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Role of RSK1 in Oltipraz-Induced Specific Phosphorylation of C/EBPβ for *GSTA2* Gene Transactivation

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Running Title: RSK1-Dependent C/EBPβ Activation by Oltipraz

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Abbreviations: C/EBPβ, CCAAT/enhancer binding protein-β; ChIP, chromatin immunoprecipitation; CTT-RSK, C-terminal truncated-RSK; EGF, epidermal growth factor; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; MEM, minimal essential medium; PDK1, 3-phosphoinositide-dependent protein kinase-1; PMSF, phenylmethylsulfonylfluoride; PI3-kinase, phosphatidylinositol 3-kinase; RSK, p90-ribosomal S6-kinase; SDS, sodium dodecylsulfate

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Abstract

Oltipraz, which has been extensively studied as a cancer chemopreventive agent, promotes phosphatidylinositol 3-kinase (PI3-kinase)-mediated activation of CCAAT/enhancer binding protein-β (C/EBPβ). Activated p90 ribosomal S6-kinase-1 (RSK1) phosphorylates major transcription factors including C/EBPβ. This study examined whether oltipraz induces phosphorylation of C/EBPβ at specific residues and if so, whether RSK1 regulates C/EBPβ phosphorylation by oltipraz for the *GSTA2* gene transactivation. Subcellular fractionation and immunoblot analyses revealed that oltipraz treatment increased the level of C/EBPβ phosphorylated at Ser^{105} in the cytoplasm, which translocated to the nucleus for DNA binding in rat H4IIE cells. Immunoprecipitation-immunoblot, chromatin-immunoprecipitation and specific mutation analyses revealed that Ser^{105}-phosphorylated C/EBPβ recruited CBP for histone acetylation and transactivation of the *GSTA2* gene. The role of RSK1 in Ser^{105}-phosphorylation of C/EBPβ by oltipraz and its gene transactivation was evidenced by transfection experiments with dominant negative mutants of RSK1. In mouse Hepa1c1c, human HepG2 cells, and rat primary hepatocytes, oltipraz induced phosphorylation of C/EBPβ at Thr^{217}, Thr^{266} and Ser^{105}, respectively, via RSK1. The experiment using small interference RNA of RSK1 confirmed the essential role of RSK1 in the gene expression. Inhibition of PI3-kinase activity prevented oltipraz-inducible Ser^{105}-phosphorylation of rat C/EBPβ. Oltipraz treatment led to increases in the catalytic activity and nuclear translocation of RSK1, which was abrogated by PI3-kinase inhibition. In summary, oltipraz induces phosphorylation of rat C/EBPβ at Ser^{105} (functionally analogous $Thr^{217/266}$ in mouse and human forms) in hepatocytes, which results in CBP recruitment for the *GSTA2* gene transactivation, and the specific C/EBPβ phosphorylation is mediated by RSK1 downstream of PI3-kinase.

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Introduction

Oltipraz (5-[2-pyrazinyl]-4-methyl-1,2-dithiol-3-thione) has been extensively studied as a cancer chemopreventive agent for malignancies including liver and colorectal cancer (Kensler, 1997; Rao et al., 1993). In experimental cancer prevention studies, oltipraz reduced tumor incidence and multiplicity (Kensler, 1997; Roebuck et al., 1991; and Bolton et al., 1993). A phase IIa randomized chemoprevention trial of oltipraz in residents of Qidong, China, supported that oltipraz might be clinically active as a chemopreventive agent (Wang et al., 1999; Jacobson et al., 1997). Comprehensive mechanistic studies suggest that oltipraz exerts cancer chemopreventive effects through the induction of glutathione S-transferase, a representative phase II detoxifying enzyme (Kensler, 1997; Jacobson et al., 1997).

The family of C/EBPs plays important roles in regulating the expression of hepatocyte-specific genes, particularly those associated with cell survival or proliferation (Buck and Chojkier, 2003; Diehl, 1998). We reported that oltipraz promotes nuclear translocation of CCAAT/enhancer binding protein-β (C/EBPβ) and its DNA binding activity for transactivation of the *GSTA2* gene, and that the pathway of phosphatidylinositol 3-kinase (PI3-kinase) regulates the activation of C/EBPβ (Kang et al., 2003; Cho and Kim, 2003). In addition, we observed that mitogen-activated protein kinases (MAPKs) including ERK1/2 were not activated by oltipraz nor involved in C/EBPβ-mediated gene expression (Kang et al., 2003). Yet, the kinase(s) responsible for the activation of C/EBPβ by oltipraz remained to be elucidated. Therefore, we proposed the hypothesis that oltipraz activates C/EBPβ by phosphorylation at specific site(s), which may be mediated by cellular kinase(s) downstream from PI3-kinase.

The members of p90 ribosomal S6-kinase (RSK) family play a critical role in mitogen-activated cell growth, differentiation or cell survival (Frodin and Gammeltoft, 1999; Bhatt and Ferrell, 1999; Gross, et al., 1999). Among the RSK isoforms, RSK1 is a major form expressed in the tissues including liver, muscle and fat (Moller et al., 1994). The RSK1 contains functionally active two distinct kinase domains, and the N-terminal kinase of activated RSK1 phosphorylates the cellular protein substrates including C/EBPβ, CREB, c-Fos, and IκB (Frodin and Gammeltoft, 1999, Buck et al., 1999; Xing, et al., 1996; Chen, et al., 1993; Schouten, et al., 1997; Ghoda, et al., 1997). Activation of RSK1 by growth factor requires extracellular signal-regulated kinase (ERK) docking near the C-terminus region (Roux et al.,

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2003), and the activated C-terminal kinase domain leads to autophosphorylation located in the linker region (Vik and Ryder, 1997). Another phosphorylation by 3-phosphoinositide-dependent protein kinase-1 (PDK1) in the activation loop of the N-terminal kinase domain allows RSK1 to phosphorylate the target proteins (Jensen, et al., 1999; Richards et al., 1999; Williams et al., 2000).

Receptor-activated signaling pathways regulate phosphorylation of C/EBPβ in its activation domain (Buck and Chojkier, 2003), which leads to the transcription of its target genes. It has been shown that RSK activated downstream from the TGFα receptor tyrosine kinase induces phosphorylation of C/EBPβ at specific residues such as Thr^{217} in the mouse form (Buck et al., 1999). Phosphorylation of Thr^{217} residue in mouse C/EBPβ (Thr^{266} in the human form) turned out to be essential for transactivation of target genes (Buck and Chojkier, 2003). Because rat C/EBPβ has evolved with a double mutation and thus lacks the phosphoacceptor, the C/EBPβ form has a compensatory Ser^{105}, whose phosphorylation is also catalyzed by RSK (Buck and Chojkier, 2003). Hence, Ser^{105} residue in rat C/EBPβ and functionally analogous residues, Thr^{217} and Thr^{266} in mouse and human forms, respectively, are the critical phosphoacceptors that are responsible for gene transactivation (Buck et al., 1999). Also, C/EBPβ phosphorylated at the specific residue by RSK1 non-transcriptionally prevents apoptosis of cells through interaction with procaspases (Buck et al., 2001). Hence, RSK1-mediated specific phosphorylation of C/EBPβ regulates cell survival.

In view of the activation of C/EBPβ by oltipraz and the essential role of Ser^{105} phosphorylation (analogous phosphorylations at $Thr^{217/266}$ in mouse and human) in gene transactivation, we were tempted to investigate whether oltipraz induces C/EBPβ phosphorylation at the residue for the *GSTA2* gene transactivation and if so, whether the phosphorylation of C/EBPβ is mediated by RSK1. We additionally determined the effects of oltipraz on Thr^{217} or Thr^{266} phosphorylation in the mouse and human forms of C/EBPβ, respectively, and the role of RSK1 in the phosphorylations. Also, we verified the specific C/EBPβ phosphorylation by RSK1 in primary cultured rat hepatocytes. Toward the end, we explored what the role of PI3-kinase is in RSK1-mediated C/EBPβ phosphorylation by oltipraz. Now, we report that oltipraz induces specific C/EBPβ phosphorylation for the *GSTA2* gene transactivation via RSK1 and that PI3-kinase contributes to the RSK1-mediated phosphorylation of C/EBPβ.

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Materials and Methods

Materials. [γ-^{32}P]ATP (3000 mCi/mmol) was purchased from PerkinElmer Life Science (Arlington Heights, IL). Anti-C/EBPα, anti-C/EBPβ, anti-C/EBPδ, anti-Ser^{105}-phosphorylated C/EBPβ (sc-16994-R), anti-Thr^{217}-phosphorylated C/EBPβ (sc-16993-R), anti-CBP, anti-RSK1, anti-HA, anti-Myc and anti-actin antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-acetylated histone antibody was purchased from Upstate (Waltham, MA). Anti-Thr^{189}-phosphorylated C/EBPβ, anti-ERK and anti-Thr$^{42/44}$-phosphorylated ERK antibodies were supplied from Cell Signaling Technology (Beverly, MA). Horseradish peroxidase-conjugated goat anti-rabbit and rabbit anti-goat IgGs were purchased from Zymed Laboratories (San Francisco, CA). U0126 was obtained from Alexis Corporation (Lausen, Switzerland). LY294002 and other reagents in the molecular studies were supplied from Calbiochem (Darmstadt, Germany). S6 rsk substrate peptide was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The plasmid of C/EBP-containing *GSTA2* promoter region (-1651 bp to +66 bp) was kindly provided from Dr. C.B. Pickett (Schering-Plough Corp Inst. Kenilworth, NJ). The plasmids encoding HA-C-terminal truncated (CTT)-RSK1 and HA-K112/464R-RSK1 were kind gifts from Dr. J. Blenis (Harvard Medical School, Boston, MA). The overexpression vector of p85 subunit of PI3-kinase was obtained from Dr. A. Toker (The Boston Biomedical Research Institute, Boston, MA). MKK1 dominant-negative mutant was a gift from Dr. N.G. Ahn (University of Colorado, Boulder, CO).

