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Article

Production of Reactive Oxygen Species and Changes in Antioxidant Enzyme Activities during Differentiation of 3T3-L1 Adipocyte

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Obesity, a major public health problem around the world, is a strong risk factor for the development of type 2 diabetes, atherosclerosis, hypertension and cardiovascular diseases. Recent research suggests that increased production of reactive oxygen species (ROS) from accumulated fat in obesity leads to elevated systemic oxidative stress and contributes to the development of obesity-linked chronic disorders. The aim of the current study was to investigate changes of key enzymes associated with antioxidant response and metabolic pathways in 3T3-L1 cell, a preadipocyte cell line that undergoes differentiation into mature adipocytes. The changes in lipid accumulation, ROS production, glucose-6-phosphate dehydrogenase (G6PDH), superoxide dismutase (SOD) and catalase (CAT) during the course of differentiation were determined. The ROS production and G6PDH activity exponentially increased as differentiation progressed. However, CAT activity showed a sharp decrease until day 2, followed by a gradual increase up to day 6 and then leveled off. Given the importance of adipocyte differentiation and ROS production in oxidation-linked diseases, these data provide a biochemical rationale for further studies to identify cellular mechanisms that can counter ROS generation and modulate cellular redox environment in adipocytes.

Key words: *antioxidant enzyme response, glucose-6-phosphate dehydrogenase, hexose monophosphate shunt, reactive oxygen species, 3T3-L1 adipocyte differentiation*

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Abbreviations: AER, antioxidant enzyme responses; BCS, bovine calf serum; CAT, catalase; DETAPAC, diethylene triaminie penta-acetic acid; DEX, dexamethasone; DMEM, dulbecco's modified Eagle's medium; ETS, electron transport system; FBS, fetal bovine serum; G6PDH, glucose-6-phosphate dehydrogenase; ROS, reactive oxygen species; HMP, hexose monophosphate; IBMX, 3-isobutyl-1-methylxanthine; NBT, nitroblue tetrazolium; NO, nitric oxide; NOX, NADPH oxidase; OD, optical density; PBS, phosphate buffered saline; P/S, penicillin-streptomycin; SOD, superoxide dismutase

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The prevalence of obesity has reached crisis levels worldwide and is projected to increase substantially over the next decade in both developed and developing countries. Obesity is associated with physiological changes that cause or contribute to the development of metabolic complications such as type 2 diabetes, insulin resistance and dyslipidemia, resulting in the metabolic syndrome and cardiovascular disease. Thus the increasing incidence of obesity is a major public health problem and a global burden on health care systems as it requires long-term expensive treatment.

There is growing evidence, suggesting that changes resulting from environmental factors, mainly diet and exercise, and their interactions with the genetic

components have contributed significantly to the rapid increase of the obesity epidemic. The weight control by lifestyle modifications through diet and exercise has been increasingly recognized as an important strategy to manage body weight as well as to promote overall health.

While the genetics is a contributing factor, this alone is unlikely to account for the recent rise in the incidence of obesity and its accompanying disorders. Whether the origin is genetic, environmental factors or the combinations, one of the key features of obesity phenotype is the increased adipose tissue mass, which is characterized by the differentiation of preadipocytes into an excessive number of mature adipocytes. Therefore, an increased number of terminally differentiated adipocytes characterize not only obesity, but also type 2 diabetes, dyslipidemia and cardiovascular disease. Preadipocyte differentiation has become an intense area of research in recent years and been studied primarily by using *in vitro* models of adipogenesis including the 3T3-L1 cell line, one of the well-characterized and reliable models. Furthermore, the use of 3T3-L1 cell line in the study of obesity and metabolic syndrome has generated considerable progresses in our understanding of adipocyte biology and the impacts of dietary factors on these metabolic disorders [Spiegelman and Flier, 1999; Furukawa *et al.*, 2004; Hsu and Yen, 2008; Rayalam *et al.*, 2008]. It has become clear that adipose tissue not only acts as a lipid storage depot, but also actively regulates its own as well as whole body energy metabolism through paracrine and endocrine actions.

