Regulation of Inhibin β Chains and Follistatin mRNA Levels During Rat Hepatocyte Growth Induced by the Peroxisome Proliferator Di-\(n\)-butyl Phthalate

Tetsu KOBAYASHI,*a Shingo NIIMI,a Masamichi FUKUOKA,b and Takao HAYAKAWAa

\*Division of Biological Chemistry and Biologicals, National Institute of Health Sciences; 1–18–1 Kamiyoga, Setagaya-ku, Tokyo 158–8501, Japan; and Departement of Clinical Pharmacology and Toxicology, Showa Pharmaceutical University; 3–3165 Higashi-Tamagawagakuen, Machida, Tokyo 194–8543, Japan. Received March 28, 2002; accepted June 24, 2002.

Peroxisome proliferators stimulate hepatocyte growth in rat liver \textit{in vivo}. Activin A, a homodimer of inhibin \(\beta_a\), inhibits DNA synthesis in hepatocytes. The inhibitory action of activin A is suppressed by follistatin, an activin-binding protein. In this paper, we investigated whether administration of \(n\)-butyl phthalate (DBP), a peroxisome proliferator, modifies the production of activin A and follistatin in rat liver by hourly monitoring of inhibin \(\beta_a\), and follistatin mRNA levels by reverse transcriptase polymerase chain reaction analysis. The mRNA levels of the other inhibin \(\beta\) chains (inhibin \(\beta_b\) and \(\beta_c\)) were examined in a similar manner. The inhibin \(\beta_a\) mRNA level decreased to about 30% by 3 h after DBP administration (8.6 mmol/kg body weight), remained low until 12 h, and returned to its original level by 24 h. The follistatin mRNA level increased to about 2 times by 6 h, and returned to its original level by 24 h. The inhibin \(\beta_b\) mRNA had started to increase by 1 h, peaked at 6 h at about 4 times its initial level, and returned to its original level by 12 h. The inhibin \(\beta_c\) mRNA level had doubled by 6 h and it returned to its original level. These results indicate that the growth stimulatory action of peroxisome proliferators may be mediated \textit{via} the decrease in activin A level and activity and suggest that the increases in follistatin as well as inhibin \(\beta_b\) and \(\beta_c\) chains may play a role in peroxisome proliferator-stimulated hepatocyte growth.

Key words inhibin \(\beta\) chain; follistatin; peroxisome proliferator; hepatocyte growth

A single dose of peroxisome proliferator is known to stimulate hepatocyte growth in rat liver \textit{in vivo},\(^{1,5}\) and transcriptional regulation, which is mediated by the peroxisome proliferator activated receptor subtypes, is one important mechanism of peroxisome proliferator action.\(^{2,3}\) The receptor-mediated process seems to be essential for stimulation of hepatocyte growth, but, as described below, the rest of the molecular mechanism has not been well clarified.

It is likely that peroxisome proliferator-stimulated hepatocyte growth is mediated \textit{via} regulation of the expression of some hepatocyte growth stimulatory and suppressive factors, because a balance between these opposite factors regulates hepatocyte growth. Various factors are known to regulate normal hepatocyte growth:\(^4\) hepatocyte growth factor and transforming growth factor-\(\alpha\) are potent hepatocyte mitogens,\(^{51}\) tumor necrosis factor-\(\beta\) triggers hepatocytes growth,\(^6\) and transforming growth factor-\(\beta I\) is a well known suppressor of hepatocyte growth.\(^{51}\) However, the mRNA levels of these stimulatory and suppressive factors in rat liver failed to increase and decrease, respectively, after a single dose of 4-chloro-\(6-(2,3\text{ xylidino})-2\text{-pyrimidinylthio (N-\(\beta\)-hydroxy) acetamide, a peroxisome proliferator.}\(^1,13,14\)

In addition to these factors, hepatocyte growth is also regulated by the activin follistatin system. Activin A, a homodimer of the inhibin \(\beta_a\) chain, inhibits hepatocyte growth, and follistatin suppresses the inhibitory action of activin A.\(^9\) We therefore, hypothesized that peroxisome proliferator-stimulated hepatocyte growth is mediated via regulation of the expression of activin A and follistatin. In this paper, we measured inhibin \(\beta_a\) and follistatin mRNA levels in the liver of rats after administration of \(n\)-butyl phthalate (DBP), a peroxisome proliferator,\(^{10–12}\) by reverse transcriptase polymerase chain reaction (RT-PCR) analysis to verify this hypothesis. We also measured the mRNA levels of the other inhibin \(\beta\) chains, inhibin \(\beta_b\) and \(\beta_c\), in a similar manner.

\section*{MATERIALS AND METHODS}

\textbf{Isolation of RNA} Male Wistar rats (150–180 g) were obtained from Sankyo Laboratory (Tokyo, Japan), and given pure DBP in a single oral dose of 8.6 mmol/kg body weight. Livers were removed at various times after administration, and total RNA was isolated with a QuickPrep Total RNA Extraction Kit (Amersham Pharmacia Biotech, Little Shalfont, U.K.).

