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Antioxidative Effects of a New Lychee Fruit-Derived Polyphenol Mixture, Oligonol, Converted into a Low-Molecular Form in Adipocytes

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In this study we investigated the antioxidative effects of Oligonol (Amino Up Chemical Co., Ltd., Sapporo, Japan), a new polyphenol, in adipocytes. The levels of reactive oxygen species (ROS) and the expression of adipokine genes decreased in HW mouse white adipocytes upon treatment with Oligonol as compared to control cells. The transcriptional activity of nuclear factor-kappaB (NF-κB) and the activation of extracellular signal-regulated kinase (ERK) 1/2 were also down-regulated by Oligonol. In addition, when C57BL/6J mice were fed a high fat diet (HFD) for 5 weeks, the levels of epididymal white adipose tissue (WAT) mass and lipid peroxidation in WAT both increased, but Oligonol intake clearly inhibited such HFD-induced increases. Furthermore, dysregulated expression of genes for adipokines in WAT of mice fed solely a HFD was attenuated by Oligonol intake. These results suggest that Oligonol has antioxidative effects and that it attenuates HFD-induced dysregulated expression of genes for adipokines in adipocytes.

Key words: adipocytes; Oligonol; antioxidative effects; adipokines; metabolic syndrome

Adipocytes secrete many adipokines, which are members of the cytokine and chemokine family. Adipokines, which include tumor necrosis factor-α (TNF-α), plasminogen activator inhibitor-1 (PAI-1), and leptin, have important effects on both lipid and glucose metabolism.[1)] Dysregulated secretion of adipokines is thought to be a causal reason for metabolic disease. For example, adipose TNF-α secretion in obese patients is increased,[2,3)] and increased secretion of TNF-α induces insulin resistance in skeletal muscles and adipocytes,[4–6)] causing a vicious circle. Furthermore, the level of PAI-1 in the plasma is also increased in obese patients, and many reports have shown that increased PAI-1 levels contribute to the development of obesity.[7)] Conversely, decreased expression of the gene for adiponectin in WAT, as well as decreased levels of adiponectin, are observed in the plasma of obese patients.[8)] Because adiponectin induces fatty acid oxidation and decreases the concentration of triglycerides in skeletal muscle and liver, reduction of adiponectin levels is implicated in type 2 diabetes.[9,10)] In addition, an inflammatory response in WAT is considered to be a cause of insulin resistance. Expression of the gene for monocyte chemoattractant protein-1 (MCP-1) is up-regulated in the WAT of genetically obese (ob/ob) mice and high fat diet (HFD)-induced obese mice, and overexpression of MCP-1 in adipocytes promotes macrophage infiltration in WAT.[11,12)]

Recently, it was reported that adipocytes generate ROS, that the levels of oxidative stress in the WAT of KKAy mice (a diabetic model) is increased, and that

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Abbreviations: ACC, acetyl-CoA carboxylase; BAT, brown adipose tissues; ERK, extracellular signal-regulated kinase; FAS, fatty acid synthase; HFD, high fat diet; LFP, lychee fruit-derived polyphenol; MCP-1, monocyte chemoattractant protein-1; NBT, nitroblue tetrazolium; NF-κB, nuclear factor-kappaB; PAI-1, plasminogen activator inhibitor-1; PPARγ-2, peroxisome proliferator-activated receptor-γ2; ROS, reactive oxygen species; SOD, superoxide dismutase; TBARS, thiobarbituric acid reactive substance; TNF-α, tumor necrosis factor-α; UCP, uncoupling protein; WAT, white adipose tissue

increased oxidative stress in adipocytes causes dysregulated expression of adipokines.[13–15] Moreover, increased oxidative stress in adipocytes has been found to impair insulin signaling. For example, prolonged exposure of 3T3-L1 adipocytes to micromolar concentrations of H_2O_2 inhibits insulin-induced translocation of glucose transporter 4 (GLUT4) to the plasma membrane, and inhibits glucose uptake.[16] These results indicate that increased oxidative stress in adipocytes is a cause of obesity-associated metabolic syndrome.

Polyphenols are a large group of natural antioxidants in the diet. They are found in fruits and vegetables, and in beverages such as tea, coffee, and wine. Current studies strongly suggest that polyphenols prevent cardiovascular diseases, cancers, neurodegenerative disease, diabetes and osteoporosis.[17,18] For example, many epidemiologic studies demonstrate beneficial effects of the intake of polyphenols in reducing cardiovascular diseases.[19,20] Furthermore, experimental studies show that polyphenols improve endothelium function.[20] On the other hand, in diabetes, a negative correlation is observed in both normal and diabetic individuals between the glycemic index and the total intake of polyphenols.[21] Other studies have shown that ingestion of walnut polyphenol reduces urinary 8-hydoroxy-2′-deoxyguanosin levels in obese db/db mice,[22] and that ingestion of Malaysian cocoa extract reduces the level of serum glucose, total cholesterol, and triglycerides in streptozodocin-induced diabetic rats.[23]

As stated above, polyphenols are powerful antioxidative substances and are used in the prevention of various diseases, but their antioxidative effects in adipocytes have not been examined. Lychee fruit pericarp contains significant amounts of polyphenolic compounds such as tannins (polymeric proanthocyanidins), and the extract of lychee fruit pericarp has been shown to have powerful antioxidant properties against fat oxidation *in vitro*.[24] Hence, in this study, we investigated the antioxidative effects of a new lychee fruit-derived polyphenol mixture, Oligonol, converted into a low-molecular form in adipocytes.

Materials and Methods

Oligonol. Oligonol was obtained by oligomerizing the polyphenol polymers in LFP using a modification of a patented technology previously described.[25] The process involves mixing proanthocyanidins with tea extract but not with L-cysteine and purifying the mixture using a column. Oligonol contains 15.7% polyphenol monomer ((+)-catechin and (−)-epicatechin *etc.*) and 13.3% polyphenol dimer (procyanidin B2 *etc.*), while LFP contains 6.4% polyphenol monomer and 9.9% polyphenol dimer. Oligonol was approved as a new dietary ingredient (NDI) by the Food and Drug Administration of the United States (FDA) an May 23, 2007, and is commercially available at present (Amino Up Chemical Co., Ltd., Sapporo, Japan).

Cell culture. HW mouse white and HB2 brown preadipocytes were kindly provided by Professor M. Saito (Tenshi University, Sapporo, Japan).[26] Both cell types were maintained in Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal calf serum. Differentiation to adipocytes was induced by treatment with 1 µM dexamethasone (DEX) and 0.5 mM 3-isobutyl-1-methylxanthine (IBMX) for 48 h. The treated cells were maintained in DMEM containing 10 µg/ml of insulin and 50 nM 3-3′-5-triod-L-thyronine (T3) for 72 h to accumulate triglyceride. Both fully differentiated cell types were treated with Oligonol or LFP (10 and 20 µg/ml respectively) for 24 h. Furthermore, to examine the effects of Oligonol on the differentiation of the two cell types, post-confluent cultures of both preadipocytes were pre-incubated with DMEM containing Oligonol or LFP (20 µg/ml each) for 1 h prior to induction of differentiation, and then the cells were differentiated as described above. Lipid droplets in each cell sample were stained using 0.3% Oil-Red-O (Sigma Chemical, St. Louis, MO) in 60% (v/v) 2-propanol in water for 1 h.

Measurement of ROS production in adipocytes. Measurement of ROS production in the two cell types was described previously.[13] ROS production was measured by nitroblue tetrazolium (NBT) reduction. The two cell cultures were incubated for 60 min in PBS (137 mM NaCl, 8.1 mM Na_2PO_4, 2.68 mM KCl, 1.47 mM KH_2PO_4) containing 0.2% NBT. Formazan was dissolved in 50% acetic acid, and the absorbance was monitored at 560 nm using a spectrophotometer.

Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis. Total RNA was isolated and purified from mouse HW cells, HB2 cells, epididymal WAT, and interscapular brown adipose tissues (BATs) by the acid guanidine phenol chloroform method using Isogen (Nippon Gene, Toyama, Japan). First-stranded cDNA was obtained by incubating total RNA (2 µg) with reverse transcriptase (Superscript III, Invitrogen, Carlsbad, CA) in the reaction mixture (18 µl). The cDNA (1 µl) was subjected to semi-quantitative PCR using Taq DNA polymerase (Applied Biosystems, Branchburg, NJ). Fifteen to 35 cycles of amplification were carried out for various target genes. The primers used are described below. The PCR products were electrophoresed in 1% agarose gels containing ethidium bromide.

TNF-α:
5′-CAGACCCTCACACTCAGATC-3′,
5′-GACTCCAAAGTAGACCTGCC-3′
MCP-1:
5′-AGGTCCCTGTCATGCTTCTG-3′,
5′-AGTTCACTGTCACACTGGTC-3′
PAI-1:
5′-CGTTTGTGTTCCAGTCACAC-3′,
5′-AGGTTTGTGGAGTGATGCAC-3′

Adiponectin:
5′-TGTTCGTCGTAGCTAAGCGC-3′,
5′-CTTCATAGCCATCAAACCTG-3′
Leptin:
5′-AAGGTTGTCCAGGGTTGATC-3′,
5′-CAGCTCAGGTTCTTTCACAC-3′
Cu,Zn-superoxide dismutase (Cu,Zn-SOD):
5′-ATGGCGATGAAAGCGGTGTG-3′,
5′-TACTGCGCAATCCCAATCAC-3′
Mn-SOD:
5′-CTGGAGCCACACATTAACGC-3′,
5′-ACTGAAGGTAGTAAGCGTGC-3′
Extracellular SOD (EC-SOD):
5′-TGCAGGGTACAACCATCAGC-3′,
5′-TTAAGTGGTCTTGCACTCGC-3′
Uncoupling protein (UCP)-1:
5′-CCTCTCTCGGAAACAAGATC-3′,
5′-ACATGATGACGTTCCAGGAC-3′
UCP-2:
5′-AGATGAGCTTTGCCTCCGTC-3′,
5′-AGCATGGTAAGGGCACAGTG-3′
UCP-3:
5′-CATTCGAATTGGCCTCTACG-3′,
5′-ATCATCACGTTCCAAGCTCC-3′
Fatty acid synthase (FAS):
5′-GTATTAACCTGGACAGCACG-3′,
5′-ACATCTCGAAGGCTACACAG-3′
Acetyl-CoA carboxylase (ACC):
5′-ATGAGATTGGCATGGTAGCC-3′,
5′-GTAAGCCCCAATACCAATGG-3′
Peroxisome proliferator-activated receptor-γ2 (PPARγ-2):
5′-ACTGCCTATGAGCACTTCAC-3′,
5′-GATGGCATTGTGAGACATCC-3′
Nuclear factor-kappaB (NF-κB):
5′-GTACTTGCCAGACACAGATG-3′,
5′-GATGCCAGGTCTGTGAACAC-3′
β-actin:
5′-CCTCATGAAGATCCTGACC-3′,
5′-ACTCATCGTACTCCTGCTTG-3′
18s rRNA:
5′-GAGAAACGGCTACCACATCC-3′,
5′-GAGAAACGGCTACCACATCC-3′

Preparation of protein extracts and Western blot analysis. Nuclear and cytosol protein extracts of HW and HB2 cells were fractionated with a Nuclear/Cytosol Fractionation kit (BioVision Research Products, Mountain View, CA) according to the manufacturer's protocol. Total protein was extracted with T-PER Tissue Protein Extraction Reagent containing 50 mM NaF (sodium fluoride), 0.5 mM Na_3VO_4, 0.5 mM PMSF (phenylmethylsulfonyl fluoride), 5 mg/ml of aprotinin, and 5 mg/ml of leupeptin. Each protein sample was subjected to 10–12% NuPAGE (Invitrogen) gradient gel electrophoresis and transferred onto a nylon membrane. The membrane was blocked using TBS-T (20 mmol/l of Tris–HCl, pH 7.5, 137 mmol/l of NaCl, and 0.1% Tween-20] containing 5% nonfat dried milk, and then probed with an anti-NF-κB, anti-phosphorylated extracellular signal-regulated kinase (ERK) 1/2 antibody (sc-372 and sc-16982-R respectively; Santa Cruz Biotechnology, Santa Cruz, CA) or anti-ERK1/2 antibody (Promega, Madison, WI). After washing with TBS-T, bound antibody was detected using the ECL system (Amersham, Buckinghamshire, UK).

Luciferase assay. pNF-κB-Luc vector (1 µg) (BD Bioscience, San Jose, CA) was transfected into HW and HB2 cells cultured in 24-well plates with Lipofect-AMINE LTX (Invitrogen) according to the manufacturer's protocol. The cell cultures were differentiated 24 h after transfection, as described above. The differentiated cell cultures were treated with Oligonol or LFP (20 µg/ml) for 24 h. Cell extracts for luciferase assay were prepared with Cell Culture Lysis Reagent (Promega). Luciferase activity was assayed with Luciferse Assay Systems (Promega) using a luminometer.

Animal care. Six-week-old male C57BL/6J mice (Sankyo Labo Service Corporation, Tokyo) were housed in cages in a temperature-controlled room at 23 °C under a 12:12 h light-dark cycle. The mice were divided randomly into four groups ($n = 4$ each): i) C mice: control mice fed normal chow for 5 weeks; ii) HFD-only mice: mice given a HFD for 5 weeks; iii, iv) HFD + Oligonol and LFP mice: mice given a HFD with Oligonol or LFP. The HFD consisted of 25.5% (w/w) protein, 2.9% fiber, 4.0% ash, 29.4% carbohydrates, 32% fat, and 6.2% water (HFD32; Clea Japan, Tokyo). HFD + Oligonol and HFD + LFP mice were given Oligonol or LFP (100 mg/kg/d each) added to their drinking water. All experiments conducted in this study were approved by the Animal Care Committee of the Kyorin University School of Medicine.

Measurement of lipid peroxidation in WAT. The measurement of lipid peroxidation in epididymal WAT was described previously.[13] Epididymal WAT was homogenized in PBS, and centrifuged, and the supernatant was used to assess lipid peroxidation. The levels of lipid peroxidation in epididymal WAT were measured as thiobarbituric acid reactive substance (TBARS) by the LPO-test (Wako, Osaka, Japan).

Blood samples and analysis. Blood samples were collected from the hearts, and plasma was separated by centrifugation at 3,000 rpm at 4 °C for 15 min. Samples were stored at −80 °C until use. The levels of plasma insulin and glucose were measured using a Mouse Insulin ELISA Kit (Shibayagi, Shibukawa, Japan) and the Glucose C2 test (Wako) respectively.

Data analysis and statistical methods. The intensities of bands from RT-PCR and Western blot analyses were quantified with the National Institute of Health Image Computer program. Values represent means ± SE.

