Angiotensin-(1–7) Administration Reduces Oxidative Stress in Diabetic Bone Marrow


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Diabetics have an increased risk of developing cardiovascular disease, in part due to oxidative stress, resulting in endothelial nitric oxide synthase (eNOS) dysfunction. Studies have demonstrated that angiotensin-(1–7) [Ang-(1–7)] can activate eNOS activity. Because the bone marrow is a primary source of a number of progenitors important in physiological homeostasis and healing, the goal of this study was to evaluate the in vivo effects of Ang-(1–7) treatment on oxidative stress and the ensuing nitrative stress in diabetic bone marrow and its potential pathways. BKS.Cg-Dock7m +/+ Leprdb/J mice and their heterozygous controls were administered Ang-(1–7) alone or combined with A-779, losartan, PD123,319, nitro-L-arginine methyl ester, or icatibant sc for 14 d. The bone marrow was then collected to measure nitric oxide levels, eNOS phosphorylation, and expression of nitric oxide synthase, superoxide dismutase, and p22-phox. Nitric oxide levels in the bone marrow were significantly decreased in diabetic mice, and Ang-(1–7) treatment was able to significantly increase these measures (P < 0.01). This effect was blocked by the coadministration of PD123,319, A-779, nitro-L-arginine methyl ester, and icatibant. In addition, Ang-(1–7) treatment reversed the paradoxical increase in eNOS and neuronal nitric oxide synthase expression and decreased the phosphorylation of eNOS at Thr495 seen in diabetic mice. Ang-(1–7) also reversed diabetes-induced production of reactive oxygen species by decreasing p22-phox expression and increasing superoxide dismutase 3 expression, leading to a significant reduction in 3-nitrotyrosine formation in diabetic bone marrow (P < 0.05). Our findings demonstrate that Ang-(1–7) administration decreases diabetes-induced oxidative stress in the bone marrow and modifies pathways involved in eNOS dysfunction. (Endocrinology 153: 2189–2197, 2012)
Unlike angiotensin II (Ang II), the beneficial effects of Ang-(1–7) are due in part to antagonism of Ang II-induced vasoconstriction of the arteries as well as the stimulation of NO release from endothelial cells and cardiomyocytes resulting in vasodilation (10–17). Cross talk between the RAS and kinin-kallikrein system has been well documented (18, 19). After binding to the Mas receptor, Ang-(1–7) also potentiates the release of bradykinin, a ligand of the bradykinin B2 receptor that results in further vasodilatory effects (20–22).

Studies have also shown that Ang-(1–7) is effective in stimulating hematopoietic progenitor cell proliferation and increasing hematopoietic recovery after chemotherapy and irradiation injury in vivo (23, 24). In addition, Ang-(1–7) can activate eNOS and increase eNOS activity as well as increase the proliferation of bone marrow-derived progenitors (17, 25, 26). The bone marrow is a primary source of progenitors responsible for important biological processes such as vasculogenesis, angiogenesis, and hematopoiesis (28). Oxidative stress and damage, such as that seen in diabetes, can directly impact cell survival and function. Exposure of bone marrow cells to these insults could lead to many of the detrimental and irreversible complications of diabetes. The aim of the current study was to investigate multiple molecular markers of oxidative stress in mice, in which Ang-(1–7) 500 μg/kg administered as a once-daily sc dose was found to be the most effective dose in diabetic mice with no observable toxicities. Plasma glucose levels were measured at necropsy; however, there were no significant changes in any of the diabetic treatment groups (data not shown). In addition, there were no significant changes in any of the parameters investigated after treatment with inhibitors alone (data not shown). After the 14-d treatment period, the mice were euthanized, and the parameters were investigated as described below to determine the effect of diabetes and the Ang-(1–7) treatment with and without the coadministration of the various inhibitors.

