Plasminogen Activator Inhibitor-1 Is Involved in Streptozotocin-Induced Bone Loss in Female Mice

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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 current study, we investigated the role of plasminogen activator inhibitor (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 in tibia as well as serum osteocalcin levels suppressed by the 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 current 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. ***Diabetes*** **62:3170–3179, 2013**

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 diabetes 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 (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. (10) reported that PAI-1 contributes to the development of diabetes. 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. (12) 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. However, the role of PAI-1 in the pathogenesis of diabetic osteoporosis has not yet been elucidated. In the current 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 injections of streptozotocin (50 mg/kg body wt i.p. 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 >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 BMD in the tibia. Mice (controls and patients with diabetes) were then fasted for 6 h and killed. All experiments were performed according to the guidelines of the National Institutes 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 nonfasting state (<144 mg/dL) for 4 weeks after induction of diabetes in female WT mice as previously described (14).

Quantitative CT analysis. For quantitative CT (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, Novi, MI). 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-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 three 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 sixwell plates and

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were maintained in Minimum Essential Medium Alpha Modification (MEM α) supplemented with 10% FBS and 100 mg/mL penicillin streptomycin and grown at 37°C with 5% CO_2.

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

Real-time PCR analysis. Bone samples were crushed in liquid nitrogen, and total RNA was extracted from the homogenized samples or cell cultures using an RNeasy 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 in 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 glyceraldehyde-3-phosphate dehydrogenase mRNA, respectively.

Protein extraction and Western blotting. Whole tibia was homogenized using homogenizer, and powder of tibia was lysed into radioimmunoprecipitation assay buffer containing 1 mmol/L phenylmethylsulfonyl fluoride. Then, Western blotting was performed as we have 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).

RESULTS

Effects of streptozotocin administration 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 (Fig. 1*A*). Four days after the final streptozotocin injection, blood glucose levels were markedly elevated in both sexes of mice (Fig. 1*B*), 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 (Fig. 1*C*). 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 (Fig. 1*C*).

The levels of PAI-1 mRNA in tibia of female mice were higher than those in tibia of male mice (Fig. 1*D*). Streptozotocin treatment did not affect the levels of PAI-1 mRNA in the lung, kidney, and heart of WT mice (Fig. 1*D*). 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 (Fig. 1*D*).

Effect of streptozotocin administration on BMD and bone strength index in male and female mice. Hematoxylin-eosin staining of tibia showed that streptozotocin treatment appeared to reduce the trabecular bone