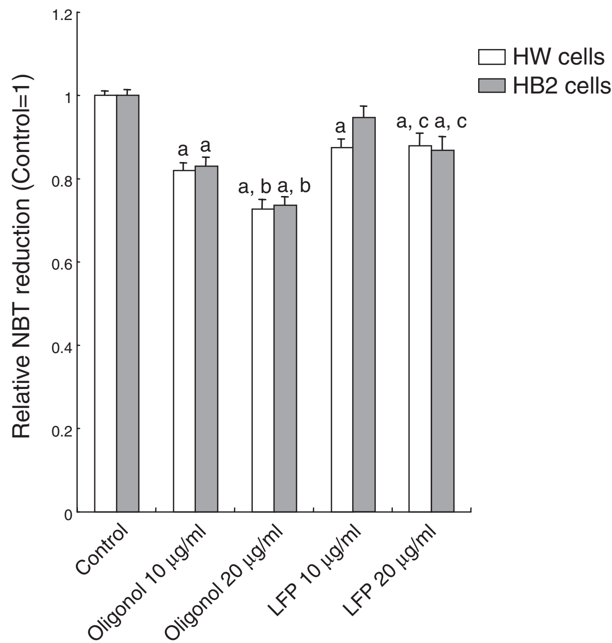

Fig. 1. Effect of Oligonol on Production of ROS in Adipocytes.
Fully differentiated HW and HB2 cells were treated with Oligonol or LFP (10 and 20 µg respectively) for 24 h. ROS production in both cell types was measured by NBT reduction. The data were expressed as a ratio to the value of the control (sets to = 1), and the mean ± SE ($n = 3$) is given. $^{a}p < 0.05$ *vs.* control; $^{b}p < 0.05$ *vs.* Oligonol 10 µg/ml; $^{c}p < 0.05$ *vs.* Oligonol 20 µg/ml.

Analysis of variance (ANOVA) was used to compare the values, and the Tukey-Kramer method was used for the *post hoc* test. Differences were considered significant at $p < 0.05$.

Results

Effect of Oligonol on ROS production in adipocytes

First we investigated the effect of Oligonol on ROS production in adipocytes by NBT assay. The levels of ROS in fully differentiated HW and HB2 cells were significantly decreased following treatment with Oligonol for 24 h as compared with the control cells (Fig. 1), suggesting that Oligonol has antioxidative effects on adipocytes. On the other hand, the antioxidative effects of LFP were weaker than those of Oligonol (Fig. 1). No cytotoxicity of Oligonol and LFP was observed when lactate dehydrogenase (LDH) released from damaged cells was measured (data not shown).

Expression of genes for adipokines, SODs, and UCPs in adipocytes

It is known that oxidative stress affects the expression of genes for adipokines and Mn-SOD, a SOD antioxidative mitochondrial isoenzyme.[13–15,27,28] As shown in Fig. 1, Oligonol has antioxidative effects in adipocytes; hence the expression levels of adipokines, SOD isoenzymes, and the UCP family, which has antioxidative effects, were investigated. Expression of the genes for adipokines (TNF-α, MCP-1, PAI-1, adiponectin, and leptin) was down-regulated in HW cells by treatment with Oligonol for 24 h (Fig. 2A). Moreover, expression of the gene for Mn-SOD was also decreased by this treatment, whereas the expression levels of other SOD isoemzymes (Cu,Zn-, and EC-SOD) were unaffected (Fig. 2A). Expression of the genes for PAI-1 and Mn-SOD in HB2 cells was significantly decreased after Oligonol treatment (Fig. 2B), whereas the expression of genes for the UCP family was essentially unaffected in both cell types by treatment with Oligonol (Fig. 2A, B). Expression of the gene for UCP-1 was below the detectable level in HW cells (data not shown). When both cell types were treated with LFP, similar results were obtained (Fig. 2A, B).

Effect of Oligonol on the transcriptional activity of NF-κB and the activation of ERK

Transcription factor NF-κB has been found to be activated by oxidative stress in various cells, and to regulate the expression of genes for adipokines, such as PAI-1 and Mn-SOD.[15,29–32] Activation of ERK is also regulated by oxidative stress, and it relates to the expression of the gene for MCP-1.[33] Hence, to identify the mediators of the Oligonol-induced expression changes of genes for adipokines and Mn-SOD, we examined the transcriptional activity of NF-κB and the activation of ERK. As shown in Fig. 3A and B, the expression of the NF-κB gene in HW and HB2 cells and the accumulation of NF-κB protein in the nuclei of both cell types treated with Oligonol significantly decreased as compared with those of the control cells, whereas the accumulation of NF-κB protein in the cytosol did not change. Moreover, the transcriptional activity of NF-κB in both cell types was also decreased by treatment with Oligonol (Fig. 3C). When HW and HB2 cells were treated with LFP, similar results were obtained (Fig. 3A–C). Moreover, phosphorylation of ERK1 (p44) and 2 (p42) in HW cells was attenuated by treatment with Oligonol, and the effects of LFP were weaker than those of Oligonol (Fig. 3D), but phosphorylation of ERK1/2 in HB2 cells was not markedly changed by treatment with Oligonol or LFP (Fig. 3D).

Effect of Oligonol on adipocyte differentiation

Recent reports indicate that tea cathechin and genistein, a soybean-derived polyphenol, inhibit adipocyte differentiation.[34,35] Hence, we investigated to determine whether Oligonol blocks adipocyte differentiation. Lipid droplets in HW and HB2 cells were stained with Oil-Red-O. Fat accumulation in both cell types treated with Oligonol or LFP during differentiation was clearly suppressed as compared with control cells (Fig. 4A). Moreover, expression of lipogenic genes, including FAS and ACC, in both HW and HB2 cells was down-regulated following treatment with Oligonol (Fig. 4B). Additionally, expression of the gene for PPAR-γ2, an important transcription factor for the expression of

