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2,3,7,8-tetrachlorodibenzo-*p*-dioxin induces insulin-like growth factor binding protein-1 gene expression and counteracts the negative effect of insulin

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List of abbreviations:

IGFBP, insulin-like growth factor binding protein; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; XRE, xenobiotic responsive element; AhR, aryl-hydrocarbon receptor; ARNT, AhR receptor nuclear translocator; CYP1A1, cytochrome P450 1A1; NQO1, NAD(P)H : quinone oxidoreductase 1; IGF, insulin-like growth factor; BiP, immunoglobulin binding protein; GRP, glucose-regulated protein; IRS, insulin responsive sequence; GRE, glucocorticoid responsive element; RT-PCR : reverse transcription PCR.

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Abstract

Recent epidemiological studies have revealed a possible correlation between exposure to high levels of dioxins or dioxin-like compounds and diabetes. Yet, the interaction between insulin and dioxins actions remains elusive. We studied the regulation of insulin-like growth factor binding protein-1 (IGFBP-1), a protein involved in glucose homeostasis and which expression is down regulated by insulin. We showed that 2,3,7,8-tetrachorodibenzo-*p*-dioxin (TCDD) specifically induced IGFBP-1 mRNA in human hepatocytes and HepG2 human hepatoma cells (2.5- and 8-fold respectively). Cellular and secreted IGFBP-1 protein levels were also up-regulated. Transfection and reporter assays showed that the IGFBP-1 promoter was activated by TCDD and that this activation was dependent on the integrity of a proximal xenobiotic responsive element (XRE). This XRE, located near the insulin-glucocorticoid regulatory region, binds the aryl-hydrocarbon receptor (AhR). In agreement with previous studies, the IGFBP-1 promoter was down-regulated by insulin (50 %); we show here that while TCDD activated the IGFBP-1 promoter 5- to 6-fold, the combination of TCDD and insulin led to an expression level of IGFBP-1 that was higher than basal level (2- to 3-fold activation). Similar regulations were observed for the endogenous IGFBP-1 mRNA. These data suggest that the xenobiotic-hormonal regulatory region of the IGFBP-1 promoter mediates an upregulation of IGFBP-1 expression by TCDD even in the presence of insulin. Since IGFBP-1 modulates blood glucose levels, the up-regulation of IGFBP-1 by dioxins might account for the disruptive effects of these pollutants on glucose metabolism.

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Introduction

A variety of human pathological conditions has been associated with professional or accidental exposure to high levels of 2,3,7,8-tetrachorodibenzo-*p*-dioxin (TCDD) or dioxin-like compounds. Studies have mostly focused on cancer, endocrine and reproductive toxicity and cardio-vascular diseases (Kogevinas, 2001; Pesatori et al., 2003) and these effects have been confirmed in several animal species. A correlation between elevated TCDD exposure, as indirectly assessed by residual TCDD levels in serum lipids, and diabetes has also been suggested (Remillard and Bunce, 2002). The latter point was of particular interest since the prevalence of type II diabetes has been continuously increasing in the last decades suggesting a possible contribution of environmental factors in addition to obvious nutritional habits (Longnecker and Daniels, 2001). The epidemiological studies focused on highly exposed individuals: residents in Seveso (Italy), Vietnam war veterans who were exposed to agent orange, chemical workers, pesticide manufacturers and applicators. In these studies, there was a positive correlation between background TCDD concentration and glucose regulation abnormalities as well as diabetes prevalence, particularly for Vietnam veterans (Kim et al., 2003; Steenland et al., 2001) and females residents in Seveso (Pesatori et al., 1998). Two studies of populations exposed to low levels of TCDD suggest that dioxin-like compounds and diabetes might be related (Cranmer et al., 2000; Longnecker and Daniels, 2001). The demonstration of a direct role of TCDD in diabetes still needs further investigations.

Few biological studies have attempted to clarify the interactions between dioxin-like compounds and insulin. It has been shown that TCDD decreases glucose transport and lipoprotein lipase activity in adipocyte cultures; furthermore, an increase in tumor necrosis alpha factor secretion was also noted in this tissue where TCDD is concentrated (Kern et al., 2002). An inhibition of adipocyte differentiation and lipid synthesis was observed (Alexander

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et al., 1998) which could contribute to insulin resistance. Despite these studies, the actual mechanisms accounting for the interaction between TCDD, glucose homeostasis and insulin signaling remain elusive. In particular, it is unclear whether, some of the genes that are regulated by TCDD could contribute to the perturbation of insulin action.

Most toxic effects of TCDD are mediated by the aryl hydrocarbon receptor (AhR) which, when activated by its ligand, is translocated into the nucleus and forms a heterodimer with the AhR receptor nuclear translocator (ARNT). The heterodimer binds to specific responsive elements called xenobiotic responsive elements (XREs) and induces the expression of several genes (Mimura and Fujii-Kuriyama, 2003). Most studies on the regulation of gene expression by TCDD have initially focused on xenobiotic metabolic enzymes genes; theses genes were designated as the “Ah battery” genes (Nebert et al., 2000). More recently, large scale gene expression studies showed that the panel of genes regulated by TCDD is much larger and comprises genes with other cellular functions including cell division, cell signaling… (Frueh et al., 2001; Puga et al., 2000). The nature of the regulated genes could shed some light on the mechanisms of TCDD toxicity, however a more complete evaluation has yet to be performed.

In the course of a large scale study of TCDD effects on gene expression in the human hepatoma cell line HepG2 (unpublished data), we noticed that the insulin-like growth factor binding protein-1 (IGFBP-1) gene was induced several folds. IGFBPs are a family of secreted proteins which display a high affinity for insulin-like growth factors, IGF-I and IGF-II. The main function of these binding proteins is to keep the level of free IGFs under control and to target the release of these highly active growth factors (Firth and Baxter, 2002). In addition to the modulation of IGF biodisponibility, IGFBPs exert IGF-independent effects (Firth and Baxter, 2002). IGFBP-1 displays tissue-specific expression and is produced primarily by hepatocytes in both rodents and humans and by decidualized stroma cells of the endometrium

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(Lee et al., 1997). IGFBP-1 is also synthesized to a lesser extent by the kidney, uterus, heart and lung. Hepatic production is maximal during fetal life, decreases rapidly after birth, and thereafter is primarily dependent on insulin secretion that inhibits its synthesis at the transcriptional level. Insulin acts through two insulin responsive sequences that are present as an inverted palindrome in the IGFBP-1 promoter (Suwanichkul et al., 1994). It is believed that IGFBP-1 regulation by insulin contributes to the metabolic response to food intake since a decrease in IGFBP-1 would increase the bioavailability of IGFs which exert insulin-like metabolic functions (Murphy, 2003). Various transgenic mice over-expressing the IGFBP-1 gene under the control of different promoters consistently display impaired glucose tolerance and abnormalities of insulin action, in addition to other growth disrupting effects (Crossey et al., 2000; Gay et al., 1997; Rajkumar et al., 1996; Schneider et al., 2000). These observations indicate that increased IGFBP-1 expression may disrupt the physiological control of glucose homeostasis, a condition that is believed to contribute to the pathogenesis of diabetes. Moreover, IGFBP-1 levels are elevated in type I diabetic patients , in some forms of acquired insulin resistance such as in type II diabetes (Monzavi and Cohen, 2002) and in diverse liver deseases (Hwang et al., 2003). Thus, the IGFBP-1 gene is a particularly relevant target of TCDD for the delineation of the interaction between TCDD signaling and insulin action. In this paper, we show that TCDD induces the IGFBP-1 gene expression in HepG2 cells and in human hepatocytes. This effect is mediated by an XRE which is in the vicinity of the negative insulin responsive sequences of the human IGFBP-1 promoter. The studies show that TCDD leads to increased IGFBP-1 expression even in the presence of insulin; this elevation of IGFBP-1 expression may contribute to the disruptive effects of TCDD on glucose homeostasis.

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Materials and methods

Chemicals **:** TCDD was purchased from LCG Promochem (Molsheim, France). Oligonucleotides were obtained from Qiagen (Les Ulis, France).

Cell culture : Human hepatocarcinoma HepG2 cells were cultured at 37°C in 50% Dulbecco's minimal essential medium complemented with non-essential amino-acids and 50% Ham's F12 medium, supplemented with 10% fetal bovine serum, 200 units/ml penicillin, 50 µg/ml streptomycin (Invitrogen, Cergy-Pontoise, France) and 0.5 mg/ml amphotericin B (Bristol Myers Squibb, Princeton, USA) in a humidified atmosphere in 5% CO_2. Human hepatocytes were a kind gift from Dr. P. Maurel (INSERM 128, Montpellier, France) and were maintained as previously described (Isom et al., 1985). The culture medium of hepatocytes contains 10^{-6} M bovine insulin.