Chemical and reagents
Ang-(1–7) (prepared using good manufacturing practices) and d-Ala2-Ang I/II (1–7) (A-779), an antagonist of the Mas receptor, were purchased from Bachem (Torrance, CA). Losartan, an angiotensin type 1 receptor (AT1) antagonist, PD123,319, an AT2 receptor antagonist, and nitro-L-arginine methyl ester (L-NAME), an inhibitor of NOS, were purchased from Sigma-Aldrich (St. Louis, MO). Icatibant, an antagonist of bradykinin B2 receptors, was purchased from Tocris Bioscience (Ellisville, MO). CellROX® Deep Red reagent for oxidative stress detection was purchased from Invitrogen (Carlsbad, CA). The mouse antinitrotyrosine, clone 1A6, Alexa Fluor® 488-conjugated monoclonal antibody and IgG2b isotype control for flow cytometry was purchased from Millipore (Billerica, MA). Primers for quantitative RT-PCR were purchased from Integrated DNA Technologies (San Diego, CA), and antibodies used for Western blotting were purchased from Cell Signaling Technology (Danvers, MA).

Study design
BKS.Cg-Dock7m+/+ Leprdb/J mice and their heterozygous controls (n = 7/group) were administered saline (control), inhibitors alone (losartan, PD123,319, A-779 or L-NAME at 10 mg/kg/d or icatibant at 0.4 mg/kg/d), Ang-(1–7) alone (500 μg/kg/d), or Ang-(1–7) 500 μg/kg/d combined with an inhibitor at the aforementioned doses for 2 wk by sc injection. The mice were weighed three times weekly and the doses adjusted accordingly. The dose of Ang-(1–7) used in this study is based on unpublished data from our laboratory following multiple dose-response studies in mice, in which Ang-(1–7) 500 μg/kg/d administered as a once-daily sc dose was found to be the most effective dose in diabetic mice with no observable toxicities. Plasma glucose levels were measured at necropsy; however, there were no significant changes in any of the diabetic treatment groups (data not shown).

Measurement of bone marrow nitrite levels
After permeabilization of aliquoted bone marrow cells with 0.1% Triton-X, NO levels were measured using the Griess reagent system (Promega, Madison, WI). Due to the instable and volatile nature of NO, this assay measures nitrite, one of the stable metabolites of NO. The assay was performed per the manufacturer protocol.

Materials and Methods

Animals
The National Institutes of Health Principles of Laboratory Animal Care were followed, and the Department of Animal Resources at the University of Southern California approved this study. Six- to 8-wk-old male BKS.Cg-Dock7m+/+ Leprdb/J mice and their heterozygous controls were purchased from Jackson Laboratories (Bar Harbor, ME). Mice homozygous for the diabetes spontaneous mutation Leprdb (BKS.Cg-Dock7m+/+ Leprdb/J), which is an obese model of type 2 diabetes due to truncation of the leptin receptor, were used in this study. The mice were quarantined for 1 wk before the initiation of the study, and all diabetic mice had verified plasma glucose levels greater than 500 mg/dl before the initiation of treatment. Food and water were available ad libitum, and all mice were kept on a 12-h light, 12-h dark cycle.

Harvesting of bone marrow
The femurs from each mouse were collected and the bone marrow was harvested by flushing with PBS containing 2% fetal calf serum. After the collection of the bone marrow and aliquoting, red blood cells were lysed with a hypotonic solution and mixed with 0.04% trypan blue, and the number of nucleated cells was assessed using a hemocytometer under light microscopy.

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Measurement of bone marrow ROS levels

Isolated bone marrow cells were incubated with CellROX® Deep Red reagent at 5 µM and incubated for 30 min at 37°C per the manufacturer protocol. ROS levels were measured via flow cytometry and values reported as median fluorescent intensity.

Preparation of bone marrow cells for flow cytometry

Bone marrow cells were suspended at 10^6 cells/ml in DMEM containing 2% fetal calf serum, 1 mM HEPES, penicillin, and streptomycin. For antibody staining, bone marrow cells were suspended in Hanks’ balanced salt solution containing 2% fetal calf serum, 1 mM HEPES, penicillin, and streptomycin at 10^8 cells/ml. An aliquot was first labeled with Alexa Fluor® 488 conjugated mouse antinitrotyrosine antibody added at 1 µg per 1 × 10^6 cells and then fixed with 4% paraformaldehyde. Flow cytometric analysis was performed on a LSR II flow cytometer using FACSDiva software (Becton Dickinson, Franklin Lakes, NJ) at the Flow Cytometry Core facility located at the University of Southern California School of Pharmacy.

