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Association of anti-obesity activity of N-acetylcysteine with metallothionein-II down-regulation

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Abbreviations: MT-II, metallothionein-II; NAC, N-acetylcysteine

Abstract

People with upper body or visceral obesity have a much higher risk of morbidity and mortality from obesity-related metabolic disorders than those with lower body obesity. In an attempt to develop therapeutic strategies targeting visceral obesity, depot-specific differences in the expression of genes in omental and subcutaneous adipose tissues were investigated by DNA array technology, and their roles in adipocyte differentiation were further examined. We found that levels of metallothionein-II (MT-II) mRNA and protein expression were higher in omental than in subcutaneous adipose tissues. The study demonstrates that MT-II may play an important role in adipocyte differentiation of 3T3L1 preadipocytes, and that N-acetylcysteine (NAC) inhibits the adipocyte differentiation of 3T3L1 cells by repressing MT-II in a time- and dose-dependent manner. Furthermore, the intraperitoneal administration of NAC to rats and mice resulted in a reduction of body weights, and a marked reduction in visceral fat tissues. These results suggest that MT-II plays important roles in adipogenesis, and that NAC may be useful as an anti-obesity drug or supplement.

Keywords: anti-obesity agents; metallothionein-II; N-acetylcysteine; obesity; oligonucleotide array sequence analysis

Introduction

Obesity is a ubiquitous health hazard in industrialized countries and is closely associated with a number of pathological disorders, e.g., non-insulin-dependent diabetes, hypertension, cancer, gallbladder disease, and atherosclerosis (Gregoire *et al.,* 1998). Moreover, obesity-associated disorders are known to be closely associated with not only the degree of excess adipose tissue but also with the distribution of body fat (Bjorntorp, 1996). Upper body or visceral obesity presents a much higher risk of morbidity and mortality from the above-mentioned metabolic disorders than lower body obesity (Wajchenberg *et al.,* 2002). Visceral (omental) and subcutaneous adipose tissues are morphologically and functionally different, which may contribute to the increased morbidity associated with visceral obesity. A variety of metabolic differences such as fatty acid turnover, lipolysis (van Harmelen *et al.,* 2002), and the effectiveness of insulin action, in omental and subcutaneous adipose tissues have been reported (Wajchenberg *et al.,* 2002). In addition, many depot-related genes have been characterized by DNA array technology (Gabrielsson *et al.,* 2002; Gabrielsson *et al.,* 2003) and by differential display PCR (Montague *et al.,* 1998). Inhibitor of apoptosis (Montague *et al.,* 1998), lipoprotein lipase (Fried *et al.,* 1993), leptin (Montague *et al.,* 1997), glucose transporter 4, glycogen synthase, peroxisome proliferator-activated receptor (PPAR)-γ (Lefebvre *et al.,* 1998), angiotensin (Rahmouni *et al.,* 2004), acylation stimulating protein, cholesterol ester transfer protein (Dusserre *et al.,* 2000), and perillipin (Arvidsson *et al.,* 2004) have all been reported to be differentially expressed in omental and subcutaneous adipose tissues.

Metallothioneins (MTs) are a family of low molecular weight (6-7 kDa), cysteine-rich metal binding proteins, with 61-68 amino acid residues (Coyle *et al.,* 2002). The induction of MTs has been shown to be dependent on cell type and to be specifically regulated. MT expression patterns depend largely on the availability of cellular zinc derived from the dietary zinc supply (Bremner, 1991). MTs may act in zinc trafficking and/or zinc donation to apoproteins, such as zinc finger proteins, which act in cellular

signaling and transcriptional regulation. The ability of MTs to exchange their zinc for other metals may explain their roles in metal toxicity. Similarly, the mobilization of zinc from MTs by oxidative stress may explain their proposed antioxidant function (Bremner and Beattie, 1990; Steven and Robert, 2000). As a result, the expression of MTs may affect a number of cellular processes including gene expression, apoptosis, proliferation and differentiation (Coyle *et al.,* 2002; Jin *et al.,* 2002). In addition, accumulating evidence indicates that MTs may play a role in adipocyte differentiation, and are induced in 3T3L1 mouse fibroblasts during adipocyte differentiation (Schmidt and Beyersmann, 1999; Trathurn *et al.,* 2000a; b). The level of MT-2A mRNA in subcutaneous fat tissues was found to be significantly higher in obese subjects (Do *et al.,* 2000). However, roles of MTs during adipocyte differentiation and of their regional preferences for omental and subcutaneous adipose tissues are not understood.

In the present study, we found that metallothionein-II is upregulated in omental adipose tissues as compared with subcutaneous adipose tissues by using DNA array technology. Our findings suggest that MT-II plays an important role in the adipocyte differentiation of 3T3L1 preadipocytes. In addition, we found that N-acetylcysteine (NAC) inhibits the adipocyte differentiation of 3T3L1 cells by repressing MT-II in a dose- and time-dependent manner. Animal studies also showed that NAC reduced body weights, especially by decreasing the visceral fat tissue amounts.

Materials and Methods

Materials

Human GDA 1.3 arrays were purchased from Incyte Genomics Inc. (Palo Alto, CA). Mouse fibroblast 3T3L1 cells were obtained from the Korean Cell-line Bank (Seoul, Korea). Dulbecco's modified Eagles Medium (DMEM) and penicillin-streptomycin-fungizone solution were purchased from Life Technologies Inc. (Gaithersburg, MD). Dexamthasone, insulin, reduced glutathione (GSH), sodium vanadate, retinoic acid, peroxidase-conjugated anti-mouse antibody, and N-acetylcysteine (NAC) were obtained from Sigma-Aldrich (St. Louis, MO). LY 294002, PD 98059, SB 203580, H9, and genistein were from Calbiochem (San Diego, CA). A mouse monoclonal antibody against metallothionein (E9) was from Dako (Hamburg, Germany), fetal bovine serum (FBS) from HyClone (Logan, UT), and the enhanced chemiluminescence detection system from Amersham-Pharmacia (Buckinghamshire, UK). Nylon membranes and nitrocellulose membranes were purchased from Schleicher and Schuell Inc. (Postfach, Germany).

Tissue harvesting

Omental and abdominal subcutaneous adipose tissue biopsies were obtained from patients undergoing elective open-abdominal surgery. All patients were fasted for at least 12 h preoperatively and all underwent general anesthesia. Tissue biopsies were obtained from 4 women (age 60.8 $\pm$ 11.2 years; BMI 24.5 $\pm$ 1.7 kg/m^2) and 5 men (age 46.2 $\pm$ 15.4 years; BMI 21.5 $\pm$ 2.6 kg/m^2). The biopsies were frozen immediately and stored at -70^{o}C until use. Of the female subjects, 3 had surgery for a common bile duct stone and one underwent partial gastrectomy for malignancy. Of the male patients, 2 underwent surgery for hemoperitonium, 2 for gastric cancer, and 1 for bezoar. Samples were obtained in compliance with a protocol approved by the Institutional Review Board for Human subjects at Yeungnam University Hospital. Written consent was obtained from all subjects after the nature of the procedure was explained.

