1. Title

Xenobiotic-Metabolizing Cytochromes P450 in Human White Adipose Tissue: Expression and Induction

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2. **Running title**

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d. **List of abbreviations:**

   - AhR: Aryl hydrocarbon receptor
   - CAR: Constitutive androstane receptor
   - CPY: Chlorpyrifos
   - CYP: Cytochrome P450
   - LIND: Lindane
   - PB: Phenobarbital
   - PRO: Prochloraz
   - PXR: Pregnane X receptor
   - RIF: Rifampicin
   - TBP: Tata binding protein
   - TCDD: 2,3,7,8-tetrachlorodibenzo-p-dioxin
   - WAT: White adipose tissue
3. Abstract

Lipophilic pollutants can accumulate in human white adipose tissue (WAT) and consequences of this accumulation are still poorly understood. Cytochromes P450 (CYP) have recently been found in rat WAT and shown to be inducible through similar mechanisms as in the liver. Our study aimed at describing the cytochrome P450 pattern and their induction mechanisms in human WAT. Explants of subcutaneous and visceral WAT and primary culture of subcutaneous adipocytes, were used as WAT models, whereas liver biopsies and primary culture of hepatocytes were used as liver models to characterize CYP expression in both tissues. The WAT and liver models were then treated with typical CYP inducers (rifampicin, phenobarbital and 2,3,7,8-tetrachlorodibenzo-p-dioxin) and lipophilic pollutants (lindane, prochloraz and chlorpyrifos) and the effects on CYP expression were studied. CYP expression was considerably lower in WAT than in the liver, except for CYP1B1 and CYP2U1, which were the most highly expressed adipose CYP in all individuals. 2,3,7,8-tetrachlorodibenzo-p-dioxin and prochloraz induced CYP1A1 and CYP1B1 expression in both tissues. The Aryl hydrocarbon receptor was also present in WAT. In contrast, neither phenobarbital nor rifampicin treatment induced CYP2 or CYP3 mRNA in WAT and constitutive androstane receptor and pregnane X receptor were almost undetectable. These results suggest that the mechanisms by which CYP of family 1 are regulated in the liver are also functional in human WAT, but those regulating CYP2 and 3 expression are not.
4. Introduction

White adipose tissue (WAT) has a very important double physiological role: through its triacylglyceride storage capacity, it is the key organ for energy homeostasis, but it is also becoming increasingly apparent that WAT is a major endocrine organ (Ahima and Flier, 2000). The adipocyte secretes many hormones and proteins which have been implicated in metabolic, neuroendocrine, immune and cardiovascular regulation. Since 70% of the mass of WAT is formed by lipids, this tissue represents a major reservoir for many different lipophilic contaminants.

Humans are indeed exposed to an increasing numbers of environmental xenobiotics. Some of these chemicals escape from the body metabolic system and tend to accumulate in WAT. Consequences of this accumulation are still poorly understood, but by modulating WAT metabolism and function, pollutants could affect the physiological role of WAT, leading for instance to the development of obesity related disorders. The mechanisms through which chemicals can modulate the differentiation, metabolism and secretory function of adipocytes are numerous (Mullerova and Kopecky, 2007). The link between exposure to endocrine disruptor xenobiotics and obesity has already been proposed (Elobeid and Allison, 2008) and some phthalates, which are highly prevalent lipophilic pollutants, have been shown to promote adipocyte differentiation, therefore supporting this hypothesis (Feige et al., 2007). Several epidemiological studies have also linked dioxin exposition to increased risk of diabetes or modified glucose metabolism (Uemura et al., 2008).

Cytochromes P450 (CYP) are a multigene family of constitutively expressed and inducible enzymes involved in the oxidative metabolic activation and detoxification of many
endogenous and exogenous compounds (Nebert and Dalton, 2006). These enzymes are mainly expressed in the liver. Several CYPs, however, contribute to drug metabolism in extrahepatic tissues, particularly in organs directly in contact with xenobiotics, such as the small intestine, the colon, the respiratory tract or the skin (Park et al., 1995; Bieche et al., 2007). Up to now, three main CYP families (1, 2 and 3), numbering 23 isoforms, have been identified as involved in xenobiotic metabolism. The expression of these enzymes is known to be regulated by physiological, pathological, genetic and environmental factors. Xenobiotics themselves play an important role as CYP inducers: enhancing CYP expression is indeed a common cellular mechanism leading to increased metabolism of xenobiotics. The mechanism of this CYP induction is mostly transcriptional, resulting in elevated mRNA levels (Dogra et al., 1998).

Indeed, typical CYP inducers, Phenobarbital (PB), rifampicin (RIF) and 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) activate nuclear receptors, the constitutive androstane receptor (CAR), the pregnane X receptor (PXR), and the aryl hydrocarbon receptor (AhR), respectively, leading to binding of these receptors to specific responsive elements in the CYP2B, CYP3A or CYP1 gene sequences.