Analysis of bone marrow mRNA expression

Total RNA was extracted from bone marrow cells using TRIzol (Invitrogen). For each sample, approximately 100 ng of RNA was reverse transcribed using Maxima reverse transcriptase (Fermentas, Glen Burnie, MD). Real-time PCR was conducted to examine expression of eNOS, neuronal NOS (nNOS), inducible NOS (iNOS), superoxide dismutase (SOD)-1, SOD2, SOD3, and p22-phox mRNA in bone marrow (for primer sequences, see Supplementary Material, published on The Endocrine Society’s Journals Online web site at http://endo.endojournals.org). Amplification of the cDNA was performed using SYBR Green PCR master mix (Applied Biosystems by Life Technologies, Carlsbad, CA) using an ABI 7300. Expressions of eNOS, nNOS, iNOS, SOD1, SOD2, SOD3, and p22-phox mRNA were normalized against 18S mRNA and expressed as fold change compared with nondiabetic controls.

Analysis of bone marrow protein expression

Protein lysates isolated from bone marrow cells for Western blot analysis (30 µg) were resolved on SDS-PAGE gels and transferred to nitrocellulose membranes by electro blotting. The membranes were incubated with monoclonal rabbit antibodies against eNOS, phospho-eNOS (Ser1177 and Thr495), nNOS, SOD3, and p22-phox, followed by antirabbit horseradish peroxidase-conjugated antibody. An antibody against β-actin was used to normalize protein loading. SuperSignal West Pico enhanced chemiluminescence substrate was used to detect the bands (Thermo Scientific, Rockfield, IL). The resultant bands were quantified using densitometry using ImageJ, version 1.45i (National Institutes of Health, Bethesda, MD). The results were expressed as the ratio of target protein band to β-actin band intensity.

Statistical analysis

GraphPad Prism, version 5.0d for Mac OS X (GraphPad Software, San Diego, CA) was used to analyze the data. One-way ANOVA followed by a Tukey’s test was used to compare data from more than two groups, and linear regression was used to determine the relationship between bone marrow nitrite levels and percentage of bone marrow tyrosine nitration. The level of statistical significance was set at 5%. Data are expressed as mean value ± SEM.

Results

Ang-(1–7) effects on bone marrow ROS and nitrite levels

Bone marrow ROS levels were significantly increased in diabetic bone marrow, whereas administration of Ang-(1–7) for 14 d significantly reduced these ROS levels (Fig. 1A). Co-administration of Ang-(1–7) with losartan, PD123,319, A-779, L-NAME, or icatibant resulted in a significant increase in ROS in diabetic bone marrow compared with treatment with Ang-(1–7) alone. Due to the integral relationship between diabetes-induced oxidative stress, NO and the production of reactive nitrogen species leading to protein tyrosine nitration and posttransla-
tional modifications, NO levels were measured in bone marrow cells isolated from both nondiabetic and diabetic mice. Bone marrow NO levels were significantly reduced in diabetic mice when compared with nondiabetic controls, whereas treatment of diabetic mice with Ang-(1–7) resulted in a significant increase in bone marrow NO levels (Fig. 1B). Administration of Ang-(1–7) to nondiabetic mice did not significantly affect bone marrow NO levels (data not shown). Administration of PD123,319, A-779, or L-NAME in combination with Ang-(1–7) in diabetic mice resulted in a significant decrease in bone marrow nitrite levels compared with diabetic mice treated with Ang-(1–7) alone. The combination of Ang-(1–7) with either losartan or icatibant resulted in a nonsignificant reduction in bone marrow nitrite levels.

Effects of Ang-(1–7) on NOS isoform expression and eNOS activation

Because other investigators have observed a paradoxical increase in eNOS expression in endothelial cells despite decreased NO levels in type 2 diabetes (29, 30), bone marrow eNOS, nNOS, and iNOS mRNA and protein expression were assessed. Bone marrow eNOS and nNOS mRNA and protein expression were significantly increased in diabetic mice (Fig. 2, A–D), whereas there were no significant changes in bone marrow iNOS expression in any group (data not shown). Treatment of diabetic mice with Ang-(1–7) significantly decreased both eNOS and nNOS mRNA and protein expression, whereas the coadministration of PD123,319, A-779, L-NAME, or icatibant with Ang-(1–7) resulted in a significant increase in eNOS and nNOS mRNA and protein expression compared with diabetic mice treated with Ang-(1–7) alone, whereas coadministration of losartan with Ang-(1–7) did not have a significant effect.

