Title: Plasminogen activator inhibitor-1 is involved in streptozotocin-induced bone loss in female mice

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Short Running Title: Role of PAI-1 in diabetic osteoporosis

Word count (3979 words), 7 Figures, and 1 Table
Abstract

In diabetic patients, the risk of fracture is high because of impaired bone formation. However, the details of the mechanisms in the development of diabetic osteoporosis remain unclear. In the present study, we investigated the role of plasminogen activator inhibitor-1 (PAI-1) in the pathogenesis of type 1 diabetic osteoporosis by using PAI-1-deficient mice. Quantitative computed tomography analysis showed that PAI-1 deficiency protected against streptozotocin-induced bone loss in female mice, but not in male mice. PAI-1-deficiency blunted the changes in the levels of Runx2, Osterix, and alkaline phosphatase (ALP) in tibia as well as serum osteocalcin levels suppressed by diabetic state in female mice only. Furthermore, the osteoclast levels in tibia, suppressed in diabetes, were also blunted by PAI-1 deficiency in female mice. Streptozotocin markedly elevated the levels of PAI-1 mRNA in liver, in female mice only. In vitro study demonstrated that treatment with active PAI-1 suppressed the levels of osteogenic genes and mineralization in primary osteoblasts from female mouse calvaria. In conclusion, the present study indicates that PAI-1 is involved in the pathogenesis of type 1 diabetic osteoporosis in females. The expression of PAI-1 in the liver and the sensitivity of bone cells to PAI-1 may be an underlying mechanism. (200 words)

Key words: Osteoporosis, Insulin Deficient Type 1 Diabetes, PAI, Streptozotocin
Introduction

Type 1 diabetes is a disease in which patients have little or no insulin secretion and hyperglycemia. A decrease in bone mineral density (BMD) and a marked increase in fracture risk have been described in patients with type 1 diabetes (1; 2). The detrimental skeletal effects of glucose toxicity, insulin deficiency and diabetic complications might partly explain the association between type 1 diabetes and osteoporosis (3-5). Previous findings suggest that a decrease in osteoblastic bone formation is a major contributor to diabetic osteoporosis (4; 6). However, the pathogenesis of this skeletal fragility and markers for the evaluation of bone metabolism in type 1 diabetic patients remain to be fully clarified.

Plasminogen activator inhibitor-1 (PAI-1) functions as the principal inhibitor of plasminogen activators, and hence fibrinolysis. PAI-1 has been of particular focus in cardiovascular disease because of strong positive correlations between serum PAI-1 levels and cardiovascular risk (7). Several reports have shown that circulating PAI-1 levels are elevated in type 1 and type 2 diabetic patients and in animal models (8; 9). Furthermore, Ma et al reported that PAI-1 contributes to the development of diabetes (10). PAI-1 has various functions, including regulating the degradation of the extracellular matrix, cell migration, and apoptosis (11), which may be related to
osteoblast differentiation and function. Daci et al previously reported that PAI-1 deficiency partially protected against bone loss in estrogen-deficient mice. These findings suggest that PAI-1 may contribute to impairment of bone remodeling and the development of osteoporosis (12). However, the role of PAI-1 in the pathogenesis of diabetic osteoporosis has not yet been elucidated.

In the present study, we examined the effects of PAI-1 deficiency on streptozotocin-induced diabetic bone loss by using wild type and PAI-1-deficient mice.

**Research Design and Methods**

**Diabetic mouse model**

Diabetes was induced in male and female wild type (PAI-1 WT) mice and PAI-1-deficient (PAI-1 KO) mice (10 weeks of age) by daily intra-peritoneal injections of streptozotocin (STZ, 50 mg/kg body weight in saline (13)), a pancreatic β-cell cytotoxin, for 4 days. Controls were injected with saline alone. Four days after the last injection (day 4), nonfasting blood glucose level was measured with a glucometer (Glutest Ace, Sanwa Kagaku Kenkyusyo, Nagoya, Japan) by using blood obtained from the tail vein. Mice with blood glucose levels greater than 300 mg/dl were considered diabetic. Animals were maintained in metabolic cages on a 12-h light, 12-h dark cycle
and they received food and water ad libitum. At 4 weeks after induction of diabetes, computed tomography (CT) analysis was performed to measure bone mineral density (BMD) in the tibia. Mice (controls and diabetics) were then fasted for 6 h, and sacrificed. All experiments were performed according to the guidelines of the National Institute of Health (NIH) and the institutional rules for the use and care of laboratory animals in Kinki University.

**Insulin treatment**

Insulin was administered by subcutaneous implantation of Linbit (Linshin, Ontario, Canada) for maintaining normal blood glucose levels in the non-fasting state (<144 mg/dl) for 4 weeks after induction of diabetes in female WT mice, as previously described (14).