Many other environmental pollutants have been described as CYP inducers. Lindane (LIND) for example, an organochlorine insecticide and fumigant which is used in agriculture and in veterinary and human medicine, has been reported as a CYP2B1/2B2, CYP1A1/1A2 and CYP2E1 inducer in the rat brain and liver (Parmar et al., 2003). Prochloraz (PRO) is an imidazole derivative that is widely used as an antifungal agent in agriculture. It has been described as a mixed inducer of CYPs in rat liver microsomes (Needham et al., 1992). Chlorpyrifos (CPY) is an organophosphorated pesticide that has been on the market since 1965 and that can bind to PXR and therefore induce CYP2B6 and CYP3A4 expression in human hepatocytes (Lemaire et al.,
2004). Residues of all of those compounds have been found in human adipose tissue samples (Russo et al., 2002; Ibarluzea Jm et al., 2004).

Two recent studies conducted by Yoshinari et al (2004, 2006) have shown that some drug-metabolizing CYP isoforms were present and functional in the rat adipose tissue, and that typical CYP inducers enhanced the expression of these enzymes, probably through the same transcriptional mechanisms as in the liver. This observation has raised the possibility that lipophilic environmental contaminants accumulated in adipose tissue may dysregulate the gene expression profile of this tissue. Since interspecies differences are numerous regarding CYP expression and regulation, we aimed at describing the CYP gene expression pattern in human WAT. Moreover, we also investigated the effects of typical CYP inducers and of various lipophilic environmental pollutants on the expression of these adipose CYPs, and we compared them with the CYP regulation mechanisms in human hepatocytes.

CYP expression in human WAT was found to be low and CYP1B1 and 2U1 were the most highly expressed CYP isoforms. The AhR-CYP1 induction pathway was also functional in this tissue.
5. **Methods**

**Materials**

Unless otherwise stated, all chemicals were purchased from Sigma-Aldrich (St-Louis, MO, USA).

**Human samples**

*Human adipose tissue explants*

The study was performed according to the Declaration of Helsinki and all the patients gave informed written consent.

Samples from human abdominal subcutaneous adipose tissue were obtained from women undergoing elective surgery. They were aged 45±15 years and had a body mass index of 29.5±4.5 kg/m². None of the subjects suffered from known metabolic or malignant diseases, nor were they taking medications known to alter WAT metabolism. Adipose tissue was cut into ±20 mg fragments in DMEM (GIBCO; Invitrogen Corp., Cergy-Pontoise, France) containing 12.5 mM glucose, 200 IU/ml penicillin, 50 mg.l⁻¹ streptomycin, 10% Bovine Serum Albumin fatty acid free and 1 µM dexamethazone. Explants were then either immediately frozen or preincubated for 1 h in a humidified 37 °C incubator with 10% CO₂ before inducer treatment.

Samples from human visceral adipose tissue were obtained from women undergoing various forms of gastrointestinal surgery at Hôpital Européen Georges Pompidou (Paris, France). They were aged 60±10 years and had a body mass index of 26.2±4.5 kg/m². Visceral fat specimens were extracted from the greater omentum and explants were prepared in the same conditions as subcutaneous adipose tissue explants.
Primary culture of adipocytes

Primary culture of preadipocytes and *in-vitro* differentiation into adipocytes were performed in UMR 5241 as previously described (Saillan-Barreau et al., 2008). Briefly stroma vascular fraction cells of the adipose tissue were isolated and seeded in plastic plates before being cultured in DMEM/F12 [1:1] supplemented with 5% new born calf serum (NCS), 100 µg/mL of pantothenic acid, 100 µM of ascorbic acid, 16 µM of biotin, 250 µg/mL of amphotericin, 5 µg/mL of streptomycin and 5 U/mL of penicillin. Preadipocytes were cultured in this medium until cells reached confluence (day 0). At day 0, adipose cell differentiation was induced using a differentiation medium DMEM/F12, 5% NCS, dexamethazone 1 µM, indomethacin 60 µM, supplemented with 450 µM of 3-isobutyl-1 methylxantine for 3 days. Differentiated adipocytes were obtained 8-10 days after induction of differentiation and then subjected to inducer treatment.

Human liver samples

cDNA from 12 human liver biopsies was a generous gift from Dr. I. Bieche (INSERM UMR735, Saint-Cloud, France). The samples originated from 4 women and 8 men. The origin and preparation of these samples has previously been described (Bieche et al., 2007). The 12 cDNA samples were pooled to perform the real time PCR reactions.

Microsomal fractions of pooled human liver were purchased from Gentest BD (Woburn, MA, USA).

Culture of human hepatocytes
Culture and induction of human hepatocytes from three donors were performed by Kaly Cell (www.kaly-cell.com). The other three primary cultures were performed at INSERM U620 as previously described (Langouet et al., 2002). The samples originated from 5 men and 1 woman.

**Treatment of cultures**

Human WAT models and hepatocytes were incubated with inducers for 48 h. Rifampicin (RIF), Phenobarbital (PB), 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), lindane (LIND), prochloraz (PRO) and chlorpyrifos (CPY) were dissolved in DMSO and added to the culture medium to give a final concentration of 50 µM, 1 mM, 10 nM, 50 µM, 25 µM and 10 µM respectively. Doses of inducers were not toxic for any cultured model. DMSO concentration in the culture medium was 0.1% (v/v). The medium was renewed every day with fresh inducers.

**Real-Time RT-PCR**

Total RNA was isolated using TriPure Isolation Reagent (Roche Applied Science, Meylan, France), submitted to DNase treatment using the RNeasy MinElute Cleanup kit (Qiagen, Courtaboeuf, France), then reverse-transcribed by using the High Capacity cDNA Archive kit (Applied biosystems, Villebon-sur-Yvette, France).

