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Niacin induces PPARγ expression and transcriptional activation in macrophages via HM74 and HM74a-mediated induction of prostaglandin synthesis pathways

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ABSTRACT

HM74 and HM74a have been identified as receptors for niacin. HM74a mediates the pharmacological anti-lipolytic effects of niacin in adipocytes by reducing intracellular cyclic AMP (cAMP) and inhibiting release of free fatty acids into the circulation. In macrophages, niacin induces peroxisome proliferator-activated receptor γ (PPARγ)-dependent and cAMP-dependent expression of genes mediating reverse cholesterol transport, although via an unidentified receptor. We describe constitutive expression of HM74a mRNA and hypoxia- and IFNγ-inducible expression of HM74 and HM74a in human monocytic cell lines and primary cells in culture. In U937 cells niacin-induced expression of 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2), the most potent endogenous ligand of PPARγ. Both niacin and the structurally distinct HM74/HM74a ligand acifran-induced nuclear expression of PPARγ protein and enhanced PPARγ transcriptional activity. Niacin-induced PPARγ transcriptional activity was pertussis toxin sensitive and required activity of phospholipase A_2 (EC 3.1.1.4), cyclo-oxygenase (EC 1.14.99.1) and prostaglandin D_2 synthase (EC 5.3.99.2). Niacin also induced PPARγ transcriptional activity in HM74 and HM74a CHO cell transfectants, although not in vector-only control cells. This was sensitive to pertussis toxin and to inhibition of phoshoplipase A_2 and cyclo-oxygenase activity. Additionally, niacin increased intracellular cAMP in U937 via a pertussis toxin and cyclo-oxygenase-sensitive mechanism. These results indicate that HM74 and HM74a can mediate macrophage responses to niacin via activation of the prostaglandin synthesis pathway and induction and activation of PPARγ. This suggests a novel mechanism(s) mediating the clinical effects of pharmacological doses of niacin.

1. Introduction

HM74 (GPR109B, GenBank accession no. NM_006018) and HM74a (GPR109A, GenBank accession no. NM_177551) are highly homologous Gi-G-protein-coupled receptors that were initially identified as receptors for niacin (nicotinic acid, Vitamin B3) [1–3]. HM74a, the human homologue of murine PUMA-G (protein upregulated in macrophages by IFN-γ [4]), is the higher affinity receptor with niacin binding and G-protein activation evident at 0.05–1 μM Kd/EC_{50} [1–3]. HM74 has 96% nucleotide sequence identity and 89% amino acid identity with HM74a, encompassing 7 predicted amino acid changes and a 24 amino acid C-terminal extension [2,5]. These changes are sufficient to give HM74 much reduced

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affinity for niacin, with ligand binding and G-protein activation generally described as non-saturable [1,2].

Despite the significantly higher affinity of niacin for HM74a than HM74, it is unlikely that it represents the endogenous ligand for either receptor [6]. However, HM74a might mediate the pharmacological anti-lipolytic effects of niacin, which can be achieved at plasma concentrations $\geq$4 μM [6]. The mechanism of niacin's anti-lipolytic effect is thought to involve an inhibitory G-protein signal that reduces production of adipocyte cyclic AMP (cAMP) via adenylyl cyclase and so reduces hormone-sensitive lipase activity due to reduced protein kinase A activation [6,7]. This inhibits release of free fatty acids (FFA) from adipose tissue into the circulation, resulting in a 15–35% increase in hepatic secretion of high density lipoprotein (HDL) and a 5–25% decrease in low density lipoprotein (LDL), the primary target for cholesterol-lowering treatment to reduce both the risk of and mortality due to coronary heart disease [7,8]. That HM74a might mediate this effect is supported by niacin-induced reduction of intracellular cAMP in HM74a-transfected CHO-K1 [3], 293EBNA [1], HEK293 and 3T3L1 adipocytes [9]. Additionally, experiments in PUMA-G-deficient mice demonstrated 75% reduction in niacin binding to adipocyte membranes associated with loss of niacin-induced inhibition of FFA release [3].

The observation that the fatty acid-derived ketone body (D)-β-hydroxybutarate ((D)-β-OHB) mediates similar anti-lipolytic effects to niacin with respect to reduction of serum FFA [10,11] and inhibition of adipocyte lipolysis [12] recently led to the identification of (D)-β-OHB as the first endogenous ligand for HM74a [13]. (D)-β-OHB suppressed FFA efflux from murine adipocytes in a PUMA-G-dependent manner and induced [^{35}S]GTPγS binding to membranes from HM74a and PUMA-G transfectants with an EC_{50} of 0.8 mM [13]. This is within the serum (D)-β-OHB concentration achieved following 2 days starvation in man [14] and suggests that HM74a additionally mediates homeostatic negative feedback mechanisms to conserve fat stores during starvation [13].

A common side-effect of niacin administration is an intense prostaglandin-mediated skin flush due to vasodilation following release of prostaglandins PGD_2 and PGE_2 from resident dermal macrophages and/or epidermal cells [15,16]. PGD_2 is non-enzymatically dehydrated to form 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2), the most potent endogenous ligand of peroxisome proliferator-activated receptor γ (PPARγ) [17]. Using the human differentiated monocytic cell line MonoMac6, it has been shown that niacin induces PPARγ expression and intracellular cAMP in monocytic cells, although the receptor(s) mediating this effect was not identified [18]. Niacin also enhanced transcription of both the oxidised LDL (oxLDL) scavenger receptor CD36 and of ABCA1, the key transporter for efflux of cellular cholesterol onto apolipoprotein A-I-containing HDL particles [18]. Niacin-induced effects on macrophage lipid regulation could present an additional mechanism whereby clinical administration of niacin might elevate HDL cholesterol levels and also reduce macrophage foam cell formation in atherogenesis [18,8].

We have identified HM74 as a hypoxia-inducible gene from cDNA array analysis of normoxic and hypoxic monocytic cell lines. Interestingly, nicotinamide, the downstream conversion product of nicotinic acid and other prinicipal form of Vitamin B3, is central to the ARCON (accelerated radiotherapy with carbogen and nicotinamide) therapeutic strategy to overcome radiotherapy resistance due to hypoxia [19]. Nicotinamide operates by reducing intermittent vascular shut-down [20] to decrease perfusion-limited tumour hypoxia [21], in analogy to the effects of niacin on vascular parameters to increase local blood flow [15,16]. Niacin and nicotinamide also have protective effects versus chemical and UV-induced carcinogenesis via a number of mechanisms including impaired immune surveillance [22,23]. HM74 might therefore provide insight into mechanism(s) of vasodilation and a link between treatments for cancer and dyslipidaemia.

