INTRODUCTION

Many non-genotoxic chemicals can induce hepatic lesions and eventually hepatocellular carcinoma (HCC) in rodents. At the early stage in the response to these chemicals, the liver often develops hepatocellular hypertrophy, which is usually accompanied by an increase in liver size and weight. Since hepatocellular hypertrophy can be a sign of adverse effects caused by chemicals or a physiological response related to the metabolism of xenobiotics, it is important to determine what other early changes occur in relation to the development of chemical hepatocarcinogenesis in order that the risk can be reliably characterized and assessed.

Sex steroid hormones have been implicated in the development of hepatocellular carcinoma (HCC) as suggested by etiological data. Recent studies in Korea and Japan indicated that elevation in serum testosterone level or imbalanced testosterone/estradiol ratio is associated with an increased risk of HCC (Ryu et al., 1997; Yu and Yuan, 2001). On the other hand, use of oral contraception also has been reported to increase the HCC risk (Yu et al., 2004). In rats, estrogen is a strong promoter for HCC when applied in the two-stage carcinogenesis model (Dragan et al., 1991). In vitro, estrogens stimulate proliferation of hepatocytes (Francavilla et al., 1989). Since the estrogen receptor (ER) and androgen receptor (AR) mediate sex steroid hormonal actions, several studies have investigated the status of these receptors in liver tissue. In rats treated with hepatocarcinogenic peroxisome proliferators, a marked decrease in ER activity and an increase in AR activity were reported in HCC tissue.
Moreover, a recent study demonstrated that mice lacking the hepatic AR developed HCC later and of lesser severity than did wild type littermates (Ma et al., 2008). Studies of human HCC specimens demonstrated low-level expression of ER in tumor tissue, while the receptor was expressed to a significantly greater degree in surrounding normal liver tissue. In contrast, higher AR expression was detectable in the HCC tissues (Mutai et al., 1990; Boix et al., 1993). Then, ER and AR expression in the liver may be useful markers for assessing HCC inducing chemicals.

In the present study, we investigated hormone receptor expression levels as well as changes in estrogen signaling during the development of hepatocellular hypertrophy induced by several types of non-genotoxic hepatocarcinogens, phenobarbital (PB), chlofibrate (CF) and piperonyl butoxide (PBO). PB is a well known hepatocarcinogen in rodent (Kolaja et al., 1996); CF is one of the peroxisome proliferators (Rao and Reddy, 1991); PBO may be an inducer of oxidative stress (Muguruma et al., 2007).

MATERIALS AND METHODS

Animal experiments
Animal experiment of hormone administration was approved by the Animal Experiment Committee of Hiroshima University (document # B05-4) and conducted in accordance with 'A Guide for the Care and Use of Laboratory Animals of Hiroshima University'. Male and female F344/DuCrj rats were purchased from Charles River Japan Co. (Kanagawa, Japan) and maintained with free access to a MF basal diet (Oriental Yeast Company, Tokyo, Japan) and tap water. At 9 weeks old, rats were divided into 3 groups (n = 4 per group, each sex); 2 of these groups were surgically gonadectomized. When the rats reached 10 weeks of age, a pellet containing 0.2 mg of estradiol benzoate (Sigma Chemical, St. Louis, MO, USA) was administered s.c. in gonadectomized groups. After 48 hr, rats were killed under ether anesthesia and liver tissues were dissected, immediately frozen in liquid nitrogen, and stored at -80°C.

An animal experiment feeding of PB, CF and BPO was previously reported (Nemoto et al., 2011) and the same liver samples were shared in the present study. Briefly, six week-old male F344/DuCrj rats were fed with PB, CF and PBO in diet at concentrations of 2500, 2500 and 20,000 ppm, respectively. Rats were sacrificed by decapitation at day 3, week 4 and week 13. Livers were weighed and pieces of the tissue were immediately frozen in liquid nitrogen and stored at -80°C. Histopathological assessment of liver tissues were performed with haematoxylin-eeosin (HE) sections.

