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Niacin receptor activation improves human microvascular endothelial cell angiogenic function during lipotoxicity

Jennifer M. Hughes-Large, Dominic K.T. Pang, Debra L. Robson, Pak Chan, Jelena Toma, Nica M. Borradaile*

Department of Physiology and Pharmacology, Schulich School of Medicine and Dentistry, Western University, London, ON N6A 5C1, Canada

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ABSTRACT

Objective: Niacin (nicotinic acid) as a monotherapy can reduce vascular disease risk, but its mechanism of action remains controversial, and may not be dependent on systemic lipid modifying effects. Niacin has recently been shown to improve endothelial function and vascular regeneration, independent of correcting dyslipidemia, in rodent models of vascular injury and metabolic disease. As a potential biosynthetic precursor for NAD^+, niacin could elicit these vascular benefits through NAD^+-dependent, sirtuin (SIRT) mediated responses. Alternatively, niacin may act through its receptor, GPR109A, to promote endothelial function, though endothelial cells are not known to express this receptor. We hypothesized that niacin directly improves endothelial cell function during exposure to lipotoxic conditions and sought to determine the potential mechanism(s) involved. **Methods and results**: Angiogenic function in excess palmitate was assessed by tube formation following treatment of human microvascular endothelial cells (HMVEC) with either a relatively low concentration of niacin (10 μM), or nicotinamide mononucleotide (NMN) (1 μM), a direct NAD^+ precursor. Although both niacin and NMN improved HMVEC tube formation during palmitate overload, only NMN increased cellular NAD^+ and SIRT1 activity. We further observed that HMVEC express GRP109A. Activation of this receptor with either acifran or MK-1903 recapitulated niacin-induced improvements in HMVEC tube formation, while GPR109A siRNA diminished the effect of niacin. **Conclusion**: Niacin, at a low concentration, improves HMVEC angiogenic function under lipotoxic conditions, likely independent of NAD^+ biosynthesis and SIRT1 activation, but rather through niacin receptor activation.

1. Introduction

Niacin (nicotinic acid) has been used clinically for decades to lower circulating triglycerides, raise HDL, and improve cardiovascular risk [1]. Despite disappointing results in recent trials of 2 g extended release niacin as an adjunct therapy to statins (AIM-HIGH [2], HPS2-THRIVE [3,4]), niacin as a monotherapy has been shown to improve vascular health in several patient populations, including those with metabolic syndrome dyslipidemia (hypertriglyceridemia, low HDL) [1]. Interestingly, retrospective meta-analyses [5], and studies in cell culture, mouse models, and patients with type 2 diabetes mellitus [6–11] suggest that the vascular benefits of niacin may not be dependent on its systemic lipid lowering effects. Moreover, recent studies of endothelial function in middle-aged and older adults indicate that higher dietary niacin intake is associated with improved brachial artery flow-mediated dilation and reduced vascular oxidative stress, with no changes in systemic lipid concentrations [12]. In further support of this concept, several studies in rodent models of endothelial dysfunction, peripheral ischemic injury, and stroke have shown that niacin can directly improve endothelial cell function and vascular regeneration [9,10,13,14].

Elevated blood lipids, particularly triglycerides, are key stressors in the vasculature during obesity metabolic syndrome. Uptake and accumulation of surplus fatty acids from both lipoprotein-derived and albumin-bound sources can lead to cell dysfunction and death, referred to as lipotoxicity, in tissues throughout the body including those of the vasculature [15–17]. Endothelial cells may be especially vulnerable to lipotoxicity because despite being continually exposed to elevated circulating lipids during metabolic disease, they are not metabolically programmed to process large quantities of lipid [18,19]. Palmitate, in particular, induces cellular dysfunction and death in endothelial cells [20–30], while

* Corresponding author.
E-mail address: nica.borradaile@schulich.uwo.ca (N.M. Borradaile).

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unsaturated fatty acids such as oleate are generally not cytotoxic [22,23,25,29]. Within hours of exposure to palmitate, reactive oxygen species are generated [20–22,24,28,30], and inflammatory and cell death signaling cascades are initiated [23,25–27]. Many of these observations in isolated endothelial cells have been confirmed in the vasculature *in vivo* [24,27,30], suggesting that lipotoxcity contributes to vascular disease during metabolic syndrome.

The mechanisms of action of niacin in vascular protection remain controversial. Niacin is a precursor for NAD^+ synthesis and, as such, can increase cellular NAD^+ content and activate sirtuins (SIRTs) [31], a seven member family of NAD^+-dependent protein deacetylases and ADP-ribosyltransferases. All seven SIRTs are expressed in human endothelial cells [32]. SIRT1 and SIRT3 in particular are known to improve cell survival under conditions of stress, and to improve glucose and lipid metabolism in many cell types, including those of the vasculature. SIRT1 has been shown to regulate endothelial cell physiology, primarily by driving cell proliferation, migration, and neovessel formation [32]. SIRT1 activation in human aortic endothelial cells also increases aerobic glycolysis and angiogenic function in high glucose [33], raising the possibility that metabolic regulation by SIRT1 may also be important for angiogenic function under conditions of lipid overload. Niacin may also elicit its effects on the vasculature through its cell surface receptor. GPR109A is robustly expressed in brown and white adipocytes, intestinal epithelium, epidermal Langerhans cells, and, of particular relevance to the vasculature, in several immune cell types [34]. GPR109A expression has not been reported in vascular endothelial cells. Here, we examined the effects of niacin on the ability of human microvascular endothelial cells (HMVEC) to form tube networks during exposure to excess fatty acids, as would be expected to occur in the vasculature during metabolic syndrome. Our data suggest that niacin, at a relatively low concentration, preserves the ability of HMVEC to form tubes under conditions of saturated fatty acid excess, and may elicit this effect through activation of GPR109A.