Obesity has been linked to accelerated systemic oxidative stress and reduced antioxidant capacity. Recent research suggests that increased reactive oxygen species (ROS) production from accumulated fat in obesity leads to elevated systemic oxidative stress, contributing to the development of obesity-linked chronic disorders (Furukawa *et al.*, 2004). Moreover, obesity-induced oxidative stress in adipose tissue may result in an increase in inflammatory signals, dysregulation of adipokines, and insulin resistance (Dandona *et al.*, 2004; Furukawa *et al.*, 2004). During differentiation of preadipocytes into adipocyte, the cellular fate of glucose is mostly directed towards biosynthetic and metabolic pathways, which can include glycerol-3-phosphate used in triglyceride and phospholipid synthesis as well as sugar phosphates via hexose monophosphate (HMP) shunt. It is also known that NADPH derived from glucose-6-phosphate dehydrogenase (G6PDH), the rate limiting enzyme of the HMP pathway, is a key system involved in maintaining the function of several important redox and antioxidant defense mechanisms. In addition, one of the major sources of cellular ROS in adipocyte is known to be NADPH oxidase (NOX), whose activity may paradoxically increase by generation of NADPH associated with enhanced HMP pathway. Although increased ROS production in cellular differentiation has been implicated, it remains largely unexplored how increased oxidative stress is managed and the potential roles of the HMP pathway and NADPH state for the redox regulation during the course of preadipocyte differentiation, a process central to the development of obesity as well as in the normal development and function of adipose tissue.

Consequently, the aim of this study was to investigate whether the changes in lipid accumulation and ROS production are associated with changes of enzymes activities linked to endogenous antioxidant enzyme responses (AER) and the HMP pathway in differentiating 3T3-L1 preadipocytes.

Materials and Methods

Cell line and materials. 3T3-L1 preadipocyes were obtained from American Type Culture Collection (ATCC, CL-173). Dulbecco's modified Eagle's medium (DMEM), bovine calf serum (BCS), fetal bovine serum (FBS), penicillin-streptomycin (P/S), phosphate buffered saline (PBS), and trypsin-EDTA were purchased from Gibco (Gaithersburg, MD). Dexamethasone (DEX), 3-isobutyl-1-methylxanthine (IBMX), insulin, Oil-red O and nitroblue tetrazolium (NBT) were acquired from Sigma (St. Louis, MO). All other chemicals were purchased from Sigma.

Cell culture. 3T3-L1 preadipocytes were cultured, maintained, and differentiated as described by Student *et al*. [1980]. Briefly, cells were plated and grown in DMEM with 3.7 g/L sodium bicarbonate, 1% P/S, and 10% BCS. Adipocyte differentiation was induced by treatment of 2 days post-confluent cells with 10% FBS and a hormonal mixture (MDI), consisting of 0.5 mM IBMX (M), 1.0 μM DEX (D), and 1.67 μM insulin (I). Two days after the initiation of differentiation, the culture medium was replaced with DMEM, supplemented with only 1.67 μM insulin and 10% FBS. This medium was then replenished every other day throughout the differentiation process.

Determination of lipid accumulation by Oil red O staining. The extent of differentiation reflected by amount of lipid accumulation was determined at 0, 2, 4, 6, 8 and 10 days after treatment by Oil red O staining. For the determination of lipids, cells were fixed in 10% formaldehyde in PBS for 1 h, washed with 60% isopropanol, and completely dried. Dried cells were then stained with 0.5% Oil red O solution in 60% isopropanol for 30 min at room temperature and washed four times with distilled water, and then dried again. The degree of

differentiation was quantified by measuring an optical density (OD) at 490 nm after eluting with 0.5 mL of isopropanol [Blumberg *et al.*, 2006].

