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Histone acetylation and steroid receptor coactivator expression during clofibrate-induced rat hepatocarcinogenesis

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Peroxisome proliferators (PPs), non-genotoxic rodent carcinogens, cause the induction of the peroxisomal fatty acid β-oxidation system, including bifunctional enzyme (BE) and 3-ketoacyl-CoA thiolase (TH), in the liver. GST M1 gene is polymorphic in Sprague–Dawley rats, NC- and KS-type. The KS-type rats showed enhanced susceptibility to ethyl-α-chlorophenoxyisobutyrate (clofibrate, CF), one of the PPs. The degree of BE induction was higher in the KS-type and preneoplastic foci developed after 6–8 weeks of treatment, whereas no foci developed in the NC-type. In the preset study, factors involved in different BE inducibility were investigated. There were no differences in hepatic peroxisome proliferator-activated receptor (PPAR) α levels between them. Among various coactivators for PPARα, only steroid receptor coactivator (SRC)-3 level was higher in the KS-type. To investigate the association between PPARα and SRC-3 or other proteins, nuclear extracts from CF-treated livers were applied to a PPARα column. In the KS-type, 110, 72, and 42 kDa proteins were bound and these were identified as SRC-3, BE, and TH, respectively. EMSA supported the binding of these proteins to PPARα associated to the BE enhancer in CF-treated KS-type, but not in the NC-type. Histone H3 acetylation was increased 11-fold in the KS-type by CF treatment but not in the NC-type. As BE and TH are responsible for acetyl-CoA production and SRC-3 possesses a histone acetyltransferase activity, these results suggest that enhanced BE induction in the KS-type livers is due to acetylation-mediated transcriptional activation and epigenetic mechanisms might be involved in CF-induced rat hepatocarcinogenesis. (*Cancer Sci* 2010; 101: 869–875)

Ethyl-α-chlorophenoxyisobutyrate, a hypolipidemic agent, and other PPs, when given to rats and mice results in a marked increase in the number and size of peroxisomes and increase in liver weight. These chemicals also induce the peroxisomal fatty acid β-oxidation enzyme system in the liver,[1–3] composed of three proteins.[4,5] The first, acyl-CoA oxidase, catalyzes dehydrogenation of fatty acyl-CoA, leading to the production of enoyl-CoA and H_2O_2. The second, BE, catalyzes the production of 3-ketoacyl-CoA from enoyl-CoA. The third, TH, is involved in the formation of acetyl-CoA.[4,5] In addition, when given long term, PPs result in the formation of rodent hepatic preneoplastic lesions and hepatocellular carcinomas.[6,7]

Glutathione transferases are a group of multifunctional enzymes that catalyse the conjugation of many electrophiles with glutathione. They have been classified into several groups, alpha, mu, pi, theta, sigma, and zeta.[8] Hepatic GSTs are involved in detoxication and conjugation reactions, and GST M1-1, M1-2, and M2-2, the major forms of rat liver GSTs in the mu class, are activated by reactive oxygen species.[9] The GST M1 gene is polymorphic in Sprague–Dawley rats, one type encoding ^{198}Asn-^{199}Cys (NC-type), and another encoding ^{198}Lys-^{199}Ser (KS-type).[10,11] In our previous study, the KS-type rats showed enhanced susceptibility and preneoplastic foci developed after CF treatment for 6–8 weeks, whereas no foci developed in the NC-type rats. Furthermore, the degree of BE induction was higher in the KS-type.[12,13] As the induction of peroxisome proliferation and the peroxisomal fatty acid β-oxidation system has been suggested as a practical short-term *in vivo* test to evaluate the carcinogenic potentials of PP,[14] it is important to clarify factors responsible for different BE inducibility in the polymorphic rats in order to develop a reliable test.

Induction of the enzymes of the peroxisomal β-oxidation system is due to transcriptional activation by PPARα, a member of the steroid hormone receptor superfamily,[15] interacting with the PPRE of genes encoding the enzymes.[16] It is becoming clear that not only PPARα but also many cofactors, such as SRC,[17,18] PBP,[19] and PPAR interacting protein,[20] are involved in the transcriptional activation of these genes. SRC possesses an intrinsic histone acetyltransferase activity[18] that catalyzes the transfer of acetyl group from acetyl-CoA to core histones.[21,22] The modification of core histones and transcription factors causes transcriptional activation.[23,24]

In the present study, factors involved in differences in BE inducibility between the polymorphic rats were investigated. Although the PPARα levels were not different, SRC-3 in nuclear extract was higher in the KS-type. Furthermore, BE, TH, and SRC-3 were bound to PPARα and histone H3 was highly acetylated in CF-treated KS-type. These results suggest that enhanced BE induction in the KS-type livers is due to acetylation-mediated transcriptional activation and the epigenetic mechanism might be involved in CF-induced rat hepatocarcinogenesis.

