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Caspase-2 Deficiency Protects Mice From Diabetes-Induced Marrow Adiposity

Lindsay M. Coe, Dennean Lippner, Gloria I. Perez, and Laura R. McCabe*

Department of Physiology and Department of Radiology, Biomedical Imaging Research Center, Michigan State University, East Lansing, Michigan 48824

ABSTRACT

Type I (T1) diabetes is an autoimmune and metabolic disease associated with bone loss. Bone formation and density are decreased in T1-diabetic mice. Correspondingly, the number of TUNEL positive, dying osteoblasts increases in bones of T1-diabetic mice. Moreover, two known mediators of osteoblast death, TNFα and ROS, are increased in T1-diabetic bone. TNFα and oxidative stress are known to activate caspase-2, a factor involved in the extrinsic apoptotic pathway. Therefore, we investigated the requirement of caspase-2 for diabetes-induced osteoblast death and bone loss. Diabetes was induced in 16-week old C57BL/6 caspase-2 deficient mice and their wild type littermates and markers of osteoblast death, bone formation and resorption, and marrow adiposity were examined. Despite its involvement in extrinsic cell death, deficiency of caspase-2 did not prevent or reduce diabetes-induced osteoblast death as evidenced by a twofold increase in TUNEL positive osteoblasts in both mouse genotypes. Similarly, deficiency of caspase-2 did not prevent T1-diabetes induced bone loss in trabecular bone (BV/TV decreased by 30 and 50%, respectively) and cortical bone (decreased cortical thickness and area with increased marrow area). Interestingly, at this age, differences in bone parameters were not seen between genotypes. However, caspase-2 deficiency attenuated diabetes-induced bone marrow adiposity and adipocyte gene expression. Taken together, our data suggest that caspase-2 deficiency may play a role in promoting marrow adiposity under stress or disease conditions, but it is not required for T1-diabetes induced bone loss. J. Cell. Biochem. 112: 2403–2411, 2011. © 2011 Wiley-Liss, Inc.

KEY WORDS: CASPASE-2; DIABETES; BONE; MARROW; ADIPOSITY

Type I (T1) diabetes affects over 800,000 men and women in the United States and is commonly characterized by elevated blood glucose levels (greater than 200 mg/dl) and little or no insulin secretion. Many known secondary complications arise from T1-diabetes including osteoporosis. Both male and female patients are vulnerable to decreased bone density and increased fracture risk (Levin et al., 1976 #607; Bouillon, 1991 #613; Forsen et al., 1999 #612; Krakauer et al., 1995 #606; Alexopoulou et al., 2006 #611; Khazai et al., 2009 #616). T1-diabetic rodent models (pharmacological and spontaneous) exhibit decreased bone density comparable to the human bone phenotype [Martin and McCabe, 2007]. To date, bone loss has been detected in all bones examined (e.g., femur, tibia, calvaria, vertebrae) in mouse models of T1-diabetes and is independent of gender [Botolin and McCabe, 2007; Martin and McCabe, 2007; McCabe, 2007], similar to humans. Although exact mechanisms of T1-diabetic bone loss remain unknown, recent studies have demonstrated a role for decreased osteoblast number and activity in diabetic bones [Botolin and McCabe, 2007; Martin and McCabe, 2007; McCabe, 2007]. Decreased bone formation can occur through multiple mechanisms including altered mesenchymal stem cell linage selection (adipocytes over osteoblasts), reduced osteoblast differentiation, and increased osteoblast apoptosis [McCabe, 2007; Coe et al., 2011]. Past studies in our laboratory have demonstrated that bone loss occurs irrespective of marrow adiposity/altered stem cell lineage selection [Botolin and McCabe, 2006; Martin and McCabe, 2007; Motyl and McCabe, 2009]. This has led us to focus on the role of osteoblast apoptosis in diabetic bone loss.

All osteoclasts and roughly 60–80% of osteoblasts [Weinstein and Manolagas, 2000] undergo programmed cell death (PCD) as an integral physiological component of bone remodeling, fracture repair, and bone rejuvenation. PCD has been implicated in bone adaptation with unloading [Aguirre et al., 2006; Swift et al., 2010], aging [Perez et al., 2007], and bone-destructive diseases such as rheumatoid arthritis, periodontal diseases, and T1-diabetes [Mogi et al., 1999; Liu et al., 2006; Graves et al., 2007; Coe et al., 2010]. Recently, our laboratory demonstrated an increase in osteoblast

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*Correspondence to: Laura R. McCabe, PhD, Departments of Physiology and Radiology, Michigan State University, 2201 Biomedical and Physical Science Bldg., East Lansing, MI 48824. E-mail: mccabel@msu.edu

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TUNEL staining during diabetes onset [Coe et al., 2010], which is consistent with the first detectable suppression of osteocalcin gene expression in bone [Motyl et al., 2009]. Therefore, increased osteoblast apoptosis may play a critical role in mediating T1-diabetic bone loss.

