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Extracellular Superoxide Dismutase in Tissues from Obese (ob/ob) Mice

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We have examined the protein content and gene expression of three superoxide dismutase (SOD) isoenzymes in eight tissues from obese ob/ob mice, particularly placing the focus on extracellular-SOD (EC-SOD) in the white adipose tissue (WAT). Obesity significantly increased EC-SOD level in liver, kidney, testis, gastrocnemius muscle, WAT, brown adipose tissue (BAT), and plasma, but significantly decreased the isoenzyme level in lung. Tumor necrosis factor-α and interleukin-1β contents in WAT were significantly higher in obese mice than in lean control mice. Immunohistochemically, both WAT and BAT from obese mice could be stained deeply with anti-mouse EC-SOD antibody compared with those from lean mice. Each primary culture *per se* almost time-dependently enhanced EC-SOD production, and overtly expressed its mRNA. The loss of heparin-binding affinity of EC-SOD type C with high affinity for heparin occurred in kidney of obese mice. These results suggest that the physiological importance of this SOD isoenzyme in WAT may be a compensatory adaptation to oxidative stress.

Keywords: Extracellular superoxide dismutase, obesity, ob/ob mouse, cytokine, affinity for heparin

INTRODUCTION

Björntorp [1] has distinguished between two types of obesity: one type in which there is an increased number of white fat cells (hyperplasia-obesity), and another in which there is a normal number of white fat cells, but each fat cell has an increased content of triglycerides (hypertrophy-obesity). Also, obesity is generally characterized by hyperinsulinemia, variable degrees of glucose intolerance, and resistance to both endogenous and exogenously administered insulin [2].

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The obese ob/ob mouse (also known as obese-hyperglycemic), which was first discovered in the Jackson Laboratory (Bar Harbor, ME, USA), is the most widely used animal in obesity research. The principle features of the mutant including hyperinsulinemia and insulin resistance as well as obesity, have led to the use in diabetes research [3]. Recently, glycation of protein and increased generation of free radicals have been proposed to explain the pathogenesis of diabetes [4,5]. For example, glycated and less active Copper, Zinc-superoxide dismutase (Cu, Zn-SOD), which is one of the most important enzymes in the antioxidant defense system, is increased in erythrocytes of patients with insulin-dependent diabetes mellitus [6,7], followed by production of reactive oxygen species (ROS), then resulting in site-specific and random fragmentation of the SOD isoenzyme [8]. Moreover, as for another SOD isoenzyme, extracellular SOD (EC-SOD), Adachi *et al.* [4] have also demonstrated that the proportion of glycated EC-SOD in serum of diabetic patients is considerably higher than in normal subjects.

Three types of SOD isoenzymes which are characterized by metal ions and their different localizations have been identified in mammals [7,9]. Cu,Zn-SOD is found in the cytosol and contains copper and zinc ions in a molecule, whereas manganese-SOD (Mn-SOD) is located in the mitochondria with a manganese ion. The third isoenzyme, copper and zinc containing EC-SOD, is a secretory glycoprotein [10,11] and is predominantly located in the extracellular space such as plasma [10] but occurs also in tissues [12,13]. In addition, one of the unique properties of EC-SOD is its affinity for heparin and analogues, *in vivo*, mediating attachment to heparan sulfate proteoglycans located on cell surfaces and in the connective tissue matrix [14,15]. Interestingly, our previous study on normal mice has revealed that white fat has a high content of EC-SOD, accompanied by relatively strong expression of its mRNA [13]; however, there is very little information on the tissue distribution of EC-SOD in obese animals. Thus, we have examined the tissue distribution of the three SOD isoenzymes in obese ob/ob mice, particularly placing the focus on EC-SOD in the white adipose tissue (WAT) and brown adipose tissue (BAT).

MATERIALS AND METHODS

Animals

Male genetically obese mice (C57BL/6J ob/ob; $n=12$) and their lean controls (C57BL/6J ?/+; $n=12$) were obtained from the Jackson Laboratory. The mice were housed under barrier conditions in the animal unit at National Defense Medical College (Tokorozawa, Japan). They were reared at 25°C under artificial lighting for 12 h from 7 a.m. to 7 p.m. daily and were given a standard laboratory diet (Oriental MF, Oriental Yeast Co., Tokyo, Japan) and tap water *ad libitum*. The animals were cared for in accordance with the Guiding Principles for the Care and Use of Animals approved by the Council of the Physiological Society of Japan, based upon the Declaration of Helsinki as revised in 1996. When the mice reached 7 or 14 weeks of age, heparinized blood samples were collected by heart puncture under anesthesia. Eight tissues (heart, lung, liver, kidney, testis, gastrocnemius muscle (GM), WAT, and BAT) were then removed quickly, rinsed in phosphate-buffered saline (PBS; 50 mM sodium phosphate and 150 mM NaCl, pH 7.4), and frozen in liquid nitrogen. Subsequently, a portion of tissues was homogenized in 9 vol of PBS with a Polytron homogenizer (Kinematika, Luzern, Switzerland) and then centrifuged at 5000*g* (4°C) for 10 min, and the supernatant was used for various assays. Protein content was determined with a BCA protein assay kit (Pierce, Rockford, IL, USA) using bovine serum albumin as a standard. The tissues from 14-week-old lean and obese mice ($n=6$ each) were used only for immunological EC-SOD and protein assays.

