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Enhanced CCAAT/Enhancer-Binding Protein β-Liver-Enriched Inhibitory Protein Production by Oltipraz, Which Accompanies CUG Repeat-Binding Protein-1 (CUGBP1) RNA-Binding Protein Activation, Leads to Inhibition of Preadipocyte Differentiation[S]

Eun Ju Bae and Sang Geon Kim

College of Pharmacy and Research Institute of Pharmaceutical Sciences, Seoul National University, Seoul, Korea

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ABSTRACT

The CCAAT/enhancer-binding protein (C/EBP) β-isoforms liver-enriched activator protein (LAP) and truncated dominant-negative liver-enriched inhibitory protein (LIP) differentially regulate adipogenesis. We previously demonstrated that oltipraz (5-[2-pyrazinyl]-4-methyl-1,2-dithiol-3-thione), a cancer-chemopreventive agent, promotes C/EBPβ-LAP activation in hepatocytes. This study investigated whether oltipraz affects adipocyte differentiation and, if so, the molecular basis for the alterations in adipogenesis. The expression of LIP notably increased 6 to 48 h after oltipraz treatment of 3T3-L1 preadipocytes, whereas that of LAP was minimally changed. Oltipraz treatment ~3-fold elevated the ratio of LIP to LAP. Immunoblot, gel-shift, and Southwestern analyses revealed that oltipraz enhanced the levels of nuclear LIP and LAP and their binding to the C/EBP-binding site. Cotransfection of predipocytes with the plasmid encoding LIP interfered with LAP-mediated luciferase expression, confirming the inhibitory role of LIP in gene expression. Likewise, LAP-mediated luciferase gene transactivation was inhibited by oltipraz, as was observed by cotransfection of a dominant-negative mutant form of C/EBP. Oltipraz enhanced cytoplasmic translocation and RNA binding of CUG repeat-binding protein-1 (CUGBP1) but not calreticulin, another RNA-binding protein that interacts with C/EBPβ mRNA. When 3T3-L1 preadipocytes were induced to differentiate by exposure to 3-isobutyl-1-methylxanthine, dexamethasone, and insulin, oltipraz markedly inhibited hormone-induced adipocyte differentiation. In primary cultured rat preadipocytes, oltipraz enhanced LIP production and inhibited adipocyte differentiation. In conclusion, oltipraz inhibits adipogenesis by promoting LIP production and activation, and the enhanced LIP production accompanies cytoplasmic translocation of CUGBP1 and its binding to the GC-rich region of C/EBPβ mRNA. Our finding holds significance in that adipogenesis can be pharmacologically controlled by LIP production.

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 (Rao et al., 1993; Kensler, 1997). In experimental cancer prevention studies, oltipraz reduced tumor incidence and multiplicity (Roebuck et al., 1991; Bolton et al., 1993). Studies from our laboratories demonstrated that oltipraz regenerates cirrhotic liver in rats with liver cirrhosis (Kang et al., 2002). We also reported that oltipraz promotes nuclear translocation of CCAAT/enhancer-binding protein β (C/EBPβ)-liver-enriched activator protein (LAP) and its DNA-binding activity for transactivation of the target gene in hepatocyte-derived cells (Kang et al., 2003). Isoforms of C/EBPβ play differential roles in cell growth and differentiation. Nevertheless, the effects of oltipraz on cell differentiation in association with the activation of C/EBPβ isoforms have never been explored.

Hormonal inducers stimulate preadipocyte differentiation, which includes synchronous reentering of the cells to the cell cycle, mitotic clonal expansion, and adipocyte phenotypic differentiation (Bernlohr et al., 1985; Cornelius et al., 1994; MacDougald and Lane, 1995; Tang et al., 2003). The transcription factors that function in the activation of adipogenic

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ABBREVIATIONS: C/EBP, CCAAT/enhancer-binding protein; LAP, liver-enriched activator protein; LIP, liver-enriched inhibitory protein; CUGBP1, CUG repeat-binding protein-1; DMEM, Dulbecco's modified Eagle's medium; DTT, dithiothreitol; GM, growth medium; FBS, fetal bovine serum; IBMX, 3-isobutyl-1-methyxanthine; MDI, IBMX, dexamethasone, and insulin; PPARγ, peroxisome proliferator activated receptor γ; CMV, cytomegalovirus; PMSF, phenylmethylsulfonyl fluoride.

genes for preadipocytes differentiation include C/EBPβ and C/EBPδ (Yeh et al., 1995; Clarke et al., 1997; Darlington et al., 1998). In particular, C/EBPβ plays an essential role in the regulation of the *peroxisome proliferator activated receptor γ* (*PPARγ*) and *C/EBPα* genes by its specific binding to the C/EBP-response elements present in the promoter regions (Christy et al., 1991; Rosen et al., 2000). Therefore, cooperative DNA binding of combinatorial C/EBPβ isoforms is important in the coordinate regulation of the steps of mitotic clonal expansion and preadipocyte differentiation.

The C/EBPβ proteins form homodimeric or heterodimeric combinations of monomers that have differential DNA-binding specificity. Hence, the combinatorial complexity of multiple C/EBPβ isoforms controls transcriptional activation of the target genes. Multiple C/EBPβ isoforms with different molecular weights are translated from a single mRNA transcribed from the *C/EBPβ* gene that has no intron (Descombes and Schibler, 1991). Because C/EBPβ-liver-enriched inhibitory protein (LIP) is translated from the third in-frame AUG start codon, LIP lacks most of transactivation domain and has a higher binding affinity for DNA compared with LAP isoforms. LIP functions as a dominant-negative mutant of LAP by formation of LIP-LAP heterodimer or competing for the binding to the promoter regions of target genes as a LIP homodimer. An increase in the ratio of LIP/LAP negatively regulates C/EBPβ-LAP-mediated gene expression. Hamm et al. (2001) has shown that LIP overexpression, resulting in a LIP/LAP ratio of ~1, exerted an inhibitory effect on adipogenesis. Given the fact that oltipraz activates C/EBPβ in hepatocytes, we were tempted to determine whether oltipraz also activates C/EBPβ-LAP or its isoform(s) in other type of cells and, if so, the role of C/EBPβ isoform expression in C/EBPβ-LAP-mediated gene transactivation. Furthermore, we were interested in the effects of oltipraz on preadipocyte differentiation for which C/EBPβ-LAP-mediated gene regulation is essential. We were surprised to find that oltipraz blocks differentiation of 3T3-L1 preadipocytes as a consequence of enhanced production of C/EBPβ-LIP. Antiadipogenic effect of oltipraz was also verified in primary cultured rat preadipocytes.

Translation of the C/EBPβ isoforms is controlled by CUG RNA-binding protein named CUG repeat-binding protein-1 (CUGBP1), which binds to the 5′-untranslated region of the C/EBPβ mRNA and regulates a leaky ribosomal scanning mechanism (Timchenko et al., 1999, 2001a; Welm et al., 2000). CUGBP1 interacts with GC-rich RNA triplet repeats and thus binds to upstream AUGs in the C/EBPβ mRNA for LIP production (Timchenko et al., 1999). Although the role of CUGBP1 in myogenesis or in proliferation of certain types of cells (e.g., epithelial cells and fibroblasts) has been studied (Timchenko et al., 2001b, 2004; Baldwin et al., 2004), the role of CUGBP1 in preadipocyte differentiation is not known. This report describes CUGBP1 activation in preadipocytes as part of the mechanistic studies on LIP production by oltipraz.

