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Sex-linked regulation of CYP1A2 in adult human cultured hepatocytes

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Abstract

CYP1A2 belongs to cytochrome P450 super family and mainly expressed in liver. C*YP1A2* contributes 11% of total drug metabolism. *CYP1A2* enzyme is involved in an NADPH-dependent electron transport chain. *CYP1A2* has been shown to metabolize nutrients, antibiotics (ciprofloxacin) and flutamide. Impacts of hormones and gender on the regulation of *CYP1A2* have not been well documented. Current study reports that *CYP1A2* expression is diminished in NF-1 and USF-1 deficient HepG2 cells and further NF-1 and USF-1 regulation is mediated through growth hormone and dexamethasone. GH and dexamethasone controlling the regulation and recruitment of NF-1 and USF-1 on *CYP1A2* proximal promoter in a gender-dependent manner in cultured hepatocytes. Additionally, DNA/Protein array identified that NF-1 and USF-1 regulation and nuclear translocation was higher in cultured female adult human hepatocytes and this process was potentiated through a combined action of growth hormone and dexamethasone. Alternatively, ChIP assay demonstrates that the nuclear translocated and *CYP1A2* promoter bound NF-1 and USF-1 was abundant in cultured human female hepatocytes in response to GH and dexamethasone regime. The expression of *CYP1A2* mRNA and protein correlated with the status of NF-1 and USF-1 in sex-dependent fashion and in response to GH and dexamethasone. Results from the study conclude that *CYP1A2* regulation is female dominant and controlled through growth hormone and dexamethasone. This article summarizes the molecular impact of sex and hormones on regulation of human *CYP1A2*. The understanding of sex-dependent regulation of CYPs may help to develop P450 mediated therapies for men and women in clinics.

Abbreviations

ChIP, chromatin immunoprecipitation; CYP, cytochrome P450; DMEM, Dulbeccos's modified Eagle's medium; NF-1, nuclear factor-1; GH, growth hormone; USF-1, upstream stimulatory factor; AP-1, activator protein 1; AP-2, activator protein 2; PVDF, polyvinylidene diflouride; rhGH, recombinant human growth hormone; Dex, dexamethasone; siRNA, small inhibitory RNA.

Introduction

The human genome encodes fifty-seven cytochrome P450s [1]. Cytochrome P450 (CYP) enzymes are the key regulators in the phase I-dependent metabolism of drugs and other xenobiotics, mostly catalyzing oxidations of the substrate, but occasionally also involved in reduction reactions [2,3]. GH secretary patterns are sexually dimorphic and are episodic in men and more continuous in women [4,5]. GH is the major determinant of human CYP regulation [6-8]. Sex-dependent and GH regulated CYPs were very well investigated in rodents models and humans [7,9-19]. Reports strongly suggest that sex dependent and continuous GH induced highest level of *CYP3A4* in human primary hepatocytes [7,10]. Similar inherent sexually dimorphic regulation in humans by the male-dependent GH pattern have been reported for IGF-1, growth and GH binding protein; male>female [20-24]. *CYP1A2* is a phase I enzyme and ranks 3rd in total content among all other human hepatic CYPs [25,26].

CYP1A2 is located on human chromosome 15q24.1 and comprising of seven introns and six exons and mainly expressed in the liver, and can be induced via the aryl hydrocarbon receptor (AhR), similarly to *CYP1A1* [27,28]. *CYP1A2* has been shown to metabolize nutrients, antibiotics (ciprofloxacin) and flutamide [29,30]. Another known substrate for *CYP1A2* is caffeine; individuals who carry one or more *CYP1A2**1C alleles are "slow" caffeine metabolizers, whereas carriers of the variant *CYP1A2**1F are "fast" caffeine metabolizers. The same amount of caffeine will therefore tend to have more stimulatory effects on *CYP1A2* slow metabolizers than on *CYP1A2* fast metabolizers. *CYP1A2* has several alleles, which includes *CYP1A2**1, *CYP1A2**1C, *CYP1A2**1D, *CYP1A2**1F, *CYP1A2**1K_-729C>T, *CYP1A2**1K_-739T>G, *CYP1A2**2_63C>G, *CYP1A2**3, *CYP1A2**4, *CYP1A2**6, *CYP1A2**7_3534G>A, *CYP1A2**8, *CYP1A2**11, *CYP1A2**15, *CYP1A2**16 and *CYP1A2*_1545T>C. Common polymorphisms with important functional effects in *CYP1A2* activity have not been identified and only a couple of very rare genetic variants, *CYP1A2**7 and *CYP1A2**11, have been studied.

GH deficiency leads to reduced activities of *CYP1A2* in children and this observation indicates that GH plays a vital role in the regulation of *CYP1A2* [31]. Sex dependent regulation of *CYP1A2* in humans and pigs has been reported, however the molecular mechanism of sex dependent and GH mediated regulation of *CYP1A2* is poorly studied [7,32,33]. Here we cultured primary hepatocytes from men and women under a continuous (feminine) GH regime to elucidate the possible molecular mechanisms associated with the regulation of *CYP1A2*. In the present study we show that the regulation of NF-1 and USF-

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1 transcription factors and their occupancy on *CYP1A2* upstream sequence are sexually dimorphic (F>M). Additionally, NF-1 and USF-1 deficient HepG2 cells demonstrate that NF-1 and USF-1 are required for transcriptional activation of the *CYP1A2* gene.

Materials and methods

Human Primary Hepatocyte and HepG2 Culture

Hepatocytes were isolated from men and women and plated on rat-tail collagen-coated flasks (T-25) containing Dulbecco's Modified Eagle's Medium (DMEM) and were obtained through the Liver Tissue Procurement and Distribution System (Pittsburgh, PA) [34]. All of the samples were obtained with donors consent and with approval of the appropriate hospital ethics committee. Male and female donors varied in age from 21 to 56 years. About 80% were Caucasian; the remaining were African American and Hispanic. Alcohol consumption, smoking and drug history as well as causes of death varied between donors. Approximately 50% of livers had some degree of steatosis (5-40%). Approximately 48 hours after isolation and plating, the primary hepatocyte cultures arrived at our laboratory. HepG2 (ATCC, Manassas, VA) cells were cultured in Eagle's Minimum Essential Medium (EMEM). The replacement medium and culture conditions were described previously [7,9,10].

Hormonal treatments

In order to replicate the more continuous feminine-like growth hormone (GH) profile shown to be favored for human CY1A2 expression, the primary hepatocytes were constantly exposed (2 ng/ml) to rhGH purchased from the National Hormone and Peptide Program (Torrance, CA) [7]. Other cells were exposed to Dexamethasone (Dex) (4 ng/ml) alone or to both Dex and GH. The medium was changed every 12 hours. After 5 days in culture, cells were harvested 60 minutes following the final change of media with hormones as previously reported [7,12]. Cells were exposed to one of the following conditions, GH or GH diluents or Dex or Dex diluents. (GH-diluent was 0.01 M sodium bicarbonate and DMSO as dexamethasone diluent.