Isolation of total RNA and nuclear RNA : Total RNA were isolated using the RNeasy midi kit from Qiagen according to the manufacturer's instructions. To prepare nuclear RNA from HepG2 cells, 4 millions HepG2 cells (treated or not with 10^{-7}M insulin (Sigma-Aldrich, Saint-Quentin Fallavier, France) and/or 25 nM TCDD) were fed with a serum free medium containing 1 g/liter fatty acid-free bovine serum albumin (Sigma-Aldrich) during the treatment. After 24 hours incubation, cells were washed twice with cold phosphate buffer saline, collected and centrifugated 5 min at 1500g. Pellets were resuspended in 1 ml NP40 lysis buffer (10 mM Tris pH 7,5, 10 mM NACl, 3mM MgCl2, 0,5% Nonidet P40 (Sigma-Aldrich)) and incubated on ice for 5 min. A centifugation at 1500g for 5 min was then performed and the supernatant were eliminated. These steps were performed twice. Then the nuclei were lysed and RNA prepared using the RNeasy mini kit from Qiagen with a DNAse treatment as recommanded. Nuclear RNA were retrotranscribed in cDNA for quantitative RT-PCR experiments.

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Northern Blot Hybridization : Northern Blots were performed using 10 µg of total RNA per lane. The probe used to detect IGFBP-1 was isolated by MscI, ClaI digestion of the pBluescript/BP-1 plasmid containing the human IGFBP-1 cDNA (a gift from Pr M. Binoux, (Gay et al., 1997)). The NQO1 probe is a 795 bp fragment (nucleotide 624 -1419) of the human NQO1 cDNA and was previously described (Marchand et al., 2004). The GRP94 probe consists of nucleotides 633 to 1033 of human GRP94 cDNA (Marchand et al., 2004). The human BiP probe corresponds to the rat BiP cDNA. The alpha-fetoprotein probe was isolated by reverse transcription-PCR from HepG2 RNA using oligonucleotides 5'-AAATACATCCAGGAGAGCCA-3' (sense strand) and 5'-CTGAGCTTGGCACAGATCCT-3' (antisense strand).

These probes were labeled using the Megaprime DNA labeling system (Amersham Biosciences, Orsay, France) and hybridization was performed using Rapid-hyb buffer (Amersham) following the manufacturer's instructions. Membranes were washed for 45 min at 65°C with 2X SSC and 0.1% SDS and for 35 mn with 0.5X SSC and 0.1% SDS. Quantifications were performed with a PhosphorImager and the ImageQuant software (Molecular Dynamics, Inc.).

Quantitative Reverse Transcription – PCR (RT-PCR) **:** Reverse transcription was performed on each RNA sample (2 µg) using the cDNA High-Capacity Archive kit from Applied Biosystems (Courtaboeuf, France) in a final reaction volume of 50 µl according to the manufacturer's instructions. All specific oligonucleotides were designed using the oligo explorer software (oligosoftware). The sequences are shown in table 1.

Quantitative RT-PCR was performed with 4 ng of the cDNA, 300 nM of each primer and SYBR-Green PCR Master Mix (Applied Biosystems), final volume : 10 µl. Quantitative RT-PCR measurements were performed on an ABI Prism 7900 Sequence Detector system (Applied Biosystems, Foster city, USA). PCR cycles proceeded as follows: Taq activation (10

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min), denaturation (15 sec, 95°C); annealing and extension (1 min, 60°C). The melting curve analysis showed the specificity of the amplifications. Threshold cycle, Ct, which inversely correlates with the target mRNA level, was measured as the cycle number at which the reporter fluorescent emission appears above the background threshold. The relative mRNA levels were estimated by the standard method using ribosomal protein L13a as the reference gene.

Cellular protein extracts and medium protein extracts : Ten million HepG2 cells were treated or not with 25 nM TCDD in 6 ml serum free medium during 24 h. Culture supernatant containing secreted IGFBP-1 was saved and protein extracts from cells were prepared. Cells were washed twice with Hank's balanced Salt solution and centrifuged at 1500 rpm for 5 min. The pellet was resupended in 0.25 M sucrose, 10 mM TrisHCl pH 7.4, 1 mM EDTA containing an antiprotease inhibitor cocktail tablet (Roche Diagnostics, Meylan, France) and lysed by sonication 30 seconds with a Vibracell (Fisher Bioblok Scientific). A centrifugation of 20 minutes at 15,000 rpm was then performed and the pellets were resuspended in 200 µl of 100 mM $NaPO_4$, 10 mM $MgCl_2$, 20% glycerol pH 7.4. Protein concentration of cell lysates and medium extracts were measured using the BCA protein assay reagent (Pierce) and bovine serum albumin as a standard.

Western Blot : each sample (40 µg cellular protein extracts or 20 µg culture cell medium) in 10% glycerol, 75 µM Tris pH 6.8, 1% SDS, 0.005% pyronine was boiled 2 min. The 9% polyacrylamide gel was run at 8 mA for 16 h. Proteins were then transferred to nitrocellulose Hybond C (Amersham Biosciences, Orsay, France) by electroblotting (3 mA/cm^2, 1h30). The blot was incubated 3 hours in PBS-0.05%Tween20 with 1% polyvinyl pyrrolidone (Sigma-Aldrich, Saint Quentin Fallavier, France) and then overnight at 4°C in the same solution with either a mouse monoclonal anti-human IGFBP-1 (Mediagnost, Reutlingen, Germany) or a mouse anti alpha-fetoprotein (tebu-bio, Le Perray en Yvelines, France) or a

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goat polyclonal BiP antibody (tebu-bio). After six washes of 5 minutes with PBS-0.05% Tween20, the blot was incubated 30 min at 37°C with the specific anti-IgG coupled to horseradish peroxidase (DAKO A/S, Denmark). After six additional washes, the peroxidase activity was measured with the enhanced chemiluminescence kit (Amersham Biosciences).

Cloning of IGFBP-1 promoter and plasmid contruction : the first 1205 bp fragment of the human IGFBP-1 promoter had already been cloned and sequenced (Suwanichkul et al., 1990). Comparison of this sequence with the GenBank database (Blast) allowed us to identify BAC #RP11-132L11 (GenBank accession number AC091524) which contains 7400 bp upstream of the transcription initiation site of the human IGFBP-1 gene. Using this sequence, we designed oligonucleotides (Primer3 input software, www.broad.mit.edu/cgi-bin/primer/primer3_www.cgi/) which allowed the amplification of various fragments of the promoter. Nucleotide numbering represents the distance 5' (negative) or 3' (positive) to the mRNA capsite (nucleotide 1).

OL-2644 : 5'-TTGCAAGCTGAGGAAGTCAG-3'

OL-1618: 5'-AAGGATGCCCCAGGATTTAT-3'

OL-1111 : 5'-TCTCCAGTTTCCCAGTTTGG-3'

OL-683 : 5'-GGATTCTGGCTCCACTGCTA-3'

OL-550 : 5' - TTGTTTTGTTGCAGGTT 3'

OL-130 : 5'-TGGGTGCACTAGCAAAACAA-3'

OL+102: 5'-CCAAACTCTGGGCAAGTGAT -3'

Different PCR fragments have been generated; they have different upstream ends in the IGFBP-1 promoter (-2644 down to -58) and the same downstream end at +102. PCR reactions were performed with the HotStar Taq DNA polymerase (Qiagen) using HepG2 genomic DNA as a matrix. PCR amplified products were purified using the Qiaquick PCR purification kit from Qiagen and each PCR fragment was inserted into pCR® II-TOPO using

the TOPO-TA® kit (Invitrogen) according to the manufacturer's instructions. Digestion by KpnI and XhoI released the promoter fragments which were subsequently gel-purified and subcloned into the KpnI-XhoI digested pGL3 Basic vector (Promega, Charbonnieres, France). The resulting p-2644-FL, p-1618-FL, p-1111-FL, p-683-FL, p-550-FL, p-130-FL plasmids were all sequenced in the promoter region. The sequence corresponds to the one present in the GeneBank database (GenBank accession number AC091524).

Site directed mutagenesis. The human IGFBP-1 promoter was mutated between nt -87 and nt -80 (which corresponds to the XRE) by a two step PCR which gave rise to p-130mutXRE-FL. The mutation was generated using four oligonucleotides : OL-130, OL+102, OLmut1 :5'-AGCTCCTA**CTACTT**GGCGC-3',
OLmut2 :5'GCGCC**AAGTAG**TAGGAGCT-3'. The PCR-amplified products OL-130/OLmut2 and OL+102/OLmut1 were purified and a second PCR reaction was carried out with the two PCR products, OL-130 and OL+102. This amplification yielded the product - 130mutXRE which was inserted into pCR® II-TOPO and subsequently into PGL3 basic vector by KpnI-XhoI digestion.