Materials and Methods

Rats and treatment with CF. Male Sprague–Dawley rats homozygous for the KS-type or NC-type GST M1 gene were used in the present study. GST M1 genotypes were confirmed by PCR with primers covering a polymorphic nucleotide sequence site followed by digestion with the restriction enzyme *Ear*I, as reported previously.[11,13] Rats were housed in plastic cages in an air-conditioned room maintained on a 12:12 h light: dark cycle in the Institute for Animal Experiments of Hirosaki University School of Medicine (Hirosaki, Japan) and maintained on a powdered diet (Oriental MF; Oriental Yeast Co., Tokyo, Japan) with or without 0.3% w/w CF (Tokyo Kasei Kogyo, Tokyo, Japan) for 4 weeks and killed by decapitation, and the livers were excised. This timepoint was selected, before the

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development of preneoplastic hepatic foci at 6 weeks or later.[13] All procedures and treatments that involved live animals were approved by the Animal Care and Use Committee, Hirosaki University.

Preparation of cytosol, peroxisomes, and nuclear extracts. Livers from KS- and NC-type rats were homogenized with 9 vol. of 20 mM Tris-HCl (pH 7.8). The supernatant obtained by centrifugation at 30 000*g* for 10 min at 4°C was further centrifuged at 105 000*g* for 45 min. Supernatant was used as cytosol fractions. Protein content was measured by Bradford's method.[25] Peroxisomes were fractionated according to the method described by de Duve *et al.*[26,27] Nuclear extract was prepared using Dignam's method.[28] For analysis of histone acetylation, nuclear extract was isolated by the method of Kang *et al.* [29]

SDS-PAGE and immunoblotting. SDS-PAGE was carried out using 10% acrylamide gel. Thirty microgram protein each was loaded on the gel. After electrophoresis, separated proteins were transferred to nitrocellulose membrane (Amersham Biosciences, Piscataway, NJ, USA). Antibody against BE and antibodies against GST A1-2 and GST M1-1 were prepared in rabbits as reported previously.[12,30] Anti-PPARα (sc-9000), anti-SRC-1 (sc-6096), anti-SRC-2 (sc-6976), anti-SRC-3 (sc-7216), and anti-PBP (sc-5334) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-histone H3 antibody (06-755) and anti-acetyl-histone H3 antibody specific for the acetylated lysine 9 or lysine 14 (06-599) were from Upstate Biotechnology (Lake Placid, NY, USA). The antigen–antibody complexes were then detected using ECL chemiluminescence (Amersham Biosciences). Detected bands were quantified with a scanning densitometer using NIH image software.

Production of anti-TH antibody. The peptide, ^{41}VHGRRTPIGRAGRGGC^{56} of rat TH,[31] was synthesized on a peptide synthesizer (model 432A; Applied Biosystems, Foster City, CA, USA). The sequence of this peptide was confirmed by fast atom bombardment mass spectrometry using model AX-505H (JEOL, Tokyo, Japan). The peptide was conjugated to keyhole limpet hemocyanin using *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester. Adult white rabbits were immunized with 200 μg conjugate at 2-week intervals.

RNA preparation and Northern blotting. Total RNA was isolated from livers using an RNeasy kit (Qiagen, Tokyo, Japan). Twenty micrograms of total RNA was electrophoresed on 1.0% formamide gel, transferred to nylon membrane, then hybridized at 42°C in a 50% formamide hybridization solution using ^{32}P-labeled BE cDNA probe and exposed to Kodak XAR-5 film at −80°C. cDNA probe for BE was generated by RT-PCR. The 5′ and 3′ primers used were 5′-AGACCACATGGTTAAAGCCA-3′ and 5′-ACTTCACGACTGCATCCAGA-3′, respectively.[32] The nucleotide sequence of the cDNA was confirmed by sequencing. Equal loading of RNA was confirmed by the intensities of 18S and 28S ribosomal RNAs. Quantitation of the results was done as described above using NIH image software.

Expression of His-tagged rat PPARα in *Escherichia coli*. To obtain rat PPARα cDNA, nested RT-PCR was carried out. cDNA was reverse-transcribed from total RNA isolated from control rat liver by incubating at 65°C for 1 min, 30°C for 5 min, 65°C for 15 min, 98°C for 5 min, and 5°C for 5 min in a reaction volume of 20 μL, containing 22 units *Bca*Best polymerase, 1× buffer, 500 μM each dNTP, 2.5 μM oligodT primer, and 40 units RNase inhibitor. The first PCR conditions were at 94°C for 2 min, followed by 25 cycles of denaturing at 94°C for 30 s, annealing at 61°C for 30 s, and extension at 72°C for 50 s, containing the RT mixture, 2.5 U *Bca*-optimized Taq, 1.5 mM $MgCl_2$, 1× buffer, 0.2 μM primers. The 5′ primer, 5′-GGATCTTAGAGGCGAGCCAA-3′, and the 3′ primer, 5′-ACGGCCTACCATCTCAGGAA-3′,[15] were used. For the second PCR, 50 pg of first-round amplified cDNA was used as the template and amplification conditions were at 94°C for 2 min, followed by 30 cycles of denaturing at 94°C for 1 min, annealing at 60°C for 2 s, and extension at 72°C for 2 min, containing 0.5 U *Taq* polymerase (Takara, Shiga, Japan), 10× buffer, 10 pmol primers, and 200 μM each dNTP. The 5′ primer was 5′-CATTCACCATGGTGGACACA-3′, and the 3′ primer, 5′-CCGCTCGAGATACATGTCTCTGTAGATCT-3′,[15] designed to contain *Nco*I and *Xho*I linkers. This PCR product was digested with *Nco*I and *Xho*I, and then cloned into an expression vector pQE-TriSystem (Qiagen, Tokyo, Japan). PPARα fused to 8× His-tag was expressed in M15 cells (Qiagen). To purify the fusion protein, bacterial extract was applied to Ni-NTA agarose beads and bound proteins were eluted with 50 mM sodium phosphate buffer, pH 8.4, containing 300 mM NaCl and 250 mM imidazole.