Analysis of the expression pattern of CYP mRNA isoforms in liver and WAT was performed using real time RT PCR as previously described (Girault et al., 2005). The TATA Binding Protein (TBP), a component of the DNA-binding protein complex TFIID, was used as an endogenous control. The expression of TBP was very stable in all WAT models, and ΔCt of TBP between WAT and liver models did not exceed 2. Moreover, its expression was not modified by any inducer treatment.
Nucleotide primer pairs for the CYP of families 1 to 3 have previously been tested and validated (Girault et al., 2005) and are available from Biopredic International (Rennes, France) (http://www.biopredic.com). Primers for nuclear receptors were designed and validated as previously performed for CYP primers. Sequences are as follows: AhR U: 5’ TAA CCC AGA CCA GAT TCC TCC AGA 3’ and L: 5’ CCC TTG GAA ATT CAT TGC CAG A 3’; CAR U: 5’ TGC CCG TCT TCC GTT CCC T 3’ and L: 5’ GTT TTG TGT TTG GAG ACA GAA AGT GGT 3’; PXR U: 5’ CCC CAG CCT GCT CAT AGG TTC T 3’ and L: 5’ GCT GGG TGT GCT GAG CAT TGA T 3’.

Each sample was normalized on the basis of its TBP content as previously described (Bieche et al. 2007), using the 2\(\Delta\)Ct method. With this method, the values displayed in the different tables of the paper do not provide absolute quantification of CYP mRNA, normalized to cell number or transcriptional activity of the tissue, but can be interpreted as relative one to each other. This means that, relying on our data, one can discuss the relative levels of a given CYP between models, and also the relative levels of different CYP isoforms.

**Western Blotting**

Homogenized explants of WAT, sonicated cultured adipocytes and hepatocytes were subjected to differential centrifugation to obtain microsomal fractions. To remove the lipid layer effectively the homogenates from WAT models were first centrifuged at 1000 g at 4 °C for 5 min and the resulting pellets and fat cakes were discarded. The resultant middle layer and the hepatocyte homogenate were then centrifuged at 9000 g at 4 °C for 20 min. The supernatant was then centrifuged at 100000 g at 4 °C for 60 min to recover the microsomal pellet. The 100000 g pellet was resuspended in a buffer containing NaH\(_2\)PO\(_4\) 100 mM, MgCl\(_2\) 10 mM and 20%
glycerol, adjusted at pH 7.4. The protein concentration was determined with a BCA kit (Pierce Biotechnology, Rockford, USA). Proteins were boiled in Laemmli buffer, subjected to electrophoresis on 10% SDS-PAGE gels, and blotted onto Hybond-C transfer membranes (Amersham Biosciences). Anti-CYP2U1 rabbit antiserum and HEK293 cells transfected with the human pCMV4-CYP2U1 vector used as positive control were a generous gift from Pr Magnus Ingelman-Sundberg (Institute of Environmental Medicine, Karolinska Institutet, Stockholm) (Karlgren et al., 2004). Polyclonal rabbit anti-CYP1A1 antibody and recombinant yeast expressing human CYP1A1 and used as positive control were produced in our laboratory (Belloc et al., 1996). Polyclonal rabbit anti-CYP1B1 was purchased from Santa-Cruz Biotechnology; recombinant yeast expressing human CYP1B1 was produced in our laboratory and extracts were used as positive control. To ensure the quantity of loaded proteins, actin protein has been quantified. Images were then analyzed using Image J software (Abramoff M.D., 2004).

**Statistical analysis**

Data were analyzed for statistically significant differences between control and treated cultures using the Friedmann non parametric test followed by Dunn’s post-test, at the 5% level of significance.
6. Results

Quantification of CYP mRNAs in human WAT and liver

To examine whether CYPs are expressed in human WAT, real-time RT-PCR was carried out on liver biopsies and on subcutaneous and visceral WAT. Relative mRNA levels of CYPs of families 1 to 3 are shown in table 1. Results discussed in this section concern uncultured models of liver and WAT, i.e. liver biopsies and WAT explants at t=0.

The CYP expression pattern was highly similar in both visceral and subcutaneous WAT. As expected, CYP mRNA levels were much lower in WAT than in the liver, except for CYP1B1 and CYP2U1. CYP1B1 was significantly more expressed in visceral WAT than in subcutaneous WAT or in the liver. CYP1A1 mRNA levels showed very important interindividual variations in WAT, since only one individual out of six had significant 1A1 expression in subcutaneous WAT (>2000 arbitrary units) while the others had almost undetectable CYP1A1 mRNA levels (<200 arbitrary units). Consistent with previous data (Hasler, 1999), CYP1A2 was detected only in the liver and the highest mRNA levels in the liver were those of CYP2E1, 3A4 and 2C9, thus validating our approach.

Detection of CYP proteins in human WAT and liver

To confirm the results obtained at the mRNA level, microsomal proteins of human liver and subcutaneous and visceral WAT were subjected to western-blotting (Figure 1). Since all CYPs, except for CYP1B1 and 2U1, were expressed at low levels in WAT compared to liver, protein expression was investigated only for those two isoforms.
When compared to liver microsomes, CYP1B1 was expressed in subcutaneous WAT 1.7±1 fold and in visceral WAT 1.8±2.2 fold, using actin as an internal standard (n=6). As expected from the CYP mRNA pattern, CYP1B1 was expressed both in subcutaneous and visceral WAT (Fig 1A) with important interindividual variations.

