Published in final edited form as:

Mol Cell Endocrinol. 2016 February 15; 422: 211–220. doi:10.1016/j.mce.2015.12.005.

Deregulation of arginase induces bone complications in high-fat/high-sucrose diet diabetic mouse model

Anil Bhatta^{2,*}, Rajnikumar Sangani^{1,*}, Ravindra Kolhe³, Haroldo A. Toque², Michael Cain¹, Abby Wong¹, Nicole Howie⁴, Rahul Shinde³, Mohammed Elsalanty⁴, Lin Yao², Norman Chutkan⁵, Monty Hunter¹, Ruth B. Caldwell⁶, Carlos Isales^{1,7}, R. William Caldwell^{2,¶}, and Sadanand Fulzele^{1,7,¶}

¹Departments of Orthopaedic Surgery, Georgia Regents University, Augusta, GA 30912

²Department of Pharmacology and Toxicology, Georgia Regents University, Augusta, GA 30912

³Departments of Pathology, Georgia Regents University, Augusta, GA 30912

⁴School of Dentistry, Georgia Regents University, Augusta, GA 30912, Augusta, GA 30912

⁵The CORE Institute, Phoenix, AZ 85023, USA

⁶Cell Biology and Anatomy and Vascular Biology Center, Georgia Regents University; Charlie Norwood VA Medical Center, Augusta, GA, 30912

⁷Institute of Regenerative and Reparative Medicine, Georgia Regents University, Augusta, GA, 30912

Abstract

A balanced diet is crucial for healthy development and prevention of musculoskeletal related diseases. Diets high in fat content are known to cause obesity, diabetes and a number of other disease states. Our group and others have previously reported that activity of the urea cycle enzyme arginase is involved in diabetes-induced dysregulation of vascular function due to decreases in nitric oxide formation. We hypothesized that diabetes may also elevate arginase activity in bone and bone marrow, which could lead to bone-related complications. To test this we determined the effects of diabetes on expression and activity of arginase, in bone and bone marrow stromal cells (BMSCs). We demonstrated that arginase 1 is abundantly present in the bone and BMSCs. We also demonstrated that arginase activity and expression in bone and bone marrow is up-regulated in models of diabetes induced by HFHS diet and streptozotocin (STZ). HFHS diet down-regulated expression of healthy bone metabolism markers (BMP2, COL-1, ALP, and RUNX2) and reduced bone mineral density, bone volume and trabecular thickness. However, treatment with an arginase inhibitor (ABH) prevented these bone-related complications of

¶Corresponding Authors: ¶Sadanand Fulzele, DVM, PhD, sfulzele@gru.edu, Department of Orthopaedic Surgery, Georgia Regents University, Augusta, GA 30904, Tel: (706) 721-4850. ¶R. William Caldwell, PhD, wcaldwel@gru.edu, Department of Pharmacology and Toxicology, Georgia Regents University, Augusta, GA 30904, Phone: (706) 721-3383.

*Equal contribution

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diabetes. In-vitro study of BMSCs showed that high glucose treatment increased arginase activity and decreased nitric oxide production. These effects were reversed by treatment with an arginase inhibitor (ABH). Our study provides evidence that deregulation of L-arginine metabolism plays a vital role in HFHS diet-induced diabetic complications and that these complications can be prevented by treatment with arginase inhibitors. The modulation of L-arginine metabolism in disease could offer a novel therapeutic approach for osteoporosis and other musculoskeletal related diseases.

Introduction

The worldwide increase in the prevalence and incidence of obesity in the last few decades has become a major public health concern [1, 2]. Obesity is associated with many serious chronic diseases, including diabetes, cardiovascular disease, hypertension, stroke and some forms of cancers [3–7]. There are also reports that obesity has a considerable effect on musculoskeletal related conditions such as osteoarthritis, osteoporosis and increased fracture rate [8–12]. Osteoporosis is a metabolic bone disease characterized by reduction in bone mass, leading to weaker bones that have an increased risk of fracture [11, 13]. The role of obesity in the development of osteoporosis is controversial [6]. Previously, obesity has been considered to have a positive impact on bone formation because of the beneficial effect of mechanical loading, exerted by high body mass, but recent studies suggest obesity is a considerable risk factor for osteoporosis [6, 8, 14–16].

