Inhibition of myeloperoxidase increases revascularization and improves blood flow in a diabetic mouse model of hindlimb ischaemia

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Abstract
Objective: Diabetes mellitus is a significant risk factor for peripheral artery disease. Diabetes mellitus induces chronic states of oxidative stress and vascular inflammation that increase neutrophil activation and release of myeloperoxidase. The goal of this study is to determine whether inhibiting myeloperoxidase reduces oxidative stress and neutrophil infiltration, increases vascularization, and improves blood flow in a diabetic murine model of hindlimb ischaemia.

Methods: Leptin receptor–deficient (db/db) mice were subjected to hindlimb ischaemia. Ischaemic mice were treated with N-acetyl-lysyltyrosylcysteine-amide (KYC) to inhibit myeloperoxidase. After ligating the femoral artery, effects of treatments were determined with respect to hindlimb blood flow, neutrophil infiltration, oxidative damage, and the capability of hindlimb extracellular matrix to support human endothelial cell proliferation and migration.

Results: KYC treatment improved hindlimb blood flow at 7 and 14 days in db/db mice; decreased the formation of advanced glycation end products, 4-hydroxynonenal, and 3-chlorotyrosine; reduced neutrophil infiltration into the hindlimbs; and improved the ability of hindlimb extracellular matrix from db/db mice to support endothelial cell proliferation and migration.

Conclusion: These results demonstrate that inhibiting myeloperoxidase reduces oxidative stress in ischaemic hindlimbs of db/db mice, which improves blood flow and reduces neutrophil infiltration such that hindlimb extracellular matrix from db/db mice supports endothelial cell proliferation and migration.

Keywords
Diabetes mellitus, peripheral artery disease, neutrophils, myeloperoxidase, limb ischaemia, blood flow

Introduction
Diabetes mellitus (DM) is a significant risk factor for peripheral artery disease (PAD). Patients with DM have two to four times the risk of developing PAD, and 20%–30% of all PAD patients have DM.1 The complex aetiology and pathophysiology of DM associated with PAD are poorly understood; however, a myriad of inflammatory mediators in DM such as advanced glycation end products (AGEs), lipid peroxidation, and oxidative stress are all considered to contribute to PAD onset and progression. The pathobiology of PAD is further exacerbated by tissue ischaemia, endothelial dysfunction, and vascular inflammation.2,3 The current treatment regimen is primarily lifestyle changes and drugs that target specific PAD/DM symptoms of cardiovascular, hypertensive, thrombotic, or limb-related comorbidities.4,5
The development of effective drug therapies requires an understanding of disease pathology, identification of critical pathway/network processes, and drug targets associated with the disease. PAD is characterized by critical peripheral ischaemia mediated by redox biology and oxidative stress. Chronic increases in oxidative stress are known to cause exaggerated polymorphonuclear neutrophil (PMN) responses to stimuli with concomitant increases in the release of myeloperoxidase (MPO), which further increases inflammation. Based on this association, we propose that peroxidase acts as a ‘bridge’ between oxidative stress and inflammation. Based on this association, we propose that peroxidase acts as a ‘bridge’ between oxidative stress and inflammation.9,10