Cell Culture. Rat H4IIE, mouse Hepa1c1c, and human HepG2 cells were obtained from American Type Culture Collection (Rockville, MD). Primary hepatocytes were isolated from male Sprague-Dawley rats according to the previously published method with slight modifications (Buck et al., 2001, Kang et al., 2003). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS), 50 units/ml penicillin, and 50 μg/ml streptomycin at 37°C in a humidified atmosphere with 5% CO_2. After deprivation of serum for 24 h, the cells were incubated with oltipraz (CJ Corp., Korea), dissolved in dimethylsulfoxide, for the indicated time period at 37°C.

Subcellular Fractionation. Total cell lysates, cytosolic fractions and nuclear extracts were prepared according to previously published methods (Park et al., 2004). Briefly, cells were centrifuged at 2,300*g*

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for 3 min and allowed to swell after the addition of the lysis buffer. The lysate samples were centrifuged at 10,000*g* for 10 min to obtain cell lysates. To prepare cytosolic fractions and nuclear extracts, cells were centrifuged at 2,300*g* for 3 min and allowed to swell after the addition of 100 μl hypotonic buffer. The lysates were incubated for 10 min on ice and then centrifuged at 7,200*g* for 5 min at 4°C. The supernatants were used as cytosolic fractions. Pellets containing crude nuclei were resuspended in 50 μl of extraction buffer. Nuclear extracts were prepared from the samples by centrifugation at 15,000*g* for 10 min and stored -70°C until use. Protein content was determined by the Bradford assay (Bio-Rad® protein assay kit, Bio-Rad, Hercules, CA).

Immunoblot Analysis. Sodium dodecylsulfate (SDS)-polyacrylamide gel electrophoresis and immunoblot analysis were performed according to previously published procedures (Kang et al., 2003).

Gel Shift Assay. A double stranded probe containing the C/EBP consensus oligonucleotide was used for gel shift analysis after end-labeling of the probe with [γ-^{32}P]ATP and T_4 polynucleotide kinase, as described previously (Park et al., 2004, Kang et al., 2003). Specificity of binding was determined by competition experiments, known as immuno-inhibition assays. For immuno-inhibition assays, anti-Ser^{105}-phosphorylated C/EBPβ, anti-Thr^{189}-phosphorylated C/EBPβ or anti-Sp1 antibody (1 μg each) was added to the reaction mixture after initial 10-min incubation, and additionally incubated with the probe for 30 min at 25°C. Samples were loaded onto 4% polyacrylamide gels at 100 V. The gels were removed, fixed and dried, followed by autoradiography.

Immunoprecipitation. To determine the physical interaction of CREB binding protein (CBP) with Ser^{105}-phosphorylated C/EBPβ, a fraction of cell lysates (100 μg proteins in 300 μl) was incubated with a polyclonal rabbit anti-CBP antibody overnight at 4°C. The antigen-antibody complex was immunoprecipitated following incubation for 2 h at 4°C with protein G-agarose. Immune complex was solubilized in 2 × Laemmli buffer and boiled for 5 min. Samples were separated and analyzed using 7.5% SDS-PAGE and then transferred to nitrocellulose membranes. The samples were then immunoblotted with antibodies directed against Ser^{105}- or Thr^{189}-phosphorylated C/EBPβ. Blots were developed using an ECL chemiluminescence detection kit.

Chromatin Immunoprecipitation (ChIP) Assays. H4IIE cells were treated with oltipraz for 12 h,

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and then formaldehyde was added to the cells to a final concentration of 1%. Ser^{105}-phosphorylated C/EBPβ, CBP or acetylated histone was cross-linked to chromatin by incubating the cells for 10 min at 37°C. The cells were washed with ice-cold phosphate-buffered saline, and lysed in the Tris-HCl buffer (50 mM, pH 8.1) containing 1% SDS and 10 mM EDTA. The lysates were sonicated and centrifuged at 10,000g for 10 min to remove debris. The supernatants containing chromatin were diluted with 10 volumes of the ChIP dilution buffer [Tris-HCl (16.7 mM, pH 8.1), 167 mM NaCl, 1.2 mM EDTA, 0.01% SDS and 1.1% Triton X-100]. One-tenth of the chromatin solution was reserved for total input. The remaining solution was precleared with protein G-agarose, subsequently incubated with each antibody (1 μg) for 12 h at 4°C with shaking, and then further incubated with protein G-agarose for 2 h. The immunoprecipitates were washed, reverse cross-linked by adding 5 M NaCl to a final concentration of 200 mM and incubated for 4 h at 65°C, as described previously (Duong et al., 2002). ChIP assay was also carried out with anti-Thr^{189}-phosphorylated C/EBPβ antibody, which was used as a negative control. DNA was phenol-chloroform-extracted. PCR was performed with specific primers flanking the C/EBP binding site in the *GSTA2* gene promoter (sense: 5'-GGACAACACACTCAGCTTTG-3', antisense: 5'-TCAGTGCAGCCTGTGAGTC-3') or flanking the *β-actin* gene promoter (sense: 5'-CGTTCCGAAATTGCCTTTTA-3', antisense: 5'-GGAGCTGCAAGGAGGTTGTA-3'). Amplified fragments (347 bp, 121 bp) were analyzed on a 2% agarose gel.

Mutagenesis Assay. C/EBPβ amplified from pCDNA3.1(+)-rat C/EBPβ (Cho and Kim, 2003) using specific primers was inserted into pGEM-T vector (Promega, Madison, WI) and subcloned into the *BamHI/HindIII* sites of the pCMV-Tag3A plasmid (Stratagene, La Jolla, CA). Specific base substitution was made by oligonucleotide-mediated mutagenesis according to the manufacturer's instruction (Stratagene, La Jolla, CA). Ser^{105} residue in rat C/EBPβ was mutated to alanine using a mutagenic primer (5'-GTAACCGTAGTCGGCCGGCTTCTTGCTCGG-3'). The DNA sequence was verified by using an automatic DNA sequence analyzer.

Transient Transfection and pGL-1651 Promoter-Luciferase Assay. To determine the activity of C/EBPβ-mediated target gene transactivation, we used the pGL1651-luciferase reporter assay system according to the previously published procedures (Cho and Kim, 2003; Park et al., 2004). Cells were

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transiently transfected with pGL1651-promoter luciferase construct in combination with the plasmid of pCMV-Tag3A-C/EBPβ or pCMV-Tag3A-C/EBPβ-S105A (Ala^{105} mutant of rat C/EBPβ). In some experiments, HA-CTT-RSK1 or HA-K112/464R-RSK1 plasmid was cotransfected with the pGL-1651 construct. Briefly, cells (5×10^5 cells/well) were re-plated in six-well plates overnight, serum-starved for 6 h, and transiently transfected with 1 μg of pGL-1651-luciferase construct and 0.3 μg of pCMV-β-galactosidase plasmid (Invitrogen, Carlsbad, CA) in the presence of Lipofectamine® Reagent (Life Technologies, Gaithersburg, MD) for 3 h. The pCMV-β-galactosidase plasmid was used to evaluate the transfection efficiency. Transfected cells were incubated in DMEM containing 1% FCS for 3 h and exposed to oltipraz for 18 h at 37°C. For β-galactosidase activity, 10 μg of cell lysates was added to the solution containing 0.88 mg/ml *o*-nitrophenyl-β-D-galactopyranoside, 100 μM $MgCl_2$, and 47 mM β-mercaptoethanol in 100 mM sodium phosphate buffer. The reaction mixture was incubated for 12 h at 37°C and the absorbance was determined at 420 nm. The relative luciferase activity was calculated by normalizing firefly luciferase activity to that of β-galactosidase.

H4IIE, Hepa1c1c, HepG2 cells or primary cultured rat hepatocytes were also transiently transfected with the plasmid encoding HA-CTT-RSK1 or HA-K112/464R-RSK1 and incubated for the indicated time period to assess the extent of C/EBPβ phosphorylation.

Knock-down Experiment using siRNA. To knock-down RSK1, HepG2 cells were transfected with the siRNA against human RSK1 [*Silencer*® Validated siRNA against RSK1, ID:354, Ambion (Austin, TX)]. pGL-1651-promoter luciferase construct was co-transfected with the RSK siRNA or a non-specific scRNA (100 pmole/ml) using Lipofectamine 2000 according to the manufacturer's instructions. On day 3 after transfection, the cells were incubated with oltipraz for 18 h. The whole lysates were used for the luciferase activity assay. Immunoblot analysis confirmed RSK1 knock-down 3 days after transfection.