We have considered whether the receptor(s) mediating macrophage responses to niacin could be HM74a and/or HM74. We have determined that HM74 and HM74a mRNA is hypoxia-inducible in human monocytic cell lines and monocyte-derived macrophages. Additionally, we show that activation of both HM74 and HM74a by niacin stimulates the prostaglandin synthesis pathway resulting in elevated expression of PPARγ and enhanced PPARγ-dependent transcription activation. This demonstrates for the first time the effect of activation of the HM74 and HM74a GPCRs on macrophage gene expression and suggests an additional mechanism whereby HM74 and HM74a could mediate the clinical effects of niacin.

2. Materials and methods

2.1. Cell culture

Cell lines were obtained from the Cancer Research UK cell service unless otherwise stated. Human monocytic cell lines THP1 (acute monocytic leukaemia) and U937 (lymphoma), 1397 and JB EBV-transformed lymphocytes (a gift from Dr. S. Holbrook, Weatherall Institute of Molecular Medicine, Oxford, UK), Jurkat (T cell acute lymphatic leukaemia), breast cancer cell lines MDA-MB-468 and MDA-MB-231, PC3 (prostate) and 293T (embryonic kidney) cells were maintained in RPMI 1640. HCT116 and HT29 (colon), HT1080 (fibrosarcoma) and U87MG (glioblastoma) cells were maintained in DMEM. CHO cells were maintained in αMEM. All media was supplemented with 10% FBS, L-glutamine (2 μM), penicillin (50 IU/ml) and streptomycin sulphate (50 μg/ml). Peripheral blood mononuclear cells (PBMC) were isolated from whole blood using Lymphoprep (Axis-Shield UK Ltd., Huntingdon, UK) as described [24], monocytes were selected by adhesion and differentiated into macrophages by adhesion over 7 days. THP1 and U937 cells were induced to differentiate by treatment with 20 nM phorbol-12-myristate 13-acetate (PMA) for 48 h. Hypoxic exposures (0.1% O_2, 5% CO_2, balance N_2) were performed in a Heto-Holten CellHouse 170 incubator (RS Biotech, Irvine, Scotland).

2.2. cDNA array

3×10^7 monocytic or PMA-treated THP1 cells were exposed to either hypoxia (0.1% O_2) or normoxia for 16 h. Total RNA was extracted using TRI Reagent (Sigma, Poole, UK), contaminating DNA removed with DnaseI and polyA$^+$ RNA selected using an Oligotex mRNA spin column (Qiagen, Crawley, UK). Cy3 and Cy5 labelled mRNA was hybridised

overnight onto a poly-L-lysine coated glass slide microarray and the resulting image was scanned with a GenePix 4000A (Molecular Devices, Sunnyvale, CA).

2.3. Ribonuclease protection assay (RPA)

Total RNA was extracted in TRI Reagent and dissolved in hybridisation buffer (80% formamide, 40 mM PIPES, 400 mM NaCl, 1 mM EDTA (pH 8)). Probes specific for HM74 (amplified with PCR primers 5′-atctgcctgccgttcg-3′ and 5′-agggccattctg-gatcagcaa-3′ corresponding to ntds +231 to +520 and confirmed as HM74 by sequencing) and a U6 small nuclear RNA internal control were labelled with [^{32}P]-CTP (Amersham Biosciences, Little Chalfont, UK) and hybridised to 20–40 μg of RNA overnight at 55 °C. Following digestion of unhybridised species, samples were analysed on a 6% polyacrylamide sequencing gel.

2.4. PCR

Total RNA was extracted in TRI Reagent, treated with DNaseI and PCR-amplified using the SuperScript™ III One-Step RT-PCR System with Platinum® *Taq* DNA Polymerase (Invitrogen, Paisley, UK). PPARγ primers were 5′-catggcaattgaatgtcgtg-3′ and 5′-cctcaaatatggagtccacg-3′ [18], HM74 primers were 5′-cgccactttgctggagcattc-3′ and 5′-ccaaagcatgtgagtctc-3′; HM74a primers were 5′-cacacctccttgctggagcat-3′ and 5′-accgtttccc-taaatcgcatt-3′. HM74 and HM74a primer specificity was confirmed by sequence analysis of a random set of PCR products cloned directly into pCRII-TOPO (Invitrogen).

2.5. PPAR-luciferase assay

Cells were seeded in six-well plates and transfected with the pHRG-TK renilla luciferase reporter (Promega, Southampton, UK) and pLFABPLuc (a gift from Dr. C. Palmer, University of Dundee, Dundee, UK). Twenty-four hours post-transfection cells were treated with niacin (Sigma) or acifran (Tocris Cookson Ltd., Bristol, UK) for a further 24 h, then lysed in 300 μl cell culture lysis reagent (Promega). Luminescence in relative light units (RLU) was read in a FLUOstar OPTIMA luminometer (BMG Labtech Ltd., Aylesbury, UK) and firefly luciferase values normalised to renilla. For inhibitor assays, cells were pre-incubated with the relevant inhibitor compound for 10 min prior to addition of niacin. Inhibitors were HQL-79 (Cayman Chemicals, Ann Arbour, MI), acetylsalicylic acid (aspirin, Sigma), U73122 and Methyl Arachidonyl Fluorophosphate (MAFP) (Calbiochem, San Diego, CA). For activator assays, cells were incubated for 24 h with ciglitizone, WY-14643 (Sigma) or GW501516 (Alexis Biochemicals, Lausen, Switzerland).

2.6. PPARγ Western blot

To prepare cytoplasmic extract, cells were incubated on ice for 20 min in 150 μl Buffer A (10 mM HEPES pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.4% NP-40 plus protease inhibitors) and centrifuged for 5 min at 5000 rpm. For nuclear extract, the pellet was resuspended in 80 μl Buffer B (20 mM HEPES pH 7.9, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT plus protease inhibitors), homogenised and incubated on ice for 10 min prior to centrifugation for 15 min at 13000 rpm. Protein concentration was determined using the BioRad DC Protein Assay (BioRad Laboratories Ltd., Hemel Hempstead, UK). Nuclear extracts (30 μg) were separated on a 10% acrylamide-glycerol gel and transferred to PVDF membrane. Primary antibodies were polyclonal rabbit anti-human PPARγ (raised against the AB domain of human PPARγ; a gift from Dr. C. Palmer, University of Dundee, Dundee, UK), mouse anti-lamin A + C (Abcam, Cambridge, UK) and mouse anti-β-tubulin (Sigma). Immunoreactivity was visualised with HRP-linked goat secondary antibodies and chemiluminescence. Quantification was achieved by densitometry.