A chronic high fat high sucrose (HFHS) diet is a well-established method to promote obesity and insulin resistance, resulting in a type 2 diabetes-like condition (hyperglycemia) in animal models [15, 17–19]. The hyperglycemic condition increases oxidative stress, which is associated with a high risk of diabetic complications in various tissue types [7, 17]. Osteoporosis also has been linked to obesity and hyperglycemia [8, 6, 11, 13]. We previously reported that STZ-induced diabetic mouse bones have low bone mineral density and are osteoporotic in nature and that diabetic bone and bone marrow have redox reaction imbalances [20]. Interestingly, our group and others also found an association between dysregulation of the redox homeostasis, excessive arginase activity and diabetic complications in retinal, vascular and erectile tissues of STZ and Akita mouse models [21–26].

Arginase is an enzyme that plays an important role in L-arginine metabolism and the urea cycle [26, 27]. L-arginine is the common substrate for arginase and nitric oxide synthase (NOS) [28–31]. Arginase hydrolyzes L-arginine to L-ornithine and urea whereas NOS utilizes L-arginine to generate NO and L-citrulline [25, 26, 31]. Elevated arginase activity limits the bioavailability of L-arginine which alters NOS activity causing it to produce more superoxide and less NO [32, 33]. In short, excessive arginase activity affects redox balance and normal cell metabolism. Little is currently known about the role of arginase in diabetes-related bone metabolism. In this study, we determined the effect of diabetes and arginase activity on bone and bone marrow metabolism. We also used an arginase inhibitor (ABH) to determine if arginase is involved in diabetes and obesity-induced bone complications. This study revealed a link between obesity, arginase and bone complications.

Material and Methods

Animal Preparation and Experimental Design

All animal protocols were approved by the Institutional Animal Care and Use Committee at Georgia Regents University. Male C57BL/6J mice (The Jackson Laboratory, Bar Harbor, ME, USA) were obtained at 8 weeks of age. Animals were housed in 12-hours light/dark cycle and had free access to food and water throughout the study. To induce obesity and type 2 diabetes, mice were fed on high fat/high sucrose (HFHS diet % of calories = 59% fat, 15% protein, 26% carbohydrate [mostly sucrose], F#1850, BioServe, USA) for 28 weeks. Control mice were fed a normal diet (ND, % of calorie = 18% fat, 24% protein, 58% carbohydrate, Harlan, USA) over the same period. Treatment with the arginase inhibitor, 2-(S)-amino-6-boronohexanoic acid (ABH, 10 mg/kg/day in drinking water) was started after one month and was continued until the end of the study. Body weight and blood glucose levels were measured every two weeks until the animals were sacrificed. After 28 weeks the mice were euthanized for the collection of serum, tibia and femurs. To model type 1 diabetes, STZ induced-diabetes animal studies were performed as per our published methods [20]. In brief, animals were given intraperitoneal (IP) injections of vehicle or freshly prepared streptozotocin in 0.01 mol/L sodium citrate buffer, pH 4.5 (45 mg/kg) after a 4-hour fast each day for 5 consecutive days. Diabetes was confirmed by fasting blood glucose levels of 250 mg/dL. Four and eight weeks after the establishment of diabetes, the mice were euthanized for the collection of serum, tibia and femurs.

The tibia and femurs were excised carefully and all the soft tissues were removed from the bones. RNA and protein analysis were performed on tibia samples while micro CT was conducted on femur samples. The epiphyses of the tibia were removed and the marrow was flushed out with phosphate-buffered saline (PBS). The diaphyses were cleaned twice with PBS and then snap-frozen in liquid nitrogen and stored at -80°C . For bone marrow cell isolation, the marrow was flushed with PBS and the cellular material harvested. The cellular material was then centrifuged, the supernatant was discarded, and the pellet was washed with PBS. The cellular material was used for western blot as per our published protocol [20].