Quantification of mRNAs by real-time RT-PCR
Total RNA was prepared from frozen liver tissues using an RNA isolation kit (NucleoSpin RNA II; Machery-Nagel GmbH & Co. KG, Düren, Germany), and 2 μg of the total RNA was reverse-transcribed as described previously (Fujimoto et al., 2004). An ABI Prism 7500 (PerkinElmer Life Sciences, Boston, MA, USA) was employed for quantitative measurement of the cDNA using Sybr premix Ex Taq II (Takara Bio, Shiga, Japan). Specific primer sets with a Tm of about 59°C were designed for each mRNA (Table 1). Prior to quantitative analysis, PCR products were prepared separately and purified by gel-electrophoresis. The DNA sequenc-

Table 1. Quantitative PCR primers for rat genes

<table>
<thead>
<tr>
<th>Gene</th>
<th>GenBank Acc#</th>
<th>5'-Primer</th>
<th>3'-Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>AR</td>
<td>NM_012502</td>
<td>5'-CACCATGCAACTTCTTCAGCA</td>
<td>5'-CGAATTGCCCTAGGTAACT</td>
</tr>
<tr>
<td>β-actin</td>
<td>X03765</td>
<td>5'-CTGCCCTGTGCTTCGTC</td>
<td>5'-TGAGTGATTGTCAGGTCC</td>
</tr>
<tr>
<td>calbD9k</td>
<td>X16635</td>
<td>5'-AACGAGCTGTCAAAGGAGGA</td>
<td>5'-CTTCTCATACCCGTCCTCA</td>
</tr>
<tr>
<td>CD36</td>
<td>NM_031561</td>
<td>5'-CTGTGGGCTATTACTGAGC</td>
<td>5'-TTTCACTACCTTGGTTTGAAGCA</td>
</tr>
<tr>
<td>CYP2C13</td>
<td>NM_138514</td>
<td>5'-CAAGAGTTTTCCCAACCCAGA</td>
<td>5'-GAATAGAAACAGCAGCTCCG</td>
</tr>
<tr>
<td>ERα</td>
<td>NM_012689</td>
<td>5'-CCAGAATGGGCGGAGAGAC</td>
<td>5'-CCAGGCCCTCATAATGGTAGG</td>
</tr>
<tr>
<td>ERβ</td>
<td>U57439</td>
<td>5'-TGCCACATCATGCTCTTCTA</td>
<td>5'-GGCACAAGTCTCCCACTAAG</td>
</tr>
<tr>
<td>IGFBP1</td>
<td>NM_013144</td>
<td>5'-ATTAGCTTGCCGCTCAACAGAA</td>
<td>5'-CCACCATCCATGGTACAC</td>
</tr>
<tr>
<td>Kinogen1</td>
<td>NM_012696</td>
<td>5'-TAAATCTCGTGACCCACCA</td>
<td>5'-GAAATGCTCCACAGCGTCTT</td>
</tr>
<tr>
<td>LIFR</td>
<td>NM_031048</td>
<td>5'-AGAAACTTTCTGGACGCT</td>
<td>5'-AGATGGATAAGGGCGG</td>
</tr>
</tbody>
</table>

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es were confirmed with a capillary DNA sequencer (ABI 310; PerkinElmer Life Sciences). The extracted fragments were used as standards for quantification. The PCR conditions were 30 sec initial denaturing followed by 40 cycles for 5 sec per cycle at 95ºC and 34 sec per cycle at 60ºC. All mRNA contents were normalized with reference to β-actin mRNA.

Statistical analysis
Statistical comparisons were made by Dunnett’s multiple comparison test.

RESULTS

Estrogen-responsive genes in the liver of 10-week-old rats
Candidate estrogen-responsive genes identified from the literature (Diel et al., 1995; Singhal et al., 2009) were examined for their response to estrogen in liver tissue. Candidates were insulin-like growth factor binding protein 1 (IGFBP1), calbindin (calb) D9k, kinogen 1, leukemia inhibitory factor receptor (LIFR), CD36 and CYP2C13. LIFR was found to be a significant estrogen-responsive gene, while CD36 also displayed weak responses (Table 2).

Estrogen and androgen receptor mRNA expression
Detectable amounts of ERα mRNA were noted in liver tissue with expression level at 17.0 ± 2.2 fg/pg β-actin while ERβ mRNA was not detected (< 0.01 fg/pg β-actin) in 10-week-old rats. The level of AR mRNA was also determined as 4.65 ± 0.74 fg/pg β-actin. ERα mRNA level was relatively low at the beginning of experiment (at 6 weeks of age) and increased to constant levels at week 4 (at 10 weeks of age) in the control group (Fig. 1). When animals were treated with test chemicals, PB administration did not alter the ERα mRNA levels. In CF and PBO groups, on the other hand, the expression of ERα mRNA was significantly reduced at weeks 4 and 13.

