Cellular carbonyl stress enhances the expression of plasminogen activator inhibitor-1 in rat white adipocytes via reactive oxygen species-dependent pathway.

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Running Title: Carbonyl Stress Activates PAI-1 Expression in Adipocytes
Carbonyl stress is one of the important mechanisms of tissue damage in vascular complications of diabetes. In the present study, we observed that the PAI-1 levels in serum and its gene expression in adipose tissue were up-regulated in aged Otsuka Long-Evans Tokushima Fatty (OLETF) rats, model animals of obese type 2 diabetes. To study the mechanism of PAI-1 up-regulation, we examined the effect of advanced glycation end products (AGEs) and the product of lipid peroxidation (4-hydroxy-2-nonenal : HNE) both of which are endogenously generated under carbonyl stress. Stimulation of primary white adipocytes by either AGE or HNE resulted in the elevation of PAI-1 in culture medium and at mRNA levels. The up-regulation of PAI-1 was also observed by incubating the cells in high glucose medium (30 mM, 48 h). The stimulatory effects by AGE or high glucose were inhibited by antioxidant, pyrroldidine dithiocarbamate (PDTC), and reactive oxygen scavenger, probucol, suggesting a pivotal role of oxidative stress in white adipocytes. We also found that the effect by HNE was inhibited by antioxidant, N-acetylcysteine (NAC), and that a specific inhibitor of glutathione biosynthesis, L-buthionine-S, R-sulfoximine, augmented the effect of subthreshold effect of HNE. Bioimaging of reactive oxygen species (ROS) by a fluorescent indicator, 6-carboxy-2’,7’-dichlorodihydrofluorescein diacetate (DCF-DA), revealed ROS production in white adipocytes treated with AGE or HNE. These results suggest that cellular carbonyl stress induced by AGEs or HNE may stimulate PAI-1 synthesis in and release from adipose tissues through ROS formation.
Adipose tissue directly secretes biologically active molecules (adipocytokines) including plasminogen activator inhibitor-1 (PAI-1) and actively affects other tissues. This is thought to be one of the risk factors for the development of obesity-linked disorders (1). High plasma PAI-1 activity, which regulates fibrinolytic as well as thrombotic processes, is a frequent finding in obesity (2-4) and/or in Type II (non-insulin-dependent) diabetes (5-7). Among the studies about PAI-1 expression in adipose tissue, biological significances of visceral fat during the development of obesity have been highlighted (8). Furthermore, Alessi et al. (9) demonstrated that human adipocytes in culture produce PAI-1 and that omental fat produces more PAI-1 than subcutaneous adipose tissue in obese or non-obese individuals. However, the mechanism leading to up-regulation of PAI-1 has not clearly been elucidated.

The pathological conditions of diabetes with obesity have been widely associated with reactive oxygen species (ROS) and reactive carbonyl species (RCS), which may be key intermediates leading to diabetic complications. RCS originates from a multitude of mechanistically related pathways, like glycation (10) and lipid peroxidation (11). Glycation, a spontaneous amino-carbonyl reaction between reducing sugars and proteins, is a major source of RCS production. The complex reaction sequence is initiated by the reversible formation of a Schiff base, which undergoes an Amadori rearrangement to form a relatively stable ketoamine product during early glycation. A series of further reactions involving sugar fragmentation and formation of α-dicarbonyl compound yield stable advanced glycation end products (AGEs) under chronic hyperglycemia (12, 13). Carbonyl stress also leads to the intracellular formation of 4-hydroxy-2-nonenal (HNE), one of the active endproducts of lipid peroxidation. Interestingly, RCS and AGEs can exert their detrimental cellular effects by increasing ROS production (14), thereby forming a vicious cycle of ROS and RCS production.

In the present study, to investigate the mechanisms underlying increased PAI-1 levels in obese diabetic conditions, we performed both in vivo and in vitro studies. For in vivo
studies, gene expression and secretion of PAI-1 were measured in visceral adipose
tissue using Otsuka Long-Evans Tokushima Fatty (OLETF) rats, model animals of
obese type II diabetes (15). In parallel with the progression of diabetes, serum levels of
both AGE and malondialdehyde (MDA) derived from lipid peroxidation were shown to
increase (16, 17). For in vitro studies, we examined the effects of AGE and HNE on the
PAI-1 activity and its gene expression in rat white adipocytes differentiated in vitro. We
focused on the role of carbonyl stress in the up-regulation of PAI-1 expression.
EXPERIMENTAL PROCEDURES

Animals---Male Otsuka Long-Evans Tokushima Fatty (OLETF) rats, model animals of Type II diabetes mellitus established in 1990 at Tokushima Research Institute (Otsuka Pharmaceutical Co., Ltd.) (15), were generously supplied at the 4-week-old age. Male Long-Evans Tokushima Otsuka (LETO) rats served as normal controls. These animals were kept in an air-conditioned room (23 ± 2 °C, 55 ± 5 % humidity) lighted 14 h a day (06:00 to 20:00) and were maintained on a standard diet and water ad libitum. These rats were sacrificed at 12, 20, 30 and 50 weeks of age and blood was collected for determination of the plasma PAI-1 and 8-hydroxy-2’-deoxyguanosine (8-OHdG). Immediately after decapitation, visceral fat was dissected out and frozen in liquid nitrogen and stored in deep freezer (-80°C) until use. At the 29-week-old age, OLETF and LETO rats were divided into 4 groups, which were injected with probucol (3, 10, 30 mg/kg s.c.) or vehicle (0.1% DMSO containing ethanol) for 7 days.