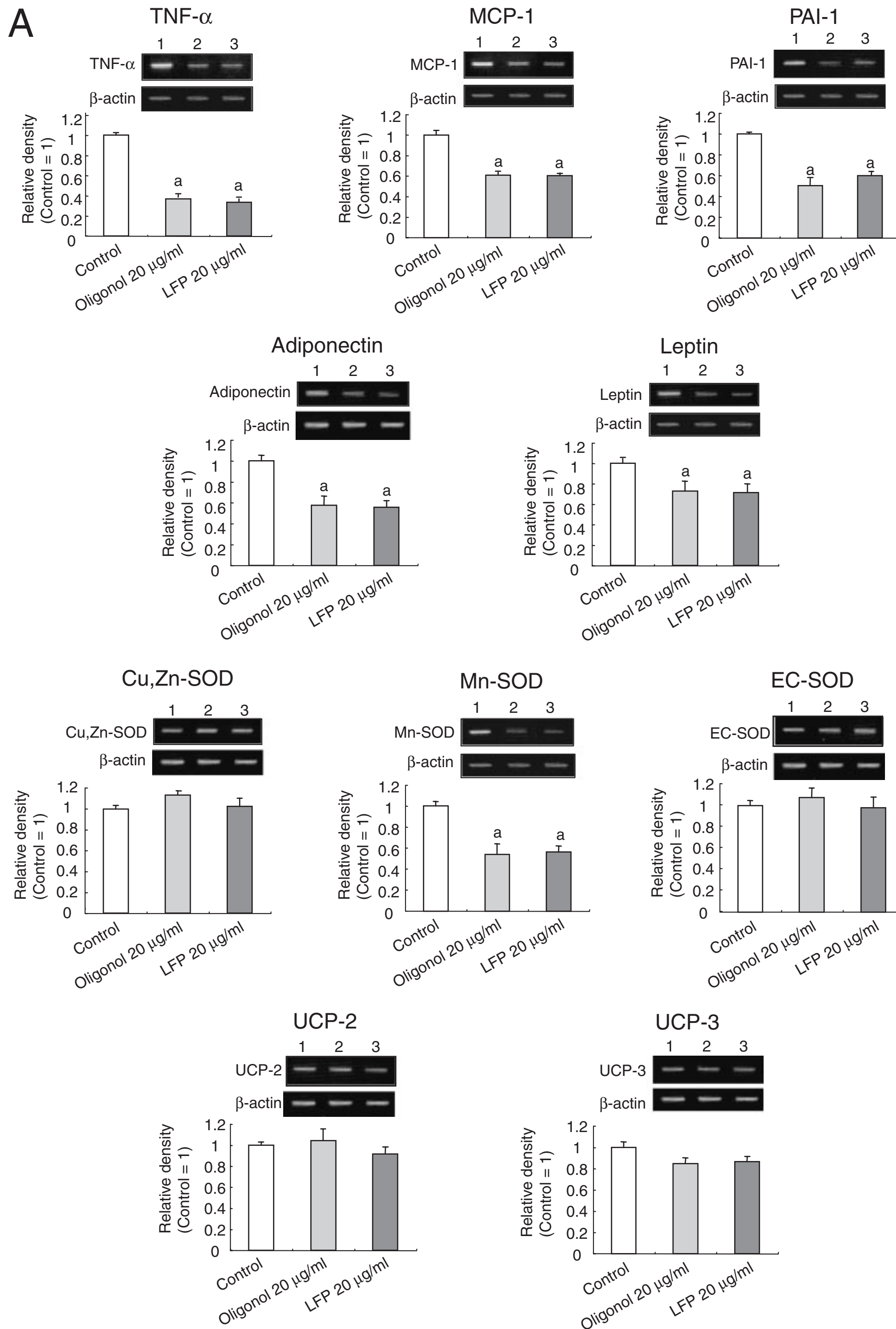

Fig. 2. *Continued.*

lipogenic genes, was also down-regulated. The inhibitory effects of LFP on the expression of the genes for FAS and ACC appeared to be lower than those of Oligonol (Fig. 4B).

Effects of HFD, Oligonol, and LFP intake on levels of WAT/BAT mass and lipid peroxidation in WAT

Next, HFD, Oligonol, and/or LFP were administered to mice for 5 weeks to investigate the antioxidative effects of Oligonol *in vivo*. The body masses of HFD-only, HFD + Oligonol, and HFD + LFP treated mice all increased significantly as compared with that of C mice, and no significant differences were observed in the body masses of the HFD-only, HFD + Oligonol, and HFD + LFP treated mice (Table 1). However, although the levels of WAT mass and TBARS in the HFD-only mice were significantly higher than those in the C mice, Oligonol clearly attenuated such HFD-

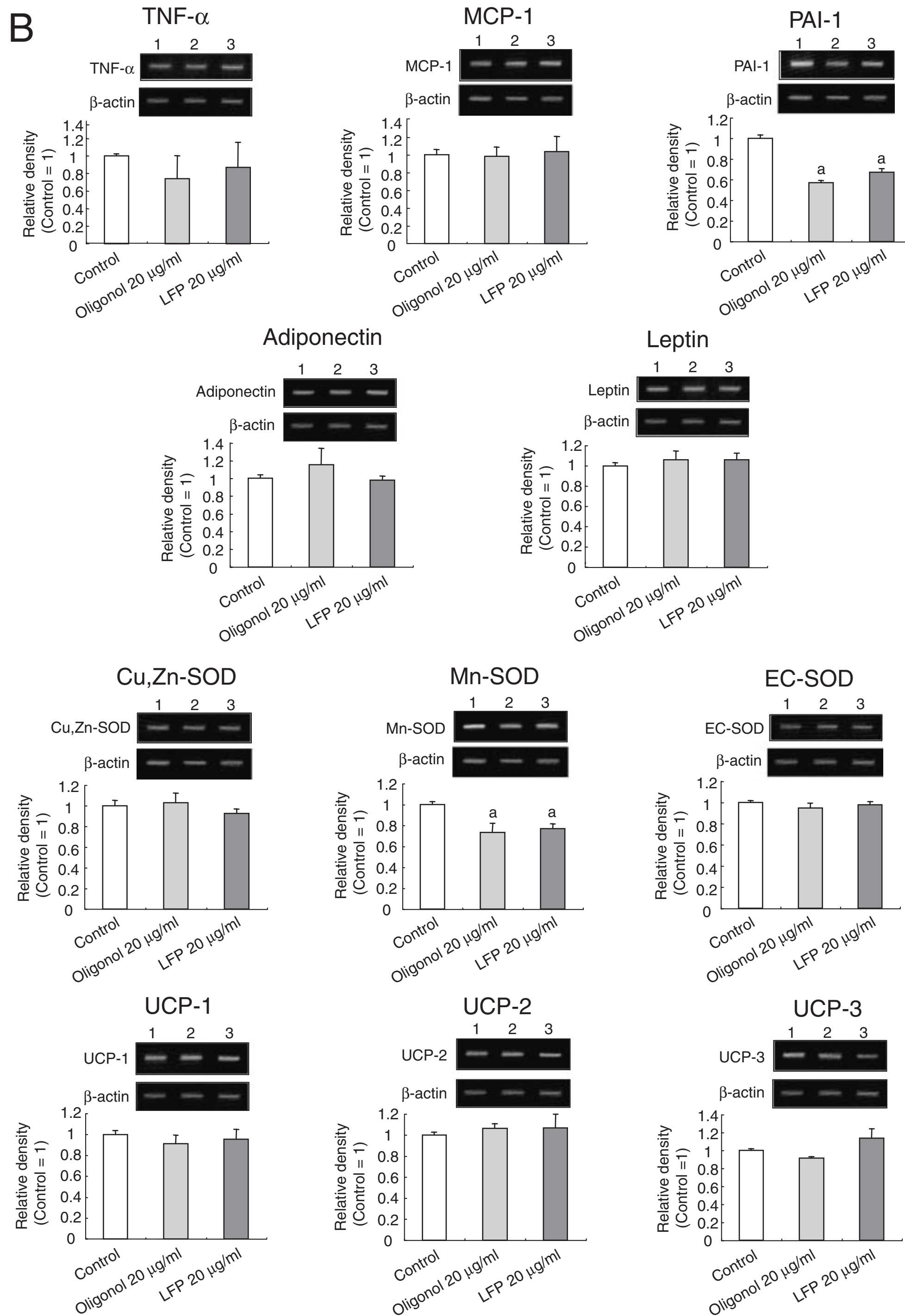

Fig. 2. Effect of Oligonol on Expression of the Genes for Adipokines, SODs and UCPs in HW and HB2 Cells.

Total RNA was extracted from fully differentiated HW (A) and HB2 cells (B) treated with Oligonol or LFP (20 μg each) for 24 h, and subjected to RT-PCR analysis. Representative data for RT-PCR analysis are shown above each bar graph (lane 1, basal; lane 2, Oligonol 20 μg/ml; lane 3, LFP 20 μg/ml). The expression level of each gene was normalized to that of the β-actin gene. The values shown by the bar graphs are related to the optical density of the control (sets to = 1), and the mean ± SE ($n = 3$) is given. [a]$p < 0.05$ *vs.* control.

induced increases, whereas the food intake of the HFD + Oligonol mice was not significantly different from that of the HFD-only mice (Table 1). The levels of WAT mass and TBARS in the HFD + LFP-treated mice did not differ from those in the HFD-only mice. In addition, the levels of BAT mass in the HFD-only mice were significantly lower than in the C mice (Table 1). The levels of plasma insulin in the HFD-only, HFD + Oligonol, and HFD + LFP mice were higher than in the C mice, although no significant differences in plasma glucose levels were observed between any of the groups (Table 1).