The control AR mRNA level was also relatively low at day 3 (at 6 weeks of age) and increased to constant levels at week 4 (Fig. 1). PB administration strongly suppressed the AR mRNA expression at week 4 and it remained low at week 13. In CF and PBO groups, AR mRNA levels were also lower than that of control but increased afterward.

Expression of LIFR and CD36
The control level of LIFR expression decreased from day 3 (at 6 weeks of age) to week 13 (at 19 weeks of age) (Fig. 2). PB administration increased LIFR expression at week 4 and it remained higher than the control level at week 13. PBO strongly suppressed LIFR expression at week 4 but returned the control level at week 13, while CF had little effect on LIFR expression. The control level of CD36 increased from day 3 to week 13. CD36 expression was suppressed by PB but elevated by CF and PBO.

DISCUSSION
Our previous study showed that well known non-genotoxic hepato-tumorigenetic agents including PB, CF (a peroxisome proliferator), and PBO (a pesticide synergist), induce hepatic hypertrophy (Nemoto et al., 2011). To varying degrees, the increase in liver weight was evident even at day 3 in all of the groups. PBO induced severe diffuse hypertrophy with doubling of the liver weight, while less severe pathological features were noted in the CF and PB groups. The present study demonstrated that the expression of ERα and an estrogen responsive gene, LIFR, was dramatically changed after administration of these chemicals.

Table 2. Expression of LIFR, CD36, kinogen 1, IGFBP1, calbD9K and Cyp2C13 in the liver of E2 treated rats

<table>
<thead>
<tr>
<th></th>
<th>LIFR</th>
<th>CD36</th>
<th>kinogen 1</th>
<th>IGFBP1</th>
<th>calbD9K</th>
<th>Cyp2C13</th>
</tr>
</thead>
<tbody>
<tr>
<td>♂ C</td>
<td>0.42 ± 0.034</td>
<td>5.0 ± 0.70**</td>
<td>0.41 ± 0.063</td>
<td>43.1 ± 10.1</td>
<td>20.8 ± 4.5</td>
<td>12.9 ± 1.11</td>
</tr>
<tr>
<td>Cast</td>
<td>0.34 ± 0.031</td>
<td>21.0 ± 0.82</td>
<td>0.37 ± 0.041</td>
<td>59.6 ± 28.2</td>
<td>15.8 ± 2.9</td>
<td>9.44 ± 1.15</td>
</tr>
<tr>
<td>Cast+E2</td>
<td>0.79 ± 0.049**</td>
<td>32.8 ± 1.38**</td>
<td>0.55 ± 0.087**</td>
<td>71.3 ± 9.4</td>
<td>24.4 ± 2.9**</td>
<td>6.93 ± 0.66</td>
</tr>
<tr>
<td>♀ C</td>
<td>24.60 ± 2.18**</td>
<td>59.3 ± 1.43**</td>
<td>1.00 ± 0.096</td>
<td>294 ± 60.6*</td>
<td>119 ± 6.2*</td>
<td>1.25 ± 0.21</td>
</tr>
<tr>
<td>Ovx</td>
<td>1.18 ± 0.24</td>
<td>36.8 ± 1.63</td>
<td>0.81 ± 0.081</td>
<td>186 ± 23.5</td>
<td>72.4 ± 7.8</td>
<td>0.75 ± 0.33</td>
</tr>
<tr>
<td>Ovx+E2</td>
<td>5.38 ± 0.98*</td>
<td>55.1 ± 9.77</td>
<td>1.17 ± 0.089**</td>
<td>249 ± 38.1*</td>
<td>89.8 ± 2.6*</td>
<td>1.13 ± 0.04</td>
</tr>
</tbody>
</table>

Mean ± S.E.M. (n = 4). *P < 0.05, **P < 0.01 vs. castration (male) or ovx (female).
Various lines of evidence suggest the involvement of sex steroid hormones in hepatocellular carcinogenesis in humans as well as in rodents. In a previous study in which rats were exposed to the peroxisome proliferator agents CF and BR931, total ER binding decreased while nuclear ER increased (Eagon et al., 1996). Interestingly, in hepatic tumors induced by 9-10 month administration of these agents, ER activity was markedly decreased. Conversely, AR binding activity did not change in the short term and increased in tumor tissue. Studies of human HCC cases have indicated significant losses of ER and retention or increase of AR (Nagasue et al., 1989; Boix et al., 1993), which is consistent with the findings in our rat model. The reduction of ER expression may be involved in the development of HCC, and the eventual loss of expression was clearly related to HCC. Then, the decrease in ER expression and altering estrogen signaling in CF- and PBO-treated rats may be indicative to hepatocarcinogenesis. Hepatocarcinogenic chemicals may potentially change steroid hormone metabolism and serum hormone levels.
Previous studies, however, have indicated no change in serum estradiol levels by PB (Mesia-Vela et al., 2006) or CF (Eagon et al., 1996).