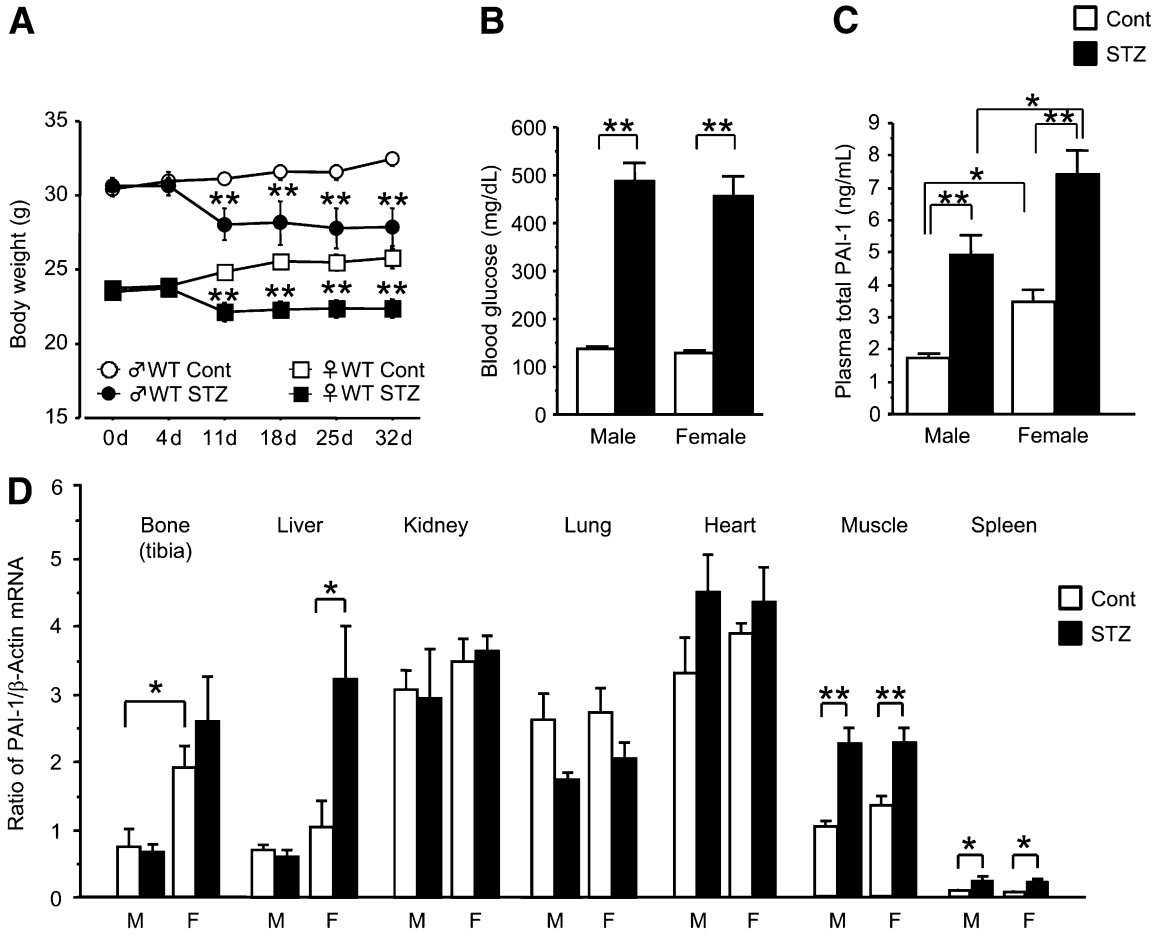

FIG. 1. Effects of streptozotocin treatment on circulating PAI-1 levels and PAI-1 expression in both sexes of WT mice. Growth curve during experiments (*A*) in control and streptozotocin-treated male and female of PAI-1 WT mice. Results are expressed as means ± SEM. **$P < 0.01$ vs. each control group ($n = 5$ in each group). Blood glucose (*B*) and plasma PAI-1 levels (*C*) in control and streptozotocin-treated 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 and streptozotocin-treated 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). Cont, control; d, day(s); F, female; M, male; STZ, streptozotocin.

in tibia from male and female WT mice (Fig. 2*A*). 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 (Fig. 2*B* and *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 (Fig. 2*B* and *E*). Though cortical thickness was not affected by streptozotocin treatment in either sex of WT mice (Fig. 2*C* and *D*), streptozotocin treatment decreased the bone strength index (second moment of minimum and polar areas) in female WT mice

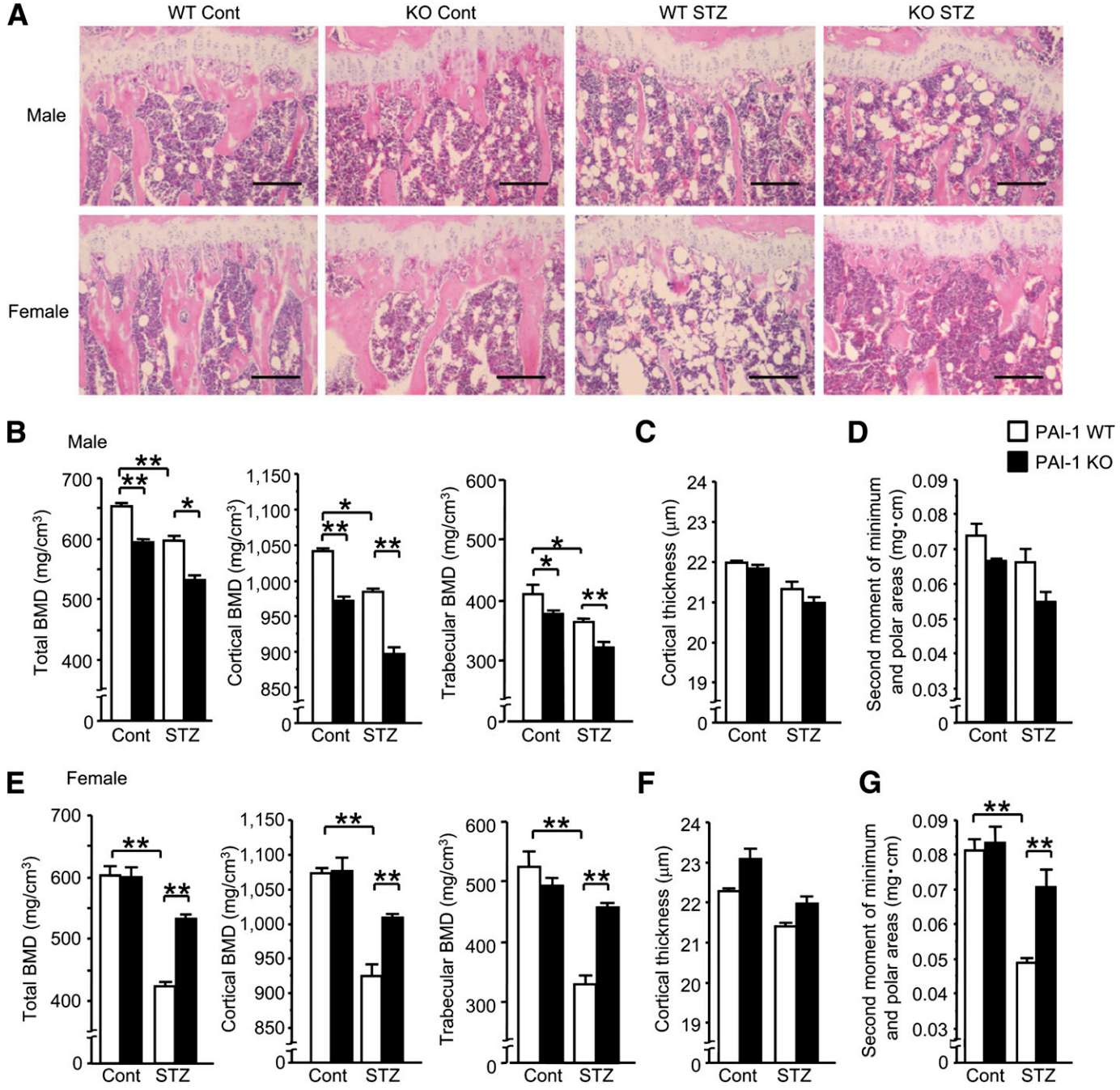

FIG. 2. Effects of PAI-1 deficiency on diabetic bone loss in both sexes of mice. Hematoxylin-eosin staining of tibia in control and streptozotocin-treated male and female PAI-1 WT and KO mice (*A*). BMD values in total, trabecular, and cortical bones (*B*); cortical thickness (*C*); and second moment of minimum and polar areas (*D*) of tibia in control and streptozotocin-treated male PAI-1 WT and KO mice. 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. For assessment of trabecular BMD, trabecular regions of interest extended from 96 um distal to the end of the proximal growth plate over 1.5 mm toward 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 9,600-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 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). Cont, control; STZ, streptozotocin.