**Quantitative computed tomography (qCT) analysis**

For qCT analysis of BMD and bone strength, mice were scanned using a LaTheta (LCT-200) experimental animal CT system (Hitachi-Aloka Medical, Tokyo, Japan).

**Blood measurements**
Blood was obtained from mice at 4 weeks after induction of diabetes. Plasma total PAI-1 was measured using a Murine Total PAI-1 ELISA kit (Molecular Innovations, MI, USA). The levels of serum creatinine and blood urea nitrogen (BUN) were analyzed by SRL, Inc. (Tokyo, Japan). Serum insulin and Gla-osteocalcin levels were measured using a mouse insulin ELISA kit (Morinaga Institute of Biological Science, Tokyo, Japan) and mouse Gla-osteocalcin high-sensitive enzyme immunoassay kit (Takara-bio, Ohtsu, Japan) respectively.

**Histological analysis**

Tibia was fixed for 16 h at 4°C in 4% paraformaldehyde, and further fixed for 7 days in 80% ethanol. After dehydration with formic acid, tibia was embedded in paraffin; 4-μm sections were stained using hematoxylin and eosin. For osteoclast staining, sections were stained using a tartrate-resistant acid phosphatase (TRAP)/alkaline phosphatase (ALP) staining kit (Wako Pure Industry, Osaka, Japan). The number of osteoclasts was counted in 10 separate fields, and expressed as number per bone perimeter (mm⁻¹). The number of osteoblasts was counted as TRAP-positive multinucleated cells with at least 3 nuclei.
Cell cultures

Mouse primary osteoblasts were prepared from the calvaria of newborn male and female wild-type mice. Newborn male and female mice were distinguished by differences in sex-specific organs such as testis and uterus. Primary osteoblasts (1 × 10^5 cells/well) were plated into 6-well plates, and were maintained in α-MEM supplemented with 10% FBS and 100 mg/ml penicillin–streptomycin, and grown at 37°C with 5% CO₂.

Mineralization assay

Mineralization of primary osteoblasts was assessed with Alizarin red staining, and quantified as previously described (15).

Real-time polymerase chain reaction (PCR) analysis

Bone samples were crushed in liquid nitrogen, and total RNA was extracted from the homogenized samples or cell cultures using an RNaseasy mini kit (Qiagen, Tokyo, Japan). Real-time PCR was performed on a StepOne Plus using Fast SYBR GREEN PCR Master Mix (Life Technologies Japan, Tokyo, Japan). Primer sets are shown as supplementary table 1. The mRNA levels in the tissues of mice and in primary
osteoblasts were normalized relative to the amount of β-Actin and GAPDH mRNA, respectively.

**Protein extraction and Western blotting**

Whole tibia was homogenized using homogenizer, and powder of tibia was lysed into RIPA buffer containing 1 mM PMSF. Then, Western blotting was performed, as we previously described (16).

**Statistical analysis**

All data were expressed as means ± SEM. Statistical significance was assessed using an unpaired t-test and one-way ANOVA. Differences with p < 0.05 were regarded as significant. All statistical analyses were performed using StatView version 5.0 software (SAS Institute; Cary, NC, USA).

**Results**

**Effects of streptozotocin treatment on circulating PAI-1 levels and the expression of PAI-1 in male and female mice**

Streptozotocin treatment decreased the body weight in both male and female mice from
7 days after the last injection of streptozotocin (Figure 1A). Four days after the final streptozotocin injection, blood glucose levels were markedly elevated in both sexes of mice (Figure 1B), indicating that streptozotocin induced diabetes equally in both sexes of mice. In the control group, plasma PAI-1 levels in female mice were higher than those in male mice (Figure 1C). Consistent with the elevation in blood glucose levels, circulating PAI-1 levels were elevated by streptozotocin treatment in both sexes of mice, whereas higher levels of plasma PAI-1 were observed in diabetic female mice than in diabetic male mice (Figure 1C).

The levels of PAI-1 mRNA in tibia of female mice were higher than those in tibia of male mice (Figure 1D). Streptozotocin treatment did not affect the levels of PAI-1 mRNA in the lung, kidney, and heart of WT mice (Figure 1D). However, the levels of PAI-1 mRNA in muscles and spleens from both sexes of mice were increased by streptozotocin treatment. Furthermore, streptozotocin treatment markedly increased the levels of PAI-1 mRNA in liver from female mice, whereas streptozotocin did not affect the levels of PAI-1 mRNA in liver of male WT mice (Figure 1D).

Effect of streptozotocin treatment on BMD and bone strength index in male and female mice
Hematoxylin eosin (HE) staining of tibia showed that streptozotocin treatment appeared to reduce the trabecular bone in tibia from male and female WT mice (Figure 2A). qCT analysis showed that streptozotocin treatment decreased the total BMD values and the trabecular and cortical bones in tibia from both male and female WT mice (Figures 2B, E), indicating that streptozotocin induces bone loss in both male and female WT mice. Furthermore, the bone loss by streptozotocin treatment was more severe in female WT mice than in male WT mice (Figures 2B, E). Though cortical thickness was not affected by streptozotocin treatment in either sex of WT mice (Figures 2C, D), streptozotocin treatment decreased the bone strength index (second moment of minimum and polar areas) in female WT mice, but not in male mice (Figures 2D, G), suggesting that streptozotocin decreases bone strength in female WT mice.

