Phenylbutyrate up-regulates the adrenoleukodystrophy-related gene as a nonclassical peroxisome proliferator

Catherine Gondcaille,1 Marianne Depreter,2 Stéphane Fourcade,1 Maria Rita Lecca,1 Sabrina Leclercq,1 Pascal G.P. Martin,2 Thierry Pineau,3 Françoise Cadepond,4 Martine ElEtr,4 Nathalie Bertrand,5 Alain Beley,5 Sandrine Duclos,1 Dirk De Craemer,2 Frank Roels,2 Stéphane Savary,1 and Maurice Bugaut1

1Laboratoire de Biologie Moléculaire et Cellulaire, Faculté des Sciences Gabriel, 21000 Dijon, France
2Department of Human Anatomy, Embryology, Histology and Medical Physics, Ghent University, 9000 Gent, Belgium
3Laboratoire de Pharmacologie et Toxicologie, Institut National de la Recherche Agronomique, 31931 Toulouse, France
4Institut National de la Santé et de la Recherche Médicale U488, 94276 Le Kremlin-Bicêtre, France
5Laboratoire de Pharmacodynamie, Faculté de Pharmacie, 21000 Dijon, France

X-linked adrenoleukodystrophy (X-ALD) is a demyelinating disease due to mutations in the ABCD1 (ALD) gene, encoding a peroxisomal ATP-binding cassette transporter (ALDP). Overexpression of adrenoleukodystrophy-related protein, an ALDP homologue encoded by the ABCD2 (adrenoleukodystrophy-related) gene, can compensate for ALDP deficiency. 4-Phenylbutyrate (PBA) has been shown to induce both ABCD2 expression and peroxisome proliferation in human fibroblasts. We show that peroxisome proliferation with unusual shapes and clusters occurred in liver of PBA-treated rodents in a PPARα-independent way. PBA activated Abcd2 in cultured glial cells, making PBA a candidate drug for therapy of X-ALD. The Abcd2 induction observed was partially PPARα independent in hepatocytes and totally independent in fibroblasts. We demonstrate that a GC box and a CCAAT box of the Abcd2 promoter are the key elements of the PBA-dependent Abcd2 induction, histone deacetylase (HDAC)1 being recruited by the GC box. Thus, PBA is a nonclassical peroxisome proliferator inducing pleiotropic effects, including effects at the peroxisomal level mainly through HDAC inhibition.

Introduction

X-linked adrenoleukodystrophy (X-ALD; OMIM 300100) is an inherited disorder characterized by progressive demyelination of the central nervous system and adrenal insufficiency. The disease is due to mutations in the ABCD1 (ALD) gene located in Xq28 (Mosser et al., 1993). ABCD1 encodes the protein ALDP, a peroxisomal member of the ATP-binding cassette family, which is thought to participate in the entry of VLCFA into the peroxisome where VLCFA are β-oxidized. ALDP is a half-transporter, which is supposed to function as a homodimer or a heterodimer, in association with one of the three other peroxisomal ATP-binding cassette half-transporters, ALDRP (adrenoleukodystrophy-related protein), which is the closest homologue of ALDP (Lombard-Platet et al., 1996), PMP70 (70-kD peroxisomal membrane protein; Kamijo et al., 1990), and PMP69 (Holzinger et al., 1997). These transporters are encoded by the ABCD2 (Adrenoleukodystrophy-related), ABCD3, and ABCD4 genes, respectively, and their function is still unclear. Overexpression of ALDRP has been demonstrated to compensate for ALDP deficiency in Abcd1−/− mice, thus preventing VLCFA accumulation and the onset of a neurological phenotype (Pujol et al., 2004). Furthermore, restoration of VLCFA β-oxidation could be obtained in X-ALD human fibroblasts transfected with Abcd2 cDNA (Braiterman et al., 1998; Kemp et al., 1998; Flavigny et al., 1999; Netik et al., 1999; Fourcade et al., 2001). Therefore, pharmacological induction of this partially redundant gene could be a therapeutic strategy for X-ALD. We have shown that fibrates up-regulate Abcd2...
expression (Albet et al., 1997, 2001; Berger et al., 1999; Fourcade et al., 2001) in the liver of rodents. Fibrates can restore β-oxidation of VLCFA in the liver of Abcd1 −/− mice (Netik et al., 1999) but not in brain, possibly due to obstacle of the blood–brain barrier (Waddell et al., 1989; Berger et al., 1999). Fibrates, commonly used as hypolipidemic drugs in human medicine, are peroxisome proliferators (PPPs) in rodents. PPPs are ligands of a member of the steroid nuclear receptor family named PPARα (PP-activated receptor α). PPARα up-regulates expression of target genes involved in lipid metabolism by binding to a DNA sequence called PPRE (PP response element). However, such a functional PPRE has not been found in the Abcd2 promoter (Gartner et al., 1998; Fourcade et al., 2001).

4-Phenylbutyrate (PBA) treatment can restore β-oxidation of VLCFA and increase Abcd2 expression in fibroblasts from X-ALD patients and Abcd1 −/− mice (Kemp et al., 1998). Furthermore, dietary PBA was shown to be efficient in vivo to reduce the VLCFA levels in the brain of Abcd1 −/− mice, but Abcd2 expression has not been analyzed (Kemp et al., 1998). Interestingly, the authors observed a 2.4-fold increase in the number of peroxisomes in 5 mM PBA-treated control or X-ALD human fibroblasts, which was not accompanied by induction of the PPARα and peroxisomal Acyl-CoA Oxidase (AOX) genes but could be linked to the observed induction of the PEX11α gene. This hypothesis was strengthened by the demonstration that PBA-mediated hepatic peroxisome proliferation is absent in Pex11α −/− mice (Li et al., 2002). Classical PPPs do not cause peroxisome proliferation in humans, and fibroblasts are not known to be the target cells for PPPs. Moreover, both peroxisome proliferation and PPARα and Aox induction occur in PPP-treated rodents. Kemp et al. (1998) reported induction of PPARα, but not of Aox, in PBA-treated mice fibroblasts. By contrast, activation of a reporter plasmid containing the rat Aox PPRE has been obtained in PBA-treated mouse hepatoma cells (Pineau et al., 1996) and the binding of PBA to PPARα has been demonstrated using C6 rat glioma cells (Liu et al., 2002). It should also be noticed that an increased expression of PPARα has been observed in PBA-treated human glioma cells (Pineau et al., 1996). Together, the data indicate that PBA, a compound structurally related to fibrates, is a PP that acts partially through noncanonical mechanisms.