Transient transfection studies : HepG2 cells (4.10^5 cells/well of 6 wells plate) were transfected in triplicate (1µg plasmid-FL/ well) with the calcium phosphate co-precipitation technique except that the glycerol shock was omitted. When insulin studies were performed, sixteen hours after transfection, cells were refed with serum free media plus 1 g/liter fatty acid-free bovine serum albumin (Sigma-Aldrich) with or w/o 10^{-7} M insulin (Sigma-Aldrich). After a 24 h incubation, cells were washed and homogenized for enzymatic assays in 200 µl of PLB 1X buffer (Promega). Firefly luciferase was assayed with the Promega kit according to the manufacturer's instructions.

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Electrophoretic Mobility Shift Assay **:** Eight millions HepG2 cells were treated for 90 minutes with 25 nM TCDD. Nuclear extracts were prepared as previously described (Gouedard et al., 2004).

Synthetic double stranded DNA probes (4 pg) were labeled with $\alpha[^{32}P]$dCTP (Amersham Biosciences) and the large Klenow fragment of DNA Polymerase I (Ozyme, Saint Quentin en Yvelines, France). Ten micrograms nuclear extracts, 50 fentomoles labeled probe (200,000 cpm), 50 fold excess of non-labeled oligonucleotides, 10 micrograms antibodies were used. The binding reactions were performed for 15 minutes at room temperature in 10 mM Hepes pH 7.9, 0.1 mM EDTA, 50 mM KCl, 10% glycerol, 2 mM DTT, 1 µg of polydI-dC (Amersham Biosciences) and 500 ng of salmon testis DNA (Sigma-Aldrich). DNA-protein complexes were separated on a 6% (w/v) polyacrylamide gel containing 2.5% glycerol, with 1X tris borate EDTA as a running buffer. DNA-proteins complexes were detected and quantified with the NIH Image 1.62 quantification software.

Several double stranded DNA sequences were used in this study: XRE-IGFBP-1: -95/-74 sequence of human IGFBP-1 gene region (TCAGCTCCTAGCGTGCGGCG), mutXRE-IGFBP-1 (TCAGCTCCTA**CTACTT**GGCG) and a consensus XRE from the CYP1A1 gene (GAGGCCTCGCGTGACTGCGAG). The monoclonal AhR antibody clone RPT9 was obtained from Affinity Bioreagents (Golden USA).

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Results

TCDD induces IGFBP-1 mRNAs in cultured human hepatocytes.

Primary cultures of human hepatocytes were treated or not for 24 h with 25 nM TCDD and the mRNAs for IGFBP-1, actin and two endoplasmic reticulum resident proteins, BiP and GRP 94 were analyzed by Northern blot. Figure 1A shows that TCDD increased IGFBP-1 mRNAs 2 to 3-fold but had no effect on the other mRNAs tested. This semi-quantitative observation was confirmed by quantitative RT-PCR since TCDD was shown to increase IGFBP-1 mRNA 2.6-fold (figure 1B). We then asked whether other components of the IGF/ IGFBP system were also regulated by TCDD. Quantitative RT-PCR showed that IGF-I, IGF-II and the IGF receptor type I mRNAs were not modified. Similarly, neither other IGFBP proteins expressed by hepatocytes nor the acid-labile subunit protein (which interacts with IGFBP-3 and IGFBP-5 to form stable ternary complex with IGFs in serum) were induced. Thus, the induction of IGFBP-1 by TCDD in hepatocytes appears to be specific. This conclusion was supported by the absence of regulation of another secreted protein, alpha-fetoprotein, the endoplasmic reticulum resident protein BiP and the stress sensor protein heat shock protein 70. As a positive control of TCDD action, we analysed the expression of two members of the "Ah gene battery": NAD(P)H : quinone oxidoreductase 1 (NQO1) and cytochrome P450 1A1 (CYP1A1). As expected, TCDD induced NQO1 mRNA about 2-fold (figure 1B) and the CYP1A1 gene was potently induced (basal level undetectable, data not shown).

TCDD induces IGFBP-1 mRNA and protein in HepG2 cells.

The effect of TCDD on IGFBP-1 mRNA was tested in the human hepatocarcinoma HepG2 cells. As shown in figure 2A, TCDD elicited an 8-fold increase in IGFBP-1 mRNA. BiP and alpha-fetoprotein mRNAs were not regulated, while, as previously described, the

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NQO1 mRNA was increased 2-fold (Marchand et al., 2004). The data indicate that the regulation of IGFBP-1 by TCDD and its specificity are similar in human hepatocytes and HepG2 cells, albeit the magnitude of the effect was stronger in the latter cells.

The regulation of the IGFBP-1 protein was assessed by Western blot. Figure 2B shows that the amount of the IGFBP-1 protein was increased in cellular extracts of HepG2 cells treated for 24 h or 32 h with TCDD (3- to 4-fold). We also followed the kinetics of IGFBP-1 secretion in the medium in the presence or absence of TCDD. The protein started to accumulate in the medium following 16 h of treatment and continued to accumulate up to 32 h. TCDD increased the amount of secreted IGFBP-1 several-fold at all time-points, reflecting its effect on the intra-cellular protein. Neither the amount of alpha-fetoprotein and BiP proteins in the cellular extracts nor the secretion of alpha-fetoprotein were modified by TCDD treatment.

Regulation of the human IGFBP-1 gene promoter

Several fragments of the human IGFBP1 gene promoter were amplified by PCR, sequenced and subcloned upstream a Firefly luciferase reporter gene in the pGL3-Basic vector (see Material and Methods). These fragments had different upstream ends in the IGFBP-1 promoter (-2644 down to -58, +1 being the transcription start site) and the same downstream end at +102. HepG2 cells were transiently transfected with these recombinant plasmids, treated or not with TCDD for 24 h, and luciferase activity was assayed. As shown in figure 3, the promoter activity of the construct comprising the (-2644/ +102) fragment was activated 5-fold by TCDD. All promoter fragments extending upstream of position -130 displayed a similar induction by TCDD (average 4- to 5-fold). Deletion of the -130/-59 region led to a dramatic decrease of promoter regulation by TCDD. Thus, the -130/-59 region of the IGFBP-1 promoter comprises the TCDD target sequence.

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Analysis of the -130/-59 region revealed the presence of a sequence (-87/-80) 5'-TAGCGTGC-3' that perfectly corresponds to the consensus xenobiotic responsive element (XRE 5'-TNGCGTGC-3' (Mimura and Fujii-Kuriyama, 2003)) (figure 4A). In order to establish the functional role of the IGFBP-1 (-87/-80) XRE, the sequence was mutated within the p-130-FL construct (p-130 mutXRE-FL). As shown in figure 4B, mutation of the (-87/-80) XRE led to a dramatic decrease in TCDD regulation of the IGFBP-1 promoter. These data indicate that the (-87/-80) XRE is the functional target of TCDD within the IGFBP-1 promoter.

Binding of the AhR to the IGFBP-1 XRE

In order to confirm the role of the IGFBP-1 XRE, electrophoretic mobility shift assays were carried out using nuclear extracts from TCDD-treated HepG2 cells. The -95/-74 sequence of the IGFBP-1 promoter (XRE-IGFBP-1 oligonucleotide) was labeled and incubated with nuclear extracts. A DNA-protein complex was observed under these conditions (figure 5A, lane 1). This complex was competed by a 50-fold excess of cold probe (lane 2), but not by the mutated mutXRE-IGFBP-1 oligonucleotide (lane 4). Furthermore, it was fully competed by an oligonucleotide containing the CYP1A1 XRE, known to bind the AhR (lane 3). A significant decrease in the complex formation was observed when the extracts were incubated with an anti-AhR antibody (lane 6) as compared to a control IgG (lane 5, figure 5B). Quantification of the data from three independent experiments confirmed these observations (figure 5C). We conclude that the AhR is able to bind the IGFBP-1 XRE and to mediate the regulation of the promoter by TCDD.

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Interaction between insulin and TCDD regulation

The identified XRE is located in the hormonal regulatory region of the IGFBP-1 promoter. Indeed, IGFBP-1 is known to be positively regulated by cAMP and glucocorticoids and negatively by insulin. The cAMP responsive region is located upstream of -130 and is therefore distinct from the TCDD responsive region. The -130/-59 region includes the two insulin responsive sequences (IRS A, IRS B) as well as one glucocorticoid responsive element (GRE 2) (Suwanichkul et al., 1994) (figure 6A). The functional IGFBP-1 XRE sequence is adjacent to the GRE 2 and is separated from IRS B by only 14 bp. We hypothesized that these sequences may be part of a composite regulatory region and we studied the interaction between insulin and TCDD effects.