Proteins bound to PPARα-agarose. Two milligrams of purified His-tagged rat PPARα was incubated at 4°C overnight with 5 mL Ni-NTA agarose beads equilibrated with 25 mM Tris-HCl, pH 7.8, and set into a column (1.5 cm × 2.0 cm). Liver nuclear extracts were applied to the column, and proteins were eluted with 1 M NaCl in 25 mM Tris-HCl, pH 7.8.

Proteins bound to GST M1-1-Sepharose. The KS- and NC-type GST M1-1 was purified from rat livers as described previously.[11] Four milligrams of purified KS- and NC-type GST M1-1 was coupled to 4 mL CNBr-activated Sepharose 4B (Amersham Biosciences). After blocking, the resins were set into columns (1.5 cm × 2.0 cm) equilibrated with 20 mM

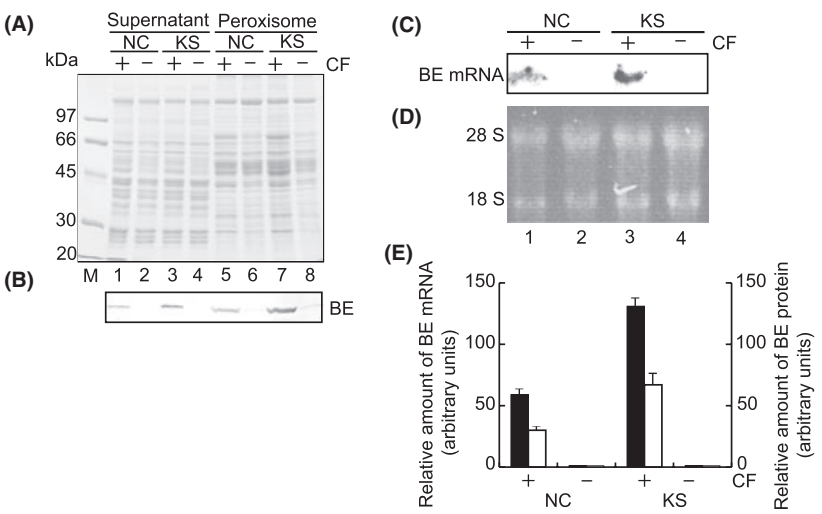

Fig. 1. Induction of bifunctional enzyme (enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase) (BE) protein and BE mRNA in NC- and KS-type rat livers by clofibrate (CF) treatment. Supernatant and peroxisome fractions from NC- and KS-type rat livers treated with or without CF were subjected to SDS-PAGE then stained with Coomassie Brilliant Blue R-250 (A) or immunoblotted with anti-BE antibody (B). Lane M, molecular mass markers. Northern blotting for BE mRNA using total RNAs from the same liver samples was carried out (C). 28S and 18S ribosomal RNA were stained with ethidium bromide (D). BE protein and mRNA quantified with a scanning densitometer were expressed relative to the value of control NC-type (E). Closed bars, BE mRNA value; open bars, BE protein value in peroxisome fraction. Values are expressed as means ± SD (n = 3).

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Tris-HCl, pH 7.8. Nuclear extracts from KS- and NC-type rat livers were applied to these columns, and bound proteins were eluted with 0.2 M NaCl in 20 mM Tris-HCl, pH 7.8.

Immunoprecipitation. Anti-BE antibody (800 μg protein) was coupled to 2 mL CNBr-activated Sepharose 4B at 4°C overnight, according to the manufacturer's instructions. Anti-BE antibody-Sepharose was incubated with liver nuclear extracts. After washing, bound proteins were eluted with SDS sample buffer.

Electrophoretic mobility shift assay. The PPRE of the rat BE gene enhancer between −2952 and −2918, 5′-AGCTT-TCTTTTGACCTATTGAACTATTACCTACATTTGAGG-3′,[33] was synthesized and labeled with [γ-^{32}P] ATP and used as a probe for EMSA. The assay was carried out according to the method described by Sanguino *et al.*[34] Supershift experiments were carried out by adding anti-PPARα (sc-9000 X), anti-BE, anti-TH, anti-SRC-3, or anti-GST M1-1 antibodies. Samples were loaded on a 4% non-denaturing polyacrylamide gel in 0.5× TBE and electrophoresed at 4°C and 200 V for 180 min. The gel was dried and visualized by autoradiography.