but not in male mice (Fig. 2*D* and *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 (Fig. 2*B*). However, PAI-1 deficiency did not affect cortical thickness and bone strength index in tibia from control male mice (Fig. 2*C* and *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 (Fig. 2*B*), 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 (Fig. 2*E*). Likewise, PAI-1 deficiency did not affect cortical thickness and bone strength index in tibia from control female mice (Figs. 2F, G). Histological analysis showed that PAI-1 deficiency markedly protected from streptozotocin-induced trabecular bone loss in female mice (Fig. 2*A*). Unstained large circular regions within marrow showed adipocytes in hematoxylin-eosin staining of tibias. Streptozotocin treatment increased adiposity in both sexes of WT mice (Fig. 2*A*). However, PAI-1 deficiency seemed to blunt adiposity in tibia of female mice but not in that of male mice (Fig. 2*A*). We show that BMD decreased by streptozotocin was strikingly blunted by PAI-1 deficiency in female mice (Fig. 2*E*). 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 (Figs. 2*F* and *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, an early marker of osteogenic differentiation, tended to be decreased by streptozotocin treatment in tibia from male mice (Fig. 3*A* and *B*), whereas levels of ALP mRNA were not altered by streptozotocin in tibia from male mice (Fig. 3*C*). PAI-1 deficiency did not affect the levels of these genes and protein in tibia from male diabetic mice (Fig. 3*A–D*).

Streptozotocin significantly reduced the levels of osteogenic differentiation markers in tibia from female PAI-1 WT mice in contrast with male mice (Fig. 3*F–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 (Fig. 3*F–I*).

Streptozotocin treatment significantly decreased the serum osteocalcin levels, a late-stage osteoblast differentiation marker, in both male and female mice (Fig. 3*E* and *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 (Fig. 3*E* and *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 (Fig. 4*A* and *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 (Fig. 4*C*). 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 (Fig. 4*A–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 with male

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

	Control		Streptozotocin	
	PAI-1 WT	PAI-1 KO	PAI-1 WT	PAI-1 KO
Male				
Body weight (g)	29.4 ± 1.1	31.7 ± 1.5	25.2 ± 1.4*	26.3 ± 1.3†
Blood glucose (mg/dL)	145.2 ± 1.8	140.5 ± 7.5	501.2 ± 24.8**	489.6 ± 35.4††
Serum insulin (ng/mL)	0.40 ± 0.07	0.34 ± 0.08	ND	ND
Serum creatinine (mg/dL)	0.07 ± 0.02	0.08 ± 0.02	0.08 ± 0.01	0.07 ± 0.01
Serum BUN (mg/dL)	31.4 ± 2.1	31.6 ± 4.1	29.5 ± 2.0	25.7 ± 1.2
Female				
Body weight (g)	25.0 ± 0.3	25.4 ± 0.6	21.8 ± 0.6*	22.1 ± 0.7†
Blood glucose (mg/dL)	127.0 ± 4.1	143.0 ± 5.2	474.6 ± 25.6**	488.9 ± 28.6††
Serum insulin (ng/mL)	0.32 ± 0.06	0.31 ± 0.05	ND	ND
Serum creatinine (mg/dL)	0.06 ± 0.01	0.06 ± 0.02	0.05 ± 0.02	0.07 ± 0.02
Serum BUN (mg/dL)	24.8 ± 1.6	21.8 ± 2.8	28.9 ± 2.3	28.5 ± 4.0

Results are expressed as means ± SEM. ND, not detected. *$P < 0.05$ vs. control PAI-1 WT, **$P < 0.01$ vs. control PAI-1 WT, †$P < 0.05$ vs. control PAI-1 KO, ††$P < 0.01$ vs. control PAI-1 KO (n = 5–7 in each group).

