

## ORIGINAL ARTICLE

# Niacin promotes revascularization and recovery of limb function in diet-induced obese mice with peripheral ischemia

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## Keywords

Inflammation, lipotoxicity, metabolic syndrome, niacin, peripheral vascular disease

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## Abstract

Niacin can reduce vascular disease risk in individuals with metabolic syndrome, but in light of recent large randomized controlled trials outcomes, its biological actions and clinical utility remain controversial. Niacin can improve endothelial function, vascular inflammation, and vascular regeneration, independent of correcting dyslipidemia, in various lean rodent models of vascular injury. Here, we tested whether niacin could directly improve endothelial cell angiogenic function during combined exposure to excess fatty acids and hypoxia, and whether intervention with niacin during continued feeding of western diet could improve revascularization and functional recovery in obese, hyperlipidemic mice with peripheral ischemia. Treatment with niacin (10  $\mu$ mol/L) increased human microvascular endothelial cell angiogenic function during exposure to high fatty acids and hypoxia (2% oxygen), as determined by tube formation on Matrigel. To assess revascularization in vivo, we used western diet-induced obese mice with unilateral hind limb femoral artery ligation and excision. Treatment for 14 days postinjury with once daily i.p. injections of a low dose of niacin (50 mg/kg) improved recovery of hind limb use, in association with enhanced revascularization and decreased inflammation of the tibialis anterior muscle. These effects were concomitant with decreased plasma triglycerides, but not increased plasma apoAI. Thus, niacin improves endothelial tube formation under lipotoxic and hypoxic conditions, and moreover, promotes revascularization and functional hind limb recovery following ischemic injury in diet-induced obese mice with hyperlipidemia. These data may have implications for niacin therapy in the treatment of peripheral ischemic vascular disease associated with metabolic syndrome.

## Abbreviations

ALT, alanine transaminase; apoAI, apolipoprotein AI; AST, aspartate transaminase; BSA, bovine serum albumin; HDL, high-density lipoprotein; HMVEC, human microvascular endothelial cells; HOMA-IR, homeostatic model assessment for insulin resistance; MOMA-2, monocyte macrophage antigen 2.

## Introduction

Vascular complications arising from insults to the endothelium, including ischemic vascular diseases, are common in the setting of metabolic syndrome (Vita and Hamburg 2010; Campia *et al.* 2012; Kim *et al.* 2012). Excess circulating triglycerides and fatty acids, which are characteristic of metabolic syndrome, can trigger endothelial cell dysfunction and subsequently limit the regenerative capabilities of the endothelium (Imrie *et al.* 2010; Symons and Abel 2013). Clinical studies over several decades have shown that niacin, which lowers triglycerides and raises high-density lipoprotein (HDL), can reduce vascular disease risk, but its mechanism of action is unclear and may not depend on its systemic lipid-modifying effects (Lavigne and Karas 2013). Negative outcomes of recent large clinical trials (Boden *et al.* 2011; Haynes *et al.* 2013; Landray *et al.* 2014) have challenged the clinical utility of high-dose niacin in combination with statins for long-term treatment of dyslipidemia in patients with characteristics of metabolic syndrome. However, niacin monotherapy improves vascular health in several patient populations (Creider *et al.* 2012), including those with metabolic syndrome dyslipidemia (Thoenes *et al.* 2007; Guyton *et al.* 2013; Toth *et al.* 2015). Intriguingly, retrospective meta-analyses (Lavigne and Karas 2013) and studies in cell culture, mouse models, healthy subjects, and patients with type 2 diabetes mellitus suggest that the vascular benefits of niacin may be driven by direct effects on endothelial and immune cells (Wu *et al.* 2010, 2012; Lukasova *et al.* 2011; Tavintharan *et al.* 2011; Digby *et al.* 2012; Chai *et al.* 2013b; Kaplon *et al.* 2014).

Uptake and accumulation of surplus fatty acids (particularly saturated species) from both lipoprotein-derived and albumin-bound sources can lead to cell dysfunction and cell damage, a process referred to as lipotoxicity, in cells and tissues throughout the body including those of the vasculature (Kim *et al.* 2012; Wende *et al.* 2012; Symons and Abel 2013). Endothelial cells may be especially vulnerable to lipotoxicity because, despite being continually exposed to elevated circulating lipids and lipids within steatotic tissues during obesity and metabolic syndrome, they are not metabolically programmed to process large quantities of fatty acids (Dagher *et al.* 2001; Helies-Toussaint *et al.* 2006). We recently reported that supplementation of culture medium with a low, but pharmacologically relevant, concentration of niacin (10  $\mu\text{mol/L}$ ) improves human microvascular endothelial cell tube formation during exposure to excess palmitate under normoxic conditions (Hughes-Large *et al.* 2014). Whether niacin can improve microvascular endothelial cell angiogenic function under combined conditions of lipid excess and hypoxia is unknown. These conditions

coexist in tissues such as skeletal muscle during peripheral ischemic vascular disease associated with obesity and metabolic syndrome, as a result of reduced blood flow and microvessel rarefaction (Chantler and Frisbee 2015) and tissue lipid accumulation (Eckardt *et al.* 2011).

Evidence of endothelial lipotoxicity in association with vascular dysfunction has been observed in rodent models of obesity and hyperlipidemia (Chinen *et al.* 2007; Kim *et al.* 2007; Maloney *et al.* 2009; Tian *et al.* 2012; Cheang *et al.* 2014; Chattopadhyay *et al.* 2015; Toral *et al.* 2015), suggesting that this process indeed contributes to vascular disease during metabolic syndrome. To assess vascular regeneration during metabolic disease, models of acute peripheral ischemic injury following unilateral femoral artery ligation and excision have been developed in obese and diabetic mice. These mice exhibit impaired restoration of blood flow, and reduced capillary and collateral vessel growth and maintenance after ischemic injury, indicating that functional vascular regeneration is limited during metabolic disease (Emanuelli *et al.* 2007; Li *et al.* 2007; Wang *et al.* 2009; Kito *et al.* 2012; Landazuri *et al.* 2012; Dokun *et al.* 2014). Previous reports show that niacin promotes angiogenesis and functional revascularization in ischemic brains of lean, healthy rats after stroke (Chen *et al.* 2007, 2009), and in lean, streptozotocin-induced diabetic mice with critical limb ischemia (Huang *et al.* 2012). It is not known whether intervention with niacin can improve revascularization and recovery of ischemic limb function in the setting of diet-induced obesity and metabolic syndrome, without frank type 2 diabetes, conditions which reflect the patient population most likely to receive niacin treatment.

In light of these knowledge gaps, we investigated the effects of low concentrations of niacin on angiogenic function in cultured human microvascular endothelial cells (HMVEC) during lipotoxicity and hypoxia, and on recovery from critical hind limb ischemia in diet-induced obese, hyperlipidemic mice. Data reported herein show that niacin improves the ability of HMVEC to form tubes under conditions of fatty acid excess and hypoxia, and that intervention with niacin enhances revascularization and functional hind limb recovery following critical ischemic injury in obese mice with characteristics of metabolic syndrome.