Stable Transfection. For the preparation of PI3-kinase p85 [p85(+)] or MKK1 dominant-negative mutant [MKK1(-)] cells, H4IIE cells were transfected with the respective plasmid and incubated for 48 h, as described previously (Kang et al., 2003, Cho and Kim, 2003). Geneticin was added to select the resistant colonies.

RSK1 Kinase Assay. Cells that had been incubated in the medium without serum for 24 h were

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treated with vehicle or oltipraz (30 μM) for the indicated time period, harvested, and lysed in the buffer containing Tris-HCl (25 mM, pH 7.4), 2 mM dithiothreitol, 10 mM $MgCl_2$, 5 mM β-glycerophosphate, 1 mM Na_3VO_4 and 1 mM PMSF. RSK1 in cell lysates (300 μg) was immunoprecipitated with anti-RSK1 antibody. Immunoprecipitates were washed three times in lysis buffer, and once in kinase buffer containing Tris-HCl (25 mM, pH 7.4), 10 mM $MgCl_2$, 25 mM β-glycerophosphate, 1 mM Na_3VO_4, 2 mM dithiothreitol, 1 mM PMSF, 1 μg/ml leupeptin and 200 μM ATP. Kinase reaction was initiated by adding S6 rsk substrate peptide (5 μg per assay) and 2 μCi of [γ-^{32}P]ATP to a 20 μl reaction mixture, and continued for 30 min at 30°C. After brief centrifugation, the supernatant of reaction mixture was spotted onto p81 phosphocellulose paper (Upstate, Waltham, MA). The paper was washed with 0.8% phosphoric acid for 5 min three times and subsequently with 90% ethanol for 5 min. The membrane was dried and transferred to 5 ml of scintillation cocktail, and the radioactivity of phosphorylated substrate was measured using a β-counter (Wallac, Gaithersburg, MD).

Statistical Analysis. Scanning densitometry of the immunoblots was performed with Image Scan & Analysis System (Alpha-Innotech Corporation, San Leandro, CA). The area of each lane was integrated using the software AlphaEase™ version 5.5, followed by background subtraction. One way analysis of variance (ANOVA) was used to assess statistical significance of differences among treatment groups. For each statistically significant effect of treatment, the Newman-Keuls test was used for comparisons between multiple group means. The data were expressed as means ± S.E. The criterion for statistical significance was set at $p<0.05$ or $p<0.01$.

Results

Induction of C/EBPβ by Oltipraz. Our previous study showed that oltipraz induces nuclear translocation of C/EBPβ and promotes C/EBPβ binding to the C/EBP binding site (Kang et al., 2003). In view of the importance of C/EBPs as transcriptional factors, we sought to determine the expression of major forms of C/EBP in H4IIE cells treated with oltipraz. Immunoblot analysis revealed that the levels of C/EBPβ were increased 12-48 h after oltipraz treatment, as compared to untreated control (Fig. 1), whereas those of C/EBPα and δ forms were unaffected. Thus, activation of C/EBPβ by oltipraz involves

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induction of C/EBPβ.

Alignment of RSK1- or ERK-phosphorylated Residues in Rat, Mouse and Human C/EBPβ. Activation of C/EBPβ for gene transcription involves phosphorylation of specific residues in its activation domain. In Fig. 2A, parts of the sequences of rat, mouse and human homologous forms of C/EBPβ were aligned and specific phosphorylation residues with numbers were indicated. RSK1 induces phosphorylation of Ser^{105} in rat C/EBPβ (Buck et al., 1999). Because Ser^{105} in rat C/EBPβ is replaced with alanine in the mouse and human homologous forms, phosphorylation residues activated by RSK1 should differ in these species. Thr^{217} is the RSK1-induced phosphorylation residue in mouse C/EBPβ (Buck et al., 1999), which is functionally analogous to Ser^{105} in rat C/EBPβ (Fig. 2B). Thr^{266} in human form of C/EBPβ is equivalent to Thr^{217} in the mouse form. Activated ERK directly phosphorylates other residues, namely Thr^{189}, Thr^{188} and Thr^{235} in rat, mouse and human forms of C/EBPβ, respectively (Fig. 2B)(Nakajima et al., 1993).

Ser^{105} Phosphorylation of C/EBPβ by Oltipraz. Immunoblot experiments were conducted with cell lysates to determine whether the levels of rat C/EBPβ (38 kDa and 35 kDa) phosphorylated at the residue of Ser^{105} or Thr^{189} were increased. Oltipraz only minimally increased Ser^{105} phosphorylation of 38 kDa C/EBPβ in lysates. Hence, in subsequent studies we focused on the phosphorylation of 35 kDa form. The levels of Ser^{105}-phosphorylated 35 kDa C/EBPβ were enhanced 6-24 h after treatment of cells with oltipraz, whereas those of Thr^{189}-phosphorylated C/EBPβ were unchanged (Fig. 3A). The results provided evidence that 35 kDa C/EBPβ was phosphorylated at Ser^{105}, but not Thr^{189}, by oltipraz treatment in H4IIE cells. Next, the localization of C/EBPβ was determined by subcellular fractionations and immunoblot analyses. Phosphorylated C/EBPβ at Ser^{105} was located predominantly in the cytoplasm of H4IIE cells treated with oltipraz for 6 h (Fig. 3A, middle). However, when cells were treated with oltipraz for 12 h, Ser^{105}-phosphorylated C/EBPβ showed nuclear localization to a greater extent. At 24 h of oltipraz treatment, Ser^{105}-phosphorylated C/EBPβ was found in both the cytoplasm and the nucleus (Fig. 3A, middle and right). The levels of Thr^{189}-phosphorylated C/EBPβ in nuclear or cytoplasmic fractions were unchanged after oltipraz treatment.

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Next, we confirmed formation of C/EBPβ-DNA binding complexes after oltipraz treatment (Fig. 3B). To determine whether increase in the band intensity by oltipraz obtained in gel shift assays occurred as a result of phosphorylation of C/EBPβ at Ser^{105}, immuno-inhibition experiment was performed with the antibody directed against Ser^{105}- or Thr^{189}-phosphorylated C/EBPβ. Presence of anti-Ser^{105}-phosphorylated C/EBPβ antibody completely abolished the band intensity of the C/EBPβ-DNA binding complex, whereas either anti-Thr^{189}-phosphorylated C/EBPβ or anti-Sp1 antibody failed to do so (Fig. 3B). The antibody competition assays verified that oltipraz-induced C/EBPβ-DNA binding activity is specifically dependent on Ser^{105}-phosphorylated C/EBPβ. These data provided evidence that oltipraz treatment led to an increase in C/EBPβ phosphorylation at Ser^{105}, but not Thr^{189}, in H4IIE cells and that Ser^{105}-phosphorylated C/EBPβ served as an active form in the formation of C/EBPβ-DNA binding complex.

Association of Ser^{105}-Phosphorylated C/EBPβ with CBP. To investigate whether increase in Ser^{105} phosphorylation of C/EBPβ enhanced recruitment of CBP coactivator for gene transactivation, H4IIE cells were serum-starved for 24 h, and then treated with oltipraz for the indicated time periods. Nuclear extracts prepared from untreated cells or cells treated with oltipraz were immunoprecipitated with anti-CBP antibody and then immunoblotted with anti-Ser^{105}-phosphorylated C/EBPβ antibody. Formation of CBP-Ser^{105}-phosphorylated C/EBPβ complex was increased after stimulation of cells with oltipraz for 12-24 h (Fig. 4A), during which time period the level of Ser^{105}-phosphorylated C/EBPβ in the nuclear fraction maximally increased (Fig. 4A, right). In contrast, Thr^{189}-phosphorylated C/EBPβ, immunoprecipitated with CBP, was unchanged. These data provide evidence that oltipraz treatment enhances the level of Ser^{105}-phosphorylated C/EBPβ that is capable of interacting with CBP coactivator.

Next, to determine association of Ser^{105}-phosphorylated C/EBPβ with CBP on the target gene promoter, we performed ChIP analysis. The DNA-protein complexes were immunoprecipitated with anti-Ser^{105}-phosphorylated C/EBPβ, anti-CBP or anti-acetylated histone antibody, followed by reversal of cross-linking and PCR amplification using primers flanking the proximal and distal regions of the DNA comprising the C/EBP binding site in the *GSTA2* gene promoter (Fig. 4B). In the cells treated with oltipraz (12 h), the intensities of the three PCR products were all distinctly higher compared with

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vehicle-treated control. The intensities of the PCR products using primers flanking the proximal and distal regions of the *β-actin* gene promoter (a house-keeping gene) were unaffected by oltipraz. In the sample immunoprecipitated with anti-acetylated histone antibody, the intensity of the PCR product from the *β-actin* gene was intense in control cells, but was not further increased after oltipraz treatment (Fig. 4B). In contrast to the result obtained with Ser^{105}-phosphorylated C/EBPβ immunoprecipitate, the band intensity in Thr^{189}-phosphorylated C/EBPβ immunoprecipitate was not enhanced by oltipraz.