Isolation of BMSCs from Mice

Murine BMSCs were isolated from the long bones of C57BL/6 mice as previously described [34]. Briefly, the marrow was flushed with PBS and the cellular material harvested. The cells were plated in 100-cm² culture plates with DMEM, supplemented with 10% heat-inactivated fetal bovine serum (FBS), 50 U/mL penicillin/streptomycin, and 2 mM L-glutamine. After 24 h, the supernatant was removed, and the adherent stromal cells were trypsinized for negative selection. A negative selection process was used to deplete hematopoietic cell lineages (T- and B-lymphocytic, myeloid and erythroid cells) using a commercially available kit (BD biosciences), thus retaining the progenitor (stem) cell population. The positive fractions were collected using the following parameters: negative for CD3e (CD3 ϵ chain), CD11b (integrin α M chain), CD45R/B220, Ly-6G and Ly-6C (Gr-1), and TER-119/ Erythroid Cells (Ly-76). Positive selections were performed using the anti-Stem cell antigen-1 (Sca-1) column magnetic bead sorting kit (Miltenyi Biotec, CA, USA).

Arginase activity assay

Plasma and bone marrow homogenates or BMSC lysates in Tris buffer (50 mmol·L⁻¹ Tris-HCl, 0.1 mmol·L⁻¹ EDTA and EGTA, pH 7.5 containing protease inhibitors) were used for arginase activity assay as previously described [35]. Briefly, 25 µL of 10 mM MnCl₂ were added to 25 µL of homogenates (cell or tissue) and heated at 57°C for 10 minutes to activate arginase. Next, 50 µL of 0.5M L-arginine was then added to the reaction tube and incubated in 37°C for 1 hour and 400 µL of acid mixture (H₂SO₄ : H₃PO₄ : H₂O in a ratio of 1:3:7) was added to stop the reaction. Then, 25 µL of 9% α-isonitrosopropiophenone (in ethanol) was added and the mixture was heated for 45 minutes at 100°C and placed in dark for 10 minutes to develop color. Arginase activity was measured by loading 200 µL of the reaction mixture in a 96-well plate and absorbance was read at 540 nm.

Nitric oxide assay

Media from the BSMC cell cultures was collected for NO measurement as previously described [36]. Total NO in the culture medium was assayed as nitrite (NO₂⁻), a stable breakdown product of NO, using a Sievers NO chemiluminescence analyzer (Analytix, Sunderland, UK). Briefly, 20 µL of supernatant was injected into the reaction chamber containing glacial acetic acid and sodium iodide where nitrite is quantitatively converted into NO which was measured by the chemiluminescence analyzer. NO values (nanomoles/mg protein) were calculated using standard curves generated by sodium nitrite and protein assays.

Isolation of RNA, synthesis of cDNA, and real-time PCR

Total RNA was isolated from the tibia of mice. Tibia bone particles were ground in liquid N₂ with a pestle and mortar, and the powdered tissue was dissolved in Trizol. RNA was isolated using the Trizol method following the manufacturer's instructions, and the quality of the RNA preparations was monitored by absorbance at 260 and 280 nm (Helios-Gamma, Thermo Spectronic, Rochester, NY). The RNA was reverse-transcribed into complementary deoxyribonucleic acid (cDNA) using iScript reagents from Bio-Rad on a programmable thermal cycler (PCR-Sprint, Thermo Electron, Milford, MA). The cDNA (50 ng) was amplified by real-time PCR using a Bio-Rad iCycler and ABgene reagents (Fisher Scientific, Pittsburgh, PA) and appropriate primers (Table 1). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the internal control for normalization.

Western blot analysis

Protein was extracted from bone marrow and cell culture lysate, subjected to SDS-PAGE and transferred to nitrocellulose membranes. Membranes were incubated with a polyclonal antibody against Glutathione peroxidase (GPX1), superoxide dismutase (SOD1) (Santa Cruz Biotechnology, Santa Cruz, CA), Arginase 1 (Santa Cruz Biotechnology, Santa Cruz, CA), and GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4°C, followed by incubation with HRP-conjugated goat anti-rabbit IgG antibody. Proteins were visualized with an ECL Western blot detection system (Thermo Scientific, Waltham, MA).

Micro-computed Tomography Analyses (μ CT)

For bone mineral density measurement and 3D morphometric analysis, 4% paraformaldehyde fixed femurs were scanned in a μ CT system (Skyscan 1172; Skyscan, Aartlesaar, Belgium) as described by us previously [20]. Scanning was performed at an image pixel size of 14.59 μ m. Reconstruction of the scanned images was done using a Skyscan Nrecon program. The reconstructed data sets were loaded into Skyscan CT-analyzer software for measurement of bone mineral density and 3D morphometric parameters. A region of interest representing the femoral head and neck was selected; the bone mineral density was measured in each region of interest after calibration with hydroxyl apatite phantoms of known density.