Preparation of whole cell and nuclear extracts

To isolate total protein for western blots, harvested hepatocytes were centrifuged (800 xg for 10 min) and the resulting cell pellets were re-suspended in lysis buffer [35]. The chromatin was cleared by sonication. Cell lysate was then centrifuged at 12,000 xg for 20 min. The supernatant (whole cell extract) was then removed and stored at -80°C until further analyses. Following, nuclei were isolated as described by a series of centrifugations, re-suspended, homogenized, dialyzed and the nuclear extracts were stored for future analyses. Protein concentrations were determined by Bio-Rad protein assay reagent (Bio-Rad, Hercules, CA) [36].

Bioinformatics analyses

Various bioinformatics tools and programs, including Match-Public (www.gene-regulation.com/cgi-bin/pub/programs/match/match.cgi), AliBaba2 (http://www.generegulation.com/pub/programs/alibaba2/index.html), and Transcription Element Search software, which utilizes the Transcription Factor Database (http://www.cbil.upenn.edu/cgi-bin/tess/tess), were used to identify transcription factor binding sites on the human *CYP1A2* upstream sequence.

NF-1 and USF-1 Knockdown in HepG2 Cells

NF-1 and USF-1 knockdown was carried out as described and following the manufacturer's instructions (Santa Cruz Inc, CA) [37-39]. After 48 hours post transfection of NF-1 siRNA or USF-1 siRNA, the HepG2 cells were treated with 2 ng/ml rhGH (NHPP, Torrance, CA) for 24 hours and medium was replaced every 12 hours [12]. On the 3rd day cells were harvested after 60 minutes following the addition of Dex or GH or combination with Dex and GH. During harvest, HepG2 cells were washed with ice-cold phosphate-buffered saline (PBS). Some of the cells were processed for RNA analysis, ChIP assay and some of the cells were used for nuclear extract preparation. In some experiments, control cells were incubated in the presence of rhGH diluent instead of the hormone or Dex diluent instead of Dex.

RNA analysis

Total RNA was isolated from cultured human hepatocytes or NF-1 or USF-1 proficient or deficient HepG2 cells (NF-1 or US-1 knockdown cells) after hormonal treatments. Five micrograms of total RNA was reverse transcribed with random hexamers. Approximately 25-50 ng of cDNA was applied in real time PCR as described [40,41]. Real time PCR was performed with an ABI Step-One apparatus using Power SYBR Green Master Mix and using *CYP1A2* mRNA forward and reverse primer sequences presented in supplemental table 1. The mRNA signals were normalized with GAPDH mRNA signals (internal control) and quantitated using CT values.

Western blotting analysis

Immunoblotting was performed as described[7,13]. Approximately 25 to 50 μg of total extracts or nuclear extracts from cultured human hepatocytes, NF-1 or USF-1 proficient and deficient HepG2 cells were resolved on 10% SDS-polyacrylamide gel electrophoresis and transferred electrophoretically onto PVDF membranes with a Bio-Rad transfer unit. The membranes were then blocked with 5% non-fat dry milk and incubated with primary antibody raised against recombinant human *CYP1A2* (kindly provided by Dr. F. Peter Guengerich), β-actin, NF-1 and USF-1 (Santa Cruz, Inc CA). The primary antibody was located by using horseradish peroxidase conjugated to anti-rabbit IgG. The blots, incubated with SuperSignal West Femto (Pierce, Rockford, IL), were visualized, captured and quantified by using an Alpha Innotech FluroChem 8800 Imager (Alpha Innotech, San Leandro, CA) with a movie mode. Signals were normalized to a positive control sample, which was repeatedly run on each blot and exhibited a concentration variant between blots of 3.2 to 6.7% for the different proteins. Lastly, blots were stripped and re-probed with loading control p97 or β-actin antibody and found to be comparable to those obtained with internal controls of the assayed samples.

Protein/DNA array hybridization and analysis

To investigate the relative binding of GH and Dex induced transcription factors to their unique consensus DNA sequences in human hepatocytes, we used TransSignal protein/DNA array (Cat # MA1210, Panomics, Redwood City, CA). In comparison to gel shift assays, which identifies few transcription factors at a time, the TransSignal-Protein/DNA array profiles multiple transcription factor-DNA interactions simultaneously. Array analysis was performed as per the manufacturer's instructions using nuclear extracts from cultured male and female cultured human hepatocytes. Nuclear extracts were prepared using the Panomics nuclear extraction kit (Cat # AY2002) as per the manufacturer's instructions. An equal amount of nuclear extracts (10 μg) from cultured male and female hepatocytes were used for hybridization with TransSignal probe mix (10 μl) containing 56 biotin-labeled double-stranded DNA oligonucleotides. The biotin-labeled DNA binding oligonucleotides were preincubated with the

nuclear extracts to allow formation of protein/DNA complexes according to the protocol suggested by the manufacturer of TranSignal Protein/DNA Array System. The protein/DNA complexes were separated from the free probes using a spin column separation system. The bound probes were extracted and hybridized to an array membrane spotted with 56 different consensus-binding sequences following a protocol suggested by the manufacturer. The biotin-labeled oligonucleotides specifically bound to the TFs were extracted and hybridized to the TransSignal array membrane spotted with 56 different consensus-binding sequences for overnight at 42°C. The blots were then washed and incubated with a horseradish peroxidase (HRP)-conjugated streptavidin according to the manufacturer's instructions. The resulting signals were visualized and captured on FluorChem TM IS-8800 Imager by using a movie mode (X-ray film was not used to avoid saturation). The spots were identified with reference provided in the TransSignal Protein/DNA/ Array I kit. The signals were quantitated using FluorChem ™ IS-8800 Imager and the signals were normalized with references provided in the TransSignal Protein/DNA Array I kit. The data were analyzed using Sigma Plot.

Chromatin immunoprecipitation assay (ChIP)

Following the hormonal regimen described above, ChIP assays (*15*) were performed in primary human hepatocytes as well as HepG2 cells (American Type Culture Collections, HB-8065, Manassa, VA) according to our previously described procedure [13,14]. Nuclei were lysed and the purified nuclei were sonicated to generate DNA fragments with an average length of 200 to 500 bp. Equal concentrations of chromatin from all treatment groups were pre-cleared with protein A agarose beads in the presence of bovine serum albumin and sonicated salmon sperm DNA to reduce the background. After removal of beads by centrifugation, 2 µg of NF-1 or USF-1 specific antibody (Santa Cruz Biotechnology) was added and incubated at 4°C for overnight on a rotary platform. The immunoprecipitates were washed sequentially, eluted and prepared as previously reported [13]. Immunoprecipitated DNA was purified using a PCR purification kit (Qiagen, Valencia, CA) and re-suspended in 50 µl of sterile water. The immunoprecipitated and input DNA were analyzed by semi-quantitative PCR using forward and reverse primers of the *CYP1A2* upstream sequence made from the *CYP1A2* gene sequence [42-44] (Supplemental Table 1). PCR products resolved on agarose gels were quantified using FluroChem IS-8800 Imager.