Next, we assessed the functional role of Ser^{105} phosphorylation of C/EBPβ by oltipraz in target gene transactivation by the specific mutagenesis assay. Oltipraz treatment was capable of increasing luciferase expression from the pGL-1651 *GSTA2* promoter that contains the C/EBP binding site (Fig. 4C, left). Similarly, the expression of Myc-C/EBPβ promoted the gene transcription. Exposure of cells transfected with the myc-C/EBPβ plasmid to oltipraz for 18 h resulted in a greater increase in luciferase expression (i.e., ~1.6-fold increase relative to C/EBPβ alone)(Fig. 4C, right). The expression of Myc-Ala^{105} mutant of C/EBPβ, compared to that of Myc-C/EBPβ, completely abolished the ability of oltipraz to promote luciferase expression from pGL-1651 (Fig. 4C, right). These results indicate that Ser^{105} phosphorylation of C/EBPβ, which is promoted by oltipraz treatment, leads to recruitment of CBP to the *GSTA2* gene promoter and enhances histone acetylation for the gene transcription.

Ser^{105} Phosphorylation of C/EBPβ by RSK1. In view of the role of RSK1 in the phosphorylation of C/EBPβ, we sought to determine whether RSK1 is responsible for Ser^{105} phosphorylation of C/EBPβ. Transfection with the KH3 plasmid, a control vector, allowed cells to phosphorylate C/EBPβ at Ser^{105} in response to oltipraz (30 μM, 12 h). In contrast, expression of HA-CTT-RSK1 or constitutively inactive kinase-dead mutant of RSK1 (HA-K112/464R-RSK1) completely inhibited oltipraz enhancement in Ser^{105} phosphorylation of C/EBPβ, as determined by immunoblot analyses in lysates (Fig. 5A). In contrast, Thr^{189} phosphorylation was unchanged by the plasmids. In parallel with this, increase in the level of nuclear Ser^{105}-phosphorylated C/EBPβ was prevented by overexpression of HA-CTT-RSK1 or HA-K112/464R-RSK1 (Fig. 5A).

Subsequently, we determined the effects of dominant negative mutants of RSK1 on oltipraz-inducible expression of the pGL-1651 luciferase reporter gene (Kang et al., 2003). As expected,

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transfection of cells with HA-CTT-RSK1 or HA-K112/464R-RSK1 entirely inhibited the ability of oltipraz to stimulate reporter gene expression from pGL-1651 (Fig. 5B). KH3, which was used as a control, did not inhibit the reporter gene expression. This finding indicates that RSK1 mediates Ser^{105} phosphorylation of C/EBPβ by oltipraz for *GSTA2* gene transactivation.

RSK1-Dependent Phosphorylation of C/EBPβ in Other Species. We then determined whether oltipraz induced phosphorylation of the residue in mouse or human C/EBPβ functionally analogous to Ser^{105} of rat C/EBPβ. Immunoblot analysis revealed that phosphorylation of C/EBPβ at Thr^{217} or Thr^{266} was promoted by oltipraz (30 μM, 12 h) in mouse Hepa1c1c cells and human HepG2 cells, respectively (Fig. 6A). Increases in C/EBPβ phosphorylation at the $Thr^{217/266}$ residues by oltipraz were abolished by transfection with the plasmid encoding HA-K112/464R-RSK1. Hence, RSK1 contributes to $Thr^{217/266}$ phosphorylation by oltipraz in mouse and human C/EBPβ. In addition, we verified the role of RSK1 in Ser^{105} phosphorylation of C/EBPβ by oltipraz in primary cultured rat hepatocytes. Oltipraz (30 μM, 12 h) was capable of inducing C/EBPβ phosphorylation at Ser^{105} in the primary hepatocytes (Fig. 6B). As expected, a dominant negative mutant of RSK1 prevented oltipraz-inducible phosphorylation of C/EBPβ.

In addition, we employed the knock-down technique to verify the functional role of RSK1 in the *GSTA2* gene transactivation by oltipraz. Knock-down of RSK1 by transfection of HepG2 cells with the siRNA that specifically catalyzes degradation of human RSK1 mRNA resulted in a substantial decrease in the luciferase expression from pGL-1651 (Fig. 6C, left). In this experiment, scrambled RNA (scRNA) was used as a nonspecific RNA. Immunoblot analysis confirmed a decrease in RSK1 expression by the siRNA (Fig. 6C, right). The human RSK1 siRNA failed to degrade rat RSK1 (supplemental data #1), which confirmed its specificity.

Effects of MKK1 Inhibition on Oltipraz Activation of C/EBPβ and Gene Expression. Activation of ERK initiates an activating process of RSK1 by epidermal growth factor (Roux et al., 2003). To study whether the MKK1/ERK pathway was involved in the increase in Ser^{105} phosphorylation of C/EBPβ by oltipraz, we assessed the effect of U0126, an MKK1 inhibitor, on the phosphorylation in lysates. U0126 weakly, but insignificantly, prevented an increase in Ser^{105} phosphorylation of C/EBPβ in the lysates prepared from H4IIE cells treated with oltipraz for 12 h (Fig. 7A, left). We also monitored the

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levels of Ser^{105}-phosphorylated C/EBPβ in H4IIE cells or cells stably transfected with dominant-negative mutant of MKK1 [MKK1(-)] (Fig. 7A, right). Increase in nuclear Ser^{105}-phosphorylated C/EBPβ by oltipraz was marginally repressed by MKK1(-) transfection. U0126 treatment or MKK1(-) transfection completely inhibited the activation of ERK1/2 by insulin-like growth factor (IGF, 100 ng/ml, 10 min). Therefore, it is unlikely that increase in Ser^{105} phosphorylation of C/EBPβ by oltipraz is under the control of ERK1/2.

We also monitored the levels of Ser^{105}-phosphorylated C/EBPβ in nuclear fractions prepared from cells treated with U0126 or MKK1(-) cells to determine whether the activity of ERK was required for nuclear translocation of Ser^{105}-phosphorylated C/EBPβ. Immunoblot analyses demonstrated that either U0126 treatment or MKK1(-) transfection only weakly blocked an increase in nuclear Ser^{105}-phosphorylated C/EBPβ by oltipraz (Fig. 7B). The extent of increase in nuclear Ser^{105}-phosphorylated C/EBPβ by oltipraz was comparable to that in lysates, indicating that the activity of ERK1/2 was unnecessary for nuclear translocation of the phosphorylated C/EBPβ by oltipraz. In addition, MKK1(-) transfection failed to prevent enhancement in pGL-1651 luciferase expression by oltipraz (Fig. 7C). These results show that Ser^{105} phosphorylation of C/EBPβ and target gene transactivation by oltipraz does not require MKK1-ERK-mediated activation process.

PI3-Kinase-Dependent Ser^{105} Phosphorylation. The pathway of PI3-kinase is involved in a number of cellular responses by growth stimuli (Katso et al., 2001). Previously, we reported that C/EBPβ activation is dependent on the activity of PI3-kinase (Kang et al., 2003). To determine whether Ser^{105} phosphorylation of C/EBPβ by oltipraz was controlled by PI3-kinase, we measured the levels of Ser^{105}-phosphorylated C/EBPβ in lysates or nuclear fractions. H4IIE cells were incubated with LY294002 (10 μM), a chemical inhibitor of PI3-kinase, for 1 h and then exposed to oltipraz (30 μM). Immunoblot analysis revealed that LY294002 blocked oltipraz-inducible phosphorylation of C/EBPβ at Ser^{105} in lysates (Fig. 8A). Also, increase in the level of nuclear Ser^{105}-phosphorylated C/EBPβ was almost completely abolished by treatment with LY294002 or stable transfection with the p85(+) subunit of PI3-kinase (Fig. 8B). Additional immunoblot assays showed that the levels of total C/EBPβ were unchanged by LY294002 treatment (data not shown), indicating that prevention of oltipraz-inducible Ser^{105}

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phosphorylation of C/EBPβ by PI3-kinase inhibition was not due to a decrease in C/EBPβ expression. Our results showed that PI3-kinase regulated Ser^{105} phosphorylation of C/EBPβ.

Role of PI3-Kinase in the Activation of RSK1 by Oltipraz. We finally determined the kinase activity of RSK1 in cells exposed to oltipraz for a variety of time periods. Treatment of H4IIE cells with oltipraz resulted in rapid increases in the catalytic activity of RSK1 toward S6 rsk substrate peptide (Fig. 9A). RSK1 activity in lyates maximally increased 1 h after treatment, which gradually decreased from the maximum at later times. Increase in RSK activity was observed at least up to 24 h. In view of the fact that Ser^{105} phosphorylation of C/EBPβ by oltipraz depended on the pathway involving PI3-kinase, we were interested in whether RSK1 activation by oltipraz was under the control of PI3-kinase. We observed that LY294002 treatment for 1 h prior to the addition of oltipraz abrogated an increase in the kinase activity of RSK1 by oltipraz (Fig. 9B). Consistently, nuclear translocation of RSK1 stimulated by oltipraz treatment (12 h) was prevented by concomitant treatment of cells with LY294002 (Fig. 9C). The role of PI3-kinase for RSK1 activation was confirmed in cells stably transfected with the plasmid encoding p110 or p85 subunit of PI3-kinase (supplemental data #2). These results provide evidence that increase in RSK1 kinase activity by oltipraz requires the basal PI3-kinase activity. Wortmannin was not used as an inhibitor in this experiment because the agent at the concentration effective for PI3-kinase inhibition elicited nuclear translocation of RSK1 (supplemental data #3).