Sodium butyrate induces a variety of alterations at the molecular and cellular levels. Transcriptional activation, as a result of the inhibition of histone deacetylase (HDAC) activity could represent the main mechanism of action of butyrate (Davie, 2003). PBA, a butyrate analogue, displays also similar pleiotropic effects in vitro and in vivo. It has been reported that PBA induces hyperacetylation of histones (Lea and Randolph, 1998; Warrell et al., 1998). Deacetylase inhibition may account for Abcd2 induction observed in PBA-treated fibroblasts and probably for PEX11α induction, which might be responsible for the PPARα-independent peroxisome proliferation.

Our main objective was to compare the effects of PBA, butyrate, and fibrate on the expression of Abcd2 and Aox and peroxisome proliferation in vivo in the liver and brain of rats and in vitro in hepatic and glial cells. The molecular mechanism of the PBA regulation of Abcd2 expression was also studied.

### Results

PBA up-regulates expression of the Abcd2 and Aox genes in liver but not in brain

Because the liver is the main target tissue for PPs and demyelination occurs in the brain of X-ALD patients, we evaluated the impact of PBA on peroxisomal gene expression in these tissues. Rats were treated per os with PBA as described by Kemp et al. (1998) (dose ×1) and also with a higher dose (dose ×1.5) during 6–9 wk. Expression of the Abcd2 and Aox genes was increased in the liver (Fig. 1 A), with marked interindividual variations as it was already seen for PPARα (Lemberger et al., 1996). No significant difference could thus be obtained between the two doses (Fig. 1 B). Considering the 10 treated animals, the average induction was 1.9- and 1.5-fold for the Abcd2 and Aox genes, respectively. Gene induction was observed neither in the brain (Fig. 1 A) nor in the spleen, kidney, and testis (unpublished data).
Induction of peroxisomal genes by PPs usually starts several hours after the beginning of a treatment (Motojima, 1997). Because a unique IP injection of PBA (400 mg/kg) was sufficient to weakly induce (1.5-fold) the expression of a PP-sensitive gene (CYP4A1; Pineau et al., 1996), we injected 720 mg of PBA per kilogram per day i.p. for 3 d and analyzed mRNA from liver, brain, intestine, muscle, spleen, kidney, and lung. No induction of the Abcd2 and Aox genes was obtained in the seven examined tissues (unpublished data).

The absence of gene induction in the brain after long- and short-term treatments, which could result from a rapid catabolism (the half-life of PBA is ~1–2 h), prompted us to perfuse PBA directly in the fourth ventricle. No induction of the two peroxisomal genes was found in the brain examined as a whole (Fig. 1 C). In the same way, no change in the gene expression was obtained when ciprofibrate was perfused under the same experimental conditions.

**PBA treatment induces peroxisome proliferation in liver along with appearance of peroxisomal phi-bodies and clusters**

Peroxisomes were observed in the liver of each control and of each per os PBA-treated rat by light microscopy (LM) and EM. When comparing the pooled data from all the treated rats (n = 10) with the pooled data from all the control rats (n = 10), peroxisome proliferation was clearly demonstrated. LM examination showed that the number of peroxisomes per cellular area was higher in the treated animals than in the control animals (×1.4; P < 0.05). By EM morphometric analysis, significantly higher numerical density (×2.3; P < 0.001), volume density (×2.3; P < 0.01), and surface density (×2.2; P < 0.01) versus controls were found in the treated rats, giving evidence of peroxisome proliferation (Fig. 2, A and B). Although the average size of peroxisomes was not significantly different in the control and treated rats, a small percentage of peroxisomes in 8 out of 10 treated animals had very large size (D-circle 2.7-fold that of control peroxisomes) and showed peculiar shapes (phi-bodies; Fig. 2 D). These phi-bodies were present in multiple fragments of the same liver and mostly located in the periportal areas. Phi-bodies were never seen in control livers. Furthermore, clusters of up to 10 peroxisomes were present in the rats treated with dose ×1.5 (Fig. 2 B), whereas in the control rats as well as in the rats treated with dose ×1, only clusters of two to three peroxisomes were seen (peroxisomes are considered to belong to a cluster when the distance between neighboring organelles is smaller than twofold of the mean diameter of the peroxisomes of the cluster). The clusters often showed nearly touching peroxisomes (Fig. 2 C). Finally, heterogeneity in the reaction product of catalase activity was detected in proliferated peroxisomes, which was not observed in control rats. AOX was located in peroxisomes by EM immunodetection (Fig. 2 E). The mean label-
ing density for AOX was increased by a factor of 1.3 in PBA-treated rats \( (n = 3) \) in comparison with control rats \( (n = 3) \). In addition, when the volume density of the peroxisomes was taken into account, the total labeling differed by a factor 2.5 between the control and treated rats. Although not significant, these differences are in agreement with RT-PCR data (Fig. 1, A and B).

A weak but significant dose effect was observed in the liver of the PBA-fed rats. The number of peroxisomes per cellular area measured by LM increased by 1.4-fold with dose \( \times 1 \) and 1.7-fold with dose \( \times 1.5 \). Furthermore, the EM morphometry values were always higher in the liver of rats \( (n = 4) \) treated with dose \( \times 1.5 \) (numerical density: \( \times 2.7 \), volume density: \( \times 3.1 \), and surface density: \( \times 2.9 \)) than in the rats \( (n = 6) \) treated with dose \( \times 1 \) (\( \times 2.1 \), \( \times 1.9 \), and \( \times 1.8 \), respectively). A peroxisome proliferation index was calculated for each treated animal \( (n = 10) \) versus the mean value of 10 control animals considering successively the number of peroxisomes per cellular area measured by LM and each of the three EM morphometry values. When the four proliferation factors of each treated animal were ranked \( (n = 40) \), the dose effect was significant \( (P < 0.001) \). In fact, the different animals did not respond to the PBA treatment to the same extent with regard to the number of peroxisomes, as it was observed already for the induction of peroxisomal genes (Fig. 1 B). In some animals, a clear proliferative effect co-occurred with a higher induction of peroxisomal genes.

