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Anti-inflammatory effects of nicotinic acid in adipocytes demonstrated by suppression of fractalkine, RANTES, and MCP-1 and upregulation of adiponectin

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ABSTRACT

Objective: A major site of action for the atheroprotective drug nicotinic acid (NA) is adipose tissue, via the G-protein-coupled receptor, GPR109A. Since, adipose tissue is an active secretory organ that contributes both positively and negatively to systemic inflammatory processes associated with cardiovascular disease, we hypothesized that NA would act directly upon adipocytes to alter the expression of pro-inflammatory chemokines, and the anti-inflammatory adipokine adiponectin.

Methods and results: TNF-α treatment (1.0 ng/mL) of 3T3-L1 adipocytes resulted in an increase in gene expression of fractalkine (9 ± 3.3-fold, $P < 0.01$); monocyte chemoattractant protein-1 (MCP-1) (24 ± 1.2-fold, $P < 0.001$), 'regulated upon activation, normal T cell expressed and secreted' (RANTES) (500 ± 55-fold, $P < 0.001$) and inducible nitric oxide synthase (iNOS) (200 ± 70-fold, $P < 0.05$). The addition of NA (10^{-4} M) to TNF-α-treated adipocytes attenuated expression of fractalkine ($50 \pm 12\%$, $P < 0.01$); MCP-1 ($50 \pm 6\%$, $P < 0.01$), RANTES ($70 \pm 3\%$, $P < 0.01$) and iNOS ($60 \pm 16\%$). This pattern was mirrored in protein released from the adipocytes into the surrounding media. The effect on gene expression was neutralised by pre-treatment with pertussis toxin. NA attenuated macrophage chemotaxis (by $27 \pm 3.5\%$, $P < 0.001$) towards adipocyte conditioned media. By contrast, NA, (10^{-6}–10^{-3} M) increased, in a dose-dependent manner, mRNA of the atheroprotective hormone adiponectin (3–5-fold $n = 6$, $P < 0.01$).

Conclusions: NA suppresses pro-atherogenic chemokines and upregulates the atheroprotective adiponectin through a G-protein-coupled pathway. Since adipose tissue has the potential to contribute to both systemic and local (perivascular) inflammation associated with atherosclerosis our results suggest a new "pleiotropic" role for NA.

1. Introduction

There is ample epidemiological evidence linking obesity, and the related 'metabolic syndrome', with vascular disease [1]. Conventionally, adipose tissue has been regarded as an inert store of triglycerides and fatty acids, but there is accumulating evidence that adipose tissue is involved in more diverse activity, including pro-inflammatory processes [2]. Several secreted factors, termed 'adipokines' influence local and distant inflammatory processes. For instance, mature adipocytes upregulate the transcriptional regulator NF-κB leading to secretion of interleukin-6 (IL-6) and tumour necrosis factor-α (TNF-α) [3] and to recruitment of macrophages [4]. Mesenteric adipose tissue has been associated with distant atherosclerosis, assessed by measurement of carotid intima thickness using ultrasound [5]. Similarly abdominal visceral fat appears to be associated with aortic [6] and carotid artery [7] stiffness and with elevated IL-6 and CRP, which have been postulated as mediators [7].

Furthermore, there is emerging evidence that perivascular adipose tissue influences vascular function and may have the potential to alter susceptibility to atherosclerosis in adjacent arteries in a paracrine manner [8]. Mazurek et al. found greater expression of pro-inflammatory cytokines in epicardial adipose tissue than in subcutaneous fat in patients undergoing coronary artery bypass grafting [9], while Henrichot et al. have identified pro-inflammatory cytokines IL-8 and monocyte chemoattractant protein-1 (MCP-1) in human peri-aortic white adipose tissue and demonstrated the potential of this tissue to promote recruitment of peripheral blood leucocytes [10].

Adipose tissue is an important target for nicotinic acid [11]. A G-protein-coupled receptor (GPCR), that binds NA, GPR109A has recently been given the HGNC approved gene symbol, NIACR1 but is also termed HM74a, in humans and 'protein upregulated in macrophages by interferon-gamma' or 'PUMA-G', in mice [12,13]. Activation of the receptor in adipocytes inhibits lipolysis via G_i mediated effects on adenylate cyclase, with decreased cellular

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cAMP levels [12], reduced lipolysis and a reduction in free fatty acids flux to the liver as substrate for VLDL synthesis. In patients nicotinic acid reduces LDL-cholesterol and increases HDL-cholesterol [14]. The observed reduction in the progression of atherosclerosis and cardiovascular morbidity with NA may be due solely to its lipid modifying effects [15]. However, niacin treatment has also been shown to increase plasma levels of the adipocyte-derived atheroprotective hormone, adiponectin [16] raising the possibility that some of the effects of NA may be mediated through lipid-independent pathways. Here, we show that NA acts directly upon adipocytes to reduce the expression of pro-inflammatory chemokines; fractalkine, MCP-1 and RANTES with inhibitory effects on monocyte chemotaxis, but with increase in the atheroprotective adipokine, adiponectin.

2. Materials and methods

2.1. Cell culture

3T3-L1 preadipocytes (ATCC, Teddington, UK) were seeded in 6-well plates at a density of 10^5 per well and cultured with Dulbecco's modified Eagle's medium (DMEM) supplemented with, L-glutamine (4 mM), 10% fetal calf serum, penicillin, 100 IU and streptomycin, 100 μg/mL, in a humidified atmosphere of 95% air/5% CO_2 at 37 °C. At confluence, cells were differentiated into adipocytes by the addition insulin (100 nM) and dexamethasone (100 nM). Morphological analysis showed that typically 80 to 90% of cells had differentiated by 10 days of incubation with differentiation media. All cell culture reagents were purchased from Sigma Aldrich (Poole, UK).