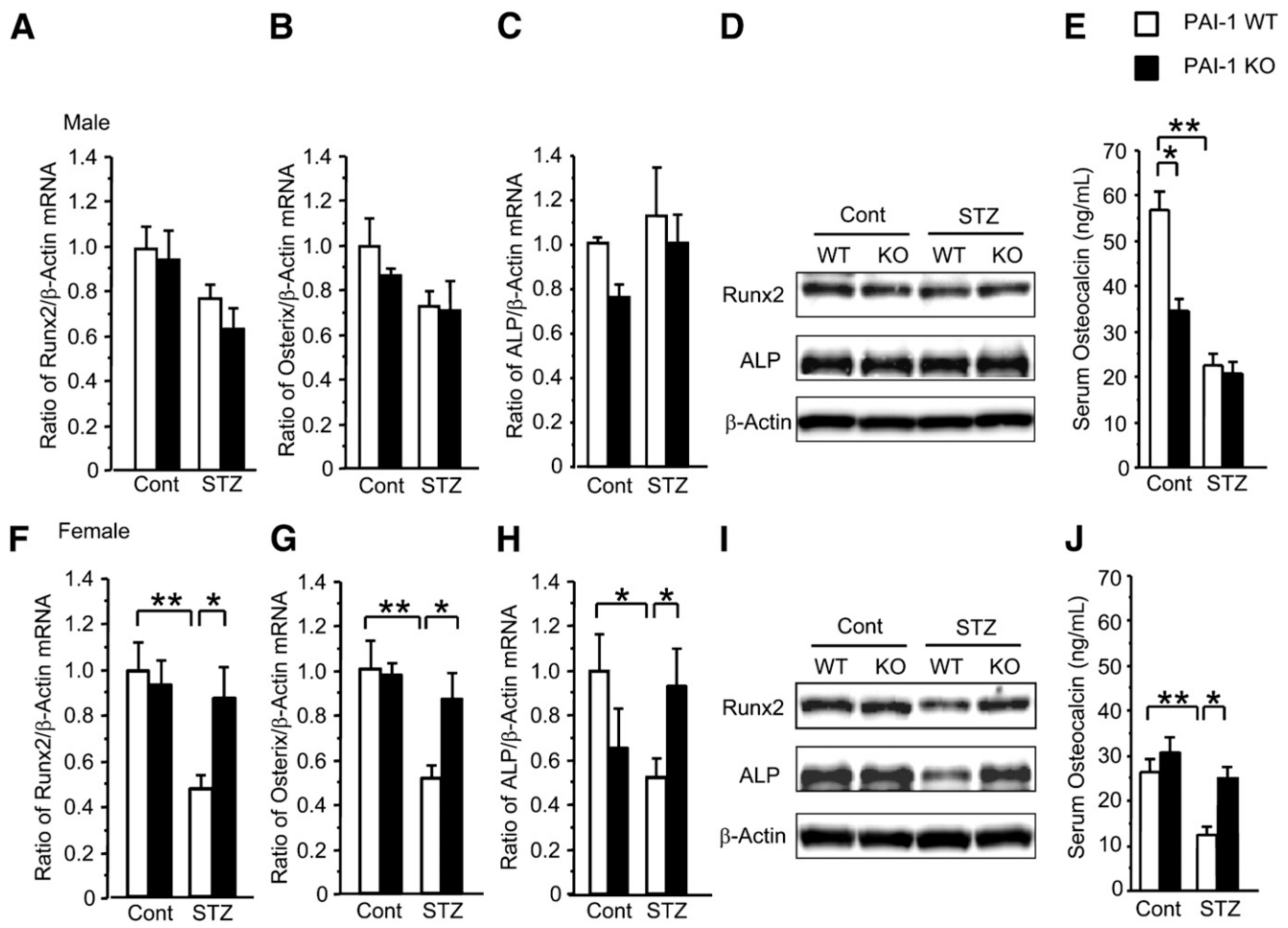

FIG. 3. Effects of PAI-1 deficiency on diabetes-induced impairment of osteogenic differentiation in both sexes of mice. mRNA levels of Runx2 (*A*), osterix (*B*), and ALP (*C*) as well as protein levels of Runx2, ALP, and β-actin (*D*) in tibia of control and streptozotocin-treated male PAI-1 WT and KO mice. 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. 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. Results are expressed as means ± SEM. *P < 0.05, **P < 0.01 (n = 5–7 in each group). Cont, control; STZ, streptozotocin.

mice (Fig. 4*D–F*). Furthermore, PAI-1 deficiency significantly blunted the osteoclast number decrease in the diabetic state in tibia from female mice (Fig. 4*D* and *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 (Fig. 4*F*). 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 (Fig. 5*A*). 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 (Fig. 5*B*). 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 (Fig. 5*C*). Alizarin red staining revealed that treatment with active PAI-1 also significantly impaired mineralization only in primary osteoblasts obtained from female mouse calvaria (Fig. 5*D*). 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) (Fig. 6*A* and *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 (Fig. 6*A* and *B*).

Streptozotocin treatment, in comparison, greatly increased the levels of adipogenic markers in tibia from female PAI-1 WT mice (Fig. 6*C* and *D*). However, the gene levels that increased in the diabetic state were strikingly suppressed in female PAI-1 KO mice (Fig. 6*C* and *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