Total RNA and mRNA purification

Total RNA in adipose tissues and cells was purified by acid-phenol-guanidium thiocyanate-chloroform extraction (Chomczynski and Sacchi, 1987). mRNA was further purified using an Oligotex mRNA purification kit (Qiagen Inc., Valencia, CA), according to the manufacturer's instructions. RNA concentrations were determined by measuring absorbance at 260 nm using a UV-spectrophotometer (Shimadzu, Japan) and stored at -70^{o}C.

cDNA array analysis

This analysis was performed using a Human GDA 1.3 array, according to the manufacturer's instruction. mRNAs purified from omental and subcutaneous adipose tissues were radio-labeled using oligo-dT and [α-^{32}P]dCTP by reverse-transcription. The ^{32}P-labeled cDNAs were then hybridized with Human GDA 1.3 membranes for 24 h at 42^{o}C. After washing, the membranes were exposed to X-ray films and analyzed.

DNA dot blotting and reverse Northern analysis

Bacterial clones containing human cDNAs, which were differentially expressed in omental and subcutaneous adipose tissues by cDNA array analysis, were purchased from Incyte Genomics Inc, and plasmid DNAs were purified from *E. coli* using plasmid DNA purification kits (I. J. Bio, Korea). Plas-

mid DNA (20 ng) was blotted on Hybond N^+ nylon membranes (Amersham Biosciences Corp., Piscataway, NJ) and membranes were hybridized with ^{32}P-labeled cDNAs prepared from the mRNAs of 9 subjects by reverse transcription. Following washing, the membranes were exposed to X-ray films and analyzed.

Cell culture

3T3L1 cells were maintained in DMEM supplemented with 10% FBS and 100 U/ml antibiotic solution at 37°C in a humidified atmosphere containing 5% CO_2 and 95% air. For adipocyte differentiation, cells grown to confluency were treated with medium containing dexamethasone (0.25 μM) and insulin (10 μg/ml). Two days later, the medium was changed to DMEM + 10% FBS with insulin, and this was replaced every second day.

Oil red O staining

Lipid accumulation in 3T3L1 cells was observed by oil red O staining (Hausman, 1981). After differentiation, cells were rinsed with phosphate-buffered saline (PBS), fixed with 10% formalin in PBS, and then stained with oil red O for 30 min. Red-colored lipid droplets in cells were visualized under a microscope and photographed.

Northern blot analysis

Ten micrograms of total RNA were separated on a 1% formaldehyde agarose gel. The RNAs were then transferred to a nylon membrane by vacuum transfer and cross-linked with UV. The membranes were then prehybridized overnight at 42°C with a hybridization buffer (0.1 M sodium phosphate pH 7.2, 0.25 M sodium chloride, 2.5 mM EDTA, 50% formamide, and 7% SDS) containing a ^{32}P-labeled MT-II probe. Membranes were then washed and exposed to X-ray films at -70°C. Equal RNA loadings were confirmed by hybridization with a ^{32}P-labeled GAPDH probe.

Inhibitor treatment

When 3T3L1 cells were confluent, 20 mM NAC, 20 μM sodium vanadate, 250 nM retinoic acid, 25 μM LY 294002, 20 μM PD 98059, 5 μM SB 203580, 2 mM GSH, 6 μM H9, or 100 μM genistein were pretreated 1 day prior to treatment with dexamethasone and insulin. Lipid accumulation was observed by oil red O staining and MT-II expression levels were analyzed by Northern and Western blotting.

Western blot analysis

3T3L1 cells were lysed with a lysis buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 10% glycerol, 1 mM PMSF, 1 mM sodium vanadate, and 5 mM NaF) and centrifuged at 15,000 rpm at 4°C for 10 min. Proteins (20 μg) were then separated on an 18% SDS-polyacrylamide gel and transferred to nitrocellulose membranes, which were soaked in 5% nonfat dried milk in TTBS (10 mM Tris-HCl pH 7.5, 150 mM NaCl, and 0.05% Tween-20) for 30 min, and then incubated overnight with a mouse monoclonal antibody against metallothionein at 4°C. After washing three times with TTBS for 10 min, membranes were incubated with a horseradish peroxidase-conjugated anti-mouse IgG for 1 h at 4°C. The antigen-antibody complex was detected by enhanced chemiluminescence.

Animal treatment

Male Sprague-Dawley (80-90 g) rats and male C57BL/6 (25-30 g) mice were purchased from Jung-Ang Lab Animal Inc. (Seoul, Korea). All animals were fed standard rat or mouse chow and housed in a climate-controlled environment (21°C) under a 12-h light-dark cycle. The protocol used was approved by the College of Medicine, Yeungnam University, Animal Care and Use Committee. NAC was dissolved in distilled water and the pH of this solution was adjusted to 7.4 with NaOH. This solution was then administered intraperitoneally at 81, 163, 326, or 816 mg/kg/day for 41 days to rats (n = 6 per group), and at 81, 163, or 326 mg/kg/day for 8 wk to mice (n = 6 per groups). Control animals were injected with an equivalent amount of NaCl. Body weights and food intake were recorded every two days in rats and every week in mice. At day 41 for rats and at week 8 for mice, animals were anesthetized with ether and various tissues, including visceral fat (epididymal, perirenal, and retroperitoneal fat pads), subcutaneous fat from the abdominal wall, gastrocnemius muscle, and kidney were removed and weighed.

Statistical analysis

All data are presented as means ± SE. The Student's t test was employed for all analyses. A P-value of < 0.05 was considered statistically significant.

Results

Upregulation of MT-II mRNA and MT protein expression in omental adipose tissues

To identify differentially expressed genes in omental and subcutaneous adipose tissues in humans, we used cDNA arrays spotted with 18,000 bacteria