Discussion

Activation of C/EBPβ, which involves the process of nuclear translocation and C/EBPβ binding to the C/EBP binding site, requires phosphorylations at specific residues by cellular kinases (Frodin and Gammeltoft, 1999). In the present study, we found that activation of C/EBPβ by oltipraz involved specific Ser^{105} phosphorylation in the rat form, and also $Thr^{217/266}$ phosphorylations in the mouse and human forms. Oltipraz did not enhance Thr^{189} phosphorylation of C/EBPβ, which is known to be catalyzed by Ras-MAPK or Cdk (Nakajima et al., 1993, Shuman et al., 2004). Lack of an increase in the Thr^{189} phosphorylation may explain no mitogenic effect of oltipraz (Ruggeri et al., 2002) because Thr^{189}-phosphorylated C/EBPβ has been implicated in the cell-cycle progression (Shuman et al., 2004). The

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level of Ser^{105}-phosphorylated C/EBPβ in nuclear fraction increased with a reciprocal decrease in its cytoplasmic content. At 6 h after oltipraz treatment, we observed a notable increase in Ser^{105} phosphorylation of C/EBPβ in the cytoplasm. In fact, the level of cytoplasmic Ser^{105}-phosphorylated C/EBPβ at the early time increased to a greater extent than that in the nucleus, whereas the nuclear form gradually increased at later times after oltipraz treatment. The early increase in cytoplasmic Ser^{105} phosphorylation suggested that the phosphorylation be mediated by the cytoplasmic enzyme activated by oltipraz. Therefore, oltipraz-inducible Ser^{105} phosphorylation of C/EBPβ is likely to occur initially in the cytoplasm and then the phosphorylated form translocates into the nucleus. Alternatively, the enzyme that activates C/EBPβ may translocate into the nucleus with C/EBPβ for phosphorylation.

The immunoprecipitation and ChIP assays demonstrated that Ser^{105}-phosphorylated C/EBPβ was functionally active in gene transcription. N-terminal transactivation domain of C/EBPβ may interact with CBP/p300 coactivator, which is critical for C/EBPβ-mediated gene transactivation (Mink et al., 1997). In the present study, we revealed that oltipraz treatment increased the level of Ser^{105}-phosphorylated C/EBPβ that is capable of binding to CBP, inducing histone acetylation for the *GSTA2* gene transactivation. The *C/EBPβ* gene contains the C/EBPβ binding site(s) in the promoter region (GenBank178567)(Mink et al., 1999). The role of Ser^{105}-phosphorylated C/EBPβ in gene transactivation was additionally supported by the finding that oltipraz specifically increased the expression of C/EBPβ, but not C/EBPα or C/EBPδ. Thus, it is highly likely that oltipraz induction of C/EBPβ after initial activation of preexisting C/EBPβ by Ser^{105} phosphorylation contributes to persistent gene transactivation. Although Thr^{189} phosphorylation of rat C/EBPβ was unchanged after oltipraz treatment, we observed that Thr^{189}-phosphorylated C/EBPβ constitutively interacted with CBP and bound to the promoter region of the *GSTA2* gene (Fig. 3). Hence, the Thr^{189} phosphorylation might be responsible for the constitutive gene expression. Activation of C/EBPβ may be mediated by multiple phosphorylations at the serine or threonine residues within the molecule (Buck and Chojkier, 2003). In the present study, the specific mutagenesis analysis of C/EBPβ lends support to the essential role of Ser^{105} phosphorylation for oltipraz's inducible gene transcription, as evidenced by the complete abrogation of oltipraz's increase in C/EBPβ-mediated gene expression in cells transfected with Myc-C/EBPβ-S105A. Our results provide

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compelling evidence that Ser^{105} plays a critical role in C/EBPβ activation by oltipraz.

Rat C/EBPβ is known to be phosphorylated at the residue of Ser^{105} by RSK1 (Buck et al., 1999). In cells transfected with the plasmid encoding truncated or kinase-dead mutant form of RSK1, oltipraz failed to induce Ser^{105} phosphorylation of C/EBPβ or *GSTA2* gene transactivation. Thus, Ser^{105} phosphorylation of 35 kDa C/EBPβ appeared to be mediated by RSK1. C/EBPβ is phosphorylated by other cellular kinases including PKC, PKA and Ras-MAPK (Buck et al., 1999; Nakajima et al., 1993; Trautwein et al., 1993, 1994; and Hanlon et al., 2001). PKC may phosphorylate rat C/EBPβ at the residue of Ser^{105}, whereas MAPK downstream from Ras phosphorylates Thr^{235} of human C/EBPβ (analogous to Thr^{189} in the rat form). GF109203 (PKC inhibitor) did not inhibit the Ser^{105} phosphorylation of C/EBPβ by oltipraz (supplemental data #3). Oltipraz's increase in Ser^{105}-phosphorylated C/EBPβ was also unaffected by pretreatment with rapamycin (inhibitor of p70 ribosomal S6-kinase, 100 μM) or H89 (PKA inhibitor, 20 μM)(data not shown), but weakly inhibited by Akt inhibitor IV (supplemental data #3). Thus, the possibility that Akt affects C/EBPβ phosphorylation was not completely excluded although oltipraz failed to stimulate Akt (Kang et al., 2003).

We showed that RSK1 regulated Ser^{105} phosphorylation of C/EBPβ by oltipraz in primary rat hepatocytes as well as H4IIE cells. Also, oltipraz enhanced phosphorylation of the mouse or human C/EBPβ at the residue of Thr^{217} or Thr^{266}, which was also catalyzed by RSK1. The role of human RSK1 in C/EBPβ-mediated gene activation was additionally supported by the RSK1 knock-down experiment. Our results provide evidence that activation of the C/EBPβ forms by oltipraz involves functionally analogous phosphorylation at the specific residues by RSK1 in the species. Oltipraz activation of C/EBPβ by specific phosphorylation would result in a conformational change of the protein for DNA binding and gene transactivation. The finding that oltipraz activates C/EBPβ via RSK1 brings insights into the role of organic compounds in activating the critical signaling pathway and cellular functions.

Previously, we showed that the pathways of MAPKs, ERK1/2, p38 kinase and c-Jun N-terminal kinase, were not responsible for C/EBPβ-mediated GST induction by oltipraz (Kang et al., 2003). In the current study, the extent of increase in Ser^{105} phosphorylation of C/EBPβ or C/EBPβ-mediated gene transactivation by oltipraz in MKK1(-) cells was almost comparable to that in control, suggesting that

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oltipraz was capable of stimulating the Ser^{105} phosphorylation independent of MKK1-ERK activity. In general, activation of ERK1/2 is necessary for RSK1 activation by EGF (Roux et al., 2003). The observations that Ser^{105} phosphorylation of C/EBPβ by oltipraz was minimally decreased by chemical inhibition of MKK1-ERK1/2 and that RSK1 regulated Ser^{105} phosphorylation of C/EBPβ by oltipraz indicate that RSK1 activation elicited by oltipraz may not need the constitutive activity of ERK1/2. This is consistent with our previous observation that oltipraz did not enhance the activity of MAPK and that activation of C/EBPβ by oltipraz was independent on the ERK activity.

RSK1 activation by growth factors requires ERK docking near the C-terminus region (Roux et al., 2003). Ser^{380} phosphorylation of RSK1 (i.e., auto-phosphorylation) is catalyzed by the C-terminal kinase domain of activated RSK1. Activation of ERK initiates a series of activating processes of RSK1 (Roux et al., 2003), which includes auto-phosphorylation of RSK1. We found that the RSK1 levels in cell lysates were unchanged after oltipraz treatment. In additional experiments, we observed that Ser^{380} phosphorylation in RSK1 was increased by oltipraz treatment, which was persistent up to 24 h. Increase in RSK1 kinase activity by oltipraz paralleled that in the Ser^{380} phosphorylation. It has been reported that RSK1 activated by EGF or GRH translocates into the nucleus (Roux et al., 2003, Shah et al., 2003). After oltipraz treatment, RSK1, which was present in the cytoplasm under the resting condition, localized in the nucleus. Taken together, our data support that oltipraz treatment results in activation of RSK1. In contrast to the activation of MAPKs as well as PI3-kinase by mitogens, oltipraz did not increase activities of the kinases (Kang et al., 2003). No change in oltipraz-inducible Ser^{105} phosphorylation of C/EBPβ by MKK1-ERK1/2 inhibition supports that the mechanistic basis of RSK1 activation by oltipraz may differ from that by growth factors. The molecular basis as to how oltipraz activates RSK1 remains to be elucidated.