2.7. 15-d-PGJ$_2$ ELISA

U937 cells were exposed to niacin for 24 h. Cell culture supernatant was collected and 15d-PGJ$_2$ levels were assayed using the 15-deoxy-$\Delta^{12,14}$-Prostaglandin J$_2$ Immunoassay kit according to the manufacturers protocol (R&D Systems, Minneapolis, MN) and adjusted according to cell number.

2.8. Generation of HM74 and HM74a transfectants

RNA was extracted from PMA-treated U937 cells exposed to 16 h hypoxia and HM74 and HM74a amplified as for PCR. HM74 primers produced a 1711 bp PCR product corresponding to ntd −59 to +1652 (relative to the ATG start codon at +1) and HM74a primers produced a 1299 bp PCR product corresponding to ntd −61 to +1238 that were cloned directly into pCRII-TOPO. The HM74 sequence contained 1 ntd difference versus the consensus sequence NM_006018 at +284 (C > G, Asp > Lys) which database analysis revealed to be the more common variant, occurring in 3/6 Genbank entries and 7/7 ESTs covering this region of HM74. HM74a differed from the consensus sequence NM_177551 at +931 (C > T, Arg > Cys) and +951 (G > A, Met > Ile), SNPs that have been reported as occurring with 20 and 29% frequency, respectively, and with a combined haplotype frequency of 20% [5]. HM74 and HM74a were sub-cloned into pcDNA3.1(+) using the *Eco*RI–*Eco*RV and the *Kpn*I–*Not*I cloning sites, respectively. The resulting plasmids were transfected into CHO cells and stable transfectants selected and maintained with 1 mg/ml G418. Individual clones were derived from single cells and selected on the basis of both strong expression of HM74/HM74a mRNA and a strong PPARγ transcriptional response to niacin as determined by PPAR-luciferase assay.

2.9. Cyclic AMP ELISA

Cells were exposed to niacin ± inhibitors for 24 h and lysed in 0.1N HCl. cAMP levels were assayed using the Cyclic AMP (low pH) Immunoassay kit according to the manufacturers protocol (R&D Systems) and adjusted according to lysate protein concentration.

2.10. Statistical analysis

Results are expressed as mean ± standard deviation (S.D.) of *n* experiments. Results were analysed by two-tailed Student's *t*-test.

3. Results

3.1. HM74 and HM74a are hypoxia-inducible in monocytic cells

We originally identified HM74 as a gene of interest in PMA-differentiated [25,26] THP1 macrophages where, by cDNA array analysis, HM74 mRNA was upregulated 2.3-fold following 16 h hypoxic exposure (0.1% O_2, data not shown). Ribonuclease protection assay (RPA) confirmed that hypoxic upregulation of HM74 mRNA was specific to PMA-treated cells in THP1 and U937 monocytic cell lines (Fig. 1A, top panel). As HM74 is also expressed in lymphocytes [2], we assayed 1397 and JB EBV-transformed lymphocytes and the Jurkat T cell acute lymphatic leukaemia cell line for hypoxic induction of HM74 mRNA. HM74 was only detected in 1397 and JB cells, with no evidence of hypoxia inducibility (Fig. 1A, top panel).

To extend and facilitate screening, we designed PCR primers specific for HM74 and the highly homologous HM74a, confirming specificity by sequencing a sample set of PCR products from each primer pair. Results for HM74 correlated with the original RPA (Fig. 1A, bottom panel). HM74a was constitutively expressed in monocytic cells but only marginally (and variably) induced by hypoxia in PMA-treated U937. It was largely absent from lymphocytic cell lines (Fig. 1A, bottom panel) as previously observed in fresh lymphocytes [2]. Expression of both HM74 and HM74a was hypoxia-inducible in primary human monocytes (Fig. 1B). In primary human macrophages, HM74 mRNA was mostly undetectable whereas HM74a was still strongly inducible by hypoxia (Fig. 1B). Further screening of a panel of epithelial cancer cell lines revealed HM74a expression to be widespread while HM74 displayed a more restricted expression profile. Neither gene was hypoxia-inducible in these cell types (Fig. 1C).

Further analysis of the hypoxic regulation of HM74 in PMA-differentiated U937 (which demonstrated the most robust hypoxia response) showed it to be rapidly induced by hypoxia and to maintain this expression level for at least 24 h (Fig. 1D). Upon reoxygenation, HM74 mRNA returned to (or dropped below) the normoxic level of expression within 8 h. Expression of HM74a was largely unaffected by either hypoxia or reoxygenation (Fig. 1D). PUMA-G, the murine homologue of HM74a, has been reported as IFNγ-inducible in ANA-1 macrophages [4]. We therefore treated PMA-differentiated U937 and lymphocytic 1397 cells for 4 h with 110 U/ml IFNγ and assayed for induction of HM74 and HM74a mRNA. Both genes were induced by IFNγ (Fig. 1E), confirming the result of Schaub et al. [4] and demonstrating for the first time that HM74 is also regulated by IFNγ.

3.2. Niacin induces PPARγ transcriptional activity, increases PPARγ expression and elevates 15d-PGJ_2 secretion in differentiated U937

HM74 and HM74a have been identified as low- and high-affinity niacin receptors, respectively, in assays of GTPγS