**Effects of PAI-1 deficiency on streptozotocin-induced bone loss in male and female mice**

Body weight was equally reduced by streptozotocin treatment in both sexes of PAI-1 WT and KO mice (Table 1). Streptozotocin treatment markedly elevated blood glucose levels and decreased serum insulin levels in both sexes of PAI-1 WT and KO mice, but there were no differences in blood glucose and serum insulin levels between diabetic
PAI-1 WT and KO mice (Table 1), indicating that PAI-1 deficiency did not affect the streptozotocin-induced diabetic state. In addition, streptozotocin treatment for 4 weeks did not affect the levels of serum creatinine and BUN in all groups (Table 1), indicating that neither PAI-1 deficiency nor the diabetic state affects renal function in mice for at least 4 weeks. In our preliminary study, there were no differences in serum 17-β estradiol levels among all groups (data not shown).

BMD in the tibia of male PAI-1 KO mice was lower than that of male PAI-1 WT mice in the control group (Figure 2B). However, PAI-1 deficiency did not affect cortical thickness and bone strength index in tibia from control male mice (Figures 2C, D). Though streptozotocin treatment did reduce BMD in tibia from both male PAI-1 WT and KO mice, these reductions were similar between male PAI-1 WT and KO mice (Figure 2B), suggesting that PAI-1 deficiency does not affect diabetic bone loss in male mice.

There were no differences in BMD values between control female PAI-1 WT and KO mice, in contrast to male mice (Figure 2E). Likewise, PAI-1 deficiency did not affect cortical thickness and bone strength index in tibia from control female mice (Figures 2F, G). Histological analysis showed that PAI-1 deficiency markedly protected from streptozotocin-induced trabecular bone loss in female mice (Figure 2A). Unstained
large circular regions within marrow showed adipocytes in HE staining of tibias. Streptozotocin treatment increased adiposity in both sexes of WT mice (Figure 2A). However, PAI-1 deficiency seemed to blunt adiposity in tibia of female mice, but not in that of male mice (Figure 2A). We show that BMD decreased by streptozotocin was strikingly blunted by PAI-1 deficiency in female mice (Figure 2E). Although PAI-1 deficiency did not affect cortical thickness in female diabetic mice, bone strength index decreased by streptozotocin was blunted by PAI-1 deficiency in female mice (Figures 2F, G). Taken together, our data indicate that PAI-1 deficiency protects from diabetic bone loss in female mice, but not in male mice.

Effect of PAI-1 deficiency on the impaired osteogenic differentiation by streptozotocin in mice

mRNA levels of osteogenic genes such as Runx2 and Osterix, a early marker of osteogenic differentiation, tended to be decreased by streptozotocin treatment in tibia from male mice (Figures 3A, B), whereas levels of ALP mRNA were not altered by streptozotocin in tibia from male mice (Figure 3C). PAI-1 deficiency did not affect the levels of these genes and protein in tibia from male diabetic mice (Figures 3A-D). Streptozotocin significantly reduced the levels of osteogenic differentiation
markers in tibia from female PAI-1 WT mice, in contrast to male mice (Figures 3F-I). However, consistent with BMD decreased by the diabetic state, PAI-1 deficiency significantly blunted the reduction in the levels of osteogenic gene and protein in tibia from female diabetic mice (Figures 3F-I).

Streptozotocin treatment significantly decreased the serum osteocalcin levels, a late-stage osteoblast differentiation marker, in both male and female mice (Figures 3E, J). Although PAI-1 deficiency did not affect the levels of serum osteocalcin in male diabetic mice, these decreases in serum osteocalcin levels were blunted by PAI-1 deficiency in female diabetic mice (Figures 3E, J). Taken together, these data indicate that PAI-1 deficiency preserves osteoblast function in diabetic female mice.

**Effects of PAI-1 deficiency on bone resorption in diabetic mice**

Streptozotocin did not affect the number of TRAP-positive multinucleated cells in tibia from male PAI-1 WT mice (Figures 4A, B), suggesting that streptozotocin-induced diabetes did not affect osteoclast formation in male mice. The levels of receptor activator of nuclear factor κB ligand (RANKL) mRNA, a crucial osteoclast differentiation factor, were also unchanged by streptozotocin treatment in tibia of male PAI-1 WT mice (Figure 4C). In addition, PAI-1 deficiency did not affect either the
number of osteoclasts or the levels of RANKL mRNA in tibia from both control and diabetic male mice (Figures 4A-C).