KYC is a novel tripeptide inhibitor of MPO toxic oxidant N-acetyl-lysyltyrosylcysteine-amide (KYC). db/db mice (n=8) were injected subcutaneously (SQ) daily with phosphate buffered saline (PBS); db/db mice (n=8) treated daily with 3 mg/kg KYC SQ; and a third group of untreated C57BL/6J mice (n=8). We have previously demonstrated that untreated C57BL/6J mice manifest identical biological endpoints compared to the same mice injected SQ with PBS. Mice were weighted and subjected to non-fasting endpoints compared to the same mice injected SQ with PBS at any time over 14 days post occlusion compared to blood flow in control animals (see Figure 1(a)). Note that in Figure 1(a), at day 0, we set the % change to 100 since this served as the baseline for comparison of blood flow changes at days 3, 7, and 14. Analysis of day 14 mice revealed that KYC-treated db/db mice showed improved blood flow in their IL hindlimbs 7 days post occlusion compared to PBS-treated db/db and C57BL/6J mice [db/db+KYC 370% ± 70% vs. db/db+PBS 166% ± 27% vs. C57BL/6J 179% ± 56% change from day 0 to day 7 in Figure 1(a)]. Blood flow continued to improve in the db/db mice treated with KYC even 14 days post occlusion compared to blood flow in control animals (db/db+KYC 499% ± 64% vs. db/db+PBS 155% ± 22% vs. C57BL/6J 435% ± 55%; D7 p < 0.01, D14 p < 0.05). Blood flow was not significantly restored in db/db mice treated with PBS at any time over the 14-day time course (day 0: 100% ± 11%; day 3: 162% ± 22%; day 7: 166% ± 27%; day 14: 155% ± 22%) (see Figure 1(a)). Note that in Figure 1(a), at day 0, we set the % change to 100 since this served as the baseline for comparison of blood flow changes at days 3, 7, and 14. Analysis of day 14 mice revealed that KYC-treated db/db mice had significantly fewer neutrophils in IL compared with the number in IL of PBS-treated db/db and C57BL/6J mice. KYC treatment significantly reduced the number of neutrophils in the CL of the db/db mice from an average of 6.4 to 0.2 neutrophils per section (six sections/animal, p < 0.01) and in IL of db/db mice from an average of relative band densities was performed using image analysis software (ImageJ, Bethesda, MD). For immunohistochemistry, gastrocnemius muscles were mounted in Tissue-Plus (Fisher Scientific, Hampton, NH), and 10 μm sections were cut and fixed using standard protocols. Sections were incubated with primary antibodies for the neutrophil biomarker NIMP-R14 (Santa Cruz Biotechnologies, Santa Cruz, CA). The effects of ECM on endothelial proliferation and migration were evaluated on human umbilical vein endothelial cells (HUVECs). These cells were cultured on fibrin gel mixed with ECM isolated from CL and IL hindlimbs from mice treated with PBS or KYC. Plasminogen-free human fibrinogen (5 mg/mL; Calbiochem, San Diego, CA) was dissolved in serum-free medium, filtered through 0.2 μm filters, and 20 μg/mL ECM was added. Fibrin matrices were prepared by polymerization using thrombin (2.5 U/mL, Sigma, Saint Louis, MO; 2 h at 37°C). After polymerization, thrombin was inactivated using culture medium containing 10% foetal bovine serum (FBS) for 2 h at 37°C. HUVECs were seeded on to 24-well plates for 5 days. Images (200×) were captured, and an electronic grid was superimposed on each image. The number of tubes intersecting the squares was counted using Nikon Element software.

Analysis of variance (ANOVA) with Bonferroni Dunn post hoc modification was used to compare blood flow values and Western blot densities for multiple comparisons. Student’s t test was used for single comparisons of band densities. A p value of <=0.05 was considered statistically significant.

Results

KYC-treated db/db mice showed improved blood flow in their IL hindlimbs 7 days post occlusion compared to PBS-treated db/db and C57BL/6J mice [db/db+KYC 370% ± 70% vs. db/db+PBS 166% ± 27% vs. C57BL/6J 179% ± 56% change from day 0 to day 7 in Figure 1(a)]. Blood flow continued to improve in the db/db mice treated with KYC even 14 days post occlusion compared to blood flow in control animals (db/db+KYC 499% ± 64% vs. db/db+PBS 155% ± 22% vs. C57BL/6J 435% ± 55%; D7 p < 0.01, D14 p < 0.05). Blood flow was not significantly restored in db/db mice treated with PBS at any time over the 14-day time course (day 0: 100% ± 11%; day 3: 162% ± 22%; day 7: 166% ± 27%; day 14: 155% ± 22%) (see Figure 1(a)). Note that in Figure 1(a), at day 0, we set the % change to 100 since this served as the baseline for comparison of blood flow changes at days 3, 7, and 14. Analysis of day 14 mice revealed that KYC-treated db/db mice had significantly fewer neutrophils in IL compared with the number in IL of PBS-treated db/db and C57BL/6J mice. KYC treatment significantly reduced the number of neutrophils in the CL of the db/db mice from an average of 6.4 to 0.2 neutrophils per section (six sections/animal, p < 0.01) and in IL of db/db mice from an average