In rat liver, the ER concentration is low before puberty and subsequently increases (Rochman et al., 1985). In the present experiment in male rats, the expression of ERα mRNA was low in 6-week old rats (experimental day 3) and increased at week 4 and beyond. Similarly, AR expression increased from day 3 to week 4 and beyond, a finding consistent with a previous study showing lower expression of AR mRNA before puberty (Song et al., 1991).

In the present study, we examined the expression of several estrogen- and androgen-responsive gene candidates to determine the possible markers of estrogen- and androgen signaling. There are a limited number of reports regarding sex steroid hormone-dependent genes in the liver, although the liver is considered to be a target organ for sex steroids. IGFBP1, calb D9k and kinogen 1 were reported to be up-regulated by E2 in the rat Fe33 hepatic tumor cell line (Diel et al., 1995). A cDNA microarray analysis of hepatic genes in E2-treated Sprague-Dawley rats showed up-regulation of LIFR, CD36, and CYP2C13 (Singhal et al., 2009). Despite the reported E2-responsiveness of all of these genes, only LIFR was confirmed to be regulated in our study, while weak E2 regulation of CD36 was noted. Candidates for androgen-responsive genes were A2MG and carbonic anhydrase 3 (Carter et al., 1984; Chatterjee et al., 1990). Since neither of the genes displayed significant androgen responsiveness in our system (data not shown), no further examinations were conducted.

Recently, LIFR has been identified as a specifically down-regulated gene in human HCC cases based on cDNA microarray analysis (Okamura et al., 2010). Promoter hypermethylation of the LIFR gene was observed in 48% of tumor tissues, and this was clearly related to the suppression of gene expression. LIFR is an integral component of the glycoprotein 130-LIFR signaling complex, which acts as a signal receptor for cytokines such as leukemia inhibitory factor (LIF) and ciliary neurotrophic factor. PTEN (phosphatase and tensin homolog deleted on chromosome 10) was found to act as a general tumor suppressor of somatic malignancies (Li et al., 1997). Since PTEN-Akt-FOXO signaling regulates the LIFR-Stat3 pathway, which plays a critical role in suppressing malignant transformation, loss of PTEN results in tumorigenesis correlating with low expression of LIFR (de la Iglesia et al., 2008). LIFR itself may therefore act as a tumor suppressor in HCC. The present study demonstrated that LIFR mRNA expression was regulated by estrogen. CF and PBO suppressed ERα mRNA expression and as a consequence, the expression of LIFR was reduced. Therefore LIFR may be a key mediator of the ability of CF and PBO to promote HCC. On the other hand, change in expression of CD36, a weak responder to estradiol, did not correlate to ER levels. The expression of CD36 probably does not primarily depend on estrogen.

PB has been studied extensively but the mechanism of its hepatocarcinogenic action in rodents is unclear. In 2-stage carcinogenesis models, the tumor-promoting activity of PB is apparent (Farinati et al., 2002). PB promotes focal hepatic lesion growth both by increasing DNA synthesis and decreasing the rate of apoptosis (Kolaja et al., 1996). Peroxisome proliferators including CF are another group of non-genotoxic chemicals that induce hepatocarcinogenesis (Rao et al., 1991). Chronic administration of these types of chemicals results in altered areas of liver, followed by neoplastic nodules, and finally HCC. The mechanism of carcinogenesis is unclear, although there appears to be a strong association with the potency of peroxisome proliferation, and increased oxidative stress may also play a role. PBO, a pesticide synergist, was not considered as a carcinogen in humans until a strong positive result was reported in a 2-year study of F344 rats (Takahashi et al., 1994). It was suggested that the carcinogenic mechanisms of PBO are similar to those of PB in terms of induction of CYP2B and inhibition of gap junctional intercellular communication (Okamiya et al., 1998). However, the present study showed that the ER-expression and estrogen-signaling profiles of PBO were similar to those of CF but differed from those of PB.

ACKNOWLEDGMENT

This work was in part by a Grant-in-Aid for the Research Program for Risk Assessment Study on Food Safety from the Food Safety Commission, Japan (No. 0703).

REFERENCES


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