CYP2U1 was hardly detected in the liver, but was expressed in both visceral and subcutaneous WAT (Fig 1B). Quantification between liver and WAT was not possible, given the very low level of CYP2U1 protein detected in human liver microsomes.

Presence of CYP2B6 and CYP3A5 proteins was also investigated for subcutaneous (n=1) and visceral (n=3) samples. Results were consistent with mRNA levels, since none of these proteins were detected in WAT (data not shown).

Detection of CYP mRNAs in cultured human WAT

Two ex-vivo models are commonly used to study human WAT metabolism and/or gene regulation: explants of whole adipose tissue and primary culture of preadipocytes which can be differentiated in-vitro. We used both of these models to investigate whether they were appropriate to study a potential xenobiotic metabolism in human WAT and/or the consequences of the exposition of this tissue to xenobiotics. Using RT PCR, we investigated whether the CYP mRNA expression pattern observed in uncultured WAT explants was stable in cultured explants of WAT and primary culture of adipocytes (table 1).

In cultured explants as in culture of adipocytes, CYP1B1 and 2U1 were the most expressed CYP isoforms, consistent with the results of uncultured tissue. However, several differences were observed between cultured and uncultured models of WAT. CYP1B1 mRNA was greatly enhanced in adipocyte culture. Explants culture increased CYP1A1 and CYP1B1
mRNA, more in subcutaneous culture (by 15 and 6 times compared to uncultured explants respectively) than in visceral explants (8 and 2 fold increase respectively).

**Induction of hepatic CYP mRNA levels by typical CYP inducers and other pollutants**

As a positive control and to allow direct comparison between CYP regulation in the liver and WAT, human hepatocytes were treated with the same compounds as human WAT models. Typical CYP inducers, RIF, PB and TCDD, which mainly induce CYP3A4, CYP2B6, and CYP1A1 respectively, were used. We also investigated the effects of environmental pollutants possibly found in human WAT –LIND, PRO and CPY- on CYP expression. Induction of CYP mRNA was investigated for the 23 isoforms of families 1, 2 and 3 using RT PCR. Results are presented in table 2.

As expected, RIF treatment increased CYP2B6 and CYP3A4 mRNAs, as did PB treatment, which also induced CYP2A6 and CYP3A7 mRNA. CYP1A1, 1A2 and 1B1 mRNA were induced by TCDD treatment. Lindane induced CYP2B6 mRNA significantly and CYP3A4 and 3A7 to a lesser extent. Prochloraz was a significant CYP1 inducer and induced CYP1A1 and CYP1A2, but induction factors were lower than with the TCDD treatment. PRO was also a strong repressor of a few CYP isoforms, such as CYP2D6, CYP2E1, CYP2J2 and CYP2U1. CPY treatment increased CYP1A1 and CYP1A2 mRNA levels. These findings confirm that the inducer doses and the experimental procedures used in this study were suitable for the observation of the CYP induction mechanisms.

**Induction of adipose CYP mRNA by typical CYP inducers and other pollutants**

To investigate whether CYP induction is also possible in human WAT, we treated our human WAT models with CYP inducers as described in the previous section.
Interestingly, for CYPs of families 2 and 3, mRNA levels were not modified by chemical treatments in WAT models. Only the TCDD and PRO treatments had effects on CYP1 family mRNA and therefore, only results of CYP1A1 and CYP1B1 are presented in figure 2.

TCDD was a significant CYP1A1 and CYP1B1 inducer in visceral WAT and subcutaneous adipocytes, although induction factors were significantly lower than in hepatocytes. In cultured adipocytes, basal level of CYP1A1 mRNA was very low and induction with TCDD was therefore important. Surprisingly subcutaneous explants barely responded to TCDD treatment. PRO was a weaker CYP1A1 inducer in visceral WAT. In subcutaneous explants, it was also a significant CYP1A1 and CYP1B1 inducer and it even displayed a stronger effect than TCDD.

Induction of adipose and hepatic CYP proteins

Only treatments by TCDD and PRO induced changes in CYP1 mRNA levels in WAT. To examine whether these changes have consequences at the protein level, microsomal fractions were prepared from WAT models and from primary culture of hepatocytes treated with TCDD or PRO. Western-Blotting was then carried out (fig 3).

CYP1A1 protein was significantly increased by TCDD treatment in hepatocytes (fig 3A). In cultured subcutaneous adipocytes, CYP1A1 protein level was below the limit of Western blotting detection, even after TCDD or PRO treatment. In subcutaneous and visceral explants of WAT, the CYP1A1 protein was detected at very low levels. Quantification was difficult and no induction was found significant, even after TCDD or PRO treatment.

TCDD treatment enhanced the amount of CYP1B1 protein in all WAT models (fig 3B). In subcutaneous explants, consistent with mRNA results, CYP1B1 protein levels were lower and quantification of the induction was then more difficult. TCDD enhanced CYP1B1 protein in this
model but this induction was not statistically significant. PRO treatment also enhanced CYP1B1 protein in all WAT models but to a lesser extent.

