non significant. Experimental details are described in the text.

Table 2. Effects of Isoflurane on PBG-D and HO activity in a mouse model of EPP

Enzyme	Tissue	Mice	Specific activity	
			Control	Isoflurane
PBG-D **(**pmol/mg protein)	Liver	+/+	3.251 ± 0.898	4.349 ± 0.729
		+/-	4.647 ± 0.763	5.072 ± 0.693
		-/-	2.714 ± 0.235	3.592 ± 0.729
	Kidney	+/+	3.065 ± 0.771	3.197 ± 0.412
		+/-	2.885 ± 0.354	2.915 ± 0.208
		'-/-	2.300 ± 0.870	3.312 ± 0.219
	Blood	+/+	0.134 ± 0.016	0.170 ± 0.066
		+/-	0.165 ± 0.046	0.170 ± 0.066
		-/-	0.232 ± 0.012 #	0.201 ± 0.067
HO (nmol/mg protein)	Liver	+/+	1.330 ± 0.280	1.265 ± 0.256
		+/-	1.491 ± 0.299	1.265 ± 0.266
		-/-	1.127 ± 0.165	1.293 ± 0.278

Three groups of 4-6 animals (8-12 weeks of age): wild-type (+/+), heterozygous (+/Fech^{m1Pas}) (+/-) and homozygous (Fech^{m1Pas}/Fech^{m1Pas})(-/-) mice received a single dose of 2 ml/kg (0.6:3, v/v. in oil) i.p, of Isoflurane and were sacrificed 20 minutes after injection (#) $P < 0.05$, significance of differences between homozygous and heterozygous or wild type animals. Data represent mean value ± S.D. Other experimental details are described in the text.

Effects of Isoflurane on ALA-S activity in the mouse model of EPP

When the mice model Fech^{m1Pas}/Fech^{m1Pas} received Isoflurane, an induction of ALA-S activity was observed in liver ($p<0.01$). No significant variations were detected in the wild-type or in the heterozygous group after anaesthesia (Figure 1). The renal enzyme was not altered either in any group by the action of isoflurane (Figure 1).

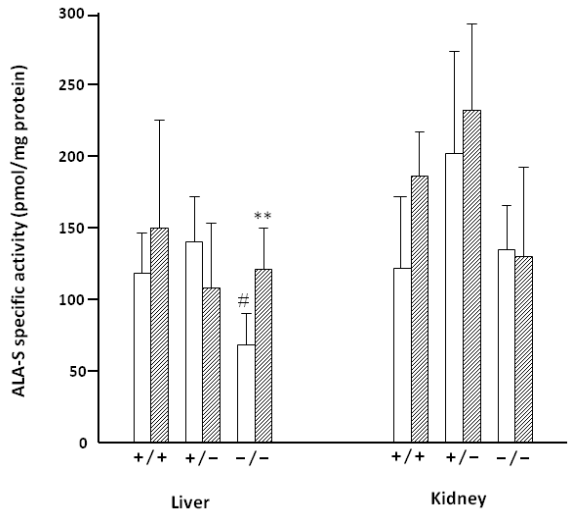

Figure 1. Effects of Isoflurane on ALA-S activity in the liver and kidney from a mouse model of EPP.
Three groups of 4-6 animals (8 -12 weeks of age): wild-type (+/+), heterozygous (+/Fech^{m1Pas}) (+/-) and homozygous (Fech^{m1Pas}/Fech^{m1Pas}) (-/-) mice received a single dose of 2 ml/kg (0.6:3, v/v. in oil) i.p, of Isoflurane and were sacrificed 20 minutes after injection (▭). The corresponding controls (▨) received oil. Data represent mean value ± S.D. (**) $P < 0.01$, significance of differences between treated and control animals. (#) $P < 0.01$, significance of differences between homozygous and heterozygous or wild type animals. Experimental details are described in the text.

Effects of Isoflurane on ALA and PBG levels in the mouse model of EPP

EPP mice injected with the Isoflurane showed an increased excretion of ALA and PBG when compared with the same animals before and after oil injection (Figure 2).

Effects of Isoflurane on PBG-D activity in the mouse model of EPP

No significant changes in PBG-D activity were detected in liver, kidney or blood when Isoflurane was administered to wild type, heterozygous or homozygous EPP mice (Table 2).