Plasma Glucose

Plasma glucose level was measured using a commercial kit (Glucose B-test wako; Wako Pure Chemical Industries, Ltd., Osaka, Japan).

ELISA

EC-SOD was purified from mouse lungs, and the rabbit antiserum against the purified mouse EC-SOD was obtained according to our previous studies [13,16]. The antibodies for rat Cu,Zn-SOD and Mn-SOD were described previously and the specificity of these antibodies to mouse SODs could be judged by Western blotting analysis [17]. To estimate the protein contents of EC-SOD, Cu,Zn-SOD and Mn-SOD, an ELISA was developed using each antibody with a sandwich method [13,18]. The tissue contents of cytokines, tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β), were also determined by an ELISA using the respective commercial kit (Endogen, Inc., Cambridge, MA, USA).

Northern Blot Analysis

Total RNAs were isolated from each tissue using a Trizol reagent (Life Technologies, Rockville, MD, USA) ($n = 3$ each). RNA (16 μg) was fractionated by electrophoresis on a denaturing formaldehyde – 1.2% agarose gel and transferred onto a positively charged nylon membrane (Hybond N+; Amersham Life Science, Arlington Heights, IL, USA) by capillary action in 20× SSC overnight (1× SSC : 150 mM NaCl/15 mM trisodium citrate, pH 7.0), followed by ultraviolet irradiation fixation. The cDNA probes for each isoenzyme were prepared according to former reports [13,18], and labelled with [^{32}P]-dCTP by the random-priming method [19] using a DNA labelling kit (Wako Pure Chemical Industries, Ltd.). The blotted membrane was prehybridized in the prehybridization solution (50% formamide/3× SSC/5× Denhardt/0.1% SDS/10 μg/ml denatured shared salmon sperm DNA/5% (w/v) dextran sulfate (Pharmacia Biotech, Uppsala, Sweden)) at 42°C for 4 h, followed by hybridization in the same buffer containing 1×10^6 cpm/ml of the [^{32}P]probe at 42°C overnight. The membrane was washed twice in 1× SSC/0.1% SDS at room temperature, and once in 0.1× SSC/0.1% SDS at 42°C, 50°C or 60°C for 15 min, and exposed to an imaging plate (Fujix, Tokyo, Japan) at room temperature for 30–60 min. Autoradiographic signals were quantified using a BAS 2000 Bioimaging Analyzer (Fujix). The density of 18S (2.1 kb) rRNA in the blots stained with ethidium bromide was quantitated using an LKB Ultrascan XL enhanced laser densitometer (Pharmacia Biotech). The degree of SOD isoenzyme mRNA was calculated after normalization to the intensity of the 18S rRNA (an internal control).

Primary Culture of Isolated Adipocytes

To investigate whether EC-SOD is primarily synthesized in adipocytes and secreted, epididymal fat pads and interscapular BAT were removed from lean control mice. White and brown adipocytes were isolated as described previously [20]. Fibroblastic preadipocytes and brown adipocyte precursor cells were inoculated with 2×10^6 cells/well or 4×10^5 cells/well in 6-well culture plates, respectively, and cultured in Medium 199 supplemented with 10–20% fetal bovine serum and antibiotics (100 units/ml penicillin G, 100 μg/ml streptomycin and 0.25 μg/ml fungizon; GIBCO, Grand Island, NY, USA) at 37°C in an atmosphere of 5% CO_2 in air.

Immunostaining of Mouse Tissues

Immunohistochemical analysis of WAT and BAT was done according to our previous study [13]. Briefly, air-dried frozen tissue sections were fixed at room temperature for 5 min in 4% paraformaldehyde/PBS (pH 7.3) and blocked in PBS containing 5% (w/v) skim milk for 10 min. Sections were incubated with 1 μg/ml affinity-purified

anti-mouse EC-SOD IgG at 4°C overnight. Bound antibodies were detected with horseradish peroxidase (HRP)-labelled donkey anti-rabbit IgG polyclonal antibody diluted 1:100 (Chemicon, Temecula, CA, USA). HRP activity was developed in a 50 mM Tris–HCl buffer (pH 7.6) containing 0.1% DAB and 0.01% H_2O_2 for 5 min. The sections were lightly washed with PBS between each step. Counterstaining was carried out using hematoxylin.

Heparin-Sepharose Column Chromatography

The chromatography was carried out at 4°C in 1 ml heparin-Sepharose column (HiTrap Heparin, Pharmacia Biotech). The lung and kidney extracts were diluted twice with a 25 mM sodium phosphate buffer, pH 6.5, and were applied to the column equilibrated with the above buffer and extensively washed with the same buffer according to the method described previously [16]. Bound components were then eluted with a linear gradient of NaCl in the buffer (0–1 M). In each 0.5 ml fraction collected, EC-SOD was determined by an ELISA.

Statistical Analysis

Data are presented as mean ± SEM. The statistical analysis of the data was accomplished with an analysis of variance (ANOVA), and then the Bonferroni *post-hoc* test was conducted when a significant F ratio was obtained. When applicable, the unpaired Student's t test was used. We calculated a correction coefficient for the linear regression analysis. A 0.05 level of significance was used.