Streptozotocin treatment significantly reduced the number of osteoclasts and the levels of RANKL mRNA in tibia from female PAI-1 WT mice, in contrast to male mice (Figures 4D-F). Furthermore, PAI-1 deficiency significantly blunted the osteoclast number decrease in the diabetic state in tibia from female mice (Figures 4D, E). The levels of RANKL mRNA in tibia from diabetic female PAI-1 KO mice were also higher than those in diabetic female PAI-1 WT mice (Figure 4F). Taken together, our data indicate that PAI-1 deficiency blunts the decrease in osteoclast formation in the diabetic condition.

Effects of PAI-1 on osteoblastic differentiation and mineralization in primary osteoblasts

Active PAI-1 treatment did not affect osteogenic gene expression, such as Runx2, Osterix, and ALP in primary osteoblasts obtained from the calvaria of male mice (Figure 5A). However, mRNA levels of these osteogenic genes were reduced by active PAI-1 treatment in a concentration-dependent manner in primary osteoblasts obtained from female WT mice (Figure 5B). Furthermore, treatment with the active form of
PAI-1 also decreased ALP activity in primary osteoblasts obtained from female mice, but not from male mice (Figure 5C). Alizarin red staining revealed that treatment with active PAI-1 also significantly impaired mineralization only in primary osteoblasts obtained from female mouse calvaria (Figure 5D). In our preliminary study, an estrogen receptor antagonist, Fulvestrant, did not affect the suppressive effects of active PAI-1 on osteogenic gene levels in primary osteoblasts obtained from female mouse calvaria (data not shown). Taken together, our data indicate that PAI-1 impairs osteoblast differentiation and mineralization only in female mice.

**Effects of PAI-1 deficiency on adipogenic differentiation in tibia from diabetic mice**

Streptozotocin treatment markedly increased the levels of adipogenic genes in tibia from male PAI-1 WT mice, such as peroxisome proliferator-activated receptor γ (PPARγ) and adipocyte protein-2 (aP2) (Figures 6A, B), suggesting that the diabetic state enhances adipogenesis in mouse bone tissue. However, there were no differences in the levels of adipogenic genes between diabetic male PAI-1 WT and KO mice (Figures 6A, B).

Streptozotocin treatment, in comparison, greatly increased the levels of adipogenic markers in tibia from female PAI-1 WT mice (Figures 6C, D). However, the
gene levels that increased in the diabetic state were strikingly suppressed in female PAI-1 KO mice (Figures 6C, D), suggesting that PAI-1 deficiency blunts the adipogenesis induced by the diabetic state in bone tissues from female mice.

**Effect of Insulin treatment on an elevation in circulating PAI-1 levels and bone loss in diabetic mice.**

Chronic insulin treatment normalized hyperglycemia (data not shown). Insulin treatment completely suppressed the levels of plasma PAI-1 and the expression of PAI-1 in liver elevated by streptozotocin treatment in female WT mice (Figures 7A, B). Furthermore, insulin treatment completely blunted BMD and bone strength index, suppressed by streptozotocin treatment, in female WT mice (Figure 7C). These data indicate that PAI-1 changes and bone loss induced by streptozotocine result from insulin insufficiency, but not from the pharmacological effect of streptozotocin itself.

**Discussion**

Hyperglycemia, caused by impaired insulin secretion, is a main feature of type 1 diabetes. A previous study suggested that hyperglycemia is a salient factor that has both direct and indirect deleterious effects on osteoblast function and bone formation (17; 18).
However, insulin deficiency may be related to the bone loss in type 1 diabetes. Several studies have indicated that insulin promotes osteoblast proliferation, collagen synthesis, and ALP production (19; 20). Furthermore, insulin treatment reverses the changes in BMD and bone turnover markers, and the impairment in bone fracture healing induced by diabetes (21; 22). These findings suggest that insulin may play a key role in type 1 diabetic osteoporosis. However, previous studies have suggested that serum insulin levels are not related to fracture risk in clinical studies of postmenopausal women with type 2 diabetes (23). In the present study, we have shown that streptozotocin treatment decreases BMD and bone strength index as assessed by qCT analysis in female WT mice. In addition, it decreased the levels of osteogenic genes, such as Runx2, Osterix, and ALP, in tibia from female PAI-1 WT mice, but not female PAI-1 KO mice, although there were no differences in the diabetic state between PAI-1 WT and KO mice. Furthermore, we have shown that active PAI-1 treatment suppresses osteogenic gene levels, ALP activity, and mineralization in primary osteoblasts obtained from female mice. These findings indicate that PAI-1 impairs osteoblast function by directly affecting osteoblasts in the diabetic state in female mice.