Materials and Methods

Oltipraz was provided from CJ Corporation (Seoul, Korea). [γ-^{32}P]ATP (3000 mCi/mmol) was purchased from PerkinElmer Life and Analytical Sciences (Boston, MA). 3-Isobutyl-1-methyxanthine (IBMX), dexamethasone, insulin, cycloheximide, transferrin, and 3,3′,5-triiodo-L-thyronine were obtained from Sigma-Aldrich (St. Louis, MO). Antibodies directed against C/EBPα, C/EBPβ, C/EBPδ, PPARγ, CUGBP1, and calreticulin were supplied from Santa Cruz Biotechnology (Santa Cruz, CA). Rosiglitazone was kindly provided by DongA Pharmaceutical Co. (Kyunggi, Korea).

Culture and Differentiation of Preadipocytes. 3T3-L1 preadipocyte cell line was obtained from American Type Culture Collection (Manassas, VA). Cells were maintained in the growth medium (GM) containing Dulbecco's modified Eagle's medium (DMEM), 10% fetal bovine serum (FBS), 50 units/ml penicillin, and 50 μg/ml streptomycin at 37°C in a humidified atmosphere with 5% CO_2 and induced to differentiate as described previously (Student et al., 1980). In brief, 2-day postconfluent preadipocytes were treated with 0.5 μM IBMX, 1 μM dexamethasone, and 1 μg/ml insulin (MDI) for 2 days in GM. Cells were then incubated in the GM containing 1 μg/ml insulin for 2 additional days and, thereafter, exposed to GM in the absence of insulin for 6 to 8 days. Cells were exposed to 30 μM oltipraz for 2 days before the addition of MDI and continuously incubated with oltipraz. In some experiments, preadipocytes were treated with oltipraz for the indicated time period. Neutral lipid content was determined by staining the cells with Oil Red O.

Primary preadipocytes were obtained from 9- to 10-week-old Sprague-Dawley rats essentially as described previously (Rodbell, 1980). The epididymal fat pads were removed, minced, and digested using collagenase at 37°C for 1 h. Isolated preadipocytes were seeded at a density of 1×10^4 cells/cm^2 in DMEM/Ham's F-12 medium containing 10% FBS. The next day, the medium was changed and, upon reaching confluence, cells were exposed to the differentiation medium containing MDI, 10 μg/ml transferrin, and 0.2 nM 3,3′,5-triiodo-L-thyronine (Rodbell, 1980). Differentiation was observed 8 days after the addition of the differentiation medium.

Subcellular Fractionation. Whole cell lysates, cytoplasmic fractions, and nuclear extracts were prepared according to the previously published methods (Park et al., 2004). In brief, cultured cells were washed twice with ice-cold phosphate-buffered saline and harvested in the buffer containing 10 mM Tris-HCl, pH 7.1, 100 mM NaCl, 1 mM EDTA, 10% glycerol, 0.5% Triton X-100, 0.5% Nonidet P-40, 1 mM dithiothreitol (DTT), and 0.5 mM phenylmethylsulfonyl fluoride (PMSF). For the preparation of nuclear extracts, harvested cells were centrifuged at 3000*g* for 3 min and allowed to swell after the addition of 100 μl of hypotonic buffer containing 10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.5% Nonidet P-40, 1 mM DTT, and 0.5 mM PMSF. The lysates were incubated for 10 min on ice and then centrifuged at 7200*g* for 5 min at 4°C. Supernatants were collected as cytoplasmic fractions and stored −70°C until use. Pellets containing crude nuclei were resuspended in 50 μl of extraction buffer containing 20 mM HEPES, pH 7.9, 400 mM NaCl, 1 mM EDTA, 10 mM DTT, and 1 mM PMSF and incubated for 1 h on ice. The samples were centrifuged at 15,000*g* for 10 min to obtain supernatants containing nuclear fractions and stored at −70°C until use. Protein content was determined by the Bradford assay (protein assay kit; Bio-Rad, Hercules, CA).

Immunoblot Analysis. SDS-polyacrylamide gel electrophoresis and immunoblot analyses were performed as described previously (Park et al., 2004). In brief, proteins in the cytoplasmic and nuclear fractions were resolved by 7.5 or 12% gel electrophoresis and electrophoretically transferred onto nitrocellulose paper. The nitrocellulose paper was incubated with the antibody of interest after incubation with horseradish peroxidase-conjugated secondary antibody (Zymed Laboratories, South San Francisco, CA). Immunoreactive protein was visualized by an ECL chemiluminescence detection kit (Amersham Biosciences UK, Ltd., Little Chalfont, Buckinghamshire, UK). Equal loading of proteins was verified by Coomassie Blue staining of gels and actin immunoblotting. Changes in the protein levels were determined via scanning densitometry of the immunoblots. At least four separate experiments were performed with different preparations to confirm changes in the protein levels.

DNA and RNA Gel-Shift Assays. Gel-shift assays for C/EBP DNA binding were carried out with the double-stranded oligonucle-

otide (5′-TGCAGATTGCGCAATCTGCA-3′) end-radiolabeled with [γ-^{32}P]ATP and T4 polynucleotide kinase. The reaction mixture contained 2 μl of 5× binding buffer [containing 50 mM Tris-HCl, pH 7.5, 20% glycerol, 5 mM $MgCl_2$, 250 mM NaCl, 2.5 mM EDTA, 2.5 mM DTT, and 0.25 mg/ml poly(dI-dC)], 10 μg of nuclear extract, and sterile water up to a total volume of 10 μl. The probe (1 μl, containing 10^6 cpm) was added to the reaction mixture, and DNA-binding reactions were carried out for 30 min at room temperature. Specificity of protein binding to DNA was determined by competition experiment, which was carried out by adding a 20-fold molar excess of an unlabeled oligonucleotide to the reaction mixture before the labeled probe was added. In other analyses known as supershift and immunoinhibition assays, anti-C/EBPβ antibody (2 or 4 μg) was added to the reaction mixture 20 min before the labeled probe was added and the reaction was continued for another 1 h at 25°C. Proteins were resolved on 4% polyacrylamide gels at 100 V. The gels were fixed, dried, and subjected to autoradiography.

The CUGBP1-binding RNA oligonucleotides, (CUG)8 and LAP-CUGBP1 (5′-AUGCACCGCCU GCUGGCCUGGGAC-3′), were purchased from Oligos Etc., Inc. (Wilsonville, OR) and labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase. Cytoplasmic proteins (15 μg each) were incubated with the RNA probes under RNase-free conditions and analyzed by polyacrylamide gels. To verify the specificity of protein binding to the RNA, 2 μg of antibody directed against CUGBP1 or calreticulin was added to the reaction mixture 20 min before the labeled probe was added. For competition experiments, reaction mixtures were preincubated for 20 min with a 20-fold molar excess of the unlabeled RNA oligonucleotide before the labeled probe was added.

Southwestern Blot Analysis. Southwestern blot analysis was carried out as described previously (An et al., 1996). In brief, nuclear proteins were resolved by 12% gel electrophoresis and electrophoretically transferred onto nitrocellulose paper. The nitrocellulose paper was blocked with 5% nonfat dry milk in binding buffer (20 mM HEPES, pH 7.9, 60 mM KCl, 1 mM DTT, 0.01% Nonidet P-40, and 10% glycerol) for 1 h at 4°C and incubated with ^{32}P-labeled double-stranded C/EBP-binding oligonucleotide in the binding buffer containing salmon sperm DNA (1 μg/ml) and 0.25% nonfat dry milk for 24 h at 4°C. The membrane was washed three times for 10 min in the binding buffer containing 0.25% nonfat dry milk. A replicate blot was also probed with SP-1-binding oligonucleotide (5′-ATTC-GATCGGGGCGGGGCGAGC-3′). The blots were autoradiographed.