RESULTS

Body Weight and Plasma Glucose

As was expected, the body weight of obese mice at 7 and 14 weeks of age (37.5 ± 0.9 and 56.5 ± 1.2 g, respectively) was significantly higher than that of lean mice (22.8 ± 0.3 and 30.7 ± 0.8 g, respectively) ($p < 0.001$). As for 7-week-old animals, plasma glucose concentration was significantly higher in obese mice (31.8 ± 3.2 mmol/l) than in lean mice (19.2 ± 2.0 mmol/l) ($p < 0.01$).

Tissue Distribution of SOD Isoenzymes

As shown in Figure 1, EC-SOD level in WAT, lung, kidney, and BAT from 7-week-old lean mice showed relatively higher values compared to that in heart, liver, testis, and GM from the same animals, being in approximate agreement with our previous findings [13]. WAT showed the highest value. Obesity significantly increased EC-SOD level in liver, kidney, testis, GM, WAT, BAT, and plasma, especially in WAT, but significantly decreased the enzyme level in lung. On the other hand, no effect of obesity was noted in heart. In addition, EC-SOD level in tissues from 14-week-old obese mice showed similar results (data not shown). Mn-SOD level in heart, liver,

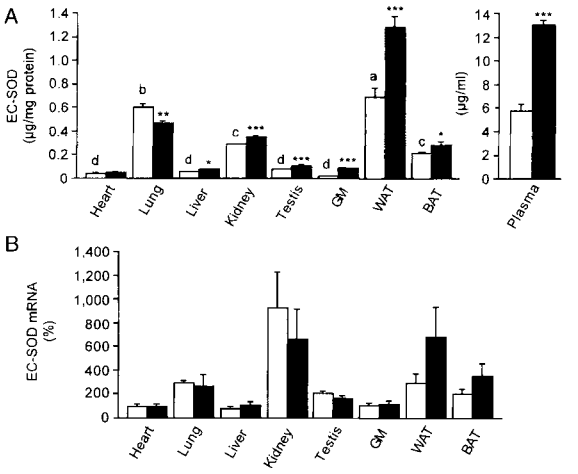

FIGURE 1 Immunoreactive EC-SOD protein content (A) and relative abundance of EC-SOD mRNA expression (B) in lean (open bars) and obese (filled bars) mouse tissues. Values are mean ± SEM. GM, gastrocnemius muscle; WAT, white adipose tissue; BAT, brown adipose tissue. (A) $n = 6$; (B) $n = 3$. a–d Values with different alphabetical letters are significantly different ($p < 0.05$) (comparisons among EC-SOD contents in lean mouse tissues). $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ vs lean mice.

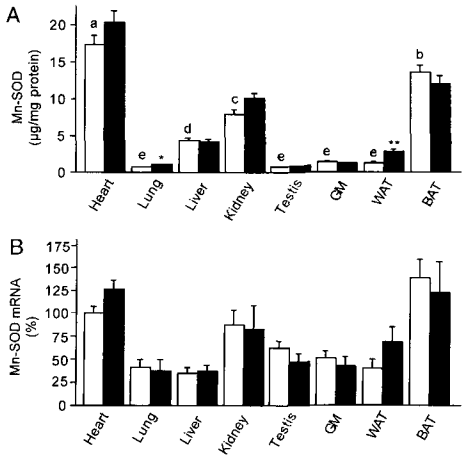

FIGURE 2 Immunoreactive Mn-SOD protein content (A) and relative abundance of its mRNA expression (B) in lean (open bars) and obese (filled bars) mouse tissues. Values are mean ± SEM. (A) $n = 6$; (B) $n = 3$. a–e Values with different alphabetical letters are significantly different ($p < 0.05$) (comparisons among Mn-SOD contents in lean mouse tissues). *$p < 0.05$; **$p < 0.01$ vs lean mice.

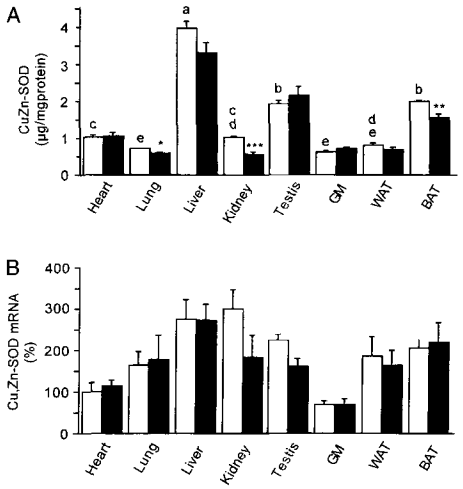

FIGURE 3 Immunoreactive Cu,Zn-SOD protein content (A) and relative abundance of its mRNA expression (B) in lean (open bars) and obese (filled bars) mouse tissues. Values are mean ± SEM. (A) $n = 6$; (B) $n = 3$. a–e Values with different alphabetical letters are significantly different ($p < 0.05$) (comparisons among Cu,Zn-SOD contents in lean mouse tissues). *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ vs lean mice.

kidney, and BAT from 7-week-old lean mice showed relatively higher values compared to that of their lung, testis, GM, and WAT. Heart showed the highest value. Mn-SOD level in lung and WAT was significantly higher in obese mice than in lean mice (Figure 2). Cu,Zn-SOD level in liver, testis, and BAT from 7-week-old lean mice showed relatively higher values compared to that of their heart, lung, kidney, GM, and WAT. Liver showed the highest value. Conversely, Cu,Zn-SOD content in lung, kidney and BAT was significantly reduced with obesity (Figure 3). The mRNA expressions of the three SOD isoenzymes in both mice appeared to roughly parallel the respective isoenzyme protein levels in tissues (Figures 1–3). Meanwhile, no overt effect of obesity on the mRNA abundance of SOD isoenzymes was noted in any of the tissues examined.