The number of peroxisomes per cellular area was studied by LM in the kidney (tubulus contortus I cells) and the intestinal epithelium, two PP-sensitive tissues, and showed no significant differences between the control rats \( (n = 3) \) and the per os PBA-treated rats \( (n = 3) \) with dose \( \times 1 \) of PBA.

In the rats i.p. injected with PBA during 3 d, LM examination revealed no peroxisome proliferation in the liver, in agreement with the absence of induction of peroxisomal genes as mentioned in the previous paragraph.

**PBA treatment modifies the peroxisome distribution in ependymal cells of the fourth ventricle**

Peroxisomes were also studied by EM in ependymal cells lining the fourth ventricle of control rats \( (n = 7) \) and per os PBA-treated rats with doses \( \times 1 \) \( (n = 3) \) or \( \times 1.5 \) \( (n = 3) \). A 1.4-fold \( (P = 0.05) \) increase in the number of peroxisomes per cytoplas-
mic area occurred together with more frequent clusters (×2.0; P < 0.05) (Fig. 3). However, EM revealed no difference in ependymal cells of the choroid plexus or lining the lateral wall of the lateral ventricle and in stem cells of the subventricular zone. These stem cells contained only very few and small catalase particles.

When PBA was infused directly into the fourth ventricle, no effect on peroxisomes was observed by EM in ependymal cells, whereas infusion of ciprofibrate caused a decrease (×0.67; P < 0.05) in the number of peroxisomes per cell profile.

**PBA and butyrate induce expression of peroxisomal genes and proliferation of peroxisomes in primary cultures of hepatocytes**

Considering the short half-life of butyrate in living animals (~6 min), primary cultures of rat hepatocytes were treated with butyrate, PBA, and ciprofibrate for 72 h to gain insight into the mechanisms involved in the regulation of gene expression and peroxisome biogenesis. PBA and butyrate displayed a similar dose-dependent activation of *Abcd2* expression (Fig. 4). However, the increase in *Aox* mRNA levels was lower after butyrate treatment than after PBA treatment (Fig. 4). Furthermore, the *Abcd2* expression level relative to *Aox* level was higher with PBA and butyrate than with ciprofibrate (Fig. 4). Therefore, the effects on peroxisomal gene expression in primary hepatocytes appeared to be different for the three compounds.

Catalase-stained peroxisomes were visualized in hepatocytes by LM. The number of peroxisomes per cellular area was increased 1.4-fold (P < 0.01) in PBA-treated cells versus controls for dose 1.25 mM and 1.8-fold (P < 0.001) for dose 2.5 mM. An increase was also seen when cells were treated with butyrate: 1.3-fold (P < 0.05) for dose 1.25 mM and 1.4-fold (P < 0.01) for dose 2.5 mM. A similar increase (×1.7; P < 0.001) was obtained after 0.25 mM ciprofibrate treatment.

**PBA and butyrate, but not ciprofibrate, induce *Abcd2* expression and peroxisome proliferation in cultured glial cells**

In the prospect of a possible pharmacological therapy of X-ALD, it is relevant to try to induce *Abcd2* expression in glial cells. In differentiated CG4 cells, pure primary astrocytes, and mixed primary cultures of glial cells, we observed a dose-dependent induction of *Abcd2* expression after PBA or butyrate treatment but no effect on *Aox* expression (Fig. 5, A and B). When the glial cells were treated with 0.25 and 0.5 mM ciprofibrate, we observed no change in the *Abcd2* and *Aox* expression in the three types of cell culture (unpublished data).

We also examined the peroxisomes by LM after immunolocalization of catalase in C6 rat glioma cells treated with 5 mM PBA or 0.5 mM ciprofibrate. The number of peroxisomes per cellular area was significantly higher in PBA-treated cells than in controls (×2.0; P < 0.01) but remained unchanged in ciprofibrate-treated cells. The PBA treatment generated a change in cell morphology, characterized by a fibroblastic aspect, which was much less marked when cultures were treated with ciprofibrate (unpublished data).

**PBA induction of the *Abcd2* gene and peroxisome proliferation does not require PPARα**

We have previously shown that fenofibrate induction of *Abcd2* and *Aox* in the liver of rodents requires the presence of PPARα (Fourcade et al., 2001). To test whether or not PBA induction of the peroxisomal genes is dependent on PPARα, fibroblasts from control and PPARα −/− mice were treated with PBA or fenofibrate and mRNA was analyzed. In contrast to hepatocytes (Fig. 4), the peroxisomal genes in control fibroblasts were not induced by fenofibrate (Fig. 6 A), as observed in glial cells (unpublished data). PBA elicited the same dose-dependent induction of *Abcd2* in both PPARα +/+ and PPARα −/−
fibroblasts, indicating that it does not require PPARα to activate Abcd2 (Fig. 6 A). The changes in expression of Aox in the PBA-treated fibroblasts were too weak (as already observed in PBA-treated normal fibroblasts by Kemp et al. [1998]) to conclude whether or not their induction by PBA is PPARα-dependent (Fig. 6 A). Similar results were obtained using hepatocytes, except that PBA induction of Abcd2 was significantly higher in PPARα+/+ cells than in PPARα−/− cells (Fig. 6 B), indicating that the extra induction of Abcd2 in normal hepatocytes is PPARα-dependent. Furthermore, the peroxisome proliferation induced by PBA in control hepatocytes (see the section PBA and butyrate induce expression of peroxisomal genes and proliferation of peroxisomes in primary cultures of hepatocytes) was maintained in PPARα+/− hepatocytes (Fig. 6, C and D) as the peroxisome number measured by LM was increased in cells treated with 2.5 (×1.7; P < 0.05) and 5.0 mM PBA (×1.4; P < 0.05). This result was confirmed using EM (×2.7 in 5.0 mM PBA-treated cells).