Preparation of Plasmid Constructs. The pGL-1.65 plasmid (GSTA2 promoter, −1651/+66 bp) containing a C/EBP-response element was used to assess C/EBPβ-LAP-mediated gene transactivation. The pGL-1.65 plasmid construct was prepared as described previously (Kang et al., 2003). In brief, the pGL-1651 reporter gene construct was generated by ligating the region 1.65 kilobases upstream of the transcription start site of the rat *GSTA2* gene to the firefly luciferase reporter gene coding sequence. The dominant-negative expression plasmid of C/EBP (AC/EBP) and pCMV500 were gifts from Dr. C. Vinson (National Institutes of Health, Bethesda, MD). pCDNA-C/EBPβ, which encodes rat C/EBPβ, was constructed as described previously (Cho and Kim, 2003). pCDNA-C/EBPβ-LAP2-M3L, in which the third start codon of C/EBPβ was mutated to the codon for leucine, was prepared by using the QuikChange site-directed mutagenesis system (Stratagene, La Jolla, CA). The coding region encoding C/EBPβ-LIP was polymerase chain reaction-amplified from the template of pCDNA-C/EBPβ-LAP2 and cloned into pCDNA. Authenticity of the constructs was verified by DNA sequencing (ABI7700 DNA cycle sequencer; Applied Biosystems, Foster City, CA).

LAP-Mediated Gene Transactivation Assay. 3T3-L1 preadipocytes were transiently transfected with the pGL-1651 using Lipofectamine 2000 reagent according to the instructions stipulated by the manufacturer (Invitrogen, Carlsbad, CA) (Kang et al., 2003). For some experiments, cells were cotransfected with pCDNA, pCDNA-C/EBPβ-LAP2-M3L, or pCDNA-C/EBPβ-LIP in combination with pGL-1651. In brief, cells were replated 24 h before transfection at a density of 5×10^5 cells in six-well plates. Cells were transfected by the addition of 1 ml of DMEM containing each plasmid ranging from 0.3 to 1.5 μg with a fixed total amount of plasmid and 2 μl of Lipofectamine 2000 (Invitrogen) and then incubated at 37°C in a humidified atmosphere of 5% CO_2 for 3 h. The cells were then incubated in DMEM containing 5% FBS for 24 h at 37°C. Control cells were transfected with an equal amount of the empty plasmid.

To assess transactivation activity from pGL-1651, we used the dual-luciferase reporter system (Promega, Madison, WI). In brief, 3T3-L1 cells (5×10^5 cells/well) were replated in six-well plates 24 h before transfection and transiently transfected with 0.5 μg of pGL-1651-luciferase construct and 0.2 μg of CMV-β-galactosidase plasmid (Invitrogen) in the presence of Lipofectamine 2000 for 3 h. The CMV-β-galactosidase plasmid was used to evaluate the efficiency of transfection. Transfected cells were incubated overnight in DMEM containing 5% FBS and then exposed to oltipraz for 24 h at 37°C. Firefly luciferase activity in cell lysates (Promega) was measured using a Luminoskan luminometer (Thermo Electron Corporation, Waltham, MA). The activity of firefly luciferase was measured by adding Luciferase Assay Reagent II (Promega) according to instructions by the manufacturer. 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.

Statistical Analysis. Scanning densitometry of the immunoblots was performed with Image Scan and Analysis System (Alpha Innotech, San Leandro, CA). The area of each lane was integrated using the software AlphaEase, version 5.5 after 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

Enhanced LIP Production by Oltipraz. We have shown that oltipraz activated C/EBPβ-LAP (LAP2) and increased pGL-1651 luciferase gene expression in hepatocyte-derived cells (Kang et al., 2003). We initially examined the effects of oltipraz on the expression patterns of LAP and LIP in preadipocytes incubated with or without FBS and differentiating hormones. Anti-C/EBPβ antibody (catalog number sc-150; Santa Cruz Biotechnology) recognized C/EBPβ isoforms, including LAP1 (38 kDa), LAP2 (35 kDa), and LIP (20 kDa). LAP2 is a major form functionally responsible for gene transcription. Immunoblot analysis revealed that the expression of LAP2 was unchanged by oltipraz treatment in preadipocytes that had been incubated in the DMEM culture medium without FBS and MDI. To our surprise, the expression of LIP began to notably increase 6 h after oltipraz treatment of preadipocytes incubated without FBS and MDI, and increase in LIP production was persistent at least up to a 48-h time point (Fig. 1A, top). C/EBPβ-LIP can dimerize with C/EBPβ-LAP, and the LIP-LAP complex functions as a dominant-negative transcription factor. To predict the antagonism of LIP against LAP-mediated transactivation, we calculated the ratio of LIP to LAP2 after scanning densitometry of the immunoblots and plotted the ratio of LIP/LAP as a function of time (Fig. 1A, bottom). The ratio of LIP to LAP

protein during the time period of 6 to 48 h after oltipraz treatment was much greater than that in vehicle-treated control cells, which indicated that formation of negative transcription factor complex was increased by oltipraz. Incubation of preadipocytes with MDI stimulated induction of both LAPs and LIP at early times (Fig. 1B). When preadipocytes were cultured with FBS and MDI in the presence of oltipraz, LIP expression increased to a greater extent than that in cells treated with MDI alone during the time period of 24 to 48 h. Our results show that oltipraz promotes the production of LIP in preadipocytes to a greater extent than that of LAP.

Nuclear Activation of LIP by Oltipraz. Given the function of LIP as a dominant-negative factor for transcriptional activity, we sought to determine whether oltipraz increases nuclear LIP in preadipocytes and promotes its DNA binding. The levels of LIP in the nuclear fractions prepared from cells treated with oltipraz for 6 to 12 h were clearly elevated compared with control (Fig. 2A). In contrast to the induction of LIP, the band intensities of LAP proteins were unchanged. Nevertheless, the electrophoretic mobilities of LAP proteins were slightly retarded by oltipraz treatment, which may have resulted from post-translational modifications (e.g., phosphorylation).

We performed gel-shift analysis next to assess protein binding to the C/EBP-binding site. The ability of proteins in nuclear extracts to bind with the C/EBP consensus oligonucleotide was greatly enhanced in cells treated with oltipraz for 6 to 12 h (Fig. 2B, left), which was in parallel with the

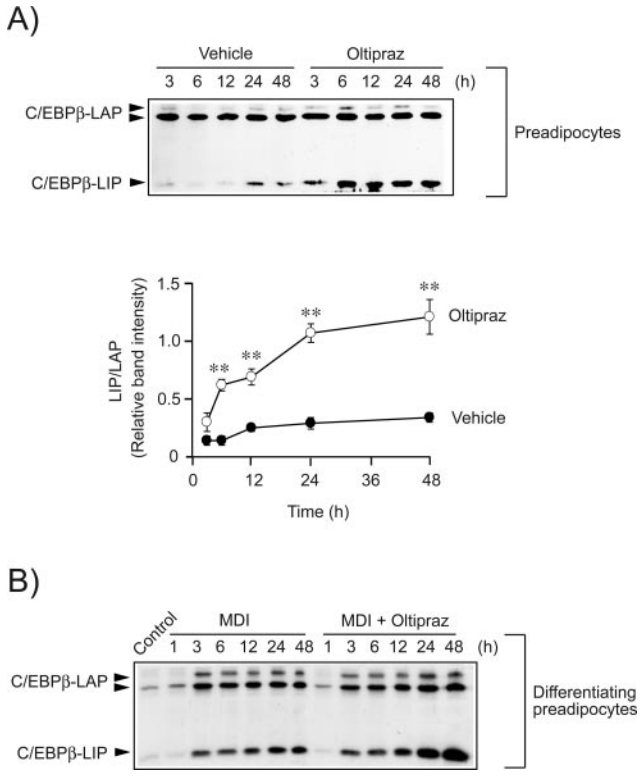

Fig. 1. The effects of oltipraz on the expression of C/EBPβ-LAP and C/EBPβ-LIP in preadipocytes. A, a representative immunoblot shows the levels of C/EBPβ-LAP and C/EBPβ-LIP in 3T3-L1 preadipocytes (top). Immunoblot analyses were performed with the lysates prepared from preadipocytes that had been incubated in the culture medium containing 30 μM oltipraz for 3 to 48 h. The ratios of C/EBPβ-LIP to C/EBPβ-LAP in preadipocytes treated with vehicle (DMSO) or oltipraz were shown as a function of time (bottom). The relative ratio of C/EBPβ-LIP to C/EBPβ-LAP was derived from the densitometric band intensities of immunoblots. The experimental value represented the mean ± S.E. with four separate experiments (significant compared with the respective vehicle-treated control; **, $p < 0.01$). B, the effects of oltipraz on C/EBPβ-LAP and C/EBPβ-LIP expression in MDI-induced differentiating preadipocytes. The levels of C/EBPβ-LAP and C/EBPβ-LIP were determined in preadipocytes incubated in the medium containing MDI in the presence or absence of oltipraz for 1 to 48 h. Results were confirmed by at least three repeated experiments.