Detection of adipose transcription factors involved in CYP induction

In human WAT, none of the CYPs of families 2 or 3 was induced by typical inducer treatment, namely RIF and PB. To explain these results, we looked for transcription factors usually involved in the induction of these CYP: mainly AhR for CYP1, CAR and PXR for CYP2 and 3 (table 3).

We found that AhR was present both in subcutaneous and visceral WAT. Interestingly, the AhR mRNA level was significantly higher in visceral WAT than in subcutaneous WAT or in the liver. On the contrary, CAR or PXR were undetected or expressed at very low levels in WAT models. This result was not due to an expression decrease during the culturing process of WAT models, since neither CAR nor PXR were detected in uncultured WAT explants.
7. Discussion:

Human WAT represents a reservoir of lipophilic environmental pollutants. Chemicals like dioxins, furans, PCB, organochlorine, organophosphorus pesticides or brominated diphenyl esters have been found in WAT samples in numerous populations (Pauwels et al., 2000; Petreas et al., 2004; Jimenez Torres et al., 2006). In rat, previous studies (Yoshinari et al., 2004) have shown that some xenobiotic-metabolizing forms of CYP were expressed in WAT and that their expression was increased by typical CYP inducers. These results demonstrated the presence of active CYPs as well as the functionality of the induction pathways of CYPs in this tissue.

However, little is known about human WAT. In this study, we demonstrated that a few CYP isoforms were expressed in human WAT, mainly CYP1B1 and CYP2U1. CYP2U1 was identified and characterized by S. Chuang et al. (Chuang et al., 2004). It was specifically detected in both the human brain and thymus, and shown to metabolize long chain fatty acids. It has been postulated that CYP2U1 plays an important role in fatty acid signaling processes in both the cerebellum and thymus, and our results suggest that this role could also be important in human WAT. CYP1B1 is expressed in many extra hepatic organs and is involved in the metabolism of 17β-estradiol and testosterone (Spink et al., 1994). These results have led to the speculation of its possible physiological function: the metabolism of steroid hormones. In mouse preadipocyte cell lines, CYP1B1 expression has been shown to rise during adipogenesis (Alexander et al., 1997), suggesting that CYP1B1 may generate or remove physiological substrates that regulate this process. CYP1B1 has also been shown to be inducible by AhR ligands and involved in activation of many human promutagens and procarcinogens (Shimada et al., 1996), and it is therefore believed to be an extrahepatic route of xenobiotic metabolism. We also detected CYP1A1, another AhR inducible CYP isoform, but interindividual variations in its expression were
significant. Some individuals had indeed high CYP1A1 mRNA levels, while in others, CYP1A1 mRNA was undetectable.

Moreover, we have found significant differences in adipose CYP expression following culturing processes. In *in-vitro* differentiated adipocytes, CYP1B1 expression was highly enriched compared to all other WAT models. This discrepancy could be explained by the fact that WAT explants contain not only mature adipocytes, but also other cells constituting stroma vascular fraction (SVF) of WAT. However, most of the cells in WAT explants are mature adipocytes and some complementary experiments, where CYP1B1 expression was compared between isolated adipocytes and SVF cells from the same individual, have shown that differences in CYP expression between adipocytes and SVF cells are not sufficient to account for this enrichment in CYP1B1 (data not shown). More probably, its expression was induced by the adipogenic hormonal mixture used to stimulated adipocyte differentiation, as described in C3H10T1/2 cells, where this mechanism was independent of the AhR (Cho et al., 2005). Here, WAT explants culture also led to CYP1A1 and 1B1 induction. WAT explants culture has already been shown to drastically change adipocyte gene expression (Gesta et al., 2003), and CYP1A1 expression to be greatly enhanced during several culturing processes, through mechanisms that were independent of the AhR. For exemple, in CaCo-2 cells, physiological compounds contained in serum induced CYP1A1 gene expression by transcriptional activation independent of the AhR pathway (Guigal et al., 2000). In our study, we cannot conclude whether the CYP1 induction that we observe in our cultured WAT models is dependant or not of the AhR. It is thus very important that CYP expression pattern was here described for the first time in uncultured human WAT samples, showing actual constitutive expression of CYP1B1 and CYP2U1. Whether their physiological role in this tissue concerns adipogenesis regulation, fatty acid, steroid or xenobiotic metabolism still needs to be elucidated.
In-vivo rat studies have shown that CYPs are regulated in WAT in the same way that they are in the liver: CYP1A1, CYP2B and CYP3A mRNA and protein inductions were indeed observed after betanaphthoflavone, PB and dexamethazone treatment respectively, both in rat epididymal WAT and liver. Nuclear receptors CAR and PXR, as well as AhR mRNA, have also been detected in rat epididymal WAT. We thus treated human WAT models with 3 typical CYP inducers that use the same receptor systems in both species: PB for CAR, RIF for PXR, and TCDD for AhR. In our human WAT models, neither PB nor RIF induced CYP2B of CYP3A mRNA, whereas treated with the same doses and preparations of inducers, human hepatocytes showed clear and statistically significant inductions of CYP2B6 and CYP3A4. Taken together with the fact that CAR and PXR mRNA were almost undetectable in our WAT samples, our results show that CYP2 and CYP3 are not regulated in human WAT in the same way that they are in the human liver or in the rat WAT. Numerous interspecies expression differences in nuclear receptor distribution have already been reported (Timsit and Negishi, 2007). It thus seems very likely that differences between rat and human concerning CYP2 and 3 regulation in WAT are due to interspecies differences in nuclear receptor expression.