Cell culture studies

BMSCs were cultured and treated with (normal glucose) NG (5mM), HG (25mM) alone or with arginase inhibitor ABH (100 μ M) for 3days. Arginase activity, and nitric oxide analysis were performed as per described above.

Statistics

GraphPad Prism 5 (La Jolla, CA) was utilized to perform ANOVA with Bonferroni pair-wise comparison or unpaired t-tests as appropriate. P values of <0.05 were considered significant.

Results

Body and serum parameters of STZ and HFHS diet-induced diabetic mice

STZ induced-diabetic animals showed significant increases in blood glucose levels and decreases in body weight (*Table.2*). Mice fed with HFHS-diet had increased body weight compared with the normal diet (ND) control mice (*Table.2*). Fasting serum glucose concentration was also significantly increased in HFHS fed mice compared to that of ND fed mice (*Table.2*). Neither weight gain nor serum glucose increase was altered by ABH treatment.

Identification of arginase isoform in bone and BMSCs

Analysis of arginase mRNA levels in mouse bones and BMSCs was performed using PCR. There are two isoforms of arginase, arginase 1 (Arg1) and arginase 2 (Arg2) in mammalian systems [25]. We found no evidence of mRNA expression of Arg2 in bone or BMSCs, (Fig. 1). However, Arg1 mRNA expression was detected in both bone and BMSCs (Lin -ve, Sca1+ve). These results showed that BMSCs and bone in mice express only the arginase 1 isoform. Total lung RNA was used as a positive control for both isoforms of arginase.

Arginase activity and expression are increased in HFHS diet and STZ induced diabetic bone and bone marrow

To investigate whether HFHS diet and STZ induced diabetes could alter L-arginine metabolism in bone marrow and bone, we measured arginase enzyme activity in bone marrow lysates and Arg1 mRNA expression in bone. Significant increases in arginase activity were observed in bone marrow of both HFHS (p 0.01) and STZ (p 0.01) induced

diabetic mice compared to their respective controls (Fig 2a&b). Arg1 mRNA expression was also up-regulated in bone from both HFHS (p 0.01) and STZ (p 0.01) induced diabetic mice (Fig 2c&d). In order to confirm the up-regulation of arginase in the HFHS-treated mice, we used 2(*S*)-amino-6-boronoheptanoic acid (ABH) to inhibit arginase activity. ABH is a highly selective competitive inhibitor of arginase and has been successfully shown to have this effect in various animal models (37). Plasma, bone marrow cell lysates and bone were analyzed for arginase activity and expression. We observed that ABH treatment blocked diabetes-induced increase in arginase activity in plasma (p 0.01) (Fig.3a) and prevented the up-regulation of Arg1 mRNA in bone (p 0.01) and bone marrow (p 0.05) (Fig.3b&c).

HFHS diet decreases endogenous antioxidant levels in bone and bone marrow and arginase inhibitor prevents this effect

Increased oxidative stress has been implicated in the pathogenesis of various musculoskeletal diseases including those involving the bone and bone marrow [8, 6, 11, 13, 38–41]. Superoxide dismutase (SOD1), glutathione peroxidase (GPX1) and peroxiredoxin-1 (PRDX1) are endogenous antioxidants which control levels of reactive oxygen species (ROS) and free radicals in pathological conditions such as diabetes [20, 42, 43]. We therefore studied expression of these antioxidants using western blot and quantitative RT-PCR. Our western blot analysis showed significant down-regulation of GPX1 (p 0.01) in the bone marrow of HFHS diet diabetic mouse indicating an increase in oxidative stress, whereas SOD1 was slightly, but not significantly, reduced (p 0.074) (Fig 4a& b). Quantitative RT-PCR showed that PRDX1 mRNA expression was also significantly down-regulated (p 0.05) in both bone marrow and bones of HFHS diet-treated animals. By contrast treatment of the HFHS fed mice with ABH caused significant increases in protein levels of both GPX1 and SOD1 as compared with the HFHS mice and significantly inhibited the HFHS diet-induced decreases in PRDX1 mRNA levels in the bone marrow (Fig 4c & d).