Comparisons between EC-SODs in Plasma and in Tissues

Except for heart and lung, EC-COD concentration in plasma from lean and obese mice correlated well with the enzyme level in the six tissues examined (Figure 4). On the other hand, a negative correlation was found between EC-SODs in plasma and lung.

Tissue Cytokines

As shown in Table I, TNF-α and IL-1β contents in WAT were significantly higher in obese mice than in lean mice. IL-1β content in testis and in GM was significantly reduced with obesity.

Comparisons between SODs and Cytokines

When EC-SOD level was compared with TNF-α level, a direct linear correlation was found in WAT and BAT from lean and obese mice (Figure 5A). There was also a definite correlation between EC-SOD and IL-1β levels in the lung and WAT (Figure 5B). Moreover, a positive correlation between Mn-SOD level on one hand, and TNF-α and IL-1β levels on the other, was observed in WAT from lean and obese mice (data not shown). There existed no significant correlation between the levels of Cu,Zn-SOD and cytokines.

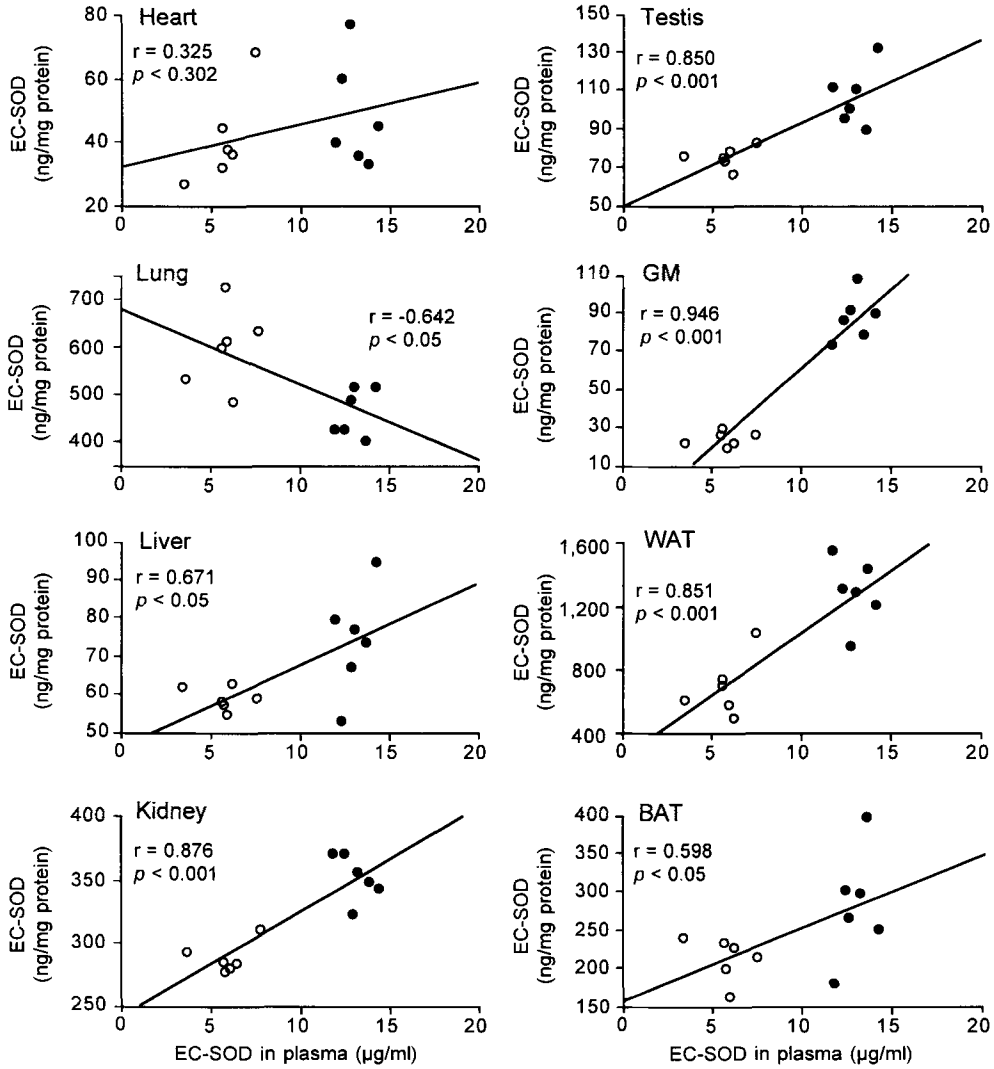

FIGURE 4 Relationships between EC-SOD levels in plasma and in tissues. ○, lean mice; ●, obese mice.