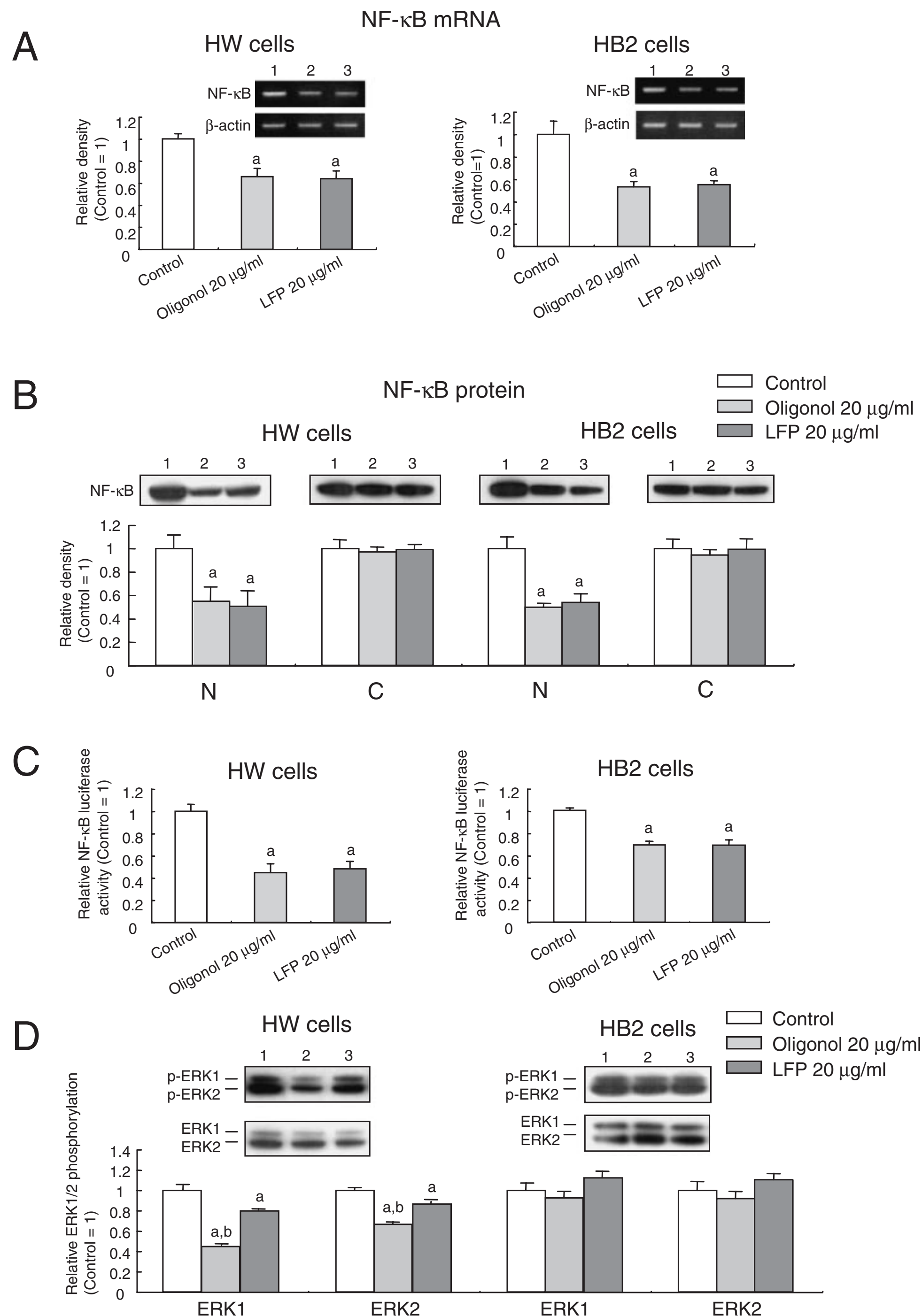

Fig. 3. Expression and Transcriptional Activity of NF-κB and Phosphorylation of ERK1/2.

Expression level of the NF-κB gene (A). Total RNA was extracted from fully differentiated HW and HB2 cells treated with Oligonol or LFP (20 µg each) for 24 h, and then subjected to RT-PCR analysis. Representative data from RT-PCR analysis are shown above each bar graph (lane 1, basal; lane 2, Oligonol 20 µg/ml; lane 3, LFP 20 µg/ml). The expression level of each gene was normalized to that of the β-actin gene. Accumulation of NF-κB protein in the nucleus and cytosol (B). Nuclear and cytosol protein samples (10 mg each) were extracted from cells treated for 24 h with Oligonol or LFP (20 µg each), then subjected to Western blot analysis. Representative data from Western blot analysis are shown above each bar graph (lane 1, basal; lane 2, Oligonol 20 µg/ml; lane 3, LFP 20 µg/ml). Transcriptional activity of NF-κB (C). HW and HB2 cells were transiently transfected with the pNF-κB-Luc vector before differentiation was induced. The transfected cells were treated with Oligonol or LFP (20 µg each) for 24 h after differentiation, then luciferase activity was measured. Phosphorylation of ERK1/2 (D). Total protein (10 µg) was extracted from both cell types treated for 24 h with Oligonol or LFP (20 µg each), and then subjected to Western blot analysis. Representative data from Western blot analysis are shown above each bar graph (lane 1, basal; lane 2, Oligonol 20 µg/ml; lane 3, LFP 20 µg/ml). Phosphorylation level of ERK1/2 was normalized to that of the expression level of ERK1/2. The data were expressed as a ratio to the value of the control (sets to = 1), and the mean ± SE ($n = 3$) is given. $^{a}p < 0.05$ *vs.* control; $^{b}p < 0.05$ *vs.* LFP.