Reactive oxygen species (ROS) production in differentiating 3T3-L1 preadipocyte. 3T3-L1 preadipocytes were grown to confluence and induced to differentiate to adipocytes as described above. ROS production was measured by NBT assay. NBT is reduced by ROS to a dark-blue, insoluble form of NBT called formazan [Furukawa *et al.*, 2004]. At days 0, 2, 4, 6, 8 and 10 after the induction, cells were incubated for 90 min in 0.2 mL of PBS containing 0.2% NBT. Formazan was dissolved in acetic acid, and the absorbance was determined at 570 nm.

Cell extraction for enzyme activity. The cells were harvested at days 0, 4, 8, and 10 after the MDI-initiated differentiation. Two mL of 50 mM potassium phosphate buffer, pH 7.5 (0.15 M NaCl and 1 mM EDTA) were added to the vials. The sample was centrifuged at 2,000× g for 10 min at 4°C and kept on ice. Pellets was resuspended in 2.0 mL of 50 mM potassium phosphate buffer, pH 7.5 followed by thorough homogenization at 2,000×g for 2 min using a tissue homogenizer (Biospec products, Bartlesville, OK). The sample was again centrifuged at 12,000×g for 15 min at 4°C and kept on ice. The supernatant was used for further enzyme activity analyses.

Total protein analysis. Protein content was determined according to the method previously described by Bradford [1976]. The dye reagent concentration (Bio-Rad protein assay kit II, Bio-Rad Laboratory, Hercules, CA) was diluted 1:4 with distilled water. Five mL of diluted dye reagent was added to 100 μL cell extract. After vortexing and incubating for 5 min, the absorbance was measured at 595 nm against a 5 mL reagent blank and 100 μL of 50 mM potassium phosphate buffer using a UV-VIS spectrophotometer (Milton Roy, Inc., Rochester, NY).

G6PDH enzyme activity assay. A modified version of the G6PDH assay described by Deutsch [1983] was used. The enzyme reaction mixture containing 5.88 μmol β-NADP, 88.5 μmol $MgCl_2$, 53.7 μmol glucose-6-phosphate, and 0.77 mmol malemide was prepared. This mixture alone was used to obtain a baseline at 340 nm wavelength. One mL of the enzyme reaction mixture was added to 50 μL of the sample. The rate of change in absorbance per minute was used to measure the enzyme activity in the mixture by employing the extinction co-efficient of NADPH (6.22 mM^{-1} cm^{-1}).

Superoxide dismutase (SOD) enzyme activity assay. A competitive inhibition assay, in which xanthine-xanthine oxidase-generated superoxide reduces NBT to blue formazan, was employed to measure SOD enzyme activity. Spectrophotometric assay of SOD activity was carried out by monitoring the reduction of NBT at 560 nm [Oberley and Spitz, 1984]. The enzyme reaction mixture contained 13.8 mL of 50 mM potassium phosphate buffer (pH 7.8) with 1.33 mM diethylene triaminie penta-acetic acid (DETAPAC), 0.5 mL of 2.45 mM NBT, 1.7 mL of 1.8 mM xanthine, and 40 IU/mL catalase. To 0.8 mL of enzyme reaction mixture, 100 μL of phosphate buffer and 100 μL of xanthine oxidase was added. The changes in absorbance at 560 nm were measured every 20 s for 2 min and the concentration of xanthine oxidase was adjusted to obtain a linear curve with a slope of 0.025 absorbance per min. The phosphate buffer was then replaced by the enzyme extract and the changes in absorbance were monitored as performed in control reaction (every 20 s for 2 min). One unit of SOD was defined as the amount of protein that inhibits NBT reduction by 50 % of the maximum.