Immunohistochemical staining. Paraffin sections were passed through xylene and a graded alcohol series and stained for BE using the avidin-biotin-peroxidase complex (ABC) method, as reported previously.[13]

Results

Enhanced BE induction in KS-type rat livers by CF treatment. To study whether the extent of BE induction by CF is different between the polymorphic rats, supernatant and peroxisome fractions from the individual rat livers were subjected to SDS-PAGE. Protein staining revealed that proteins with subunit molecular mass 72 kDa, 48 kDa, 42 kDa, 32 kDa, and 30 kDa were increased in the peroxisome fraction of CF-treated rats, as compared with the control rats (Fig. 1A). The extents of induction of these proteins were higher in the KS-type rats than in NC-type rats. Immunoblotting with anti-BE antibody revealed the 72 kDa protein to be BE (Fig. 1B, lanes 5 and 7). BE mRNA levels evaluated by Northern blotting (Fig. 1C,D) were induced 131-fold in the KS-type and 59-fold in NC-type (Fig. 1E, closed bars). By densitometry, BE protein was induced 67-fold in the KS-type and 30-fold in the NC-type (Fig. 1E, open bars).

To clarify factors involved in different BE induction between the KS- and NC-type rat livers, PPARα levels in nuclear extract were examined by immunoblotting (Fig. 2A,B). The levels were induced 2.5–3-fold by CF treatment in both types, but not different between them. The levels of coactivators for PPARα were also analyzed. SRC-1, SRC-2, and PBP were not induced by CF treatment and their levels were not different between the polymorphic rats (Fig. 2C,D,F). On the other hand, SRC-3 was higher in the control KS-type than in NC-type (Fig. 2E, lanes 3 and 4), but not increased by CF treatment. Both BE and GST M1-1 were detected in the nuclear extract of CF-treated livers and the amounts were higher in the KS-type than in the NC-type (Fig. 2G,H, lanes 1 and 3). The other GST, subunits A1 or A2, were not detected in either type (Fig. 2I). Quantitative data of PPARα and SRC-3 were presented in Fig. 2J.

Binding of SRC-3, BE, and TH to PPARα in CF-treated KS-type livers. To investigate proteins with an affinity for PPARα, nuclear extracts from CF-treated livers were applied to a PPARα-agarose column. Bound proteins were subjected to SDS-PAGE. Protein bands with the molecular mass of 110, 72, and 42 kDa (indicated with asterisks in Fig. 3A) were detected in the CF-treated but not control KS-type. These proteins were identified as SRC-3 (Fig. 3B), BE (Fig. 3C), and TH (Fig. 3D), respectively, by immunoblotting. Low amounts of BE and TH were detected in CF-treated NC-type. GST M1-1 of either type was not bound to PPARα (Fig. 3E). To confirm the binding of BE to PPARα, immunoprecipitates of nuclear extracts from CF-treated livers using anti-BE antibody-Sepharose were examined by immunoblotting with anti-PPARα antibody. The result confirmed their binding (Fig. 3F). However, the intensity of PPARα band was less in CF-treated KS-type livers than in NC-type. This finding might reflect the higher amount of free BE, but the lower amount of PPARα-bound BE, in the KS-type. Analysis