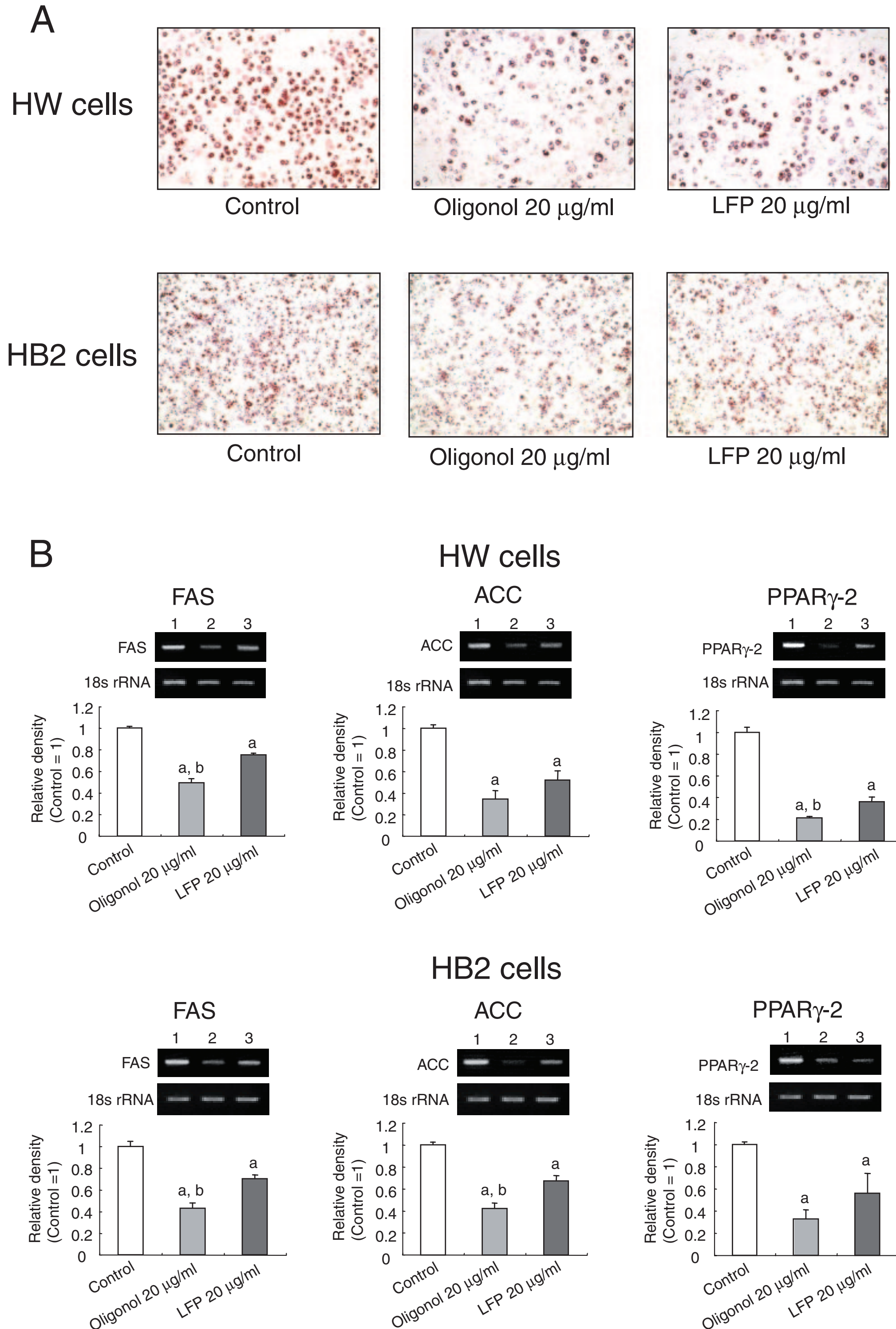

Fig. 4. Effect of Oligonol on Adipocyte Differentiation.

Lipid droplets in HW and HB2 cells were stained red using an Oil-Red-O solution. Representative photomicrographs of both cell types are shown (A). Red stains indicate lipid droplets in the HW and HB2 cells. Expression of adipocyte differentiation-related genes (B). Total RNA was extracted from HW and HB2 cells treated with Oligonol or LFP (20 µg each) during differentiation, and subjected to RT-PCR analysis. Representative data from RT-PCR analysis are shown above each bar graph (lane 1, basal; lane 2, Oligonol 20 µg/ml; lane 3, LFP 20 µg/ml). The expression level of each gene was normalized to that of 18s rRNA. The values shown by the bar graphs are related to the optical density of the control (sets to = 1), and the mean ± SE ($n = 3$) is given. $^{a}p < 0.05$ *vs.* control; $^{b}p < 0.05$ *vs.* LFP.

Table 1. Effect of HFD, Oligonol, and LFP Intake on the Levels of WAT/BAT Mass and TBARS in WAT

	Control	HFD-only	HFD + Oligonol	HFD + LFP
Body weight (g)	24.7 ± 0.2	35.8 ± 1.1^{a}	32.5 ± 0.8^{a}	34.4 ± 0.8^{a}
Food intake (g/4 mice/d)	11.3 ± 0.3	10.2 ± 0.2	10.1 ± 0.4	10.1 ± 0.2
Plasma insulin (ng/ml)	1.11 ± 0.23	6.53 ± 1.74^{a}	6.13 ± 1.40^{a}	4.52 ± 0.69^{a}
Plasma glucose (mg/dl)	226 ± 8	286 ± 26	277 ± 19	303 ± 26
Epididymal white adipose tissue/body weight (%)	1.57 ± 0.07	5.81 ± 0.23^{a}	$4.37 \pm 0.29^{a,b}$	5.07 ± 0.24^{a}
Interscapular brown adipose tissue/body weight (%)	0.73 ± 0.03	0.48 ± 0.02^{a}	0.59 ± 0.03	0.49 ± 0.06
Epididymal white adipose tissue TBARS (nmol MDA/mg protein)	0.67 ± 0.10	1.88 ± 0.19^{a}	$1.25 \pm 0.15^{a,b}$	1.59 ± 0.15^{a}

$n = 4$ for each group, Mean $\pm$ SE, $^{a}p < 0.05$ *vs.* Control, $^{b}p < 0.05$ *vs.* HFD only

Expression of genes for adipokines, SODs, and UCPs in the WAT and BAT of the various groups

Expression of the TNF-α gene in the WAT of HFD-only mice was significantly higher than that in the C mice, but no increased expression of this gene was observed in the WAT of the HFD + Oligonol and HFD + LFP mice (Fig. 5A). Similarly, although expression of the gene for MCP-1 in the WAT of HFD-only mice was also significantly higher than in the C mice, only Oligonol intake attenuated the increase in expression of this gene (Fig. 5A). Conversely, expression of the gene for adiponectin was suppressed in the HFD-only mice as compared with the C mice, but not in the HFD + Oligonol or HFD + LFP-treated mice. Moreover, expression of the gene for PAI-1 in the WAT of the HFD + Oligonol mice was significantly lower than in the HFD-only mice (Fig. 5A). In contrast, the expression of the gene for EC-SOD was enhanced in the WAT of the HFD + Oligonol mice (Fig. 5A). Expression of genes for adipokines in BAT was not significantly affected in any of the groups, but expression of the genes for EC-SOD and UCP-1 was increased in the BAT of the HFD + Oligonol and HFD + LFP mice as compared with the C and HFD-only mice (Fig. 5B). Expression of the gene for UCP-1 in WAT was below the detectable level (data not shown).

Discussion

Many recent reports have addressed the role of oxidative stress in metabolic disease. In adipocytes, increased oxidative stress in the WAT of diabetic KKAy mice is higher than that of control C57BL/6 mice, and dysregulated expression of genes for metabolic syndrome-related adipokines, including TNF-α and adiponectin, has been observed in 3T3-L1 adipocytes exposed to oxidative stress agents such as H_2O_2.[13,14] Moreover, increased oxidative stress in adipocytes leads to interruption of insulin-induced GLUT4 translocation in 3T3-L1 adipocytes.[16] These reports indicate that the development of drugs and/or supplements which have antioxidative effects in adipocytes is required. Polyphenols are well known to be potent natural antioxidants in the diet. Hence, polyphenols are considered to be good candidates for antioxidants in adipocytes. Oligonol, used in the current study, is a new lychee fruit-derived polyphenol that is converted into a low-molecular form. Generally, it is thought that high-molecular forms of polyphenols are more difficult to absorb than low-molecular ones. For example, Fujii *et al.*[25] found that oligomerization of purified grape seed polyphenols is more conducive to higher absorption rates *in vivo* than the non-oligomerized form. Hence, it is speculated that Oligonol is also more rapidly absorbed than LFP. In fact, in our study, Oligonol showed antioxidative effects in adipocytes both *in vitro* and *vivo* and these antioxidative effects were stronger than those of LFP, suggesting that the absorption rate of polyphenols affects their antioxidative effects in adipocytes.