Southern blotting analysis

The conventional Southern blotting procedure was applied to confirm the USF-1 and NF-1 binding sites within the ChIP PCR products. Southern blotting was carried out as described before to confirm the NF-1 and USF-1 binding motif in the ChIP PCR products [13,14]. Following, we synthesized the oligonucleotides encompassing the binding sites on the *CYP1A2* upstream sequence and labeled this oligonucleotide with γ-^{32}P [42] (Supplemental Table 1). The γ-^{32}P labeled radioactive oligonucleotide served as a probe in Southern blot analysis. Meanwhile, the PCR amplified DNA from the ChIP assay was separated by agarose gels and DNA from the agarose gel was transferred to a nylon membrane; the membrane was baked at 80°C for 2 hours. After 8 hours of pre-hybridization, the membrane was incubated with denatured single-stranded radiolabeled probe and hybridized for overnight at 42°C. Membranes were washed with specific buffers and the signals were scanned and quantitated using a FluroChem TM IS-8800 Imager. The signals were normalized to positive controls, which were repeatedly run on each blot

Statistics

All data were subject to analysis of variance. Significant differences were determined with *t* statistics and the Bonferroni procedure for multiple comparisons.

Results

Mapping of the transcription factor binding sites on the human *CYP1A2* upstream sequence

1 Kb *CYP1A2* upstream sequence was analyzed using bioinformatics tools to identify the transcription factor binding sites. The bioinformatics tools identified NF-1 and USF-1 (USF-1 close to +1 site and another USF-1 is far from +1 site) putative binding sites along with other transcription factors are presented in the supplemental figure 1. Human CYP1A2 gene consists of seven introns and six exons. NF-1 and USF-1 transcription factors regulate

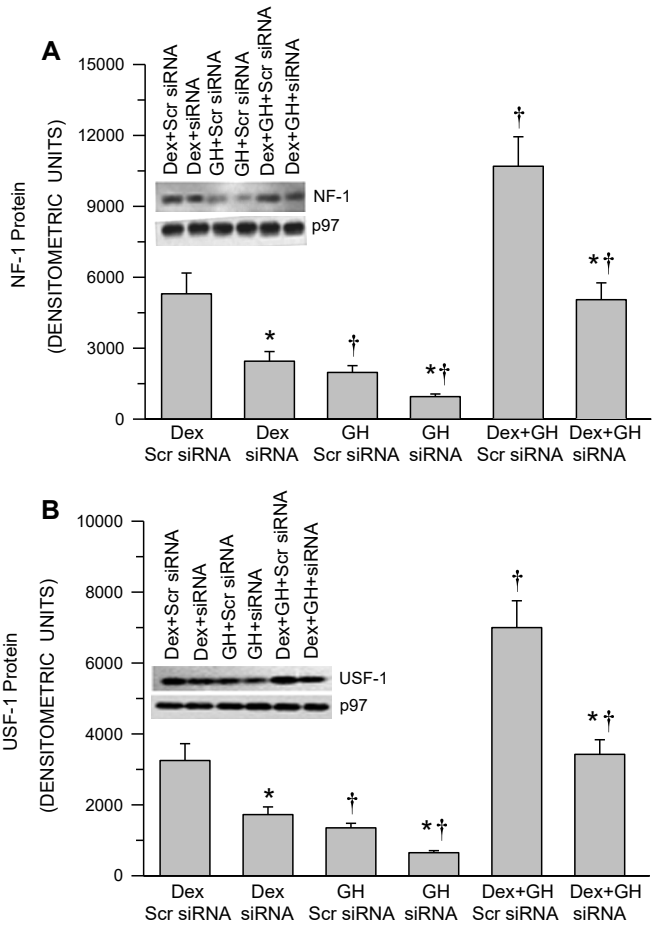

Figure 1. Analysis of hormonal regulation of nuclear NF-1 and USF-1 levels in NF-1 and USF-1 proficient and deficient HepG2 cells. Following 50–70% confluency, HepG2 cells were transfected with either the control non-specific scrambled (Scr) siRNA (proficient) or NF-1 or USF-1 knockdown siRNA (deficient) and 24 hours later the cells were exposed to either continuous dexamethasone alone (Dex) or continuous GH alone (GH) or both hormones (Dex+GH) for 2 days after which the cells were harvested and analyzed. **(A)** A graphic representation of nuclear NF-1 levels and a representative immunoblot of NF-1 and their respective loading controls. **(B)** A graphic representation of nuclear USF-1 levels and a representative immunoblot of USF-1 and their respective loading controls. Positive controls for (NF-1 and USF-1) were repeatedly run on all blots for procedural integrity (not shown). Each data point is a mean ± S.D. from 5 or more independent experiments. *P < 0.01 compared the knockdown siRNA with the control cells exposed to the same hormone treatment. †P < 0.01 compared with dexamethasone alone in cells transfected with the same siRNA.

human *CYP1A2* [43,44]. Accordingly, we examined the effectiveness of siRNA targeted to NF-1 or USF-1 in HepG2 cells. HepG2 cells transfected with nonspecific (scrambled) siRNA in the presence of Dex and GH combination exhibited the highest level of NF-1 and USF-1 expression and their nuclear translocation. Cells transfected with NF-1 siRNA or USF-1 siRNA showed ~50-60% reduction in NF-1 or USF-1 expression and nuclear translocation and responsiveness to Dex and GH (Figure 1A,B). Similarly, HepG2 cells exposed to Dex alone showed a modest increase in the expression and nuclear translocation of NF-1 or USF-1. NF-1 siRNA or USF-1 siRNA knockdown showed both a ~50-60% lesser expression and nuclear NF-1 or USF-1 under Dex condition in the nuclear extracts. HepG2 cells exposed to GH alone showed the smallest increase in expression and nuclear translocation of NF-1 or UF-1, whereas NF-1 siRNA or USF-1 siRNA produced a ~50-60% reduction of expression and nuclear accumulation of NF-1 or USF-1 under GH alone growth condition (Figure 1A,B). In vehicle treated cells, no measurable levels of nuclear NF-1 or USF-1 were observed (data not presented) in nuclear extracts. NF-1 or USF-1 siRNA transfected HepG2 reduced the effectiveness of each hormone treatment to stimulate NF-1 and USF-1 expression and nuclear accumulation by ~ 60%. While this level of knockdown is somewhat modest when compared to the effects of other species of siRNA, it is in agreement with reports specifically using siRNA against NF-1 and USF-1 [38,39,45-47]. In general, exposure of the HepG2 cells to the NF-1 or USF-1 siRNA knockdown reduced the effectiveness of each hormone treatment to stimulate NF-1 or USF-1 nuclear translocation by about 60%.

NF-1 or USF-1 deficiency fails to recruit NF-1 or USF-1 on to the *CYP1A2* upstream sequence

GH regulated expression levels of NF-1 and USF-1 directly correlated with the nuclear translocated NF-1 or USF-1 and occupancy of these transcription factors on the *CYP1A2* upstream sequence. Regardless of the treatment, there was a direct correlation of nuclear levels and the promoter binding levels of NF-1 or USF-1. The greatest binding affinity was observed in Dex and GH combination than other treatment groups (Dex+GH is greater than Dex alone and is greater than GH alone (Figure 2A,3B). NF-1 or USF-1 deficiency leads to weaker recruitment of NF-1 or USF-1 to the CYP1A2 upstream sequence. Further, Southern blotting confirms similar levels of the occupied NF-1 and USF-1 binding motifs of the *CYP1A2* upstream sequence as seen in the ChIP assays (Figure 2B,3B). These results clearly demonstrate the effectiveness of NF-1 siRNA and USF-1 siRNA to reduce promoter binding of the nuclear factors by ~ 60%.