Cell culture---White fat precursor cells were isolated from epididymal fat pad of 4-week-old male Wistar-Imamichi rats. Briefly, the minced tissue was incubated in a HEPES-buffered solution (pH 7.4) containing 0.2% collagenase type II (Sigma Chemical, St. Louis, MO, USA) for 20 min at 37°C with vortexing every 5 min. After incubation, the tissue remnants were filtered through a 250-µm nylon screen into plastic test tubes. The tubes were placed on ice for at least 30 min to allow the mature white fat cells and lipid droplets to float on the surface of the cell suspension. The infranatant (stromal-vascular fraction) was collected and filtered through a 25-µm nylon screen to remove cell aggregates. The stromal-vascular fraction containing precursor cells was pelleted by a centrifugation for 10 min at 700 x g, and resuspended in the culture medium. The cells were plated in six-well multiplates and cultured in DMEM (containing 5.55 mM glucose) supplemented with 10% fetal bovine serum, 4 nM insulin, 25 mg/ml sodium ascorbate, 10 mM HEPES (pH 7.4), 4 mM glutamine, 50
IU/ml penicillin and 50 µg/ml streptomycin. The cells were grown at 37°C under an atmosphere of 5% CO₂ in air and characterized morphologically on day 11-13 after plating. At this stage, cells were recognized to be differentiated to mature white adipocytes. AGE-BSA at the concentrations of 0.01, 0.03, 0.1 and 0.3 mg/ml and nonglycated BSA as a control at the concentrations of 0.1 and 0.3 mg/ml were added to the cells. Cells were exposed to AGE-BSA or BSA for 2, 4, 8, 12 and 16 h. Antioxidants such as 10 µM pyrrolidinedithiocarbamate (PDTC) and 50 µM probucol were added to the culture medium 9 h prior to the harvest of the cells. 4-hydroxy-2-nonenal (HNE) dissolved with 0.1% DMSO at the concentrations of 3, 10, 30 and 100 µM were added to the cells. Cells were exposed to HNE for 2, 4, 8 and 16 h. N-acetylcysteine (NAC), a precursor of glutathione and L-buthionine-S, R-sulfoximine (BSO), a γ-glutamylcysteine synthetase inhibitor were added to the cells for 24 h at the concentrations of 1, 5, 20 mM and 10, 50, 100 µM, respectively.

Preparation of AGE---AGEs were prepared by incubating 50 mg/ml bovine serum albumin (BSA) (fraction V, fatty acid-free, low endotoxin; Sigma Chemical, St. Louis, MO, USA) with 0.5 M glucose-6-phosphate in phosphate-buffered saline (PBS) (10 mM, pH 7.4) at 37°C for 4 weeks as described (18, 19) with a slight modification. Unmodified BSA was incubated under the same conditions without glucose-6-phosphate as controls. Unincorporated sugar was removed by dialysis against phosphate-buffered saline. The concentrations of glycated BSA and control BSA was measured by the method of Lowry et al. (20).

Measurement of PAI-1 levels in serum and culture medium---PAI-1 levels were determined by measuring of active PAI-1 in the culture medium as follows. After 9-11 days of culture, the medium was replaced with 1 ml of fresh medium with 0.1% instead of 10% fetal bovine serum. AGE-BSA or HNE was added at concentrations of 0.01, 0.03, 0.1, 0.3 mg/ml or 3, 10, 30, 100 µM, respectively. In some cells, antioxidants (10
μM PDTC or 50 μM probucol), a glutathione precursor (1, 5, 20 mM NAC) or a γ-glutamylcysteine synthetase inhibitor (10, 50, 100 μM BSO) was added to the culture medium 9 h or 24 h prior to the harvest of cells. For the high glucose experiments, cells were incubated in 30 mM glucose for 48 h. During the last 24 h of incubation, the medium was replaced with 1 ml of fresh medium containing 30 mM glucose with 0.1% instead of 10% fetal bovine serum. In some experiments, the cells were incubated in 30 mM glucose in combination with antioxidants (10 μM PDTC or 50 μM probucol) for 48 h. After incubation, PAI-1 concentrations of the culture medium were determined using rat PAI-1 ELISA kit (Molecular Innovations, Royal Oak, MI, USA) and were expressed as nanograms of active PAI-1 released from white adipocytes per mg of cell protein. Protein contents of the cells were determined as follows. Cells were rinsed twice with ice-cold phosphate-buffered saline and scraped from dishes with rubber policemen into 1 ml phosphate-buffered saline and were sonicated for 15s. After precipitation with 10% Trichloroacetic acid, proteins were measured by the method of Lowry et al. (20).