Prevention of bone loss in HFHS diet diabetic animals by treatment with arginase inhibitor (ABH)

To examine if increased arginase activity/expression in bone marrow and bone have a pathological effect on bone health, we analyzed levels of mRNA for specific markers of healthy bone metabolism. As expected, significant decreases in bone morphogenetic protein 2 (BMP2), Collagen type I, alkaline phosphatase (ALP) and Runt-related transcription factor 2 (RUNX2) were observed in HFHS diet group compared to that in normal diet (ND). Osteocalcin expression was not affected by the HFHS diet. Interestingly, the ABH treated group had significantly higher expression of the bone metabolism markers as compared with the vehicle-treated HFHS diet group (Fig.5).

Next, we studied the effect of HFHS diet on bone microstructure using the microcomputed tomography (microCT) technique. MicroCT was performed on the femur to measure bone mineral density (BMD), bone volume/total volume (BV/TV), trabecular thickness (TbTh) and trabecular separation (Tb.Sp). Our data show significant decreases in bone mineral density, bone volume and trabecular thickness in HFHS mice compared to the controls (Fig. 6). There was no significant change in trabecular separation in HFHS mice. Treatment with the arginase inhibitor ABH prevented each of these alterations associated with the HFHS

diet. These results demonstrate that elevated arginase activity is involved in HFHS-induced bone loss.

Prevention of high glucose (HG) induced decreases in NO production in BMSCs by arginase inhibitor (ABH)

Further studies were performed to assess the potential involvement of arginase in high glucose-induced alterations in BMSCs physiology. BMSCs are progenitor cells which differentiate into osteoblasts, osteocytes, adipocytes, and cartilage [44, 45]. We found that treatment with high-glucose (HG, 25mM) alone significantly increased arginase activity and (Arg1) protein expression and ABH treatment significantly inhibited these effects ($P = 0.001$) (Fig 7a & b). Nitric oxide (NO) analysis showed the reverse effect, HG reduced NO formation and ABH treatment significantly ($P = 0.001$) prevented this decrease (Fig 7c). Our results suggest that hyperglycemia is responsible for inducing an increase in arginase activity and a reduction of NO formation in BMSCs.

HFHS diet elevated iNOS levels in bone and arginase inhibitor (ABH) prevents this effect

Chronic oxidative and inflammatory stress is associated with activation of inducible nitric oxide synthase (iNOS) in many disease conditions including diabetes. To examine the potential roles of arginase and NOS in HFHS diet-induced diabetic bone complications, we determined the effects of ABH on the expression of iNOS and endothelial NOS (eNOS) in bones of the HFHS diet-treated mice. Our data demonstrated that iNOS expression was significantly ($P = 0.01$) elevated in bones of HFHS diabetic animals compared to normal diet control mice. Treatment with the arginase inhibitor prevented this effect ($P = 0.01$) (Fig.8). Levels of eNOS mRNA were not significantly altered by either HFHS or ABH treatment.

Discussion

High calorie diet intake in adults and children increases the risk of obesity dependent diabetes and musculoskeletal related complications [1–5, 9–12]. We previously reported that STZ-induced diabetic condition leads to a decline in bone quality [20]. The objective of this study was to assess effects of HFHS diet-induced diabetes on bone quality and role of arginase in the pathogenesis of any diabetes-induced alterations. In doing so, this study is the first to identify arginase 1 as the isoform present in bone and BMSCs. Additionally; this study demonstrated the efficacy of pharmacologically inhibiting arginase in preventing HFHS-induced pathologies in bone and bone marrow.

Arginase, an enzyme of the urea cycle, plays a major role in L-arginine metabolism. There are two distinct isoforms of arginase, arginase 1 (Arg1) and arginase 2 (Arg2) [25]. Although both isoforms of arginase are present in mammalian cells, our mRNA analysis showed that bone and BMSCs only express arginase 1 (Arg1). Our group and others have previously reported that arginase activity is involved in hyperglycemia-induced dysregulation of retinal and vascular endothelial function [21–22–24–27]. We hypothesized that obesity-induced diabetes may elevate arginase activity in bone and bone marrow, which may lead to bone-related complications. Our results show that this is indeed the case. Arginase activity significantly increases in a diabetic bone marrow environment. We