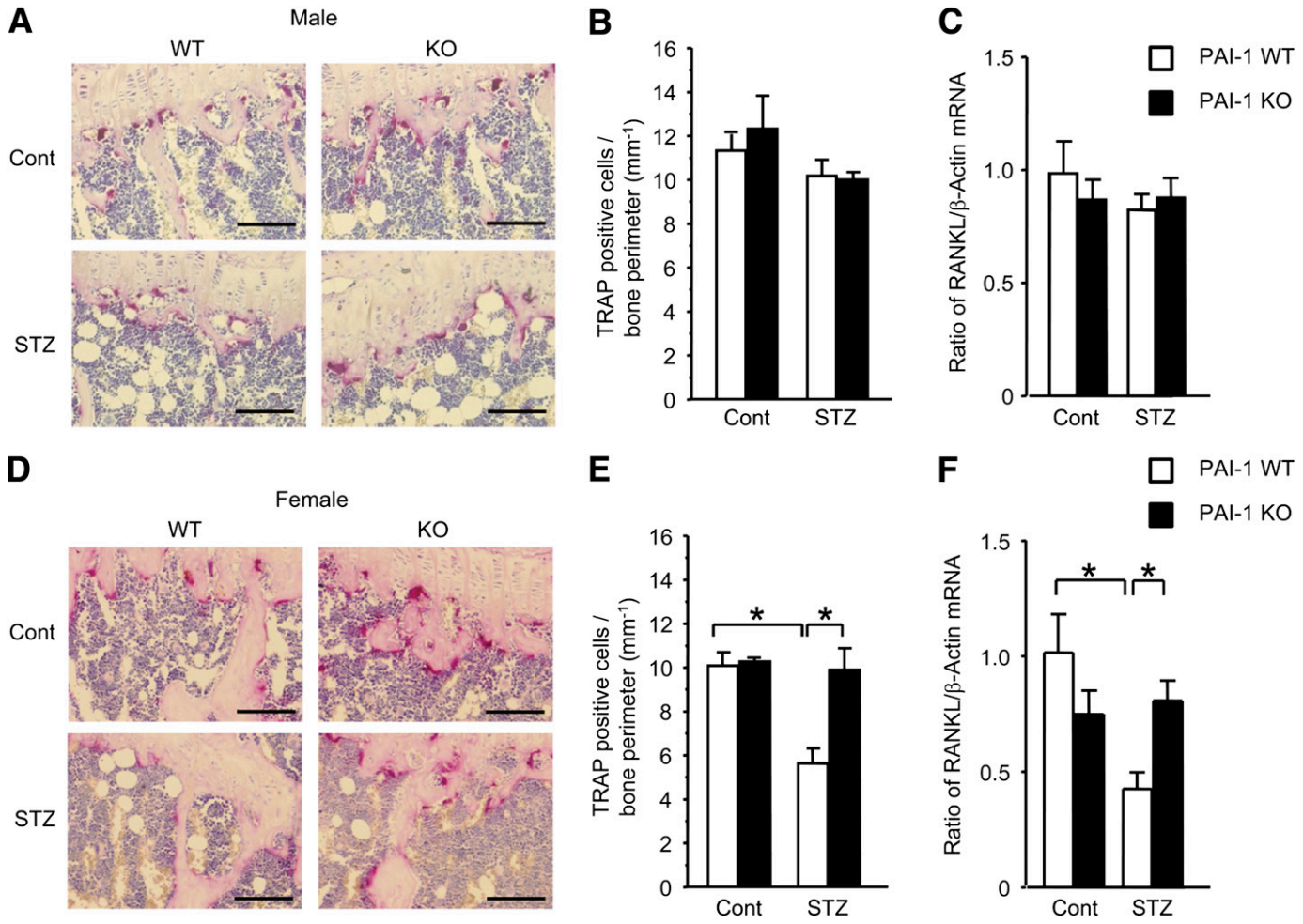

FIG. 4. Effects of PAI-1 deficiency on bone resorption in tibia of both sexes of diabetic mice. TRAP staining (*A*) and number of TRAP-positive multinucleated cells (*B*) in tibia of control and streptozotocin-treated male PAI-1 WT and KO mice. Results are expressed as means ± SEM. **P* < 0.05 (*n* =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

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 (Fig. 7*A* and *B*). Furthermore, insulin treatment completely blunted BMD and bone strength index, suppressed by streptozotocin treatment, in female WT mice (Fig. 7*C*). These data indicate that PAI-1 changes and bone loss induced by streptozotocin 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 current 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).

