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 perilliprin (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...
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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 (Breman 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). Dexamethasone, 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 metallothioneine (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 ± 11.2 years; BMI 24.5 ± 1.7 kg/m²) and 5 men (age 46.2 ± 15.4 years; BMI 21.5 ± 2.6 kg/m²). The biopsies were frozen immediately and stored at -70°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°C.

cDNA array analysis

This analysis was performed using a Human GDA 1.3 array, according to the manufacturer’s instructions. mRNAs purified from omental and subcutaneous adipose tissues were radio-labeled using oligo-dT and [α-32P]dCTP by reverse-transcription. The 32P-labeled cDNAs were then hybridized with Human GDA 1.3 membranes for 24 h at 42°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\(^\circ\)C in a humidified atmosphere containing 5% CO\(_2\) and 95% air. For adipocyte differentiation, cells grown to confluence were treated with medium containing dexamethasone (0.25 \(\mu\)M) and insulin (10 \(\mu\)g/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\(^\circ\)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\(^\circ\)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 \(\mu\)M sodium vanadate, 250 nM retinoic acid, 25 \(\mu\)M LY 294002, 20 \(\mu\)M PD 98059, 5 \(\mu\)M SB 203580, 2 mM GSH, 6 \(\mu\)M H\(_2\), or 100 \(\mu\)M 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\(^\circ\)C for 10 min. Proteins (20 \(\mu\)g) 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\(^\circ\)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\(^\circ\)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\(^\circ\)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...
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
kinase inhibitor) (Engelman et al., 1998), LY 294002 (a phosphatidylinositol 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
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 ± 0.03, 0.93 ± 0.14, 0.84 ± 0.02, and 0.78 ± 0.04 respectively; these differences were statistically significant at all treated concentrations \( (P < 0.01) \) (Figure 5D).

**Discussion**

This study shows that levels of MT-II mRNA and 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
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, phosphatiidylinositol 3-kinase (PI3K), MAPKs, cAMP, steroid hormones, and protein kinase C (Farese et al., 1992; Frevert and Kahn, 1998; 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 carcinoogen-DNA adducts (De Vries and De Flora,
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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 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).
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.

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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).
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