PAI-1 levels in the serum were detected using the same ELISA kit. Since we used ELISA kit plated on t-PA antigen, the amount and activity of PAI-1 are simultaneously monitored.

Measurement of 8-OHdG in serum---Serum samples were centrifuged at 10,000 x g for 15 min, and the supernatant was used for determination of 8-hydroxy-2’-deoxyguanosine (8-OHdG) by a competitive ELISA (8-OHdG Check, Japan Institute for Control of Aging, Fukuroi, Shizuoka, Japan).

Detection of PAI-1 mRNA by Northern blotting---Total RNAs was isolated from visceral adipose tissue or white fat cells by using Isogen (Nippongene, Toyama, Japan) and 10 μg of total RNAs per lane were separated by electrophoresis on 1% agarose gel containing 6% formaldehyde, followed by blotting onto nylon filters (Hybond-N, Amersham). The blots were prehybridized in Expresshybri solution (Invitrogen,
Carlsbad, CA, USA) for 30 min at 68°C, then hybridized with 32P-labeled rat PAI-1 cDNA probe for 1 h at the same temperature. Ribosomal RNAs of 28S stained with ethidium bromide were utilized as an internal control for the amounts of total RNAs loaded. PAI-1 mRNA levels and 28S ribosomal RNAs were quantified on autoradiograms using Fluor-S laser scanning densitometry (Fluor-S MultiImager, Biorad). The levels of PAI-1 mRNA were expressed as fold increases over controls after correcting with 28S ribosomal RNA levels.

**Intracellular ROS fluorescence imaging**---To detect intracellular ROS, 6-carboxy-2′,7′-dichlorodihydrofluorescein diacetate (DCF-DA) (Molecular Probe, Eugene, OR, USA) was used. DCF-DA diffuses into cells and is hydrolyzed by intracellular esterases to polar 6-carboxy-2′,7′-dichlorodihydrofluorescein. This nonfluorescent fluorescein analog is trapped in cells and can be oxidized to highly fluorescent 6-carboxy-2′,7′-dichlorofluorescein by intracellular oxidants. Cells on day 9 of culture were exposed to 0.1 mg/ml AGE-BSA or 30 µM HNE for 8 h. After washing 2 times with PBS(+), 10 µM of DCF-DA was loaded for cells for 30 min. Some cells were pretreated with 10 µM of PDTC for 9 h or 20 mM of NAC for 24 h. Then cells were examined under an inverted fluorescent microscope (IXE70, Olympus, Tokyo, Japan), equipped with an excitation (470-490 nm) and emission (515 nm) filter for fluorescein. Digital images of the fluorescent microscope images were obtained with a CCD camera (Hamamatsu Photonics, Hamamatsu, Japan) and stored in a Macintosh G3 (Apple Japan, Tokyo, Japan).

**Detection of IκB-α by Western blotting**---Rat white adipocytes on day 9 were exposed to 0.1 mg/ml of AGE for 15 min or 30 µM of HNE for 15 min and were washed twice with phosphate-buffered saline (pH 7.4) and lysed with lysis buffers (50 mM Tris-HCl (pH 7.5), 2% SDS, 6% β-mercaptoethanol, 1% glycerol). Each whole cell lysate was then treated with sample buffers for 5 min at 100°C. The samples were
run on 10% SDS-PAGE slab gels. The gel was transblotted on a nitrocellulose membrane (Hybond ECL, Amersham) and incubated with skim milk (10 mg/ml) for blocking. After washing, the nitrocellulose membranes were immunoblotted with a polyclonal anti-IκB-α (Santa Cruz Bio-technology, Santa Cruz, CA, USA) or monoclonal anti-actin (Chemicon international, Temecula, CA, USA) and then with polyclonal peroxidase-conjugated anti-rabbit antibody (Amersham) or anti-mouse antibody (Amersham) as a secondary antibody. Bound antibodies were detected by enhanced chemiluminescence using ECL Western blotting detection reagents (Amersham) and visualized by exposure of the membrane to autoradiography films. The intensity of each band was quantitatively analysed using Fluor-S laser scanning densitometry (Flour-S MultiImager, Biorad).
RESULTS

Enhanced expression of PAI-1 in adipose tissue from OLETF rats---We examined the serum levels of PAI and its gene expression in adipose tissue from OLETF rats. The serum PAI-1 levels in OLETF rats began to increase at the age of 20 weeks and reached a maximum at the age of 50 weeks, whereas those in control LETO rats remained unchanged (Fig.1 A). The PAI-1 mRNA levels in visceral fat of OLETF rats, but not in LETO rats increased in parallel with the progression of serum PAI-1 (Fig.1 B). The serum glucose levels of OLETF rats at the ages of 12, 20, 30 and 50 weeks were 120.2±5.6, 160.7±15.2, 397.4±30.5 and 367.5±66.8 mg/dl, respectively, whereas there were no changes in those of LETO rats (123.2±6.5, 121.3±7.6, 123.3±2.9, 117.2±10.4, respectively). Thus, we confirmed that OLETF rats developed spontaneous and persistent hyperglycemia after the age of 20 weeks as originally described (15), which we found correlated well with the increased expression of PAI-1 in visceral fat.