Catalase (CAT) enzyme activity assay. A method originally described by Beers and Sizer [1952] was empoyed for catalase activity assay. One mL of 0.059 M hydrogen peroxide (Merck's Superoxol or equivalent grade) in 0.05 M potassium phosphate (pH 7.0) was added to 1.9 mL of distilled water. This mixture was incubated in a spectrophotometer for 4-5 min to achieve temperature equilibration and to establish blank rate. To this mixture, 0.1 mL of diluted enzyme was added and the disappearance of peroxide was determined spectrophotometrically by monitoring the decrease in absorbance at 240 nm for 2-3 min. The change in absorbance ΔA_{240}/min from the initial (45 s) linear portion of the curve was calculated. One unit of catalase activity was defined as amount that decomposes one ìmole of H_2O_2.

Results and Discussion

Changes of lipid accumulation and ROS production during the differentiation of 3T3-L1 preadipocytes. The changes of lipid accumulation and ROS production were evaluated during the differentiation of 3T3-L1 preadipocyte using Oil Red O staining and NBT assay, respectively (Fig. 1). When preadipocytes were differentiated into adipocytes, morphological alterations were observed due to the accumulation of lipid droplets in the cytoplasm. As determined by Oil Red O staining, 3T3-L1 adipocytes at day 10 significantly increased lipid accumulation compared to preadipocyte at day 0 (Fig. 1A). In addition, ROS production markedly increased during differentiation of 3T3-L1 preadipocyte into adipocyte, suggesting that ROS production increased in parallel with fat accumulation (Fig. 1B). These results are

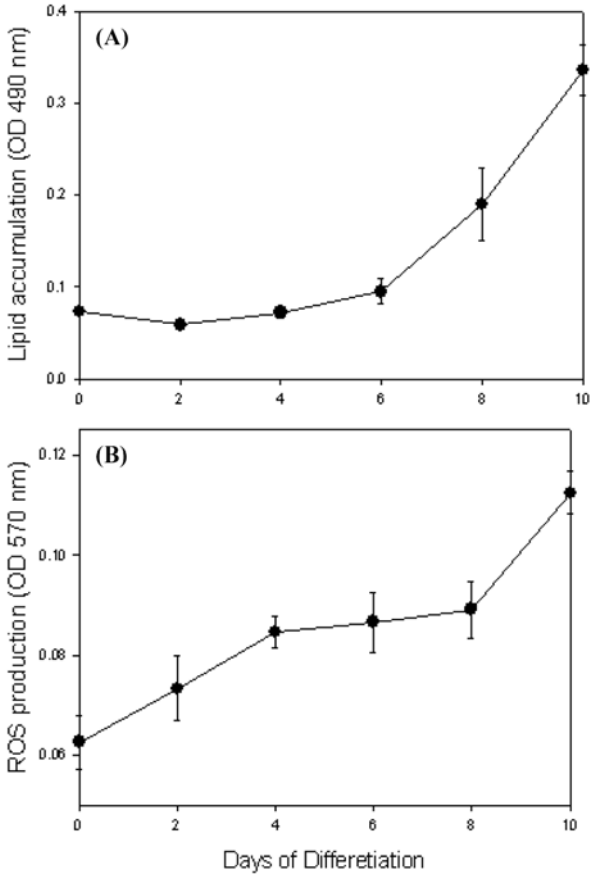

Fig. 1. Changes of lipid accumulation (A) and ROS production (B) during differentiation in 3T3-L1 preadipocyte. Lipid accumulation was determined by observing the absorbance at 490 nm while ROS production was assayed at 570 nm.

in line with recent studies showing that accumulated fat in obesity is associated with increased oxidative stress and metabolic syndrome [Furukawa *et al.*, 2004].

There are several sources of ROS production during adipocyte differentiation. One of the ROS sources can be from oxidation of the accumulated lipid molecules in adipocytes. Also, the major source of cellular ROS is known to be from the electron transport system (ETS) of mitochondria, and thus altered function of mitochondria can increase mitochondrial ROS generation, thereby contributing to several pathological conditions, such as vascular complications of diabetes, neurodegenerative diseases, and cellular senescence. Another source of cellular ROS is pro-oxidative enzymes such as NADPH oxidase (NOX) and inducible nitric oxide (iNO) systems. The increased activity of these enzymes has also been shown to stimulate oxidative stress [Park *et al.*, 2005]. A recent report strongly suggests that NOX is a major source of ROS in adipocyte, and that augmented NOX enzyme seems to contribute to increased ROS production in obesity [Furukawa *et al.*, 2004; Park *et al.*, 2006].