TCDD is well known to accumulate in WAT, its half-life being estimated between 5.8 and 9.6 years in humans (Michalek et al., 1992). In in-vivo animal studies in WAT, the inhibition of glucose transport (Enan et al., 1992), lipoprotein lipase activity (Brewster and Matsumura, 1988) and fatty acid synthesis (Lakshman et al., 1989) have been reported as consequences of the presence of TCDD. The effects of TCDD are mediated through AhR, for which depletion occurs during adipose differentiation in 3T3-L1 cells (Shimba et al., 1998), raising the hypothesis that mature adipocytes could lose their functional response to TCDD. In this work, we detected AhR mRNA in all models of subcutaneous and visceral WAT, at levels that are comparable to and sometimes even higher than those that we found in hepatocytes. Moreover, TCDD treatment
significantly increased CYP1A1 and CYP1B1 mRNA and proteins in most WAT models. Only subcutaneous explants barely responded to TCDD, at mRNA level, while cultured subcutaneous adipocytes did. AhR expression was comparable in both models and can therefore not account for this large difference. One possible explanation could be that stromal vascular adipose cells do not respond to TCDD, as shown in rat epididymal WAT (Yoshinari et al., 2006). As discussed previously, important CYP1 inductions occurred during culturing processes of WAT explants, and more importantly in subcutaneous than in visceral WAT. Whether these inductions depend on the AhR is not clear. However, it is possible that such inductions nearly saturated the AhR pathway in this model, lowering the sensitivity to AhR inducers. But even though we were not in optimal conditions, CYP1 inductions were still observable in most WAT models after TCDD treatment, and it has previously been demonstrated that AhR mediated the CYP1A1 induction by betanaphthoflavone in rat WAT (Yoshinari et al., 2006). Our results thus suggest the existence of a constitutive functional AhR-CYP1 induction pathway in human WAT and in human adipocytes.

Interestingly, AhR mRNA levels were significantly higher in visceral than in subcutaneous adipose tissue, as well as CYP1A1 and CYP1B1 induction. Other differences were observed between subcutaneous and visceral WAT, such as more important CYP1B1 and CYP2U1 expression in visceral samples. Differences in endocrine and biochemical characteristics have already been reported between subcutaneous and visceral fat (Lafontan and Berlan, 2003). Due to their different anatomic location and subsequent different vascularization, one can think that xenobiotic distribution would vary according to the fat deposit localization. Our results suggest that subcutaneous and visceral adipose tissues may react differently after xenobiotic exposition.
Human WAT models were also treated with pesticides, lindane, prochloraz and chlorpyrifos, which have all been found in human WAT and which have been reported to be potent CYP inducers. Lindane has been described as a CYP1A1, 2B1/2 and 2E1 inducer in rat liver microsomes (Parmar et al., 2003) and a PXR ligand inducing CYP2B6 and CYP3A4 expression in human hepatocytes (Lemaire et al., 2004). Our findings in human hepatocytes confirm these results, but no significant changes in adipose CYPs were observed. On the other hand, prochloraz showed some significant effects on adipose CYPs. Prochloraz has been shown to cause CYP1A induction in a rainbow trout cell line (Babin et al., 2005) and to have a mixed induction profile close to the one of phenobarbital in rat liver enzymes (Needham et al., 1992). In human hepatocytes, we found that PRO is indeed a CYP2B6 inducer, but mostly an important CYP1A1/2 inducer. These results suggest that PRO could be a ligand for the AhR. However, the fact that subcutaneous explants of WAT do respond to PRO and not to TCDD and that PRO does not induce CYP1B1 mRNA in hepatocytes also suggest that other mechanisms could be involved in PRO induction pathway. Nevertheless, these results highlight the fact that not only the very potent AhR ligand TCDD, but also some less documented environmental pollutants could modify the CYP1 expression pattern in human WAT.

In conclusion, we have identified the expression of xenobiotic metabolizing CYPs in human WAT. We have also demonstrated that some, but not all, of the CYP hepatic regulation mechanisms are functional in this tissue. This point raises the possibility that such CYPs could play a role in in-situ xenobiotic elimination and in the half-life time of lipophilic pollutants in the human body. By activating the functional receptors of WAT, some pollutants can indeed modify the expression pattern of xenobiotic metabolizing enzymes in this physiologically important tissue.
8. **Acknowledgments**

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9. References


10. Figure legends

Figure 1: CYP proteins in human liver and WAT. Microsomal proteins (30µg per lane) from pooled human liver, subcutaneous WAT (SC WAT) and visceral WAT (VS WAT) were subjected to western-blotting with anti-CYP1B1 (A) and anti-CYP2U1 (B) antibodies. Actin was used as an internal standard.

Figure 2: Induction of CYP1A1 and CYP1B1 mRNA in human hepatocytes and WAT models. Explants of various adipose tissues (subcutaneous, SC EXP or visceral, VS EXP) or primary cells from human liver (hepatocytes, HEP) or from human subcutaneous WAT (subcutaneous adipocytes, SC AD) were treated with 2,3,7,8-terachloro-p-dibenzodioxin (TCDD, 10nM) or prochloraz (PRO, 25µM) for 48h. Levels of CYP1A1 (A) and CYP1B1 (B) mRNA were investigated by RT-PCR. Numbers above bars indicate fold induction compared to DMSO treated models. Results are presented as mRNA arbitrary units (mean ± SD for n=6). DMSO: white bars, TCDD: black bars, PRO: striped bars.