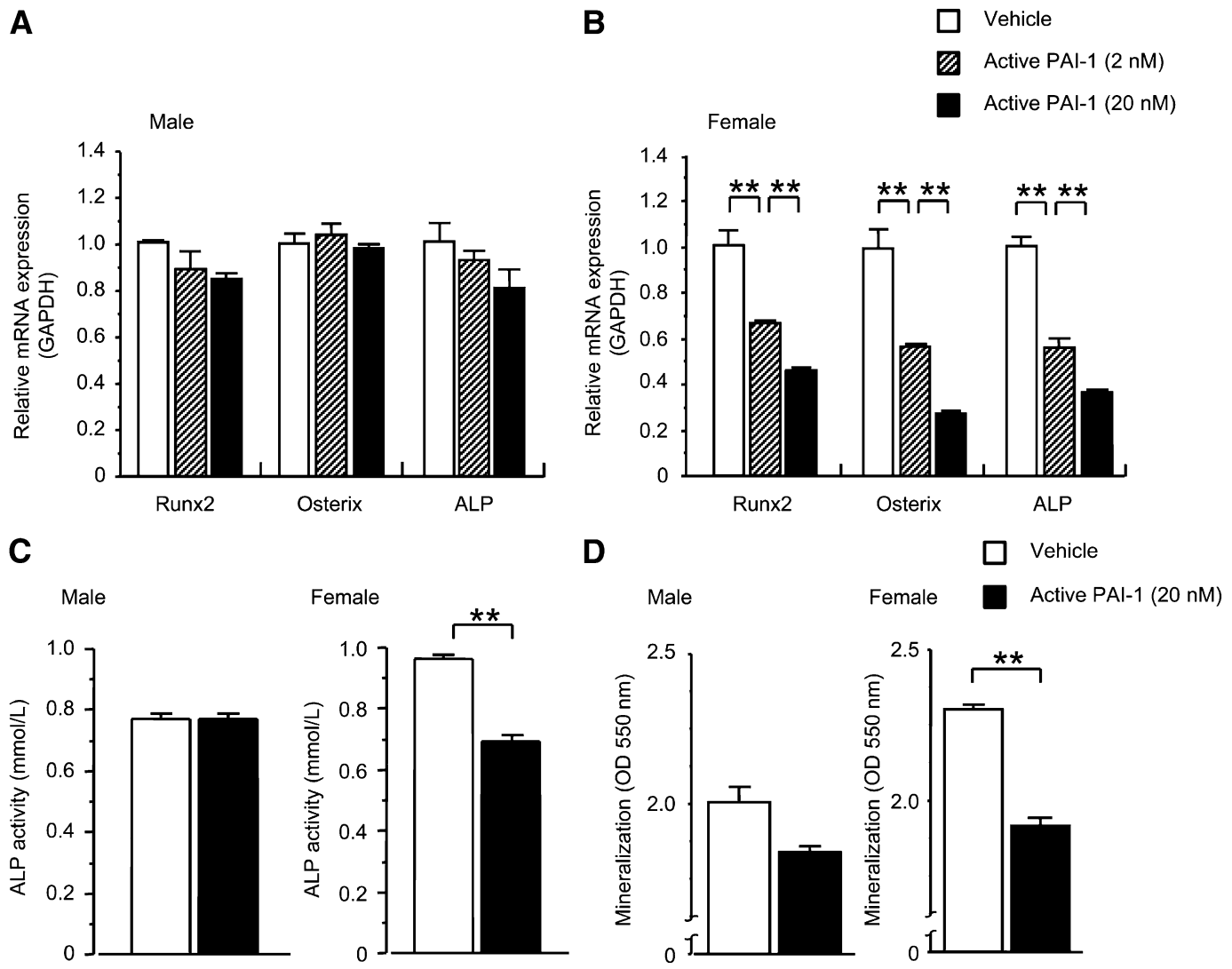

FIG. 5. Effects of active PAI-1 treatment on osteoblast differentiation and mineralization in primary osteoblasts from WT mouse calvaria. For RNA analysis and measurement of ALP activity, primary osteoblasts were treated with either vehicle or active PAI-1 (2 nmol/L and 20 nmol/L; Molecular Innovations) 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 or active PAI-1 (2 nmol/L or 20 nmol/L) for 24 h. Data are expressed relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA values and expressed as means ± SEM. ***P* < 0.01 (*n* = 3 in each group). ALP activity (*C*) in primary osteoblasts from male and female WT mouse calvaria treated with vehicle or active PAI-1 (20 nmol/L) 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 (MEM α, containing 10 mmol/L β-glycerophosphate and 50 μg/mL ascorbic acid) for 21 days treated with vehicle or active PAI-1 (20 nmol/L). Results are expressed as means ± SEM. ***P* < 0.01 (*n* = 3 in each group).

The current 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 the diabetic state. However, Daci et al. (12) reported that PAI-1 deficiency improved estrogen deficiency–induced bone loss in female mice using an ovariectomy model. 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 current 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 evidence has 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 that regulates adipogenesis (10,28–30). In the current study, we have shown that PAI-1 deficiency blunted the change in the levels of adipogenic genes such as PPARγ

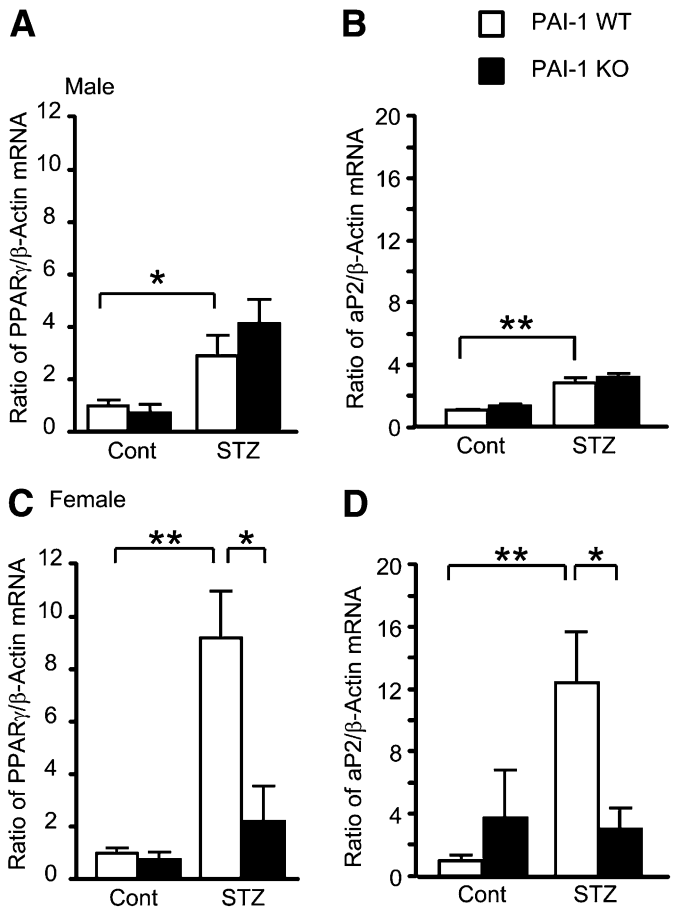

FIG. 6. Effects of PAI-1 deficiency on adipogenesis in tibia of both sexes of diabetic mice. Levels of PPARγ mRNA (*A*) and aP2 mRNA (*B*) in control and streptozotocin-treated male PAI-1 WT and KO mice. Levels of PPARγ mRNA (*C*) and aP2 mRNA (*D*) in control and streptozotocin-treated female PAI-1 WT and 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). Cont, control; STZ, streptozotocin.

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 the diabetic state only in female mice—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 β-cell apoptosis in female mice (31). However, in the current study, we show 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 β-cell apoptosis is not involved in the sex differences in the effect of PAI-1 deficiency on diabetic bone loss. In the current 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 that mechanisms other than body weight are responsible for sex differences in the effect of PAI-1 deficiency on diabetic bone loss in mice. We reveal 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 show 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 nonspecific vessel endothelial cells are responsible for the major source of PAI-1 produced by the diabetic state in female mice. DiMusto et al. (32) reported that induction of PAI-1 expression was higher in abdominal aortic aneurysm formation of female mice than in male mice, 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 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 have 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. (36) reported a sex difference in osteogenic response to vitamin D treatment in primary osteoblasts. 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 current 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 Fig. 7*D*. A 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 (Fig. 7*D*). 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 current study, PAI-1 deficiency slightly but significantly decreased BMD only in male mice—not in female mice—which is compatible with the previous