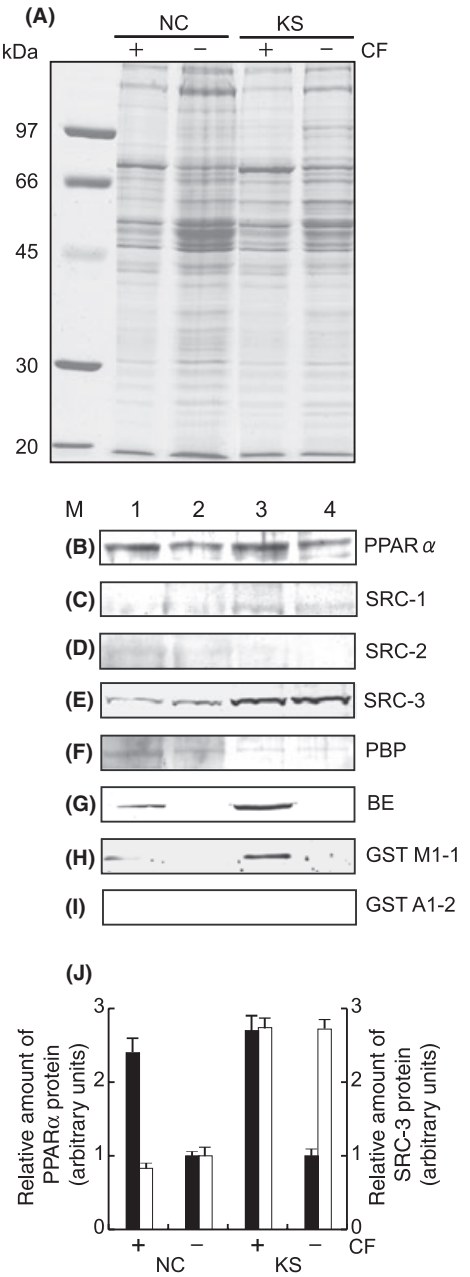

Fig. 2. Protein levels of peroxisome proliferator-activated receptor α (PPARα), coactivators, and GST M1-1 in KS- and NC-type rat liver nuclei. Nuclear extracts prepared from NC- and KS-type livers were subjected to SDS-PAGE and stained with Coomassie Brilliant Blue R-250 (A) or immunoblotted with anti-PPARα antibody (B), anti-steroid receptor coactivator (SRC)-1 antibody (C), anti-SRC-2 antibody (D), anti-SRC-3 antibody (E), anti-PPAR binding protein (PBP) antibody (F), anti-bifunctional enzyme (enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase) (BE) antibody (G), anti-GST M1-1 antibody (H), and anti-GST A1/A2 antibody (I). Lane M, molecular mass markers. PPARα and SRC-3 bands in immunoblotting were quantified (J). Closed bars, PPARα protein; open bars, SRC-3 protein. Values are expressed as means ± SD (n = 3).

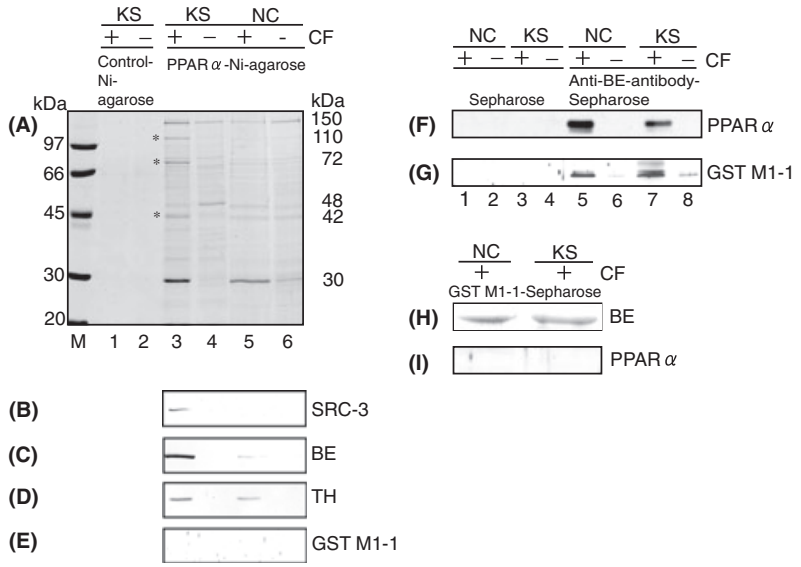

Fig. 3. Binding of steroid receptor coactivator (SRC)-3, bifunctional enzyme (enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase) (BE) and 3-ketoacyl-CoA thiolase (TH) to peroxisome proliferator-activated receptor α (PPARα) in clofibrate (CF)-treated KS-type rat livers. Nuclear extracts from CF-treated KS- and NC-type livers were applied to His-tagged PPARα-agarose column, and bound proteins were subjected to SDS-PAGE and stained with Coomassie Brilliant Blue R-250 (A), or immunoblotted with anti-SRC-3 antibody (B), anti-BE antibody (C), anti-TH antibody (D), or anti-GST M1-1 antibody (E). Lane M, molecular mass markers. (F,G) Binding of PPARα or GST M1-1 to BE. A pull-down assay with anti-BE antibody was subjected to immunoblotting with anti-PPARα antibody (F) and anti-GST M1-1 antibody (G). (H,I) Binding of BE to GST M1-1. Peroxisome fractions from CF-treated NC- and KS-type livers were applied to KS-type GST M1-1-Sepharose column. Bound fractions were subjected to immunoblotting with anti-BE antibody (H). Nuclear extracts from CF-treated NC and KS-type livers were applied to the column and bound fractions were subjected to immunoblotting with anti-PPARα antibody (I).

using the same antibody-Sepharose also indicated the binding of both KS- and NC-type GST M1-1 to BE (Fig. 3G, lanes 5 and 7). To confirm their association, nuclear extracts of CF-treated livers were applied to the KS-type or NC-type GST M1-1-Sepharose column and bound fractions were examined by immunoblotting with anti-BE antibody. The results confirmed the binding of BE to the KS (Fig. 3H) and NC-type GST M1-1 (data not shown), whereas PPARα was not bound to either type of GST M1-1 (Fig. 3I).

Binding of BE, TH, and SRC-3 to PPARα–PPRE of BE enhancer. PPARα binds to the PPRE of BE enhancer to activate its transcription. To examine whether BE and SRC-3 can bind to the PPARα–PPRE complex, their binding was evaluated by EMSA. Nuclear extracts from CF-treated KS- and NC-type livers were subjected to EMSA with a radiolabeled oligonucleotide probe for PPARα. Of two bands designated 1 and 2, band 2 was increased 6-fold in both types, compared with the respective control (Fig. 4). Both bands were specific for the PPRE, because these were lost with increasing amounts of unlabeled probe. To determine whether the bands include PPARα and BE, EMSA was carried out in the presence of specific antibodies. Band 2 was barely decreased by anti-PPARα antibody or anti-BE antibody in CF-treated NC-type (Fig. 5A,D), but was decreased 32–40% in the KS-type (Fig. 5-B,C,E,F). Addition of anti-TH antibody (data not shown) or anti-SRC-3 antibody showed similar results as the case of anti-BE antibody, with a selective decrease in the KS-type (Fig. 5G,H,J). Thus, although band 2 showed similar electrophoretic mobility between the polymorphic rats (Fig. 4), the band of KS-type seemed to contain SRC-3, BE, and TH, whereas that of NC-type did not. The partial inhibition in these supershift assays might reflect steric hindrance by bound proteins, including BE and SRC-3. The band was not altered in either the KS or NC-type by anti-GST M1-1 antibody (data not shown). In the control KS-type, anti-SRC-3 antibody did not affect the band (Fig. 5I).