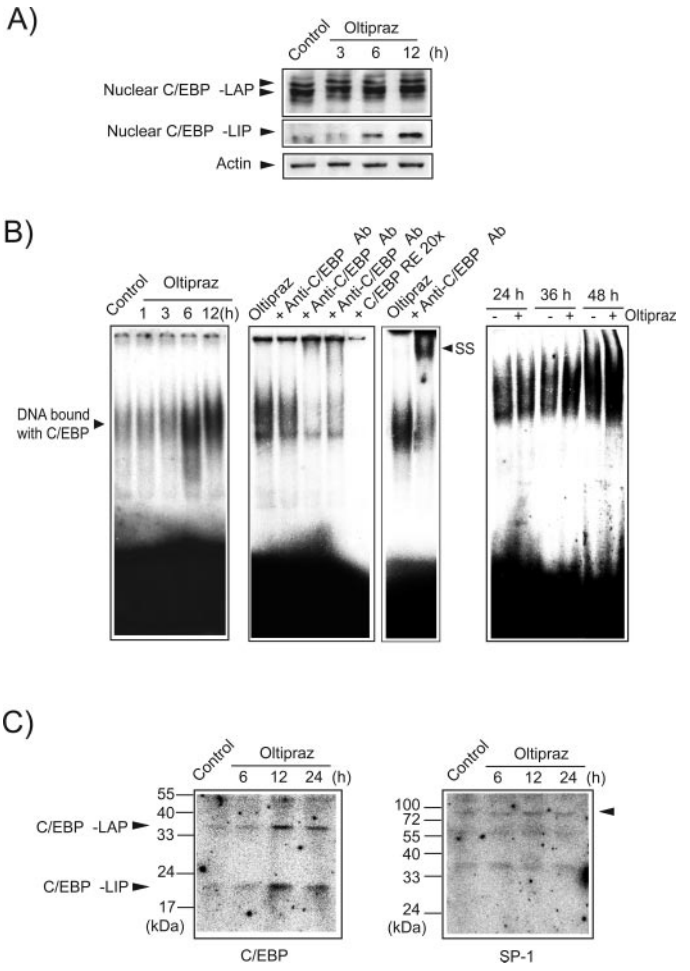

Fig. 2. Activation of C/EBPβ-LIP by oltipraz in preadipocytes. A, immunoblot analyses of nuclear C/EBPβ-LAP and LIP. LAP and LIP proteins were immunoblotted in the nuclear fractions prepared from 3T3-L1 preadipocytes that had been incubated with 30 μM oltipraz for 3 to 12 h. Each lane contained 10 μg of nuclear proteins. Equal loading of proteins was verified by probing the replicate blot for actin. B, gel-shift analyses of protein binding to the consensus C/EBP-binding DNA oligonucleotide. Nuclear extracts were prepared from 3T3-L1 preadipocytes cultured with oltipraz for 1 to 12 h. All lanes contained 10 μg of nuclear extract and 5 ng of labeled C/EBP-binding DNA. Immunodepletion or supershift assays were carried out by incubating the nuclear extract (oltipraz, 12 h) with the polyclonal antibody (2 μg each) directed against C/EBPα, C/EBPβ, and C/EBPδ, whereas a competition assay was performed with unlabeled C/EBP oligonucleotide (a 20-fold molar excess). Anti-C/EBPβ antibody exhibited partial immunodepletion and/or supershift effect. SS indicates supershifted DNA in the sample incubated with 4 μg of anti-C/EBPβ antibody. Additional gel-shift analyses were performed with nuclear extracts prepared from preadipocytes treated with or without oltipraz for 24 to 48 h. C, Southwestern analysis of nuclear proteins that bind to C/EBP-binding DNA oligonucleotide. Nuclear proteins prepared from preadipocytes untreated (control) or treated with oltipraz for 6 to 24 h were subjected to 12% SDS-polyacrylamide gel electrophoresis, blotted onto nitrocellulose paper, and probed with ^{32}P-radiolabled C/EBP or SP-1-binding consensus oligonucleotide (10^6 cpm). The locations of the molecular size standards are shown on the left. Arrowheads indicate C/EBPβ-LAP and C/EBPβ-LIP (left). Results were confirmed by at least three repeated experiments.

results of immunoblot analyses. Competition experiments with the antibodies directed against C/EBPα, C/EBPβ, and C/EBPδ supported that oltipraz-induced C/EBP DNA-binding activity in preadipocytes was mainly dependent on C/EBPβ. Because a specific antibody that recognizes endogenous LIP is not available, we used the anti-C/EBPβ antibody that interacts with both LIP and LAP forms. The anti-C/EBPβ antibody almost completely reduced the band intensity of the C/EBP DNA-binding complex, whereas anti-C/EBPδ antibody decreased the band intensity to a lesser extent (Fig. 2B, middle). Anti-C/EBPβ antibody immunodepleted and/or supershifted the C/EBP DNA complex. The experiment suggested that the C/EBPβ forms, presumably including LIP, play a major role in the C/EBP DNA-binding activity induced by oltipraz. The addition of a 20-fold excess of an unlabeled C/EBP-binding oligonucleotide to the nuclear extract completely abolished the protein DNA-binding activity. An extended experiment showed that the band intensities of C/EBP binding to the consensus oligonucleotide were greatly increased 36 to 48 h after oltipraz treatment (Fig. 2B, right). The data are consistent with the sustained increase in LIP by oltipraz in preadipocytes.

To verify that nuclear C/EBPβ-LIP activated by oltipraz binds to the C/EBP-binding DNA, we performed Southwestern blot analyses. Nuclear extracts prepared from preadipocytes treated with vehicle or oltipraz were subjected to SDS-polyacrylamide gel electrophoresis analysis and transferred to nitrocellulose membrane, which was probed with the ^{32}P-labeled double-stranded C/EBP-binding DNA. The band intensity of C/EBPβ-LIP binding to the double-stranded C/EBP-binding oligonucleotide was greatly enhanced in cells treated with oltipraz for 12 or 24 h compared with control (Fig. 2C). The binding of LIP (20 kDa) to the C/EBP-binding oligonucleotide was minimal in vehicle-treated control. The band intensity of C/EBPβ-LAP2 (35 kDa) was also increased by oltipraz treatment. Nevertheless, comparable increases in the intensities of LIP and LAP bands by oltipraz suggested to us that the stoichiometry of activated LIP/LAP be no less than one at the time points examined. Protein binding to SP-1 consensus oligonucleotide, which was examined as a comparative control, was not notably changed by oltipraz treatment. Our data provide strong evidence that oltipraz activates both LAP and LIP and promotes LIP binding, presumably as a LIP and LAP heterodimer or LIP homodimer, to the C/EBP-binding DNA element.