Hormonal regulation of *CYP1A2* in NF-1 or USF-1 proficient and deficient HepG2 Cells

Using NF-1 and USF-1 deficient cells, we sought to correlate NF-1 or USF-1 binding on the *CYP1A2* upstream sequence with expression levels of *CYP1A2* in HepG2 cells under the hormone regimes. CYP1A2 expression was directly correlated with the status of NF-1 or USF-1 in response to DEX and GH as evidenced via CYP1A2 qRT-PCR mRNA analysis (Figure 4A,B), Whereas hormone treatments (Dex combination with GH was higher than (more than two fold) Dex alone or GH alone) stimulated nuclear accumulation and promoter bound NF-1 or USF-1 in NF-1 or USF-1 proficient and deficient HepG2 cells (Figure 1A,B), no measurable NF-1 or USF-1 binding to the *CYP1A2* upstream sequence was observed in control (diluents) treatment by ChIP assay (data not shown). Nevertheless, interference with NF-1 or USF-1 expression by siRNA knockdown repressed the hormone-induced *CYP1A2* mRNA by real time RT-PCR accounts ~60% in

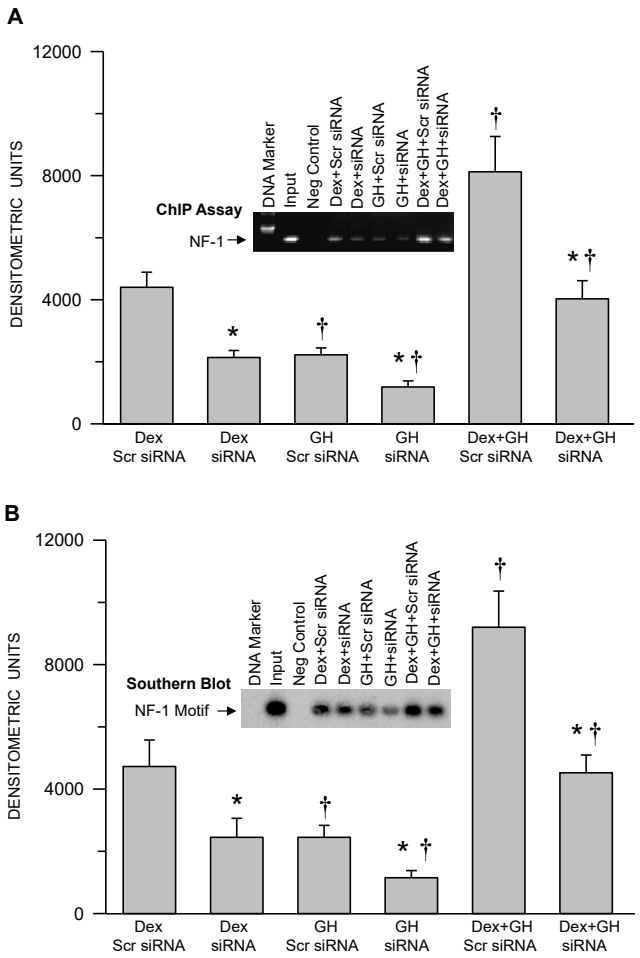

Figure 2. Hormonal regulation of NF-1 binding to the *CYP1A2* upstream sequence and confirmation of the occupied NF-1 binding motif in the *CYP1A2* upstream sequence in NF-1 proficient and deficient HepG2 cells. Following 50–70% confluency, HepG2 cells were transfected with either control non-specific scrambled (Scr) siRNA (proficient) or NF-1 knockdown siRNA (deficient) and 24 hours later the cells were exposed to either continuous dexamethasone alone (Dex) or continuous growth hormone alone (GH) or both hormones (Dex+GH) for 2 days, after which the cells were fixed with formaldehyde, lysed and the chromatin was sheared and processed for further analysis. **(A)** A graphic representation of the ChIP-PCR amplified signal and a representative agarose gel picture of the ChIP assay. **(B)** A graphic representation of the occupied NF-1 binding motif and a representative Southern blot image (insert). Rabbit IgG served as a negative control and input chromatin as a positive control. Each data point is a mean ± S.D. from 5 or more independent experiments. *P < 0.01 compared the effects of the knockdown siRNA with control cells exposed to the same hormone treatment. †P < 0.01 compared with dexamethasone alone in cells transfected with the same siRNA.

HepG2 cells at all hormone regimens (Figure 4A,B). GH alone induced the smallest increase in *CYP1A2* mRNA, whereas Dex was more than twice as effective and the combined hormone treatment was clearly the most effective. The signals were normalized to GH alone.

Sex dependent hormonal regulation of *CYP1A2* in cultured human hepatocytes

Having demonstrated *CYP1A2* regulation is mediated by Dex and GH in HepG2 cells, we wanted to see if the same was true in normal hepatocytes from adult men and women. In agreement with HepG2 cells (Figure 4A,B), hepatic *CYP1A2* mRNA by real time qRT-PCR and protein expression was more than two fold when hepatocytes were exposed to the combined treatment of continuous Dex and GH (Figure 5A,B). GH alone was moderately inductive and dexamethasone itself

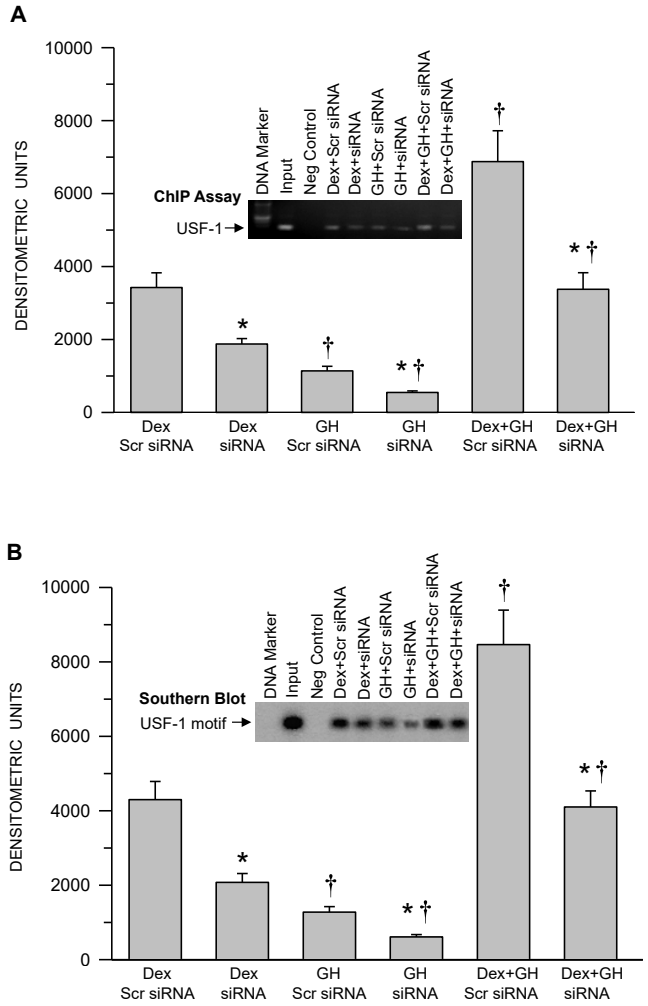

Figure 3. Hormonal regulation of USF-1 binding to the *CYP1A2* upstream sequence and confirmation of the occupied USF-1 binding motif in the *CYP1A2* upstream sequence in USF-1 proficient and deficient HepG2 cells. Following 50–70% confluency, HepG2 cells were transfected with either control non-specific scrambled (Scr) siRNA (proficient) or USF-1 knockdown siRNA (deficient) and 24 hours. Later the cells were exposed to either continuous dexamethasone alone (Dex) or continuous growth hormone alone (GH) or both hormones (Dex+GH) for 2 days after which the cells were fixed with formaldehyde, lysed and the chromatin was sheared and processed for further analysis. **(A)** A graphic representation of ChIP-PCR amplified signal and a representative agarose gel picture of the ChIP assay. **(B)** A graphic representation of the occupied USF-1 binding motif and a representative Southern blot image (insert). Rabbit IgG served as a negative control and the input chromatin as a positive control. Each data point is a mean ± S.D. from 5 or more independent experiments. *P < 0.01 compared the effects of the knockdown siRNA with control cells exposed to the same hormone treatment. †P < 0.01 compared with dexamethasone alone in cells transfected with the same siRNA.