Methods

This study was carried out in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and was approved by the Medical College of Wisconsin’s Institutional Animal Care and Use Committee. All animals were purchased from Jackson Laboratory (Bar Harbor, ME). The cohorts of mice were all 8-week-old males and consisted of db/db mice (n=8) injected subcutaneously (SQ) daily with phosphate buffered saline (PBS); db/db mice (n=8) treated daily with 3 mg/kg KYC SQ; and a third group of untreated C57BL/6J mice (n=8). We have previously demonstrated that untreated C57BL/6J mice manifest identical biological endpoints compared to the same mice injected SQ with PBS. Mice were weighted and subjected to non-fasting blood glucose determination using a Accu-Chek Instant glucometer (Roche, Indianapolis, IN). All mice underwent unilateral ligation of the femoral artery while under 1% isoflurane anaesthesia. Relative blood flow was measured under similar conditions using laser Doppler imaging (LDI; Moor Instruments, Wilmington, DE) immediately after surgery (day 0) and on postoperative days 3, 7, and 14. All mice were euthanized at day 14, and the gastrocnemius muscles from both ischaemic (IL) and control (CL) hindlimbs were harvested for Western blot analysis, immunohistochemistry, and isolation of hindlimb extracellular matrix (ECM).

In order to perform immunoblotting, tissues were lysed and analysed for AGEs (AbD Serotec, Raleigh, NC), 4-hydroxynonenal (4-HNE) (Millipore, Kankakee, IL), and 3-chlorotyrosine (3-Cl-Tyr) (Abcam, Cambridge, MA) using standard Western blot methods. Determination of relative band densities was performed using image analysis software (ImageJ, Bethesda, MD). For immunohistochemistry, gastrocnemius muscles were mounted in Tissue-Plus (Fisher Scientific, Hampton, NH), and 10 μm sections were cut and fixed using standard protocols. Sections were incubated with primary antibodies for the neutrophil biomarker NIMP-R14 (Santa Cruz Biotechnologies, Santa Cruz, CA). The effects of ECM on endothelial proliferation and migration were evaluated on human umbilical vein endothelial cells (HUVECs). These cells were cultured on fibrin gel mixed with ECM isolated from CL and IL hindlimbs from mice treated with PBS or KYC. Plasminogen-free human fibrinogen (5 mg/mL; Calbiochem, San Diego, CA) was dissolved in serum-free medium, filtered through 0.2 μm filters, and 20 μg/mL ECM was added. Fibrin matrices were prepared by polymerization using thrombin (2.5 U/mL, Sigma, Saint Louis, MO; 2 h at 37°C). After polymerization, thrombin was inactivated using culture medium containing 10% foetal bovine serum (FBS) for 2 h at 37°C. HUVECs were seeded on to 24-well plates for 5 days. Images (200×) were captured, and an electronic grid was superimposed on each image. The number of tubes intersecting the squares was counted using Nikon Element software.

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of 12.6 to 3.0 neutrophils per section (six sections/animal, \( p < 0.001 \)) (Figure 1(b)). KYC treatment also reduced oxidative stress in \( \text{db/db} \) mice based on marked decreases in AGE (Figure 1(c)), 4-HNE (Figure 1(d)), and 3-Cl-Tyr (Figure 1(e)) in the IL of \( \text{db/db} \) mice. Reductions in these biomarkers in IL of KYC-treated \( \text{db/db} \) mice compared to levels in IL of PBS-treated \( \text{db/db} \) mice were statistically significant ranging from 106.2\% for AGE (\( p < 0.01 \)), 55.5\% for 4-HNE (\( p < 0.01 \)), and 62\% for 3-Cl-Tyr (\( p < 0.001 \)). Metabolic measurements between PBS- and KYC-treated \( \text{db/db} \) mice were not significantly altered (body weight: PBS 59 ± 1.23 g and KYC 62.33 ± 2.96 g, blood glucose: PBS 298.85 ± 30.03 mg/dL and KYC 218.33 ± 8.92 mg/dL).