## Materials and Methods

### Endothelial cell culture and treatments

HMVEC are the closest representation of endothelial cells likely to experience low oxygen (Chantler and Frisbee 2015), and to participate in revascularization of the tibialis anterior muscle in response to lower limb ischemia

(Shireman, 2007). Primary HMVEC (Lonza, Walkersville, MD, USA) were maintained in Medium 199 (Life Technologies, Carlsbad, CA, USA) supplemented with EGM-2MV SingleQuots (Lonza), and subcultured as recommended by the supplier. For experiments, cells from two different donors were used, between subcultures 4 and 8. Hypoxic conditions were induced by incubation at 2% oxygen. For fatty acid treatments, growth medium was supplemented with 0.5 mmol/L palmitate, or 0.5 mmol/L oleate, or a combination of palmitate and oleate (1:1 ratio, 0.25 mmol/L palmitate plus 0.25 mmol/L oleate to achieve a total concentration of 0.5 mmol/L fatty acids). All fatty acids were complexed to bovine serum albumin (BSA) at molar ratio of 2:1 prior to addition to culture medium (Hughes-Large *et al.* 2014). Concentrations of fatty acids used reflect high physiological to pathophysiological concentrations, as would be observed during obesity, and metabolic syndrome (Gordon 1960; Soriguer *et al.* 2009). The combination of palmitate plus oleate was used to reflect the ratio of saturated and unsaturated fatty acids in western diets. Experimental media were supplemented with 10  $\mu\text{mol/L}$  niacin (Fluka BioChemika, St. Louis, MO, USA), where indicated, solubilized in cell culture grade water. The concentration of niacin used (10  $\mu\text{mol/L}$ ) reflects the low end of the range of plasma concentrations that can be achieved following oral administration of high-dose niacin (2 g), which has been shown to be approximately 10  $\mu\text{mol/L}$ –240  $\mu\text{mol/L}$  within 8 h of dosing (Menon *et al.*, 2007a; Menon *et al.*, 2007b). Based on this previous work, plasma concentrations achieved from recommended daily intake of niacin (14–16 mg/day), would be predicted to reach a maximum of approximately 2  $\mu\text{mol/L}$ .

### Tube formation

Formation of tube networks was assessed using a growth factor replete basement membrane matrix formulation (Matrigel, BD Biosciences, Bedford, MA, USA), as previously described (Borradaile and Pickering 2009; Hughes-Large *et al.* 2014). Tube networks formed on Matrigel within 18 h were visualized by light microscopy using an Olympus IX71 inverted microscope. Total tube lengths per field of view were quantified using ImageJ. A tube was defined as an apparently three-dimensional (tube-like), elongated structure stretching between branch points, with a width large enough along its entire length to permit the passage of an erythrocyte.

### Fatty acid oxidation

Palmitate oxidation was assessed by measuring conversion of  $^3\text{H}$ -palmitate oxidized to  $^3\text{H}_2\text{O}$ , as previously described

(Borradaile and Pickering 2009; Hughes-Large *et al.* 2014). The carnitine palmitoyltransferase-1 inhibitor, etomoxir (200  $\mu\text{mol/L}$ ) (Sigma, St. Louis, MO, USA), was used as a control.

### Mouse model of diet-induced obesity and metabolic syndrome

All mouse experiments were approved by the Animal Care and Use Subcommittee at Western University (protocol number 2011–044) in accordance with the requirements of the Canadian Council on Animal Care ([http://www.ccac.ca/en\\_](http://www.ccac.ca/en_)). Five-week-old male 129S6 mice (Taconic, Germantown, NY, USA) were fed western diet containing 42% of calories from animal fat (Harlan Teklad, Toronto, ON, Canada) ad libitum for 17 weeks. The fatty acid composition of this diet is approximately 65% saturated and 35% unsaturated species. Control mice were maintained on chow diet with 14% of calories from fat (Harlan Teklad). Mice were fasted for 6 h prior to sacrifice, and body weight and blood glucose measurements were performed immediately prior to euthanasia. All remaining parameters were determined postmortem. Liver and plasma cholesterol and triglycerides were determined by enzymatic, colorimetric assays (Roche Diagnostics, Indianapolis, IN, USA, and Wako Diagnostics, Richmond, VA, USA) (Assini *et al.* 2013). Plasma liver enzymes (alanine transaminase [ALT] and aspartate transaminase [AST]) were measured by enzymatic rate assays (Roche Diagnostics) using a Cobas autoanalyzer at the London Health Sciences Centre Core Laboratory. Plasma apoAI was measured using a mouse enzyme-linked immunosorbent assay (ELISA) (Cloud-clone, Houston, TX, USA). Blood glucose was determined with an Ascensia Elite glucometer (Bayer, Mississauga, ON, Canada) and plasma insulin was measured using a mouse ultrasensitive insulin ELISA (Alpco Diagnostics, Salem, NH, USA). HOMA-IR was calculated using blood glucose and plasma insulin values. All plasma and tissue biochemical measurements, with the exception of ALT and AST, were performed through the Metabolic Phenotyping Laboratory at Robarts Research Institute, Western University.

### Hind limb ischemia and niacin intervention

After 15 weeks of control chow or western diet, mice underwent right femoral and saphenous artery ligation, followed by complete excision of the femoral artery (Frontini *et al.* 2011). Briefly, mice were anesthetized using a combination of *i.p.* ketamine (150 mg/kg) and xylazine (5 mg/kg), followed by exposure of the femoral artery through a small incision in the skin. The proximal end of the femoral artery and the distal end of the

saphenous artery were ligated, and the artery excised. The overlying skin was then sutured and mice were given one i.p. dose of meloxicam (1 mg/kg), followed by a second dose 24 h postsurgery. For 14 days following surgery, lean control mice (chow) received once daily i.p. injections of vehicle, while obese mice (western diet) were randomized into two groups and received once daily i.p. injections of either vehicle or niacin (50 mg/kg). This dose of niacin is approximately equivalent to a human dose of 250 mg (Reagan-Shaw *et al.*, 2008), and has previously been shown to decrease plasma triglycerides in mice (Tunaru *et al.*, 2003). All mice were maintained on their respective diets for the 14-day treatment period. All mice were euthanized on day 15 by carbon dioxide overdose, followed by cardiac puncture to collect blood.

### Gait analyses

Limb function was assessed on postsurgery days 4, 9, and 15 by gait analyses using a Catwalk system (Noldus, Leesburg, VA, USA) (Neurobehavioural Core Facility, Robarts Research Institute, Western University) to observe paw contact times and intensities (Frontini *et al.* 2011). As mice traversed an illuminated glass walkway, the duration (sec) of surface contact for each paw was digitally recorded and illustrated as contact duration maps. Hind limb use ratios were calculated from values for right (injured) and left (uninjured) hind limbs. Since healthy mice use both hind limbs equally, right hind limb to left hind limb ratios <1.0, for either contact time or intensity, indicate decreased use of the injured limb.