Repression of LAP-Mediated Gene Transactivation by Oltipraz. To functionally determine whether increases in band intensities obtained in gel-shift assays occurred as a result of C/EBPβ-LIP binding to the target promoter, the pGL-1651 luciferase gene transactivation was monitored in 3T3-L1 preadipocytes. First, we assessed the effect of LIP on the gene expression. The mutant plasmid of C/EBPβ-LAP2-M3L that encodes an active LAP2 but not LIP, in which the third AUG site required for LIP translation was mutated to the codon for leucine, was prepared (Fig. 3A, top). As preadipocytes were transfected with the C/EBPβ-LAP2-M3L plasmid in combination with increasing amounts of the C/EBPβ-LIP plasmid, luciferase expression from pGL-1651 gradually decreased (Fig. 3A, bottom). Immunoblot analysis verified the expression of LAP and LIP in the cells. These data showed that C/EBPβ-LIP expressed ectopically was capable of repressing LAP-mediated gene expression from pGL-1651 in preadipocytes.

Second, we examined whether oltipraz down-regulates pGL-1651 gene expression in preadipocytes. Exposure of preadipocytes transfected with an empty vector (pCMV500) to oltipraz resulted in a 50% decrease in luciferase activity compared with untreated control (Fig. 3B). AC/EBP is a

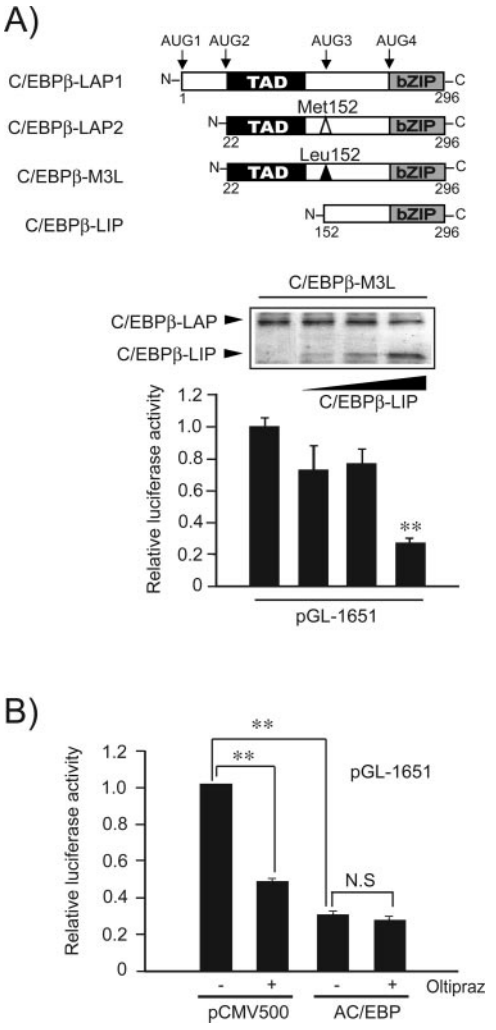

Fig. 3. The effects of C/EBPβ-LIP or oltipraz on the C/EBPβ-LAP-mediated gene transactivation. A, the effect of C/EBPβ-LIP on luciferase expression from pGL-1651. The sizes of C/EBPβ-LAP1, C/EBPβ-LAP2, C/EBPβ-LAP2-M3L (M3L), and C/EBPβ-LIP proteins were compared at the top. In the plasmid of C/EBPβ-LAP mutant M3L, the third AUG site required for LIP translation was mutated to UUG for the prevention of C/EBPβ-LIP expression. Luciferase expression from pGL-1651 was monitored in 3T3-L1 preadipocytes that had been transfected with the M3L plasmid alone (0.3 μg) or the M3L plasmid in combination with increasing amounts of the plasmid encoding for LIP (0.3~1.5 μg). Overexpression of M3L and/or LIP in the cells was confirmed by immunoblot analysis (luciferase activity in cells transfected with the plasmid encoding C/EBPβ-M3L = 1). B, suppression of C/EBPβ-LAP-mediated reporter gene expression by oltipraz or AC/EBP. 3T3-L1 preadipocytes that had been cotransfected with pGL-1651 (firefly luciferase) in combination with an empty vector (pCMV500) or the plasmid encoding for a dominant-negative mutant of C/EBPβ (AC/EBP) were treated with vehicle or 30 μM oltipraz for 18 h. Luciferase activities were measured in cell lysates. Activation of the reporter gene was calculated by normalizing firefly luciferase activity to that of β-galactosidase. Data represent the mean $\pm$ S.E. with four separate experiments (significant compared with pCMV500 without oltipraz treatment; **, $p < 0.01$; N.S., not significant; luciferase activity in cells transfected with pCMV500 alone without oltipraz treatment = 1).

dominant-negative mutant form of C/EBPβ that prevents nuclear translocation and DNA binding of C/EBPβ-LAP and thus inhibits adipogenesis (Zhang et al., 2004). As expected, transfection of preadipocytes with the AC/EBP plasmid inhibited the gene expression (Fig. 3B). Luciferase expression was not further decreased by oltipraz in the cells transfected with the AC/EBP plasmid. Our data supported the conclusion that the observed induction of LIP by oltipraz in preadipocytes leads to repression of the target gene transactivation. Thus, it is highly probable that functionally active LIP enhanced by oltipraz treatment inhibits adipogenesis, as in the case of AC/EBP (Zhang et al., 2004).

Activation of CUGBP1 by Oltipraz. Because CUGBP1 has been identified as an RNA-binding protein and plays a key role in C/EBPβ-LIP expression (Timchenko et al., 1999, 2001a; Welm et al., 2000), we hypothesized that the induction of LIP by oltipraz might be mediated by activation of CUGBP1. As part of the studies on CUGBP1 activation, we determined the subcellular distribution of CUGBP1 before and after oltipraz treatment in preadipocytes. Subcellular fractionation and immunoblot analysis revealed that CUGBP1 was predominantly located in the nuclear fractions of preadipocytes under basal conditions and gradually translocated to the cytoplasmic fractions after stimulation (Fig. 4A). In particular, CUGBP1 showed greater extents of cytoplasmic localization 12 to 24 h after oltipraz treatment than at earlier time points. The levels of CUGBP1 in total cell lysates were unchanged by oltipraz (Fig. 4A). These data indicate that oltipraz induces gradual time-dependent translocation of CUGBP1 into the cytoplasm with no change in the protein expression.

Calreticulin is another RNA-binding protein that interacts with GC-rich sequences. The binding of calreticulin to GC-rich stem-loop structures causes inhibition of mRNA translation (Timchenko et al., 2002). Therefore, we examined calreticulin levels in the cytoplasmic fractions prepared from cells untreated or treated with oltipraz for 3 to 24 h. Oltipraz did not change the levels of calreticulin in the cytoplasm (Fig. 4B). This was in parallel with the gel-shift data [i.e., no inhibition of oltipraz-inducible protein-(CUG)8 RNA binding by anti-calreticulin antibody]. These results indicate that calreticulin is not activated by oltipraz.

Next, to determine whether cytoplasmic translocation of CUGBP1 by oltipraz accompanies changes in the RNA-binding capacity, we conducted RNA gel-shift assays. Cytoplasmic proteins prepared from preadipocytes treated with oltipraz (12 h) showed increased binding to the RNA oligonucleotide (CUG)8 (Fig. 4C). Immunoinhibition experiments using anti-CUGBP1 antibody or anti-calreticulin antibody confirmed that increase in protein binding to (CUG)8 was attributed to CUGBP1. Addition of a 20-fold excess of unlabeled (CUG)8 RNA oligonucleotide or LAP-CUGBP-binding RNA oligonucleotide (i.e., LAP-CUGBP) to the cytoplasmic proteins completely abolished the binding activity (Fig. 4C). The cytoplasmic translocation of CUGBP1 and increase in its RNA-binding activity by oltipraz suggested that the signaling pathway activated by oltipraz regulates CUGBP1 activation, which would then stimulate LIP production in preadipocytes. In an additional experiment, we found that CUGBP1 overexpression (i.e., stable transfection of CUGBP1 in 3T3-L1 preadipocytes) alone failed to stimulate LIP production in preadipocytes (data not shown), suggesting that increase in CUGBP1 binding to RNA by oltipraz may involve post-translational modification(s).