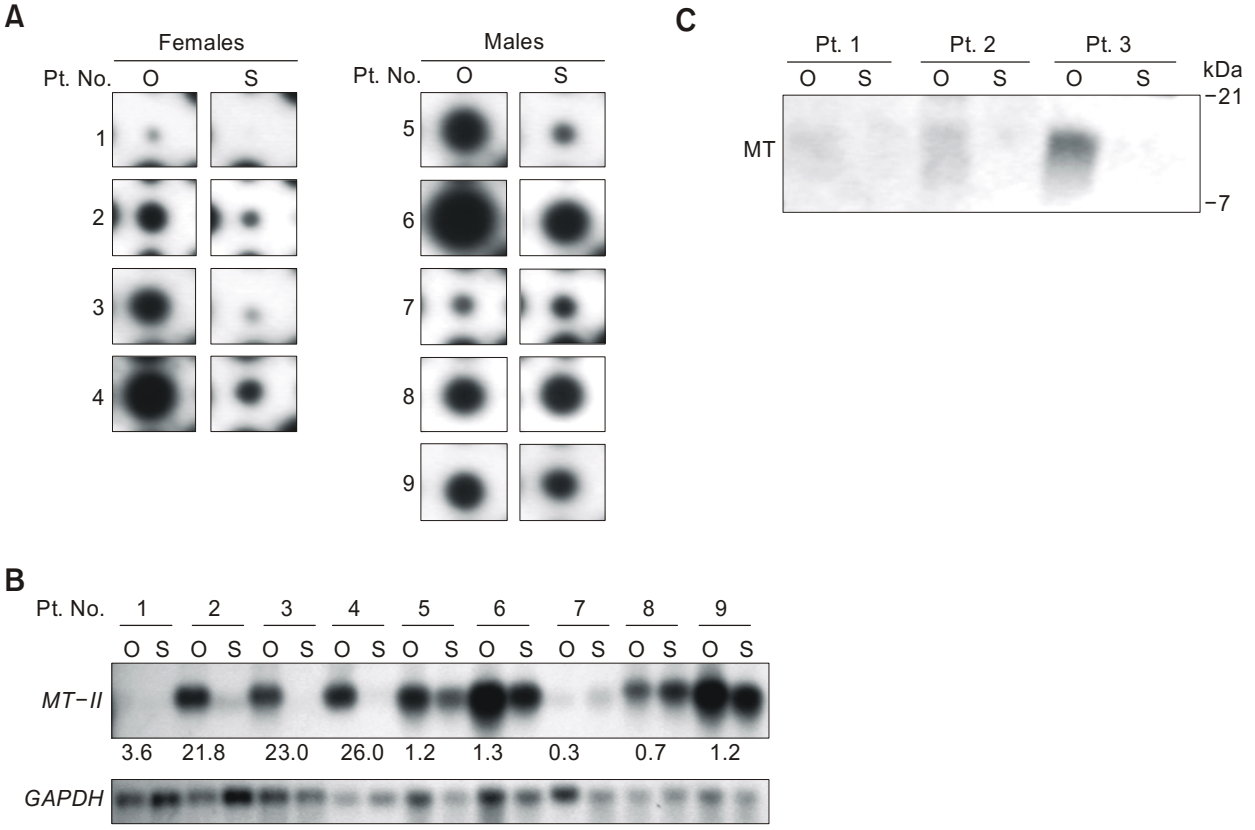

Figure 1. Up-regulation of MT-II in omental adipose tissues. (A) Dot blot and reverse Northern blot analysis of MT-II in omental and subcutaneous adipose tissues. MT-II cDNA (20 ng) was blotted on nylon membranes, which were hybridized with ^{32}P-labeled cDNA prepared from the total RNAs of adipose tissues. (B) Northern blot analysis of MT-II in adipose tissues. Numbers are fold differences in MT-II expression in omental and subcutaneous adipose tissues, normalized versus GAPDH expression levels. (C) Western blot analysis of MT. O, omental adipose tissues; S, subcutaneous adipose tissues. The figures show representative data from 3 independent experiments.

containing human cDNA plasmids. After we initially analyzed the DNA array expression data in one subject (Patient No. 1), 50 genes shown differential expression in omental and subcutaneous tissues were selected. The expression levels of 50 genes were further evaluated in the other subjects. Reverse Northern blot analysis showed that the expression levels of MT-II among 50 genes were higher in the omental adipose tissues than in the subcutaneous adipose tissues of all women and of 3 men (Figure 1A). The upregulation of MT-II expression in omental adipose tissues was also confirmed by Northern blot analysis (Figure 1B). Differences in the levels of MT-II mRNA expression between omental and subcutaneous fat tissues were greater in women than in men. Since an antibody against the mouse MT-II was not commercially available, we used a mouse monoclonal antibody (E9) against human MT, which crossreacts with mouse MTs, to examine the level of MT-II protein expression. MT protein levels were also higher in omental adipose tissues, as confirmed by Western blot analysis (Figure 1C). These results suggest that MT-II is a depot specific gene in omental and subcutaneous adipose tissues.

Roles of MT-II in the adipocyte differentiation of 3T3L1 preadipocytes

Since MT levels are known to be elevated during the adipocyte differentiation of 3T3L1 cells, we determined the level of MT-II expression and examined the effects of MT-II on adipocyte differentiation. MT-II mRNA levels were elevated during the adipocyte differentiation of 3T3L1 cells treated with dexamethasone and insulin, and peaked one day after differentiation (Figure 2A). MT protein expression was also induced and peaked on day 4 (Figure 2B). In an attempt to find a critical signaling pathway involved in adipose differentiation in 3T3L1 cells, a variety of inhibitors, namely, PD 98059 (an MEK inhibitor) (Aubert *et al.,* 1999), SB 203580 (a p38

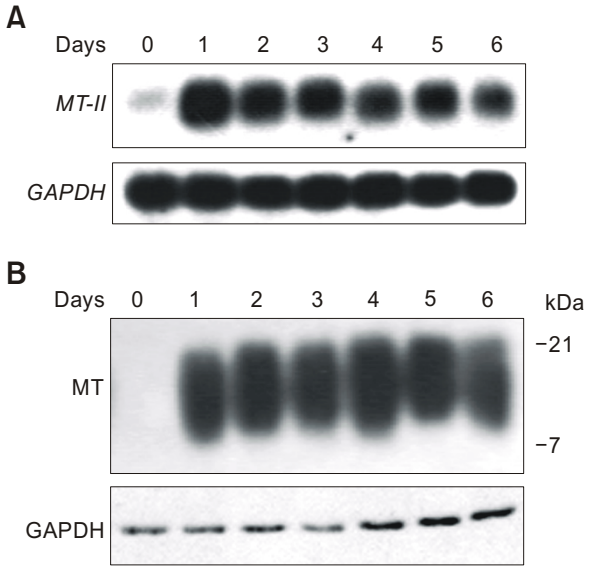

Figure 2. MT-II expression in 3T3L1 cells during adipocyte differentiation. (A) Northern blot analysis. Ten micrograms of RNA was hybridized with a ^{32}P-labeled MT-II probe. (B) Western blot analysis. Proteins (20 μg) were Western-blotted with an MT antibody. The figures show representative data from 3 independent experiments.