higher than GH alone. Very low *CYP1A2* mRNA levels were measured in vehicle treated men and women hepatocytes, but no measurable CYP1A2 protein levels were observed (data not shown). The signals were normalized to male GH alone. Sex differences in the induction of *CYP1A2* were indicated by significantly greater than two fold in female hepatocytes than male hepatocytes following all hormonal exposures (Figure 5A,B**).** The mRNA and protein expressions correlate with each other and exhibit sex-dependent regulation of CYP1A2**.**

Sex dependent regulation of transcription factor levels and binding to nuclear DNA in human hepatocyte culture

In this experiment we investigated whether the hormonally induced transcription factors are sex dependent for their nuclear accumulation and DNA binding activity. We observed that the nuclear concentrations of DNA associated transcription factors AP-1, AP-2, NF-1 and USF-1 were highest levels in female nuclear extracts compared to males exposed to combined GH and Dex (Figure 6A). The Dex treatment alone was modest and the GH was observed as lowest. In all treatment groups the DNA/protein association levels were observed as men < women. Similarly, we observed the nuclear accumulation of NF-1 and USF-1 was greatest in women hepatocytes compared to men exposed to the combined Dex and GH (Figure 6B,C). Induction and nuclear accumulation of NF-1 and USF-1 was modest in Dex alone

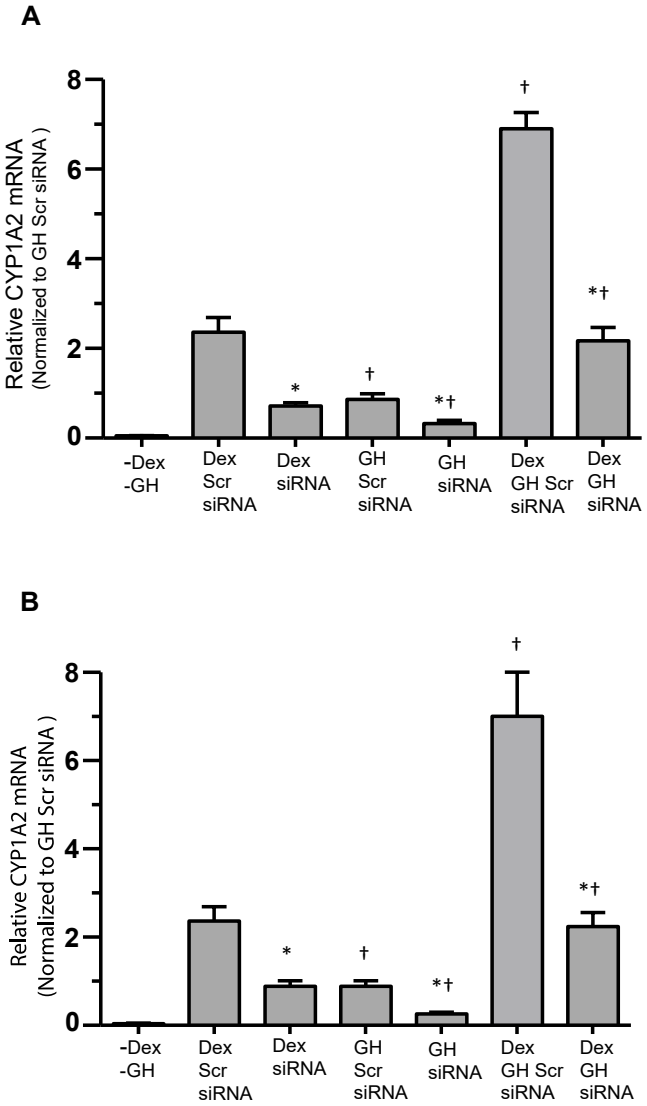

Figure 4. Hormonal regulation of *CYP1A2* mRNA levels in NF-1 and USF-1 proficient and deficient HepG2 cells. Following 50–70% confluency, HepG2 cells were transfected with either the control non-specific scrambled (Scr) siRNA (proficient) or NF-1 or USF-1 knockdown siRNA (deficient) and 24 hours later cells were either exposed to continuous dexamethasone alone (Dex) or continuous growth hormone alone (GH) or both hormones (Dex+GH) for 2 days after which the cells were harvested and analyzed. (**A**) graphic representation of *CYP1A2* mRNA levels (qRT-PCR) in NF-1 proficient and deficient HepG2 cells and the signals were normalized with GAPDH mRNA (internal control). **(B)** A graphic representation of *CYP1A2* mRNA levels (qRT-PCR) in USF-1 proficient and deficient HepG2 cells and the signals were normalized with GAPDH mRNA (internal control). Each data point is a mean ± S.D. from 5 or more independent experiments. *P < 0.01 compared the effects of the knockdown siRNA with control cells exposed to the same hormone treatment. †P < 0.01 compared with dexamethasone alone in cells transfected with the same siRNA.