Trichostatin A (TSA), a potent HDAC inhibitor, increases Abcd2 expression in hepatocytes only at high doses

From these results, we hypothesized that in hepatocytes only part of PBA induction of Abcd2 can be ascribed to HDAC inhibition, and the other part to PPARα activation. To test this hypothesis, we treated rat primary hepatocytes with TSA, a specific inhibitor of HDAC at nanomolar concentrations, using different doses (0.03–3 μM) and times (24, 48, and 72 h). Expression of Abcd2 was enhanced only at high doses (1–3 μM) of TSA, and the response was limited (×1.6 on average; Fig. 7).

Dose-dependent induction of the Abcd2 promoter by PBA or butyrate requires only the basic promoter

The rat Abcd2 promoter contains a CCAAT box (−53) surrounded by two putative GC boxes respectively located at −65 (GC2: CCGCCC) and −35 (GC1: GGGTGG), the three motifs being very well conserved in mouse and human (Fourcade et al., 2001). To identify the molecular mechanism of induction by PBA, COS-7 cells were transiently transfected with a plasmid construct (p277) containing the first 83 bp of the promoter (Fourcade et al., 2001). In the presence of PBA or butyrate, we observed a strong dose-dependent induction (Fig. 8 A). Similar results were obtained with the p2206 construct containing 2 kb of the
promoter (p2206) (unpublished data), indicating that the basic promoter (83 bp) is sufficient to mediate full induction of the Abcd2 gene by PBA.

PBA-response and basal activity of the Abcd2 promoter depends on a GC box and a CCAAT box

It has been reported that mutation or deletion of a GC box can abolish the response to butyrate (Lu and Lotan, 1999) and that HDAC1 can repress transcription by direct interaction with Sp1 and Sp3 (Doetzlhofer et al., 1999; Davie, 2003). The CCAAT box has also been demonstrated to play an important role in gene activation by HDAC inhibitors through the CCAAT-binding protein NF-Y (a trimeric association of NF-YA, NF-YB, and NF-YC) and recruitment of histone acetyltransferase (HAT) activity (McCaffrey et al., 1997; Jin and Scotto, 1998). Therefore, we further analyzed the role of the GC and CCAAT boxes in the basal activity of the Abcd2 promoter and in the context of PBA induction.

We first compared in independent experiments the activity of p277 with the activities of p277A1, p277Δ2, and p277Δ1,2 (where one or both GC boxes is mutated; Fig. 8 B) or with the activities of p243 (construct devoid of 34 bp of the 5′ region containing the GC2 and CCAAT′ boxes), p277Δ5UTR, and p243Δ5UTR (constructs devoid of 52 bp of the 5′ untranslated region) (Fig. 8 C). Mutation of the GC1 box markedly decreased the basal activity of the reporter gene (3.6- and 5.6-fold reduction for p277Δ1 and p277Δ1,2, respectively), underlining the functional importance of this GC box for basal Abcd2 expression (Fig. 8 B). By contrast, mutation of the GC2 box caused a minor decrease in basal activity (Fig. 8 B). Deletion of the 34 bp containing the GC2 and CCAAT′ boxes displayed a 3.2-fold decrease in the basal activity, which is more pronounced than the one observed with p277Δ2 (1.3-fold; Fig. 8 C). This observation suggests a functional role for the CCAAT box in the basal activity of the promoter. Moreover, basal activity of p277Δ5UTR was 1.5-fold higher, suggesting that some repression was mediated through the deleted 5′-UTR sequence. However, deletion of the 5′-UTR sequence in the p243 construct did not show any difference (Fig. 8 C).

Interestingly, the effect of PBA and butyrate treatment was abolished by the mutation of the GC1 box, indicating that the GC1 box is not only essential for basal activity but is also the key element of the activation by PBA or butyrate (Fig. 8 B). This capability of induction was also decreased in the p243 construct (1.8-fold induction) showing that the presence of the GC1 box is sufficient to mediate PBA induction but suggesting that the presence of the CCAAT box (which is not sufficient alone; see p277Δ1,2) is necessary to mediate full induction by PBA (Fig. 8 C). Deletion of the 5′-UTR sequences did not alter significantly the levels of induction by PBA or butyrate (Fig. 8 C).

The GC1 and CCAAT boxes bind their cognate factors, and HDAC-1 is recruited through the GC1 box

To explore the capability of the GC1 and CCAAT boxes of the Abcd2 promoter to bind in vitro Sp1 or Sp3 and NF-Y, respectively, we first performed electrophoretic mobility shift assay (EMSA) with the corresponding radiolabeled oligonucleotides. Several DNA–protein complexes were formed in the presence of nuclear extracts from rat liver (Fig. 9 A, lanes 2 and 10). These complexes were sequence-specific because they disappeared in the presence of an excess of unlabeled homologous oligonucleotide (Fig. 9 A, lanes 3 and 11) but not in the presence of an excess of unlabeled mutated oligonucleotide (Fig. 9 A, lanes 4 and 12). To investigate if the retarded complexes result from the binding of Sp1/Sp3 and NF-Y, we performed EMSA in the presence of the specific antibodies. Incubation with the anti-Sp1 and/or anti-Sp3 antibodies resulted in specific supershifted complexes for both antibodies, indicating that the GC1 box binds Sp1 and Sp3 (Fig. 9 A, lanes 5–7). A supershift was also obtained with the anti–NF-YA antibody
and the CCAAT probe (Fig. 9 A, lane 13), demonstrating that the CCAAT box binds NF-Y.

We further investigated by DNA affinity precipitation assay (DAPA) if the GC1 motif could be involved in the recruitment of HDAC1. We used the biotinylated oligonucleotides corresponding to the GC1 and mutated GC1 sequences used in EMSA. The presence of HDAC1 was detected in the precipitated complexes obtained with the normal GC1 box but not with the mutated motif (Fig. 9 B). This finding indicates that HDAC-1 is recruited through interaction with the Sp1/Sp3 factors bound to the GC1 box of the Abcd2 promoter.