Correlation between serum PAI-1 and serum 8-OHdG---The relationship between the serum PAI-1 and the serum 8-OHdG (8-hydroxy-2’-deoxyguanosine) levels is represented in Fig.2 A. In OLETF rats, a significant correlation was observed between the serum PAI-1 and the 8-OHdG levels (Fig.2 A; r = 0.65, P <0.001). In contrast, there was no correlation between those two parameters in control LETO rats (Fig.2 A; r = 0.10, not significant :NS). Thus, serum PAI-1 could be associated with oxidative stress during the development of diabetes in OLETF rats. Therefore, we examined the effect of probucol, a potent antioxidant, on PAI-1 activity, its gene expression, and serum 8-OHdG concentration. At the age of 30 weeks, the increased levels of serum PAI-1, 8-OHdG and adipose tissue PAI-1 mRNA in OLETF rats were suppressed by successively injected probucol in a dose-dependent manner. However, in LETO control rats, there was no increase in either serum PAI-1 or 8-OHdG level, which was not affected by probucol. Moreover, enhanced expression of PAI-1 mRNA in visceral fat was never observed in vehicle-injected 30-week-old LETO rats (Fig.2, B and C).
Up-regulation of PAI-1 expression in white adipocytes by AGEs---As up-regulation of PAI-1 was observed in in vivo studies using OLETF rats, we performed in vitro studies by directly examining the effect of AGEs on PAI-1 expression in white fat cells in primary culture. AGE-BSA increased PAI-1 activity in culture medium released from white adipocytes differentiated in culture. As shown in Fig. 3A, white adipocytes on day 9-11 exposed to AGE-BSA for 8 h showed a concentration-dependent increase in PAI-1 with a significant elevation at concentrations of 0.1 mg/ml and 0.3 mg/ml. Time-course studies indicated that cells treated with 0.1 mg/ml AGE-BSA showed a steady increase in PAI-1 accumulation in the media by 8 h and the rate of PAI-1 accumulation was reduced markedly thereafter (Fig. 3B). The PAI-1 accumulation in AGE-BSA-treated cells during 8 h incubation was approximately twice as high as that in BSA-treated cells. To clarify whether or not the AGE-BSA-induced stimulation of PAI-1 secretion involves activated transcription, PAI-1 mRNA expression was studied by Northern blotting. AGE-BSA induced a concentration-dependent increase in PAI-1 mRNA in white adipocytes (Fig. 3C). White adipocytes exposed to 0.1 mg/ml of AGE-BSA showed a time-dependent increase in PAI-1 mRNA (Fig. 3D).

Inhibition of AGE- and high glucose-induced increase in PAI-1 expression by antioxidant (PDTC) and reactive oxygen scavenger (probucol)---We further investigated the involvement of oxidative stress in the up-regulation of PAI-1 expression by AGE. As shown in Fig. 4, AGE enhanced PAI-1 expression at both protein and mRNA levels, which could be inhibited by either PDTC (antioxidant) or probucol (reactive oxygen scavenger). Moreover, to examine if this effect of AGE can be observed also by hyperglycemia, cells were incubated in high glucose for 48 h. Thirty mM glucose induced an increase in PAI-1 activity and its gene expression compared with the control cells incubated in conventional medium (5.55 mM glucose). This enhancement by high glucose was also inhibited by PDTC or probucol (Fig. 5).

An increase in PAI-1 expression in white adipocytes by 4-hydroxy-2-nonenal---As
up-regulation of PAI-1 expression was observed in white adipocytes in response to AGE-BSA, we tested to see if the most prominent intermediate of lipid peroxidation, 4-hydorxy-2-nonenal (HNE) can induce up-regulation of PAI-1. The effect of an increasing concentration of HNE on PAI-1 expression was determined (Fig.6 A and C). HNE at concentrations of 30 and 100 µM increased PAI-1 expression 8 h after addition. Time-course studies indicated that DMSO-treated control cells showed a steady increase in PAI-1 accumulation in the media by 8 h and the rate of PAI-1 accumulation was reduced markedly thereafter. Addition of HNE (30 µM) significantly increased the accumulation of PAI-1 during 8 h incubation twice as high as DMSO-treated cells (Fig. 6B). The PAI-1 mRNA levels began to increase 4 h after addition of HNE, and reached maximum at 8 h (Fig. 6 D).