To investigate the effect of diabetes and determine the impact of Ang-(1–7) on the activation of eNOS, we measured phosphorylation of eNOS at the primary activation (Ser1177) and inactivation sites (Thr495). Bone marrow eNOS protein isolated from diabetic mice exhibited significantly lower phosphorylation at Ser1177 and higher phosphorylation at Thr495 when compared with nondiabetic controls (Fig. 3, A and B). Ang-(1–7) treatment in diabetic mice resulted in a significant increase in eNOS phosphorylation at Ser1177 and a significant decrease in eNOS phosphorylation at Thr495. When PD123,319, A-779, L-NAME, or icatibant was coadministered with Ang-(1–7) in diabetic mice, the Ang-(1–7)-mediated effects on eNOS phosphorylation were blocked. No changes in eNOS phosphorylation were observed when Ang-(1–7) was coadministered with losartan.

Effect of Ang-(1–7) on SOD isoforms in the bone marrow

Diabetes causes a decreased expression of various SOD isoforms, resulting in a subsequent increase in oxidative stress (31). In this study, we measured SOD1 (Cu-Zn SOD), SOD2 (mitochondrial SOD), and SOD3 (extracellular SOD) mRNA expression, in which no significant changes in SOD1 expression was detected among the groups evaluated. In contrast, both SOD2 and SOD3 mRNA expression was significantly reduced in diabetic mice when compared with nondiabetic controls (Fig. 4, A–C). When diabetic mice were treated with Ang-(1–7), a significant increase in SOD3 expression was seen; however, changes in SOD2 mRNA expression were not statistically significant. These results were verified using Western blotting to deter-
mine protein expression (Fig. 4D). The ability of Ang-(1–7) to increase mRNA and protein expression of SOD3 was completely blocked when coadministered with PD123,319, A-779, L-NAME, or icatibant (P<0.01) but not with losartan.

Ang-(1–7) treatment decreases bone marrow p22-phox expression in diabetes

Hyperglycemia resulting from diabetes induces the expression of nicotinamide adenine dinucleotide phosphate oxidase (NADPH) oxidase through protein kinase C activation, resulting in an increased production of superoxide (32, 33). This can initiate a cascade that ultimately leads to eNOS dysfunction. Bone marrow p22-phox (a subunit of the heterodimeric, membrane bound portion of NADPH oxidase) was significantly higher in diabetic mice compared with nondiabetic controls. When treated with Ang-(1–7), the expression of p22-phox was reduced for 14 d resulted in a significant reduction in tyrosine nitration in the bone marrow. Coadministration of Ang-(1–7) with A-779, PD123,319, L-NAME, or icatibant blocked the Ang-(1–7)-mediated decreases of nitrotyrosine found in the bone marrow. However, coadministration of Ang-(1–7) with losartan did not block the effect seen after Ang-(1–7) administration alone.

To demonstrate the potential role of eNOS dysfunction in diabetic bone marrow, NO levels measured in all groups were correlated with tyrosine nitration. There was a significant negative correlation between bone marrow NO levels and protein tyrosine nitration levels, in which lower bone marrow NO levels were associated with increased bone marrow protein tyrosine nitration (Fig. 6B). Diabetic mice treated with Ang-(1–7) for 14 d showed significantly increased bone marrow NO levels and decreased bone marrow protein tyrosine nitration, suggesting a potential
reversal of the negative effects of eNOS dysfunction observed in diabetes, including increased superoxide formation, a subsequent decrease in NO levels, and increases in protein tyrosine nitration.