discovered that Arg1 expression is specifically up-regulated in bones of HFHS diet-induced diabetic mice. We also found that mRNA levels for markers of healthy bone metabolism were down regulated in bones of mice with HFHS diet-induced diabetes. Furthermore, the bones of HFHS diet-induced diabetic mice showed signs of osteoporosis. MicroCT analysis demonstrated low bone mineral density, low bone mass and increased trabecular separation, which are all indicative of osteoporotic bone. Similar bone-related complications were previously reported in studies of obesity and diabetes in humans and animal models [46–50]. Interestingly, our published results also demonstrated low bone quality in the STZ-induced diabetic mouse model [20]. We were interested to know if the increase in arginase activity and expression in bone and bone marrow was only limited to HFHS diet-induced diabetes. Our results showed that arginase activity is also increased in bone marrow from STZ induced diabetic mice. Furthermore, we observed increased Arg1 expression in bones of STZ diabetic mice. Our findings in the HFHS diet-induced diabetes model in conjuncture with the results in the STZ-induced diabetic mouse model and the results in the high glucose-treated BMSCs indicate that the increases in arginase activity result from a hyperglycemic environment.

Chronic hyperglycemia is a hallmark of diabetes (both type 1 and type 2) and is known to induce chronic oxidative stress, which leads to secondary complications [51]. Chronic oxidative stress weakens body defense mechanisms and endogenous antioxidant systems. Our analyses of bone marrow from HFHS diet animals showed decreased expression levels of endogenous antioxidants. We previously demonstrated a similar effect in bone and bone marrow from STZ-induced diabetic animals [20]. Our group and others previously reported that the diabetes-induced increase in arginase activity was blocked by ABH (2(*S*)-amino-6-boronohexanoic acid) treatment [21, 23]. ABH is a slow-binding competitive inhibitor of arginase, which has shown beneficial effects in limiting disease-related pathology in various systems [52, 53]. We hypothesized that the effect of HFHS diet-induced diabetes on bone complications can be prevented or minimized by ABH treatment. In this study, we observed that blocking the diabetes-induced increase in arginase activity resulted in a marked improvement in bone quality. Bone mineral density, bone volume and markers of healthy bone metabolism were reduced with HFHS diet and significantly improved with ABH treatment. Most importantly, we observed decreases in anti-oxidant protein or mRNA expression in bone marrow of HFHS-induced diabetic mice that were prevented by ABH treatment.

The parallel increases in arginase activity and oxidative stress parameters in diabetic bone and bone marrow suggest involvement of arginase-induced redox imbalance in the pathology. Based on these observations, we hypothesized that diabetes-induced increases in arginase activity may interfere with healthy bone and bone marrow metabolism by limiting L-arginine bioavailability and reducing NO production in bone forming cells. To test the hypothesis that hyperglycemic conditions alter L-arginine metabolism in bone forming cells, we used bone marrow stromal cells (BMSCs) as an in-vitro system. BMSCs are the progenitor cells which differentiate into osteoblasts and osteocytes [44, 45]. Our results demonstrated that high glucose (HG) treatment of BMSCs increased arginase activity and decreased NO production. These changes were prevented by ABH, indicating the involvement of arginase in BMSCs pathophysiology in the diabetes-like hyperglycemic

condition. It has been previously reported that NO plays an important role in BMSCs biology [54–58]. Ding et al (55) and others [54, 56] have reported that NO supports osteogenic differentiation. It has also been reported that NO is critical for BMSCs migration, survival, and proliferation [56–58]. Our study indicates that NO bioavailability is substantially diminished in diabetes-like hyperglycemic conditions suggesting that NOS is uncoupled. Furthermore, the decline in NO is blocked by inhibiting arginase with ABH, suggesting the involvement of arginase-induced NOS uncoupling in the pathology. Ours is the first study to show that in BMSCs, NO bioavailability is reduced in hyperglycemic condition and that this effect is blocked by an arginase inhibitor.