Inhibition of HNE-induced increase in PAI-1 expression by antioxidant (NAC) and augmentation of subthreshold effect of HNE on PAI-1 expression by a glutathione synthesis inhibitor (BSO)---As shown in Fig.7, the increases in PAI-1 activity and PAI-1 mRNA by 30 µM HNE were inhibited dose-dependently by antioxidant, N-acetylcysteine (NAC). On the other hand, at the concentration of 10-100 µM a glutathione synthesis inhibitor, L-buthionine-S, R-sulfoximine which favors intracellular redox environment to reduced state, augmented the effect of subthreshold concentration of HNE (3 µM) on PAI-1 expression (Fig. 8).

AGE- or HNE-induced intracellular ROS generation results in the activation of the transcription factor NF-κB---ROS production from white adipocytes was studied using a membrane-permeable fluorescent indicator, DCF-DA (6-carboxy-2’,7’-dichlorodihydrofluorescein diacetate). The fluorescent microscopic images are shown in Fig. 9 A and B. Cells stimulated with 0.1 mg/ml AGE-BSA for 8 hr showed substantial fluorescence 30 min after addition of 10 µM DCF-DA, whereas minimum fluorescence was observed in unglycated BSA-treated cells. The increase in the fluorescence of white adipocytes stimulated by AGE-BSA was markedly inhibited by 10 µMPDTC
Moreover, the stimulatory action of AGE was mimicked by exogenously added HNE. The generation of ROS stimulated by HNE in white adipocytes was blocked by pretreatment with antioxidant, NAC (Fig. 9 B). Furthermore, to examine if the ROS generation under enhanced carbonyl stress could activate the transcription factor NF-κB, we monitored the degradation of IκB-α, an inhibitory subunit of NF-κB, in rat white adipocytes exposed to AGE or HNE. As shown in Fig. 9 C, Western blotting revealed that both AGE and HNE markedly reduced IκB-α levels, which were blocked by pretreatment with PDTC or NAC, respectively.
DISCUSSION

A variety of observations suggests that elevated levels of plasma and adipose tissue PAI-1 are frequently found in obese humans (7, 9) and rodents (21, 22) and correlate strongly with visceral fat mass (8). Increased plasma PAI-1 levels may contribute to the impaired endogenous fibrinolysis and the increased cardiovascular risk of patients with visceral fat accumulation and/or non-insulin-dependent diabetes mellitus. Despite this well-documented link between elevated PAI-1 levels and obesity, little is known about the origin of this plasma inhibitor in obesity, or about signals that control its biosynthesis. In the present study, we first showed that circulating PAI-1 was progressively increased during the development of diabetes and hyperglycemia in Otsuka Long-Evans Tokushima Fatty (OLETF) rats and this elevation of PAI-1 accompanied a concomitant increase in PAI-1 mRNA from visceral fat, suggesting that visceral fat tissue can be an important source of elevated PAI-1 in circulation.

A considerable number of reports have accumulated regarding the association between oxidative stress and diabetic vascular complications (23, 24). There are several biomarkers of oxidative stress elevated in circulation or tissues of diabetic patients. Indeed, several lines of evidence showed increases of molecules in diabetic states, including lysophosphatidylcholine, oxidized phospholipid (25), 8-hydroxy-2′-deoxyguanosine, an indicator of oxidative damage of DNA (26-28) and hydroperoxides (29), resulting products of lipid peroxidation. We also observed that in the OLETF rats, plasma PAI-1 levels correlated well with plasma 8-OHdG, which serves as a good indicator of oxidative damage to DNA to monitor oxidative state in the whole body. Moreover, we noticed that successively injected probucol resulted in significantly reduced PAI-1 both in plasma and at mRNA levels in adipose tissue, when OLETF rats remained hyperglycemic and PAI-1 up-regulated after 30 weeks of age. These in vivo experiments suggest that up-regulation of PAI-1 in OLETF rats is strongly regulated by cellular oxidative stress.
There are increasing lines of evidence that reactive carbonyl compounds (reactive aldehyde) generated endogenously seem to contribute to the long-term complications of diabetes (30). Typical reactions yielding reactive carbonyl compounds are glycation of proteins especially under chronic hyperglycemia (24, 31), and lipid peroxidation (30). Elevation of circulating AGE (16) and a lipid peroxidation product such as malondialdehyde (MDA) (17) was observed in OLETF rats.

We found for the first time that AGEs lead to up-regulation of PAI-1 formation in rat white adipocytes via a pathway involving oxidative stress. There were some reports showing that AGEs interact with cells through specific receptors (RAGE) (32-39) found in a variety of cells including endothelial cells (40-43), mononuclear phagocytes (33), macrophage (39), smooth muscle cells (44), mesangial cells (45, 46), and neuroblastoma cells (47, 48). However, little is known about those receptors in fat cells. Schmidt et al. showed in their infusion/uptake studies using 125I-labeled AGE-albumin that fat slightly but appreciably incorporates AGE-albumin (35). Our detection of RAGE mRNAs in rat white adipocytes (data not shown) supports this information. Inhibition of AGE- and diabetic obesity (OLETF rats)-mediated PAI-1 up-regulation by antioxidants, both in vitro and in vivo, suggested the central role of oxidant stress in the enhanced PAI-1 production in adipocytes.