### Immunohistochemistry

At sacrifice, tibialis anterior muscles were dissected, immersed in zinc fixation buffer (0.1 mol/L Tris-HCl buffer (pH 7.4) containing 0.5 g/L calcium acetate, 5.0 g/L zinc acetate, 5.0 g/L zinc chloride), embedded in paraffin, and subsequently cross-sectioned (5  $\mu$ m thickness) to generate four serial sections at three equally spaced locations spanning the length of the muscle. This muscle is distal to the site of injury and undergoes significant necrosis and inflammation, followed by angiogenesis (rather than collateral vessel formation) and muscle regeneration, in response to femoral artery ligation and excision (Shireman, 2007). For determination of vessel densities, sections were analyzed after double immunostaining for CD31 and smooth muscle  $\alpha$ -actin (van der Veer *et al.* 2005; Frontini *et al.* 2011). Total vessel densities (CD31 positive) and smooth muscle  $\alpha$ -actin positive vessels (arterioles) were counted. For assessment of muscle architecture, sections were analyzed after staining with hematoxylin and eosin. Areas of

mature muscle (intense myofiber eosin staining with peripheral nuclei), regenerating muscle (intense myofiber eosin staining with central nuclei), necrotic muscle (pale myofiber eosin staining with absent nuclei), and adipose (absent eosin staining with peripheral nuclei) were determined. For assessment of tissue inflammation, sections were analyzed after immunostaining for MOMA-2 (monocyte macrophage antigen 2) (Bojic *et al.* 2014). Although commonly used for identifying vascular inflammation, the protein moiety corresponding to this antigen has not been identified. MOMA-2 staining generally shows close correlation with acid phosphatase staining, which can be a marker of autophagy, and also appears to be a lysosomal factor (Ohmi *et al.*, 2003). To accurately quantitate inflammation, MOMA-2 positive inflammatory foci (clusters of >2 nucleated MOMA-2 positive cells) were counted. All light microscopy was performed using an Olympus BX51, and all analyses were performed for 12 fields of view per muscle using ImageJ.

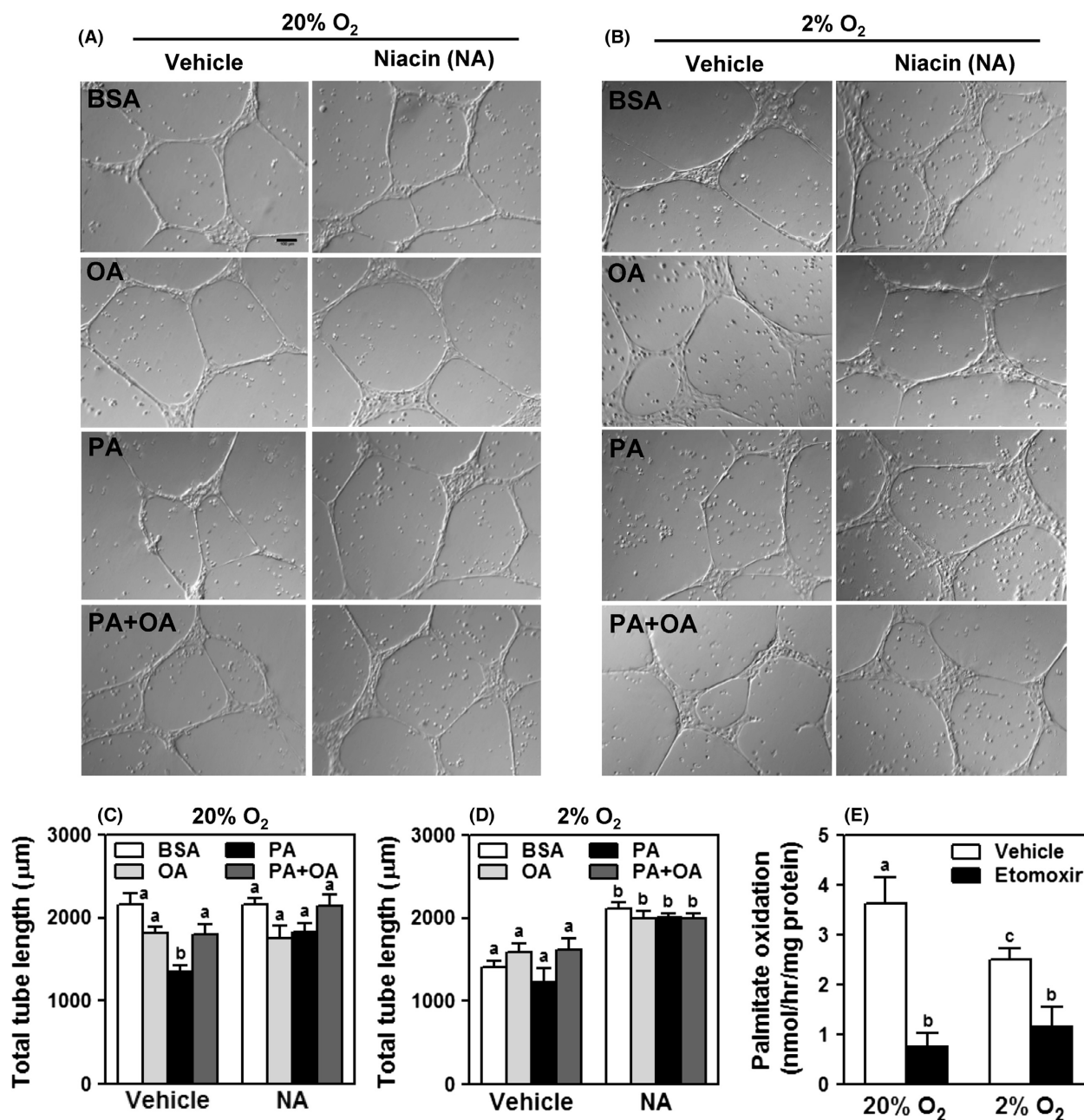
### Compliance with design and statistical analysis requirements

All cell culture experiments were performed in duplicate for  $n = 4-5$ . All experimental mouse groups included six animals. No data normalizations or transformations were performed. Statistical analyses were performed using GraphPad Prism 5. Unless indicated otherwise (as in Fig. 2D), differences in means were assessed using either two-way ANOVA (cell experiments) or one-way ANOVA (mouse experiments), followed by Bonferroni multiple comparison post hoc tests comparing all groups. Differences in means were considered statistically significant at  $P < 0.05$ . In graphs and tables, statistically significant differences are indicated with lower case letters. Bars or values with different lower case letters are significantly different from each other at  $P < 0.05$ , while those that share the same lower case letter are not significantly different. For example, a value labeled "a" is different from one labeled "b," but a value labeled "ab" is different from neither "a" nor "b."