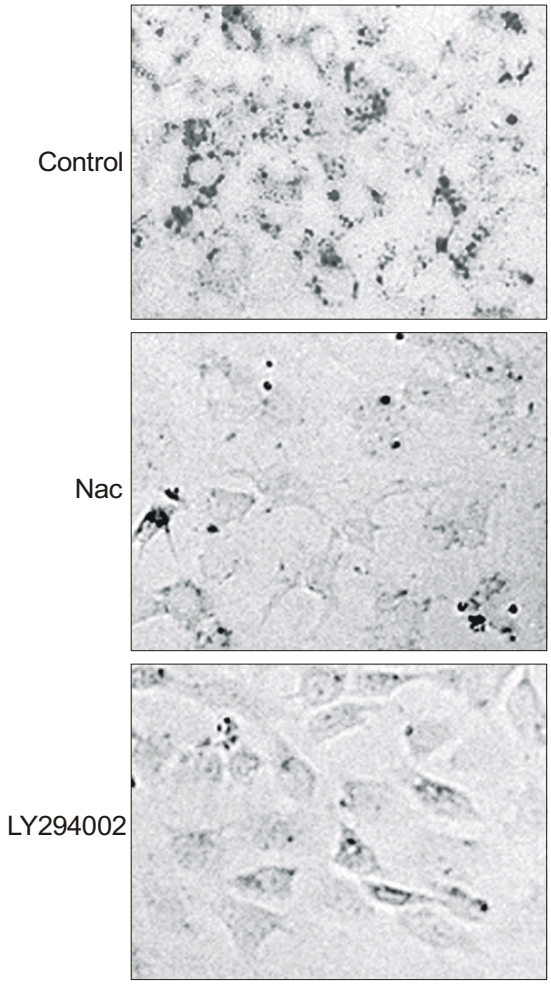

Figure 3. Inhibition of adipocyte differentiation in 3T3L1 by NAC and LY 294002. 3T3L1 cells were grown to confluency and treated with inhibitors (20 mM NAC or 25 μM LY 294002) for 1 day prior to inducing differentiation with dexamethasone and insulin. Lipid droplets were identified by oil red O staining at 4 days after induction. The figures shown are representative data from 3 independent experiments (×100).

kinase inhibitor) (Engelman *et al.,* 1998), LY 294002 (a phosphatidyinositol 3-kinase inhibitor) (Gregoire *et al.,* 1998), H9 (a protein kinase A and protein kinase C inhibitor) (Farese *et al.,* 1992; Zhang *et al.,* 2002), hydroxyurea (a G1/G0 arrest inhibitor) (Tang *et al.,* 2003), and genistein (a tyrosine kinase inhibitor) (Harmon *et al.,* 2002), and antioxidants (Mahadev *et al.,* 2002), such as NAC, GSH, vitamin C, and retinoic acid, were pretreated and lipid droplet formation was estimated by oil red O staining. Notably, NAC and LY 294002 inhibited lipid droplet formation in 3T3L1 cells (Figure 3). Since LY 294002 is known to suppress lipid droplet formation in 3T3L1 cells (Xia and Serrero, 1999), the effects of NAC on adipocyte differentiation were further investigated. The levels of MT-II expression, induced during adipose differentiation, were also found to be downregulated by NAC in a dose- and time-dependent manner (Figure 4). These results suggest that MT-II plays an important role in the adipocyte differentiation of 3T3L1 cells and that NAC is utilized to inhibit adipose differentiation in animal models.

Effects of NAC on growth rates and body weights in rats and mice

The facts that NAC inhibited adipocyte differentiation and MT-II expression in 3T3L1 cells prompted us further to investigate whether the administration of NAC reduces the amount of fat tissues. Growth curves and body weights of rats treated with or without NAC were measured (Figure 5A), and the growth rates of rats treated with NAC were found to be reduced in a dose dependent manner. Moreover, treatment with NAC at 816 mg/kg/day resulted in a significant reduction in body weights from day 16 ($P < 0.01$). The ratios of the body weights of rats treated with 0, 81, 163, 326, or 816 mg/kg/day of NAC on day 41 and day 1 were; 4.53 ± 0.21, 4.48 ± 0.29, 4.47 ± 0.08 4.27 ± 0.27, and 3.72 ± 0.22, respectively; these differences were statistically significant at 326 mg/kg/day ($P < 0.05$) and 816 mg/kg/day ($P < 0.01$). No difference was observed with respect to the food intake of rats treated with or without NAC (Figure 5B). To investigate effects of

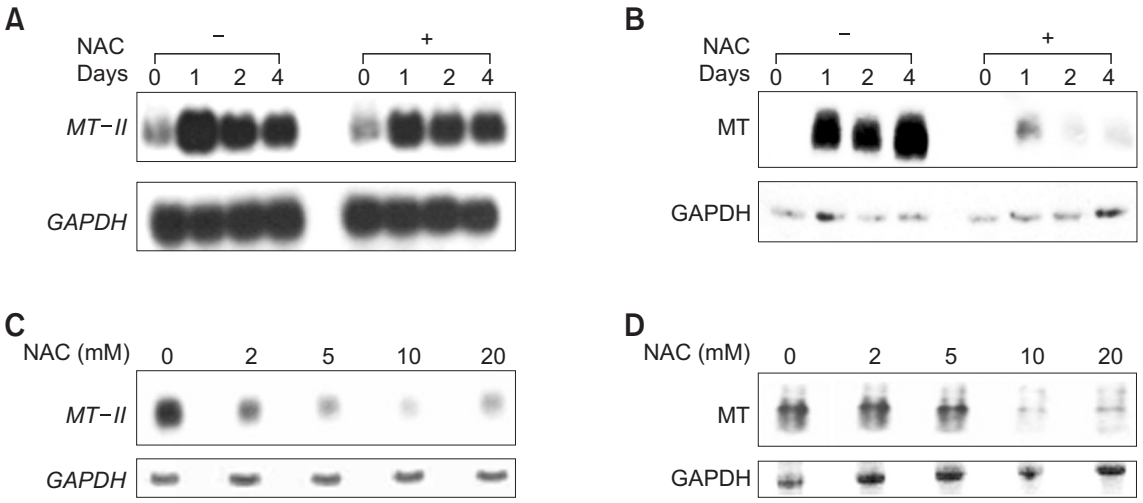

Figure 4. Effects of NAC on MT-II induction during the adipose differentiation of 3T3L1 cells. Cells were pretreated with NAC (20 mM) for 1 day and adipocyte differentiation was induced. The cells were harvested at the indicated times postinduction. MT-II expression was measured by Northern blotting using a ^{32}P-labeled MT-II probe (A) and by Western blotting using an MT antibody (B). With increasing concentrations of NAC, MT-II expression was also observed by Northern (C) and Western blotting (D) 4 days after induction.

NAC on the body weights of adult animals, C57BL/6 mice (25-30 g) were also intraperitoneally administered with NAC. Treatment with NAC was found to cause a statistically significant reduction ($P < 0.01$) in body weight in a dose dependent manner over a 3 wk period (Figure 5C). The ratios of the body weights of mice treated with 0, 81, 163, and 326 mg/kg/day of NAC at week 8 and week 0 were; 1.18 $\pm$ 0.03, 0.93 $\pm$ 0.14, 0.84 $\pm$ 0.02, and 0.78 $\pm$ 0.04 respectively; these differences were statistically significant at all treated concentrations ($P < 0.01$) (Figure 5D).

Effects of NAC on visceral and subcutaneous adipose tissues in rats and mice

In order to examine whether reductions in body weight due to NAC treatment resulted from reductions in fat tissue quantity, visceral and subcutaneous adipose tissues in animals were excised and weighed. The amounts of visceral fat tissue, including retroperitoneal, perirenal, and epididymal fat pads, were incrementally reduced by treating with increasing concentrations of NAC in rats and mice (Figure 6), whereas subcutaneous fatty tissues, gastrocnemius muscle, and kidney were unaffected by NAC treatment (Figure 7). These results show that reductions in body weight due to NAC treatment is primarily a result of reduced visceral fat by NAC, thus suggesting its use as an anti-obesity drug or supplement.