Quantitative RT-PCR experiments using nuclear RNA from HepG2 cells treated with either insulin, dioxin or both for 24 hours were performed to study the regulation of the endogenous IGFBP-1 gene. As shown in figure 6B, insulin inhibited by 60% nuclear IGFBP-1 mRNA, whereas TCDD increased IGFBP-1 mRNA 7- to 8- fold. Moreover, in presence of both insulin and TCDD, a 2- to 3-fold induction of IGFBP-1 mRNA is observed. These results indicate that TCDD exposure increases IGFBP-1 expression even in presence of insulin.

To confirm that TCDD and insulin both act at the transcriptionnal level, HepG2 cells were transfected with the p-130-FL vector which includes the -130/-59 region of IGFBP-1 promoter (see figure 6C). The regulation of the promoter activity was similar to that of the endogenous IGFBP-1 mRNA levels. Indeed, insulin inhibited the basal promoter activity by almost 50% whereas TCDD induced a 6-fold increase in luciferase activity. When insulin and TCDD were combined, a resulting 3-fold increase of promoter activity was observed. These

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data show that the regulation of IGFBP-1 mRNA by dioxin and insulin is mainly mediated by the -130/-59 promoter region.

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Discussion

Until recently, TCDD as well as dioxin-like compounds were believed to regulate primarily a subset of genes encoding drug-metabolizing enzymes called the "Ah gene battery" (Nebert et al., 2000). The induction of these genes was shown to be mediated by the AhR and corresponds to the adaptative response to this class of pollutants (Whitlock, 1999). Although the beneficial effects of xenobiotic metabolizing enzymes were well established, these enzymes can also induce some toxicity through the generation of toxic metabolites, oxidative and other cellular stresses (Marchand et al., 2004; Nebert et al., 2000). However, it is unlikely that these genes could be responsible for all the observed toxicity of dioxins (apoptosis, cellular proliferation or inflammation…). Studies on individual genes as well as more recent large scale gene expression studies revealed that a number of other genes was regulated by dioxin-like compounds and, in some cases, the AhR was shown to be involved (Frueh et al., 2001; Puga et al., 2000; Thomsen et al., 2004); these novel gene targets may be implicated in the toxic effects of TCDD or related compounds.

Several studies have shown that TCDD is an endocrine disruptor. Most of them focused on the interaction between TCDD and estrogen signaling (Ohtake et al., 2003). However, because of the epidemiological correlation between diabetes and TCDD contamination (Remillard and Bunce, 2002), the possibility that dioxins could counteract insulin effects was investigated. We hypothesized that among the variety of genes that are targeted by TCDD, some may lead to the disruption of the insulin effects. We show here that TCDD induces IGFBP-1 gene expression in human hepatocytes and HepG2 hepatoma cell line, which in turn results in increased IGFBP-1 production and secretion. We noticed that IGFBP-1 gene induction by TCDD was lower in hepatocytes than HepG2 cells (2.5-fold versus 8-fold). The presence of insulin in the culture medium of hepatocytes (10^{-6} M) is probably responsible for the lower effect of TCDD since insulin inhibits IGFBP-1 expression.

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Increased serum IGFBP-1 has been shown to elicit metabolic effects that are opposite to insulin action, probably through the sequestration of IGFs (Lee et al., 1997; Murphy, 2003). Over-expression of IGFBP-1 in transgenic mice has provided additional insights into the physiological role of IGFBP-1 in glucose metabolism: in most strains, an attenuated hypoglycemic response to exogenous IGF-I has been observed. Transgenic mice expressing high levels of the rat IGFBP-1 gene are hyperinsulinemic in the first week of life and gradually develop fasting hyperglycemia (Rajkumar et al., 1996). Mice expressing the human IGFBP-1 gene under the control of its own promoter exhibit fasting hyperglycemia and hyperinsulinemia as well as glucose intolerance in later life (Crossey et al., 2000). Thus, induction of IGFBP-1 could represent one of the mechanisms accounting for the metabolic effects of TCDD related to insulin resistance. Our recent experiments using mice treated with 3-methyl cholanthrene (another AhR ligand) revealed an induction of IGFBP-1 mRNA content in the liver (3-fold, data not shown). This indicates that upregulation of IGFBP-1 gene expression by AhR ligands is not restricted to human, but is also observed in other species.

The IGFBP-1 gene is a well known target of insulin which downregulates its expression while TCDD is a potent inducer. We found that, in HepG2 cells, the effect of insulin (10^{-7} M) is not dominant over that of TCDD (25 nM); indeed, the IGFBP-1 promoter activity in the presence of both effectors is intermediate between the activities observed with each compound alone. Moreover, since human hepatocytes are cultured in presence of 10^{-6}M insulin, a TCDD treatment of these cells mimics the HepG2 treatment with both compounds. Thus, a TCDD-upregulation of IGFBP-1 expression is still observed even in the presence of insulin. This could produce, *in vivo*, a persistant overexpression of secreted IGFBP-1 which may contribute to the metabolic disruption of glucose regulation.

In addition to its metabolic effects, IGFBP-1 has been shown to modulate cell growth and migration (Firth and Baxter, 2002). Increased IGFBP-1 affects fertility in females

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and both ante- and post-natal development (Froment et al., 2002; Gay et al., 1997); IGFBP-1 is also a secreted product of decidual endometrium and a major constituant of amniotic fluid. Interestingly, decidual IGFBP-1 overexpression has a marked effect on placental development (Crossey et al., 2002). The role of IGFBP-1 in growth and reproduction suggests that the induction of IGFBP-1 could be involved in the TCDD-elicited developmental toxicity and teratogenicity observed in various animal species (Couture et al., 1990).

The regulation of other members of the IGFBP family by TCDD has also been investigated. IGFBP-2 and IGFBP-4 are not regulated in human hepatocytes treated by TCDD (see figure 1B), IGFBP-3 has been shown to be induced in rat intestin by a polychlorinated biphenyl although the mechanism of this regulation has not been further elucidated (Lee et al., 2000). In recent studies, TCDD has been shown to induce IGFBP-6 mRNA expression in mice lymphoid organs (Park et al., 2001).

The liver specific expression and hormonal regulation of IGFBP-1 gene involves a complex regulatory region of the proximal promoter. Indeed, this region comprises two insulin responsive sequence, a glucocorticoid responsive element and an hepatic nuclear factor-1 binding sequence (Suwanichkul et al., 1994). IGFBP-1 gene was shown to be induced by additional stress conditions such as hypoxia (Tazuke et al., 1998), cytokines and reactive oxygen species (Lang et al., 1999) and by amino acids starvation (Jousse et al., 1998) but the DNA targets of these effectors appear to be either localized outside the proximal hormonal regulatory region (hypoxia responsive elements are located in the first intron of the human IGFBP-1 gene (Tazuke et al., 1998)) or have not been characterized (Jousse et al., 1998; Lang et al., 1999). We showed here, that the proximal hormonal regulatory region also contains a typical XRE which mediates TCDD induction of IGFBP-1. Down regulation of IGFBP-1 gene expression was recently observed in CYP1A2 knockout mice (Smith et al., 2003). Since CYP1A2 is a dioxin inducible gene, this suggests that, in addition to the direct

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AhR-dependent induction of the IGFBP-1 gene described in our study, an indirect mechanism involving the inducible CYP1A2 gene could also be implicated. In conclusion, this study has shown that, in addition to previously identified hormonal and stress regulations, IGFBP-1 gene expression is sensitive to dioxin exposure. IGFBP-1 gene upregulation by dioxins might be involved in some of the deleterious effects of these compounds.

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Footnotes

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Figure 1 : Specific induction of IGFBP-1 mRNA by TCDD in cultured human hepatocytes.

Human hepatocytes were treated or not with 25 nM TCDD for 24 hours. **A.** Northern Blot analysis. The blot was hybridized with probes coding for IGFBP-1, BiP and GRP94 (two endoplasmic reticulum resident proteins), actin and with 18S ribosomal RNA probe. Following phosphorImager quantification, mRNA levels were normalized to the 18S ribosomal RNA signals and 100% corresponds to the mRNA ratio in untreated cells (control). **B.** Quantitative RT-PCR analysis. Levels of mRNAs encoding IGFBP-1, IGFBP-2 and IGFBP-4 (two other members of the IGFBP family expressed in human hepatocytes), IGF-I, IGF-II, acid-labile subunit (ALS) and IGF receptor type I (IGF-IR) (other components of the IGF/IGFBP system) were studied. The expression of alpha-fetoprotein (alpha-FP, another secreted protein), BiP, heat shock protein 70 (HSP70, a global stress sensor) and NQO1 (a gene induced by TCDD) was also measured. The values were corrected using ribosomal protein L13a as a control gene. Data shown are the means +/- SEM of three quantitative RT-PCR experiments.