**Discussion**

Because PBA induces peroxisome proliferation in human fibroblasts, PBA has been described as a new PP (Kemp et al., 1998). However, the fact that AOX and PPARα expression was unchanged in these cells indicates that PBA may induce peroxisome proliferation through a mechanism not shared by classical PPs such as fibrates. To clarify this point, we studied the effects of PBA in rodent tissues and cells. Induction of the Abcd2 and Aox expression and of the peroxisome proliferation in the liver of rats was moderate and occurred only after a long-term (6–9-wk) treatment with high doses of PBA. The observed alterations in the shape (phi-bodies) and in the distribution (clusters and nearly touching organelles) of peroxisomes are reminiscent of PP-induced modifications already described in rat liver (Svoboda and Azarnoff, 1966; Baumgart et al., 1989; Gorgas and Krisans, 1989; Roels, 1991) and rat hepatoma cells (Duclos et al., 1997). It has been shown that overexpression of Pex11p in rodent or human cells causes peroxisome proliferation, characterized by initial conversion of peroxisomes from spherical vesicles into elongated tubules followed by appearance of numerous small vesicular peroxisomes (Passreiter et al., 1998; Schrader et al., 1998; Li and Gould, 2002). In the liver of rodents treated with PPs, peroxisome proliferation and peroxisomal gene induction occur early. In contrast, PBA had no effect when a short-term treatment (3 d) was given to rats, suggesting a different mode of action. However, the absence of effect could result from the rapid metabolism of PBA to phenylacetate leading to ineffective PBA plasma levels. Therefore, we exposed primary hepatocytes to high concentrations of PBA for 3 d and observed induction of the peroxisomal genes and peroxisome proliferation hardly higher than in vivo, indicating that PBA is a low-potent PP. It is well known that the peroxisome proliferation in the rodent liver due to classical PPs is totally suppressed in PPARα−/− mice (Lee et al., 1995). In contrast, the present work shows that the PBA-induced peroxisome proliferation is maintained in PPARα−/− hepatocytes. Both PBA and classical PPs induce Pex11a expression in mammals (Abe and Fujiki, 1998; Abe et al., 1998; Kemp et al., 1998; Schrader et al., 1998; Depreter et al., 2002). Surprisingly, Pex11a is dispensable for peroxisome proliferation mediated by PPARα activators, whereas Pex11α is required for peroxisome proliferation in response to PBA (Li et al., 2002). Furthermore, PBA but not fibrates induces peroxisome proliferation in nonhepatic cells (where PPARα expression is lower than in hepatocytes), namely fibroblasts (Kemp et al., 1998) and C6 cells (the present study). In summary, the requirement of PPARα and Pex11α for peroxisome proliferation and the cell type where peroxisome proliferation can occur are different for PBA and classical PPs. This finding indicates that the mechanism of peroxisome proliferation in response to PBA is distinct from that used by PPs.

X-ALD is characterized by demyelination in the brain. In the context of a pharmacological therapy based on the partial functional redundancy of the peroxisomal transporter ALDRP there is considerable interest to explore the effects of PBA in the brain. PBA is likely to cross the blood–brain barrier because it is effective in lowering VLCFA levels in the brain of Abcd1−/− mice (Kemp et al., 1998). Indeed, modifications at the peroxisomal level were detected in ependymal cells of the fourth ventricle in PBA-treated rats. Unfortunately, no induction in peroxisomal gene expression was seen in the brain examined as a whole. Nevertheless, the induction of Abcd2 that we observed in PBA-treated glial cells might account for the restoration of VLCFA peroxisomal β-oxidation in the brain of PBA-treated Abcd1−/− mice described by Kemp et al. (1998).

The knowledge of the molecular mechanism underlying the PBA effects on Abcd2 expression may provide leads in searching for new drugs for X-ALD therapy. We compared PBA with ciprofibrate or fenofibrate (classical powerful PPs) and with butyrate (an HDAC inhibitor) for their effects on peroxisomal gene expression in hepatic or nonhepatic cell cul-
tures. First, in fibroblasts and glial cells, fibrate was unable to induce Abcd2, suggesting that a cofactor of PPARα is missing or that the PPARα level is too low (Lemberger et al., 1996). In contrast, Abcd2 was activated by PBA or butyrate in these cells, and we demonstrated that the activation is PPARα independent. Furthermore, in hepatocytes, Abcd2 and Aox were induced by PBA, butyrate, and ciprofibrate, with some differences for the three compounds. We had observed that the induction of these genes by fibrate was completely abolished in the liver of PPARα−/− mice (Fourcade et al., 2001). Here, we show that PBA induction of Abcd2 is only reduced in PPARα−/− hepatocytes, suggesting that part of the induction is PPARα dependent and that the other part would originate in the same mechanism underlying the activation of Abcd2 by PBA and butyrate in nonhepatic cells. Similar conclusions can be drawn from our experiments on TSA-treated hepatocytes. Altogether, the results indicate that PBA and butyrate would mainly activate Abcd2 by HDAC inhibition, whereas in hepatocytes an extra induction occurs through PPARα. This PPARα-dependent induction is in agreement with the relatively high level of PPARα in hepatocytes and the capacity of PBA to bind PPARα (Liu et al., 2002).

HDAC is known to interact with the transcription complex and the chromatin in a DNA region surrounding the transcription start, resulting in enhanced transcription rate (Na-
cayama and Takami, 2001). A basic promoter should be
scription start, resulting in enhanced transcription rate (Na-

Altogether, the results indicate that PBA and butyrate would mainly activate Abcd2 by HDAC inhibition, whereas in hepatocytes an extra induction occurs through PPARα. This PPARα-dependent induction is in agreement with the relatively high level of PPARα in hepatocytes and the capacity of PBA to bind PPARα (Liu et al., 2002).