Lipid peroxidation of polyunsaturated fatty acids in cell membrane yields reactive carbonyl compounds such as 4-hydroxy-2-nonenal (HNE) (49-51). HNE has been considered as the end product of lipid peroxidation (30). However, this compound is still active and exhibits a high reactivity with various molecules (30, 50). In contrast to AGE, HNE may have no specific receptors and directly react with cell surface proteins and penetrate into cells, causing secondary alterations of the structure and functions of cells. Then we investigated the effect of HNE exogenously added to rat white adipocytes on PAI-1 activity and its gene expression. We demonstrated that exogenously added HNE
mimicked the stimulatory action of AGE, which was blocked by NAC and BSO. Recently, it is reported that HNE has been shown to induce intracellular peroxide production in cultured hepatocytes (52). This finding is consistent with another study showing that HNE itself induces lipid peroxidation, as judged by increased levels of malondialdehyde (53). Given the rapid reactivity of HNE with various amino acids (54), we cannot rule out the possibility that the PAI-1 expression is due to ROS-independent modification of certain signaling molecules. Thus, reactive carbonyl compounds such as AGE and HNE once formed or activated induce diverse aspects of severe cellular stress, including peroxide formation (oxidative stress).

On the other hand, it has been shown that reactive carbonyl stress also affects the NF-κB-signaling pathway. NF-κB, one of the most extensively studied molecules affected by cellular redox status, has been suggested to play an important role in gene regulations (55). Both AGE and HNE induced activation of NF-κB signaling pathway in white adipocytes, which is consistent with the previous reports that NF-κB resides in ROS signaling pathways and stimulates gene expressions. Activation of the PAI-1 promoter through the transcription factor Sp1 in vascular smooth muscle cells (56) and bovine aortic endothelial cells (57) exposed to high glucose has been reported. Further studies are needed to uncover the specific usage of transcription factors by hyperglycemia, AGE, and HNE in adipocytes.

Many biochemical pathways in diabetic complications associated with hyperglycemia are thought to be able to increase the production of free radicals (58). This glucose-related ROS production seems to occur in AGE-treated white adipocytes (in vitro) and in OLETF rats (in vivo). Based on our results, possible links among carbonyl stress (AGE, HNE), ROS, and PAI-1 up-regulation in white adipocytes were proposed (Fig. 10). It was reported that hyperglycemia induces superoxide overproduction in mitochondria (57, 58). ROS, represented by superoxide, may cause vicious cycles: one involving AGE by glycation, the other involving lipid peroxidation. The self-
amplifying ROS signals potentially activate transcription factor such as NF-κB leading to up-regulation of PAI-1. In conclusion, we demonstrated for the first time that AGE and lipid peroxidation products such as HNE could induce synthesis and release of PAI-1 in rat white adipocytes through ROS-dependent pathway.
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FIGURE LEGENDS

Fig. 1. Serum level of PAI-1 and its gene expression of visceral fat in OLETF (obese and diabetic) and LETO (control) rats. A, At the age of 12, 20, 30 and 50-weeks, serum levels of PAI-1 in OLETF and LETO rats were measured by ELISA. Values are means ± S.E.M. (bars) obtained from 5 experiments. ** P < 0.01, *** P < 0.001 vs. 12-week-old OLETF rats. B, PAI-1 mRNA levels in visceral fat were determined by Northern blotting with 10 µg of total RNAs. The Northern blots were exposed for 20 hr followed by autoradiography and quantitative densitometric scanning. PAI-1 mRNA/28S ribosomal RNA ratios were normalized to those of 12-week-old LETO rats which was set to unity. Values are means ± S.E.M. (bars) obtained from 5 experiments. * P < 0.05, *** P < 0.001 vs. 12-week-old OLETF rats. The bottom panel shows ethidium bromide staining of ribosomal RNAs (rRNA).