2.2. Cell treatments

Prior to treatments, cells were cultured in media without insulin and dexamethasone for 24 h then serum starved for 4 h. Cells were treated for 4 or 24 h with nicotinic acid (10^{-3}–10^{-6} M, Sigma Aldrich, Poole, UK), and/or TNF-α 0.1, 0.5 1,0 or 10 ng/mL (R and D Systems, Abingdon, UK). For the pertussis treatments, cells were incubated with pertussis toxin (Calbiochem, MERK, Nottingham, UK) for 16 h to activate the toxin, then treated with nicotinic acid and/or TNF-α for 4 h. At the end of the incubation times, the surrounding cell culture media, 'adipocyte conditioned media' (ACM) was collected and snap frozen then stored at −80 °C until analysis. Cells were lysed on ice in RNA lysis buffer supplied with the Qiagen RNEasy mini kit (Qiagen, Crawley, UK).

2.3. Measurement of adipokines and chemokine gene expression

Total RNA was prepared using Qiagen RNEasy mini columns and 1 μg was reverse transcribed using a QuantiTect® Reverse Transcription Kit using Oligo dT's and random hexamers as primers. Real-time PCR was carried out with 1 μL of cDNA in a 10 μL reaction mix consisting of Sybr Green Mastermix (Applied Biosystems, Warrington, UK) and sense and antisense primers (0.25 μM final concentration). Primer sequences are shown in Table 1. Cycling parameters were as follows: activation of Taq polymerase, 10 min at 95 °C, then 40 cycles at 95 °C for 15 s, then extension at 60 °C for 1 min, followed by a melt curve analysis.

2.4. Measurement of secreted adipokines and chemokines

Secreted chemokines, MCP-1, fractalkine, RANTES and adiponectin were measured in the media removed from adipocytes after 24 h incubation by a Luminex™ Multiplex bead-based system using Milliplex™ MAP kits, from the mouse cytokine/chemokine panel according to manufacturer's instructions.

Table 1
Primer sequences for quantitative real-time RT-PCR.

Gene	Primer sequence
Cyclophilin	Sense, 5′-GGCCGATGACGAGCCC-3′ Antisense, 5′-TGTCTTTGGAACTTTGTCTGCAA-3′
Adiponectin	Sense, 5′-GTTGCAAGCTCTCCTGTTCC-3′ Antisense, 5′-ATCCAACCTGCACAAGTTCC-3′
CCL5	Sense, 5′-TCCAATCTTGCAGTCGTGTTTG-3′ Antisense, 5′-TCTGGGTTGGCACACACTTG-3′
MCP-1	Sense, 5ĩ-TTCCTCCACCACCATGCAG-3ĩ Antisense, 5ĩ-CCAGCCGGCAACTGTGA-3ĩ
Fractalkine	Sense, 5′-CCAAGACGCCATGAAGCAT-3′, Antisense, 5′-TCAAACTTGCCACCATTTTTAGTG-3′

2.5. Chemotaxis and chemokinesis assays

To investigate the biological response resulting from of nicotinic acid treatment, we used a chemotaxis assay to measure macrophage migration towards ACM from differentiated treated 3T3-L1 adipocytes. Cells were exposed to TNF-α (1.0 ng/mL) with or without nicotinic acid 10^{-3} M for 24 h, then media collected and stored at −80 °C prior to chemotaxis transwell assays.

Murine macrophages were harvested by peritoneal lavage with PBS and 5 mM EDTA 4–5 days after intraperitoneal injection of 2% Bio-gel in PBS. Chemotaxis was measured using pooled macrophages obtained from two C57/BL6 mice subjected to peritoneal lavage and repeated on three separate experiments.

For the chemotaxis assays, cells were suspended in chemotaxis buffer; RPMI with HEPES (25 mM), and 0.1% BSA, and applied to a 96-well Neuroprobe ChemoTx™ membrane (Receptor Technologies, Adderbury, UK), 8 μM pore size at a density of approximately 400,000 cells per well. The lower chamber contained either 1:3 diluted ACM or chemotaxis buffer alone. As negative controls, wells included chemotaxis migration buffer only, and migration buffer with TNF-α (1.0 ng/mL) to ensure that there was no chemotaxis to TNF-α alone. For the chemokinesis assay, macrophages were suspended in ACM from TNF-α-treated cells and placed on the upper side of the membrane with the lower chamber containing the same concentration of ACM. After 4-h incubation at 37 °C in a 5% CO_2 cell culture incubator, the cells on the upper layer of the membrane were removed with a cotton swab and the membrane rinsed with PBS. Migrated cells attached to the lower area of the membrane were fixed in paraformaldehyde (4%) then mounted with mounting media containing DAPI. Migration of the cells was quantified by taking 2 images under a fluorescent microscope from each membrane with a minimum of 4 membranes per treatment. Stained nuclei were then counted using image software Image Pro Plus™ (Media Cybernetics, Silver Spring, Maryland).

2.6. Cell viability assay

Please see Supplementary methods and figures.

2.7. iNOS mRNA expression

Please see Supplementary methods and figures.

2.8. Demonstration of GPR109a gene expression in 3T3-L1 adipocytes

Please see Supplementary methods and figures.