\textbf{Reverse Transcription} The first strand cDNA was synthesized from total RNA (3.2 \(\mu\)g) with RTG You-Prime First Strand Beads (Amersham Pharmacia Biotech) supplemented with random hexanucleotide primer in a volume of 34 \(\mu\)l.

\textbf{PCR} The oligonucleotides used are shown in Table 1.\(^{13,14}\) The first strand of cDNA, corresponding to 22–88 ng of template RNA, was amplified by using each 5' oligo primer at 200 nm in 30 \(\mu\)l of reaction mixture containing 0.2 mM dNTPs, 1/10 volume of 10X reaction mixture and 25 units/ml of Taq polymerase (Amersham Pharmacia Biotech). The reaction mixtures were subjected to 15–30 cycles of denaturation, annealing, and extension.

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|}
\hline
\textbf{Genes} & \textbf{Sequences of 5' oligo} & \textbf{Sequences of 3' oligo} \\
\hline
Inhibin \(\beta_a\) & tcaacagctattaacactacagctg & agccacactctcccaactcttg \\
Inhibin \(\beta_b\) & agccacagctgtaggactgtag & agccacactctcccaactcttg \\
Inhibin \(\beta_c\) & gctagccagctcgactaatct & gctagccagctcgactaatct \\
Follistatin & gctagccagctcgactaatct & gctagccagctcgactaatct \\
GAPDH & agccacagctgtaggactgtag & agccacactctcccaactcttg \\
\hline
\end{tabular}
\caption{Sequences for PCR Primers}
\end{table}
RESULTS AND DISCUSSION

Figure 1 shows the time course of inhibin $\beta_A$, and follistatin mRNA levels in the liver after administration of DBP. The inhibin $\beta_A$ mRNA level had declined to approximately 30% of its initial level by 3 h, remained almost constant until 12 h, and returned to its original level by 24 h (Figs. 1A, B). Conversely, the follistatin mRNA level had doubled by 6 h, and returned to its initial level by 12 h (Figs. 1A, C).

Nafenopin, a peroxisome proliferator, increases cyclin dependent kinase 4 (CDK-4) in primary cultured hepatocytes, and CDK-4 cooperates with cyclin D in phosphorylation of the retinoblastoma protein during the G1 phase, thus stimulating entry into the S phase of the cell cycle. In human HepG2 hepatoma cells, activin A decreases CDK-4 protein and increases p21$^{WAF1/CIP1}$, which inhibits CDK4 activity. Follistatin is known to inactivate activin A by binding to it. Thus, peroxisome proliferator-stimulated hepatocyte growth may be mediated via the decrease in activin A and the increase in follistatin.

On the other hand, as shown in Fig. 2, the patterns of regulation of inhibin $\beta_B$ and $\beta_C$ mRNA levels were almost the same as that of follistatin. The inhibin $\beta_B$ mRNA level had started to increase by 1 h, peaked at 6 h at approximately 4 times its initial level, and returned to its original level by 12 h (Figs. 2A, B). The inhibin $\beta_C$ mRNA level had doubled by 6 h, and it then returned to its original level by 12 h (Figs. 2A, C).

Inhibin $\beta_B$ chain complexes with either inhibin $\beta_A$ or inhibin $\beta_C$, forming activin AB ($\beta_A\beta_B$) and activin B ($\beta_B\beta_B$), respectively. We found that activin A is more potent than activin AB in inhibiting epidermal growth factor-stimulated DNA synthesis in cultured rat hepatocytes, and no inhibitory effect of activin B was observed. Therefore, the increase in inhibin $\beta_B$ may result in the decrease in activin A by increasing activin AB, followed by the reduction of activin A-mediated inhibition of hepatocyte growth. In addition, activin C ($\beta_C\beta_C$) has no effect on DNA synthesis in HepG2 hepatoma cells. Similarly, the increase in inhibin $\beta_C$ may result in the decrease of activin A by increasing other activin composed of inhibin $\beta_A$ and $\beta_C$ by formation of complex of inhibin $\beta_A$ and inhibin $\beta_C$, followed by reduction of activin A-mediated inhibition of hepatocyte growth, although it is uncertain whether inhibin $\beta_A$ complexes with inhibin $\beta_C$.

We recently found that inhibin $\beta_A$, $\beta_B$, and follistatin mRNA levels change in the carbon tetrachloride induced rat liver regeneration model, and the patterns of changes are almost the same as observed in the present study. Thus, these mRNA levels may be regulated by similar mechanisms in these two different hepatocyte growth models.

In conclusion, the mRNA level of inhibin $\beta_A$ decreased in
the liver of rats given DBP. And the mRNA levels of inhibin \( \beta_B \), \( \beta_C \), and follistatin increased. These results indicate that the growth stimulatory action of peroxisome proliferators may be mediated via the decrease in activin A level and activity and suggest that the increases in follistatin as well as inhibin \( \beta_B \) and \( \beta_C \) chains may play a role in peroxisome proliferator-stimulated hepatocyte growth.

REFERENCES