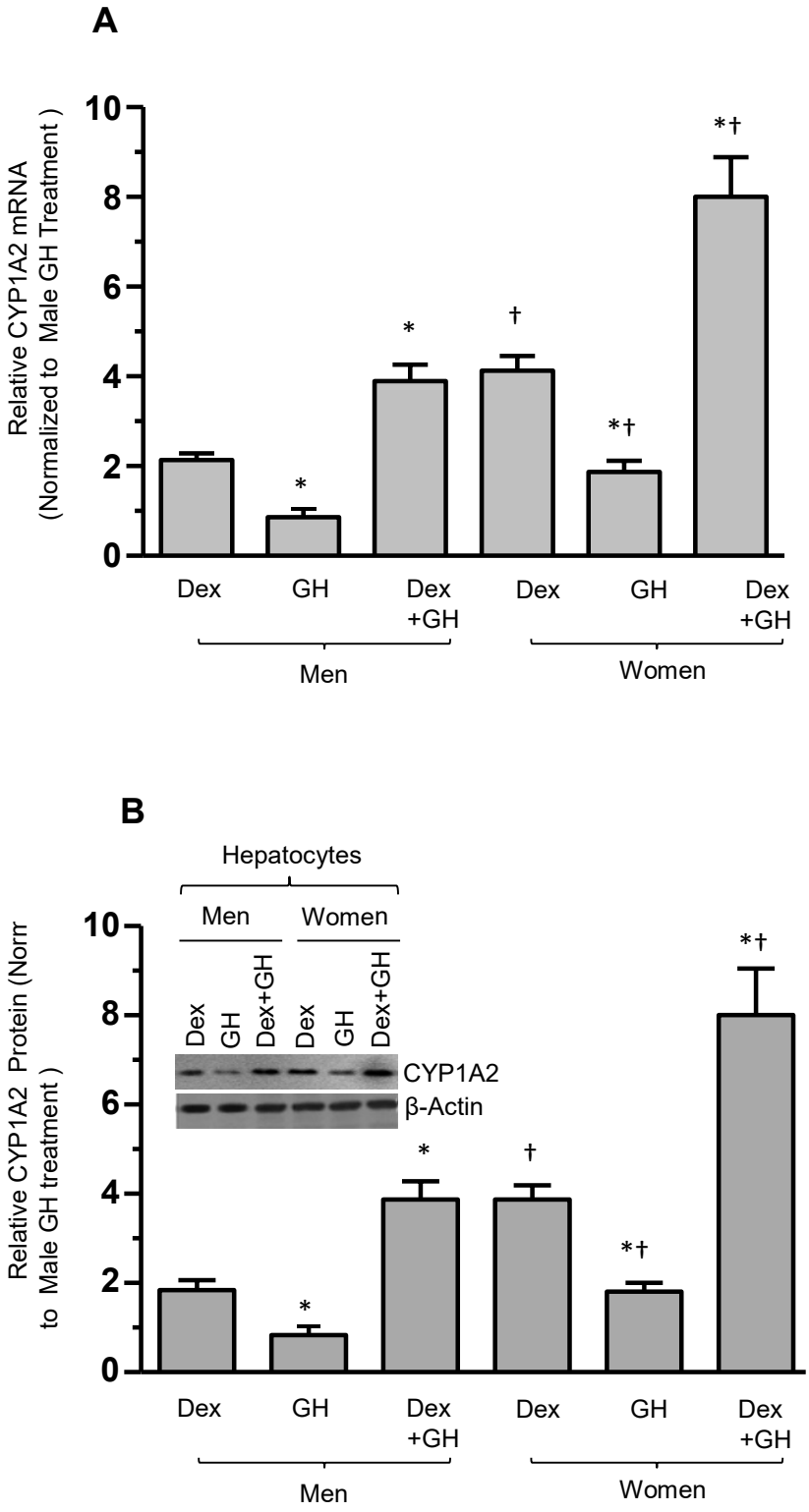

Figure 5. Growth hormone and dexamethasone effects on sex-dependent regulation of human *CYP1A2* in cultured primary human hepatocytes. Adult hepatocytes from men and women were either exposed to continuous dexamethasone alone (Dex) or continuous growth hormone alone (GH) or both hormones (Dex+GH) or vehicle (-Dex-GH) alone for 5 days in culture, after which the cells were harvested and processed for further analysis**. (A)** A graphic representation of relative *CYP1A2* mRNA levels hepatocytes and the signals were normalized with GAPDH mRNA (internal control). **(B)** A graphic representation of relative *CYP1A2* protein levels and a representative immunoblot image of *CYP1A2* protein (insert). The protein signal values for vehicle (-Dex-GH) treatments were so low as to be barely detectable and are not included in the figure. Respective loading controls (β-actin) are presented in the figure. Each data point is a mean ± S.D. for cells from 5 or more individuals. *P<0.01 compares to the dexamethasone alone treatment of the same sex. †P<0.01 compares women with men exposed to the same dexamethasone treatment.