## Results

### Niacin improves HMVEC angiogenic function during exposure to high fatty acids and hypoxia

We previously found that supplementation of culture medium with a relatively low concentration of niacin (10  $\mu$ mol/L) preserves HMVEC tube formation during exposure to excess palmitate under normoxic conditions



**Figure 1.** Niacin improves tube formation during exposure to high fatty acids under hypoxic conditions. (A, B) Human microvascular endothelial cells were pretreated for 24 h in culture medium supplemented with either vehicle (water) or 10  $\mu\text{mol/L}$  niacin (NA), and then seeded onto growth factor replete Matrigel in media containing BSA, 0.5 mmol/L oleate (OA), 0.5 mmol/L palmitate (PA), or palmitate plus oleate (PA + OA, 1:1 ratio, 0.5 mmol/L total fatty acid concentration), with or without NA. Tube networks at (A) 20% O<sub>2</sub> and (B) 2% O<sub>2</sub> are shown. Scale bar = 100  $\mu\text{m}$ . (C, D) For quantification, three fields of view per condition were assessed for total tube length. (E) To confirm functional hypoxia, palmitate  $\beta$ -oxidation was assessed by conversion of <sup>3</sup>H-palmitate to water. Cells were incubated at 20% or 2% O<sub>2</sub> for 18 h. Etomoxir (CPT1 inhibitor) was used as a control. Statistically significant differences were determined by two-way ANOVA followed by Bonferroni multiple comparison tests comparing all conditions. Bars with different lower case letters are significantly different at  $P < 0.05$ , while those that share the same lower case letter are not significantly different. Data are means  $\pm$  SEM for  $n = 4-5$ .

(Hughes-Large et al. 2014). This earlier observation was reproduced (Fig. 1A) in order to compare the effects of niacin on tube formation during both lipotoxic and

hypoxic conditions (Fig. 1B). Pretreatment for 24 h with culture medium containing 10  $\mu\text{mol/L}$  niacin increased subsequent endothelial tube formation under hypoxic

conditions (2% oxygen) in all cells, regardless of exposure to high concentrations of fatty acids (Fig. 1B and D). Interestingly, these data differed from observations obtained under normoxic conditions (Fig. 1A and C) in several ways. First, total tube lengths achieved during exposure to 2% oxygen were lower than those achieved during normoxia (20% oxygen). Second, the toxic effect of palmitate compared to control conditions (BSA alone or plus oleate) was blunted compared to its effect under normoxic conditions, possibly as a result of decreased  $\beta$ -oxidation. And third, niacin increased tube formation during hypoxia to the same extent regardless of the presence of surplus saturated or unsaturated species of fatty acids. However, similar to our previous observations in normoxia (Hughes-Large *et al.* 2014), niacin did not increase HMVEC proliferation during hypoxia. Population doublings per day for control cells and niacin-treated cells were  $0.22 \pm 0.08$  and  $0.20 \pm 0.07$ , respectively. Palmitate oxidation was decreased in HMVEC incubated at 2% oxygen, confirming that our low-oxygen incubation conditions induced functional hypoxia (Fig. 1E). In both 20% and 2% oxygen conditions, inhibition of CPT1 with etomoxir significantly decreased palmitate oxidation indicating that the assay was measuring  $\beta$ -oxidation (Fig. 1E).

### Niacin improves recovery of hind limb function after ischemic injury in obese mice with metabolic syndrome

To determine whether beneficial effects of niacin could be observed in a model of obesity, hyperlipidemia, and ischemic vascular injury, we used western diet-fed 129S6 mice. After 15 weeks of diet induction, all mice underwent right femoral artery ligation and excision, followed by 14 days of recovery with once daily i.p. injections of either vehicle (sterile water) or niacin (50 mg/kg) while being maintained on their respective diets. After a total of 17 weeks of ad libitum feeding of western diet, 129S6 mice clearly exhibited characteristics consistent with metabolic syndrome in this strain (Biddinger *et al.* 2005), including increased adiposity, hypertriglyceridemia, mildly elevated fasting blood glucose, hyperinsulinemia, and modestly increased HOMA-IR compared to mice maintained on chow diet (Table 1). Values for fasting blood glucose were below those considered to reflect frank type 2 diabetes in mouse models ( $>8.3$  mmol/L) (King 2012). Treatment with niacin did not result in decreased caloric intake, but was associated with statistically significant reductions in adipose mass, liver triglycerides, fasting plasma triglycerides, cholesterol and insulin, and HOMA-IR (Table 1). Remarkably, plasma apoAI was not increased with niacin within this time frame (Table 1).

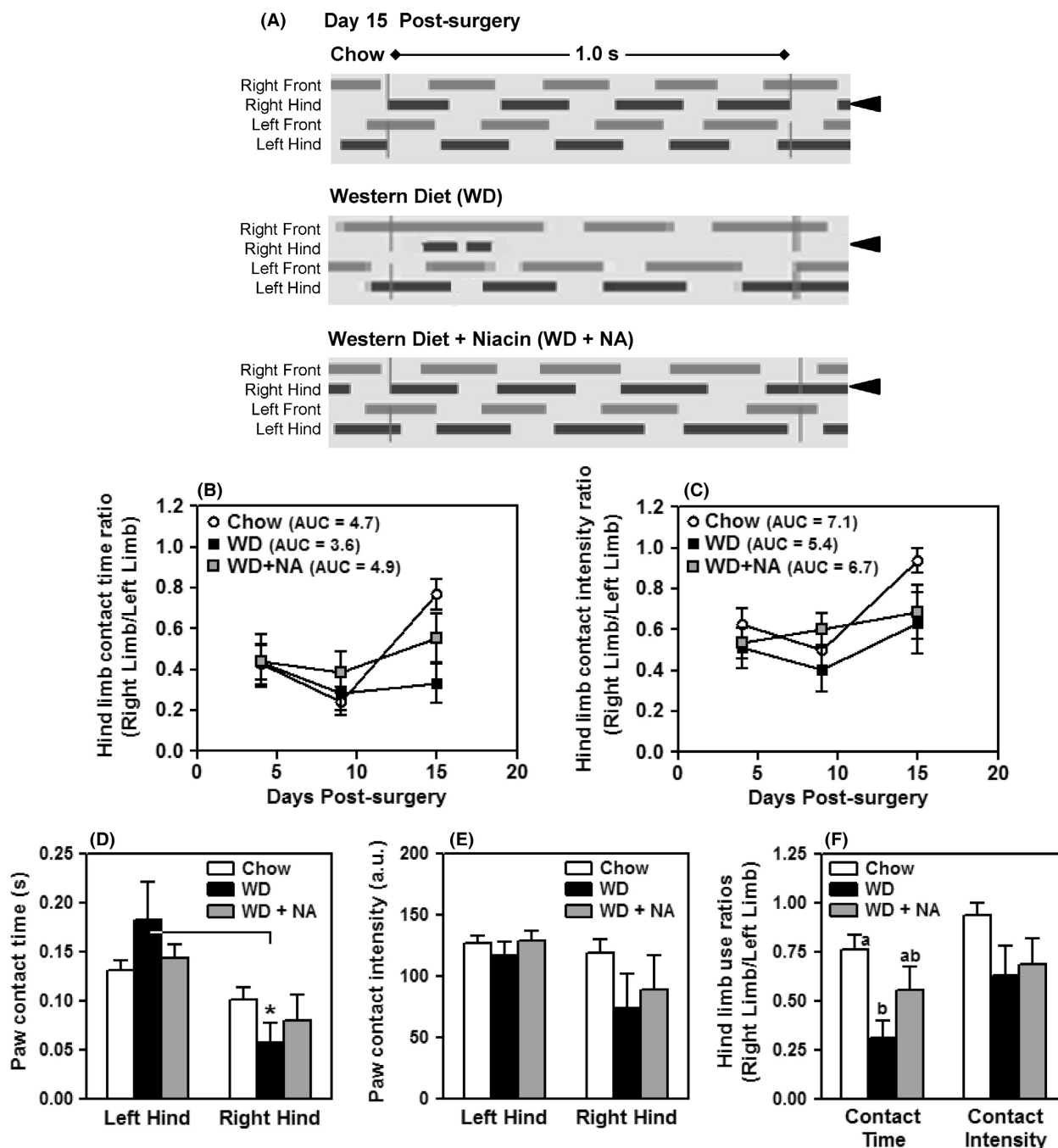
**Table 1.** Parameters of metabolic syndrome in 129S6 mice.