*: p<0.05, **: p<0.01, using the Friedman non parametric test followed by Dunn’s post-test.

Figure 3: Induction of CYP proteins in human hepatocytes and WAT models. Explants and primary culture of adipocytes of subcutaneous WAT (SC WAT), explants of visceral WAT (VS WAT) and primary culture of hepatocytes (HEP) were prepared as described earlier and treated with TCDD and PRO as described in the legend of fig 2. Microsomal proteins (30µg) were then submitted to western-blotting with anti-1A1 (A) and anti-1B1 (B) antibodies. Fold change in protein levels were quantified using actin as an internal standard and results are presented as fold change compared to DMSO treated models (mean ± SD for n=3). TCDD: black bars, PRO: striped bars.

*: p<0.05, **: p<0.01, using the Friedman non parametric test followed by Dunn’s post-test.
Table 1: Relative mRNA levels of CYPs in human liver and WAT

<table>
<thead>
<tr>
<th>CYP</th>
<th>Liver Biopsies</th>
<th>Subcutaneous WAT</th>
<th>Visceral WAT</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Explants (t=0)</td>
<td>Explants (t=48h)</td>
</tr>
<tr>
<td>1A1</td>
<td>24432</td>
<td>571±1080</td>
<td>8561±6494</td>
</tr>
<tr>
<td>1A2</td>
<td>323914</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>1B1</td>
<td>1884</td>
<td>1251±564</td>
<td>8396±3793</td>
</tr>
<tr>
<td>2A6</td>
<td>144272</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>2A7</td>
<td>28956</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>2B6</td>
<td>49353</td>
<td>50±54</td>
<td>192±304</td>
</tr>
<tr>
<td>2C18</td>
<td>54865</td>
<td>219±121</td>
<td>ND</td>
</tr>
<tr>
<td>2C19</td>
<td>74445</td>
<td>60±50</td>
<td>ND</td>
</tr>
<tr>
<td>2C8</td>
<td>360803</td>
<td>41±31</td>
<td>116±139</td>
</tr>
<tr>
<td>2C9</td>
<td>494491</td>
<td>9±18</td>
<td>ND</td>
</tr>
<tr>
<td>2D6</td>
<td>86014</td>
<td>145±22</td>
<td>139±64</td>
</tr>
<tr>
<td>2E1</td>
<td>3244323</td>
<td>155±40</td>
<td>98±25</td>
</tr>
<tr>
<td>2J2</td>
<td>50999</td>
<td>17±24</td>
<td>7±5</td>
</tr>
<tr>
<td>2R1</td>
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<td>133±90</td>
<td>510±186</td>
</tr>
<tr>
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<td>895</td>
<td>201±153</td>
<td>97±54</td>
</tr>
<tr>
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<td>2610</td>
<td>2135±803</td>
<td>1750±846</td>
</tr>
<tr>
<td>3A4</td>
<td>505805</td>
<td>116±83</td>
<td>6±6</td>
</tr>
<tr>
<td>3A43</td>
<td>5094</td>
<td>18±14</td>
<td>ND</td>
</tr>
<tr>
<td>3A5</td>
<td>75422</td>
<td>426±358</td>
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<tr>
<td>3A7</td>
<td>16355</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

ND: Not detected (Ct>33)

Relative mRNA levels of CYP were measured using RT PCR in uncultured (explants t=0, n=6) or cultured (explants t=48h, n=6, and primary culture of adipocytes, n=6) WAT and liver biopsies (pool, n=12). Relative mRNA levels were calculated by dividing CYP levels by TBP levels and are means ± SD. CYP2F1 and 2W1 mRNA were not detected and are therefore not shown in this table.
Table 2: Induction of CYPs of families 1 to 3 in human hepatocytes