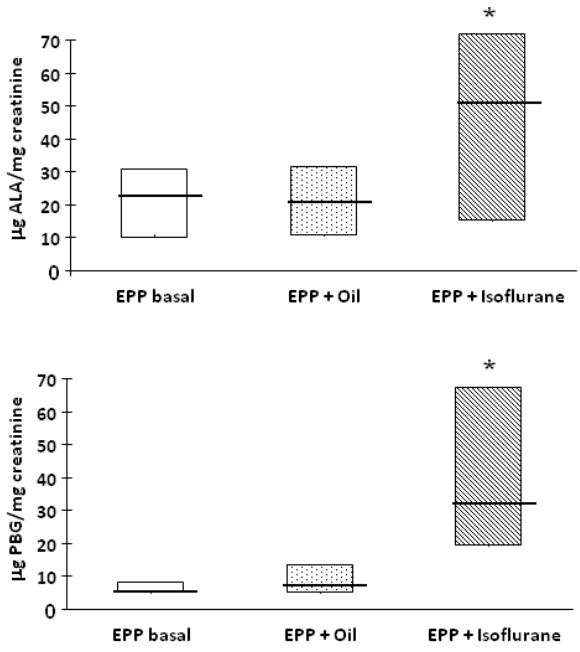

Figure 2. Effects of Isoflurane on ALA and PBG urine excretion in the mouse model of EPP.
Five EPP males were lodged in metabolic cages to collect 24-h. urine samples. The first day the aninals received saline serum. On the second day, mice were intraperitoneally injected with 3 doses of oil (vehicle) every 4 hours, and on the last day animals received fresh prepared Isoflurane oil mixture in the same protocol as vehicle. Data were transformed logarithmically [log(1+X)] before Paired t test analysis and represent mean value ± S.D. (*) P <0.05, significance of differences following vehicle and Isoflurane administration. Experimental details are described in the text.

Effects of Isoflurane on HO activity in the mouse model of EPP

No significant alterations were observed either in HO activity after Isoflurane administration (Table 2).

Effects of Isoflurane on CYP levels and CYP2E1 activity in the mouse model of EPP

CYP levels were reduced in Fech^{m1Pas}/Fech^{m1Pas} as compared to the wild type group, with no differences in CYP2E1 activity. After Isoflurane administration, no changes in the CYP content of homozygous mice were observed. The anaesthetic diminished CYP levels in the wild type and in the heterozygous mice reaching values similar to those found in the EPP mice. CYP2E1 activity was more than 100% ($p<0.05$) induced after Isoflurane administration independent of the group analyzed (Figure 3).

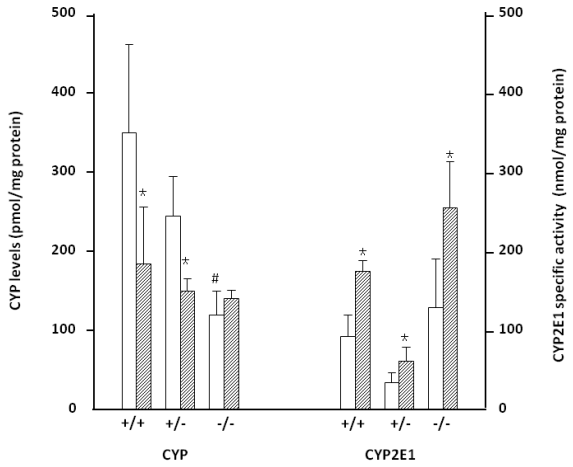

Figure 3. Effects of Isoflurane on CYP levels and CYP2E1 activity in the liver from a mouse model of EPP. Three groups of 4-6 animals (8-12 weeks of age): wild-type (+/+), heterozygous (+/Fech^{m1Pas}) (+/-) and homozygous (Fech^{m1Pas}/Fech^{m1Pas}) (-/-) mice received a single dose of 2 ml/kg (0.6:3, v/v. in oil) i.p, of Isoflurane and were sacrificed 20 minutes after injection (▨). The corresponding controls (□) received oil. Data represent mean value ± S.D. (*) $P < 0.05$, significance of differences between treated and control animals. (#) $P < 0.01$, significance of differences between homozygous and heterozygous or wild type animals. Experimental details are described in the text.

Effects of Isoflurane on GSH levels in the mouse model of EPP

GSH levels were reduced in the homozygous mice with respect to the wild type and the heterozygous group, although differences did not reach statistical significance (Figure 4). After Isoflurane administration, GSH levels were strikingly diminished (between 40-50%, p<0.05) in all three groups of animals.

DISCUSSION

Anaesthetics, like other porphyrinogenic drugs that exacerbate porphyria (19), have the capacity to induce hepatic ALAS-1, which is closely associated with the induction of CYP, a process increasing the demand for heme synthesis in the liver (11). In our experience in mice, a single dose of Isoflurane induces a great increase in ALA-S activity and a decrease in PBG-D activity at earlier times of anaesthesia, resulting in the accumulation of the heme precursors ALA and PBG (9). A potential role for heme precursors as neurotoxic agents has been suggested by the development of neuropathy in patients with acute porphyria (14).