Expression of genes for metabolic syndrome-related adipokines, such as TNF-α and PAI-1, was down-regulated by Oligonol in HW and HB2 cells (Fig. 2). To determine the molecular mechanisms of Oligonol-induced down-regulation of these genes, we focused on transcription factor NF-κB. There are many reports addressing the influence of oxidative stress on the activation of NF-κB. Treatment with H_2O_2 and other agents that cause oxidative stress, such as ultraviolet (UV) radiation, causes activation of NF-κB.[29] Conversely, N-acetyl-cysteine (NAC), a potent antioxidant, inhibits NF-κB activation and TNF-α-induced increased expression of the gene for PAI-1 in 3T3-L1 adipocytes.[14,36] Moreover, it has been found that NF-κB modulates expression of the genes for MCP-1 and Mn-

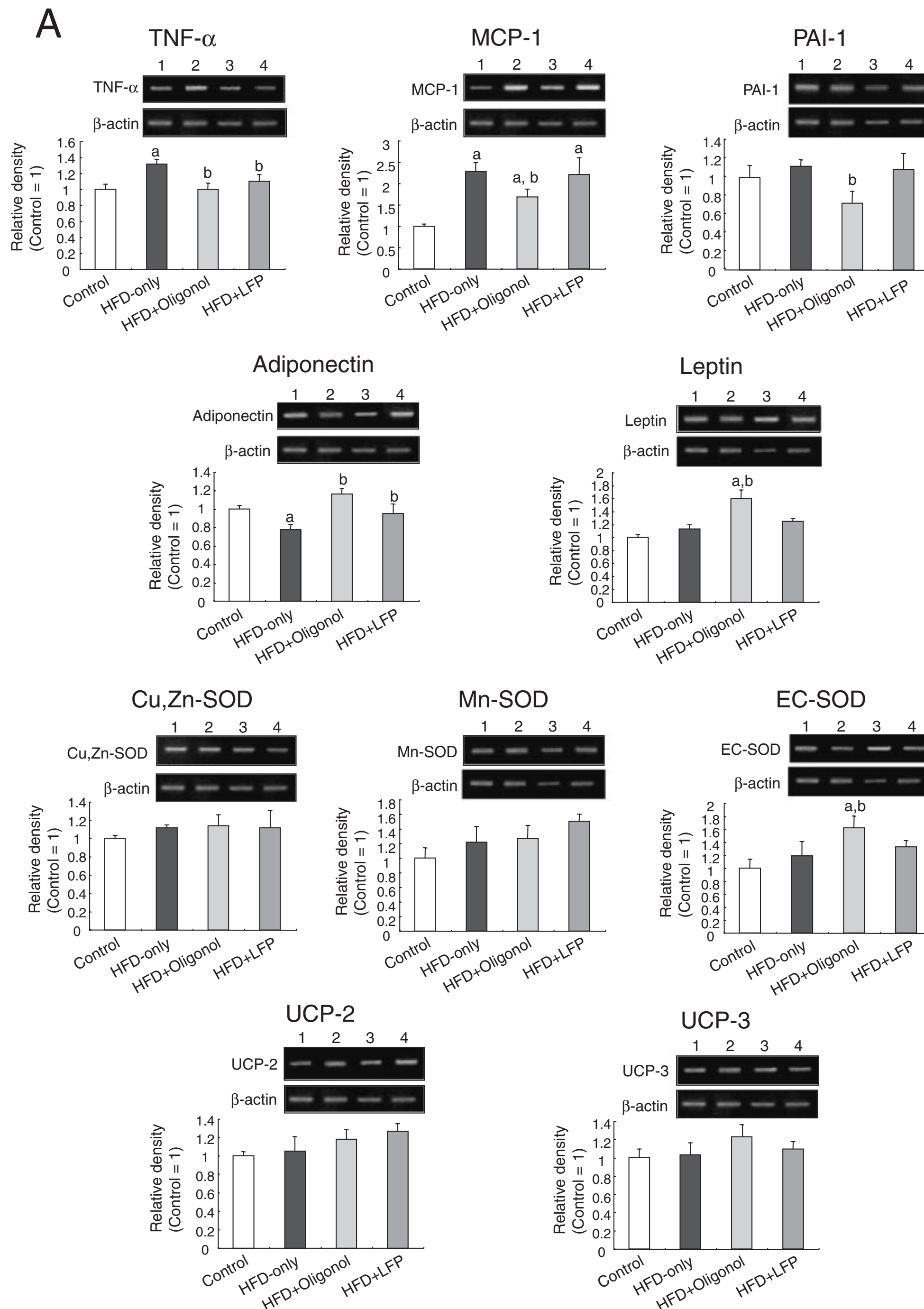

Fig. 5. *Continued.*

SOD in certain cell types.[30,31,37] In our study, Oligonol decreased the accumulation of NF-κB protein in the nucleus, but not in the cytosol (Fig. 3). These results suggest that Oligonol not only attenuates the expression of NF-κB, but also inhibits nuclear import of NF-κB. Hence, it is conceivable that the transcriptional activity of NF-κB is down-regulated additionally in HW and HB2 cells, and that expression of the genes for PAI-1 and Mn-SOD is down-regulated through the decrease in transcriptional activity of NF-κB due to Oligonol.

Mitogen-activated protein kinases (MAPKs), including ERK, c-Jun N-terminal kinase (JNK), stress-activated protein kinase (SAPK), and the p38 subfamilies, are important regulatory proteins that transduce various extracellular signals into intracellular events. It has been reported that activation of MAPKs is enhanced by oxidative stress, and that activation of ERK is implicated in the expression of the MCP-1 gene in 3T3-L1 adipocytes.[33,38] Phosphorylation of ERK1/2 in Oligonol-treated HW cells was weaker than that in the control and LFP-treated HW cells (Fig. 3D), indicating that not only the decreased transcriptional activity of NF-κB but