HDAC is known to interact with the transcription complex and the chromatin in a DNA region surrounding the transcription start, resulting in enhanced transcription rate (Nakayama and Takami, 2001). A basic promoter should be sufficient so that HDAC activity can take place. Support for this concept was obtained from our work demonstrating that the first 49 bp of the Abcd2 promoter enable PBA and butyrate to activate a reporter gene and that the first 83 bp enable full induction. In transient transfection experiments, we demonstrated that the PBA-dependent induction and the basal level of expression of Abcd2 depends on the GC1 and CCAAT boxes. Both motifs were shown to bind their cognate factors Sp1/Sp3 and NF-Y, respectively, and we demonstrated that HDAC-1 is recruited through the GC1 box in agreement with previous papers (Doetzlhofer et al., 1999). Expression of Abcd2 probably results from the balanced action of HAT and HDAC activities. The CCAAT box, which binds NF-Y, likely allows HAT activity to be recruited and to antagonize the effect of HDAC-1 (Jin and Scotto, 1998). The recruitment of HAT activity may depend not only on NF-Y but also on Sp1, as it has already been described for the promoter of HDAC1 for instance (Schutterengrub et al., 2003). It would explain why basal activity of the Abcd2 promoter is reduced when the GC1 box is mutated, whereas it might have been expected that this mutation results in enhanced promoter activity because HDAC1 is not recruited anymore. This would also be in agreement with the up-regulation of HAT activity by physical interaction with Sp1 (Soutoglou et al., 2001). Besides, it was shown that the effect of PBA on Abcd2 expression strictly depends on the presence of the GC1 box and consequently on the recruitment of HDAC1. However, full induction has been observed only when the CCAAT box is present, suggesting a synergistic effect of the two motifs. HDAC inhibition could increase recruitment of HAT to the CCAAT box as it has already been hypothesized for the TBRII gene (Park et al., 2002). Further work would be necessary to determine more precisely the complexes containing HAT and HDAC activities and to show if other HDACs of the class I (Marks et al., 2003) can be involved in Abcd2 expression.

We have shown that fibrates induce Abcd2 expression in the liver of rodents in a PPARα-dependent way but not in the brain (Albet et al., 1997; Berger et al., 1999; Fourcade et al., 2001). It was believed that the absence of effects in brain was due to their inability to cross the blood–brain barrier. Actually, the present study revealed that fibrate, a powerful PP, has no effect in glial cells and fibroblasts. This lack of effect suggests that any drug, which requires PPARα, is likely to be ineffective in inducing Abcd2 expression and in restoring VLCFA β-oxidation in the brain of Abcd1−/− mice or X-ALD patients. PBA might be a better candidate for a pharmacological therapy of X-ALD because we showed that it can induce Abcd2 expression in glial cells and cause peroxisome proliferation. An increased number of peroxisomes could contribute to improve VLCFA metabolism in X-ALD patients. However, it is still unclear whether or not the correction of VLCFA levels observed in the brain of Abcd1−/− mice after PBA treatment (Kemp et al., 1998) results from enhanced Abcd2 expression. A high effective dose and a decreased response in long-term studies are two stumbling blocks in using PBA for a therapy (McGuinness et al., 2001). In an effort to identify nontoxic agents effective at a low dose and with a long-term effect, we searched for the mechanism of pharmacological induction of Abcd2 by PBA. We show that PBA acts at the peroxisomal level probably by inhibiting HDAC activity, like butyrate, suggesting that other HDAC inhibitors should be tested. Two compounds structurally related to PBA (styrlyacetate and benzoxylacetate) were shown to be as effective as PBA in increasing VLCFA peroxi-

There is a priori concern regarding the use of HDAC inhibitors because of their nonspecific effects on gene expression, but in clinical trials several HDAC inhibitors appeared to be well tolerated (Marks et al., 2003). Investigation of pharmacological agents acting through another molecular mechanism should also be considered. A more comprehensive understanding of the expression of the Abcd2 gene is needed to identify other drugs for the development of an effective pharmacological therapy.

Materials and methods

Animals and treatments

Male Wistar rats of ~200 g (Charles River Laboratories) were kept at 22°C with equal periods of darkness and light and had free access to water and food. PBA (Sigma-Aldrich) was delivered to rats (n = 6) in their chow (7.5 g/kg) and water (10 g/L) corresponding to dose ×1 for 6–7 wk. A higher dose of PBA (dose ×1.5), corresponding to 11.3 g/kg of chow and 15 g/L of water, was also given to rats (n = 4) for 6–9 wk. The daily PBA uptake was estimated to be ~0.4–0.5 g per day per rat. The daily increase in body weight was significantly lower in treated animals (2.9 g) than in controls (5.3 g). Moreover, rats (n = 3) received 720 mg PBA per kilogram of body weight per day for 3 d via i.p. injections with an aqueous solution of PBA (40 mg/ml) twice a day. They were killed 12 h after the last injection. Rats (400 g) were anesthetized with pentobarbital (60 mg/kg, i.p.). Drugs were administered at a delivery rate of 100 μl/h through a cannula stereotaxically implanted into the fourth ventricle. Rats received ei
ther 2 mg of sodium ciprofibrate (n = 4) or 20 mg of PBA (n = 4) dissolved in a total volume of 100 μl of sterile water (pH adjusted at 7.3). Control rats (n = 4) received equimolar amounts of NaCl (100 μl). The rats were killed 48 h after injection.

Cell culture
Rat primary hepatocytes were prepared as described previously (Fourcade et al., 2001). Mouse primary hepatocytes were obtained according to Maslansky and Williams (1982), and a transfer on a cushion of Percoll yielded 90% viability. Differentiated CG4 rat glial cells, pure primary astrocytes, and mixed primary culture of oligodendrocytes and astrocytes were obtained and cultured as described previously (Fourcade et al., 2003). Embryonic fibroblasts were prepared from 14-day fetuses of PPARα +/+ and −/− mice as described previously (Doetschman et al., 1985) and cultured in DME supplemented with 10% FCS. COS-7 cells were grown in DME supplemented with 10% FCS in the absence of antibiotics.

Northern blot analysis
Total RNA was extracted from rat tissues using a standard protocol. The GenElute Mammalian Total RNA kit (Sigma-Aldrich) was used to prepare RNA from cultured cells. Membranes containing 20 μg per lane of RNA were hybridized with α-[32P]-labeled cDNA probe as described previously (Albet et al., 2001).