Previous studies suggest an impairment of osteoclastic bone resorption in type 1 diabetic osteoporosis (24-27). The present study revealed that PAI-1 deficiency blunts
the suppression in osteoclast numbers and RANKL mRNA levels in the diabetic state in tibia from female mice. Taken together, PAI-1 deficiency is believed to blunt the decrease in osteoclastic bone resorption induced by diabetic state. However, Daci et al reported that PAI-1 deficiency improved estrogen deficiency-induced bone loss in female mice using an ovariectomy model (12). They speculated that PAI-1 deficiency suppresses bone remodeling enhanced by ovariectomy, resulting in the protection from bone loss. On the other hand, our data suggest that PAI-1 deficiency normalizes reduced bone remodeling of diabetic female mice. Our preliminary study revealed that there were no differences in serum levels of 17-β-estradiol among all groups in the present study, and that an estrogen receptor antagonist, Fulvestrant, did not affect the suppressive effects of active PAI-1 on osteogenic gene levels in primary osteoblasts from WT female mouse calvaria (data not shown). Estrogen might not be responsible for diabetic bone loss in female mice, and there might be differences in the role of PAI-1 in bone loss between type 1 diabetic models and estrogen-deficiency models.

Osteoblasts differentiate from mesenchymal stem cells, which have the ability to differentiate into adipocytes and chondrocytes. Previous evidences have shown that an altered mesenchymal stem cell lineage selection toward adipocytes rather than osteoblasts is related to the mechanism of diabetic bone loss (6). Bone marrow adiposity
is observed in aged and type 1 diabetic bone tissues, which may be associated with a decrease in BMD (28). Several studies suggest that PPARγ is a key transcriptional factor, which regulates adipogenesis (10; 28-30). In the present study, we have shown that PAI-1 deficiency blunted the change in the levels of adipogenic genes such as PPARγ and aP2 that were increased in the diabetic state in tibia from female mice. PAI-1 deficiency also seemed to suppress the increase in the adiposity induced by the diabetic state in tibia of female mice. These findings show that PAI-1 may promote the differentiation of mesenchymal stem cells toward adipogenesis, but not into osteoblastogenesis in bone tissues in the diabetic state, thus resulting in an impairment of bone formation leading to diabetic osteoporosis in female mice.

We demonstrate that PAI-1 deficiency is involved in bone loss, impaired osteoblast differentiation, and enhanced adipogenesis induced by diabetic state only in female mice, but not in male mice. Sex differences in susceptibility of mice to streptozotocin have been reported, which are due to the protective effect of estrogen on streptozotocin-induced beta cell apoptosis in female mice (31). However, in the present study, we showed that there were no sex differences in the levels of blood glucose and serum insulin (Table 1) in streptozotocin-treated PAI-1 WT and KO mice, suggesting that the protective effect of estrogen on beta cell apoptosis is not involved in the sex
differences in the effect of PAI-1 deficiency on diabetic bone loss. In the present study, streptozotocin-treated female WT mice showed lower body weight and BMD than streptozotocin-treated male WT mice. This sex difference in streptozotocin-induced bone loss might be partially due to a decrease in body weight. However, PAI-1 deficiency protected from diabetic bone loss only in female mice with lower body weight, suggesting mechanisms other than body weight are responsible for sex differences in the effect of PAI-1 deficiency on diabetic bone loss in mice. We revealed that levels of circulating PAI-1 induced by streptozotocin treatment were higher in female mice, compared with those in male mice. Furthermore, the levels of PAI-1 mRNA in tibia of control female WT mice were higher than those in control male WT mice. These might be related to sex differences in the role of PAI-1 in diabetic bone loss. We also showed that streptozotocin treatment markedly increased the levels of PAI-1 in liver tissues only from female mice, and that streptozotocin treatment significantly increased the levels of PAI-1 mRNA in muscle and spleen from both sexes of mice. However, there were no sex differences in the levels of PAI-1 mRNA in muscle and spleen from streptozotocin-treated mice. Therefore, PAI-1, which is secreted from liver, may be involved in the diabetic bone loss in female mice. PAI-1 is expressed abundantly in endothelial cells. Although we could not specifically assay endothelial cells for PAI-1,
we analyzed lung tissues which contain a high density of vessels. Then, we found that streptozotocin did not affect the levels of PAI-1 mRNA in lung tissues. Therefore, it does not seem to be probable that non-specific vessel endothelial cells are responsible for the major source of PAI-1 produced by the diabetic state in female mice. DiMusto et al reported that induction of PAI-1 expression was higher in abdominal aortic aneurysm formation of female mice than in male mice (32), suggesting that PAI-1 might be more potently induced by the pathological state in females. We also found that PAI-1 impaired osteoblast differentiation and mineralization in primary osteoblasts from female mice calvaria, but not in male mice. These findings indicate that PAI-1 is involved in the pathogenesis of diabetic osteoporosis only in female mice, partly due to sex differences in response to PAI-1 in osteoblasts. Numerous studies suggested that a protein linked to the sex chromosomes is associated with the sex differences in the prevalence of osteoporosis (33). The deficiency of biglycan on chromosome X strongly affects male bones (34; 35). Furthermore, Olivares-Navarrete et al reported sex difference in osteogenic response to vitamin D treatment in primary osteoblasts (36). These findings suggest that a protein linked to sex chromosome might be responsible for the sex difference observed in response to PAI-1 in primary osteoblasts in the present study. However, further studies are necessary to clarify these issues.
On the basis of the present data, we propose the following hypothesis for the role of PAI-1 in the pathogenesis of diabetic osteoporosis in female mice, as shown in Figure 7D. Diabetic state, such as hyperglycemia, insulin insufficiency, and an elevation in advanced glycation end-products, increases PAI-1 expression in the liver, resulting in an elevation in the circulating PAI-1 levels. The elevated PAI-1 impairs osteoblast differentiation, mineralization, and bone resorption as well as promotes adipogenesis in bone tissues. These cascades may lead to type 1 diabetic osteoporosis in female mice (Figure 7D). However, further studies will be necessary to clarify the precise roles of PAI-1 in the pathogenesis of diabetic osteoporosis and its sex differences. In the present study, PAI-1 deficiency slightly, but significantly decreased BMD only in male mice, but not in female mice, which is compatible with the previous evidence that plasminogen activator deficiency increases bone mass in mice (37). Why PAI-1 deficiency decreased BMD only in male mice is unknown.