TABLE I Cytokine levels in eight mouse tissues

Tissue	TNF-α (ng/mg protein)			IL-1β (pg/mg protein)		
	n	Lean	Obese	*n*	Lean	Obese
Heart	5	0.105+0.008	0.102 ± 0.009	4	12.4 ± 2.1	11.6 ± 1.3
Lung	5	0.091 ± 0.077	0.013 ± 0.023	5	6.44 ± 0.86	4.16 ± 0.62
Liver	5	0.143 ± 0.010	0.205 ± 0.027	5	36.3 ± 1.1	44.1 ± 3.7
Kidney	5	3.03 ± 0.46	4.19 ± 0.69	5	11.6 ± 2.6	28.0 ± 7.4
Testis	5	0.026 ± 0.005	0.023 ± 0.001	5	3.26 ± 0.61	1.69 ± 0.25[a]
GM	5	0.106 ± 0.012	0.115 ± 0.013	5	7.62 ± 1.18	3.87 ± 0.52[a]
WAT	5	14.2 ± 4.0	35.0 ± 3.5[b]	5	96.5 ± 18.3	176 ± 14[b]
BAT	5	7.74 ± 0.53	9.10 ± 1.40	5	46.6 ± 7.9	43.2 ± 5.4

Values are mean ± SEM. [a]$p < 0.05$; [b]$p < 0.01$ vs lean mice.

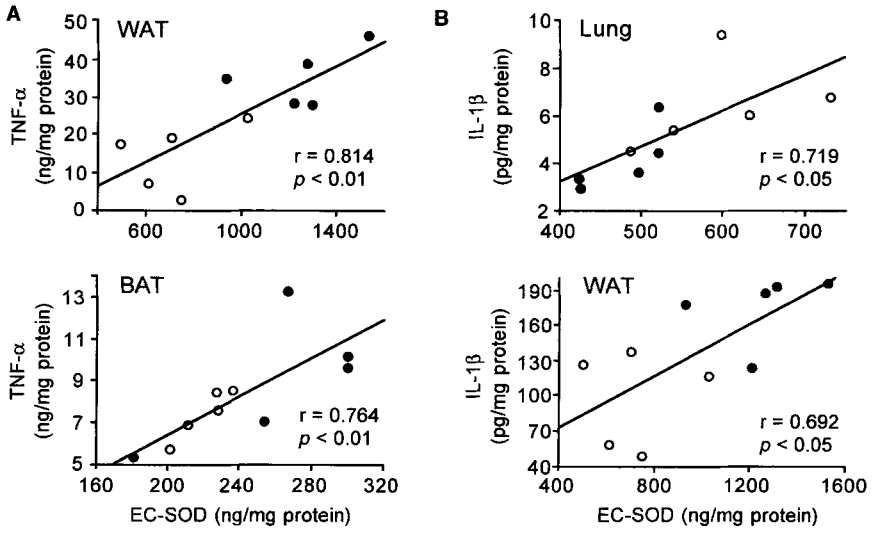

FIGURE 5 Relationships between EC-SOD and TNF-α levels in WAT and BAT (A), as well as between EC-SOD and IL-1β levels in lung and WAT (B). ○, lean mice; ●, obese mice.

Immunohistochemistry

To determine the intracellular localization of EC-SOD in adipocytes, we carried out an immunohistochemical analysis in WAT and BAT from lean and obese mice. All the adipocytes examined could be deeply stained with affinity-purified anti-mouse EC-SOD IgG in the extracellular portions (and/or the cytoplasm) and nuclei (Figure 6), the staining in obese mice being definitely deeper than that in lean mice, which was in keeping with the findings by an ELISA. Because the bulk of the content of the white and brown adipocytes from both lean and obese mice was occupied by lipid droplets, the nucleus was displaced to the edge of each cell, and the extracellular portions and/or cytoplasm was reduced to a thin rim around the lipid droplets. Both white and brown adipocytes from obese mice contained numerous larger lipid droplets compared to those from lean mice. In addition to the results of ELISA and Northern blot analysis, the immunohistochemical results also suggest that EC-SOD is actually synthesized within the adipocytes.

Primary Culture of White and Brown Adipocytes

To assess whether both WAT and BAT in lean mice indeed produce EC-SOD, white and brown adipocytes were primarily cultured. In a time course experiment (Figure 7A), each primary culture *per se* almost linearly enhanced EC-SOD production with time (on and after the third day), there being no significant difference in EC-SOD level between both cultured cells. Also, the expression of EC-SOD mRNA could be overtly found in the confluent cultures of white and brown adipocytes (Figure 7B).

Heparin Affinity of EC-SOD in Tissue Homogenates

As already described [4,15], EC-SOD is heterogeneous with regard to heparin affinity and can be divided into three fractions, A that lacks affinity, B with intermediate affinity and C with high affinity. Figure 8 shows a heparin-Sepharose column chromatography of kidney homogenates from lean and obese mice. EC-SOD could be