Recent studies indicate that nitric oxide synthase signaling plays vital role in oxidative and inflammatory stress induced bone loss [59–60]. Inducible nitric oxide synthase (iNOS) is the major nitric oxide synthase expressed in osteoblast and osteocytes during stress conditions [59–61]. Endothelial NOS (eNOS) is also expressed in bone cells [61–62]. To further evaluate a relationship between NO, arginase and NOS in the animal model, iNOS and eNOS mRNA were analyzed in HFHS bone. We found that iNOS expression was significantly elevated in bones of HFHS diabetic animals and that arginase inhibitor treatment prevents this effect. We did not observe any change in eNOS expression. Other studies have demonstrated similar findings that iNOS is elevated in several osteoporosis models [59, 63–64]. We speculate that the increase in iNOS increases the burden of oxidative stress due to decreased bioavailability of the NOS substrate L-arginine. Further studies to clarify the roles L-arginine metabolism and nitric oxide signaling in bone pathophysiology are imperative.

In conclusion, this study revealed the role of arginase deregulation in diabetes-induced bone complications, and more importantly, that these complications can be prevented by an arginase inhibitor. Limiting arginase activity is a novel therapeutic approach not only for osteoporosis in the setting of diabetes and obesity but also for other musculoskeletal diseases. Further studies are needed to elucidate the molecular mechanisms behind these processes.

Acknowledgments

This work was supported by National Institutes of Health Grants R24 DK094765 (RWC and RBC), R01 HL070215 (RWC and RBC), R01 EY011766 (RBC and RWC) and NIA-AG036675 (CI).

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Highlights

- Arginase 1 is abundantly present in the bone and BMSCs.
- HFHS diet induced Arginase activity and expression in bone and bone marrow.
- HFHS diet induced bone complications in mouse model.
- Supplementation of an arginase inhibitor (ABH) prevented bone-related complications.
- High glucose treatment regulates arginase activity and nitric oxide in BMSCs

Figure 1. Expression of arginase 1 and arginase 2 mRNA in mouse bone and bone marrow stromal cells

(a) Lane 1: positive control (Lung), Lane 2: bone, Lane 3: bone marrow stromal cells (BMSCs), and Lane 4: negative control.

Figure 2. Diabetes increases arginase activity and mRNA expression in bone marrow and bone Diabetes was induced in mice with two different methods (HFHS diet and STZ) as mentioned in material and methods section. Arginase activity was determined using an assay for urea formation in bone marrow lysates from (a) HFHS diabetic, (b) STZ induced diabetic mice. Relative levels of Arg1 mRNA in bones from (c) HFHS and (d) STZ induced-diabetic mice. Data for each sample were normalized to GAPDH mRNA and represented as the fold change in expression compared to control mice. Results are means $\pm$ SD ($n = 5-6$ * $p < 0.05$, # $p < 0.01$), data were analyzed using an unpaired t-test.

Figure 3. Effect of the arginase inhibitor (ABH) on arginase activity in HFHS diabetic mice ABH treatments were done as described in material and methods section. (a) Arginase activity in plasma was determined by arginase activity assay. Arg1 mRNA expression was analyzed in (b) bones and (c) bone marrow from mice fed normal diet or HFHS diet and treated with ABH or vehicle. Data for each sample were normalized to levels of GAPDH mRNA and represented as the fold change in expression compared to control mice. Results are means $\pm$ SD ($n = 5-6$). Data were analyzed by ANOVA followed by Bonferroni post hoc test or t-test ($*p < 0.05$).

Figure 4. Effect of the arginase inhibitor (ABH) on HFHS diet-induced decreases in endogenous antioxidant protein and mRNA

(a) Western blot and (b) western blot quantification of SOD and GPX1 in total bone marrow of diabetic animals. PRDX1 mRNA levels were determined for (c) bone and (d) bone marrow samples using quantitative RT-PCR. Data for each sample were normalized to levels of GAPDH mRNA and represented as the fold change in expression compared to control mice. Results are means $\pm$ SD ($n = 5-6$). Data were analyzed by ANOVA followed by Bonferroni post hoc test or t-test (* $p < 0.05$).

Figure 5. Effect of the arginase inhibitor (ABH) on HFHS diet-induced decreases in bone marker mRNA

Quantitative RT-PCR analysis of bone from HFHS diet-induced diabetic animals showed decreased levels of mRNA for BMP2, Collagen type 1, RUNX-2, ALP and osteocalcin and ABH treatment prevented this change. Data for each sample were normalized to levels of GAPDH mRNA and represented as the fold change in expression compared to control mice. Results are means $\pm$ SD ($n = 5-6$). Data were analyzed by ANOVA followed by Bonferroni post hoc test or t-test ($*p < 0.05$).