To better understand the mechanisms for why KYC treatment increased blood flow in IL of KYC-treated
db/db mice, we cultured HUVECs on ECM isolated from the hindlimbs of C57BL/6J, db/db mice, and db/db mice treated with KYC and assessed HUVEC proliferation and migration. The proliferation of HUVECs on PBS-treated db/db ECM was reduced compared to the proliferation of HUVECs cultured on ECM isolated from C57BL/6J control mice (Figure 1(f)). This trend was reversed when HUVECs were cultured on ECM isolated from db/db mice treated with KYC. When HUVECs were cultured on fibrin gels containing ECM from IL of db/db mice, they failed to proliferate. In contrast, HUVECs cultured on fibrin gels containing ECM isolated from IL from KYC-treated db/db mice did proliferate to about 50% of the levels of HUVECs cultured on fibrin gels containing ECM from control C57BL/6J mice. In HUVECs cultured on ECM from both IL and CL, KYC treatment significantly increased proliferation compared to PBS-treated (p < 0.01). Migration was also impaired when HUVECs were cultured on fibrin gels containing ECM isolated from hindlimbs of db/db mice (Figure 1(g)) and increased when HUVECs were cultured on fibrin gels containing ECM isolated from hindlimbs of db/db mice treated with KYC regardless of whether the ECM was isolated from IL or CL (p < 0.01).

Discussion

The goal of this study was to ascertain the role of MPO in DM-mediated PAD in a db/db mouse model of type-2 DM. Our data show that MPO generates toxic oxidants that induce oxidative damage, increase PMN recruitment, and alter ECM in the ischemic hindlimbs such that the matrix no longer supports EC proliferation and migration. These two latter functions are essential for new vascular growth. KYC treatment of the db/db mice increased blood flow and reduced PMN recruitment in the ischemic hindlimbs by reducing oxidative damage of the ECM such that after isolation from ischemic hindlimbs, the matrix can now support EC proliferation and migration. These data, taken together, suggest that inhibiting MPO toxic oxidant production is key to restoring the mechanisms mediating revascularization in db/db mice. One of the ways KYC may reduce oxidative stress in db/db mice is by promoting MPO hydrogen peroxide consumption. As an MPO substrate, KYC is oxidized into KYC thyl radicals that readily auto-scavenge with another KYC or glutathione to form harmless disulfide dimers. In this way, KYC converts MPO into a quasi-catalase that should reduce H$_2$O$_2$-dependent oxidative damage. Although the exact mechanisms by which KYC improves blood flow were not determined, collateral growth, angiogenesis, and vasculogenesis may all be involved.

Our observations have important implications for the care of DM patients with PAD because they clearly show that MPO is a therapeutic target for improving revascularization in an established murine model of DM. People with DM who have PAD are at greater risk of cardiovascular disease than people with DM without PAD. If MPO plays a role in PAD in people with DM in the same way as MPO impairs blood flow in the ischemic hindlimbs of db/db mice, then KYC may be an effective adjunct therapy for treating PAD by reducing MPO-dependent oxidative damage and improving blood flow in the lower legs and feet.

Declaration of conflicting interests

The author(s) declared the following potential conflicts of interest with respect to the research, authorship, and/or publication of this article: Dr Dustin P Martin, Dr Stephen Naylor and Dr Kirkwood A Pritchard Jr declared that they have ownership stakes in an Elm Grove–based company ReNeuroGen, LLC that is developing KYC for therapeutic use in sickle cell disease and multiple sclerosis, but not diabetes-associated PAD. All other authors declared no potential conflicts of interest with respect to the research, authorship or publication of this article.

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