Discussion

This study shows that levels of MT-II mRNA and of MT protein expression are elevated in omental adipose tissues versus subcutaneous adipose tissues. MT-I and MT-II were found to be expressed in all tissues and during developmental processes, and to be induced by metals, glucocorticoids (Karin and Herschman, 1979), and inflammatory responses (Coyle *et al.,* 2002). Although MT-I and -II null mice showed obesity and hyperleptinemia (Beattie *et al.,* 1998), the roles of MTs in obesity and energy metabolism in adipose tissues are not fully understood (Palmiter, 1998).

Bujalska *et al.,* (1997) and Stewart *et al.,* (1999) reported that adipose cells from omental fat, but not from subcutaneous fat can generate active cortisol from inactive cortisone by expressing 11β-hydroxysteroid dehydrogenase type 1. Glucocorticoid receptor expression levels were also greater in omental adipocytes than in subcutaneous cells (Wajchenberg *et al.,* 2002). Therefore, given that the regulation of MT expression is mediated through glucocorticoid receptors by glucocorticoids (Karin and Herschman, 1979; 1980), the upregulation of MT-II in omental fat tissues might be associated with the increased conversion of inactive cortisone to active cortisol in omental fat tissues. In addition, differences in the levels of MT-II expression in omental and subcutaneous fat tissues are greater in women than in men, suggesting that sex hormones might also contribute to depot-specific differences.

MT is known to be induced during the adipocyte

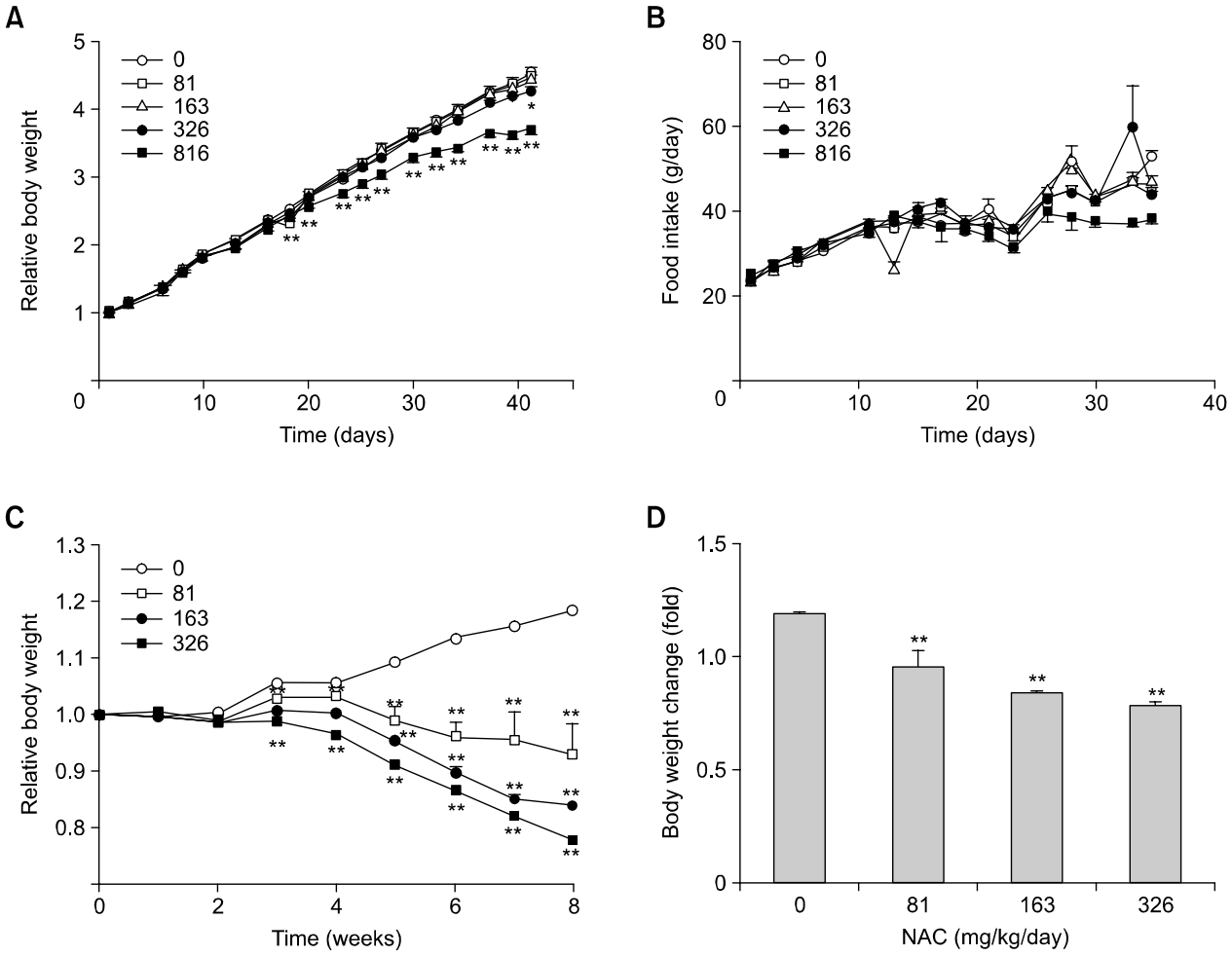

Figure 5. Effects of NAC on body weights in rats and mice. (A) Changes in body weights in rats with or without NAC administration. (B) Food intake in control and NAC-treated rats. (C) Changes of body weights in mice administered or not administered NAC. (D) Body weight changes in mice at week 8 administered the indicated amounts of NAC. Values are means ± SE of 6 animals and statistical significances were determined using the Student's *t* test (*$P < 0.05$ and **$P < 0.01$).

differentiation of 3T3L1 preadipocytes (Schmidt and Beyersmann, 1999; Trayhurn *et al.,* 2000b). We found for the first time that MT-II is also induced during adipocyte differentiation and that pretreatment with NAC inhibits adipocyte differentiation by reducing MT-II expression, suggesting that MT-II plays an important role in the adipocyte differentiation of 3T3L1 cells. The adipocyte differentiation of 3T3L1 cells is known to be mediated through various signal transduction pathways by hormones, growth factors, and cytokines (Ailhaud, 1997; Kim *et al.,* 2001; Koutnikova and Auwerx, 2001). Transcription factors, such as CCAAT/enhancer binding proteins and peroxisome proliferator-activated receptors were also found to be involved in adipocyte differentiation (Holst and Grimaldi, 2002), through many different signal transduction pathways, involving, phosphatidylinositol 3-kinase (PI3K), MAPKs, cAMP, steroid hormones, and protein kinase C (Farese *et al.,* 1992; Frevert and Kahn, 1996; Engelman *et al.,* 1998; Aubert *et al.,* 1999). Of the inhibitors used to block specific signal transduction pathways, we found that NAC and LY 294002 effectively inhibited lipid droplet formation and MT-II induction during adipocyte differentiation in 3T3L1 cells. A PI3K signal transduction pathway was previously found to be of importance in insulin-mediated adipocyte differentiation (Miki *et al.,* 2001). Moreover, NAC is known to exert antioxidative protective effects, including, the extracellular inhibition of mutagenic agents from exogenous and endogenous sources, the inhibition of the ROS genotoxicity, modulation of the metabolism coordinated with the blocking of reactive metabolites, protection of DNA and nuclear enzymes, and with the prevention of the formation of carcinogen-DNA adducts (De Vries and De Flora,