The PI3-kinase pathway affects cell growth, survival and motility. Previously, we showed that PI3-kinase regulates C/EBPβ translocation, thus controlling C/EBPβ activation in response to oltipraz or HGF (Kang et al., 2003, Cho and Kim, 2003). In this study, we found for the first time that oltipraz increased the activity of RSK1 for C/EBPβ activation, and that increases in the metabolic activity and nuclear translocation of RSK1 were dependent on the PI3-kinase activity. In general, full activation of

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RSK1 requires phosphorylation by PDK1, a constitutively active kinase downstream of PI3-kinase (Richards et al., 2001, Casamayor et al., 1999). After activation by PDK1, RSK1 translocates to the nucleus (Shah et al., 2003). In the current study, PI3-kinase inhibition prevented nuclear translocation of RSK1 by oltipraz. We observed that either chemical inhibition of PI3-kinase or stable transfection with the plasmid encoding p85 regulatory subunit almost completely inhibited an increase in Ser^{105}-phosphorylated C/EBPβ in the nuclear fraction, which was in line with the PI3-kinase dependence of RSK1 activation. It is likely that interruption of C/EBPβ activation by PI3-kinase inhibition, presumably through PDK1 (and/or Akt) inhibition, results from no phosphorylation in C/EBPβ at Ser^{105}. This is consistent with our previous observation (Kang et al., 2003) and also with the report that full RSK1 activation requires PDK1 activity downstream from PI3-kinase. The data indicate that RSK1-mediated Ser^{105} phosphorylation of C/EBPβ by oltipraz requires the constitutive activity of PI3-kinase.

In conclusion, oltipraz induces phosphorylation of rat C/EBPβ form at Ser^{105} and also the mouse and human forms at $Thr^{217/266}$. RSK1 activation by oltipraz, which is dependent on PI3-kinase, but not ERK1/2, contributes to the specific phosphorylation of C/EBPβ that leads to recruitment of CBP coactivator for *GSTA2* gene transactivation. RSK1-mediated phosphorylation of C/EBPβ at specific residues by a pharmacological agent and its gene transactivation holds a significant implication for the molecular target of oltipraz.

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Footnotes

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FIGURE LEGENDS

Fig. 1. Expression of C/EBP isoforms in cells treated with oltipraz. The levels of C/EBPβ, C/EBPα and C/EBPδ isoforms were determined in the lysates of cells treated with oltipraz for 3-48 h. Each lane was loaded with 20 μg of cell lysates. Actin was used as a control. Results were confirmed by repeated experiments.

Fig. 2. Alignment of RSK1- or ERK-phosphorylated residues in rat, mouse and human homologous forms of C/EBPβ. **(A)** Alignment of the partial sequences of rat, mouse and human C/EBPβ. The residues that are equivalent to Ser^{105}, Thr^{189} and Ala^{218} in rat C/EBPβ were high lightened for comparison. **(B)** The specific residues that are phosphorylated by RSK1 or ERK. The residues in rat, mouse and human homologous forms of C/EBPβ phosphorylated by RSK1 or ERK were indicated.

Fig. 3. Oltipraz activation of C/EBPβ by Ser^{105} phosphorylation. **(A)** Immunoblot analysis of phosphorylated C/EBPβ. The levels of C/EBPβ phosphorylated at Ser^{105} or Thr^{189} were determined by immunoblot analyses in lysates prepared from H4IIE cells treated with oltipraz for 3-48 h (left). The levels of phosphorylated C/EBPβ were also determined in the cytosolic and nuclear fractions of oltipraz-treated cells (3-24 h) (middle and right). Equal loading of proteins was verified by probing the replicate blots for actin. Each lane contained 20 μg of lysate (or cytosolic) proteins or 10 μg of nuclear proteins. **(B)** Gel shift analysis of Ser^{105}-phosphorylated C/EBPβ binding to the C/EBP binding site. Nuclear extracts were prepared from H4IIE cells cultured with 30 μM oltipraz for 12 h. All lanes contained 10 μg of nuclear extracts and 5 ng of labeled C/EBP consensus oligonucleotide. Immuno-inhibition assays were carried out by incubating the nuclear extracts (oltipraz, 12 h) with the polyclonal antibody directed against Ser^{105}- or Thr^{189}-phosphorylated C/EBPβ or Sp1. Arrowheads indicate shifted DNA bound with Ser^{105}-phosphorylated C/EBPβ. Results were confirmed by repeated experiments.

Fig. 4. Recruitment of CBP to Ser^{105}-phosphorylated C/EBPβ. **(A)** Association of CBP with Ser^{105}-phosphorylated C/EBPβ. Interaction of CBP with Ser^{105}- or Thr^{189}-phosphorylated C/EBPβ was

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determined in H4IIE cells treated with 30 μM oltipraz for 3-24 h. Whole cell lysates were precipitated with anti-CBP antibody and immunocomplexes were immunoblotted with anti-Ser^{105}-phosphorylated or anti-Thr^{189}-phosphorylated C/EBPβ antibody. Aliquots from the input were loaded for immunoblot of actin. Results were confirmed by repeated experiments. **(B)** Chromatin immunoprecipitation (ChIP) assays. The DNA-protein complexes prepared from cells treated with vehicle or oltipraz (30 μM, 12 h) were immunoprecipitated with anti-Ser^{105}-phosphorylated C/EBPβ, anti-CBP, anti-acetylated histone or anti-Thr^{189}-phosphorylated C/EBPβ antibody. The samples were PCR-amplified using primers flanking the proximal and distal regions of the DNA comprising the C/EBP binding site in the *GSTA2* promoter. A set of control experiment was carried out for the *β-actin* gene. One tenth of the total input was used as a loading control. Results were confirmed by repeated experiments. **(C)** Specific mutagenesis assay. The effect of specific mutation of Ser^{105} residue in C/EBPβ on oltipraz-inducible luciferase expression from pGL-1651 *GSTA2* promoter was assessed in H4IIE cells. Luciferase activities were measured in cells treated with vehicle or oltipraz (30 μM, 18 h), or cells transfected with the plasmid encoding Myc-C/EBPβ. Data represented the mean ± S.E. with 4 separate experiments (significant as compared to vehicle, *$p<0.05$). In another set of experiment, oltipraz-inducible change in luciferase expression was determined in cells transfected with the plasmid encoding Myc-C/EBPβ or Myc-C/EBPβ-S105A. Values were expressed as the changes relative to the respective vehicle-treated control and represented the mean ± S.E. with 4-5 separate experiments (significant as compared to Myc-C/EBPβ, $^{\#}p<0.05$). Immunoblot analysis confirmed the expression of Myc-C/EBPβ or Myc-C/EBPβ-S105A.

Fig. 5. The role of RSK1 in Ser^{105} phosphorylation of C/EBPβ and gene transactivation by oltipraz. **(A)** The levels of Ser^{105}-phosphorylated C/EBPβ. The levels of C/EBPβ phosphorylated at Ser^{105} or Thr^{189} were determined by immunoblot analyses in lysates or nuclear fractions prepared from H4IIE cells that had been treated with oltipraz (12 h) following transfection with the HA-tagged plasmid encoding a truncated RSK1 (CTT-RSK1) or a kinase-dead mutant form of RSK1 (K112/464R-RSK1). Each lane was loaded with 20 μg of lysates or 10 μg of nuclear proteins. Expression of HA-CTT-RSK1 or HA-K112/464R-RSK1 was verified by immunoblotting for HA. Equal loading of proteins in each lane was

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verified by probing the replicate blot for actin. **(B)** Repression of C/EBPβ-mediated promoter luciferase activity by dominant negative mutants of RSK1. Cells were co-transfected with the pGL-1651 and CMV-β-galactosidase plasmids (33:1) in combination with KH3 (empty vector), HA-CTT-RSK1 or HA-K112/464R-RSK1 plasmid at a ratio of 1:1 and the cells were exposed to oltipraz for 18 h. Activation of the reporter gene was calculated as a relative change to β-galactosidase activity, The value for luciferase activity was expressed as relative luciferase unit of cell lysates and represented the mean ± S.E. with 4 separate experiments (significant as compared to KH3 in untreated cells, *$p<0.05$, KH3 in untreated cells = 100%).

Fig. 6. The functional role of RSK1 in C/EBPβ phosphorylation in mouse, human cell lines or primary hepatocytes. **(A)** RSK1-mediated $Thr^{217/266}$ phosphorylation by oltipraz in mouse and human C/EBPβ. The levels of Thr^{217}- or Thr^{266}-phosphorylated C/EBPβ were assessed by immunoblot analyses in lysates prepared from mouse Hepa1c1c or human HepG2 cells that had been treated with oltipraz (30 μM, 12 h) following transfection with the plasmid encoding HA-K112/464R-RSK1. **(B)** The role of RSK1 in Ser^{105} phosphorylation of C/EBPβ by oltipraz in primary cultured rat hepatocytes. Rat hepatocytes were incubated in the medium containing 10% FCS for 24 h, transiently transfected with the plasmid encoding HA-K112/464R-RSK1 using Lipofectamine® 2000 and then treated with oltipraz for 12 h, as described in Methods. Control cells were transfected with KH3 an empty plasmid. Equal loading of proteins in each lane was verified by probing the replicate blot for actin. Expression of HA-K112/464R-RSK1 was verified by immunoblotting for HA. Results were confirmed by separate experiments. **(C)** The effect of RSK1 knock-down on the C/EBPβ-mediated gene transactivation by oltipraz. HepG2 cells were transfected with RSK1 siRNA or scRNA (RNA with scrambled sequence) in combination with pGL-1651, incubated for 3 days and then treated with vehicle or 10 μM oltipraz for 18 h. The luciferase expression from pGL-1651 was analyzed in cell lysates. Aliquots of the samples were subjected to immunoblot analyses. Values were expressed as the changes relative to the respective vehicle-treated control and represented the mean ± S.E. with 3 separate experiments (significant as compared to scRNA transfection, **$p<0.01$).