TNFα and reactive oxygen species (ROS) are two known mediators of osteoblast death (Graves, 1999 #797; Hock et al., 2001 #790; Xing and Boyce, 2005 #806; Manolagas, 2010 #782; Rached et al., 2010b #566; Rached et al., 2010a #573). Several studies have identified increased TNFα and ROS in diabetic bone [Hill et al., 1997 #789; Hamada et al., 2007; Hamada et al., 2009; Motyl et al., 2009; Roszer, 2011] suggesting a possible role for either one of these factors in mediating diabetes induced osteoblast death. TNFα levels have been shown to be elevated in diabetic serum, but more importantly, TNFα mRNA levels/expression are also elevated in the bone microenvironment during diabetes onset [Motyl et al., 2009; Coe et al., 2011]; this is the time when increased osteoblast death and reduced osteocalcin expression are first observed [Motyl et al., 2009]. Furthermore, the increased osteoblast death induced by isolated diabetic marrow cells is suppressed by treatment with TNFα neutralizing antibodies [Coe et al., 2011]. Correspondingly, entanercept or pegsunercept, TNFα inhibitors used clinically, suppress diabetes-enhanced death of fibroblasts, bone-lining cells and osteoblasts arising from periodontal disease in vivo [Liu et al., 2006; Alblowi et al., 2009; Kayal et al., 2010]. Similarly, ROS can contribute to diabetic complications and cell death. Past studies demonstrate that ROS levels are elevated in bones of T1-diabetic mice [Hamada et al.] as well as in spontaneously developing T2-diabetic Torii rats [Fujii et al., 2008]. Hyperglycemia, advanced glycation end products (AGE) accumulation, superoxide production, and/or pro-inflammatory cytokines (TNFα and INFγ) are thought to contribute to the elevated ROS levels [Hamada et al., 2009; Manolagas, 2010]. When oxidative stress is reduced, a notable reduction in osteoblast apoptosis is observed [Rached et al., 2010a; Rached et al., 2010b]. Reduction of oxidative stress in diabetic mice, by overexpressing an intracellular antioxidant, thioredoxin-1 (TRX), attenuates bone loss [Hamada et al., 2007; Hamada et al., 2009]. Taken together, both TNFα and ROS likely contribute, to some extent, to the diabetic bone phenotype.

Cells can die by two main apoptotic regulatory pathways: Intrinsic and extrinsic. Intrinsic apoptosis, also known as mitochondria-mediated cell death, is regulated through the balance of pro-apoptotic factors (bax, bak, bim, bid) and anti-apoptotic factors (bcl-2 and bcl-xl). The extrinsic pathway can be activated by extracellular ligands, including TNFα and Fas, which bind membrane bound receptors (i.e., TNFR) and trigger a cascade of caspase activations [Xing and Boyce, 2005]. ROS enhances intracellular oxidative stress and thereby triggers a cascade of caspase activations [Prasad et al., 2006]. Studies show that both ROS and TNFα activate multiple caspases including, caspase-2, -3, -8, and -9 [Prasad et al., 2006; Kim et al., 2010]. Caspases are a family of proteins that contain a cysteine residue capable of cleaving an aspartic acid-containing motif on the next downstream caspase. Two different caspase groups are involved in PCD: Initiators and effectors. Initiator caspases (i.e., caspase-2, -8, -9) are responsible for activation of the effector caspases (i.e., caspase-3, -7) that cleave an array of cellular substrates [Elmore, 2007].

Little is known about which caspase contribute to cell death in diabetic bone. Here we focused on the role of caspase-2, which is the most conserved caspase, sharing sequence homology with most initiator caspases but functioning more closely to the effector caspases, which makes it an intriguing caspase to study [Vakifahmetoglu-Norberg and Zhivotovsky, 2010]. Caspase-2 can be activated in response to cell stress, oxidative stress and TNFα treatment (Troy and Shelanski, 2003 #741; Braga et al., 2008 #738; Troy and Ribe, 2008 #743), all conditions that are elevated in diabetic mouse bone. Additionally, caspase-2 deficient mouse embryonic fibroblasts, derived from mesenchymal stem cells (which can differentiate into osteoblasts), showed protection from drug induced cell death, indicating a role for caspase-2 in mesenchymal-derived cell death [Ho et al., 2008]. Here we demonstrate that caspase-2 is not required for type I diabetes induced osteoblast death or bone loss, but surprisingly caspase-2 deficiency attenuates diabetes-induced marrow adiposity.

MATERIALS AND METHODS

ANIMALS

Diabetes was induced in C57BL/6 adult (15–16 week old) caspase-2 deficient and wild type littermate male mice by five consecutive daily intraperitoneal injections of streptozotocin (50 mg/kg body weight in 0.1 M citrate buffer, pH 4.5). Corresponding genotype controls were given citrate buffer alone. Mice were maintained on a 12-h light, 12-h dark cycle at 23°C, given standard lab chow and water ad libitum. Diabetes was confirmed by blood glucose measurements (>300 mg/dl) obtained with an Accu-Check compact glucometer (Roche Diagnostics Corporation, Indianapolis, IN). Mice were euthanized at 5 or 40 days after diabetes induction and total body, tibialis anterior, and subcutaneous femoral fat pad mass were recorded. Animal procedures were approved by the Institutional Animal Care and Use Committee at Michigan State University.

GENOTYPING

DNA was obtained from tail snips of wild type and caspase-2 deficient mice. Caspase-2 deficient mice have a mutation that deletes the exon encoding the QACRG active site of the enzyme and a portion of the subsequent exon that encodes for the caspase-2 short isoform. This deletion inactivates both the long and short form of caspase-2. Genotyping assessed the presence or absence of this region as previously described [Bergeron et al., 1998]. PCR products were separated on a 1.5% agarose gel to ensure the presence or absence of an amplicon band.