Diet (treatment)	Chow (vehicle)	Western (vehicle)	Western (niacin)
Body weight (g)	$26.0 \pm 0.7^a$	$32.7 \pm 1.6^b$	$31.1 \pm 0.5^b$
Epididymal fat weight (g)	$0.37 \pm 0.04^a$	$1.44 \pm 0.09^b$	$1.03 \pm 0.11^c$
Liver triglycerides (mg/g)	$13.3 \pm 2.0^a$	$168.4 \pm 12.6^b$	$66.9 \pm 13.3^c$
Plasma ALT (U/L)	$35.0 \pm 9.3$	$46.7 \pm 9.3$	$20.3 \pm 1.3$
Plasma AST (U/L)	$106.0 \pm 16.3$	$119.0 \pm 19.9$	$91.2 \pm 14.4$
Plasma triglycerides (mmol/L)	$0.82 \pm 0.04^{ab}$	$0.98 \pm 0.05^a$	$0.77 \pm 0.06^b$
Plasma free fatty acids (mmol/L)	$0.34 \pm 0.02$	$0.30 \pm 0.02$	$0.26 \pm 0.02$
Plasma cholesterol (mmol/L)	$2.85 \pm 0.12^a$	$5.24 \pm 0.21^b$	$3.88 \pm 0.09^c$
Plasma ApoAI (mg/mL)	$1.86 \pm 0.23^a$	$3.31 \pm 0.31^b$	$2.51 \pm 0.18^{ab}$
Blood glucose (mmol/L)	$5.2 \pm 0.2^a$	$6.3 \pm 0.1^b$	$5.4 \pm 0.4^{ab}$
Insulin (pmol/L)	$63.7 \pm 10.3^a$	$127.4 \pm 6.9^b$	$80.9 \pm 15.5^a$
HOMA-IR	$1.20 \pm 0.01^a$	$2.47 \pm 0.003^b$	$1.53 \pm 0.02^c$

Five-week-old male mice were maintained on chow (14% calories from fat) or western diet (42% calories from fat) for 15 weeks, followed by unilateral femoral artery ligation and excision surgery. Mice were maintained for a further 14 days with daily i.p. injections of either sterile water (vehicle) or niacin (50 mg/kg). Body weight and blood glucose measurements were performed immediately prior to sacrifice (day 15). All remaining parameters were determined post-mortem. HOMA-IR was calculated using blood glucose and plasma insulin values. Statistically significant differences were determined by one-way ANOVA followed by Bonferroni multiple comparison tests comparing all groups. Values with different lower case letters are significantly different at  $P < 0.05$ , while those that share the same lower case letter are not significantly different. Data are means  $\pm$  SEM for  $n = 6$ .

ALT, alanine transaminase; AST, aspartate transaminase.

Gait analyses (Fig. 2A) performed on postsurgery days 4, 9, and 15 suggested trends for increased use of the injured (right) hind limb in niacin-treated obese mice compared to vehicle-treated obese mice, particularly after day 9 (Fig. 2B). By day 15, only vehicle-treated obese mice (WD) exhibited a statistically significant difference between left (uninjured) and right (injured) hind limb use, as measured by paw contact times (Fig. 2D). Furthermore, hind limb use ratios for niacin-treated mice, based on paw contact times, were intermediate between lean control (Chow) and vehicle-treated obese (WD) mice (Fig. 2F) (Chow vs. WD,  $P = 0.002$ ; Chow versus WD plus niacin,  $P = 0.10$ ; WD versus WD plus niacin,  $P = 0.07$ ). These data indicate that short-term interven-



**Figure 2.** Niacin improves recovery of hind limb function after ischemic injury in obese mice with metabolic syndrome. Lean (Chow) and western diet-induced obese (WD) mice underwent right hind limb femoral artery excision surgery, followed by 14 days with daily i.p. injections of either vehicle or niacin (NA). (A) On days 4, 9, and 15 postsurgery, gait analyses were performed using a Catwalk system. Representative contact duration maps over a 1.0-sec time frame are shown for day 15. Black arrows indicate the injured limb. (B, C) Mean paw contact times and intensities were used to calculate hind limb use ratios at each postsurgery time point. Right limb to left limb ratios  $<1.0$ , for either contact time or intensity, indicate decreased use of the right hind limb. Areas under the curves (AUC) were calculated. (D) Mean paw contact times, (E) mean paw contact intensities, and (F) ratios of right hind limb to left hind limb use on day 15 are shown. For paw contact times and intensities, differences between left and right hind limbs within groups were determined by Student's *t* test, \* indicates  $P < 0.05$ . For hind limb use ratios, statistically significant differences between groups were determined by one-way ANOVA followed by Bonferroni multiple comparison tests comparing all groups. Bars with different lower case letters are significantly different at  $P < 0.05$ , while those that share the same lower case letter are not significantly different. Data are means  $\pm$  SEM for  $n = 6$ .