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Fig. 7. The role of ERK in C/EBPβ activation by oltipraz. **(A)** The effects of MKK1 inhibition on Ser^{105} phosphorylation of C/EBPβ in cell lysates. The effect of ERK in Ser^{105} phosphorylation of C/EBPβ was assessed by using U0126 or MKK1(-) stable transfection. H4IIE cells that had been treated with U0126 (10 μM, 1 h) were further incubated with oltipraz (30 μM, 12 h) in the continuing presence of U0126. Cells were stably transfected with the plasmid encoding MKK1(-). Inhibition of ERK activation by U0126 treatment or MKK1(-) transfection was confirmed by immunoblotting phosphorylated ERK1/2 and total ERK1/2 (p-ERK and ERK, respectively) in cells exposed to insulin-like growth factor (IGF, 100 ng/ml, 10 min). **(B)** The role of ERK in nuclear translocation of Ser^{105}-phosphorylated C/EBPβ. Immunoblot analyses were performed with the nuclear fractions prepared from cells treated as described in panel A. Data represent the mean ± S.E. with 4 separate experiments (significant as compared to control, *$p<0.05$, **$p<0.01$)(N.S., not significant). **(C)** The effect of MKK1(-) transfection on oltipraz-inducible luciferase-reporter activity. Luciferase activity was measured in lysates of H4IIE cells treated with vehicle or oltipraz (18 h) following transfection with PCMV (empty vector) or MKK1(-). Cells were transfected with pGL-1651-luciferase reporter plasmid, as described in Fig. 3C. Data represented the mean ± S.E. with 4 separate experiments (significant as compared to control, *$p<0.05$).

Fig. 8. PI3-kinase-dependent Ser^{105}-phosphorylation of C/EBPβ by oltipraz. **(A)** The levels of Ser^{105}-phosphorylated C/EBPβ in cell lysates. H4IIE cells that had been serum-starved for 24 h were pretreated with LY294002 (10 μM) for 1 h, and further incubated with 30 μM oltipraz for 12 h in the continuing presence of LY294002. Ser^{105}-phosphorylated C/EBPβ was assessed by immunoblot analysis in cell lysates. Equal loading of proteins was verified by probing the replicate blots for actin. **(B)** The levels of nuclear Ser^{105}-phosphorylated C/EBPβ. Nuclear proteins obtained from cells treated with oltipraz (30 μM, 12 h) in the presence or absence of 10 μM LY294002 were subjected to immunoblot analysis. Cells stably expressing the p85 subunit of PI3-kinase were used to assess the role of PI3-kinase in Ser^{105} phosphorylation of C/EBPβ by oltipraz. The relative levels of Ser^{105}-phosphorylated C/EBPβ were assessed by scanning densitometry of the immunoblots. Data represent the mean ± S.E. with 3 separate

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experiments (significant as compared to control, **p<0.01; significant as compared to oltipraz alone, ##p<0.01; control level = 1) [p85(+), overexpression of p85 subunit].

Fig. 9. PI3-kinase-dependent RSK1 activation by oltipraz. **(A)** Increase in the kinase activity of RSK1 by oltipraz. H4IIE cells were incubated with 30 μM oltipraz for the indicated time periods. RSK1 activity toward S6 rsk substrate peptide was determined in cell lyates by monitoring ^{32}P-radioactivity. Data represent the mean ± S.E. with 3 separate experiments (significant as compared to control, *p<0.05, **p<0.01; zero-time control = 1). **(B)** The effect of PI3-kinase inhibition on the kinase activity of RSK1. H4IIE cells were treated with vehicle or oltipraz in the presence or absence of LY294002 (10 μM) for 1 h. The kinase activity was measured as described above. Values are expressed as the change in RSK1 activity relative to vehicle-treated control and represented the mean ± S.E. with 4 separate experiments (significant as compared to control, *p<0.05; significant as compared to oltipraz treatment, #p<0.05). **(C)** The effect of PI3-kinase inhibition on the nuclear translocation of RSK1 induced by oltipraz. Immunoblot analysis was performed in the nuclear fractions prepared from cells treated with oltipraz in the presence or absence of LY294002 (10 μM) for 12 h. Equal loading of proteins was verified by probing the replicate blot for actin. Each lane contained 10 μg of proteins. Results were confirmed by three separate experiments and a representative blot is shown.

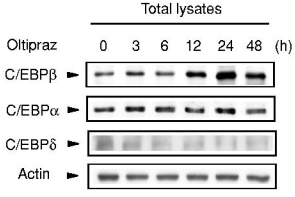
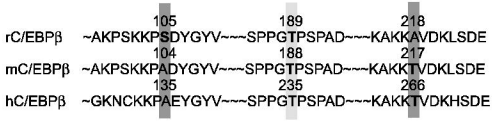

Total lysates
Oltipraz
0
3
6
12
24
48
(h)
C/EBPβ
C/EBPα
C/EBPδ
Actin

Fig. 1

A)


105
189
218
rC/EBPβ ~AKPSKKPSDYGYV~~~SPPGTPSPAD~~~KAKKAVDKLSDE
104
188
217
mC/EBPβ ~AKPSKKPADYGYV~~~SPPGTPSPAD~~~KAKKTVDKLSDE
135
235
266
hC/EBPβ ~GKNCKKPAEYGYV~~~SPPGTPSPAD~~~KAKKTVDKHSDE

B)

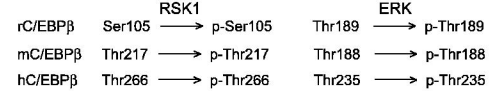

RSK1
ERK
rC/EBPβ Ser105 → p-Ser105 Thr189 → p-Thr189
mC/EBPβ Thr217 → p-Thr217 Thr188 → p-Thr188
hC/EBPβ Thr266 → p-Thr266 Thr235 → p-Thr235

Fig. 2

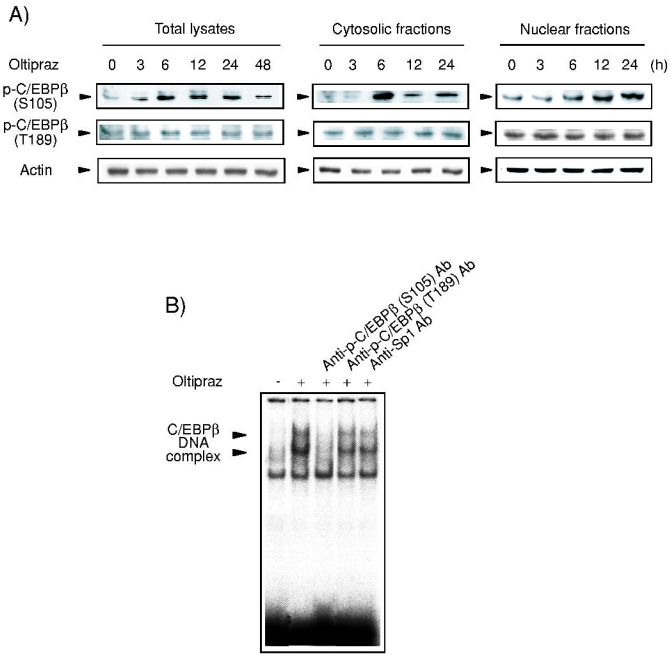

A)
Total lysates
Cytosolic fractions
Nuclear fractions
Oltipraz
0 3 6 12 24 48
0 3 6 12 24
0 3 6 12 24 (h)
p-C/EBPβ (S105)
p-C/EBPβ (T189)
Actin
B)
Anti-p-C/EBPβ (S105) Ab
Anti-p-C/EBPβ (T189) Ab
Anti-Sp1 Ab
Oltipraz
- + + + +
C/EBPβ DNA complex

Fig. 3

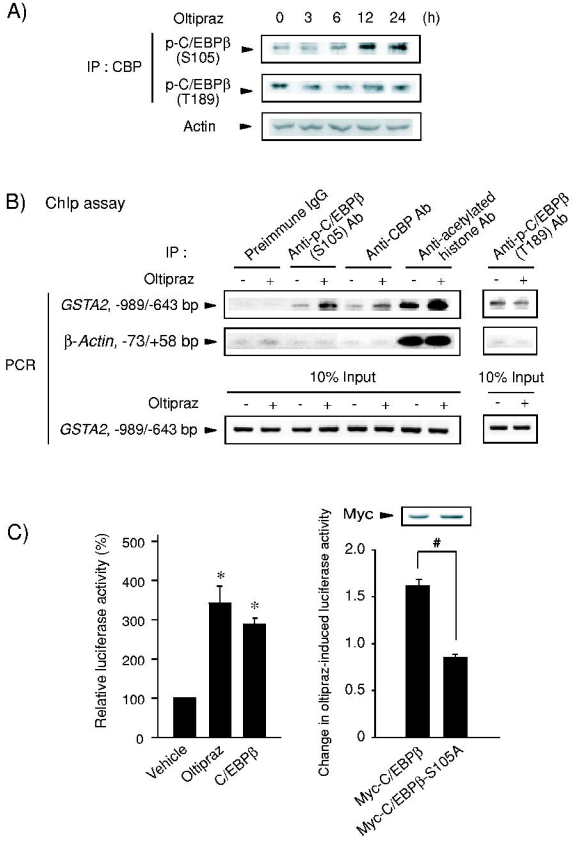

A)
Oltipraz
0 3 6 12 24 (h)
IP : CBP
p-C/EBPβ (S105)
p-C/EBPβ (T189)
Actin
B) ChIp assay
IP :
Preimmune IgG
Anti-p-C/EBPβ (S105) Ab
Anti-CBP Ab
Anti-acetylated histone Ab
Anti-p-C/EBPβ (T189) Ab
Oltipraz
- + - + - + - + - +
PCR
GSTA2, -989/-643 bp
β-Actin, -73/+58 bp
10% Input
10% Input
Oltipraz
- + - + - + - + - +
GSTA2, -989/-643 bp
C)
Relative luciferase activity (%)
500
400
300
200
100
0
*
*
Vehicle
Oltipraz
C/EBPβ
Myc
Change in oltipraz-induced luciferase activity
2.0
1.5
1.0
0.5
0
#
Myc-C/EBPβ
Myc-C/EBPβ-S105A