In conclusion, we demonstrate that PAI-1 deficiency protects against diabetic bone loss in female mice. Our data suggests that PAI-1 plays an important role in the pathogenesis of type 1 diabetic osteoporosis, and that this pathological importance may be sex-dependent. Production of PAI-1 from liver tissues and the sensitivity of bone cells to PAI-1 may be responsible for this mechanism of pathogenesis.
**Acknowledgments**

This study was supported by Grants-in-aid 24790227 and 24590289 from the Ministry of Science, Education, and Culture of Japan (to Y. T. and to H. K., respectively), a grant from Yokoyama Foundation for Clinical Pharmacology and a grant from Kinki University.

No potential conflicts of interest relevant to this article were reported.

Y. T. researched data, contributed to the discussion, and wrote the manuscript. N. K., K. O., M. Y., K. O., and O. M. contributed to the discussion. H. K. contributed to the discussion and wrote the manuscript. All authors reviewed and edited the manuscript. H. K. is the guarantor of this work and, as such, had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.
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dependent regulation of osteoblast response to implant surface properties by systemic hormones. *Biol Sex Differ* 1:4, 2010

Table 1. Characteristics of both sexes of control or streptozotocin-treated PAI-1 WT and KO mice at 4 weeks after induction of diabetes.

<table>
<thead>
<tr>
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<th>Control</th>
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<tr>
<td><strong>Male</strong></td>
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<tr>
<td>Body weight (g)</td>
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<td>Blood glucose (mg/dl)</td>
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<td>Serum BUN (mg/dl)</td>
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<td>Body weight (g)</td>
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<td>Serum BUN (mg/dl)</td>
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Results are expressed as means ± SEM. *: p < 0.05, **: p < 0.01 vs. Control PAI-1 WT, †: p < 0.05, ††: p < 0.01 vs. Control PAI-1 KO (n = 5–7 in each group). ND: not
detected.
Figure legends

Figure 1. Effects of streptozotocin treatment on circulating PAI-1 levels and PAI-1 expression in both sexes of WT mice. Growth curve during experiments (A) in control and streptozotocin-treated male and female of PAI-1 WT mice (open circle: male control WT mice, closed circle: male STZ-treated WT mice, open square: female control WT mice, closed square: female STZ-treated WT mice). Results are expressed as means ± SEM. **: p < 0.01 vs. each control group (n = 5 in each group). Blood glucose (B) and plasma PAI-1 levels (C) in control (open bar) and streptozotocin-treated (closed bar) male and female WT mice. Results are expressed as means ± SEM. *: p < 0.05, **: p < 0.01 (n = 5 in each group). Levels of PAI-1 mRNA in bone (tibia), liver, kidney, lung, heart, muscle, and spleen (D) in control (open bar) and streptozotocin-treated (closed bar) male and female WT mice. Results are expressed relative to β-Actin mRNA values, and expressed as means ± SEM. *: p < 0.05, **: p < 0.01 (n = 5 in each group).

Figure 2. Effects of PAI-1 deficiency on diabetic bone loss in both sexes of mice.