Figure 2 : Effect of TCDD on IGFBP-1 mRNA and protein levels in HepG2 cells.

Human hepatocarcinoma HepG2 cells were treated or not with 25 nM TCDD for 24 hours. **A.** Northern Blots were hybridized with IGFBP-1, alpha-fetoprotein (alpha-FP), BiP, NQO1 and with 18S ribosomal RNA probes. Following phosphorImager quantification, IGFBP-1 mRNA levels were normalized to 18S ribosomal RNA levels. 100% corresponds to the mRNA ratio in untreated cells (control). Data shown are the means +/- SEM of four independent experiments. **B.** Kinetics of IGFBP-1 protein production by TCDD. Western Blot analysis of protein extracts and culture medium from HepG2 cells treated or not with 25 nM TCDD for 8 to 32 hours. Samples of medium were recovered every 8 hours. Forty µg (cellular extracts) or

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20 µg (cell medium) proteins were loaded per lane. The blot was hybridized with anti-human IGFBP-1, anti-alpha-fetoprotein and anti-BiP IgG.

Figure 3 : Induction of IGFBP-1 gene expression by TCDD is mediated by the -130/-59 fragment of the promoter. HepG2 cells were transiently transfected with luciferase reporter gene constructs containing different fragments of IGFBP-1 promoter. Sixteen hours after transfection, cells were treated or not with 25 nM TCDD for 24 hours and then luciferase activities were assayed. The fold induction of promoter activity by TCDD are presented. Data shown are the means +/- SEM of three independent experiments.

Figure 4 : Mutational analysis of the XRE site in the IGFBP-1 gene promoter. A. Alignment of different XRE sequences : wild type IGFBP-1 XRE, mutated IGFBP-1 XRE and consensus XRE. **B.** HepG2 cells were transfected with the native p-130-FL or the mutated XRE vector, p-130mutXRE-FL. Sixteen hours after transfection, cells were treated or not with 25 nM TCDD for 24 hours and then luciferase activities were assayed. The fold induction of promoter activity by TCDD are presented. Data shown are the means +/- SEM of six independent experiments.

Figure 5 : Ah receptor binding to the IGFBP-1 XRE. Electrophoretic mobility shift assays were performed using a 20-pb ^{32}P-labeled oligonucleotide containing the (-87/-80) XRE site of the IGFBP-1 gene and 10 µg of HepG2 nuclear extracts prepared after TCDD treatment (25 nM TCDD, 90 min). **A.** Competitions experiments were performed in the presence of a 50-fold excess of unlabeled IGFBP-1-XRE, CYP1A1-XRE or XREmut-IGFBP-1 oligonucleotides. **B.** Binding reactions were carried out in the presence of antibody directed toward the Ah receptor (anti-AhR) ; control experiments were conducted with a rabbit IgG. **C.**

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Quantification of the signals. Signals of free probes and bound probes were quantitated using a phophorImager. For each DNA-protein complexe, the ratio of the complex to the corresponding probe was calculated. Data are expressed as percent of the ratio obtained in the control sample (absence of added unlabeled oligonucleotide or antibodies). The average values +/- SEM of three independent experiments are shown.

Figure 6 : TCDD and insulin regulation of IGFBP-1 expression.

A. Schematic representation of the proximal IGFBP-1 promoter. Location of characterized elements : CRE : cAMP response element, GRE 1, GRE 2 : glucocorticoid response elements, IRS A and IRS B : insulin response sequences, HNF1 (hepatic nuclear factor-1) binding site, and XRE. **B.** Quantitative RT-PCR analysis of IGFBP-1 nuclear mRNA. HepG2 cells were treated or not with 25 nM TCDD, 10^{-7} M insulin or both compounds for 24 h in a serum free medium containing 0.1% bovine serum albumin and nuclear RNA were prepared. The values of mRNA levels encoding IGFBP-1 were normalized to ribosomal protein L13a RNA levels. Each bar represents the average of two independent experiments. The values for each experiments are indicated by a dot. **C.** Regulation of IGFBP-1 promoter activity by TCDD and insulin. HepG2 cells were transfected with p-130-FL. Sixteen hours after transfection, cells were refed with a serum free medium containing 0.1% bovine serum albumin and treated for 24 hours. Luciferase activities were assayed; the fold induction over basal activity are presented. 100% corresponds to the luciferase activity in untreated cells (control). Data are the mean +/- SEM of three independent experiments.

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Table 1: sequences of oligonucleotides used for quantitative RT-PCR

NQO1	NQO1(F)	5´-GCAGACCTTGTGATATTCCAG-3´
	NQO1(R)	5´-CCTATGAACACTCGCTCAAAC-3´
BiP	BiP (F)	5'-CTGGGTACATTTGATCTGACTGG-3'
	BiP (R)	5'-TCCTTGAGCTTTTTGTCTTCCT-3'
acid-labile subunit	acid-labile subunit (F)	5'-CTGTGGCTGGAGGGTAAC-3'
	acid-labile subunit (R)	5'-GCAGGGAGCAAAGTGGGC-3'
IGFBP-1	IGFBP-1 (F)	5'-TATGATGGCTCGAAGGCTCT-3'
	IGFBP-1 (R)	5'-TAGACGCACCAGCAGAGTC-3'
IGFBP-2	IGFBP-2 (F)	5'-CTGCGGCTGCTGCTCGGT-3'
	IGFBP-2 (R)	5'-CCTCCTTCTGAGTGGTCAT-3'
IGFBP-4	IGFBP-4 (F)	5'-AGTGGGGGCAAGATGAAG-3'
	IGFBP-4 (R)	5'-GGGGATGGGGATGAAGTAG-3'
IGF receptor type I	IGF receptor type I (F)	5´-GTGTGTGGTGGGGAGAAAG-3´
	IGF receptor type I (R)	5´-GGGTCGGTGATGTTGTAGG-3´
insulin-like growth factor I	IGF-I (F)	5'-GCTCTTCAGTTCGTGTGTG- 3'
	IGF-I (R)	5'-GACTTGGCAGGCTTGAGG -3'
insulin-like growth factor II	IGF-II (F)	5´-TCGTTGAGGAGTGCTGTTTC-3´
	IGF-II (R)	5´-GGGTATCTGGGGAAGTTGTC-3´
alpha-fetoprotein	alpha-fetoprotein (F)	5'-CGAACTTTCCAAGCCATAAC-3'
	alpha-fetoprotein (R)	5'-CAGACAATCCAGCACATCTC-3'
CYP1A1	CYP1A1(F)	5´-GACCACAACCACCAAGAAC-3´
	CYP1A1(R)	5´-AGCGAAGAATAGGGATGAAG-3´
heat shock protein 70 kDa	heat shock protein 70 (F)	5'-AGGTGCAGGTGAGCTACAAGG-3'
	heat shock protein 70 (R)	5'-GGTCAGCACCATGGACGAG-3'
ribosomal protein L13 a	ribosomal protein L13a (F)	5'-CCTGGAGGAGAAGAGGAAAGAGA-3'
	ribosomal protein L13a (R)	5'-GAGGACCTCTGTGTATTTGTCAA-3'