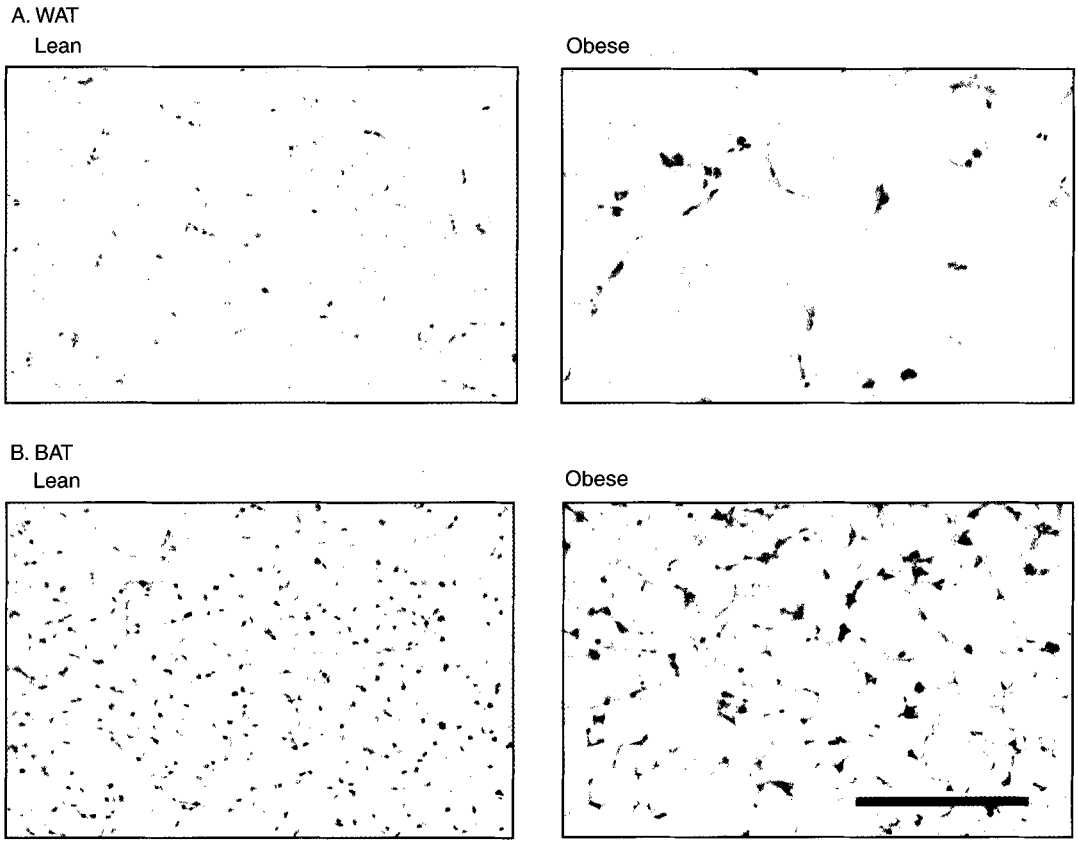

FIGURE 6 Light microscopic immunohistochemical localization of EC-SOD. WAT (A) and BAT (B) in lean and obese mice were stained with affinity-purified anti-mouse EC-SOD IgG. Nuclear counterstaining was performed with hematoxylin. Bar, 100 μm. (See Color plate III at the end of this issue.)

separated into the three factions: the loss of heparin affinity of EC-SOD C occurred in kidney of obese mice, whereas there appeared to be no disparity in heparin affinity between lung homogenates from lean and obese mice (data not shown).

DISCUSSION

As for lean mice, EC-SOD was predominantly located in lung, kidney, WAT, and BAT, Mn-SOD in heart, kidney, and BAT, and Cu,Zn-SOD in liver, testis, and BAT, as depicted in Figures 1–3, indicating that the three SOD isoenzymes occur independently in tissues. These findings were in good agreement with those by previous reports [12,13,21,22]. It should, in particular, be emphasized that EC-SOD teems in WAT, which is characteristically different from the other SOD isoenzymes [13]. Relatively strong expressions of EC-SOD mRNA were also observed in lung, kidney, WAT, and BAT, being in approximate agreement with the results of our previous study [13]. Also, the mRNA expressions of Mn-SOD and Cu,Zn-SOD roughly paralleled the respective isoenzyme protein levels in tissues.

On the other hand, compared with lean mice, significant increases in EC-SOD level were found in liver, kidney, testis, GM, WAT, and BAT of obese mice, whereas the enzyme level in the lung decreased significantly. Moreover, plasma EC-SOD concentration was significantly higher

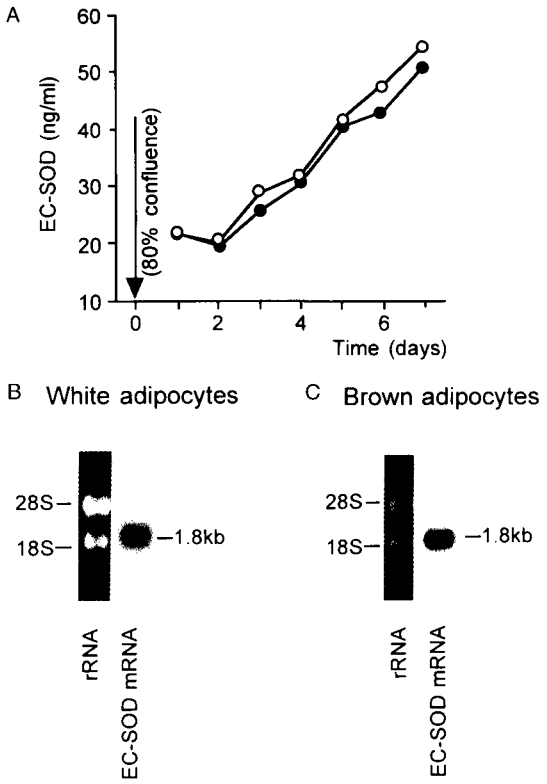

FIGURE 7 Time course of EC-SOD formation in lean mouse white and brown adipocyte primary cultures (A), and its mRNA expression in the white (B) and brown (C) adipocyte confluent cultures. ○–○, WAT; ●–●, BAT.