RNA ANALYSIS

Immediately after euthanasia, tibias were cleaned of soft tissue and one was snap frozen in liquid nitrogen and stored at −80°C. RNA was extracted from the bone using Tri Reagent (Molecular Research Center, Inc., Cincinnati, OH). RNA integrity was determined by formaldehyde–agarose gel electrophoresis. cDNA was synthesized using Superscript II Reverse Transcriptase Kit and oligo dT (12–18) primers (Invitrogen, Carlsbad, CA). cDNA was amplified by

quantitative real time PCR with iQ SYBR Green Supermix (Bio-Rad, Hercules, CA) and gene-specific primers. Primers for fatty acid binding protein (aP2), Bcl-2 associated protein-X (Bax), B-cell lymphoma-2 (Bcl-2), hypoxanthine-guanine phosphoribosyl transferase (HPRT), osteocalcin, tumor necrosis factor-α (TNFα), tartrate resistant acid phosphatase 5 (TRAP5) are previously described [Coe et al., 2010]. HPRT served as a housekeeping gene; its levels were not altered in bone by diabetes. Each amplification cycle consisted of 95°C for 15 s, 60°C for 30 s (except osteocalcin which had an annealing temperature of 65°C) and 72°C for 30 s. RNA-free samples were used as a negative control and did not produce amplicons. PCR products were separated on 1.5% agarose gel electrophoresis and sequenced to verify that the desired gene is being amplified.

MICRO-COMPUTED TOMOGRAPHY (MCT) ANALYSIS

Fixed femurs (stored in 70% alcohol) were scanned using the GE Explore μCT system at a voxel resolution of 20 μm from 720 views with a beam strength of 80 kvp and 450 μA and an integration time of 2,000 ms. Scans included bones from each condition and a phantom bone to standardize the grayscale values and maintain consistency between runs. Using the system's auto-threshold (1,000) and an isosurface analysis, trabecular bone densities (bone volume fraction (BV/TV), bone mineral density (BMD) and content (BMC), trabecular number (Tb. N), and trabecular thickness (Tb. Th) were measured in the trabecular region defined at 0.17 mm under the growth plate of the femur extending 2 mm toward the diaphysis, and excluding the outer cortical shell. Cortical bone measurements were determined with a 2 mm^3 region of interest (ROI) in the mid-diaphysis, with the exception of cortical bone mineral content and bone mineral density, which were measured using a smaller 0.1 mm^3 ROI. Cortical BMC, BMD, moment of inertia, thickness, perimeters and areas, and trabecular densities were computed by the GE Microview Software for visualization and analysis of volumetric image data.

BONE HISTOLOGY AND HISTOMORPHOMETRY

Bones fixed in formalin underwent dehydration, clearing, and infiltration on a routine overnight processing schedule. Samples were then paraffin embedded and sectioned at 5 μm using a rotary microtome. Osteoclasts were identified, by staining for TRAP (Sigma). Osteoclasts, osteoblasts, and adipocytes were identified in the trabecular region of the femur, defined at 0.17 mm under the growth plate and extending 2 mm toward to diaphysis. Osteoclast and osteoblast surfaces were measured in three trabecular regions for each mouse and expressed as a percent of total trabecular surface. Visible adipocytes, greater than 30 μm, were counted in the same trabecular region.

Cell death was determined using a TACS•XL® Basic In Situ Apoptosis Detection Kit (TUNEL, Trevigen Inc., Gaithersburg, MD) on femur sections; osteoblasts with positive nuclei were counted and expressed as a percentage of total osteoblasts counted per bone. Positive controls included slides incubated with nuclease. Five trabecular regions were examined for each mouse. Total osteoblasts counted ranged between 83 and 190 per bone. Over 500 total osteoblasts were counted per condition.

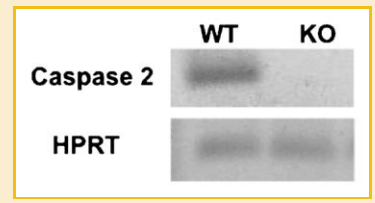

Fig. 1. The absence of detectable caspase-2 mRNA in tibias RNA isolates from deficient mice. Caspase-2 mRNA expression was examined in wild type (left) and caspase-2 deficient (right) bone. PCR products were subsequently run on a 1.5% agarose gel for the absence of caspase-2. RNA levels are expressed relative to the housekeeping gene HPRT.

STATISTICAL ANALYSIS

All data are presented as mean ± standard error. Statistical significance (P-value < 0.05) of main effects (genotype difference or diabetes) as well as genotype × diabetes interaction (which determines if diabetes alters the genotype effect or vise versa) was determined using factorial analysis of variance (ANOVA) with SPSS statistical software (Chicago, IL). Student's t-test was also used to determine significance where necessary.