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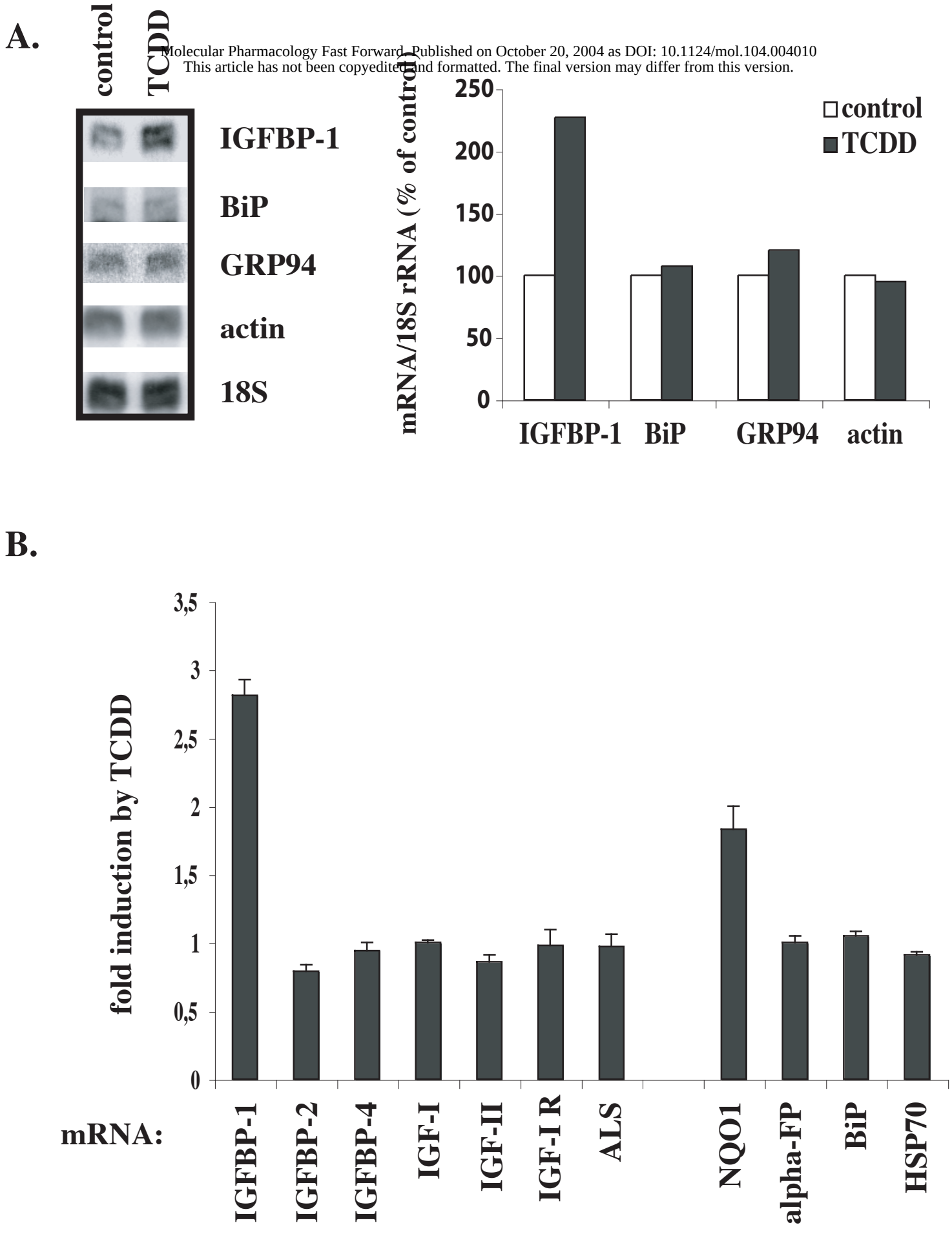

Figure 1

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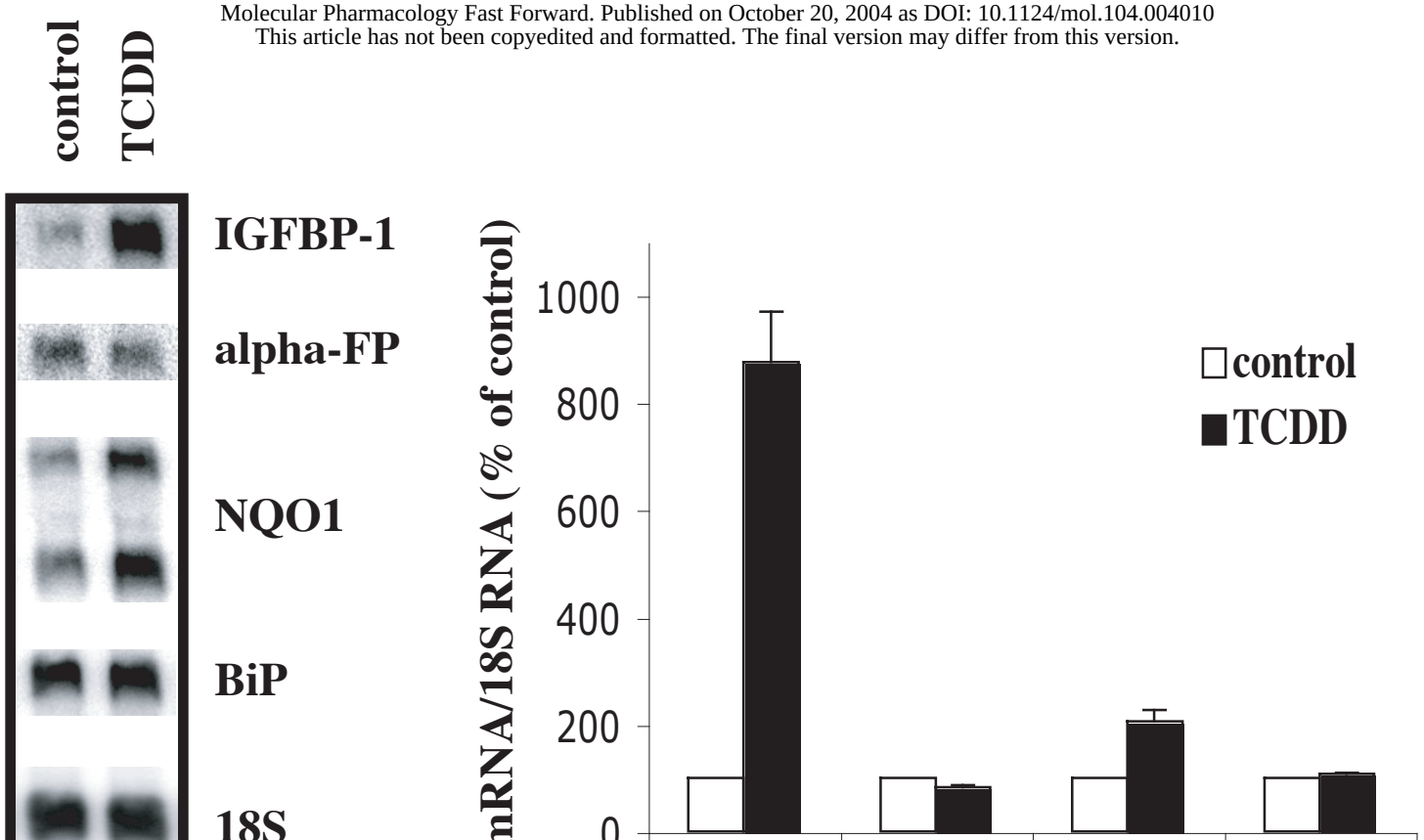