Fig. 2. Reversal of up-regulation of PAI-1 in OLETF rats by antioxidant, probucol. A, Scatter plots showing correlation between serum PAI-1 and 8-OHdG concentrations at the age of 12, 20, 30 and 50 weeks in OLETF and LETO rats measured by ELISA. PAI-1 versus 8-OHdG in OLETF rats (closed circle) and in LETO rats (open circle). B, Reversal of elevated serum PAI-1 and 8-OHdG in OLETF rats by probucol. At 29-week-old age, OLETF and LETO rats were injected with probucol (3, 10, 30 mg/kg s.c.) or vehicle (V) for 7 days and serum PAI-1 (I OLETF, i LETO) and 8-OHdG (² OLETF, ³ LETO) concentrations were determined at the age of 30 weeks. C, Reversal of enhanced expression of PAI-1 mRNA in visceral fat derived from OLETF rats by probucol. PAI-1 mRNA levels in visceral fat were determined by Northern blotting with 10 µg of total RNAs as shown in Fig.1B. *** P < 0.001 vs. OLETF vehicle-injected rats. The bottom panel shows ethidium bromide staining of ribosomal RNAs (rRNA).
FIG. 3. **Effect of AGEs on PAI-1 expression in rat white adipocytes.** Rat white adipocytes on day 9-11 were treated with AGE-BSA or BSA. PAI-1 concentrations in culture media were measured by ELISA (*A*, *B*) and 10 µg of total RNAs extracted from cells were subjected to Northern blotting (*C*, *D*). *A* and *C*; Cells were treated with indicated concentrations of AGE-BSA or BSA for 8 h. Values are means ± S.E.M. (bars) obtained from 5-6 experiments. ** *P* < 0.01, *** *P* < 0.001 vs. BSA 0.3 mg/ml (Student t-test). Quantitative densitometric scanning of PAI-1 mRNA shows means ± S.E.M. (bars) obtained from 4 experiments. *** *P* < 0.001 vs. BSA 0.03 mg/ml (Friedmans rank test followed by Mann-Whitney *U*-test). *B* and *D*; Cells were treated for indicated periods of time with 0.1 mg/ml AGE-BSA or 0.1 mg/ml BSA. At time 0 h culture medium was changed to fresh medium in which PAI-1 activity was under detection. Values are means ± S.E.M. (bars) obtained from 6 experiments. * *P* < 0.05, *** *P* < 0.001 vs. 0.1 mg/ml BSA (Student t-test). Quantitative densitometric scanning of PAI-1 mRNA shows means ± S.E.M. (bars) obtained from 4 experiments. * *P* < 0.05, *** *P* < 0.001 vs. 0.1 mg/ml BSA (Friedmans rank test followed by Mann-Whitney *U*-test).

FIG. 4. **Inhibition of AGE-induced increase in PAI-1 expression by antioxidant (PDTC) and reactive oxygen scavenger (probucol).** Cells on day 9-11 were treated with 0.1 mg/ml AGE-BSA or 0.1 mg/ml BSA for 8 h. Ten µM PDTC or 50 µM probucol was added to the culture 1 hr prior to the addition of AGE-BSA. After 9 h incubation, PAI-1 concentrations in culture media were measured by ELISA and total RNAs extracted from cells were subjected to Northern blotting. *A*, inhibitory effects of PDTC and probucol on AGE-BSA-induced increase in PAI-1 concentrations. Values are means ± S.E.M. (bars) obtained from 5-6 experiments. ** *P* < 0.01 (Student t-test). *B*, inhibitory effects of PDTC and probucol on AGE-BSA-induced increase in PAI-1 mRNAs. Values are means ± S.E.M. (bars)
obtained from 5-6 experiments. *** $P < 0.001$. (Friedman’s rank test followed by Mann-Whitney $U$-test).

**FIG. 5.** Inhibition of hyperglycemia-induced increase in PAI-1 expression by antioxidant (PDTC) and reactive oxygen scavenger (probucol). Cells on day 9-11 were incubated with 30 mM glucose (hyperglycemia) or 5.55 mM glucose (normoglycemia) for 48 h. Ten $\mu$M PDTC or 50 $\mu$M probucol was simultaneously added to the culture. During the last 24 h of incubation, the medium was replaced with 1 ml fresh medium containing 30 mM glucose and PDTC or probucol with 0.1% instead of 10% fetal bovine serum. PAI-1 concentrations in culture media were measured by ELISA and total RNAs extracted from cells were subjected to Northern blotting. $A$, inhibitory effects of PDTC and probucol on hyperglycemia-induced increase in PAI-1 concentrations. Values are means ± S.E.M. (bars) obtained from 5-6 experiments. ** $P < 0.01$ (Student $t$-test). $B$, inhibitory effects of PDTC and probucol on hyperglycemia-induced increase in PAI-1 mRNAs. Values are means ± S.E.M. (bars) obtained from 3-4 experiments. *** $P < 0.001$. (Friedman’s rank test followed by Mann-Whitney $U$-test).