Antiadipogenesis by Oltipraz. Given the enhanced activation of C/EBPβ-LIP by oltipraz, we were interested in whether oltipraz inhibits differentiation of preadipocytes. 3T3-L1 preadipocytes were exposed to vehicle or oltipraz for 2 days and induced to differentiate with MDI for 8 days in the continuing presence of oltipraz in the growth medium containing 10% FBS (GM) (Fig. 5A). Eight days after vehicle or oltipraz treatment in the presence of differentiation inducers, the cells were subjected to Oil Red O staining. Preadipocytes with vehicle treatment well differentiated, as evidenced by the accumulation of cytoplasmic triglycerides stained with Oil Red O (Fig. 5, B and C). In contrast, differentiation of

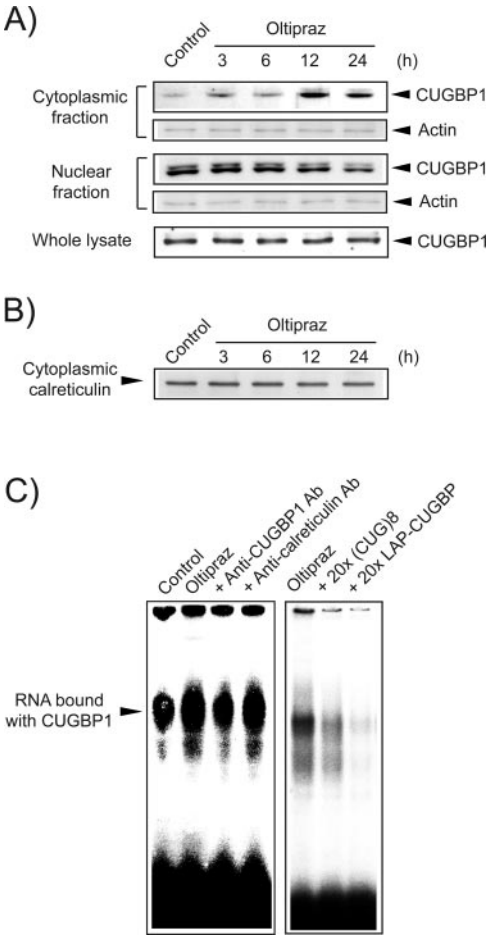

Fig. 4. Activation of CUGBP1 by oltipraz in 3T3-L1 preadipocytes. A, cytoplasmic translocation of CUGBP1 by oltipraz. The levels of CUGBP1 were determined by immunoblot analyses in cytoplasmic (or nuclear) fractions or lysates prepared from 3T3-L1 preadipocytes that had been treated with oltipraz for 3 to 24 h. Equal loading of proteins was verified by probing the replicate blots for actin. Each lane contained 10 μg of lysate (or cytoplasmic) proteins or 5 μg of nuclear proteins. B, immunoblot analysis of cytoplasmic calreticulin. Calreticulin in the cytoplasmic fraction was immunoblotted with anti-calreticulin antibody. C, increase by oltipraz in cytoplasmic CUGBP1 binding to (CUG)8 RNA oligonucleotide. The binding of cytoplasmic proteins (15 μg each), prepared from cells incubated with vehicle or oltipraz for 12 h, to radiolabeled (CUG)8 RNA was determined by RNA gel-shift assay. Specific binding of CUGBP1 to the RNA probe was verified by immunoinhibition analysis using anti-CUGBP1 antibody or anti-calreticulin antibody (2 μg each). Competition assays were also performed with a 20-fold molar excess of unlabeled (CUG)8 or LAP-CUGBP-binding RNA oligonucleotide (LAP-CUGBP). The results were confirmed by at least two repeated experiments.

preadipocytes treated with oltipraz was significantly inhibited. Rosiglitazone, a PPARγ agonist, was used as a positive control. Cells treated with rosiglitazone showed a slightly greater extent of differentiation compared with control. We next determined the expression of major transcription factors, including C/EBPβ-LAP, C/EBPβ-LIP, C/EBPα, and PPARγ, during the adipocyte differentiation program. Immunoblot analyses showed that the levels of C/EBPβ-LAP and C/EBPβ-LIP initially increased in the cells treated with MDI for 2 days with gradual decreases at later times, whereas those of mature adipocyte marker proteins, 42 kDa C/EBPα, 30 kDa C/EBPα, and PPARγ, greatly increased 5 to 10 days after hormone stimulation, verifying the adipogenic program. In contrast, the expression of C/EBPα or PPARγ was virtually abolished in cells treated with oltipraz (Fig. 5D). Incubation of 3T3-L1 cells for an extended period of time (i.e., 14–15 days) under the differentiation program failed to overcome antiadipogenic effect of oltipraz (data not shown). It was noteworthy that the band intensities of C/EBPβ-LIP were increased by oltipraz treatment at days 2 and 5 after exposure to MDI (Fig. 5D).

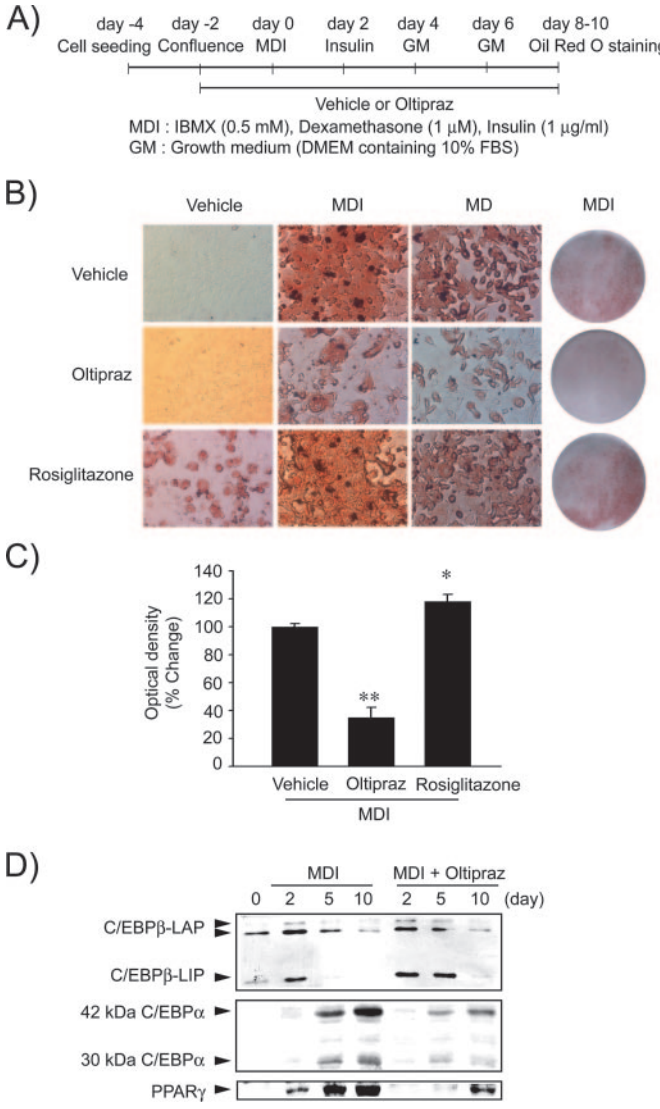

Fig. 5. Inhibition of 3T3-L1 preadipocyte differentiation by oltipraz. A, differentiation program of 3T3-L1 preadipocytes. Preadipocytes were seeded and cultured until 2 days after confluence. Differentiation was induced by incubating the 3T3-L1 preadipocytes with the GM containing 10% FBS in the presence of MDI or IBMX and dexamethasone, as described under *Materials and Methods*. Oltipraz (30 μM) was added to the incubation medium 2 days before the induction of differentiation with MDI. B, representative photographs of the cell monolayers stained with Oil Red O (left, 100; right, dishes). Rosiglitazone (1 μM) was used as an agent that induced adipocyte differentiation. C, the relative levels of optical density of Oil Red O extracted from the cells treated with vehicle, oltipraz, or rosiglitazone. Data represent the mean ± S.E. with five separate experiments (significant compared with control; *, $p < 0.05$; **, $p < 0.01$; Oil Red O content in cells incubated with MDI + vehicle, 100%). D, immunoblot analyses of C/EBPβ-LAP, C/EBPβ-LIP, 42-kDa C/EBPα, 30-kDa C/EBPα, and PPARγ. Lysates prepared from undifferentiated or differentiating preadipocytes (2–10 day postdifferentiation induction with or without oltipraz) were subjected to immunoblot analyses with the respective specific antibody.