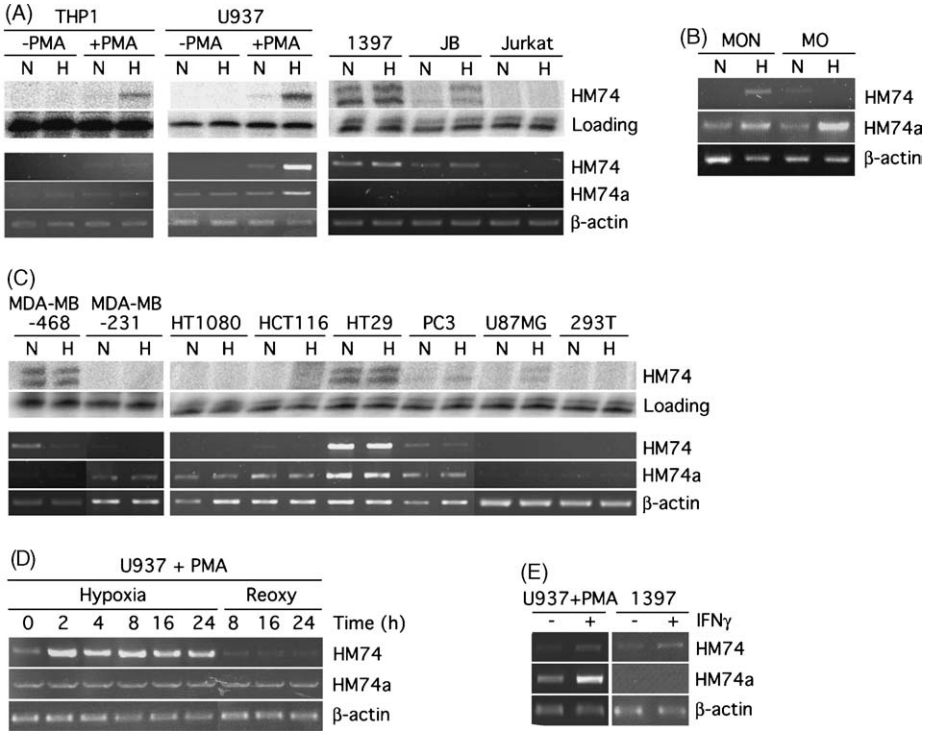

Fig. 1 – Expression analysis of HM74 and HM74a induction by hypoxia and IFNγ. RPA (top panel) and PCR (bottom panel) analysis of HM74 and HM74a mRNA in (A) PMA-differentiated monocytic cell lines and lymphocytic cells, (B) PBMC-derived primary monocytes (MON) and adhesion-differentiated macrophages (MO) and (C) a panel of cancer cell lines following 16 h exposure to hypoxia (H, 0.1% O_2) or normoxia (N). (D) PCR timecourse of hypoxic induction and the effect of reoxygenation on expression of HM74 and HM74a. (E) Effect of 4 h exposure to 110 U/ml IFNγ on expression of HM74 and HM74a in monocytic and lymphocytic cell lines.

binding [1,2], radioligand binding [1–3] and Ca^{2+} mobilisation [1,3]. We hypothesised that HM74 and/or HM74a might be the receptor(s) mediating macrophage PGD_2 release in response to niacin, with subsequent formation of 15d-PGJ_2 and activation of PPARγ. We transiently transfected monocytic and PMA-differentiated U937 with the luciferase reporter pLFABPLuc (containing four PPAR response elements from the human liver FABP gene inserted upstream of the HSV-TK promoter and firefly luciferase gene [27]) and exposed the cells to 0.1 μM to 1 mM niacin ± hypoxia for 24 h (as determined from time-course experiments, data not shown). No niacin-inducible PPAR activity was evident in monocytic U937, although PMA-differentiated cells demonstrated dose-dependent induction of PPAR activity peaking with 1 μM niacin ($P < 0.001$, Fig. 2A). Hypoxia did not significantly alter the magnitude of the PPAR response, suggesting either that the predominant receptor mediating this response is constitutively expressed (e.g. HM74a) or that maximal induction of PPAR activity via this pathway had already been achieved [28]. As previously reported, expression of PPARγ protein was induced by differentiation (Fig. 2B, upper panel) [29,30] and PPARγ mRNA and protein were dramatically upregulated by niacin in both monocytic and differentiated U937 (Fig. 2B) [18]. Basal levels of secreted 15d-PGJ_2 similarly increased 2.0-fold (±0.5) on PMA treatment of U937 cells ($P < 0.05$) and a further 2.2-fold (±0.6) following 24 h incubation with 1 μM niacin ($P < 0.05$, Fig. 2C). To determine whether the observed increase in PPAR activity was mediated via PPARγ as predicted, we transfected PMA-treated U937 with pLFABPLuc and applied agonists specific to PPARγ (ciglitizone), PPARδ (GW501516) and PPARα (WY-14643). Only ciglitizone induced PPAR transcriptional activity, suggesting that only PPARγ is present and active in these cells (Fig. 2D).

Acifran is a synthetic ligand of both HM74 and HM74a [2] and the most structurally distinct from niacin to bind the receptors to date [6]. Acifran also induced dose-dependent PPARγ activity (Fig. 2E) and induced PPARγ mRNA (Fig. 2F, left panel) and protein (Fig. 2F, right panel, 1.5-fold induction) in PMA-differentiated U937. These results demonstrate that both niacin and acifran induce PPARγ activity and/or expression in U937 cells.