2. Methods

2.1. Endothelial cell culture and treatments

Primary HMVEC (Lonza) were maintained in Medium 199 (Life Technologies) supplemented with EGM-2MV SingleQuots (Lonza), and subcultured as recommended by the supplier. For fatty acid treatments, growth medium was supplemented with 0.5 mM palmitate, 0.5 mM oleate or a combination of palmitate and oleate (1:1 ratio, total concentration of 0.5 mM) complexed to bovine serum albumin (BSA) at molar ratio of 2:1. Concentrations of fatty acids used reflect high physiological to pathophysiological concentrations, as would be observed during obesity, metabolic syndrome, and type 2 diabetes [35,36]. For niacin (nicotinic acid, NA) and nicotinamide mononucleotide (NMN) treatments, experimental media were supplemented with 10 μM NA (Fluka BioChemika) or 1 μM NMN (Sigma) solubilized in cell culture grade water. For GPR109A agonist treatments, growth medium was supplemented with either 1 μM acifran or 13 nM MK-1903 (Tocris Bioscience) solubilized in DMSO.

2.2. Tube formation and cell migration

Formation of tube networks and cell migration were assessed by Matrigel (BD Biosciences) and scratch assays respectively, as previously described [33,37]. Tube networks on Matrigel and scratched cell monolayers were visualized by light microscopy using an Olympus IX71 inverted microscope. Tube network branch points and total tube length per field of view at 18 h were quantified using Image J. A tube was defined as an apparently 3-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. Cell monolayer scratch areas at 0 h, 4 h, and 8 h were determined using Image J. Migration areas at each time point were calculated by subtracting scratch areas at 4 h and 8 h from those at 0 h.

2.3. Cell death and cell proliferation

Apoptosis and cell death were assessed by Alexa Fluor 488 annexin V staining and membrane permeability to propidium iodide, respectively, using a Dead Cell Apoptosis Kit (Molecular Probes) according to the manufacturer's protocol. HMVEC proliferation rates over 7 days were assessed by determination of population doublings per day as previously described [33].

2.4. NAD^+ measurements and SIRT activity

Cellular NAD^+ content was assessed using a colorimetric kit (BioVision) according to the protocol provided by the manufacturer, as previously described [33]. SIRT1 and SIRT2/3 activation were assessed using fluorimetric kits for SIRT1 and SIRT3 (BIOMOL), with modifications for assessment of SIRT activity in live cells [38], and as used previously in vascular smooth muscle and human aortic endothelial cells [33,39]. Briefly, confluent HMVEC monolayers were incubated in OPTI-MEM I medium (Life Technologies) with or without niacin or NMN, followed by further incubation with the addition of cell permeable Fluor de Lys SIRT substrates. Trichostain A was included in all incubations to ensure inhibition of class I and class II histone deacetylases. Deacetylated substrates appear to be efficiently transported out of cells [38], allowing fluorescence intensities from deacetylated substrates to be detected in cell culture media, using developer reagents according to the protocol provided by the manufacturer. The Fluor de Lys peptide included in the SIRT3 activity kit is a known substrate for both SIRT2 and SIRT3.

2.5. Lipid metabolism

Accumulation of cytosolic neutral lipid droplets was assessed by staining HMVEC grown on glass coverslips with Oil Red O (Sigma), as previously described [40]. Coverslips were mounted onto glass slides with mounting media containing DAPI to visualize nuclei (ProLong Gold Antifade Reagent, Molecular Probes). Cells were imaged by fluorescence microscopy on an Olympus BX51 microscope. Lipid droplet content was analyzed using Image J by quantification of red (Oil Red O) and blue (DAPI) pixel densities. Palmitate oxidation was assessed by measuring conversion of ^{3}H-palmitate oxidized to ^3H_2O, as previously described [33].

2.6. RNA and protein expression

RNA transcript levels were determined by quantitative real time PCR using TaqMan assays from Applied Biosystems for *MTTP* (Hs00165177_m1), *GPR109A* (Hs02341584_s1), *GPR109B* (Hs02341102_s1), and *GPER1* (Hs01922715_s1). *GAPDH* was used as the internal reference transcript. Protein expression was determined by immunoblotting whole cell lysates prepared as previously described [33]. GPR109A and GAPDH were detected using rabbit polyclonal (Abcam) and mouse polyclonal (Enzo) antibodies, respectively. Bands were quantified by densitometry using Quantity One (Biorad).