Antiadipogenic Effect in Primary Cultured Preadipocytes. In the subsequent experiments, the antiadipogenic effect of oltipraz was confirmed in the preadipocytes isolated from rat epididymal fat pads. Freshly isolated preadipocytes were plated in dishes and incubated with or without oltipraz. LIP expression was markedly increased in primary cultured preadipocytes 12 to 36 h after oltipraz treatment compared with control (Fig. 6A, top). The ratio of LIP to LAP was significantly greater at 24 or 48 h after oltipraz treatment than vehicle-treated control (Fig. 6A, bottom). Postconfluent preadipocytes were induced to differentiate by the methods described in detail under *Materials and Methods*. Microscopic analysis showed that the primary cultured preadipocytes were well differentiated to adipocytes by MDI stimulation (Fig. 6B). In contrast, oltipraz treatment with the differentiation inducers inhibited cell differentiation, as evidenced by the decrease in the accumulation of lipid droplets. Immunoblot assay verified that oltipraz treatment inhibited the expression of C/EBPα, a differentiation marker in the cells incubated with MDI for 8 days (Fig. 6C).

These results collectively corroborate that oltipraz exerts antiadipogenesis through LIP production and that LIP induction is stimulated by oltipraz in preadipocytes presumably through activation of CUGBP1.

Discussion

A full-length C/EBPβ-LAP1 mediates chromatin remodeling (Kowenz-Leutz and Leutz, 1999), whereas LAP2 plays a major role in gene transactivation (Descombes and Schibler, 1991). LIP retains the C-terminal basic leucine zipper and DNA-binding region but lacks most of the N-terminal transactivation domain of the full-length C/EBPβ-LAP. LIP interacts with C/EBPβ isoforms for the formation of homodimeric or heterodimeric inhibitory transcription complexes. The ratio of LIP to LAP can be changed according to cellular conditions for the regulation of adipogenesis (Karagiannides et al., 2001). In the process of adipogenic differentiation, LIP functions as a dominant-negative isoform (Hamm et al., 2001; Karagiannides et al., 2001). We demonstrated for the first time that oltipraz treatment increased LIP production in preadipocytes beginning at an early time point (6 h) and that the ratio of LIP to LAP was enhanced at least up to the 48-h time point. Increases in the LIP/LAP ratio by oltipraz were also observed in preadipocytes incubated with differentiating hormones.

Oltipraz persistently enhanced protein binding to the C/EBP-binding oligonucleotide after treatment (up to 48 h). The binding proteins include C/EBPβ and, to a minor extent,

C/EBPδ. The C/EBPβ forms interacting with DNA would include C/EBPβ-LAP and LIP. Of the isoforms, LIP has the higher DNA-binding affinity (Descombes and Schibler, 1991). Because gel-shift assay was not sufficiently sensitive to monitor the difference in the migration and/or the affinity of LIP and LAP binding to DNA, we also performed Southwestern blot analysis. This assay allowed us to show that LIP in the nuclear extracts prepared from oltipraz-treated preadipocytes could bind to the C/EBP-binding site. C/EBPβ-LAP protein (LAP2) was also capable of interacting with the C/EBP-binding DNA. However, C/EBPδ binding to the C/EBP-binding DNA was not detected by Southwestern assay, which was consistent in part with the result of the gel-shift immunoinhibition analysis. Similar increases in the band intensities of C/EBPβ-LIP and LAP by oltipraz indicate that LIP induced by oltipraz can heterodimerize with LAP for the formation of inhibitory transcription complex. This result raised the possibility that the activating process for LIP is similar to that of LAP (e.g., nuclear translocation and/or phosphorylation).

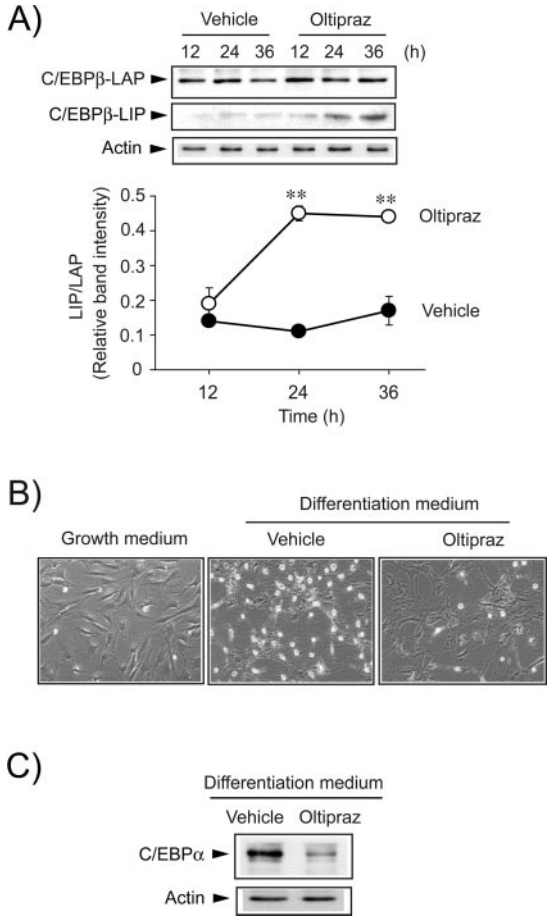

Fig. 6. Inhibition of primary cultured preadipocyte differentiation by oltipraz. A, enhanced LIP production by oltipraz in primary cultured rat preadipocytes. The levels of C/EBPβ-LAP and C/EBPβ-LIP were immunoblotted in the lysates prepared from freshly isolated rat preadipocytes that had been incubated in the growth medium with vehicle or 30 μM oltipraz for 12 to 36 h. The ratio of LIP to LAP was determined, as described in the legend to Fig. 1. B, the phase-contrast microphotographs of rat preadipocytes whose adipogenic induction was stimulated by MDI in the presence or absence of oltipraz (×100). Differentiation of primary cultured preadipocytes was induced by MDI in combination with transferrin and 3,3′,5-triiodo-L-thyronine (differentiation medium) as described under *Materials and Methods*. C, immunoblot analysis of C/EBPα. C/EBPα, a representative adipocyte marker, was immunoblotted in the lysates of rat preadipocytes that were incubated in the growth medium containing MDI, transferrin, and 3,3′,5-triiodo-L-thyronine (differentiation medium) with vehicle or oltipraz for 8 days. Equal loading of proteins was confirmed by actin immunoblotting.

The results of luciferase expression from the reporter plasmid that contains the C/EBP-binding site lends support to the conclusion that LIP whose expression was promoted by oltipraz indeed prevents LAP-mediated gene expression in preadipocytes. This was comparable with the extent of the gene repression by AC/EBP. It has been shown that AC/EBP heterodimerizes with C/EBPβ isoforms and inhibits the formation of functional LAP homodimers presumably by preventing nuclear localization signals located in its basic DNA-binding domain (Greenwel et al., 2000; Rishi and Vinson, 2003). This is in agreement with our observation that AC/EBP also inhibits LAP-mediated gene transcription. Although the inhibitory mechanism of LIP differs from that of AC/EBP (i.e., nuclear translocation and DNA binding), repression of the target gene transactivation by LIP was comparable with that of AC/EBP. Our data showed that LIP induced by oltipraz treatment was functionally active in the inhibition of LAP-mediated gene expression in preadipocytes.