Fig. 4

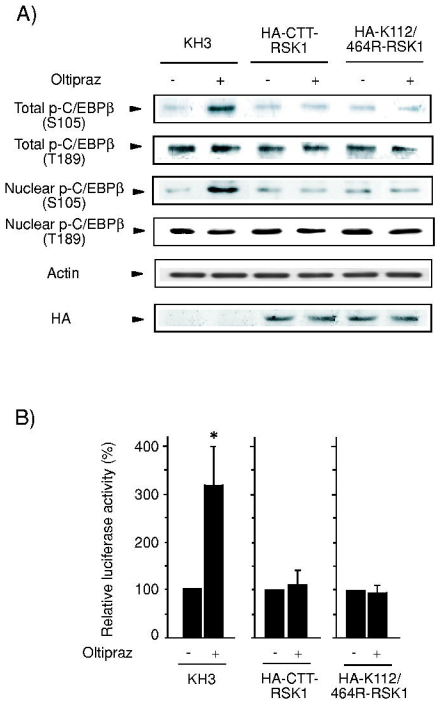

A)
KH3
HA-CTT-RSK1
HA-K112/464R-RSK1
Oltipraz
- + - + - +
Total p-C/EBPβ (S105)
Total p-C/EBPβ (T189)
Nuclear p-C/EBPβ (S105)
Nuclear p-C/EBPβ (T189)
Actin
HA
B)
Relative luciferase activity (%)
400
300
200
100
0
*
Oltipraz
- + - + - +
KH3
HA-CTT-RSK1
HA-K112/464R-RSK1

Fig. 5

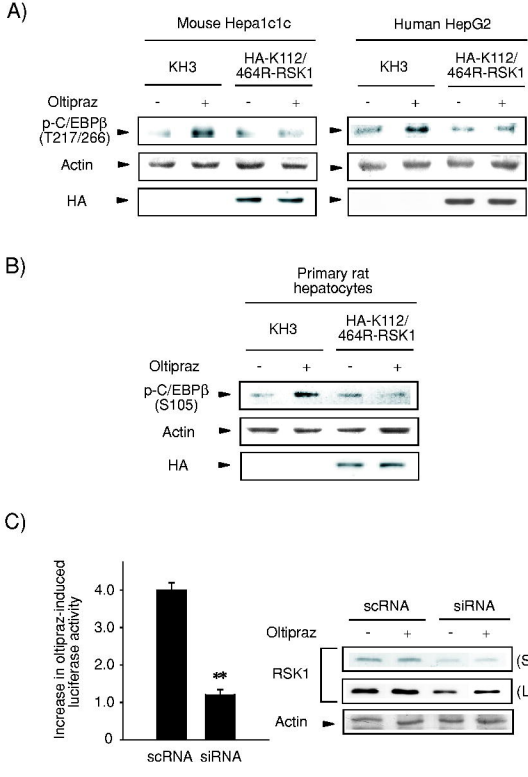

A)
Mouse Hepa1c1c
Human HepG2
KH3
HA-K112/
464R-RSK1
KH3
HA-K112/
464R-RSK1
Oltipraz
- + - + - + - +
p-C/EBPβ
(T217/266)
Actin
HA
B)
Primary rat
hepatocytes
KH3
HA-K112/
464R-RSK1
Oltipraz
- + - +
p-C/EBPβ
(S105)
Actin
HA
C)
Increase in oltipraz-induced
luciferase activity
4.0
3.0
2.0
1.0
0
**
scRNA siRNA
scRNA
siRNA
Oltipraz
- + - +
RSK1
(Short exposure)
(Long exposure)
Actin

Fig. 6

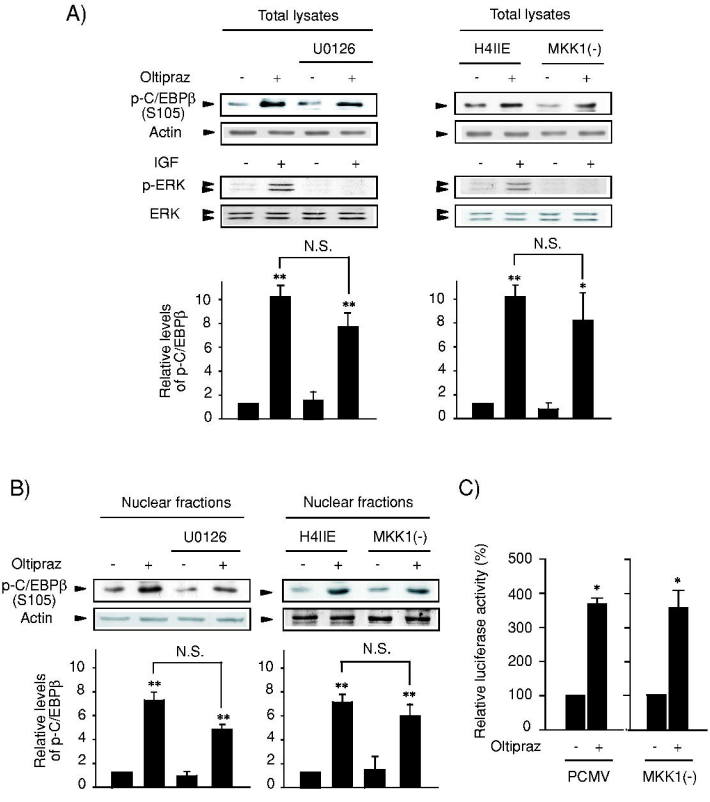

A)
Total lysates
U0126
Oltipraz
p-C/EBPβ (S105)
Actin
IGF
p-ERK
ERK
Total lysates
H4IIE
MKK1(-)
N.S.
Relative levels of p-C/EBPβ
B)
Nuclear fractions
U0126
Oltipraz
p-C/EBPβ (S105)
Actin
H4IIE
MKK1(-)
N.S.
Relative levels of p-C/EBPβ
C)
Relative luciferase activity (%)
Oltipraz
PCMV
MKK1(-)

Fig. 7

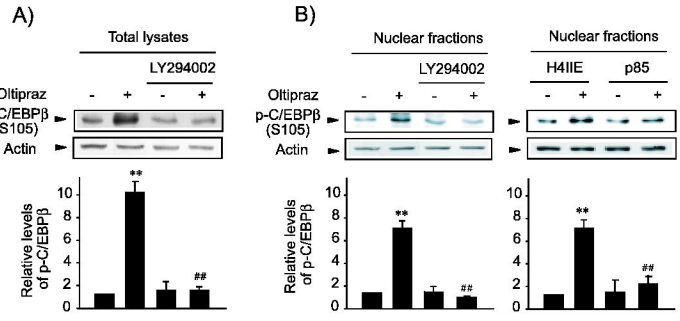
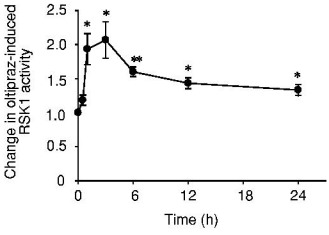
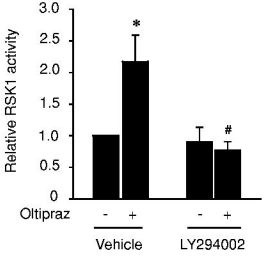

A)
Total lysates
LY294002
Oltipraz - + - +
p-C/EBPβ (S105)
Actin
Relative levels of p-C/EBPβ
10
8
6
4
2
0
**
##
B)
Nuclear fractions
LY294002
Oltipraz - + - +
p-C/EBPβ (S105)
Actin
Relative levels of p-C/EBPβ
10
8
6
4
2
0
**
##
Nuclear fractions
H4IIE
p85
- + - +
- + - +
p-C/EBPβ (S105)
Actin
10
8
6
4
2
0
**
##

Fig. 8

A)


Change in oltipraz-induced RSK1 activity
2.5
2.0
1.5
1.0
0.5
0
0
6
12
18
24
Time (h)

B)


Relative RSK1 activity
3.0
2.5
2.0
1.5
1.0
0.5
0
Oltipraz
-
+
-
+
Vehicle
LY294002

C)

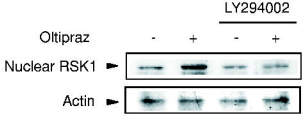

LY294002
Oltipraz
-
+
-
+
Nuclear RSK1
Actin

Fig. 9