Discussion

Our study demonstrates the in vivo effects of Ang-(1–7) on markers of oxidative and nitrative stress in a murine model of type 2 diabetes. We showed that Ang-(1–7) administra-

tion decreased ROS levels in the bone marrow of db/db mice, coupled by a decrease in p22-phox expression and an increase in SOD3 expression. In addition, Ang-(1–7) increased NO levels. These changes were linked with increased eNOS phosphorylation at Ser1177 and reduced protein tyrosine nitration in the bone marrow of db/db mice. The effects of Ang-(1–7) were modulated by a Mas-, AT2-, and bradykinin B2 receptor-dependent mechanism.

Previous studies have shown that hyperglycemia can increase NADPH oxidase expression via protein kinase C activation (32, 33), in which increased NADPH oxidase expression results in an increased production of ROS. Elevated production of ROS through NADPH oxidase combined with a decrease in superoxide-removal systems, specifically SOD3, could further increase oxidative stress. The SOD family of enzymes is part of a critical defense mechanism for managing superoxide levels, in which down-regulation of this system can increase cytotoxic levels of ROS, ultimately leading to tissue damage and the long-term complications seen in diabetes.

Similar to studies examining the effects of diabetes in other tissues, we observed a decrease in NO levels and an increase in eNOS and nNOS mRNA and protein expression in the bone marrow, which is often a direct result of NOS dysfunction (6, 34). The end result is a decrease in the production of NO and an increase in the production of superoxide anions via eNOS due to the dissociation of the heme ferrous-dioxygen complex in the oxygenase domain of eNOS. Phosphorylation of eNOS was also altered in diabetic bone marrow, in which an increase in phosphorylation at Thr495 and a decrease in phosphorylation at Ser1177 was observed. Phosphorylation of eNOS at Ser1177 results in activation, whereas Thr495 phosphorylation inactivates eNOS.

Lastly, increased oxidative stress in diabetes, specifically an increased production of superoxide anions, can result in the formation of peroxynitrite, an oxidant and nitrating agent. Peroxynitrite can cause protein tyrosine

FIG. 4. A–E, Effects of Ang-(1–7) on bone marrow SOD expression. There were no significant differences in bone marrow SOD1 mRNA expression between any groups (A). Both bone marrow SOD2 (B) and SOD3 (C) mRNA expression were significantly reduced in diabetic mice compared with nondiabetic controls (P < 0.01). Although treatment with Ang-(1–7) for 14 d did not have a significant effect on SOD2 mRNA expression, treatment with Ang-(1–7) significantly increased bone marrow SOD3 mRNA expression in diabetic mice. Coadministration of PD123,319, A-779, L-NAME, or icatibant with Ang-(1–7) resulted in a significant decrease in bone marrow SOD3 mRNA expression in diabetic mice compared to treatment with Ang-(1–7) alone (P < 0.01). SOD3 protein expression (D and E) was significantly reduced in diabetic mice compared with nondiabetic controls (P < 0.01), and the administration of Ang-(1–7) to diabetic mice significantly increased bone marrow SOD3 protein expression (P < 0.01). Coadministration of PD123,319, A-779, L-NAME, or icatibant with Ang-(1–7) significantly inhibited its effect in diabetic mice (P < 0.01). **, P < 0.01; ††, P < 0.01 compared to the db + Ang-(1–7) group.
nitrination, a posttranslational modification that can ultimately result in altered protein structure and function, and may also be the cause of many of the long-term complications of diabetes (4, 35, 36). Indeed, we observed an increase in protein tyrosine nitration in the bone marrow of \textit{db/db} mice. In addition to the commonly referred to circulating or systemic RAS, tissue-specific RAS also exist in the body, including in the pancreas, kidneys, heart, skin, and bone marrow (37). Both \textit{in vitro} and \textit{in vivo} studies have shown an important role for the RAS in the bone marrow microenvironment, especially in hematopoiesis. For example, Ang-(1–7) itself has been demonstrated to stimulate hematopoietic progenitor cells after chemotherapy in patients diagnosed with cancer (24, 38). The bone marrow plays a vital role in the generation of progenitor cells responsible for multiple functions including wound healing, neovascularization, and immune function, all of which are compromised in diabetes. Evidence points to the role of oxidative stress in cellular damage, which may result in many of the long-term complications of diabetes such as cardiovascular and immune dysfunction.