RESULTS

Cellular caspase-2 can be activated by oxidative stress and TNFα signaling, all of which are present in T1-diabetes. To test if caspase-2 is required for the induction of diabetic bone pathology, and in turn could be a therapeutic target, we induced diabetes in caspase-2 deficient and littermate wild type mice. The absence of caspase-2 was determined by genotyping and confirmed by the lack of detectable caspase-2 mRNA in bone RNA isolates from deficient mice (Fig. 1). At 15–16 weeks of age wild type and caspase-2 deficient mice were injected with streptozotocin (to induce diabetes) or vehicle (controls). Blood glucose levels illustrated that caspase-2 deficiency did not impact diabetes induction or severity. Specifically, diabetic wild type and diabetic caspase-2 deficient mice displayed similar elevations in blood glucose levels, averaging 459 and 474 mg/dl, respectively (Table I). Consistent with previous studies, T1-diabetes caused decreases in total body mass (12 and 17%, respectively) and femoral fat pad mass (64 and 60%, respectively) in both wild type and caspase-2 deficient mice. Although there was a trend toward decreased lean muscle mass in diabetic mice, it did not reach statistical significance (unlike previous studies). Taken together, caspase-2 deficiency did not

TABLE I. Type 1 Diabetic Mouse Parameters

	Wild Type		Caspase-$2^{-/-}$	
	Control	Diabetic	Control	Diabetic
Total body Mass (g)	31.0 ± 0.8	27.3 ± 1*	29.2 ± 1	24.3 ± 1.6*
Blood Glucose (mg/dl)	163 ± 6	459 ± 18*	158 ± 8	474 ± 31*
Tibialis Anterior Mass (mg)	80 ± 4	75 ± 4	76 ± 5	67 ± 5
Femoral Fat Pad Mass (mg)	360 ± 37	128 ± 18*	322 ± 31	128 ± 31*

n ≥ 10 mice per group.
*$P < 0.05$.

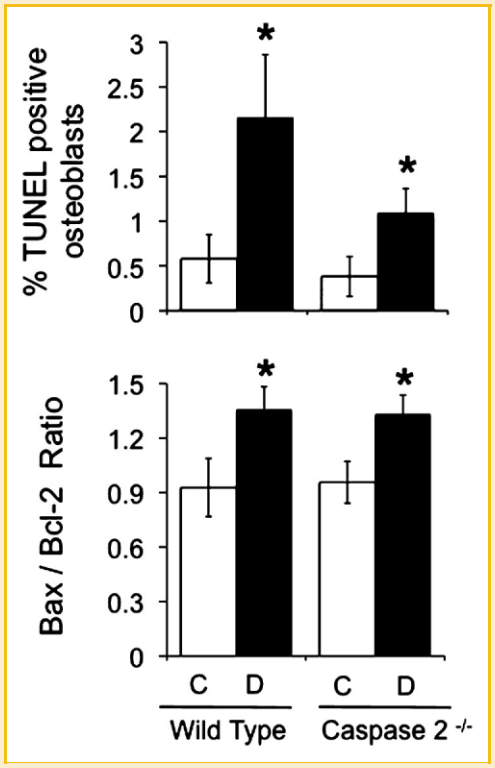

Fig. 2. Caspase-2 deficiency does not prevent T1-diabetes-induced osteoblast death. Percentage TUNEL positive osteoblasts and BAX/Bcl-2 RNA ratio from bone of control (white bars) and streptozotocin-induced diabetes (black bars) in caspase-2 deficient and wild type littermate mice at 5 days post injection (dpi) is shown. RNA levels are calculated relative to the housekeeping gene HPRT. Bars represent the average value ± SE ($n \geq 5$ per group); * denotes $P < 0.05$. For TUNEL assay $n > 500$ osteoblasts per condition were counted.

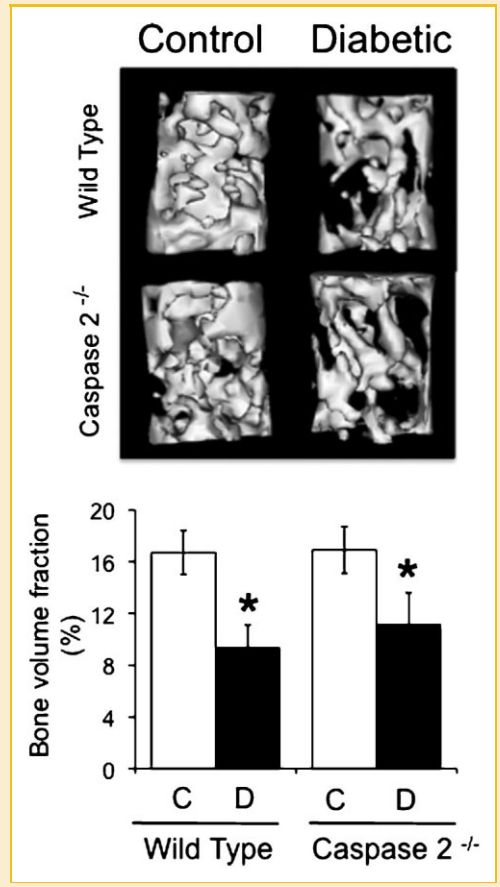

Fig. 3. Decreased bone volume fraction in wild type and caspase-2 deficient diabetic mice. Representative μCT isosurface images of trabecular bone volume in distal femurs were obtained from control and diabetic caspase-2 deficient and wild type mice. Graphical representation of bone volume fraction is displayed from control (white bars) and diabetic (black bars) caspase-2 knockout and wild type mice. Bars represent the average value ± SE ($n \geq 8$ per group); * denotes $P < 0.05$.

modify general physiologic responses to T1-diabetes.played an increase in TUNEL positive osteoblasts compared to caspase-2 deficient controls, however, it was somewhat less than in wild type mice. Statistical analyses, however, found no significant difference in the percentage of TUNEL positive osteoblasts between genotypes. We also examined the ratio of Bax/Bcl-2 mRNA levels, which positively correlates with increased cell death. The ratio was elevated in both diabetic wild type and diabetic caspase-2 deficient mice compared to their corresponding genotype control mice, but did not differ between wild type and deficient control mice, consistent with TUNEL analyses.