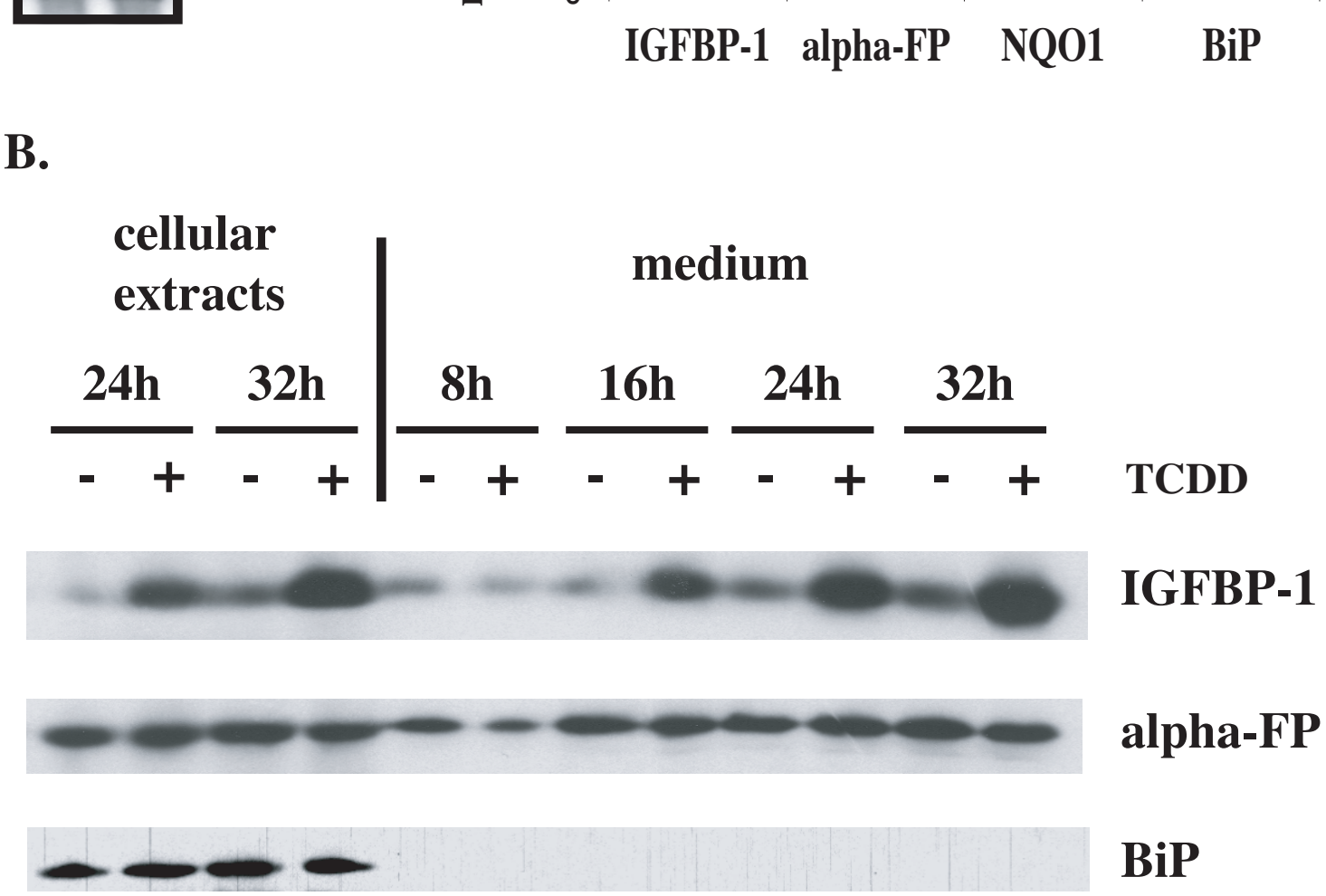

Figure 2

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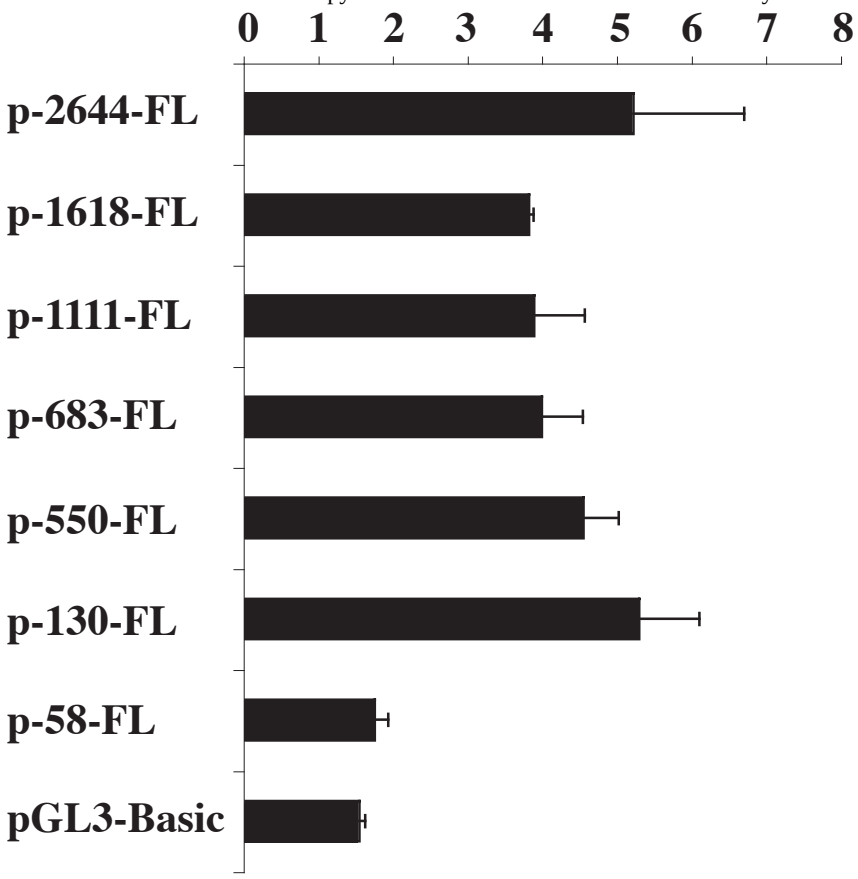

Figure 3

A.

```
consensus XRE :      TNGCGTGC
                     | | | | | | | |
IGFBP-1(-87/-80) XRE: TAGCGTGC
                     | |
IGFBP-1 mutXRE:      TACTACTT
```

B.

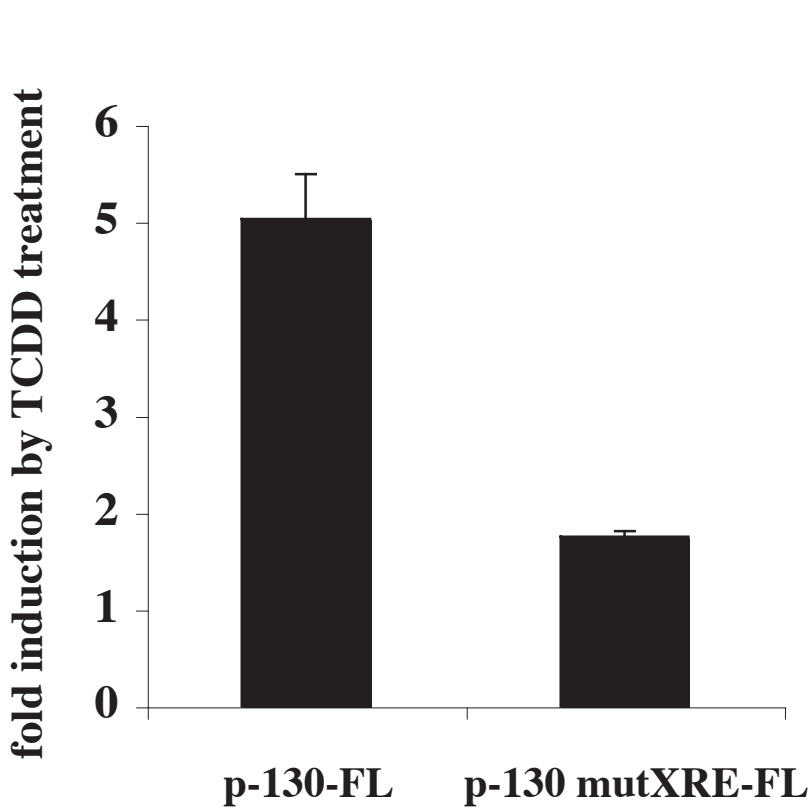

Figure 4

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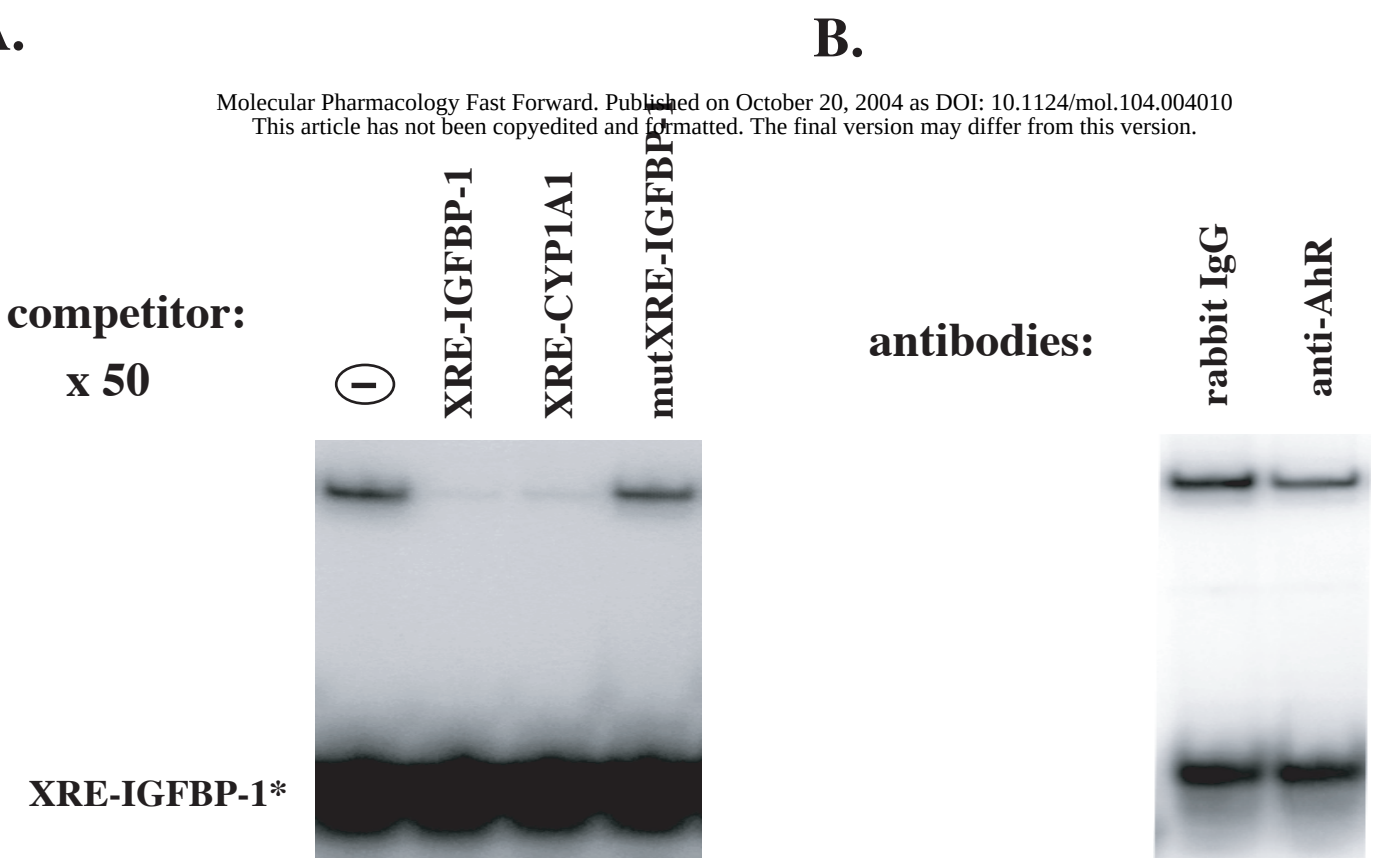