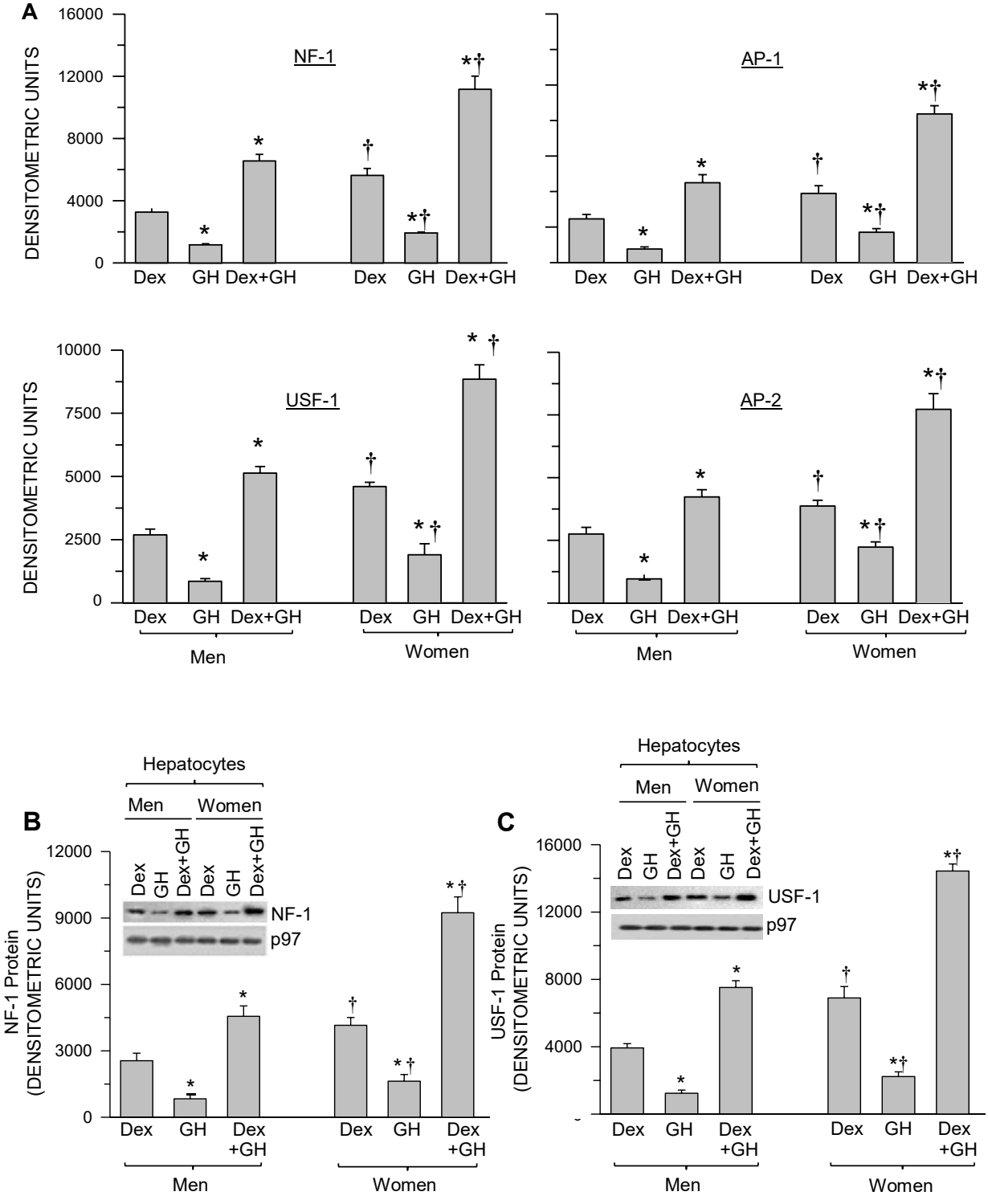

Figure 6. Hormonally regulated, nuclear DNA bound NF-1, USF-1, AP-1 and AP-2 transcription factors in hepatocyte nuclei and nuclear protein levels of NF-1 and USF-1 in cultured hepatocytes from men and women. Adult hepatocytes from men and women were either exposed to continuous dexamethasone alone (Dex) or continuous growth hormone alone (GH) or both hormones (Dex+GH) or vehicle (-Dex-GH) alone for 5 days in culture, after which the cells were harvested and nuclear extract was isolated and processed for further analysis. **(A)** Graphic representations of nuclear DNA associated transcription factors NF- 1, USF-1, AP-1 and AP-2 levels (Protein DNA array) in the nuclear extracts of cultured male and female hepatocytes (the signals were normalized with the internal controls included in the assay kit). **(B)** A Graphic representation of nuclear NF-1 protein concentrations and a representative immunoblot image of nuclear NF-1 protein (insert). **(C)** A Graphic representation of nuclear USF-1 protein and a representative immunoblot image of nuclear USF-1 protein (insert). Respective loading control (p97) for **B** and **C** are included in the figure. The protein signal values for vehicle (-Dex-GH) treatment were so low as to be barely detectable and are not included in the figure. Each data point is a mean ± S.D. for cells from 5 or more individuals. *P<0.01 compares to the dexamethasone alone treatment of the same sex. †P<0.01 compares women with men exposed to the same treatment.

and least in GH alone. Regardless of treatment, the induction and nuclear accumulation of NF-1 and USF-1 was observed as highest in women hepatocytes. There were no measurable NF-1 and USF-1 in no dexamethasone and no growth hormone growth condition. (-Dex-GH).

Sex-dependent hormonally regulated NF-1 recruitment to the *CYP1A2* upstream sequence in men and women hepatocytes

Consistent with the Figure 6A, Dex and GH treatment induced the greatest levels of NF-1 binding to *CYP1A2* upstream sequence than either Dex or GH alone (the latter, the least effective) (Figure 7B). NF-1 binding to the *CYP1A2* upstream sequence exhibited a sexual dimorphism in all hormone treatments (F>M). In confirmation using Southern blotting, we observed very similar levels of the occupied NF-1 binding motif on the *CYP1A2* upstream sequence (Figure 7C) as evidenced by chromatin immunoprecipitation (Figure 7B).

Sex-dependent hormone regulated USF-1 recruitment to the *CYP1A2* upstream sequence in cultured men and women hepatocytes

Consistent with the figure 7B, Dex and GH treatments induced the greatest levels of USF-1 binding to the *CYP1A2* upstream sequence than either Dex or GH alone (the latter, the least effective) (Figure 8B). USF-1 binding to the *CYP1A2* upstream sequence exhibited a sexual dimorphism between all hormone treatments (F>M). In confirmation using Southern blotting, we observed very similar levels of the occupied USF-1 binding motif on the *CYP1A2* as evidenced by chromatin immunoprecipitation (Figure 8B).

Discussion

GH acting through the GH receptor, regulates a complex sexually dimorphic transcriptional program of regulating CYP genes in humans and rodents [7,9-14,48]. The molecular mechanism of GH regulated sex dependent CYP 2A, 2C, and 3A has been well explored [13,49,50]. Human CYP3A4 and CYP3A5 exhibits a female predominance when determined in liver (in vivo) extracts and also in cultured hepatocytes [7,9,10, 51-53]. The molecular mechanism of sex dependent regulation of human CYP1A2 is largely unknown and with few reports, which do not describe the sex dependent regulation of CYP1A2 in liver [54,55]. CYP1A2 exhibits several alleles. The present study provides a novel molecular mechanism for growth hormone regulated sexually dimorphic expression of CYP1A2. The present study establishes five major key findings; (i) Identification of NF-1 and USF-1 in CYP1A2 gene regulation in response to hormones in HepG2 cells (in vitro). (ii) Activation of NF-1 and USF-1 regulation in response to GH and Dex in adult human hepatocytes. (iii) Identification of sex determined hormonally regulated binding of the NF-1 and USF-1 transcription factors on the CYP1A2 upstream sequence in human hepatocytes. (iv) Confirmation of the NF-1 and USF-1 binding motif on the CYP1A2 upstream sequence. (v) Expression of CYP1A2 transcript and protein expression levels correlates with the relative binding of NF-1 and USF-1 transcription factors in response to sex and hormones.

In the present study, we initially identified the transcription factor binding sites including USF-1 and NF-1 on the CYP1A2 upstream sequence and further we assessed their functional role in the CYP1A2 regulation in response to Dex and GH (Supplemental figure 1). The recruitment of USF-1 and NF-1 to the CYP1A2 upstream sequence are hormonally regulated and their DNA binding are in agreement with earlier findings (Figure 2,3) [42-44]. Interestingly, USF-1 and NF-1 deficiency dictates the repressed expression of CYP1A2 and our data