Semiquantitative RT-PCR
Total RNA was reverse-transcribed by random priming. To study Aox expression, PCR was performed as described previously (Fourcade et al., 2001). Abcd2 cDNA was amplified using the forward (F) and reverse (R) primers 5′-GTGATGCCGCTGCTAAAG-3′ and 5′-TCACTGCCAGGGCAGCTG-3′, respectively, for 27 cycles (30 cycles when RNA was extracted from primary hepatocytes) and 56°C as the annealing temperature. Amplification of 36bd cDNA was performed using the primers 5′-AYGTGGGCTCCAGGATG-3′ and 5′-GGAGATGTTCAYCATGT-3′, for 17 cycles and 60°C as the annealing temperature. PCR products were analyzed on agarose gels and quantified by digital imaging, and the relative abundance of mRNA was determined by comparison with 36bd mRNA level.

Microscopy
Rats were anesthetized and the tissues were fixed through intracardial perfusion with fixative. Peroxisomes were visualized by staining for catalase activity with DAB (Roels et al., 1995). Immunolocalization of AOX (anti-body provided by A. Völkli, University of Heidelberg, Heidelberg, Germany) or catalse for LM or EM was performed according to Espeel and Van Limbergen (1995). LM images were photographed on film on an Aris-toplan microscope [Leitz) using 63×, 1.4 NA, and 40×, 0.75 NA, oil immersion lenses [Leitz] for bright field and phase contrast and on an in-verted microscope (model DMRB: Leica) using a Leica PL APO 63×, 1.32–0.6 NA oil immersion lens, and the Leica MPS-60 photomat for immuno-fluorescence. On randomly taken LM micrographs, the number of peroxisomes per cellular area was determined by counting catalase-stained peroxisomes and measuring cellular area excluding blood vessels with a semi-automatic image analysis system (MiniMap; Kontron). EM images were photographed on a transmission electron microscope (model JEM-100B, JEOL). Morphometry of peroxisomes was performed on random EM micrographs as described previously (Kerrcaert et al., 1995). Gold parti-cles per peroxisome were counted and organellar area was measured with the Mini-Map apparatus. A nonparametric ranking test (Mann-Whitney) was used for statistical analysis of microscopy data. For publication, micrographs were scanned and imported into Adobe Photoshop.

Plasmid constructs
The first 83 bp of the rat Abcd2 promoter (relative to the transcription start site located at −194 from the translation start site) were cloned directionally into pGL3-Basic (Promega) to yield p277 as described previously (Fourcade et al., 2001). The p243 construct was prepared as p277 except that the inserted PCR fragment was amplified using forward primer 5′-tagctCCGTTGCAGCTCAGG-3′ and reverse primer 5′-CAATAATCCAGATGCAAGTCAG-3′ with the GC boxes are shown in bold and mutations are underlined.

Transient transfection experiments
COS-7 cells were transfected using Exen 500 liposome (Euromedex) with 100 ng of pcMV-HisG (CLONTECH Laboratories, Inc.), 650 ng of pGUC [a modified pGL2 vector] or empty pGL3-Basic or construct, and 250 ng of empty pSG5 (Stratagene) as described previously (Fourcade et al., 2001). The transfected cells were incubated with or without PBA or butyrate for 36 h and assayed for luciferase activity (Fourcade et al., 2001). Luciferase activity was corrected for transfection efficiency using β-galactosidase activity.

EMSA
The following pairs of oligonucleotides used as probes in EMSA were designed from the rat ABCD2 promoter: GC1 [F, 5′-agctctCGTGAGGTTGAGGACCTC-3′; R, 5′-gatccCGAGCTTCACCCTGACGC-3′], GC1 mut [F, 5′-agctctCGTGAGGTTGAGGACCTC-3′; R, 5′-gatccCGAGCTTCACCCTGACGC-3′], CCAAT [F, 5′-agctctCGAGCTTCACCCTGACGC-3′; R, 5′-gatccCGAGCTTCACCCTGACGC-3′], CCAAT mut [F, 5′-agctctCGAGCTTCACCCTGACGC-3′; R, 5′-agctctCGAGCTTCACCCTGACGC-3′]. GC1 and CCAAT boxes are shown in bold; mutations are underlined; lower case letters are modified nucleotides from the original sequence to create compatible ends for cloning and filling labeling. Labeling of probes and binding experiments in the presence of 10 μg of nuclear extracts from rat liver were performed as described previously (Fourcade et al., 2001). Competition and supershift experiments were performed by adding a 50-fold molar excess of unlabeled oligonucleotides or antibodies (Santa Cruz Biotechnology, Inc.; anti-Sp1, anti-Sp3, and anti–NF-YA) or a rabbit serum (control) 30 min before the addition of the radiolabeled probes. The samples were analyzed by electrophoresis at 4°C on 4% polyacrylamide gels in 0.5 × TBE (45 mM Tris-borate and 1 mM EDTA).

DAPA
Complementary oligonucleotides corresponding to the normal (5′-TGAAGGTGGGTGAGGGTGGCCTGCGG-3′) or mutated (5′-TGAAGGTGGGTGAGGGTGGCCTGAGG-3′) promoter: GC1 (F, 5′-agctctCGTGAGGTTGAGGACCTC-3′; R, 5′-gatccCGAGCTTCACCCTGACGC-3′). GC1 box was synthesized, biotinylated at their 5′ termini, and annealed. The DAPA was performed in a total volume of 400 μl by mixing 4 μg of biotinylated DNA probe with 50 μg of nuclear extracts from rat liver in binding buffer (20 mM Hepes, pH 7.9, 10% glycerol, 50 mM KCl, 0.2 mM EDTA, 1.5 mM MgCl2, 10 μM ZnCl2, 1 mM DTT, and 0.25% Triton X-100). After a 30-min preincu-bation on ice, 50 μl of streptavidin-garosse beads (Novagen) were added and incubated for 2 h under agitation. Beads were collected by a brief centrifugation and washed twice in binding buffer. Proteins were eluted in 15 μl of SDS-PAGE loading buffer by heating for 5 min at 95°C. After centrifugation, the supernatant was loaded on SDS-PAGE and Western blot analysis was performed using anti-HDAC1 antibody (Santa Cruz Biotechnology, Inc.).