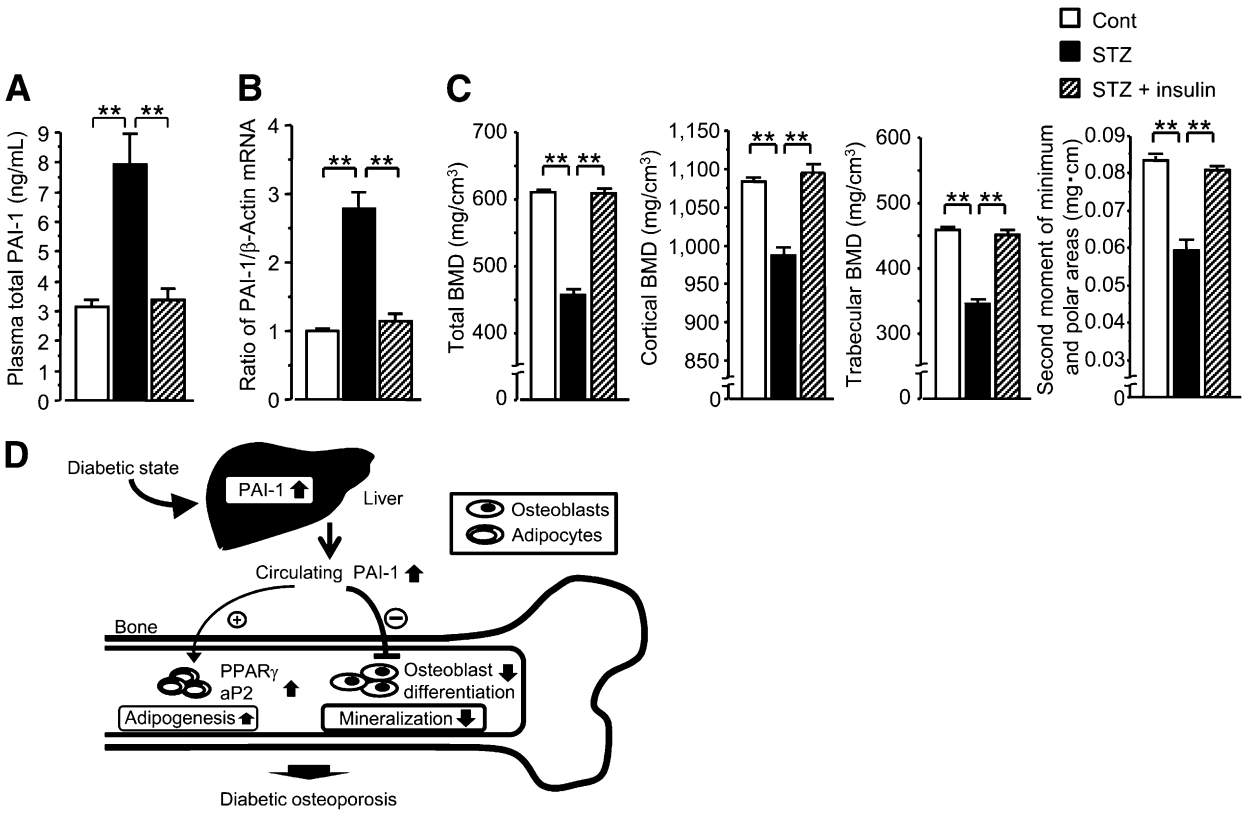

FIG. 7. Effect of insulin treatment on streptozotocin-induced bone loss in female mice. The levels of plasma PAI-1 (*A*) and PAI-1 mRNA in liver (*B*), BMDs (total, trabecular, and cortical), and bone strength index in tibia (*C*) in control female mice and diabetic female mice treated with or without insulin for 4 weeks. Results are expressed relative to β-actin mRNA values and expressed as means ± SEM. **$P < 0.01$ ($n = 6$ 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.

evidence that PAI 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 suggest 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.

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Y.T. researched data, contributed to the discussion, wrote the manuscript, and reviewed and edited the manuscript. N.K., K.Oka., M.Y., K.Oku., and O.M. contributed to the discussion and reviewed and edited the manuscript. H.K. contributed to the discussion, wrote the manuscript, and reviewed and edited the manuscript. H.K. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

REFERENCES

1. Vestergaard P. Discrepancies in bone mineral density and fracture risk in patients with type 1 and type 2 diabetes—a meta-analysis. Osteoporos Int 2007;18:427–444
2. Janghorbani M, Van Dam RM, Willett WC, Hu FB. Systematic review of type 1 and type 2 diabetes mellitus and risk of fracture. Am J Epidemiol 2007;166:495–505
3. Hofbauer LC, Brueck CC, Singh SK, Dobnig H. Osteoporosis in patients with diabetes mellitus. J Bone Miner Res 2007;22:1317–1328
4. Merlotti D, Gennari L, Dotta F, Lauro D, Nuti R. Mechanisms of impaired bone strength in type 1 and 2 diabetes. Nutr Metab Cardiovasc Dis 2010;20:683–690
5. Wongdee K, Charoenphandhu N. Osteoporosis in diabetes mellitus: Possible cellular and molecular mechanisms. World J Diabetes 2011;2:41–48
6. McCabe LR. Understanding the pathology and mechanisms of type I diabetic bone loss. J Cell Biochem 2007;102:1343–1357
7. Hoekstra T, Geleijnse JM, Schouten EG, Kluft C. Plasminogen activator inhibitor-type 1: its plasma determinants and relation with cardiovascular risk. Thromb Haemost 2004;91:861–872
8. Mathieu P, Lemieux I, Després JP. Obesity, inflammation, and cardiovascular risk. Clin Pharmacol Ther 2010;87:407–416
9. Oishi K, Ohkura N, Kasamatsu M, et al. Tissue-specific augmentation of circadian PAI-1 expression in mice with streptozotocin-induced diabetes. Thromb Res 2004;114:129–135
10. Ma LJ, Mao SL, Taylor KL, et al. Prevention of obesity and insulin resistance in mice lacking plasminogen activator inhibitor 1. Diabetes 2004;53:336–346
11. Czekay RP, Wilkins-Port CE, Higgins SP, et al. PAI-1: an integrator of cell signaling and migration. Int J Cell Biol 2011;2011:562481
12. Daci E, Verstuyf A, Moermans K, Bouillon R, Carmeliet G. Mice lacking the plasminogen activator inhibitor 1 are protected from trabecular bone loss induced by estrogen deficiency. J Bone Miner Res 2000;15:1510–1516