In humans, EPP has a benign course and most of the affected patients only suffer from photosensitivity. Hepatobiliary disease is a rare complication occurring in 1% - 5% of patients, for whom liver transplantation is often required. The fact that some of these patients develop motor neuropathy after liver transplantation (18, 22, 29) would suggest that the administration of the anaesthesia to individuals who have a partial deficiency of ferrochelatase could enhance heme precursor accumulation and produce an acute crisis in these patients. Knowledge of the anaesthetic used and its management in these patients is thus very important (3, 34). In this study, the effect of the administration of Isoflurane on heme metabolism was studied in a genetic model of EPP (Fech^{m1pas}/Fech^{m1pas}). The clinical characteristics of the Fech ^{m1pas}/Fech ^{m1pas} mouse mutation mimic those of human EPP, even though they are more severe since hepatic disease constitutes a major and constant feature (16).

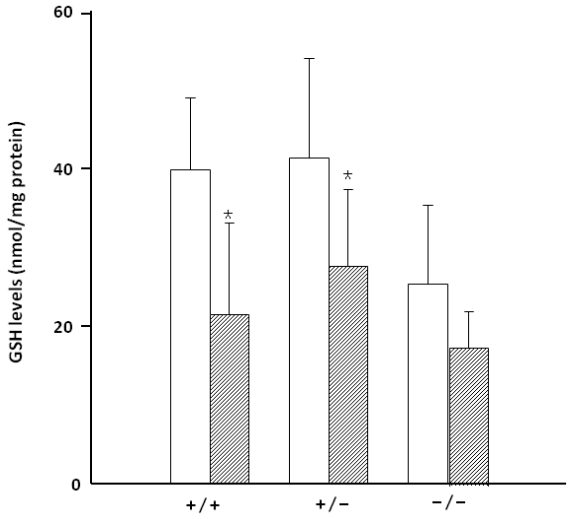

Figure 4. Effects of Isoflurane on GSH levels in the liver from a mouse model of EPP.
Three groups of 4-6 animals (8-12 weeks of age): wild-type (+/+), heterozygous (+/Fech^{m1Pas}) (+/-) and homozygous (Fech^{m1Pas}/Fech^{m1Pas}) (-/-) mice received a single dose of 2 ml/kg (0.6:3, v/v. in oil) i.p, of Isoflurane and were sacrificed 20 minutes after injection (▨). The corresponding controls (□) received oil. Data represent mean value ± S.D. (*) $P < 0.05$, significance of differences between treated and control animals. Other experimental details are described in the text.

Davies et al. (13) used the same mice model and studied the dysfunction of heme synthesis and oxidative stress. These authors observed that total CYP and CYP2E1 expression was reduced in Fech ^{m1pas}/Fech ^{m1pas} mice as compared to wild type and that, despite PP accumulation in the liver, expression of ALA-S

and HO1 was only modestly affected. Navarro et al. (25) observed no alterations in GSH levels in the same homozygous Fech ^{m1pas} mice.

In Fech ^{m1pas}/Fech ^{m1pas} mice, the regulatory enzyme of heme synthesis, ALA-S was induced by Isoflurane anaesthesia in the liver. Considering that in the homozygous Fech ^{m1pas} mice, ALA-D activity was also reduced in the liver**,** the possibility of accumulation of neurotoxic ALA is likely.

In our study, as expected, CYP2E1 activity was induced in all groups receiving the anaesthetic. Reduction of GSH levels after Isoflurane would indicate that oxidative stress has been produced by this anaesthetic. We have previously observed a similar response after Isoflurane administration to *CF1* mice (9). However, HO activity, an enzyme frequently induced against oxidative stress (23) did not change.

Moreover, Enflurane belongs to the same family as Isoflurane (28). We have previously reported that both anaesthetics produced similar effects when given to CF1 control mice. Even though both xenobiotics would appear to act through a different mechanism, these produce the same final effect: that is deregulation of the heme pathway (11). Thus, the same advice applies to Enflurane. Supporting this proposal, an induction of ALA-S was observed when we studied the effects of both anaesthetics added to a B-lymphocyte cell line established from hepatoerythropoietic porphyria patients (12) another non acute porphyria.

In conclusion, if a drug is considered potentially porphyrinogenic when its administration produces alterations in ALA-S activity or depletes the regulatory free heme pool in the liver, the findings here described, suggest that Isoflurane could be an unsafe anaesthetic if administered to individuals with the syndrome of non acute porphyria.

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