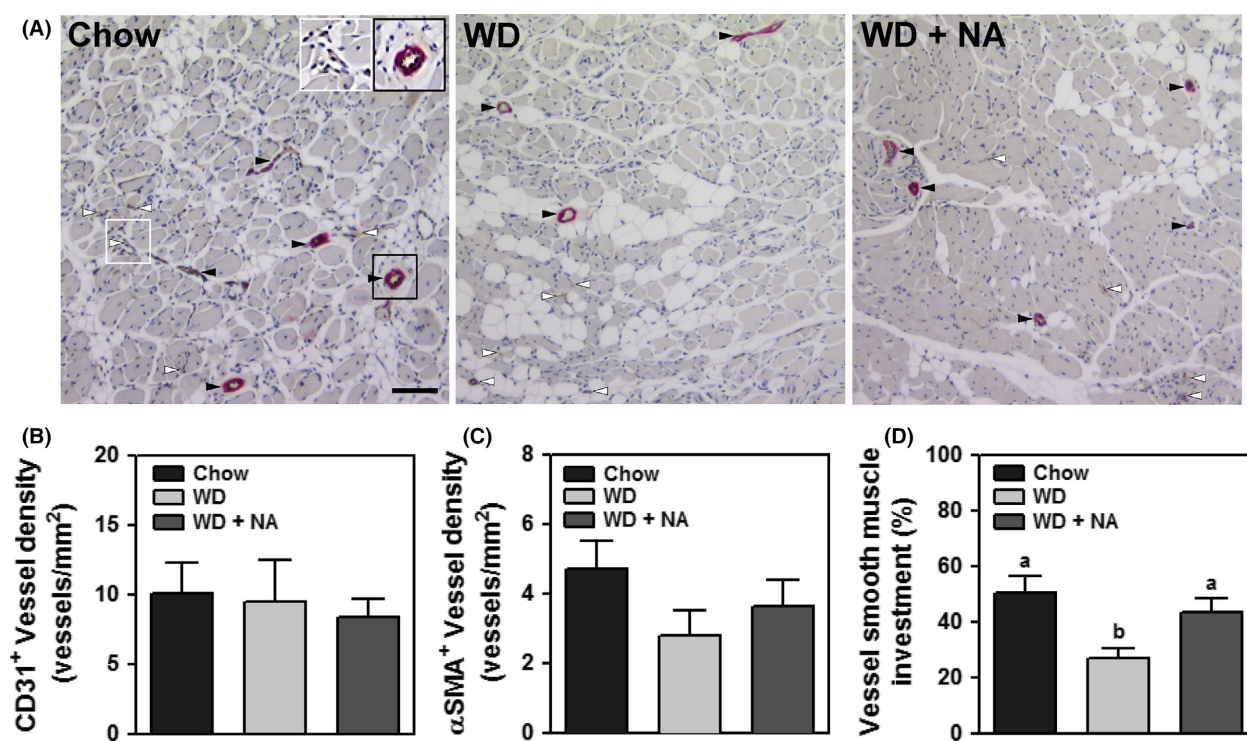
tion with niacin after ischemic injury improves recovery of limb function in a mouse model of metabolic syndrome. Consistent with previous work in 129S2 mice, lean control (Chow) mice exhibited near complete recovery of hind limb use within 15 days (Helisch *et al.* 2006).

### Niacin improves revascularization and tissue architecture of the tibialis anterior muscle after ischemic injury in obese mice with metabolic syndrome

Given that pretreatment with niacin has been shown to promote angiogenesis and revascularization in lean diabetic mice with critical limb ischemia (Huang *et al.* 2012), we determined vessel densities by immunohistochemistry in tibialis anterior muscles (Fig. 3A). Total vessel (CD31 positive) densities and arteriole (smooth muscle  $\alpha$  actin positive) densities per section area were not statistically different between groups (Fig. 3B and C). However, the proportion of total vessels invested with smooth muscle in

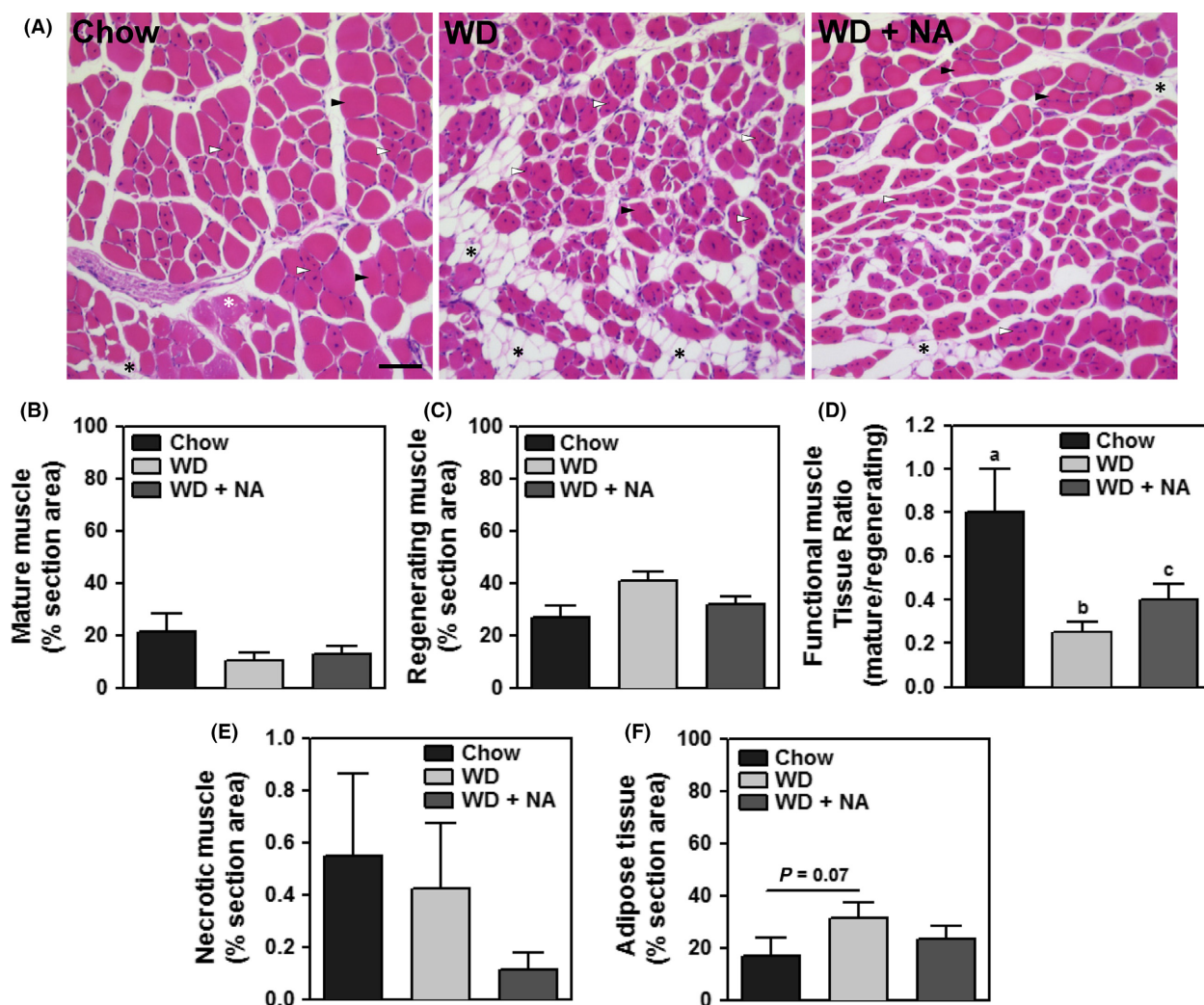
niacin-treated mice was significantly increased compared to vehicle-treated obese mice (Fig. 3D) suggesting that the quality and/or stability of newly formed vasculature may be improved with niacin.