Next, we examined the impact of caspase-2 deficiency on diabetic bone loss at 40 days post-diabetes induction, a time point when mice lose a significant amount of bone [Botolin and McCabe, 2007; McCabe, 2007]. Femurs were imaged by micro-computed tomography; representative three-dimensional isosurface images are shown (Fig. 3). As expected, wild type diabetic mice lost an average of 50% BV/TV. Caspase-2 deficient diabetic mice also lost BV/TV although it was somewhat less (30%), but not significantly different from wild type diabetic mice. Cortical bone parameters did not differ between genotypes (Fig. 4, Table II). However, T1-diabetes did have a significant effect on cortical bone parameters of both wild type and caspase-2 deficient mice. Specifically, cortical thickness was decreased and the inner (marrow/endosteal) cortical perimeter was increased (Table II). These changes resulted in a larger bone marrow area; this response was apparent in both diabetic genotypes.

Histomorphometry and molecular phenotyping of osteoblasts and osteoclasts indicated that T1-diabetes suppressed osteoblast parameters. Tibia osteocalcin mRNA levels and the percentage of osteoblasts per total trabecular surface area were reduced in wild type and caspase-2 deficient mice (Fig. 5), consistent with an increase in TUNEL positive osteoblasts early in disease progression (Fig. 1). Osteoblast parameters did not differ between control wild type and caspase-2 deficient mice, as previously reported [Zhang et al., 2007], although, osteoblast surface displayed a trend to decrease in caspase-2 deficient ($P < 0.06$). Markers of resorption indicated that osteoclast surface area was not significantly altered by diabetes (Fig. 6). However, caspase-2 deficiency alone decreased TRAP5 mRNA levels (expressed by matures osteoclasts) in control mice.

Previous reports demonstrated elevated marrow adiposity in T1-diabetic animal models [Botolin and McCabe, 2007; Martin and McCabe, 2007; McCabe, 2007]; therefore, we also investigated

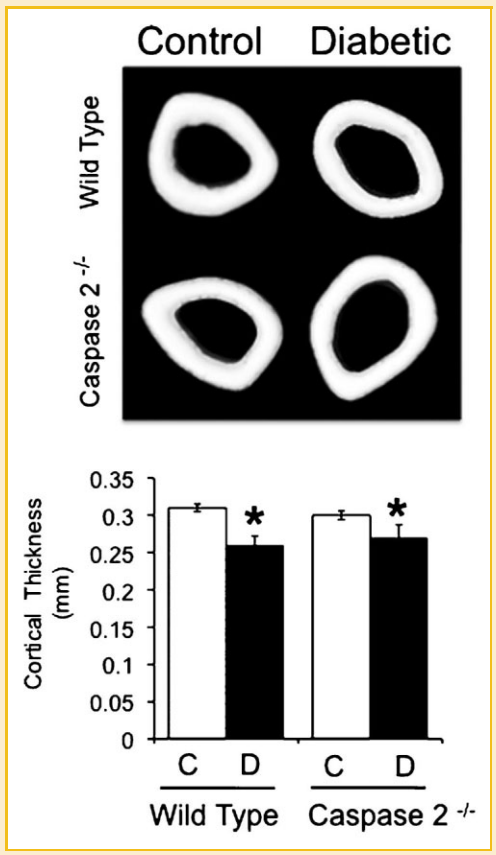

Fig. 4. Decreased cortical bone parameters in wild type and caspase-2 deficient diabetic mice. Representative μCT isosurface slices of the cortical bone (mid-diaphysis) in femurs obtained from control and diabetic caspase-2 deficient and wild type mice. Graphical representation of cortical bone thickness at 40 dpi from control (white bars) and diabetic (black bars) caspase-2 knockout and wild type mice. Bars represent the average value ± SE ($n \geq 8$ per group); * denotes $P < 0.05$.