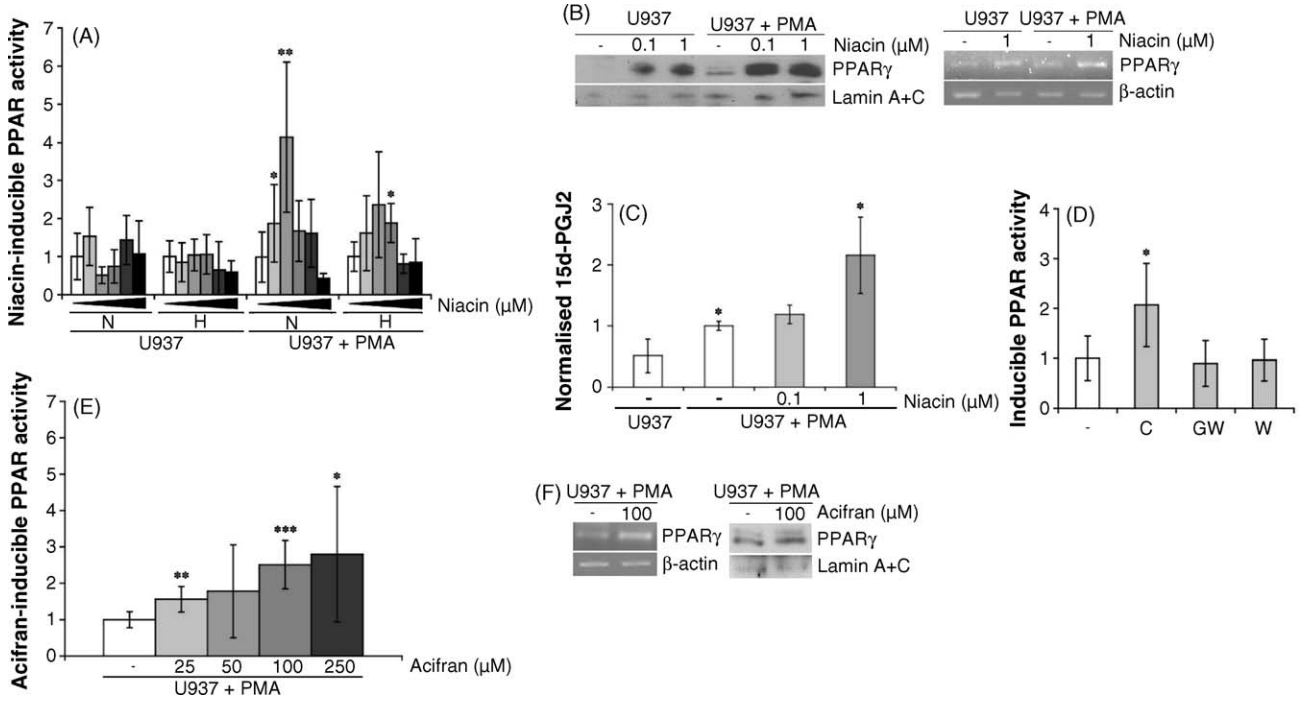

Fig. 2 – Analysis of PPARγ transcriptional activity and PPARγ expression induced by niacin and acifran in U937. (A) Activation of PPAR-luciferase activity of the pFABPLuc reporter by log-fold concentrations of niacin (0.1–1000 μM, 24 h). N = normoxia, H = hypoxia (24 h, 0.1% O_2). Luciferase readings are in relative light units (rlu) normalised to renilla and expressed as a value relative to the untreated control (solvent only, un-shaded bars). Each point represents the mean ± S.D. of at least three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.001$ compared with untreated controls. (B) Nuclear expression of PPARγ protein (with lamin A + C nuclear loading control, upper panel) and PPARγ mRNA (with β-actin loading control, lower panel) following 24 h exposure to 0.1 μM to 1 mM niacin. (C) Secreted 15d-PGJ_2 assayed following 24 h exposure to 0.1 and 1 μM niacin. Each point represents the mean ± S.D. of three independent experiments. $^{*}P < 0.05$ for U937 vs. U937 + PMA and for 1 μM niacin vs. untreated control. (D) PPAR-luciferase activity following 24 h exposure to 50 μM ciglitizone (C), 1 μM GW501516 (GW) or 50 μM WY-14643 (W). Luciferase readings normalised and expressed as for (A). Each point represents the mean ± S.D. of at least three independent experiments. $^{*}P < 0.001$. (E) Activation of pFABPLuc PPAR-luciferase activity by 24 h exposure to acifran. Luciferase readings normalised and expressed as for (A). Each point represents the mean ± S.D. of at least three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. (F) PPARγ mRNA (with β-actin loading control, left panel) and nuclear expression of PPARγ protein (with lamin A + C nuclear loading control, right panel) following 24 h exposure to100 μM acifran.

3.3. *Niacin induces PPARγ transcriptional activity via both HM74 and HM74a*

CHO cell stable transfectants for HM74 (HM74/1), HM74a (HM74a/3) and a vector-only control (HM74(a)-) were generated in order to determine whether niacin and acifran-induced PPARγ activation is mediated via these receptors. Specific mRNA level expression within the clones was confirmed by PCR (data not shown). Transient transfection of the pLFABPLuc reporter followed by 24 h exposure to log-fold concentrations of niacin demonstrated significant induction of PPAR activity in HM74a/3 cells by 100 μM ($P < 0.001$) and 1 mM niacin ($P < 0.005$) and in HM74/1 by 10 mM niacin ($P < 0.05$) (Fig. 3A). No induction of PPAR activity was observed in control cells at any concentration. Acifran-induced significant PPAR activity in HM74a/3 cells at concentrations over 25 μM (Fig. 3B), although no discernable effect was evident in HM74/1. To determine whether similarly high concentrations of niacin were necessary for HM74-mediated induction of PPAR activity in a cell line naturally expressing the receptor, we transiently transfected 1397 cells, which do not express HM74a, with pLFABPLuc. No significant niacin-mediated induction of PPAR activity was observed, although 10 mM niacin produced a non-significant 1.5-fold increase (Fig. 3C). As for PMA-treated U937, CHO cell transfectants were treated with agonists specific to PPARγ (ciglitizone), PPARδ (GW501516) and PPARα (WY-14643). All agonists induced significant PPAR transcriptional activity, although the greatest proportion of the total PPAR transcriptional readout was obtained with ciglitizone (Fig. 3D).

Considering that PPARγ is active in CHO cells and was the specific PPAR isoform mediating the response to niacin in PMA-treated U937, we analysed PPARγ expression following administration of niacin at a dose sufficient to enhance PPAR activity. PPARγ protein was induced two-fold in HM74a/3 cells by 100 μM to 1 mM niacin (Fig. 3E, top panel). No effect was evident in control cells (Fig. 3E, top panel) or in HM74/1 transfectants, even at the higher doses required for PPAR