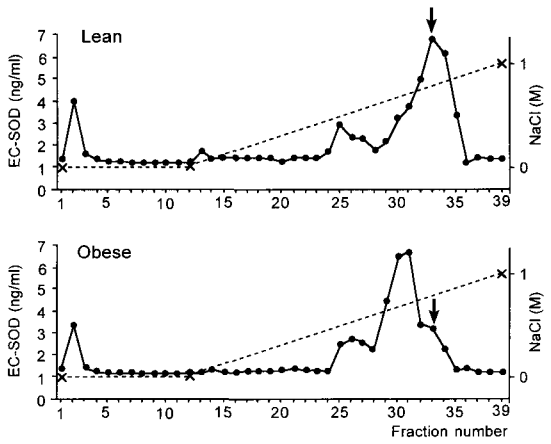

FIGURE 8 Heparin-Sepharose column chromatography of kidney homogenates from lean and obese mice. The chromatography was carried out at 4°C on 1 ml heparin-Sepharose column equilibrated with 25 mM sodium phosphate buffer, pH 6.5. Tissue homogenates containing 200 ng EC-SOD were applied at a flow rate of 0.5 ml/min, and the bound components were eluted with a linear gradient of NaCl in the buffer (0–1 M). Arrow represents 725 mM NaCl. ●–●, EC-SOD; ×---×, NaCl in the buffer.

in obese mice than in lean mice. The findings on EC-SOD level were also confirmed in both lean and obese mice of 14 weeks old. Oury *et al.* [23] have postulated that the high levels of EC-SOD in blood vessels in the lung may be important in maintaining low extracellular superoxide concentrations and may, thus, prevent superoxide-mediated inactivation of endothelial derived relaxing factor (EDRF). On the other hand, the lower EC-SOD content in the lung tissues of obese mice observed in the current study might be associated with the declined levels of physical activity and breathing exercise due to their extreme obesity, although the precise meaning still remains in doubt. The physiological role of high EC-SOD content in WAT is not fully understood, however, it would not presumably be denied that the WAT plays an important role in a supply of EC-SOD to plasma and interstitial tissues, because of its high expression and widely spread distribution within the whole body.

As already stated, it has been reported that the proportion of glycated EC-SOD in serum of diabetic patients is considerably higher than in normal subjects, probably because of the decreased heparin affinity in the glycated fraction [4]. Likewise, analysis of EC-SOD in plasma samples from 504 healthy blood donors revealed a common (2.2%) phenotypic variant displaying 8- to 10-fold increased plasma EC-SOD level, the EC-SOD in the plasma of these individuals also displaying a reduced heparin affinity when compared with samples from normal individuals [24]. Sandström *et al.* [24] have indicated that such high plasma levels can be explained by an accelerated release from the tissue interstitium heparan sulfate to the vasculature and should thus be accompanied by significantly reduced tissue EC-SOD levels. The mice used in the current study, however, showed

a high level of EC-SOD not only in blood but also in tissues.

Previous immunohistochemical and immunocytochemical studies have demonstrated that in mouse lung deep staining can be observed in extracellular portions such as connective tissue around blood vessels and bronchi, alveolar septa, and vascular walls [13], and that in human lung EC-SOD is predominantly located around larger vessels and airways, some EC-SOD being found in bronchiolar epithelial cell junctions and around the surface of vascular and airway smooth muscle cells [25]. In the current study immunohistochemical examination was done in WAT and BAT. As the result, deeper staining could be seen in both tissues from obese mice than in those from lean mice especially around lipid droplets. Actually, expression of EC-SOD occurred in the primary culture of white and brown adipocytes from lean mice, accompanied by that of its mRNA, thereby indicating that both adipocytes themselves produced EC-SOD. The increased levels of EC-SOD in both fat tissues, which were observed in the current study, were, thus, considered to be derived from the respective tissues *per se*. Although there were several reports on expression of EC-SOD in human cell lines, such as fibroblast cell lines [21,26] and glia-cell lines [26], the current study was the first to demonstrate expression of the enzyme and its mRNA in either adipocyte primary culture.

Inoue *et al.* [27] constructed a fusion gene encoding a hybrid SOD (HB-SOD) consisting of human Cu,Zn-SOD and a C-terminal basic peptide that binds to heparin-like proteoglycans and highly purified the resulting HB-SOD. When injected intravenously to rats, ^{125}I-labelled HB-SOD disappeared rapidly from the circulation; the rate of disappearance was decreased by heparin, and immunohistochemical studies revealed that HB-SOD predominantly bound to heparin-like proteoglycans on endothelial cells of the artery and other tissues. On the other hand, since EC-SOD is a secretory protein, the enzyme excreted into the extracellular space including blood retains its enzyme activity. Also, it would not probably be denied that EC-SOD excreted into blood binds to heparin-like proteoglycans on vascular endothelial cells, due to its affinity for heparin/heparan sulfate [11,28]. Moreover, except for heart, there existed a significant correlation between EC-SOD levels in plasma and in each tissue, thereby suggesting that EC-SOD level in plasma was in equilibrium with that in such tissues. It seemed likely, thus, that the increased levels of EC-SOD in plasma from obese mice directly reflected the respective tissue levels (especially the WAT level) of the enzyme (excluding heart).