To determine whether the increased smooth muscle investment of vessels we observed with niacin was associated with improved muscle tissue architecture, we measured areas of mature, regenerating, and necrotic myofibers in sections stained with hematoxylin and eosin (Fig. 4A). Only healthy, mature myofibers with peripheral nuclei are believed to be fully functional, as the central nuclei present in regenerating fibers interfere with optimal contraction (Folker and Baylies 2013). Similar to our observations for vessel densities, percent section areas for mature and regenerating muscle were not statistically different between groups (Fig. 4B and C). However, ratios of functional (mature) to regenerating muscle were modestly increased in niacin-treated mice compared to vehicle-treated obese mice (Fig. 4D). Evidence of muscle necrosis at this time point (day 15) was limited (consistently <1.0%



**Figure 3.** Niacin increases smooth muscle investment in vessels within the tibialis anterior muscle after ischemic injury in obese mice with metabolic syndrome. Lean (Chow) and western diet-induced obese (WD) mice underwent right hind limb femoral artery excision surgery, followed by 14 days with daily i.p. injections of either vehicle or niacin (NA). (A) Sections were immunostained for CD31 (brown; white arrows, white outlined inset), to identify endothelial cells, and smooth muscle  $\alpha$ -actin ( $\alpha$ SMA) (red; black arrows, black outlined inset). Scale bar represents 100  $\mu$ m. (B) Total vessel density was determined by counting CD31<sup>+</sup> vessels. (C) Arteriole density was determined by counting  $\alpha$ SMA<sup>+</sup> vessels. (D) Smooth muscle investment was calculated as the percentage of all vessels that incorporated  $\alpha$ SMA<sup>+</sup> cells. Statistically significant differences were determined by one-way ANOVA followed by Bonferroni multiple comparison tests comparing all groups. Bars with different lower case letters are significantly different at  $P < 0.05$ , while those that share the same lower case letter are not significantly different. Twelve fields of view were analyzed per muscle. Data are means  $\pm$  SEM for  $n = 6$ .





**Figure 4.** Niacin modestly improves tibialis anterior muscle architecture after ischemic injury in obese mice with metabolic syndrome. Lean (Chow) and western diet-induced obese (WD) mice underwent right hind limb femoral artery excision surgery, followed by 14 days with daily i.p. injections of either vehicle or niacin (NA). (A) Sections were stained with hematoxylin and eosin. Scale bar represents 100  $\mu$ m. (B) Mature myofibers were identified by intense eosin staining and the presence of peripheral nuclei (black arrows). (C) Regenerating myofibers were identified by intense eosin staining and central nuclei (white arrows). (D) The ratio of functional muscle tissue was calculated from percent section areas of mature and regenerating tissue. (E) Necrotic myofibers were identified by pale eosin staining and absent nuclei (white asterisk in A). (F) Adipocytes were identified by the absence of eosin staining and peripheral nuclei (black asterisks). Statistically significant differences were determined by one-way ANOVA followed by Bonferroni multiple comparison tests comparing all groups. Bars with different lower case letters are significantly different from each other at  $P < 0.05$ . All section areas were determined using ImageJ. Twelve fields of view were analyzed per muscle. Data are means  $\pm$  SEM for  $n = 6$ .

of section areas) in all groups (Fig. 4E). Interestingly, we observed a trend for increased adipose interspersed between myofibers in obese mice, which was not significantly changed by treatment with niacin (Fig. 4F). By virtue of occupying tissue area, the presence of adipocytes would be expected to contribute to changes observed in functional muscle tissue ratios, particularly between lean and obese mice (Fig. 4D).

Taken together, these data suggest that the improved functional recovery we observed in obese niacin-treated

mice (Fig. 2) is related to improved quality of newly formed vessels and improved tissue architecture of tibialis anterior muscles in these animals.

### Niacin decreases inflammation within the tibialis anterior muscle after ischemic injury in obese mice with metabolic syndrome

Accumulating evidence suggests that niacin can promote vascular function and repair by modulating the activity of

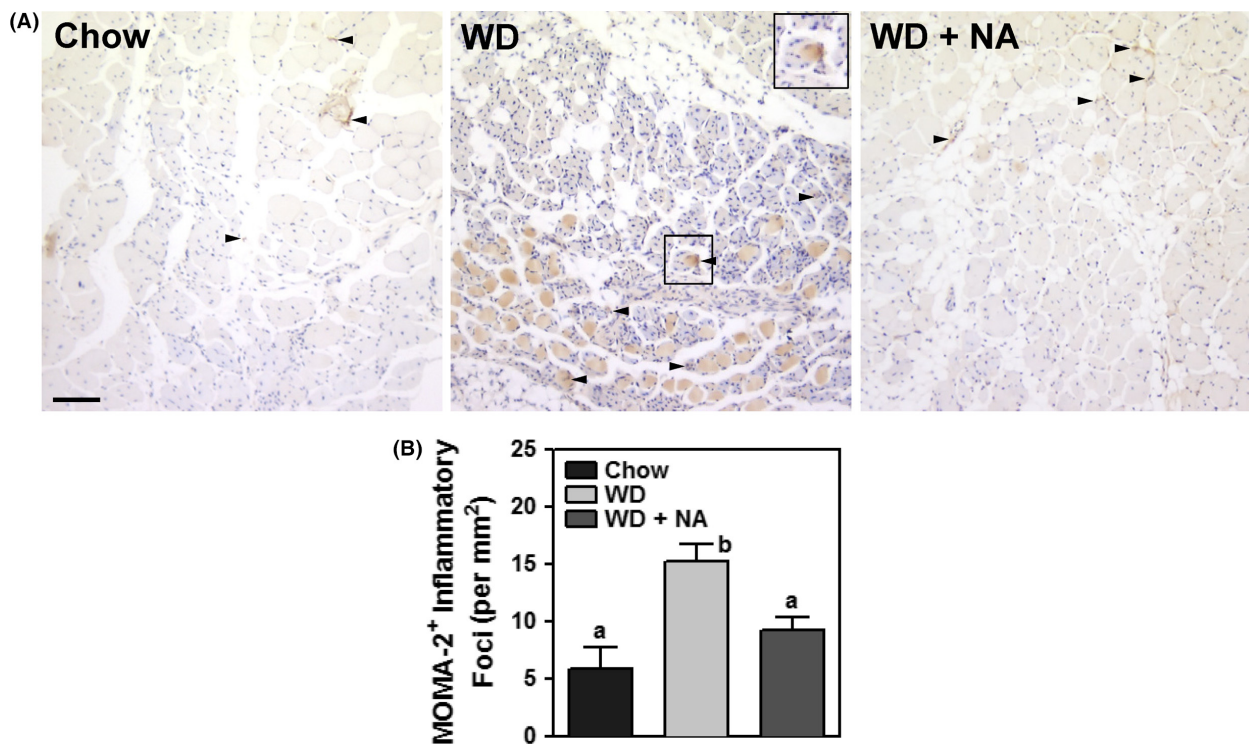
immune cells and endothelial cells (Ganji *et al.* 2009; Chai *et al.* 2013a; Hughes-Large *et al.* 2014). Immunostaining of tibialis anterior muscle sections for monocyte macrophage antigen 2 (MOMA-2) showed intermittent areas of diffuse, but intense positive staining in vehicle-treated obese mice (Fig. 5A). At higher magnification, we confirmed that the diffuse staining of myofibers in tissue sections from niacin-treated mice was not associated with individual nucleated cells, as would be expected for inflammatory infiltrates. Counting the number of MOMA-2 positive inflammatory foci, defined as clusters of >2 nucleated MOMA-2 positive cells per section area (Fig. 5A, inset), revealed that inflammation was increased in muscle sections from vehicle-treated obese mice compared to lean control mice – an observation that was reversed to near lean control levels with niacin treatment (Fig. 5B).

