Identification of multiple, oxygen-stable HIF1 alpha isoforms, and augmented expression of adrenomedullin in rheumatoid arthritis

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Abstract

Objective

To identify and quantitate hypoxia inducible factor 1 alpha (HIF1 α) isoforms in circulating peripheral blood mononuclear cells (PBMCs), and to assess their effects on target gene expression in rheumatoid arthritis (RA) patients.

Method

PBMCs from healthy controls and from RA patients were analysed ex-vivo for expression of HIF isoforms, and target genes were assessed by RT-PCR.

Results

Transcripts of multiple HIF1α isoforms exist in circulating PBMCs. Expression of all these isoforms is dramatically, and maximally, augmented by foreign surface recognition. However, HIF1α protein stabilisation requires additional cell activation with phorbol ester. No difference in the expression or regulation of the HIF1α isoforms was seen between patients with active RA and healthy controls. However, analysis of a panel of HIF1α target genes revealed increased basal expression of the adrenomedullin gene in RA PBMCs, with resulting loss of further induction upon cell activation.

Conclusion

Even in normoxia PBMCs express stable HIF1α protein on cell activation. Whilst multiple HIF1α isoforms exist in PBMCs no differences in expression were seen in RA compared with healthy controls.

RA causes constitutive adrenomedullin expression in PBMCs that is not explicable by altered HIF expression, or stabilisation. Adrenomedullin has a variety of potential biological roles in RA, including regulation of angiogenesis, and aberrant gene regulation may be relevant in RA pathogenesis.

Key words

normoxia, HIF1α, isoforms, adrenomedullin, rheumatoid arthritis

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Introduction

HIF-1 consists of an α and β-subunit, both of which are constitutively expressed in normoxic and hypoxic conditions. However, the stability of the α subunit is tightly regulated (1-3). In normoxia an oxygen dependent degradation domain (ODD) in HIF1α is targeted by prolyl-hydroxylases (PHD). Hydroxylation of HIF1α in turn facilitates interaction with the E3 ligase VHL which targets the protein for degradation by the proteasome. In hypoxia, HIF1α degradation is prevented by inhibition of PHD activity, resulting in stabilisation of HIF1α and activation of HIF1 target genes. Hypoxic stabilisation of HIF1α occurs in RA joints and is thought to potentiate inflammation (4, 5). Furthermore, we have previously shown that HIF1α is the principal transactivator of the proinflammatory mediator macrophage migration inhibitory factor (MIF), a protein elevated in the serum and synovial fluid of RA patients, and implicated in disease progression (6).

In addition to the well-described stabilisation of HIF1α protein in hypoxia HIF1α protein is reported to accumulate under normoxia in response to different inflammatory stimuli. HIF1α protein can be stabilised by nitric oxide, a near-universal accompaniment to inflammation, by inhibition of PHD activity (7-9), and also by pro-inflammatory cytokines, including IL1β, and TNFα (10). HIF1α gene expression is strongly induced by the NFkB transcription factor (11), but the requirement for additional signals to stabilise the resulting protein product remains unclear (12, 13).

Several splice variants which impact on HIF1α function have been described in cell lines (Fig. 1a). Alternative splicing of HIF1α is mainly restricted to the 3' end of the transcript; the isoforms being defined by missing exons as follows: HIF1α Δ12, HIF1α Δ11&12, HIF1α Δ14 and HIF1α Δ11). This results in splice variants lacking exons encoding parts of the ODD domain and/ or one or more transactivation domains (CTAD and/or NTAD) which are required for effective transcriptional activation of HIF target genes. These isoforms are predicted to be stable in normoxia and / or have altered transcriptional activity (13-18). In addition, alternative splicing of the HIF1α transcript occurs between exons 1 and 2, with insertion of three bases, TAG, causing substitution of Lysine 12 for Asparagine 12 and insertion of an additional Arginine HIF1α TAG) (19). However, the functional significance of this isoform is currently unclear. An alternative upstream translational start site, resulting in a protein 24 amino acids longer at the N terminus than full length HIF1α, has also been reported (HIF1α Alt1) (17). HIF1α also has two paralogs, HIF2α and HIF3α which show conservation with HIF1α in both the ODD domain and basic helix loop helix (Fig. 1b), allowing tight regulation of protein stability and dimerisation with HIF1β and DNA binding. HIF2α is mainly expressed in lung, kidney and liver. The function of HIF3α remains unclear (20, 21).

Research to date has focused on the function of full length HIF1α in transformed cell lines.

We have investigated HIF1α expression in normoxia using primary immune cells and identify oxygen-stable HIF1α splice variants. This discovery is accompanied by expression of oxygen-stable HIF1α protein in PBMCs. Furthermore, we identify deficient induction by cellular activation of the HIF target adrenomedullin (ADM), which results from increased basal expression of the gene.

Methods

Human volunteers

Patients with RA were recruited from the rheumatology clinic at Manchester Royal Infirmary. Healthy control volunteers were recruited by local advertisement. These individuals were staff and students of the University of Manchester, and from the Manchester Royal Infirmary. Patients and controls were recruited contemporaneously, and to protocol, which specified an age range (18 to 80 yrs). There was no significant difference in the ages of the patients and the controls, but the controls did tend to be younger. The demographics of the RA patient and controls groups are shown in Table I.

Table I.