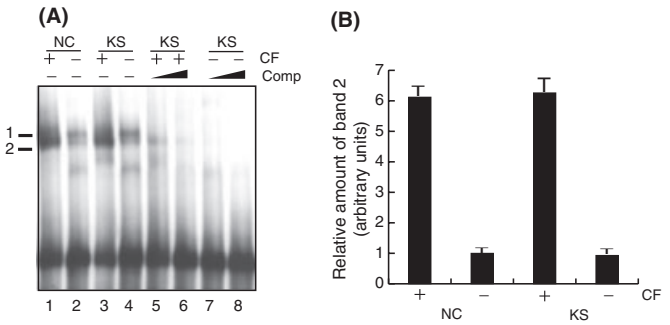

Fig. 4. Electrophoretic mobility-shift assay of peroxisome proliferator responsive element (PPRE) with nuclear extracts from NC- and KS-type rat livers. (A) EMSA was carried out with a ^{32}P-labeled PPRE oligonucleotide and liver nuclear extracts. The bands 1 and 2 denote the shifted bands. Lanes 5–8 denote the competition (Comp) experiments carried out in the presence of 50- and 200-fold excess of unlabeled oligonucleotide. (B) Relative intensities of band 2, expressed relative to the value of control NC-type. Values are expressed as means ± SD (n = 3). CF, clofibrate.

Enhanced acetylation of histone H3 in CF-treated KS-type livers. The peroxisomal fatty acid β-oxidation enzymes, including BE and TH, are involved in acetyl-CoA production. Furthermore, SRC-3 possesses histone acetyltransferase activity, using acetyl-CoA as a substrate. Thus, the binding of BE, TH, and SRC-3 to the PPARα–PPRE complex in CF-treated KS-type livers raised the possibility of histone acetylation. To examine this, acetylation of histone H3 at lysine 9 or lysine 14 was evaluated by immunoblotting with specific antibody. By densitometry, histone H3 levels were increased 1.5–2-fold by CF treatment in both types (Fig. 6A,C, open bars), whereas acetyl-histone H3 was increased 11-fold in the KS-type but not in NC-type (Fig. 6B,C, closed bars).

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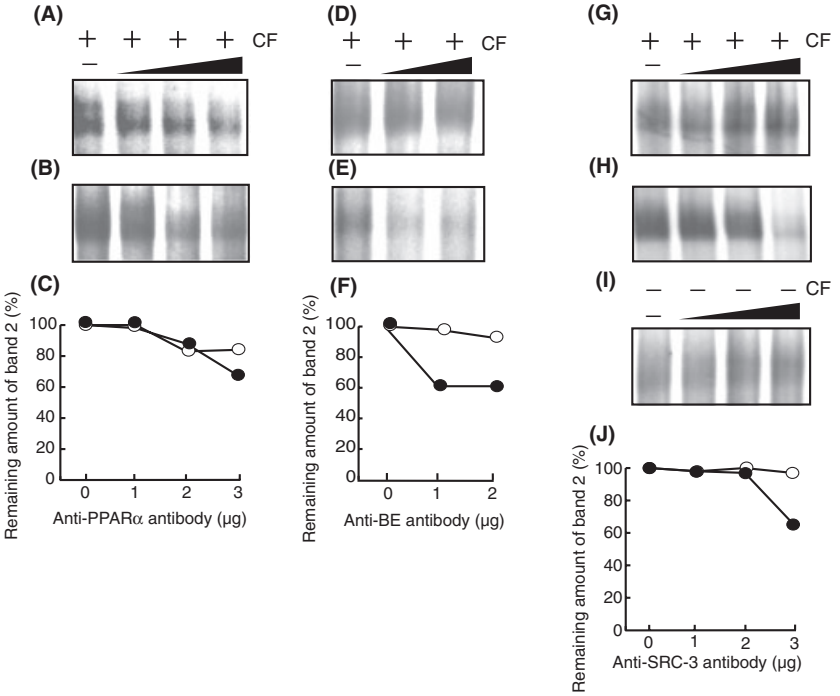

Fig. 5. Binding of steroid receptor coactivator (SRC)-3 and bifunctional enzyme (enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase) (BE) to peroxisome proliferator-activated receptor α (PPARα)–peroxisome proliferator responsive element (PPRE) complex in clofibrate (CF)-treated KS-type rat livers. EMSA using nuclear extracts from CF-treated NC- and KS-type livers was carried out in the presence of anti-PPARα antibody (A,B), anti-BE antibody (D,E), or anti-SRC-3 antibody (G,H). A, D, and G represent EMSA from the NC-type; B, E, and H from the KS-type. In the control, non-immune sera were added in place of specific antibodies. The same experiment using nuclear extract from control KS-type was carried out in the presence of anti-SRC-3 antibody (I). Changes in intensity of band 2 by the addition of anti-PPARα antibody (C), anti-BE antibody (F), or anti-SRC-3 antibody (J) were quantified. Closed circles, KS-type; open circles, NC-type.