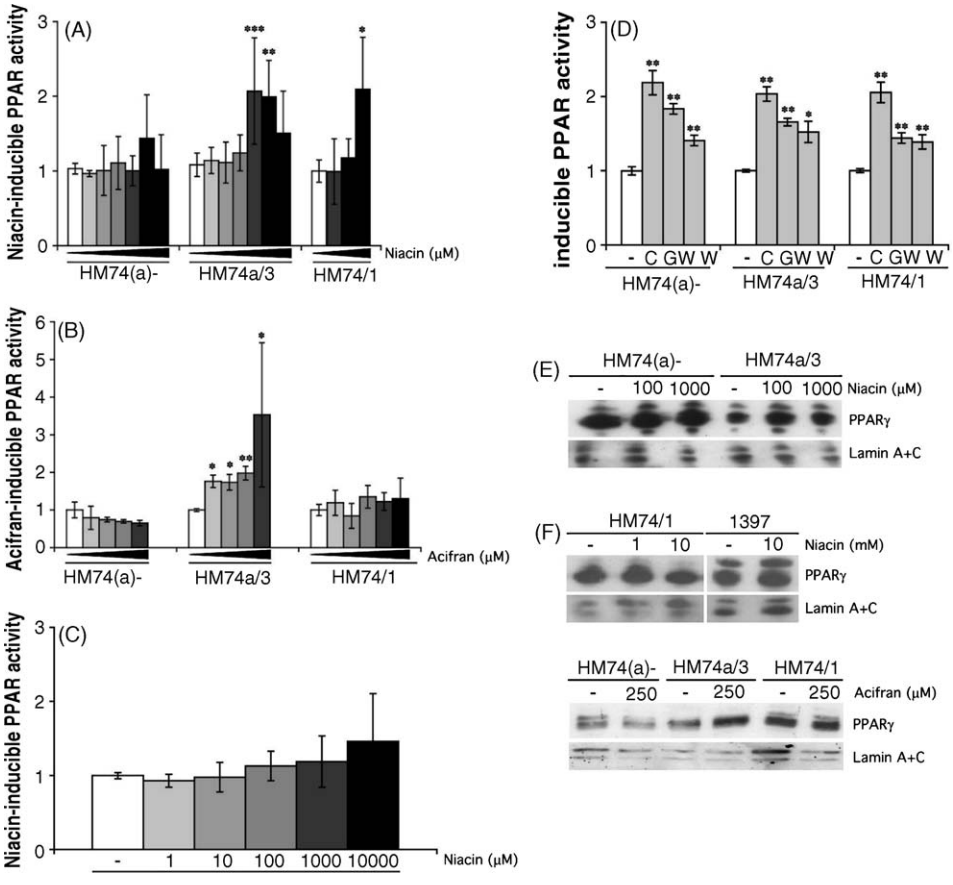

Fig. 3 – Analysis of PPAR transcriptional activity and PPARγ expression induced by niacin and acifran in HM74 and HM74a CHO cell transfectants. Activation of PPAR-luciferase activity of the pFABPLuc reporter by (A) log-fold concentrations of niacin (0.1–10,000 μM, 24 h, $^{*}P < 0.05$, $^{**}P < 0.005$, $^{***}P < 0.001$ vs. untreated controls) and (B) 24 h exposure to 25, 50, 100, 250 and 500 μM acifran ($^{*}P < 0.01$, $^{**}P < 0.005$ vs. untreated controls) in CHO cell transfectants. (C) Effect of niacin on PPAR-luciferase activity in 1397 cells. (D) Twenty-four hours exposure to 50 μM ciglitizone (C), 1 μM GW501516 (GW) or 50 μM WY-14643 (W). $^{*}P < 0.005$, $^{**}P < 0.001$. All luciferase readings are in relative light units (rlu) normalised to renilla and expressed as a value relative to the untreated control (solvent only, un-shaded bars). Each point represents the mean ± S.D. of at least three independent experiments. (E) Nuclear expression of PPARγ following 24 h exposure to 100 and 1000 μM niacin (CHO HM74(a)-, HM74a/3, top panel) or 1 and 10 mM niacin (CHO HM74/1, 1397, bottom panel) or (F) 250 μM acifran with lamin A + C nuclear loading control.

activation in these cells (Fig. 3E, bottom panel). In line with this result, 10 mM niacin did not clearly increase PPARγ expression in 1397 lymphocytes (Fig. 3E, bottom panel). Acifran markedly increased PPARγ expression in HM74a/3 and to a lesser (and more variable) extent in HM74/1 transfectants, again with no effect evident in control cells (Fig. 3F).

These results demonstrate that niacin-induced activation of PPAR activity in the CHO transfectants is also mediated via PPARγ (although we do not rule out additional possible effects via PPARα and/or PPARδ). PPAR activation was mediated by the HM74a receptor at concentrations at least 2 logs lower than that required for activation of the same pathway via HM74.

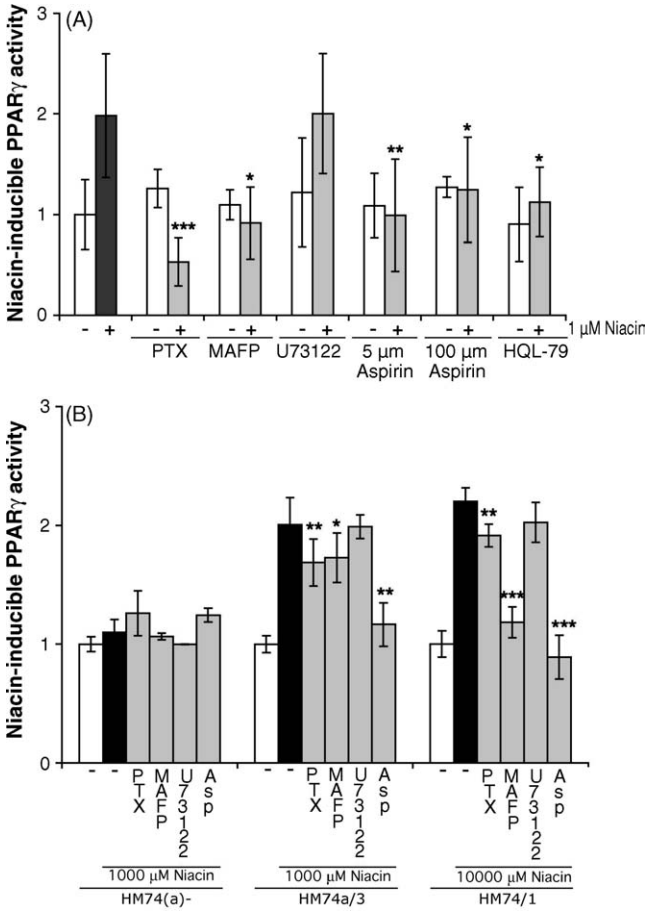

Fig. 4 – Effect of inhibitors of prostaglandin synthesis on niacin-induced PPARγ transcriptional activity. PPARγ-luciferase activity induced by 24 h exposure to niacin in (A) PMA-treated U937 ($^{*}P < 0.05$, $^{**}P < 0.005$, $^{***}P < 0.001$ relative to niacin-treated control) and (B) CHO cell transfectants ($^{*}P < 0.01$, $^{**}P < 0.005$, $^{***}P < 0.001$ relative to niacin-treated control) was assayed after 10 min pre-treatment with 100 ng/ml pertussis toxin (PTX), 2 μM MAFP, 5 μM U73122, 5 μM or 100 μM aspirin and 75 μM HQL-79. Luciferase readings are in relative light units (rlu) normalised to renilla and expressed as a value relative to the untreated control. Each point represents the mean ± S.D. of at least three independent experiments.

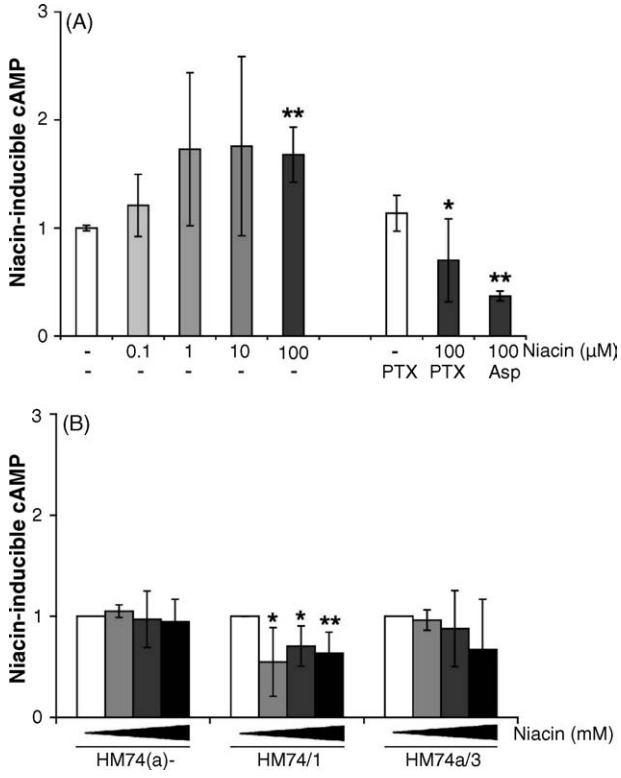

Fig. 5 – Analysis of cAMP levels in PMA-treated U937 and CHO cell transfectants. Intracellular cAMP measured following 24 h exposure to log-fold concentrations of niacin in (A) PMA-treated U937 ($^{**}P < 0.005$ vs. untreated control) and (B) CHO cell transfectants (0.1–10 mM niacin, $^{*}P < 0.05$, $^{**}P < 0.01$ vs. untreated control) and normalised to lysate total protein concentration. Cells in (A) were pre-treated for 10 min with inhibitors prior to 24 h exposure to niacin ($^{*}P < 0.01$, $^{**}P < 0.001$ v.s 100 μM niacin control). Each point represents the mean ± S.D. of at least three independent experiments.