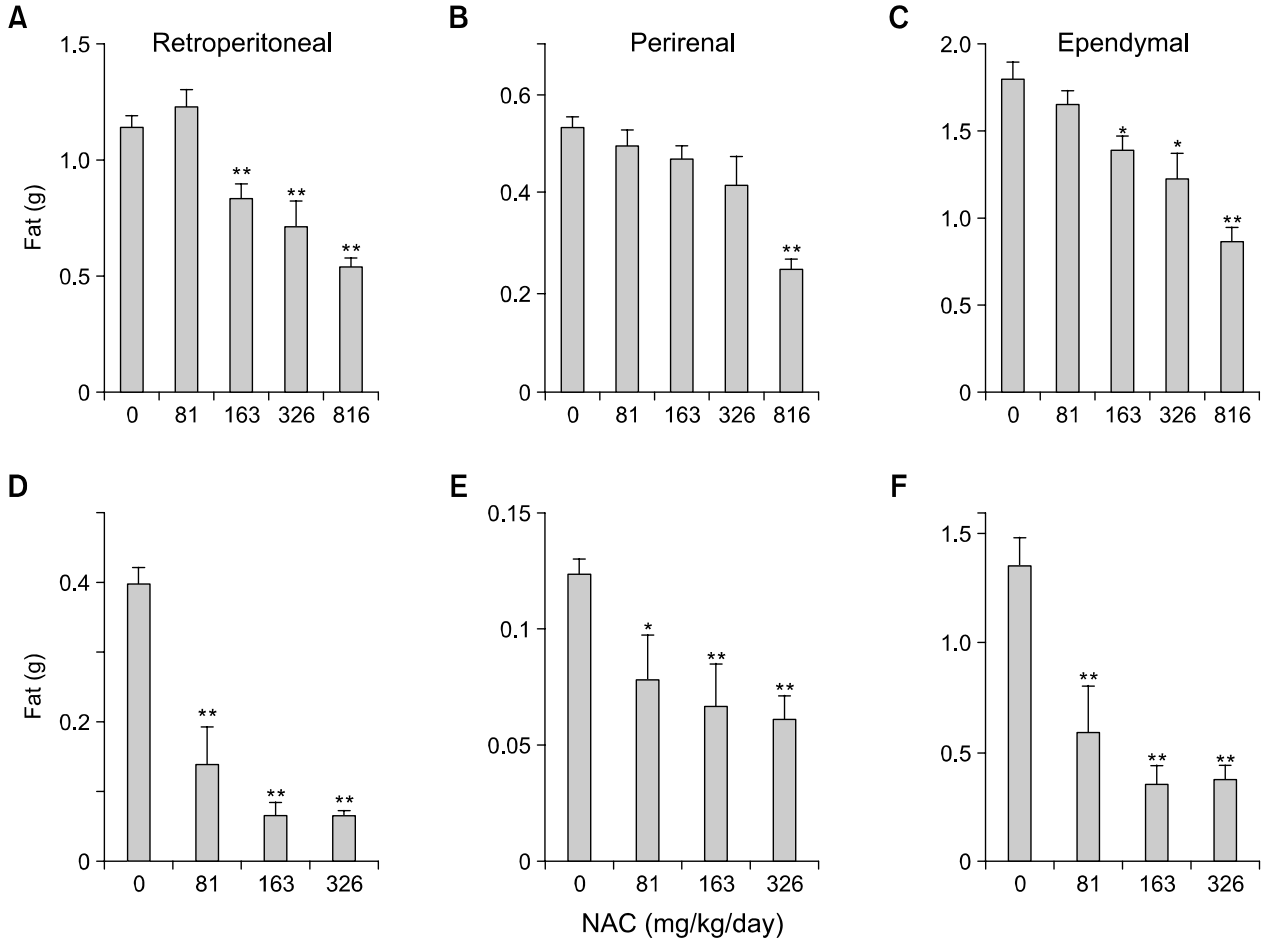

Figure 6. Effects of NAC on visceral fat mass in rats and mice. After sacrifice at 41 days (rats) or 8 wk (mice), visceral adipose tissues were excised and weighed. (A-C) rats, (D-F) mice. Values are means ± SE (n = 6); statistical significance was determined using the Student's t test (*$P < 0.05$ and **$P < 0.01$).

1993). ROS play an important role in the adipocyte differentiation of 3T3L1 cells, and mediate insulin-dependent signal transduction (Mahadev *et al.,* 2001). Therefore, the inhibition of lipid droplet formation by NAC in 3T3L1 cells may be the result of the removal of ROS generated during adipocyte differentiation. Since other antioxidants, GSH, ascorbic acid, and retinoic acid had no effects on lipid droplet formation in 3T3L1 cells, further study is needed to identify the mechanisms by which NAC inhibits adipocyte differentiation in 3T3L1 cells. Recently, α-lipoic acid, a well-known antioxidant, has been reported to inhibit adipocyte differentiation in 3T3L1 cells (Cho *et al.,* 2003) and to have anti-obesity effects in rats by suppression of hypothalamic AMP-acitvated protein kinase (Kim *et al.,* 2004).

In addition, we found that the intraperitoneal administration of NAC reduced body weights in rats and mice, primarily, by reducing the amount of visceral fat. No differences were observed in subcutaneous fat, gastrocnemius muscle, or kidney weights in rats and mice treated with or without NAC, suggesting that NAC act specifically on visceral adipose tissues to reduce of visceral fat mass. NAC is also known to have therapeutic value in terms of reducing endothelial dysfunction, inflammation, fibrosis, invasion, cartilage erosion, and acetaminophen detoxification, and to promote transplant life (Zafarullah *et al.,* 2003). NAC is now widely available in supplement form and is being recommended for preventing and treating a wide variety of ailments that may respond to its antioxidant properties. The LD50 of NAC is 7888 mg/kg in mice and > 6000 mg/kg in rats when administered orally. In animal fertility studies, no adverse effects were reported for doses up to 250 mg/kg, and no teratogenic effects were observed at doses as high as 2000 mg/kg (Kelly, 1998). In our study, the administrations of 163 mg/kg of NAC in rats and 80