It has been well known that IL-1β and TNF-α both stimulate Mn-SOD gene transcription by different pathways [29]. The expression of EC-SOD is also influenced by cytokines; for example, the expression is markedly stimulated by interferon-γ (IFN-γ), stimulated or depressed by IL-1α, intermediately depressed by TNF-α, or notably depressed by transforming growth factor-β, albeit different among cell types [21]. Cytokines are mainly produced by leukocytes (such as lymphocytes and macrophages) and partly by other cell types [30–32]. For instance, endogenous TNF-α mRNA expression is evident in WAT and spleen, but not in liver, kidney, or skeletal muscle [32]; in particular, an induction of TNF-α mRNA expression is observed in WAT from four different rodent models of obesity and diabetes. TNF-α protein is also found locally or systemically [33]. In the current study, although there was a wide difference in TNF-α and IL-1β levels among the tissues examined, both cytokines could be detected in all of them. The difference among EC-SOD levels in the tissues was also great. In addition, EC-SOD levels in WAT and BAT from lean and obese mice correlated well with TNF-α levels in the respective tissues. As shown in Figure 6, a tendency to become WAT due to the deposit of fat was apparent in BAT of obese mice, probably resulting in a marked decrease in the thermogenic capacity and activity [34], which is one of the major causes for obesity. Nevertheless,

unlike WAT, TNF-α level in BAT of obese mice was not higher than in that of lean mice. However, since there existed a definite correlation between EC-SOD and TNF-α levels in lean and obese mice, it would not presumably be denied that, even in BAT of obese mice, TNF-α induced EC-SOD expression to a certain extent.

Such findings on TNF-α in WAT appeared to be true for IL-1β. On the other hand, the decreases in EC-SOD level in lung of obese mice might be attributable, in part, to those in both TNF-α and IL-1β levels, particularly in the latter, albeit not statistically significant. However, although IL-1β level in testis and GM of obese mice showed a significant reduction as compared with that in the respective tissues of lean mice, their EC-SOD levels showed a small but significant increase. It seemed likely, thus, that an interplay between EC-SOD on one hand, and TNF-α and IL-1β on the other in various tissues from lean and obese mice was different from one another.

Interestingly, a significant correlation was noted between the levels of Mn-SOD and TNF-α or IL-1β only in WAT of lean and obese mice. Moreover, considering that the levels of both cytokines in WAT were markedly higher in obese mice than in lean mice, the bulk of the increased levels of Mn-SOD in WAT of obese mice would probably be due to such increments in these cytokines. It is known that SOD is also induced by increased exposure to oxygen free radicals in various organs and tissues [9,35]. If the current study were the case, nuclear factor-κB (NF-κB) in WAT of obese mice would probably induce transcription of SODs, in particular Mn-SOD, because oxidative stress is known to activate NF-κB [36] and Mn-SOD is a protective enzyme against cytotoxicity of TNF and IL-1β [37–39]. The precise mechanism, however, must await further study.

As already stated, inflammatory cytokines have been shown to upregulate secretion of EC-SOD, which functions to protect cells and connective tissue from extracellular superoxide ($O_2^{\bullet -}$). Inflammatory cytokines, such as TNF-α and IFN-γ, also stimulate production of nitric oxide ($^{\bullet}NO$) through upregulation of inducible nitric oxide synthase (iNOS or NOS II) transcription [37]. Brady *et al.* [40] have also demonstrated that transcription of both EC-SOD and iNOS genes is linked by activating NF-κB. The dismutation of $O_2^{\bullet -}$ by EC-SOD is of prime importance not only because $O_2^{\bullet -}$ itself is a damaging radical but also because $O_2^{\bullet -}$ can rapidly react with $^{\bullet}NO$ forming the injurious peroxynitrite anion ($ONOO^-$). It was considered, thus, that the disparity noted in EC-SOD content and/or distribution among the eight tissues measured might be attributable, in part, to varying degrees of the $^{\bullet}NO$-related factors.

As was anticipated, plasma glucose concentration was significantly higher in obese mice than in lean mice also in the current study. Adachi *et al.* [4] have shown that glycation decreases the cell-surface-associated EC-SOD in diabetic patients, resulting in an increase in the susceptibility of cells to superoxide radicals produced in the extracellular space. In the current study, thus, lung and kidney specimens containing a high content of EC-SOD were examined for heparin affinity, as depicted in Figure 8. While there appeared to be no difference in heparin affinity between lungs from lean and obese mice, the loss of heparin affinity of EC-SOD C (the high-heparin-affinity type) occurred in kidney of obese mice, probably modifications of the C-terminal heparin-binding domains in the EC-SOD C owing to the glycation reaction having weakened the binding to heparin, thereby suggesting the conversion into EC-SOD B (the weak-heparin-affinity type). Sandström *et al.* [15] have speculated that such a phenomenon facilitates entrance to the vasculature through capillaries and lymph flow, and finally results in the heterogeneous plasma EC-SOD pattern. The current results suggest that obesity contributes to the heterogeneity in heparin affinity of EC-SOD in tissues (especially kidney) of mice. However, because EC-SOD level in kidney was significantly higher in obese mice than in lean mice, it would not always follow that

the decreased heparin-binding affinity induces the excretion of EC-SOD from tissues, i.e., decreases in EC-SOD level in the tissues. In addition, the decreased level of EC-SOD in lung of obese mice appeared not to be attributable to changes in the heparin-binding affinity. The precise meanings of modifications of the affinity of EC-SOD for heparin and analogues, however, remain to be clarified.

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