3.4. *Niacin signalling via HM74 and HM74a activates prostaglandin synthesis pathways*

In order to confirm the hypothesis that HM74 and HM74a are inducing PPARγ transcriptional activity via the prostaglandin synthesis pathway, we studied the effect of inhibitors of this pathway on niacin-mediated PPARγ activation. Initially, we demonstrated in PMA-treated U937 that PPARγ activity induced by 1 μM niacin is pertussis toxin sensitive (Fig. 4A, $P < 0.001$) in accordance with HM74 and HM74a being coupled to Gi-type G-proteins [1–3]. Niacin-induced PPARγ activity was also reduced to unstimulated levels by inhibition of phospholipase A2 (MAFP, $P < 0.05$), the cyclo-oxygenase enzyme COX-1 (5 μM aspirin, $P < 0.005$) and prostaglandin D_2 synthase (HQL-79, $P < 0.05$, Fig. 4A). No effect was observed by inhibition of phospholipase C (U73122, EC 3.1.4.3) nor any additive effect of inhibition of COX-2 + COX-1 (100 μM aspirin).

Similar experiments in the CHO cell transfectants showed moderate but significant inhibition of niacin-induced PPAR activity by pertussis toxin in both HM74a/3 and HM74/1 cells (Fig. 4B, $P < 0.005$). HM74-mediated PPARγ activity was more

sensitive to phospholipase A2 inhibition than HM74a (85% inhibition versus 28%), while COX-1 inhibition (5 μM aspirin) reduced PPARγ activity to unstimulated levels in both cell types (Fig. 4B).

3.5. *Niacin-induced cAMP in U937 macrophages is pertussis toxin sensitive*

As well as mediating prostaglandin-dependent gene expression in monocytic MonoMac6, niacin has also been demonstrated to increase intracellular levels of cAMP [18]. We therefore investigated whether this effect might also be mediated via HM74 and/or HM74a. In agreement with Rubic et al. [18], 100 μM niacin-induced a 1.7-fold increase in intracellular cAMP in PMA-treated U937 ($P < 0.005$, Fig. 5A). This induction was returned to baseline levels of cAMP (or below) by pre-treatment with either pertussis toxin ($P < 0.01$) or 5 μM aspirin ($P < 0.001$, Fig. 5A), in a similar manner to that observed for effects on niacin-inducible PPARγ activity in these cells (Fig. 4A). However, both HM74/1 and HM74a/3 CHO cell transfectants showed evidence of a niacin-dependent decrease in intracellular cAMP compared with control cells (Fig. 5B). This has previously been observed in a number of non-monocytoid HM74a transfectants [1,3,9], although not in HM74-expressing cell lines, suggesting that HM74a and/or HM74 might also mediate monocyte/macrophage-specific respones to niacin via induction of cAMP.

4. Discussion

The identification of HM74 and HM74a as niacin receptors has provided opportunity to determine the molecular mechanism(s) of niacin's clinical effects. It has recently been shown that niacin stimulates PPARγ- and cAMP-dependent mechanisms of reverse cholesterol transport in macrophages [18]. Our data now demonstrates that human macrophage cell lines and primary cells in culture express both HM74 and HM74a and that these receptors can directly mediate macrophage responses to niacin via activation of the prostaglandin synthesis pathway and via increased expression and activity of PPARγ.

Prior to this study, no significant expression of either HM74 or HM74a had been detected in human monocytic populations [6]. This may have been due to the state of activation and/or differentiation of the cells studied. However, HM74 was originally cloned from a GPCR screen of a human monocyte cDNA library [31] while murine RAW 264.7 macrophages display specific [^{3}H]niacin binding [32] and PUMA-G is IFNγ, TNFα and LPS-inducible in murine ANA-1 macrophages [4]. We have identified consistent basal expression of HM74a mRNA in human monocytic cell lines and primary human monocytes/macrophages that was strongly hypoxia-inducible in the primary cells (although this was not a strong or consistent feature of the cell lines studied). HM74 mRNA was even more strikingly hypoxia-inducible in primary monocytes and PMA-differentiated U937 and THP1 cells. The reason for hypoxic induction of HM74 in apparently opposing differentiation states in cell lines versus primary cells is unclear, but may again be due to the specific state of activation/differentiation achieved with PMA-treatment [25,26] compared with that of PBMCs and the subsequent adhesion-differentiated macrophages [24]. Considering that HM74 is expressed at barely detectable levels under normoxic conditions (in comparison to the more ubiquitously expressed HM74a), the hypoxia-inducible nature of this receptor is likely to be of considerable interest if/when a physiological ligand is identified.

The recent report of niacin mediating PPARγ- and cAMP-dependent responses in macrophages via an unidentified receptor [18] prompted us to study activation of these pathways downstream of HM74 and HM74a. An association with PPARγ was additionally implied by the strikingly similar expression patterns observed for HM74/HM74a mRNA (predominantly in the adrenal glands, adipose tissue, spleen, lung and lymphocytes [1–3] and IFNγ-, TNFα-, LPS- [4] and now hypoxia-inducible in macrophages) and that described for PPARγ [33,34], which is also upregulated following activation [4,29,30,35] or differentiation [29] of monocytes/macrophages. Niacin-induced PPAR transcriptional activity in CHO cells via both HM74 and HM74a, although requiring 100-fold greater concentrations to activate pathways downstream of HM74. This is similar to previous studies of radioligand binding and G-protein activation that describes niacin effects on HM74 as ≥1000-fold less potent than HM74a [1–3]. It is possible that our transfectants display closer affinities for niacin due to use of the genetic variants expressed in U937 cells, and that single nucleotide polymorphisms within the two genes may contribute to cell or tissue-type variations in affinity for niacin [5]. That the absolute niacin concentration required to activate HM74a in CHO cells was significantly higher than the 0.1–1 μM EC_{50} described for G-protein activation [1–3] is likely to be an artefact of either altered receptor configuration or of over-expression of the receptor in a cell type containing sub-optimal levels of downstream pathway component(s). This was supported by experiments in the PMA-treated U937 from which the receptors were cloned, where maximal activation of PPARγ was observed with 1 μM niacin. Results with acifran differed in that both PMA-treated U937 and HM74a/3 CHO cells induced PPARγ activity at 25 μM acifran. HM74/1 did not induce PPAR transcriptional activation with even 500 μM acifran, despite reports of only a 10-fold difference in EC_{50} with respect to GTPγS binding [2].