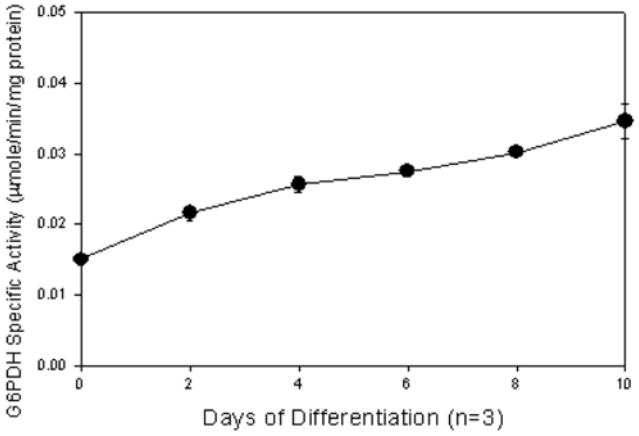

Fig. 2. Changes of G6PDH enzyme activity during the differentiation of 3T3-L1 preadipocytes. G6PDH, glucose-6-phosphate dehydrogenase

Changes of G6PDH enzyme activity. G6PDH, the first rate-limiting enzyme of the HMP shunt, is a major intracellular source of NADPH generation. NADPH produced by G6PDH is required as a cofactor for both NOX enzyme as well as lipid synthesis. Fig. 2 shows the changes of G6PDH enzyme activity during differentiation of 3T3-L1 preadipocytes. G6PDH enzyme activity was significantly increased during the differentiation of 3T3-L1 preadipocytes in a time-dependent manner with the same trend observed in lipid accumulation and ROS production (Fig. 1). These results suggest that increased G6PDH activity leads to the formation of NADPH to support lipid synthesis as preadipocytes are differentiating into mature adipocytes. Paradoxically, NADPH generated via the same HMP shunt could be an important component of ROS production by pro-oxidant enzymes, such as NADPH oxidase. It is reasonable to speculate that G6PDH is a major source of NADPH, which is linked to ROS production by NOX enzyme as recently reported [Park *et al.*, 2005]. However, it is not currently known whether this is a consequence of the differentiation per se or a cause of adipocyte differentiation.

Changes of SOD and CAT enzyme activity. Generally, superoxide anion is converted to oxygen and hydrogen peroxide by superoxide dismutase (SOD) or reacts with nitric oxide (NO) to form peroxynitrite. Hydrogen peroxide can be converted to water and a molecule of oxygen by catalase. Superoxide anion is thus believed to be the cause of other ROS formations such as hydrogen peroxide, peroxynitrite, and hydroxyl radicals. Therefore, we examined the activity of total SOD and CAT activity during differentiation of 3T3-L1 preadipocytes.

The SOD activity in cells was significantly down-regulated by day 2 and then maintained throughout the course of differentiation of 3T3-L1 preadipocytes (Fig.