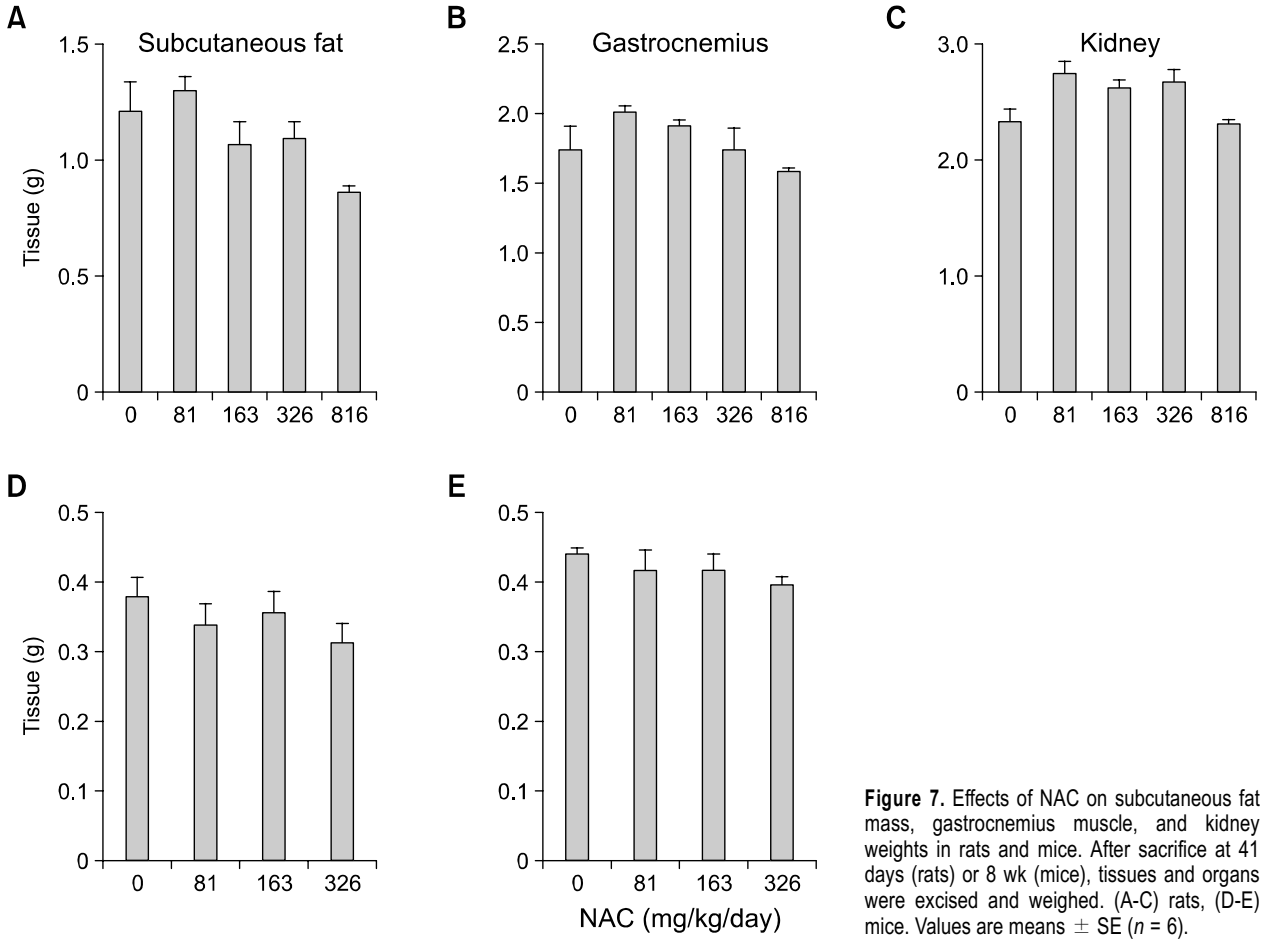

Figure 7. Effects of NAC on subcutaneous fat mass, gastrocnemius muscle, and kidney weights in rats and mice. After sacrifice at 41 days (rats) or 8 wk (mice), tissues and organs were excised and weighed. (A-C) rats, (D-E) mice. Values are means ± SE (n = 6).

mg/kg in mice were found to significantly reduce body weights and visceral fat mass. Thus, our data suggest that NAC is a useful anti-obesity drug and food supplement.

Acknowledgement

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References

Ailhaud G. Molecular mechanisms of adipocyte differentiation. J Endocrinol 1997;155:201-2

Arvidsson E, Blomqvist L, Ryden M. Depot-specific differences in perilipin mRNA but not protein expression in obesity. J Intern Med 2004;255:595-601

Aubert J, Belmonte N, Dani C. Role of pathways for signal transducers and activators of transcription, and mitogen-activated protein kinase in adipocyte differentiation. Cell Mol Life Sci 1999;56:538-42

Beattie JH, Wood AM, Newman AM, Bremner I, Choo KH, Michalska AE, Duncan JS, Trayhurn P. Obesity and hyperleptinemia in metallothionein (-I and -II) null mice. Proc Natl Acad Sci USA 1998;95:358-63

Bjorntorp P. The regulation of adipose tissue distribution in humans. Int J Obes Relat Metab Disord 1996;20:291-302

Bremner I, Beattie JH. Metallothinein and the trace minerals. Annu Rev Nutr 1990;10:63-83

Bremner I. Nutritional and physiologic significance of metallothionein. Methods Enzymol 1991;205:25-35

Bujalska IJ, Kumar S, Stewart PM. Does central obesity reflect "Cushing's disease of the omentum"? Lancet 1997; 349:1210-3

Cho KJ, Moon HE, Moini H, Packer L, Yoon DY, Chung AS. α-lipoic acid inhibits adipocyte differentiation by regulating pro-adipogenic transcription factors *via* mitogen-activated protein kinase pathways. J Biol Chem 2003;278:34823-33

Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 1987;162:156-9

Coyle P, Philcox JC, Carey LC, Rofe AM. Metallothionein: the multipurpose protein. Cell Mol Life Sci 2002;59:627-47

De Vries N, De Flora S. N-acetyl-l-cysteine. J Cell Biochem 1993;Suppl 17:270-7

Do MS, Nam SY, Hong SE, Kim KW, Duncan JS, Beattie JH, Trayhurn P. Metallothionein gene expression in human adipose tissue from lean and obese subjects. Horm Metab Res 2000;34:348-51

Dusserre E, Moulin P, Vidal H. Differences in mRNA expression of the proteins secreted by the adipocytes in human subcutaneous and visceral adipose tissues. Biochim Biophys Acta 2000;1500:88-96

Engelman JA, Lisanti MP, Scherer PE. Specific inhibitors of p38 mitogen-activated protein kinase block 3T3-L1 adipogenesis. J Biol Chem 1998;273:32111-20

Farese RV, Standaert ML, Francois AJ, Ways K, Arnold TP, Hernandez H, Cooper DR. Effects of insulin and phorbol esters on subcellular distribution of protein kinase C isoforms in rat adipocytes. Biochem J 1992;288:319-23