Many of studies have shown that LIP is induced by alternative translation of the C/EBPβ mRNA via the leaky ribosomal scanning mechanism (Descombes and Schibler, 1991; An et al., 1996; Welm et al., 1999). Among the isoforms of C/EBPβ, LIP is selectively induced by epidermal growth factor (Baldwin et al., 2004). The role of protein synthesis in LIP expression was evidenced by increase in the incorporation of [^{35}S]methionine to the protein. Other studies raised the possibility that LIP is also produced from C/EBPβ-LAP via proteolysis (i.e., N-terminal truncation) (Welm et al., 1999; Baer and Johnson, 2000). Northern blot analysis showed that the level of C/EBPβ mRNA was not increased but rather decreased by oltipraz in preadipocytes (data not shown). LIP activation by oltipraz may repress the *C/EBPβ* gene transcription, because the promoter region comprises the C/EBP-binding site. No increase in the level of C/EBPβ mRNA supports the hypothesis that LIP induction by oltipraz results from post-transcriptional regulation. LIP induction by oltipraz would result from the alternative use of in-frame translation start sites present in the C/EBPβ mRNA, presumably under the control of RNA-binding protein. To verify that LIP production was the result of protein synthesis, we treated preadipocytes with oltipraz in combination with 20 μg/ml cycloheximide. Oltipraz-inducible LIP production in cells treated with cycloheximide was attenuated compared with that of LAP (data not shown). This experiment supports the hypothesis that oltipraz enhances LIP production by translational activation. It is less likely that LIP is proteolytically generated from LAP, because LAP expression was not decreased by oltipraz during LIP induction despite the decrease in C/EBPβ mRNA.

CUGBP1 directly interacts with the GCN repeats located within mRNA. The genes whose transcripts are translationally regulated by CUGBP1 include the C/EBPβ, C/EBPα, p21, and myocyte enhancer factor 2A (Timchenko et al., 1999, 2004; Iakova et al., 2004). Activation of CUGBP1 is critical for LIP production in certain types of cells (i.e., mammary epithelial cells and fibroblasts) and in the liver tissue (Timchenko et al., 1999; Welm et al., 2000; Baldwin et al., 2004). We found that the levels of cytoplasmic CUGBP1 and protein

binding to (CUG)8 RNA oligonucleotide were both enhanced by oltipraz in preadipocytes. This finding renders us to predict that CUGBP1 activation contributes to the production of LIP that determines the preadipocyte fate. To our knowledge, oltipraz is the first pharmacological agent that activates CUGBP1 and induces CUGBP1-mediated LIP production. CUGBP1 was located in the nuclear fractions of preadipocytes under basal conditions and gradually translocated to the cytoplasm after oltipraz stimulation. In contrast, CUGBP1 was largely localized in the cytoplasm of hepatocyte-derived H4IIE cells under the resting state, whereas oltipraz treatment caused nuclear translocation of CUGBP1 (E. J. Bae and S. G. Kim, unpublished data). Thus, the cellular location and oltipraz regulation of CUGBP1 seemed to be cell type-specific. Calreticulin, which also binds to the GCN repeats of the C/EBPβ mRNA, was not activated by oltipraz. Therefore, oltipraz-activated RNA-binding protein that interacts with the C/EBPβ mRNA in preadipocytes is CUGBP1, not calreticulin. Our observation that overexpression of CUGBP1 did not stimulate LIP production in preadipocytes in conjunction with no increase in CUGBP1 expression by oltipraz indicates that activation of CUGBP1 by oltipraz may be mediated by post-translational changes.

Phosphorylation of CUGBP1 is also critical for its binding activity to mRNA, as supported by the finding that dephosphorylation of CUGBP1 abolished its interaction with C/EBPβ mRNA and inhibited LIP translation (Welm et al., 2000; Baldwin et al., 2004). It has been shown that hypophosphorylation of CUGBP1 prevented cytoplasmic translocation of CUGBP1 with its nuclear accumulation (Roberts et al., 1997). Our data show that localization of CUGBP1 is distinctly changed by oltipraz treatment, which may be controlled by its phosphorylation status. Activation of CUGBP1 by oltipraz is likely to be mediated by phosphorylation that is catalyzed by specific kinase(s). Despite the presence of the putative multiple phosphorylation sites in CUGBP1, the kinase responsible for the phosphorylation remains to be established.

The adipogenic proteins PPARγ and C/EBPα are induced during the adipogenic differentiation program. C/EBPβ-LAP regulates the PPARγ (PPARγ2 is a predominantly inducible form in adipocytes) and C/EBPα genes by its specific binding to the C/EBP response elements of the genes, coordinately regulating the essential steps of adipogenesis (Christy et al., 1991; Yeh et al., 1995; Darlington et al., 1998; Tang et al., 2003). Ectopic expression of LIP prevented the induction of the adipogenic genes such as C/EBPα, PPARγ2, and adipocyte protein-2/fatty acid-binding protein (Hamm et al., 2001), which was accompanied by the inhibition of mitotic clonal expansion. In the present study, we showed that oltipraz disrupted the program of preadipocyte differentiation induced by hormones via LIP production, which was evidenced by significant decreases in both the accumulation of lipid droplets and the expression of C/EBPα and PPARγ. Oltipraz exerted antiadipogenic effects in 3T3-L1 preadipocytes. C/EBPβ-LIP enhanced by oltipraz presumably binds to the promoter region of the C/EBPα gene, leading to repression of the gene. Repression of C/EBPα by oltipraz may also be responsible for the antiadipogenic effect. Previous studies showed that the concentration of oltipraz used in this study was comparable with that observed in the adipose tissue after administration of 30 mg/kg oltipraz, a possible clinically relevant dose, to rats (Bae et al., 2004). In primary cultured rat preadipocytes, oltipraz also increased LIP production and inhibited adipocyte differentiation. Hence, the antiadipogenic effect of oltipraz may be extended to physiological situations.

LIP expression enhanced by oltipraz (30 μM, 24 h) was maintained at least up to 6 h after washout of oltipraz (see Supplemental Figure), suggesting that the signaling pathway activated by oltipraz for LIP production was sustained after removal of oltipraz. An additional experiment was conducted to determine whether oltipraz treatment altered the stability of LIP protein. Incubation with cycloheximide, a protein synthesis inhibitor, after the washout prevented the cells from inducing LIP. LIP protein enhanced by oltipraz ~50% decreased 3 h after cycloheximide (20 μg/ml) treatment and completely disappeared in 6 h. Re-exposure (3 h) of the cells to oltipraz 1 h after washout resulted in a greater increase in LIP expression, which was also suppressed by concomitant treatment of the cells with cycloheximide. Our data indicate that the induction of LIP by oltipraz may not result from protein stabilization and corroborate that enhanced protein synthesis plays a role in the LIP induction.

In conclusion, the antiadipogenic effect of oltipraz was mediated by the promotion of LIP production and activated LIP binding to the C/EBP-binding site. In addition, the enhanced LIP production by oltipraz accompanied cytoplasmic translocation of CUGBP1 and its binding to the GC-rich region of C/EBPβ mRNA. Our finding holds a significant implication that adipogenic differentiation may be controlled by pharmacological manipulation of LIP production.

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Address correspondence to: Dr. Sang Geon Kim, College of Pharmacy, Seoul National University, Sillim-dong, Kwanak-gu, Seoul 151-742, South Korea. E-mail: sgk@snu.ac.kr