Hematoxylin eosin staining of tibia in control and streptozotocin-treated male and female PAI-1 WT and KO mice (A). BMD values in total, trabecular, and cortical bones
(B), cortical thickness (C), and second moment of minimum and polar areas (D) of tibia in control and streptozotocin-treated male PAI-1 WT and KO mice (open bar: PAI-1 WT mice, closed bar: PAI-1 KO mice). BMD values in total, trabecular, and cortical bones (E), cortical thickness (F), and second moment of minimum and polar areas (G) of tibia in control and streptozotocin-treated female PAI-1 WT and KO mice (open bar: PAI-1 WT mice, closed bar: PAI-1 KO mice). For assessment of trabecular BMD, trabecular regions of interest (ROIs) extended from 96 um distal to the end of the proximal growth plate over 1.5 mm towards the diaphysis. For assessment of cortical BMD and thickness, cortical ROIs were defined as 2.0-mm segments of the mid-diaphysis tibia. For assessment of total BMD and bone strength index (second moment of minimum and polar areas: index of bending strength), ROIs were defined as 9600-µm segment (100 slices) from distal end of proximal growth plate of tibia. Parameters used for the CT scans were as follows: tube voltage, 50 kVp; tube current, 500 µA; integration time, 3.6 ms; axial field of view, 48 mm; and the voxel size of 48 × 96 µm with a slice thickness of 96 µm. Bone parameters were analyzed using the LaTheta software (version 3.40). Results are expressed as means ± SEM. *: p < 0.05, **: p < 0.01 (n = 5–7 in each group).

Figure 3. Effects of PAI-1 deficiency on diabetes-induced impairment of osteogenic
differentiation in both sexes of mice. mRNA levels of Runx2 (A), Osterix (B), and ALP (C) as well as protein levels of Runx2, ALP and β-Actin (D) in tibia of control and streptozotocin-treated male PAI-1 WT and KO mice (open bar: PAI-1 WT mice, closed bar: PAI-1 KO mice). mRNA levels of Runx2 (F), Osterix (G), and ALP (H) as well as protein levels of Runx2, ALP and β-Actin (I) in tibia of control and streptozotocin-treated female PAI-1 WT and KO mice (open bar: PAI-1 WT mice, closed bar: PAI-1 KO mice). Data are expressed relative to β-Actin mRNA values, and expressed as means ± SEM. *: p < 0.05, **: p < 0.01 (n = 5–7 in each group). The levels of Serum osteocalcin in control and streptozotocin-treated male (E) and female (J) PAI-1 WT and KO mice (open bar: PAI-1 WT mice, closed bar: PAI-1 KO mice). Results are expressed as means ± SEM. *: p < 0.05, **: p < 0.01 (n = 5–7 in each group).

**Figure 4. Effects of PAI-1 deficiency on bone resorption in tibia of both sexes of diabetic mice.** TRAP staining (A) and number of TRAP-positive multinucleated cells (B) in tibia of control and streptozotocin-treated male PAI-1 WT and KO mice (open bar: PAI-1 WT mice, closed bar: PAI-1 KO mice). Results are expressed as means ± SEM. *: p < 0.05 (n = 5–7 in each group). Levels of RANKL mRNA (C) in tibia of control and streptozotocin-treated male PAI-1 WT and KO mice (open bar: PAI-1 WT
mice, closed bar: PAI-1 KO mice). Results are expressed relative to β-Actin mRNA values, and expressed as means ± SEM. *: p < 0.05; **: p < 0.01 (n = 5–7 in each group).

TRAP staining (D) and number of TRAP-positive multinucleated cells (E) in tibia of control and streptozotocin-treated female PAI-1 WT and KO mice (open bar: PAI-1 WT mice, closed bar: PAI-1 KO mice). Results are expressed as means ± SEM. *: p < 0.05 (n = 5–7 in each group). Levels of RANKL mRNA (F) in tibia of control and streptozotocin-treated female PAI-1 WT and KO mice (open bar: PAI-1 WT mice, closed bar: PAI-1 KO mice). Results are expressed relative to β-Actin mRNA values, and expressed as means ± SEM. *: p < 0.05, **: p < 0.01 (n = 5–7 in each group).

**Figure 5.** Effects of active PAI-1 treatment on osteoblast differentiation and mineralization in primary osteoblasts from WT mouse calvaria. For RNA analysis and measurement of ALP activity, primary osteoblasts were treated with either vehicle or active PAI-1 (2 nM and 20 nM, Molecular Innovations, MI, USA) for 24 h. Then, total cellular RNA was extracted for gene expression analysis by real-time PCR. ALP activity was measured using Labassay™ ALP (Wako Pure Industry, Osaka, Japan).

Levels of Runx2, Osterix, and ALP mRNA in primary osteoblasts from male (A) and female (B) WT mice treated with vehicle (open bar) or active PAI-1 (2 nM (hatch
marks) or 20 nM (closed bar) for 24 h. Data are expressed relative to GAPDH mRNA values, and expressed as means ± SEM. *: p < 0.05, **: p < 0.01 (n = 3 in each group).