13. Chen X, Larson CS, West J, Zhang X, Kaufman DB. In vivo detection of extrapancreatic insulin gene expression in diabetic mice by bioluminescence imaging. PLoS ONE 2010;5:e9397
14. Watkins CC, Sawa A, Jaffrey S, et al. Insulin restores neuronal nitric oxide synthase expression and function that is lost in diabetic gastropathy. J Clin Invest 2000;106:803
15. Ogawa N, Yamaguchi T, Yano S, Yamauchi M, Yamamoto M, Sugimoto T. The combination of high glucose and advanced glycation end-products (AGEs) inhibits the mineralization of osteoblastic MC3T3-E1 cells through glucose-induced increase in the receptor for AGEs. Horm Metab Res 2007; 39:871–875
16. Hisa I, Inoue Y, Hendy GN, et al. Parathyroid hormone-responsive Smad3-related factor, Tmem119, promotes osteoblast differentiation and interacts with the bone morphogenetic protein-Runx2 pathway. J Biol Chem 2011; 286:9787–9796
17. Mazziotti G, Bilezikian J, Canalis E, Cocchi D, Giustina A. New understanding and treatments for osteoporosis. Endocrine 2012;41:58–69
18. Wang W, Zhang X, Zheng J, Yang J. High glucose stimulates adipogenic and inhibits osteogenic differentiation in MG-63 cells through cAMP/protein kinase A/extracellular signal-regulated kinase pathway. Mol Cell Biochem 2010;338:115–122
19. Pun KK, Lau P, Ho PW. The characterization, regulation, and function of insulin receptors on osteoblast-like clonal osteosarcoma cell line. J Bone Miner Res 1989;4:853–862
20. Yang J, Zhang X, Wang W, Liu J. Insulin stimulates osteoblast proliferation and differentiation through ERK and PI3K in MG-63 cells. Cell Biochem Funct 2010;28:334–341
21. Follak N, Klöting L, Wolf E, Merk H. Delayed remodeling in the early period of fracture healing in spontaneously diabetic BB/OK rats depending on the diabetic metabolic state. Histol Histopathol 2004;19:473–486
22. Follak N, Klöting I, Merk H. Influence of diabetic metabolic state on fracture healing in spontaneously diabetic rats. Diabetes Metab Res Rev 2005;21:288–296
23. Koroglu BK, Kiris F, Ersoy IH, et al. Relation of leptin, adiponectin and insulin resistance to bone mineral density in type 2 diabetic postmenopausal women. Endokrynol Pol 2011;62:429–435
24. Cloos C, Wahl P, Hasslacher C, et al. Urinary glycosylated, free and total pyridinoline and free and total deoxypyridinoline in diabetes mellitus. Clin Endocrinol (Oxf) 1998;48:317–323
25. Gunczler P, Lanes R, Paz-Martinez V, et al. Decreased lumbar spine bone mass and low bone turnover in children and adolescents with insulin dependent diabetes mellitus followed longitudinally. J Pediatr Endocrinol Metab 1998;11:413–419
26. Herrero S, Calvo OM, García-Moreno C, et al. Low bone density with normal bone turnover in ovariectomized and streptozotocin-induced diabetic rats. Calcif Tissue Int 1998;62:260–265
27. Verhaeghe J, Thomsen JS, van Bree R, van Herck E, Bouillon R, Mosekilde L. Effects of exercise and disuse on bone remodeling, bone mass, and biomechanical competence in spontaneously diabetic female rats. Bone 2000;27:249–256
28. Rosen CJ, Ackert-Bicknell C, Rodriguez JP, Pino AM. Marrow fat and the bone microenvironment: developmental, functional, and pathological implications. Crit Rev Eukaryot Gene Expr 2009;19:109–124
29. Akune T, Ohba S, Kamekura S, et al. PPARgamma insufficiency enhances osteogenesis through osteoblast formation from bone marrow progenitors. J Clin Invest 2004;113:846–855
30. Crandall DL, Quinet EM, El Ayachi S, et al. Modulation of adipose tissue development by pharmacological inhibition of PAI-1. Arterioscler Thromb Vasc Biol 2006;26:2209–2215
31. Le May C, Chu K, Hu M, et al. Estrogens protect pancreatic beta-cells from apoptosis and prevent insulin-deficient diabetes mellitus in mice. Proc Natl Acad Sci USA 2006;103:9232–9237
32. DiMusto PD, Lu G, Ghosh A, et al. Increased PAI-1 in females compared with males is protective for abdominal aortic aneurysm formation in a rodent model. Am J Physiol Heart Circ Physiol 2012;302:H1378–H1386
33. Karasik D, Ferrari SL. Contribution of gender-specific genetic factors to osteoporosis risk. Ann Hum Genet 2008;72:696–714
34. Wallace JM, Rajachar RM, Chen XD, et al. The mechanical phenotype of biglycan-deficient mice is bone- and gender-specific. Bone 2006;39:106–116
35. Nielsen KL, Allen MR, Bloomfield SA, et al. Biglycan deficiency interferes with ovariectomy-induced bone loss. J Bone Miner Res 2003;18:2152–2158
36. Olivares-Navarrete R, Hyzy SL, Chaudhri RA, Zhao G, Boyan BD, Schwartz Z. Sex dependent regulation of osteoblast response to implant surface properties by systemic hormones. Biol Sex Differ 2010;1:4
37. Daci E, Everts V, Torrekens S, et al. Increased bone formation in mice lacking plasminogen activators. J Bone Miner Res 2003;18:1167–1176