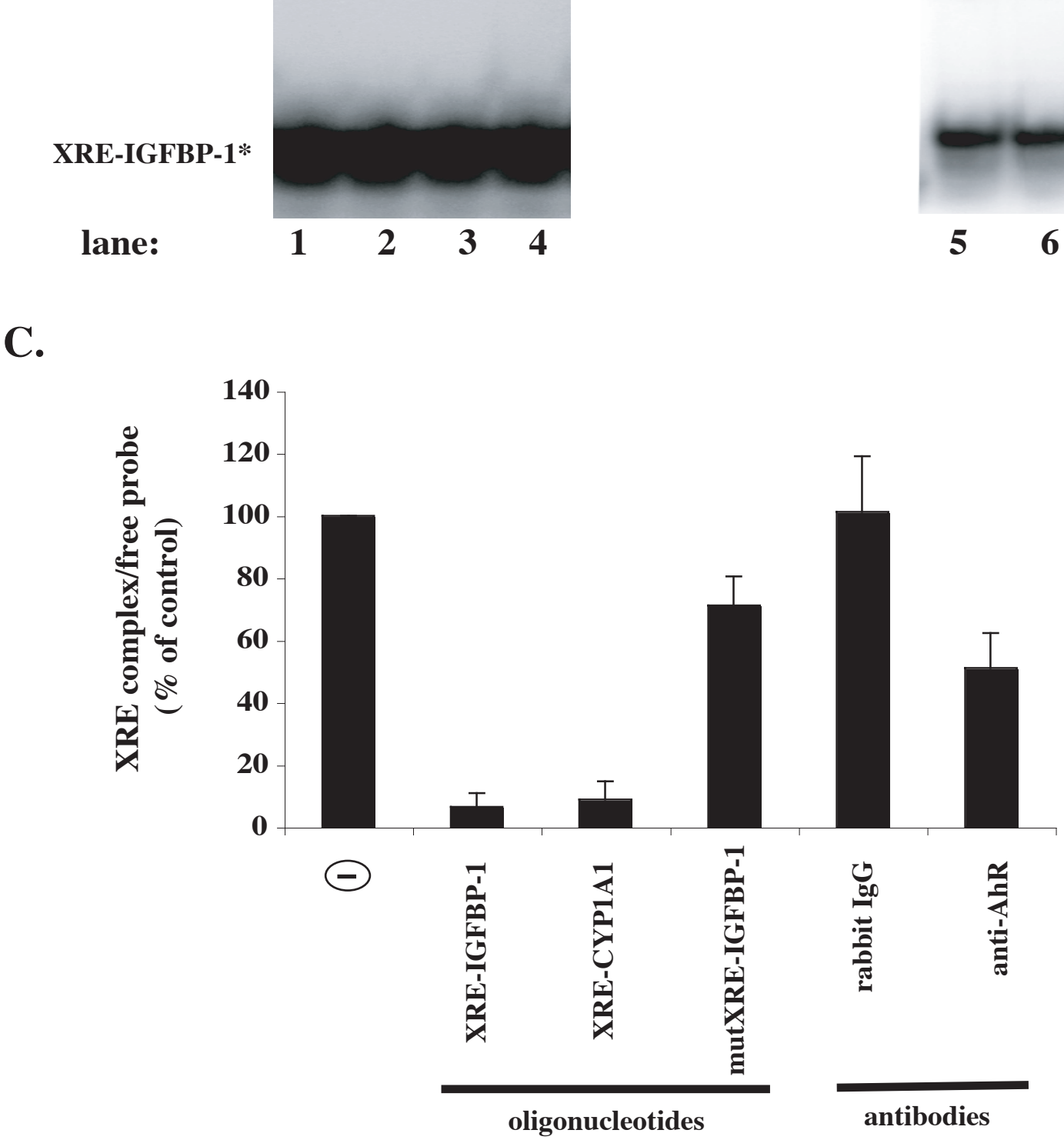
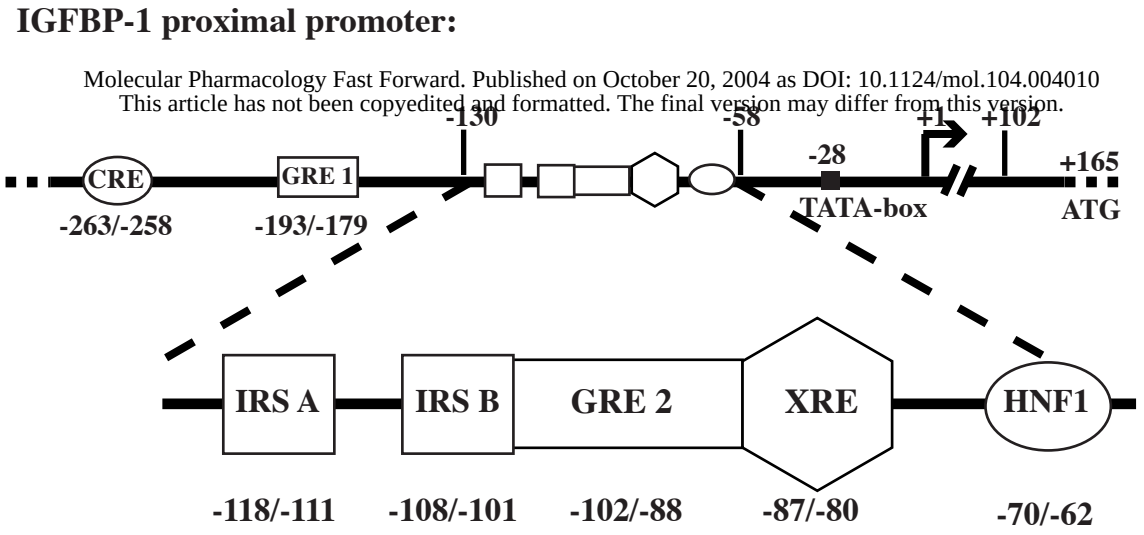


Figure 5

A.



B.

C.

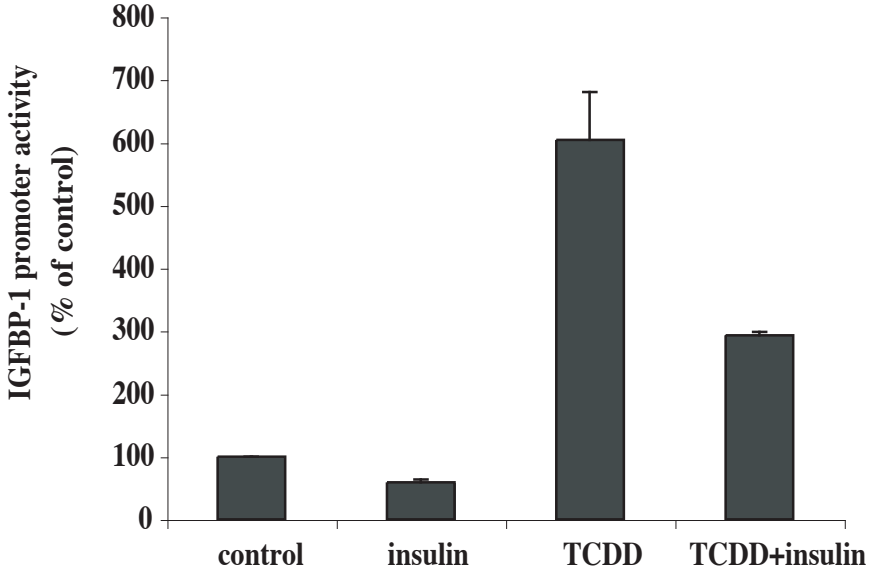


Figure 6