Frevert EU, Kahn BB. Protein kinase C isoforms epsilon, eta, delta and zeta in murine adipocytes: expression, subcellular localization and tissue-specific regulation in insulin-resistant states. Biochem J 1996;316:865-71

Fried SK, Russell CD, Grauso NL, Brolin RE. Lipoprotein lipase regulation by insulin and glucocorticoid in subcutaneous and omental adipose tissues of obese women and men. J Clin Invest 1993;92:2191-8

Gabrielsson BG, Johansson JM, Jennische E, Jernas M, Itoh Y, Peltonen M, Olbers T, Lonn L, Lonroth H, Sjostrom L, Carlsson B, Carlsson LM, Lonn M. Depot-specific expression of fibroblast growth factors in human adipose tissue. Obes Res 2002;10:608-16

Gabrielsson BG, Johansson JM, Lonn M, Jernas M, Olbers T, Peltonen M, Larsson I, Lonn L, Sjostrom L, Carlsson B, Carlsson LM. High expression of complement components in omental adipose tissue in obese men. Obes Res 2003;11: 699-708

Gregoire FM, Smas CM, Sul HS. Understaining Adipocyte Differentiation. Physiol Rev 1998;78:783-809

Harmon AW, Patel YM, Harp JB. Genistein inhibits CCAAT/enhancer-binding protein β (C/EBPβ) activity and 3T3-L1 adipogenesis by increasing C/EBP homologous protein expression. Biochem J 2002;367(Pt 1):203-8

Hausman GJ. Techniques for studying adipocytes. Stain Technol 1981;56:149-54

Holst D, Grimaldi PA. New Factors in the regulation of adipose differentiation and metabolism. Current Opinion in Lipidology 2002;13:241-5

Jin R, Chow VT, Tan PH, Dheen ST, Duan W, Bay BH. Metallothionein 2A expression is associated with cell proliferation in breast cancer. Carcinogenesis 2002;23:81-6

Karin M, Herschman HR. Dexamethasone stimulation of metallothionein synthesis in HeLa cell cultures. Science 1979;204:176-7

Karin M, Herschman HR. Glucocorticoid hormone receptor mediated induction of metallothionein synthesis in HeLa cells. J Cell Physiol 1980;103:35-40

Kelly GS. Clinical applications of N-acetylcysteine. Altern Med Rev 1998;3:114-27

Kim MS, Park JY, Namkoong C, Jang PG, Ryu JW, Song HS, Yun JY, Namgoong IS, Ha J, Park IS, Lee IK, Viollet B, Youn JH, Lee HK, Lee KU. Anti-obesity effects of α-lipoic acid mediated by suppression of hypothalamic AMP-activated protein kinase. Nat Med 2004;10:727-33

Kim SW, Muise AM, Lyons PJ, Ro HS. Regulation of adipogenesis by a transcriptional repressor that modulates MAPK activation. J Biol Chem 2001;276:10199-206

Koutnikova H, Auwerx J. Regulation of adipocyte differentiation. Ann Med 2001;33:556-61

Lefebvre AM, Laville M, Vega N, Riou JP, van Gaal L, Auwerx J, Vidal H. Depot-specific differences in adipose tissue gene expression in lean and obese subjects. Diabetes 1998;47:98-103

Mahadev K, Wu X, Zilbering A, Zhu L, Lawrence JT, Goldstein BJ. Hydrogen peroxide generated during cellular insulin stimulation is integral to activation of the distal insulin signaling cascade in 3T3-L1 adipocytes. J Biol Chem 2001;276:48662-9

Miki H, Yamauchi T, Suzuki R, Komeda K, Tsuchida A, Kubota N, Terauchi Y, Kamon J, Kaburagi Y, Matsui J, Akanuma Y, Nagai R, Kimura S, Tobe K, Kadowaki T. Essential Role of Insulin Receptor Substrate 1 and IRS2 in Adipocyte Differentiation. Mol Cellular Biol 2001;21:2521-32

Montague CT, Prins JB, Sanders L, Digby JE, O'Rahilly S. Depot- and sex-specific differences in human leptin mRNA expression: implications for the control of regional fat distribution. Diabetes 1997;46:342-7

Montague CT, Prins JB, Sanders L, Zhang J, Sewter CP, Digby J, Byrne CD, O'Rahilly S. Depot-related gene expression in human subcutaneous and omental adipocytes. Diabetes 1998;47:1384-91

Palmiter RD. The elusive function of metallothioneins. Proc Natl Acad Sci USA 1998;95:8428-30

Rahmouni K, Mark AL, Haynes WG, Sigmund CD. Adipose depot-specific modulation of angiotensinogen gene expression in diet-induced obesity. Am J Physiol Endocrinol Metab 2004;286:E891-5

Schmidt C, Beyersmann D. Transient peaks in zinc and metallothionein levels during differentiation of 3T3L1 cells. Arch Biochem Biophys 1999;364:91-8

Steven RD, Robert JC. Metallothionein Expression in Animals: A Physiological Perspectuve on Function. J Nutr 2000;130:1085-8

Stewart PM, Boulton A, Kumar S, Clark PM, Shackleton CH. Cortisol metabolism in human obesity: impaired cortisone to cortisol conversion in subjects with central adiposity. Clin Endocrinol Metab 1999;84:1022-7

Tang QQ, Otto TC, Lane MD. Mitotic clonal expansion: a synchronous process required for adipogenesis. Proc Natl Acad Sci USA 2003;100:44-9

Trathurn P, Duncan JS, Wood AM, Beattie JH. Regulation of metallothionein gene expression and secretion in rat adipo-

cytes differentiated from preadipocytes in primary culture. Horm Metab Res 2000a;32:542-7

Trayhurn P, Duncan JS, Wood AM, Beattie JH. Metallothionein gene expression and secretion in white adipose tissue. Am J Physiol Regul Integr Comp Physiol 2000b;279:R2329-35

van Harmelen V, Dicker A, Ryden M, Hauner H, Lonnqvist F, Naslund E, Arner P. Increased lipolysis and decreased leptin production by human omental as compared with subcutaneous preadipocytes. Diabetes 2002;51:2029-36

Wajchenberg BL, Giannella-Neto D, da Silva ME, Santos RF. Depot-specific hormonal characteristics of subcutaneous and visceral adipose tissue and their relation to the metabolic syndrome. Horm Metab Res 2002;34:616-21

Xia X, Serrero G. Inhibition of adipose differentiation by phosphatidylinositol 3-kinase inhibitors. J Cell Physiol 1999;178:9-16

Zafarullah M, Li WQ, Sylvester J, Ahmad M. Molecular mechanisms of N-acetylcysteine actions. Cell Mol Life Sci 2003;60:6-20

Zhang HH, Halbleib M, Ahmad F, Manganiello VC, Greenberg AS. Tumor necrosis factor-α stimulates lipolysis in differentiated human adipocytes through activation of extracellular signal-related kinase and elevation of intracellular cAMP. Diabetes 2002;51:2929-35