## Discussion

Our previous work (Hughes-Large *et al.* 2014), and the work of others (Wu *et al.* 2010, 2012; Lukasova *et al.*

2011; Tavintharan *et al.* 2011; Digby *et al.* 2012; Chai *et al.* 2013b; Kaplon *et al.* 2014), supports the general concept that niacin can act directly on endothelial and immune cells to improve vascular health. Here, we provide evidence that niacin can directly improve HMVEC angiogenic function during combined lipotoxicity and hypoxia, conditions which are thought to be particularly relevant to peripheral vascular disease in tissues such as skeletal muscle during metabolic syndrome (Eckardt *et al.* 2011; Chantler and Frisbee 2015). We further showed *in vivo* that short-term intervention with niacin can improve revascularization and recovery from ischemic injury in diet-induced obese mice with characteristics of metabolic syndrome without overt type 2 diabetes. Remarkably, this benefit with niacin was evident despite continued feeding of western diet.

Several classes of cardiovascular drugs, including extended release niacin, have been associated with worsening of blood glucose control in meta-analyses and large clinical trials (Ong *et al.* 2014), raising concern over the use of these agents in patients with metabolic syndrome, prediabetes, and overt diabetes. In the case of niacin,



**Figure 5.** Niacin decreases inflammation within the tibialis anterior muscle after ischemic injury in obese mice with metabolic syndrome. Lean (Chow) and western diet-induced obese (WD) mice underwent right hind limb femoral artery excision surgery, followed by 14 days with daily *i.p.* injections of either vehicle or niacin (NA). (A) Sections were immunostained for the monocyte/macrophage antigen, MOMA-2 (brown; black arrows). Scale bar represents 100  $\mu$ m. (B) Inflammation was quantitated by counting the number of MOMA-2 positive inflammatory foci (clusters of >2 nucleated brown cells; black outlined inset). Statistically significant differences were determined by one-way ANOVA followed by Bonferroni multiple comparison tests comparing all groups. Bars with different lower case letters are significantly different at  $P < 0.05$ , while those that share the same lower case letter are not significantly different. Twelve fields of view were analyzed per muscle. Data are means  $\pm$  SEM for  $n = 6$ .

however, this adverse effect may be related to dosing regimen (Kroon *et al.* 2015). Recent work in Zucker obese rats clearly demonstrated that intermittent dosing, but not continuous infusion (comparable to extended release), with niacin decreased liver triglycerides and increased insulin sensitivity (Kroon *et al.* 2015). The decreased liver triglycerides, decreased insulin, and decreased HOMA-IR we observed in obese mice treated once daily with niacin are consistent with this previous work, and importantly, suggest that significant vascular benefit can be achieved using this type of dosing schedule while also improving metabolic control.

The improved revascularization and tissue architecture of ischemic tibialis anterior muscles we observed with niacin was not apparently associated with increased vessel numbers, but rather with increased muscularization of vessels. Niacin is a known precursor for NAD<sup>+</sup> biosynthesis, and this observation of increased vessel stabilization is consistent with our earlier work showing that expression of the rate-limiting enzyme for NAD<sup>+</sup> synthesis (NAMPT) increases the longevity of endothelial cell tubes in high glucose (Borradaile and Pickering 2009) and promotes vascular smooth cell maturation and resistance to stress (van der Veer *et al.* 2005, 2007). These data are of particular interest given that impaired revascularization following ischemic injury in diabetes has been associated with decreased stability of newly formed vessels (Landazuri *et al.* 2012). Although we observed only modest increases in functional muscle tissue ratios, we also cannot exclude the possibility that niacin may directly promote skeletal muscle maturation or resistance to ischemic damage. In fact, niacin has recently been shown to alter gene expression, including factors involved in Wnt signaling, in skeletal muscle of obese Zucker rats (Couturier *et al.* 2014).

The vascular anti-inflammatory properties of niacin have been linked to direct inhibitory effects on immune cells (Lukasova *et al.* 2011; Tavintharan *et al.* 2011; Digby *et al.* 2012; Chai *et al.* 2013b), to inhibition of inflammatory chemokine and adhesion molecule expression in endothelial cells (Wu *et al.* 2010, 2012), and to increased circulating HDL which itself has anti-inflammatory functions (Chen *et al.* 2007; Yvan-Charvet *et al.* 2010). Neither plasma apoAI nor cholesterol were increased by treatment with niacin in our study, suggesting that the decreased tissue inflammation we observed was not related to increased HDL. Rather, it is likely that niacin decreased inflammation by direct effects on immune and vascular cells, and by decreasing plasma triglycerides and subsequent lipotoxicity associated inflammation. The modest decrease in apoAI we observed with niacin was unexpected, but is inconclusive with regard to increased cardiovascular risk in wild-type mice which carry plasma

cholesterol predominantly in HDL. The contribution of improved glucose homeostasis (Dokun *et al.* 2014) to the effects of niacin on revascularization is likely small given the mild hyperglycemia in our model. Whether decreased inflammation is an absolute requirement for the beneficial effects of niacin on recovery from peripheral ischemia may warrant further investigation.

Supplementation with niacin, either at pharmacological doses or through increased dietary intake, may elicit vascular benefits not entirely dependent on modifying systemic lipid and lipoprotein levels (Lavigne and Karas 2013; Kaplon *et al.* 2014). The observations we report here, of enhanced human microvascular endothelial cell angiogenic function with a low concentration of niacin, made under conditions of fatty acid excess and hypoxia, support this possibility. The subsequent finding that intervention with low-dose niacin enhances revascularization and recovery of limb function following ischemic injury in diet-induced obese, hyperlipidemic mice, may have implications for the use of low dose niacin in the treatment of peripheral ischemic vascular disease in the setting of obesity and metabolic syndrome.

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## Author Contributions

N. Borradaile designed the research study; D. Pang, Z. Nong, B. Sutherland, C. Sawyez, D. Robson, J. Toma, and N. Borradaile performed the experiments and statistical analyses; J. G. Pickering contributed vascular expertise and assessment of experimental design; D. Pang and N. Borradaile wrote the paper; J.G. Pickering critically reviewed the manuscript.

## Disclosures

None declared.

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