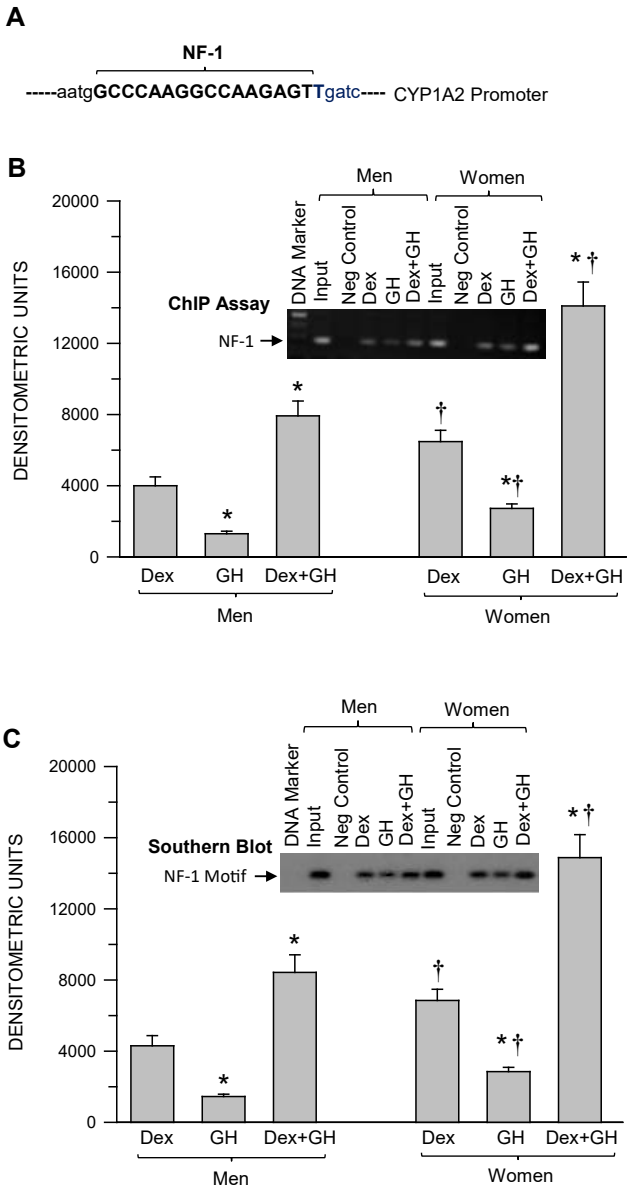

Figure 7. Mapping the NF-1 binding region on human *CYP1A2* upstream sequence and sex-dependent hormonal regulation of nuclear factor-1 (NF-1) binding to the *CYP1A2* upstream sequence as well as confirmation of the occupied NF-1 binding motif in hepatocytes derived from adult men and women. Adult hepatocytes from men and women were either exposed to continuous dexamethasone alone (Dex) or continuous growth hormone alone (GH) or combination of Dex and GH or vehicle (-Dex-GH) alone for 5 days in culture. Next, the cells were fixed with formaldehyde, lysed and the chromatin was sheared and processed for further analysis. (A) NF-1 binding location on the *CYP1A2* upstream sequence (Bold). (B) A graphic representation of the ChIP-PCR amplified signal and a representative agarose gel image of the ChIP assay. (C) A graphic representation of the occupied NF-1 binding motif and a representative Southern blot image (insert). Rabbit IgG served as a negative control and input chromatin as a positive control. The ChIP signal values for vehicle (-Dex-GH) treatment were so low as to be barely detectable and are not included in the figure. Each data point is a mean ± S.D. for cells from 5 or more individuals. *P<0.01 compares to the dexamethasone alone treatment of the same sex. †P<0.01 compares women with men exposed to the same treatment.

proposes that USF-1 and NF-1 are hormonally regulated and their role is required for CYP1A2 regulation (Figure 4A,B).

In the adult human hepatocyte culture model system, results were in agreement with in HepG2 cells as CYP1A2 regulation was mediated through Dex and GH as well as sex (Figure 5A,B). In agreement with

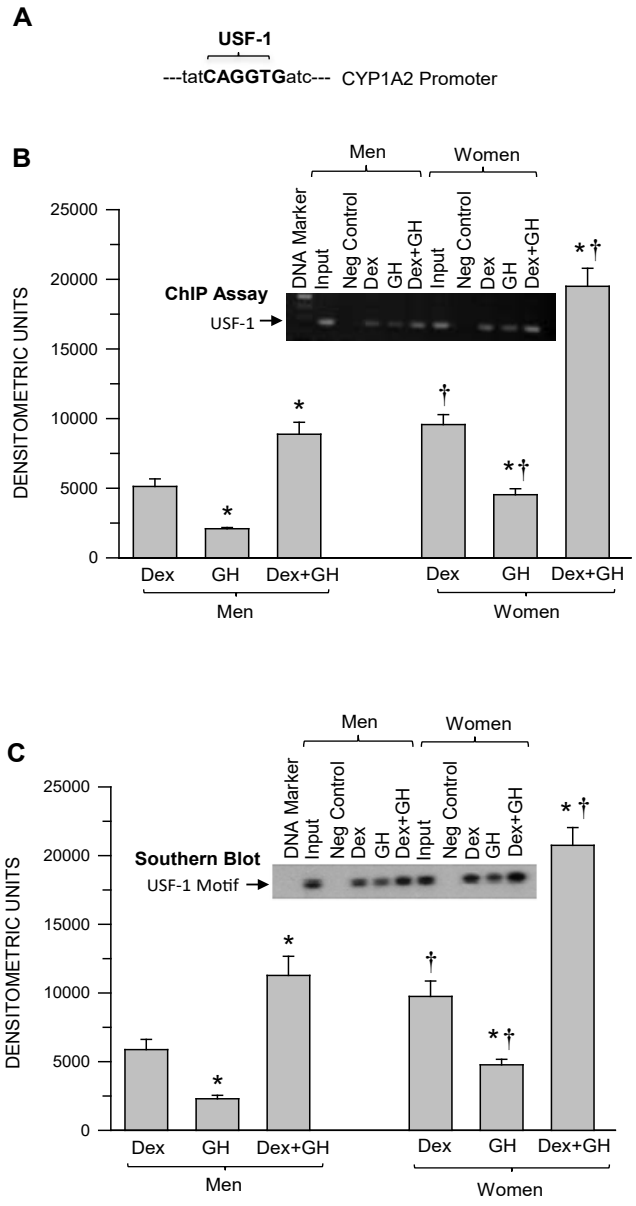

Figure 8. Mapping the USF-1 binding region on the human *CYP1A2* upstream sequence and sex-dependent hormonal regulation of USF-1binding to the CYP1A2 upstream sequence and confirmation of the occupied USF-1 binding motif in the *CYP1A2* upstream sequence in hepatocytes derived from adult men and women. Adult hepatocytes from men and women were either exposed to continuous dexamethasone alone (Dex) or continuous growth hormone alone (GH) or combination of Dex and GH or vehicle (-Dex-GH) alone for 5 days in culture. Next, the cells were fixed with formaldehyde, lysed and the chromatin was sheared and processed for further analysis. **(A)** USF-1 binding location on the *CYP1A2* upstream sequence (Bold). **(B)** A graphic representation of the ChIP-PCR amplified signal and a representative agarose gel image of the ChIP assay. **(C)** A graphic representation of the occupied USF-1 binding motif and a representative Southern blot image (insert). Rabbit IgG served as a negative control and input chromatin as a positive control. The ChIP signal values for vehicle (-Dex-GH) treatment were so low as to be barely detectable and are not included in the figure. Each data point is a mean ± S.D. for cells from 5 or more individuals. *P<0.01 compares to the dexamethasone alone treatment of the same sex. †P<0.01 compares women with men exposed to the same treatment.

earlier reports, the expression levels of hormonally regulated CYP1A2 are female dominant (F>M) [7,32,33]. These findings support our earlier sex-dependent findings of CYP3A4 and CYP3A5 [9,10]. Nuclear translocation of NF-1 and USF-1 and the association of transcription factors NF-1, USF-1, AP-1 and AP-2 to DNA are Dex and GH regulated and female predominant (Figure 6 A-C). Lastly, the recruitment of NF-1 and USF-1 to the CYP1A2 upstream sequence was female predominant and hormonally regulated (Figure 7 & 8). Both the universal human derived HepG2 model and human primary hepatocyte culture model systems demonstrate that CYP1A2 regulation is mediated through hormonally regulated NF-1 and USF-1 in a sex dependent manner. In addition, several inherent GH-dependent sexually dimorphic responses to non-CYP functions in humans have also been reported (20-23,56). Whereas any possible justification(s) for these intrinsic sexual dimorphisms is speculative, the present study has identified possible mechanisms responsible for CYP1A2 regulation. In summary female-dominant expression of human CYP1A2 appears to be irreversibly inherently expressed. Hormonally regulated and sex dependent activation of transcriptional pathways appear responsible for the sex-dependent expression of CYP1A2. These findings may contribute to the development of sex-dependent therapies.

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Author's contributions

Conception and design: C. Thangavel, E. Boopathi and BH. Shapiro; Development of methodology: C. Thangavel and E. Boopathi; Acquisition of data: C. Thangavel and E. Boopathi; Analysis and interpretation of data: C. Thangavel, E. Boopathi and BH. Shapiro; Computational analysis: C. Thangavel and BH. Shapiro; Writing and review of the manuscript: C. Thangavel and BH. Shapiro.

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