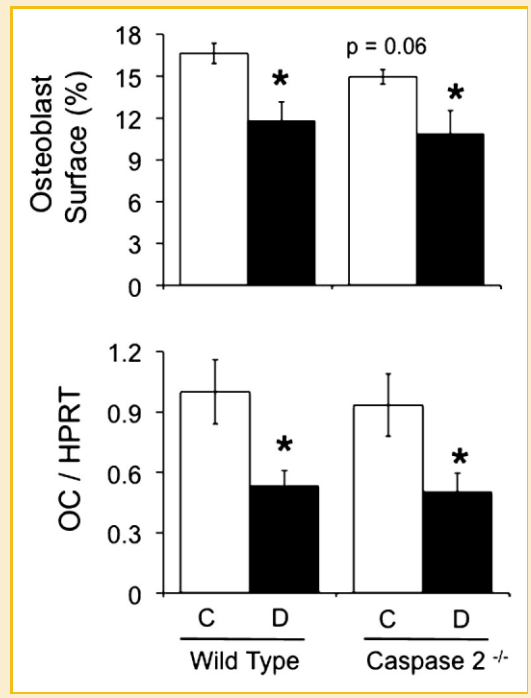

Fig. 5. T1-diabetes decreased bone formation markers in caspase-2 deficient and wild type mice. Control (white bars) and diabetic (black bars) caspase-2 knockout and wild type littermates were examined at 40 dpi for the percent osteoblast surface per total trabecular surface and tibia osteocalcin mRNA expression. RNA levels are calculated relative to the housekeeping gene HPRT. Bars represent the average value ± SE ($n \geq 8$ per group); * denotes $P < 0.05$.

markers of adiposity. As expected, wild type T1-diabetic mice displayed a twofold increase in marrow adipocyte number and elevated tibia PPARγ (early adipocyte marker) and aP2 mRNA levels (mature adipocyte marker), by 40 and 67%, respectively (Fig. 7). Interestingly, caspase-2 deficiency prevented diabetic-induced marrow adipocyte number and gene expression (Fig. 7). We also observed that caspase-2 deficiency effectively reduced bone PPARγ levels in control mice (Fig. 7B).

TABLE II. Trabecular and Cortical μCT Bone Parameters

	Wild Type		Caspase-$2^{-/-}$	
	Control (n = 14)	Diabetic (n = 11)	Control (n = 12)	Diabetic (n = 7)
Trabecular				
BMC (mg)	0.63 ± 0.03	0.51 ± 1.04*	0.66 ± 0.03	0.54 ± 0.05*
BMD (mg/cc)	210 ± 10	164 ± 12*	217 ± 10	178 ± 17*
BV/TV (%)	16.7 ± 1.7	9.4 ± 1.7*	16.9 ± 0.2	11.2 ± 2.4*
Tb Th (μm)	39.8 ± 1.2	31.3 ± 1.8*	40.3 ± 1.19	34.7 ± 2.38*
Tb N (mm^{-1})	4.06 ± 0.30	2.79 ± 0.37*	4.13 ± 0.41	3.35 ± 0.50
Cortical				
Th (mm)	0.31 ± 0.005	0.26 ± 0.012*	0.3 ± 0.006	0.27 ± 0.017*
MOI (mm^4)	0.26 ± 0.02	0.25 ± 0.02	0.26 ± 0.02	0.28 ± 0.03
Inner P (mm)	3.23 ± 0.06	3.48 ± 0.08*	3.25 ± 0.06	3.54 ± 0.09*
Outer P (mm)	5.26 ± 0.07	5.21 ± 0.06	5.24 ± 0.06	5.32 ± 0.09
Marrow Area (mm^2)	0.75 ± 0.03	0.87 ± 0.04*	0.77 ± 0.03	0.90 ± 0.04*
Cortical Area (mm^2)	1.25 ± 0.03	1.08 ± 0.06*	1.24 ± 0.03	1.15 ± 0.09
Total Area (mm^2)	1.99 ± 0.05	1.94 ± 0.07	2.00 ± 0.05	2.05 ± 0.06
BMD (mg/cc)	1093 ± 28	1041 ± 40	1159 ± 23**	1047 ± 42*

Abbreviations: BMC, bone mineral content; BMD, bone mineral density; BVF, bone volume fraction; Tb. Th, trabecular thickness; Tb. N, trabecular number; Th, thickness; MOI, moment of inertia; P, perimeter.
*$P < 0.05$ compared to genotype control.
**$P < 0.05$ compared to wild type control.

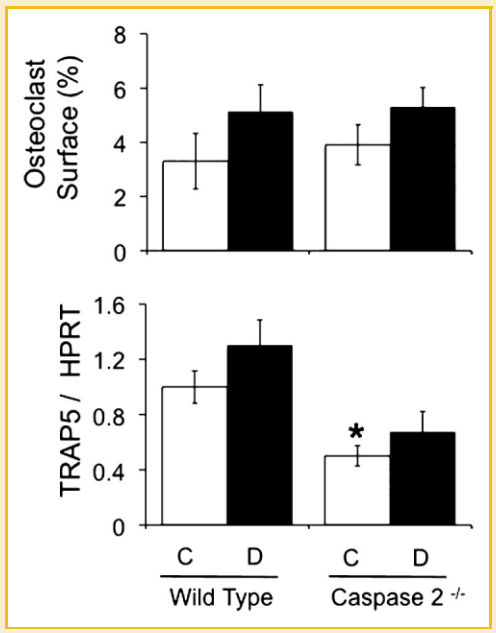

Fig. 6. Bone resorption parameters unchanged in caspase-2 deficient and wild type mice in response to T1-diabetes. Control (white bars) and diabetic (black bars) caspase-2 knockout and wild type littermates were examined at 40 dpi for percent osteoclast surface per total trabecular surface and bone Trap5 mRNA expression. RNA levels are calculated relative to the housekeeping gene HPRT. Bars represent the average value ± SE ($n \geq 8$ per group); * denotes $P < 0.05$.