**FIG. 6.** Effect of HNE on PAI-1 expression in rat white adipocytes. Rat white adipocytes on day 9-11 were treated with HNE or vehicle DMSO. PAI-1 concentrations in culture media were measured by ELISA ($A$, $B$) and 10 $\mu$g of total RNAs extracted from cells were subjected to Northern blotting as shown in Figure 3. $A$ and $C$; Cells were treated with indicated concentrations of HNE or DMSO for 8 h. Values are means ± S.E.M. (bars) obtained from 5-6 experiments. ** $P < 0.01$, *** $P < 0.001$ vs. DMSO (Student $t$-test). Quantitative densitometric scanning of PAI-1 mRNA shows means ± S.E.M. (bars) obtained from 4 experiments. *** $P < 0.001$ vs. DMSO (Friedman’s rank test followed by Mann-Whitney $U$-test). $B$ and $D$; Cells were treated for indicated
periods of time with 30 μM HNE or DMSO. At time 0 h culture medium was changed to fresh medium in which PAI-1 activity was under detection. Values are means ± S.E.M. (bars) obtained from 6 experiments. ** P <0.01, *** P <0.001 vs. DMSO (Student t-test). Quantitative densitometric scanning of PAI-1 mRNA shows means ± S.E.M. (bars) obtained from 4 experiments. * P < 0.05, *** P < 0.001 vs. DMSO (Friedmans rank test followed by Mann-Whitney U-test).

**Fig. 7. Inhibition of HNE-induced increase in PAI-1 expression by antioxidant, N-acetylcysteine (NAC).** Cells on day 9-11 were treated with 30 μM HNE or 0.1% DMSO for 8 h. NAC at the concentration 1, 5 and 20 mM was added to the culture for 24 h. PAI-1 concentrations in culture media were measured by ELISA and total RNAs extracted from cells were subjected to Northern blotting. A, Inhibitory effects of NAC on HNE-induced increase in PAI-1 levels. Values are means ± S.E.M. (bars) obtained from 5-6 experiments. ** P <0.01, *** P <0.001 (Student t-test). B, Inhibitory effects of NAC on HNE-induced increase in PAI-1 mRNAs. Values are means ± S.E.M. (bars) obtained from 4 experiments. ** P <0.01, *** P <0.001 . (Friedmans rank test followed by Mann-Whitney U-test).

**Fig. 8. Augmentation of the effect of HNE in PAI-1 activity and its gene expression by a glutathione synthesis inhibitor, L-buthionine-S, R-sulfoximine (BSO).** Cells on day 9-11 were treated with 30 μM HNE or 0.1% DMSO for 8 h. BSO at concentrations of 10, 50 and 100 μM was added to the culture for 24 h. PAI-1 concentrations in culture media were measured by ELISA and total RNAs extracted from cells were subjected to Northern blotting. A, Augmenting effects of BSO on PAI-1 concentrations. Values are means ± S.E.M. (bars) obtained from 5-6 experiments. * P <0.05, ** P <0.01 (Student t-test). B, Augmenting effects of BSO on PAI-1 mRNA. Values are means ± S.E.M. (bars) obtained from 4 experiments. ** P <0.01, *** P <0.001. (Friedmans rank test...
followed by Mann-Whitney U-test).

**FIG. 9. AGE- or HNE-induced intracellular ROS generation and NF-κB activation.**

Fluorescence microscopic analyses of the 6-carboxy-2,7-dichlorodihydrofluorescein diacetate (DCF-DA) loaded rat white adipocytes treated with AGE-BSA (A) or HNE (B) were performed. Eight hours after 0.1 mg/ml AGE-BSA or 30 μM HNE, cells were washed twice with PBS(+) and the media were changed to PBS(+) containing 1 μM DCF-DA followed by incubation for 30 min at 37 °C for dye loading (A, B; upper panels). In lower panels in A and B, cells were treated with 10 μM PDTC for 9 h or 20 mM NAC for 24 h. C, Detection of IκB-α by Western blotting. Rat white adipocytes on day 9 were exposed to 0.1 mg/ml AGE for 15 min or 30 μM HNE for 15 min and whole cell lysates were subjected to immunoblotting. The ratios of IκB-α/actin (internal control for protein loading) were normalized to those of unglycated BSA- or DMSO-treated cells which were set to unity. Values are means ± S.E.M. (bars) obtained from 4 different experiments.

**FIG 10. ROS-mediated vicious cycles for PAI-1 expression in rat white adipocytes.**

Chronic hyperglycemia induces ROS production in rat white adipocytes. ROS may cause vicious cycles; one involving AGE by glycation, the other involving lipid peroxidation. The self-amplifying ROS signals potentially activate transcription factors such as NF-κB leading to up-regulation of PAI-1.
Figure 1
Figure 2
Figure 3
Figure 4

A

B

PAI-1 (ng/mg protein)

PAI-1 mRNA/28S ratio

PDTC (10 µM, 9h)
probucol (50 µM, 9h)
AGE-BSA (0.1 mg/ml, 8h)
Figure 5
Figure 6
Figure 7
Figure 8
Figure 9
Figure 10

Chronic hyperglycemia → Glycation AGE → ROS formation → RAGE ? → NF-κB → PAI-1 up-regulation

ROS formation → Lipid peroxidation HNE
Cellular carbonyl stress enhances the expression of plasminogen activator inhibitor-1 in rat white adipocytes via reactive oxygen species-dependent pathway
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