ALP activity (C) in primary osteoblasts from male and female WT mouse calvaria treated with vehicle (open bar) or active PAI-1 (20 nM: closed bar) for 24 h. Mineralization as assessed by alizarin red staining of primary osteoblasts (D) from male and female WT mouse calvaria cultured in osteogenic medium (α-MEM containing 10 mM β-glycerophosphate and 50 µg/ml ascorbic acid) for 21 d treated with vehicle (open bar) or active PAI-1 (20 nM: closed bar). Results are expressed as means ± SEM. *: p < 0.05, **: p < 0.01 (n = 3 in each group).

Figure 6. Effects of PAI-1 deficiency on adipogenesis in tibia of both sexes of diabetic mice. Levels of PPARγ mRNA (A) and aP2 mRNA (B) in control and streptozotocin-treated male PAI-1 WT and KO mice (open bar: PAI-1 WT mice, closed bar: PAI-1 KO mice). Levels of PPARγ mRNA (C) and aP2 mRNA (D) in control and streptozotocin-treated female PAI-1 WT and KO mice (open bar: PAI-1 WT mice, closed bar: PAI-1 KO mice). Data are expressed relative to β-Actin mRNA values, and are expressed as means ± SEM. *: p < 0.05, **: p < 0.01 (n = 5–7 in each group).
**Figure 7. Effect of insulin treatment on streptozotocin-induced bone loss in female mice.** The levels of plasma PAI-1 (A), and PAI-1 mRNA in liver (B), BMDs (total, trabecular, cortical) and bone strength index in tibia (C) in control female mice (open bar) and diabetic female mice treated with (hatch marks) or without insulin (closed bar) for 4 weeks. Results are expressed relative to β-Actin mRNA values, and expressed as means ± SEM. *: p < 0.05, **: p < 0.01 (n = 6 in each group). Proposed hypothesis for the role of PAI-1 in the pathogenesis of diabetic osteoporosis in female mice (D); Diabetic state induces an increase in PAI-1 expression in the liver, resulting in an elevation of circulating PAI-1 levels in female mice. An elevated circulating PAI-1 impairs osteoblast differentiation and mineralization. Furthermore, it promotes adipogenesis in bone tissues. These cascades may lead to diabetic osteoporosis in female mice.
Figure 1

Figure 1. Effects of streptozotocin treatment on circulating PAI-1 levels and PAI-1 expression in both sexes of WT mice. Growth curve during experiments (A) in control and streptozotocin-treated male and female of PAI-1 WT mice (open circle: male control WT mice, closed circle: male STZ-treated WT mice, open square: female control WT mice, closed square: female STZ-treated WT mice). Results are expressed as means ± SEM. **: p < 0.01 vs. each control group (n = 5 in each group). Blood glucose (B) and plasma PAI-1 levels (C) in control (open bar) and streptozotocin-treated (closed bar) male and female WT mice. Results are expressed as means ± SEM. *: p < 0.05, **: p < 0.01 (n = 5 in each group). Levels of PAI-1 mRNA in bone (tibia), liver, kidney, lung, heart, muscle, and spleen (D) in control (open bar) and streptozotocin-treated (closed bar) male and female WT mice. Results are expressed relative to β-Actin mRNA values, and expressed as means ± SEM. *: p < 0.05, **: p < 0.01 (n = 5 in each group).
Figure 2

Figure 2. Effects of PAI-1 deficiency on diabetic bone loss in both sexes of mice. Hematoxylin eosin staining of tibia in control and streptozotocin-treated male and female PAI-1 WT and KO mice (A). BMD values in total, trabecular, and cortical bones (B), cortical thickness (C), and second moment of minimum and polar areas (D) of tibia in control and streptozotocin-treated male PAI-1 WT and KO mice (open bar: PAI-1 WT mice, closed bar: PAI-1 KO mice). BMD values in total, trabecular, and cortical bones (E), cortical thickness (F), and second moment of minimum and polar areas (G) of tibia in control and streptozotocin-treated female PAI-1 WT and KO mice (open bar: PAI-1 WT mice, closed bar: PAI-1 KO mice). For assessment of trabecular BMD, trabecular regions of interest (ROIs) extended from 96 um distal to the end of the proximal growth plate over 1.5 mm towards the diaphysis. For assessment of cortical BMD and thickness, cortical ROIs were defined as 2.0-mm segments of the mid-diaphysis tibia. For assessment of total BMD and bone strength index (second moment of minimum and polar areas: index of bending strength), ROIs were defined as 9600-µm segment (100 slices) from distal end of proximal growth plate of tibia. Parameters used for the CT scans were as follows: tube voltage, 50 kVp; tube current, 500 µA; integration time, 3.6 ms; axial field of view, 48 mm; and the voxel size of 48 × 96 µm with a slice thickness of 96 µm. Bone parameters were analyzed using the LaTheta software (version 3.40). Results are expressed as means ± SEM. *: p < 0.05,
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150x172mm (300 x 300 DPI)
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146x125mm (300 x 300 DPI)
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**Supplementary Table 1.** Primers used for real-time PCR

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<td>PAI-1</td>
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