DISCUSSION

Enhanced cellular apoptosis is associated with diabetic complications including retinopathy [Mohr et al., 2002; Romeo et al., 2002; Al-Mashat et al., 2006; Motyl et al., 2009], nephropathy [Kumar et al., 2004], and bone loss [Coe et al., 2010] and its repair [Al-Mashat et al., 2006; Liu et al., 2006]. The association of T1-diabetes with increased pro-inflammatory cytokines, (i.e., TNFα and IL-1β) [Motyl et al., 2009] and cell and oxidative stress [Hamada et al., 2009] led us to test the requirement of caspase-2 for the development of T1-diabetic bone pathology. Here we demonstrate that caspase-2 deficiency does not change trabecular or cortical bone parameters under control or diabetic conditions, compared to wild type mouse responses. Surprisingly, caspase-2 deficiency reduced diabetes induced marrow adiposity, suggesting a role for caspase-2 in promoting marrow fat under stress/disease conditions.

Little is known about the molecular mechanisms leading to increased osteoblast death in T1-diabetes. Our studies are the first to investigate the requirement of caspase-2 in this pathology. Although important for cell death, caspase-2 appears not to be a main contributor to T1-diabetes-induced osteoblast death. Redundancy and/or compensation from another caspase, e.g., caspase-8 or -12, as previously reported in neurons [Troy et al., 2001], and germ cells [Takai et al., 2007] could address why deletion of caspase-2 resulted in osteoblast death. However, we did not observe a change in caspase-8 mRNA levels between caspase-2 deficient and wild type control and diabetic tibias (data not shown). It is also possible that disrupting caspase-2 may not prevent activation of an intrinsic apoptotic mediator like Bax or Bid, both of which are suggested to be linked to activation of downstream caspases (e.g., caspase-3 and -9) in AGE and ROS induced osteoblast death [Roszer, 2010].

The lack of a genotype difference in bone density is consistent with previous reports demonstrating that older 14-month-old caspase-2 deficient mice display similar trabecular BMD to wild type mice [Zhang et al., 2007]. Interestingly, previous reports suggest that caspase-2 deficiency may compromise removal of oxidatively damaged cells [Zhang et al., 2007], which could contribute to an overall elevation in oxidative stress components (e.g., ROS) and have a negative impact on organ health. Consistent with this idea, caspase-2 deficient mice at 24–26 months of age exhibit lower BMD than wild type mice as a result of increased bone resorption [Zhang et al., 2007].

Surprisingly, caspase-2 deficiency did not significantly alter diabetes-induced changes in trabecular and cortical bone density parameters. While the percentage of trabecular bone loss was slightly decreased in caspase-2 deficient mice, the actual values were not significantly different between diabetic wild type and caspase-2 deficient mice and are consistent with previous reports in diabetic mice [Martin and McCabe, 2007; McCabe, 2007]. Cortical bone parameters were also altered as a result of diabetes, consistent with previous studies in rodent models [Silva et al., 2009] and humans [Santiago et al., 1977; Saha et al., 2009], and was not genotype dependent. If caspase-2 were required for diabetes-induced osteoblast cell death, deficient mice would have exhibited altered osteoblast markers and reduced bone loss. Interestingly, caspase-2 may have positive effects on bone, as seen in old mice [Zhang et al., 2007], can its loss could have compounded diabetic bone loss through increased bone resorption. We may not have observed this because our mice are young and diabetes can suppress osteoclast activity and bone turnover in general.

What is the role of caspase-2 in osteoblasts? Our studies suggest that it is not critical for mediating osteoblast apoptosis and may exhibit redundancy. Capsase-2 may also act in other cellular functions. Similarities between cell death and cell cycle progression suggest that some apoptosis mediators may participate in cell cycle regulation [Vermeulen et al., 2003]. Caspase-2 is demonstrated to participate in cell cycle regulation, DNA repair, and tumor suppression, to even a greater extent than PCD [Ho et al., 2008; Kitevska et al., 2009; Shi et al., 2009; Vakifahmetoglu-Norberg and Zhivotovsky, 2010]. Cells deficient in caspase-2 had partial loss of the G2/M checkpoint modulator resulting in cells continuing to cycle within this timeframe [Kitevska et al., 2009; Shi et al., 2009]. Thus, it may be possible that adipocyte precursors have reduced differentiation potential.

Interestingly, caspase-2 deficient diabetic mice were protected from increases in marrow adiposity. Although elevated marrow adiposity is commonly observed in both pharmacological and spontaneously diabetic mice [Botolin and McCabe, 2007; Martin and McCabe, 2007], several studies have reported that inhibition of marrow adiposity did not prevent diabetes-induced bone loss. These findings suggest that the inverse relationship between marrow adiposity and bone loss does not always occur [Botolin and McCabe, 2006; Motyl and McCabe, 2009]. Since early markers of adipocyte