We dedicate this paper to the memory of Professor Maurice Bugaut, who initiated this project and who died an untimely death while the manuscript was being revised. We thank Professor Alfred Volki for acetylCoA oxidase antibody. We also thank Betty De Prest, Dominique Jacobus, Hubert Stevens, Simonne Van Hulle, and Guido Van Limbergen (all from Ghent University) for their technical assistance.

This work was supported by grants from the European Association against Leukodystrophies (ELA) and the Regional Council of Burgundy. S. Fourcade and S. Leclercq were supported by a fellowship from ELA. S. Fourcade was also sup-port by a fellowship from Fondation pour la Recherche Médicale.

Submitted: 7 January 2005
Accepted: 3 March 2005

References
Abe, I., and Y. Fujiki. 1998. cDNA cloning and characterization of a constitutively expressed isoform of the human peroxin Pex11p. Biochem. Biophys. Res. Commun. 252:529–533.
Abe, I., K. Okamoto, S. Tamura, and Y. Fujiki. 1998. Clofibrate-inducible, 28-kDa peroxisomal integral membrane protein is encoded by PEX11. FEBS Lett. 431:468–472.
Albet, S., C. Causoret, M. Bentejac, J.L. Mandal, P. Aubourg, and M. Bugaut.
Duclos, S., J. Bride, L.C. Ramirez, and P. Bournot. 1997. Peroxisome prolifera-

Baumgart, E., A. Volkl, T. Hashimoto, and H.D. Fahimi. 1989. Biogenesis of

Fourcade, S., S. Savary, S. Albet, D. Gauthe, C. Gondcaille, T. Pineau, J. Bel-

Holzinger, A., S. Kammerer, and A.A. Roscher. 1997. Primary structure of hu-

Jin, S., and K.W. Scotto. 1998. Transcriptional regulation of the MDR1 gene by

Fernandez-Salgueiro, H. Westphal, and F.G. Gonzalez. 1995. Targeted disruption of the alpha isoform of the peroxisome proliferator-activated receptor gene in mice results in abolishment of the pleiotropic effects of peroxisome proliferators. Mol. Cell. Biol. 15:3012–3022.

Lemerber, T., O. Braissant, C. Juge-Aubry, H. Kelller, R. Saladin, B. Staels, J. Auwers, A.G. Burger, C.A. Meier, and W. Wahl. 1996. PPAR tissue distribution and interactions with other hormone-signaling pathways. Ann. NY Acad. Sci. 804:231–251.

Li, X., and S.J. Gould. 2002. PEX11 promotes peroxisome division indepen-
dently of peroxisome metabolism. J. Cell Biol. 156:643–651.

Li, X., E. Baumgart, G.-X. Dong, J.C. Morrell, G. Jimenez-Sanchez, D. Valle, K.D. Smith, and S.J. Gould. 2002. PEX11b is required for peroxisome proliferation in response to 4-phenylbutyrate but is dispensable for per-
oxisome proliferator-activated receptor-α-mediated peroxisome prolifer-

Niu, L., W. Qiang, X. Kuang, P. Thullierre, W.S. Lynn, and P.K.Y. Wong. 2002. The peroxisome proliferator phenylbutyric acid (PEPA) protects as-
trocytes from t1s1 MolMuV-induced oxidative cell death. J. Neurovirol. 8:318–325.

Lombard-Platet, G., S. Savary, C.O. Sarde, J.L. Mandel, and G. Chinnimi. 1996. A close relative of the adrenoleukodystrophy (ALD) gene codes for a peroxisomal protein with a specific expression pattern. Proc. Natl. Acad. Sci. USA. 93:1265–1269.

Lu, Y., and R. Lotan. 1999. Transcriptional regulation by butyrate of mouse ga-

ElEtr, B. Molzer, and M. Bugaut. 2003. Thyroid hormone induction of

Park, S.H, S.R. Lee, B.C. Kim, E.A. Cho, S.P. Patel, H.B. Kang, E.A. Sausville, O. Nakashiki, J.B. Trepel, B.I. Lee, S.J. Kim. 2002. Transcriptional regu-

Stroh, D. Valle, T.A. Schroer, and S.J. Gould. 1998. Expression of

Maslansky, C.J., and G.M. Williams. 1982. Primary cultures and the levels of

J. Biol. Chem. 268:3490–3500.

Netik, A., S. Forss-Petter, A. Holzinger, B. Molzer, G. Unterrainer, and J.

Motojima, K. 1997. Peroxisome proliferator-activated receptor (PPAR)-depen-
dent and -independent transcriptional modulation of several non-peroxi-

REGULATION OF ABCD2 BY PHENYL BUTYRATE • GONDCAILLE ET AL. 103
Schuettengruber, B., E. Simboek, H. Khier, and C. Seiser. 2003. Autoregulation of mouse histone deacetylase 1 expression. *Mol. Cell. Biol.* 23:6993–7004.

Soutoglou, E., B. Viollet, M. Vaxillaire, M. Yaniv, M. Pontoglio, and I. Talianidis. 2001. Transcription factor-dependent regulation of CBP and P/CAF histone acetyltransferase activity. *EMBO J.* 20:1984–1992.

Svoboda, D.J., and D.L. Azarnoff. 1966. Response of hepatic microbodies to a hypolipidemic agent, ethyl chlorphenoxyisobutyrate (CPIB). *J. Cell Biol.* 30:442–450.

Waddell, W.J., C. Marlowe, M.S. Rao, and J.K. Reddy. 1989. In vivo distribution of a carcinogenic hepatic peroxisome proliferator: whole-body autoradiography of [14C]ciprofibrate in the mouse. *Carcinogenesis.* 10:221–223.

Warrell, R.P.J., L.Z. He, V. Richon, E. Calleja, and P.P. Pandolfi. 1998. Therapeutic targeting of transcription in acute promyelocytic leukemia by use of an inhibitor of histone deacetylase. *J. Natl. Cancer Inst.* 90:1621–1625.