Nuclear expression of PPARγ was induced by niacin in both monocytic and PMA-treated U937 despite no evidence of niacin-induced PPARγ transcriptional activation in the monocytic cells. Transcriptional interaction of PPARγ with motifs in the PPRE requires recruitment of co-factors such as C/EBF, CBP/p300, steroid receptor co-activator 1 (SRC-1), receptor interacting G-protein 140 (RIP140) and PPARγ-binding G-protein (PBP) [36]. Differentiation increases the expression (RIP140 [37]) or nuclear localisation (CBP/P300 and SRC-1 [38]) of a number of such factors. Considering that PPARγ itself is not abundant in monocytic cells, it is possible that induction of PPARγ protein in monocytic U937 by niacin is unable to induce PPARγ transcriptional activity due to sub-optimal nuclear levels of the co-factors required for binding to the PPRE. Conversely, induction of PPARγ protein with no associated evidence of PPAR transcriptional activity at higher doses in PMA-treated U937 may potentially be an effect of niacin toxicity (although no evidence of loss of cell viability was observed). Niacin also induced PPARγ expression in HM74a/3

CHO cells, but not in HM74/1 or 1397 cells, whereas acifran increased PPARγ expression in both the HM74a and, to a lesser extent, the HM74 transfectants. We have not investigated the mechanism behind preferential induction of nuclear PPARγ following activation of HM74a, although it may relate to the greater affinity of HM74a for niacin and acifran and potentially therefore greater amplification of downstream target gene expression.

Niacin-induced PPAR activity mediated by HM74/HM74a was reduced or ablated by blocking prostaglandin synthesis by inhibition of PLA_2 [39,40] and COX-1/COX-2 [41,42] and additionally via Gi-G-protein inhibition with pertussis toxin. Agonist-induced phospholipase activation and COX-catalysed generation of PGH_2 are common to most cell types, while tissue-specific isomerases generate the different bioactive prostaglandin derivatives of PGH_2 [42]. Specific blockade of PGD_2 synthesis via inhibition of PGD_2 synthase [43,44] ablated niacin-induced PPARγ activity in U937 cells but had variable effects in the CHO cell transfectants (data not shown). This clearly demonstrates that niacin binding to HM74 and HM74a activates prostaglandin synthesis and that in monocytic cells this results in PGD_2 synthesis and formation of the PPARγ ligand 15d-PGJ_2. Considering that PPARγ, PPARδ and PPARα are present and active in CHO cells, it is possible that niacin-mediated activation of PPARγ via HM74/HM74a in the CHO cell transfectants is either mediated by very low level PGD_2 synthesis and/or that other prostaglandins can influence activity of the pLFABPLuc PPAR response element, for example via PPARδ activation by PGI_2 [27,45].

Niacin also induces intracellular cAMP in monocytic cells [18] in a manner we have demonstrated to be both pertussis toxin sensitive and COX-dependent. Attempts to determine whether this effect was mediated via HM74 or HM74a were unsuccessful as HM74/1, and to a lesser extent HM74a/3, CHO cell transfectants demonstrated a significant reduction in intracellular cAMP due to niacin. Several groups have observed niacin-mediated inhibition of the adipocyte cAMP response [46,47] and a similar effect in HM74a-transfected CHO-K1 [3], 293EBNA [1], HEK293 and 3T3L1 adipocytes [9], at least partially ascribed to inhibition of adenylate cyclase [43]. Our results differ in that HM74/1 transfectants showed greater sensitivity to niacin with respect to reduction in cAMP than HM74a/3. This may again be due to the genetic variants used [5] but also implies that HM74 and HM74a could be coupled to different downstream pathways; HM74 primarily mediating cAMP responses and HM74a the PPARγ response. Whether niacin has different effects on monocytic adenylate cyclase or whether stimulation of other signal transduction pathways to increase cAMP over-rides this response in macrophages is not clear. However, PGI_2 analogues and PGE_2 are known to induce cAMP [48,49], potentially explaining why COX inhibition attenuated the effect of niacin and maintaining the possibility that this effect could also be mediated via HM74 and/or HM74a.

Our findings are of particular interest regarding the clinical use of niacin, although due to the relative affinities of the two receptors, it is likely that HM74a would mediate the majority of effects. Considering the hypoxia and IFNγ-inducible nature of the receptor, macrophage expression of HM74a might be evident in clinical conditions manifesting hypoxia and/or lymphocytic infiltrates. PPARγ is expressed in macrophage foam cells of human atherosclerotic lesions [30] where it stimulates uptake of oxLDL which further induces PPARγ expression and activation via a reciprocal positive feedback mechanism [29,30,50]. Although this may be expected to further foam cell formation, instead PPARγ ligands generally promote fatty streak regression by enhancing removal of cholesterol from macrophages via stimulation of reverse cholesterol transport [18,51]. Atherosclerotic plaques express high levels of T cell-derived IFNγ [52,53], implying that macrophage foam cells might express HM74a. Monocytes/macrophages within the plaques also express COX-2 [42] and cytoplasmic 15d-PGJ_2 [54]. It will be of interest to investigate in vivo whether niacin might target such cells and enhance reverse cholesterol transport via ligand binding to HM74a.

As regards effects in cancer, PPARγ ligands are generally anti-angiogenic [36,55], pro-apoptotic [36,56], anti-proliferative [36,57] and can cause reversion of dedifferentiated neoplastic cells back to a less malignant state [36,58]. There is no data regarding the role of PPARγ in tumour-associated macrophages (TAM), although TAM accumulates in areas of hypoxia [59–61] where they might be induced to express HM74a. Both PPARγ and 15d-PGJ_2 are negative regulators of macrophage function with respect to cytotoxic [35,62] and inflammatory [28,35,62] responses to various stimuli. However, prostaglandins PGE_2 and PGD_2 have been implicated in pro-angiogenic and vasodilatory mechanisms [15,16,63,64]. It would therefore be of interest to determine whether niacin-induced pro- or anti-tumourigenic mechanisms in HM74-expressing macrophages in this setting.

In summary, we have shown that niacin induces PPARγ expression and increases PPARγ transcriptional activity via activation of the prostaglandin synthesis pathway downstream of HM74 and HM74a. This provides further insight into the effects of pharmacological doses of niacin.

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