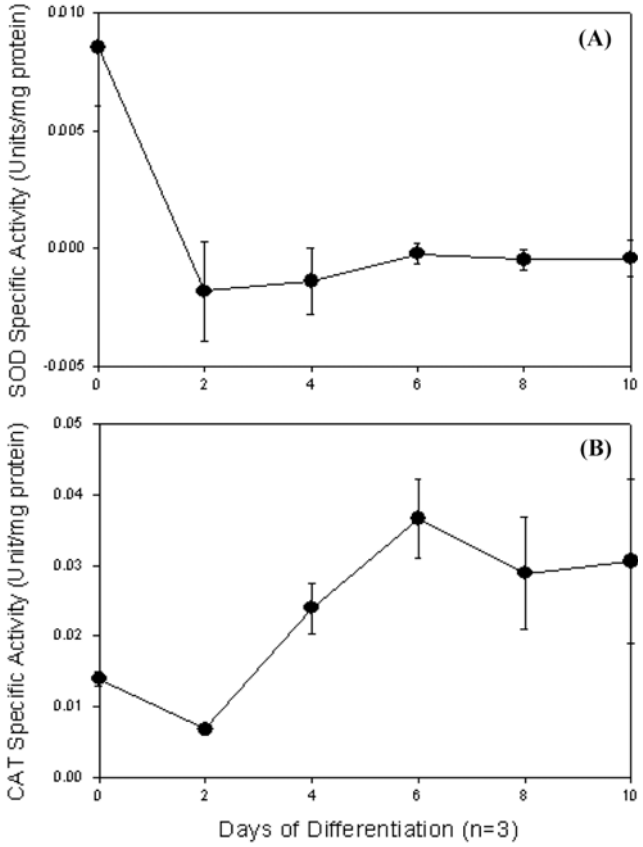

Fig. 3. Changes of SOD (A) and CAT (B) activities during the differentiation of 3T3-L1 preadipocytes. SOD, superoxide-dismutase; CAT, catalase

3A). Therefore, the high ROS level observed in mature adipocytes (Fig. 1) at day 10 may be in part mediated by decreased SOD activity. However, CAT activity showed a sharp decrease until day 2, followed by a gradual increase up to day 6 and then leveled off (Fig. 3B). An acute reduction of both enzyme activities in the early stage of differentiation may reflect a sharp increase in the production of ROS although other peroxidases and perhaps HMP shunt-driven endogenous antioxidant enzyme systems could have also contributed to the detoxification of free radicals produced. Our data are also in good agreement with Araki *et al*. [2006], in which the increase of TNF-α reduced SOD enzyme activity during the differentiation of 3T3-L1 preadipocytes.

Obesity, lipodystrophy, and alternations in the secretary function of adipose tissue are commonly associated with diverse pathologies, including diabetes, cardiovascular diseases, and immune suppression. In this regard, it is important to identify the role of ROS production presumably through NOX activity and its link to cellular antioxidant response during adipocyte differentiation. A hypothetical model for the production and maintenance of ROS as well as antioxidant responses via HMP shunt is

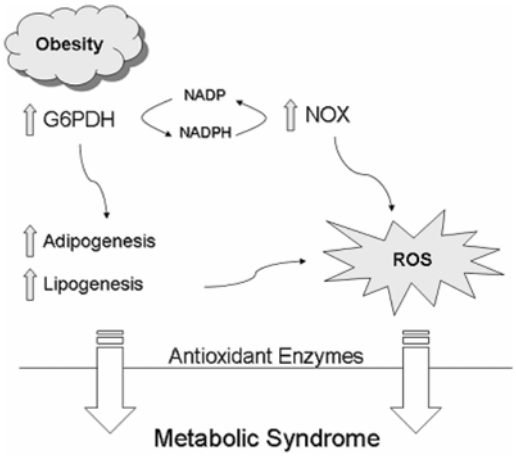

Fig. 4. A proposed pathway for the redox state and antioxidant responses during adipogenic differentiation of 3T3-L1 cell.

proposed (Fig. 4). We hypothesize that, in differentiating preadipocytes, lipid accumulation parallels ROS production and this increased oxidative stress can be countered by enhanced G6PDH activity and/or endogenous antioxidant enzyme systems like SOD, CAT, or other peroxidases, whereas this cellular redox homeostasis may be dysfunctional or lost in pathological conditions such as obesity and metabolic syndrome. This study also provides an interesting biochemical rationale for future studies to identify the relationship between dietary antioxidants, oxidative stress, and their modulation of cellular redox environment in adipocytes.

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