<table>
<thead>
<tr>
<th>CYP</th>
<th>RIF</th>
<th>PB</th>
<th>TCDD</th>
<th>LIND</th>
<th>PRO</th>
<th>CPY</th>
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</thead>
<tbody>
<tr>
<td>1A1</td>
<td>3.9±2</td>
<td>2±0.7</td>
<td>1135±748***</td>
<td>1.9±0.9</td>
<td>84±68**</td>
<td>27±44*</td>
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<tr>
<td>1A2</td>
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<td>341±306**</td>
<td>0.6±0.4</td>
<td>13±16</td>
<td>12±8</td>
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<tr>
<td>1B1</td>
<td>11.1±0.3</td>
<td>11.1±0.3</td>
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<td>1.7±0.8</td>
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<tr>
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<td>3±1.8*</td>
<td>1.1±0.7</td>
<td>1.8±1.5</td>
<td>0.9±0.7</td>
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</tr>
<tr>
<td>2A7</td>
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<td>0.2±0.1</td>
<td>1.5±0.4</td>
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<tr>
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<td>4.9±1.3*</td>
<td>7.5±4.9**</td>
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<td>7±5.4**</td>
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<td>0.8±0.2</td>
<td>0.9±0.2</td>
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<tr>
<td>2C19</td>
<td>1.4±0.5</td>
<td>1.2±0.5</td>
<td>1±0.3</td>
<td>0.9±0.2</td>
<td>0.6±0.3</td>
<td>0.9±0.2</td>
</tr>
<tr>
<td>2C8</td>
<td>2.4±0.9</td>
<td>2.3±1.2</td>
<td>0.8±0.3</td>
<td>1.1±0.8</td>
<td>0.3±0.2</td>
<td>1.3±0.3</td>
</tr>
<tr>
<td>2C9</td>
<td>1.8±0.3</td>
<td>1.7±0.2</td>
<td>0.8±0.2</td>
<td>1.1±0.6</td>
<td>0.7±0.3</td>
<td>1.3±0.4</td>
</tr>
<tr>
<td>2D6</td>
<td>0.7±0.1</td>
<td>0.7±0.2</td>
<td>0.9±0.4</td>
<td>0.5±0.2**</td>
<td>0.4±0.2***</td>
<td>0.7±0.2</td>
</tr>
<tr>
<td>2E1</td>
<td>0.4±0.2*</td>
<td>0.3±0.2*</td>
<td>0.4±0.2</td>
<td>0.2±0.2***</td>
<td>0.3±0.2***</td>
<td>0.5±0.1</td>
</tr>
<tr>
<td>2J2</td>
<td>0.7±0.1*</td>
<td>0.7±0.1*</td>
<td>0.9±0.1</td>
<td>0.7±0.1</td>
<td>0.6±0.2**</td>
<td>0.8±0.1</td>
</tr>
<tr>
<td>2R1</td>
<td>0.9±0.1</td>
<td>0.8±0.1</td>
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<td>1.1±0.2</td>
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<tr>
<td>2S1</td>
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<td>0.7±0.4b</td>
<td>0.9±0.7</td>
<td>1.2±0.9</td>
<td>1.2±1</td>
<td>0.8±0.4</td>
</tr>
<tr>
<td>2U1</td>
<td>0.9±0.1</td>
<td>0.9±0.1</td>
<td>0.7±0.1*</td>
<td>0.8±0.1</td>
<td>0.6±0.1*</td>
<td>0.9±0.1</td>
</tr>
<tr>
<td>3A4</td>
<td>6.9±4.9**</td>
<td>5.7±3.4*</td>
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<td>3±1.5</td>
<td>0.7±0.5</td>
<td>2.6±1.5</td>
</tr>
<tr>
<td>3A43</td>
<td>1.4±0.4</td>
<td>1.4±0.5</td>
<td>0.6±0.4</td>
<td>1±0.4</td>
<td>0.4±0.2</td>
<td>1±0.2</td>
</tr>
<tr>
<td>3A5</td>
<td>1.3±0.2</td>
<td>1.3±0.1</td>
<td>0.8±0.2</td>
<td>1.4±0.2</td>
<td>0.9±0.3</td>
<td>1.1±0.2</td>
</tr>
<tr>
<td>3A7</td>
<td>3.1±4.6</td>
<td>4.3±6.7*</td>
<td>1±0.5</td>
<td>2.9±5.3</td>
<td>0.9±1.2</td>
<td>2.5±3.2</td>
</tr>
</tbody>
</table>

*: p<0.05, **: p<0.01, ***: p<0.001 using the Friedman non parametric test followed by Dunn’s post-test.

Human hepatocytes (n=6) were treated with rifampicin (RIF, 50µM), phenobarbital (PB, 1mM), 2,3,7,8-terachloro-p-dibenzodioxin (TCDD, 10nM), lindane (LIND, 50µM), prochloraz (PRO, 25µM) or chlorpyrifos (CPY, 10µM) for 48h. Levels of CYP mRNA were investigated by RT-PCR. Results indicate mRNA fold induction compared to vehicle treated cells (DMSO 0.1%) and are mean ± SD. CYP2F1 and 2W1 mRNA were not detected even after inducer treatments and are therefore not shown.
Table 3: Relative mRNA levels of transcription factors involved in CYP induction in human liver and WAT

<table>
<thead>
<tr>
<th>Gene</th>
<th>Liver Biopsies (t=0)</th>
<th>Hepatocytes (t=0)</th>
<th>Subcutaneous WAT Explants (t=48h)</th>
<th>Visceral WAT Explants (t=0)</th>
<th>Explants (t=48h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AhR</td>
<td>8871</td>
<td>6431±1342</td>
<td>3960±1264</td>
<td>17881±6790</td>
<td>8744±3328</td>
</tr>
<tr>
<td>CAR</td>
<td>18618</td>
<td>5390±1743</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>PXR</td>
<td>13612</td>
<td>13153±2570</td>
<td>ND</td>
<td>2±1</td>
<td>7±4</td>
</tr>
</tbody>
</table>

ND: Not detected (Ct>33)

Relative mRNA levels of CYP were measured using RT PCR as described in table 1 legend. Relative mRNA levels were calculated by dividing receptors levels by TBP levels and are means ± SD.
Fig 1: CYP proteins in human liver and WAT

A. CYP1B1

B. CYP2U1
Figure 2: Induction of CYP1A1 and CYP1B1 mRNA in human hepatocytes and WAT models

A. CYP1A1

B. CYP1B1
Fig 3: Induction of CYP1A1 and CYP1B1 protein in human hepatocytes and WAT models

A. CYP1A1

B. CYP1B1

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