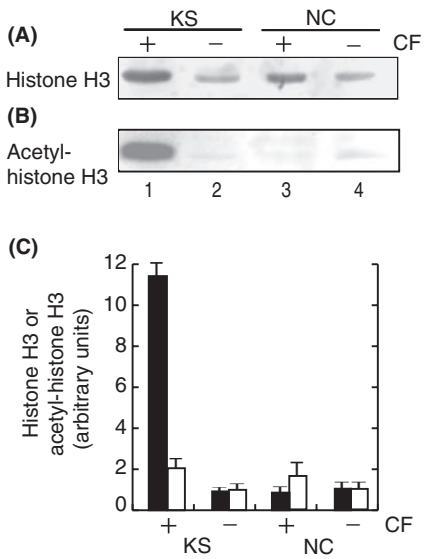

Fig. 6. Histone H3 acetylated at Lys 9 or Lys 14 in clofibrate (CF)-treated KS-type rat livers. Immunoblotting of nuclear extract was done with anti-histone H3 antibody (A), or anti-acetyl-histone H3 at Lys 9 or 14 antibody (B). Histone H3 and acetylated histone H3 bands in immunoblotting were quantified and expressed relative to the value of control NC-type (C). Open bars, histone H3; closed bars, acetyl-histone H3. Values are expressed as means ± SD (n = 3).

Nuclear localization of BE in CF-treated KS-type hepatocytes. BE is a peroxisomal enzyme, so immunohistochemistry was used to determine its nuclear localization. This confirmed enhanced BE expression in hepatocytes of CF-treated KS-type (Fig. 7A), as compared with those of the NC-type (Fig. 7B). The nuclei of some hepatocytes in the KS-type were heavily stained, but hardly stained in the NC-type. Control rats were negative in the KS-type (Fig. 7C) and NC-type (Fig. 7D). When pre-immune serum was used instead of anti-BE antibody, both the KS- and NC-type livers showed negative results (data not shown).

Discussion

In the present study, the extent of BE protein and mRNA induction by CF was higher in KS-type rats than in NC-type. PPARα was similarly induced in both types (Fig. 2B), so its level could not explain differences between them in BE induction. The SRC-3 level in the nuclear extract was higher in the KS-type, although it was not increased by CF treatment (Fig. 2E). The *SRC-3* gene is often overexpressed or amplified in breast and ovarian cancers,[35] and SRC-3 stimulates cell growth in prostate cancer cell lines.[36] SRC-3, BE, and TH in CF-treated KS-type livers were bound to PPARα, whereas those in the NC-type were hardly bound (Fig. 3). Furthermore, histone H3 was acetylated in CF-treated KS-type livers, but not in NC-type (Fig. 6). Because BE and TH are responsible for acetyl-CoA production[4,5] and SRC-3 catalyzes histone acetylation,[17,18] a higher BE induction in the KS-type seemed to be due to the acetylation-mediated transcriptional activation through the binding of BE, TH, and SRC-3 to PPARα. Although BE and TH are peroxisomal enzymes, nuclear localization of BE was determined by immunohistochemistry (Fig. 7). BE protein is reported to bind to PPARα to stimulate its transcriptional activity.[37] Because BE expression is regulated by PPARα,[15] the present results suggest that BE and TH proteins, by their ability to produce acetyl-CoA, enhance the transcriptional activity of PPARα, leading to further BE induction by the positive feedback mechanism.

BE and TH are peroxisomal enzymes, so they need to enter the nucleus to interact with PPARα. Therefore, some modifications of the peroxisomal enzymes or additional factors might be required for their migration into the nucleus. Higher amounts of GST M1-1 and BE were observed in the nuclear extracts of the KS-type than in the NC-type after CF treatment (Fig. 2G,H). Furthermore, GST M1-1 was associated with BE (Fig. 3G,H), but not with PPARα (Fig. 3I). These results suggest that higher amount of GST M1-1 migrates from cytoplasm to nuclei in the KS-type, with simultaneous BE movement by their association.