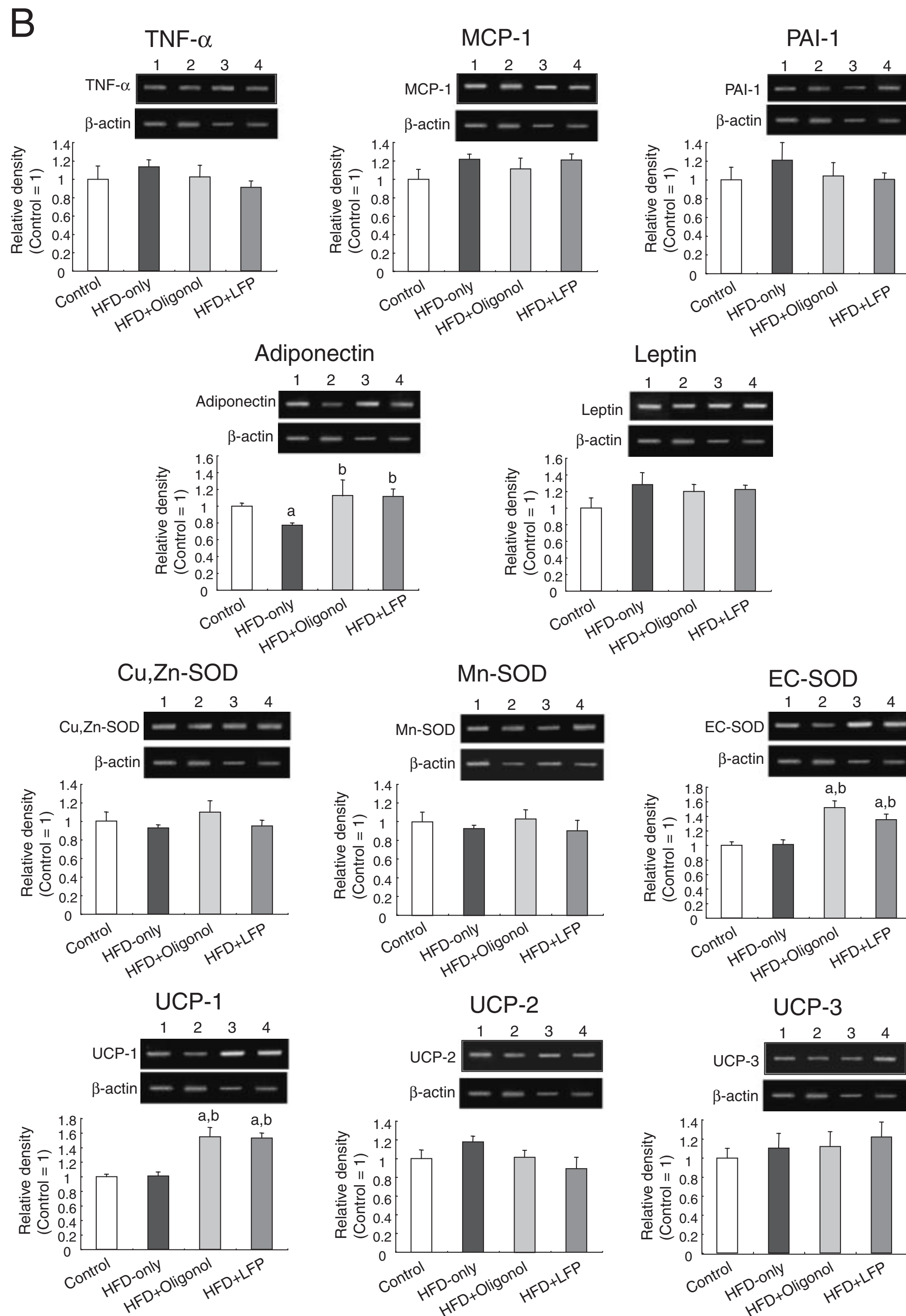

Fig. 5. Effect of Oligonol on the Expression of Genes for Adipokines, SODs, and UCPs in WAT and BAT.

Total RNA was extracted from epididymal WAT (A) and interscapular BAT (B) of each mouse and subjected to RT-PCR analysis. Representative data from RT-PCR analysis are shown above each bar graph (lane 1, Control; lane 2, HFD only; lane 3, HFD + Oligonol; lane 4, HFD + LFP). The expression level of each gene was normalized to that of the β-actin gene. The values shown by the bar graphs are related to the optical density of the control (sets to = 1), and the mean ± SE ($n = 4$) is given. $^{a}p < 0.05$ *vs.* control.

also the attenuated phosphorylation of ERK1/2 due to Oligonol acts to down-regulate MCP-1 gene expression. In addition, the difference in strength of the antioxidant as between Oligonol and LFP is reflected in its effect on the phosphorylation of ERK1/2, which is different from its effect on the transcriptional activity of NF-κB in HW cells, but Oligonol treatment did not affect the phosphorylation of ERK1/2 in HB2 cells (Fig. 3D), while Oligonol showed antioxidative effects in HB2 cells (Fig. 1). Although the reason for this difference between HW and HB2 cells is unknown and requires further study, we speculate that expression of the MCP-1 gene

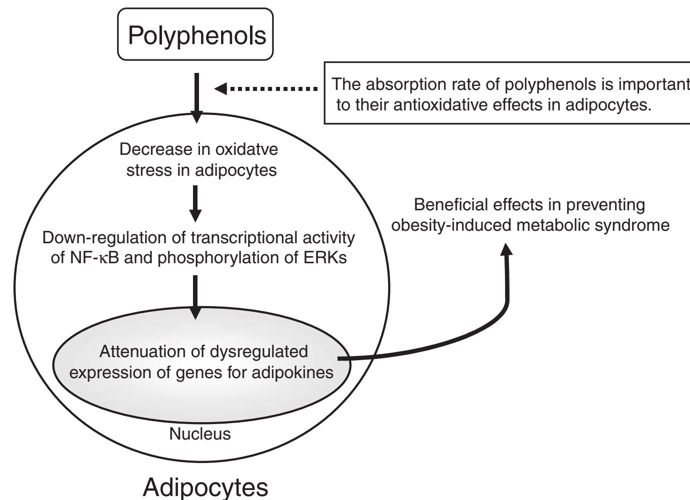

Fig. 6. Schematic Model for the Signaling Cascade Induced by Polyphenols in Adipocytes.

Treatment with polyphenol results in deceased oxidative stress in adipocytes. This decease in oxidative stress attenuates dysregulated expression of genes for metabolic syndrome-related adipokines by down-regulating the transcriptional activity of NF-κB and the phosphorylation of ERKs. Thus, polyphenols have beneficial effects in preventing obesity-induced metabolic syndrome. Moreover, the absorption rate of polyphenols is important to their antioxidative effects in adipocytes.

was not down-regulated in HB2 cells because the phosphorylation of ERK1/2 was not changed by Oligonol.

Current studies demonstrate that polyphenols can block adipocyte differentiation. For instance, grape seed-derived procyandins and tea-derived catechin suppress adipogenesis.[34,39] As the present results indicate, Oligonol also suppresses adipocyte differentiation and down-regulates the expression of adipocyte differentiation-related genes (Fig. 4). Hence, suppression of adipocyte differentiation by Oligonol might reflect inhibition of fat accumulation in the epididymal WAT of the HFD + Oligonol-treated mice.

The low-grade inflammatory response in WAT induced by inflammatory adipokines such as TNF-α and MCP-1 is quite probably a cause of insulin resistance.[40] For example, MCP-1 is implicated in macrophage infiltration in WAT[12] and is thought to be the key molecule in the inflammatory response in WAT. Furthermore, because oxidative stress has been shown to induce expression of the genes for TNF-α and MCP-1,[13,37] it is thought that suppressing oxidative stress is important in inhibiting the inflammatory response in WAT. Oligonol intake with HFD attenuated HFD-induced up-regulation of the TNF-α and MCP-1 genes (Fig. 5A). Hence, it is possible that Oligonol intake prevents metabolic syndrome not only by inhibiting the HFD-induced decrease in expression of the adiponectin gene, but also by attenuating the inflammatory response in WAT. Furthermore, since the HFD-induced increase in oxidative stress and the up-regulation of MCP-1 gene expression in WAT were not attenuated by LFP intake (Table 1 and Fig. 5A), it is speculated that the intensity of the antioxidative effects of polyphenols in adipocytes is clearly reflected in the expression of genes for adipokines *in vivo* rather than *in vitro*, but the effects of Oligonol on expression of the genes for adiponectin and leptin in white adipocytes were opposite *in vitro* and *vivo* (Fig. 2A and 5A). Although we could not resolve these discrepancies in the present study, differences in the effects of antioxidants on basal expression of the adiponectin gene were observed. In fact, the NAC dose did not affect the basal expression of the adiponectin gene in 3T3-L1 cells, whereas (−)-catechin enhanced expression.[36,41] In addition, because the effects of Oligonol *in vivo* were observed under HFD conditions, the different results *in vitro* and *vivo* might have been due to differences in experimental conditions.

Oligonol intake also resulted in up-regulation of the gene for EC-SOD in WAT, and the genes for EC-SOD and UCP-1 in BAT (Fig. 5A, B). We have found that EC-SOD is abundant in WAT and BAT, and there are indications that EC-SOD is physiologically important in adaptation to oxidative stress in WAT.[42] In addition, Vincent *et al.*[43] found that UCP-1 has antioxidative effects. These results suggest that although lipid peroxidation in the BAT from each mouse could not be measured due to insufficient sample size, Oligonol might attenuate oxidative stress not only by its direct antioxidative effects, but also through indirect effects such as up-regulation of EC-SOD and UCP-1 in WAT and BAT.

In conclusion, our results suggest that polyphenols have beneficial effects in preventing obesity-induced metabolic syndrome through their antioxidative effects, that they attenuate dysregulated expression of adipokines in adipocytes, and that the absorption rate of polyphenols is important for their antioxidative effects in adipocytes. Our findings are summarized in the schematic model depicted in Fig. 6.

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