Figure 6. Effect of the arginase inhibitor (ABH) on HFHS diet-induced bone loss
 Microcomputed tomography (μCT) was performed on mouse femurs. Bone mineral density (BMD), bone volume/total volume (BV/TV), and trabecular thickness (TbTh) were significantly reduced in femurs from HFHS treated mice compared to control and ABH treatment prevented this effect. Results are means $\pm$ SD ($n = 5-7$). *Significant $p < 0.05$ determined by ANOVA, Bonferroni post hoc test or t test.

Fig 7. Effect of high glucose on arginase activity, Arg 1 expression and NO formation in BMSCs BMSC were incubated in DMEM medium (2% FBS, 50 mM L-arginine) containing 5.5mM d-glucose (NG), 25mM d-glucose (HG) or 25mM d-glucose (HG) + ABH (100 μ M) for 72h. (a) Arginase activity in cell lysate was determined by arginase activity assay (* p < 0.05, n = 4–6). (b) Levels of arginase 1 protein expression in the cell lysate were determined by Western blot analysis (n = 3–4) and (c) The nitrite level released into the conditioned medium was determined by NO analyzer (# p < 0.01, n = 7–10). Data were analyzed by ANOVA followed by Bonferroni post hoc test or t-test.

Figure 8. Effect of the arginase inhibitor (ABH) on nitric oxide synthase mRNA expression in bone of HFHS diabetic mice

Quantitative RT-PCR was used to determine levels of mRNA for (a) inducible nitric oxide synthase (iNOS) and (b) endothelial nitric oxide synthase (eNOS). Data for each sample were normalized with GAPDH mRNA and represented as the fold change in expression compared to control animals. Results are means $\pm$ SD ($n = 5-6$). Data were analyzed by ANOVA followed by Bonferroni post hoc test or t-test (* $p < 0.05$).

Table 1

Nucleotide sequences of mouse primers used for RT-PCR

Gene	Primer	Reference/Accession Number
GAPDH	CAT GGC CTC CAA GGA GTA AGA GAG GGA GAT GCT CAG TGT TGG	[20]
Arginase 1	CTT GCG AGA CGT AGA CCC TG TTG AGT TCC GAA GCA AGC CA	NM_007482.3
Arginase 2	ATT CCT TGC GTC CTG ACG AG GCA AGC CAG CTT CTC GAA TG	NM_009705.3
ALP	AGA GTA CGC TCC CGC CAC T CCT TAC CTG CAG GCA CTC GT	[20]
Collagen type I	GCC CAT TAG CCG GTA TGT TAT TA TCC CTG GTA CCT ATG GAG ACT GT	[20]
BMP-2	TGT TTG GCC TGA AGC AGA GA TGA GTG CCT GCG GTA CAG AT	[20]
RUNX-2	GGA AAG GCA CTG ACT GAC CTA ACA AAT TCT AAG CTT GGG AGG A	[20]
Osteocalcin	ATT TAG GAC CTG TGC TGC CCT A GGA GCT GCT GTG ACA TCC ATA C	[20]
PRDX1	ACA CCC AAG AAA CAA GGA GGA TT CAA CGG GAA GAT CGT TTA TTG TTA	NM_011034.4
iNOS	ACC CAA GGT CTA CGT TCA GGA AAT GTA GAG GTG GCC CTG CT	NM_010927
eNOS	AGA TGC CCA ACC CAA ACC TT CAG AGA GGT GTC TGG GAC TCA	NM_008713

Table 2

Measurements of body weight and blood glucose level in STZ and HFHS diet-include diabetic animals

Diabetic Model	Body Weight (g)	Blood Glucose (mg/dL)
STZ Model		
Control 4weeks	30.2±2.6	113.2±5.2
STZ 4 weeks	26.58±2.14 *	476.8±68.08 *
Control 8 weeks	33.4±2.13	114±7.4
STZ 8 weeks	26.80±1.09 *	528.6±74.5 *
HFHS diet-diabetic Model		
Control/ND	37.7±1.4	110.8±3.4
HFHS	51.9±1.9 *	201.4±16.9 *
HFHS+ABH	49.6±1.6	195.7±18.4

Note: Body weight and blood glucose levels of mice in STZ and HFHS diet-induced diabetic. Values are represented as mean ± SEM, n=5–8 mice/group.

* P< 0.05 vs. Controls.