Ang-(1–7) has been shown to bind to the G protein-coupled receptor Mas, which results in an increased production of both NO and bradykinin. However, there is evidence for the involvement of the AT$_2$ receptor. In agreement with our present results, a recent paper showed that in spontaneously hypertensive rats, Ang-(1–7) administration up-regulated phosphorylated eNOS (Ser1177)/eNOS protein expression in cardiac tissues, an effect that was blocked by bradykinin B$_2$ receptor and AT$_2$ receptor antagonism (26). Because AT$_2$ receptors are up-regulated after injury (e.g. damage from increased oxidative stress), there may be a role for AT$_2$ receptors coupled with Mas receptors in diabetes. This role could potentially involve heterodimerization of the two receptors (39). In addition, other Ang-(1–7) signaling pathways that could potentially explain our results have been suggested in previous studies. This includes the counterregulation of Ang II signaling by Ang-(1–7), show-
ing specifically that the activation of NADPH oxidase by Ang II is attenuated by Ang-(1–7) treatment in vitro (40). Studies have also shown that Ang-(1–7), through binding to the Mas receptor, activates Akt-dependent pathways including the stimulation of Akt phosphorylation via Akt kinase (41, 42). These pathways ultimately lead to eNOS activation and increases in NO production through mechanisms similar to those seen in our study, specifically phosphorylation of eNOS at Ser1177 and dephosphorylation of eNOS at Thr495 (41).

An interesting result was seen when measuring ROS in the bone marrow of db/db mice, in which blockade of the AT1 receptor using losartan inhibited the effects of Ang-(1–7). This was the only parameter measured that was affected by Ang-(1–7) that was blocked by losartan in this study. There are a few potential explanations for this effect. First, RAS receptor expression may be altered in diabetic bone marrow, which could change the dynamics of AT1 receptor blockade or Mas and AT2 receptor activation. Although we did not investigate bone marrow RAS expression in the current study, other groups have demonstrated that AT1 receptor expression can be altered by various factors. Specifically, decreased circulating Ang II levels have been shown to up-regulate AT1 receptor expression (43). Losartan administration itself has also been shown to decrease myocardial AT1 receptor expression (44). In addition, other non-RAS factors can alter AT1 receptor expression, including the cytokines IL-1β, IL-1, and TNF-α, which are increased in diabetes (45). The second explanation could be the dose used for Ang-(1–7) and length of treatment in this study, which was chosen based on unpublished in vivo dose escalation studies in our laboratory examining progenitor cell counts in diabetic bone marrow. These studies were performed using doses ranging from 100 to 1000 μg/kg/d for 14 d, in which 500 μg/kg/d was shown to be the most effective at increasing progenitor cell numbers. However, the effects of various doses of Ang-(1–7) on oxidative stress markers were not determined. In vitro studies from other groups have shown that at higher doses, Ang-(1–7) may have some agonist activity at AT1 receptors (27). Additional studies will have to be performed to thoroughly investigate the effects of various Ang-(1–7) doses and study durations on RAS expression and oxidative stress markers in diabetic bone marrow. In addition, Ang-(1–7)-associated increases in bone marrow nitrite levels were not significantly blocked by coadministration with icatibant, although icatibant did block the effects of all other changes in oxidative stress markers caused by Ang-(1–7). Based on other published studies and our data, the bradykinin B2 receptor may play an integral role in the mechanism of action of Ang-(1–7), and the results in this study may be a reflection of the high variation of nitrite levels often seen in tissue samples from in vivo studies.

In conclusion, our findings demonstrate that sc Ang-(1–7) treatment for 14 d decreased oxidative stress in the bone marrow in a murine model of type 2 diabetes and had significant effects on cellular components, including NADPH oxidase and SOD. Although additional in vitro and in vivo studies will need to be undertaken to further dissect these mechanisms and signaling pathways, pharmacological treatment with Ang-(1–7) along with other first-line therapies may prove useful to treat diabetes-induced oxidative stress and prevent its associated long-term complications.

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References

8. Santos RA, Simoes e Silva AC, Maric C, Silva DM, Machado RP, de Buhr I, Heringer-Walter S, Pinheiro SV, Lopes MT, Bader M,


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