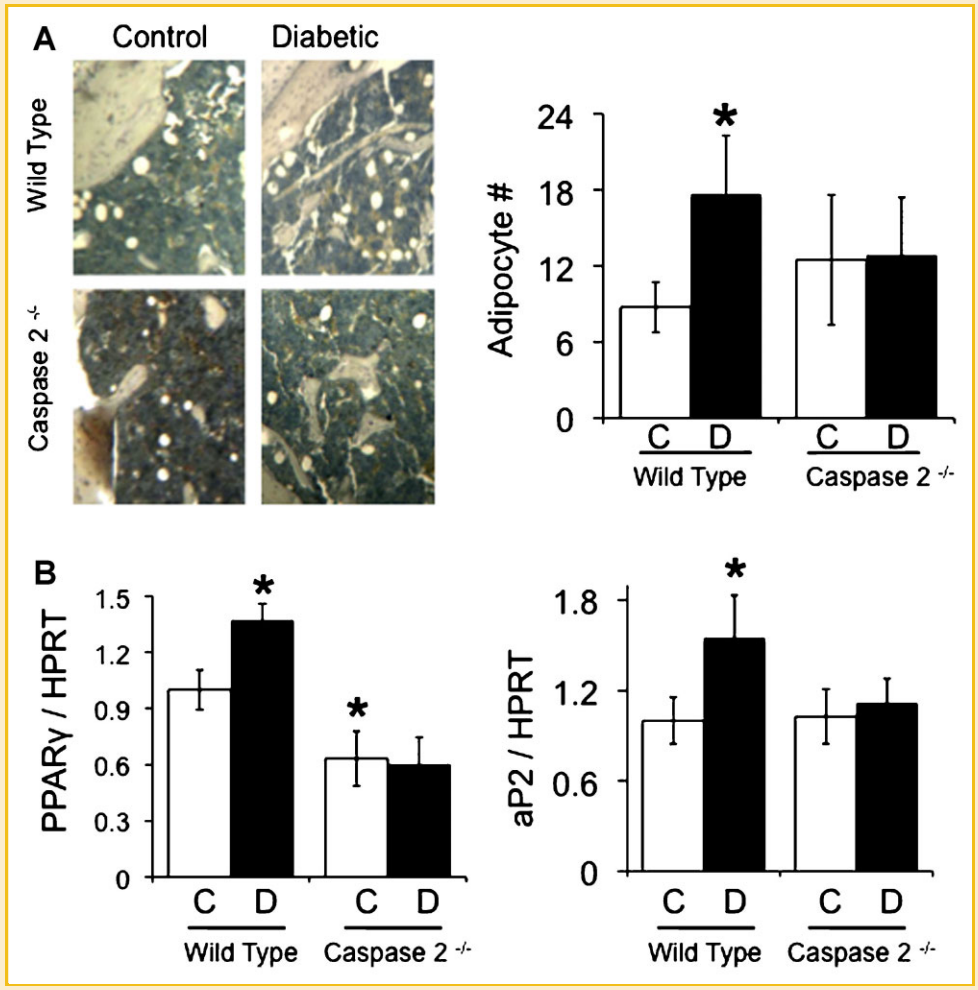

Fig. 7. Caspase-2 deficiency prevents T1-diabetes induced marrow adiposity. A: Representative adipocyte images were obtained from femurs of control and diabetic caspase-2 deficient and wild type mice. Graphical representation of adipocyte numbers in control (white bars) and diabetic (black bars) caspase-2 knockout and wild type mice. B: Expression of early and late adipocyte mRNA markers, PPARγ (early marker measured at 5 dpi to capture changes in differentiation) and aP2 (expressed in mature adipocytes measured at 40 dpi), were examined and calculated relative to the housekeeping gene HPRT. Bars represent the average value ± SE ($n \geq 5$–8 per group). Statistical analyses by factorial analysis of variance (ANOVA) was used to determine the effects of genotype, diabetes, and genotype × diabetes on adipocyte parameters, * denotes $P < 0.05$ by student *t*-test.

differentiation (PPARγ) are decreased in caspase-2 deficient mice, one hypothesis is that caspase-2 expression is involved in adipocyte differentiation. Sterol regulatory element binding protein (formally named adipocyte determination and differentiation factor 1), is required for adipocyte differentiation [Logette et al., 2005a,b] and is thought to mediate increased caspase-2 activity in these cells. Disrupting caspase-2 by siRNA impairs lipid levels in human adipocytes [Logette et al., 2005a,b], suggesting a role for caspase-2 in fatty acid and triglyceride synthesis. A second hypothesis is that caspase-2 is involved in modifying (cleaving) PPARγ and affecting its ability to regulate adipogenesis and adipocyte differentiation [He et al., 2008; Guilherme et al., 2009]. Several studies demonstrate that TNFα can activate many caspases, including 1 through 9, in adipocytes and lead to PPARγ cleavage [He et al., 2008; Guilherme et al., 2009]. Consistent with these findings, broad-spectrum caspase inhibitors decrease TNFα-mediated PPARγ cleavage and fragmentation [He et al., 2008; Guilherme et al., 2009]. Taken together, eliminating caspase-2 could result in altered transcriptional effects of PPARγ that in turn decrease adipogenesis and adipocyte differentiation, thus preventing T1-diabetes-induced marrow adiposity.

In summary, inhibition of caspase-2 prevented T1-diabetes induced marrow adiposity, but did not attenuate diabetes induced osteoblast death or bone loss. Our results suggest that caspase-2 is not involved in diabetes-mediated osteoblast death and may play a more vital role in other cell functions. Further investigation is needed to understand the mechanisms mediating T1-diabetes associated osteoblast death.

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