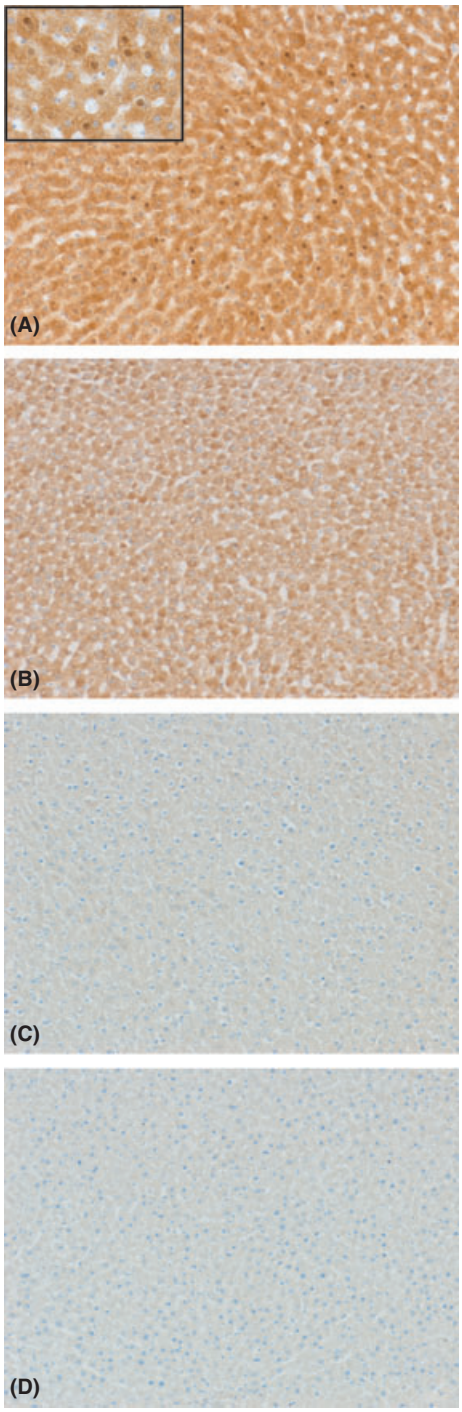

Fig. 7. Immunohistochemical staining of bifunctional enzyme (enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase) in clofibrate-treated KS-type (A) and NC-type rat livers (B), and control KS-type (C) and NC-type rat livers (D). Magnification, ×100; inset of (A), ×400.

For migration into the nucleus, post-translational modifications of KS-type GST M1-1 might be required. CF treatment results in the enhanced production of hydrogen peroxide,[4,5] and the KS-type GST M1-1 is activated by reactive oxygen species. However, the NC-type enzyme is not,[10] suggesting that the KS-type enzyme is sensitive to such modification. SRC-3 of the control KS-type was not bound to PPARα, therefore its binding (Fig. 3) might be dependent on some modifications of SRC-3 induced by CF treatment. SRC-3 is noted to be phosphorylated by MAPK[38] or IκB kinase and the phosphorylation promotes nuclear localization and transcriptional activation.[39]

Epigenetic alterations, including DNA methylation and modification of histones, are increasingly being recognized for their roles in carcinogenesis.[40] Acetylation of histones on the promoters of various proto-oncogenes might cause their transcriptional activation, whereas deacetylation of those on tumor suppressor genes results in transcriptional silencing. Thus, epigenetic alterations might mimic the effects of mutations of proto-oncogenes or tumor suppressor genes, leading to carcinogenesis.[41,42] Expression analysis of these genes in CF-treated rats is now in progress in our laboratory. Although the sample number is still small, the levels of *Myc*, *Jun*, *Hras*, *Kras*, *Tp53*, and *Rb1* are unlikely to differ between the polymorphic rats but further study is needed to establish such a relationship.

A recent study by Pogribny *et al.* reported the epigenetic effects of a PP on mouse liver DNA and histones: global hypomethylation of DNA and the methylation of both lysine 9 of histone H3 and lysine 20 of histone H4.[43] The present study indicates the acetylation of histone H3 by CF treatment and raises a possible link between the extent of histone modification and different BE inducibility in the polymorphic rats. PPs are non-genotoxic rodent carcinogens and their carcinogenic processes are suggested to differ from the mutagenic processes of genotoxic carcinogens.[44,45] Oxidative DNA damage is proposed as a causative event for PP-induced carcinogenesis.[46,47] The present results raise a possible relationship between the activating histone modification and CF-induced hepatocarcinogenesis.

Acetylation of core histones plays important roles for transcriptional activation. However, it remains to be clarified how acetyl-CoA, a substrate for the acetylation reaction, is produced in or transported into the nucleus. A cytosolic enzyme, ATP-citrate lyase, was recently proposed as a candidate for acetyl-CoA production for histone acetylation in response to growth factor stimulation.[48] The present study suggests that BE and TH in the nucleus might be involved in its production in CF-treated rat livers.

In conclusion, the binding of BE, TH, and SRC-3 to PPARα and histone acetylation are shown in CF-treated KS-type livers and this might be involved in marked BE induction. The present findings suggest that epigenetic mechanisms play an important role in PP-induced rat hepatocarcinogenesis.

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Abbreviations

BE	bifunctional enzyme (enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase)
CF	clofibrate
KS	^{198}Lys-^{199}Ser type of rat GST M1
NC	^{198}Asn-^{199}Cys type of rat GST M1
PBP	PPAR binding protein
PP	peroxisome proliferator
PPARα	PP-activated receptor α
PPRE	PP responsive element
SRC	steroid receptor coactivator
TH	3-ketoacyl-CoA thiolase

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