Group	Age (yrs) [median (95%CI)]	DAS28 [median (95%CI)]	RF [positive:negative]	Gender [F:M]
RA	64 (25-76)	5 (4-8)	11:6	15:2
Control	32 (24-51)	0	0:24	11:13

Table II.

HIF1α Δ11 forward primer GATGGAAGCACTAGACAAAGTTCAC,
HIF1α Δ11 reverse primer CTTTGAGGACTTGCGCTTTCAG,
HIF1α Δ11 probe CTGAGGACACAGATTTA;
HIF1α Δ 11&12 forward primer ACACCTAGTCCTTCCGATGGA,
HIF1α Δ11&12 reverse primer GCATTCTGCAAAGCTAGTATCTTTGGA,
HIF1α Δ 11&12 probe AAGTTCACCTGAGAACTAC;
HIF1α Δ 14 forward primer GCTTTGCAGAATGCTCAGAGAAA,
HIF1α Δ 14 reverse primer CTGGTCAGCTGTGGTAATCCA,
HIF1α Δ 14 probe TCTACATGCTAAATAATTCC;
HIF1α Alt1 forward primer GGTCTAGGAAACTCAAAACCTGAAGA,
HIF1α Alt1 reverse primer CTGCATCTCGAGACTTTTCTTTTCG,
HIF1α Alt1 probe CAGAACTTATCCTACCATTTTC;
HIF1α Δ12 forward primer CTGAAGACACAGAAGCAAAGAAC,
HIF1α Δ12 reverse primer GCATTCTGCAAAGCTAGTATCTTTGGA,
HIF1α Δ12 probe CCATTTTCTACTCAGAACTAC;
HIF1α TAG forward primer GGGACCGATTCACCATGGA,
HIF1α TAG reverse primer CGAGACTTTTCTTTTCGACGTTCAG,
HIF1α TAG probe ATCCTATTTTTCTTGTCGTTCGCG;

Catalogue numbers for Taqman reagents used:
Total HIF1α Hs00153153_m1; **IL6** Hs00174131_m1; **HK2** Hs00606086_m1; **HIF3A** Hs00541709_m1; **IL1A** Hs00899848_m1; **ADM** Hs00181605_m1; **VEGFA** Hs00173626_m1; **HIF2α** Hs00208298_m1. **Beta actin** 4326315E

Whole blood was obtained from patients with active RA, as assessed by INB (DAS28 score >4), as well as healthy volunteers, after full, informed consent was obtained. This study protocol was approved by LREC, number 09/H1013/6.

Cell culture

Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood by Ficoll gradient centrifugation and cultured in 60mm cell culture dishes at 0.8 x 10^6/ ml in RPMI 1640 medium with 5mM L-glutamine (PAA), supplemented with 10% FBS (Gibco) and 100mg/ml penicillin-streptomycin (PAA). The cells were either incubated at 37ºC for 4 hours before harvesting (4hrs plated), with 100nM phorbol 12-myristate 13-acetate (PMA) where indicated (4hrs plated + PMA), or harvested at time zero with or without prior plating (zero plated and zero spun respectively). Non-adherent PBMCs were collected by centrifugation and combined with adherent PBMCs lysed in appropriate lysis buffer.

HEK 293 cells were cultured with DMEM medium (PAA) supplemented with 10% v/v FBS (Gibco) and 5mM L-glutamine (PAA).

RNA extraction and cDNA synthesis

Total RNA was prepared using the QIAGEN RNeasy Minielute kit, followed by treatment with DNase I (NEB) as per manufacturer's instructions and cDNA prepared using Applied Biosystems High Capacity RNA-to-cDNA Kit as per manufacturer's instructions and quality was assessed using a Bioanalyser.

Semi-quantitative RT-PCR

Primers used for semi quantitative RT PCR were as follows: HIF1α S1, and A2, beta-actin. PCR was performed under the following conditions: HIF1α exons 10–15: 95ºC for 5 m, then 40 cycles at 95ºC for 30s, 53ºC for 30s, 72ºC for 1min, followed by 72ºC 7min. Beta actin: 95ºC for 5min, 20 cycles at 95ºC for 30s, 58.4ºC for 1min, 72ºC for 1min, followed by 72ºC 7min. PCR products were analysed by gel electrophoresis, excised and subject to gel extraction using Qiagen's gel extraction kit, as per manufacturer's instructions. Purified PCR products were then directly sequenced.

Real-time RT-PCR

Real time PCR was performed using Applied Biosystems Step-one plus system and Taqman assay reagents as per manufacturer's instructions. Primers and probes for analysis of HIF1α isoforms are in Table II.

Results were analysed using the comparative Ct method, normalising results to beta actin for ΔCT, and against zero-spin for ΔΔCT.

Cloning and synthesis of HIF1α isoforms

Full length HIF1α, from PBMCs isolated from a healthy volunteer was amplified with Phusion DNA polymerase using the following primers: S3 and A2, under the following conditions 98ºC for 5min, then 40 cycles at 95ºC for 30s, 53ºC for 30s, 72ºC for 1min, followed by 72ºC 7min. The HIF1α amplicon was A tailed by incubating 7ul of the amplicon with 1X Taq DNA pol reaction buffer, 0.2mM dATP and 5U Taq and was sub cloned into the pGEM®-T Easy vector (Promega) as per manufacturer's instructions. The amplicon was then cloned into pCDNA5/FRT vector using Not I restriction enzyme (NEB) sites and ligase as per manufacturers instructions. Expression vectors containing HIF1α TAG, HIF1α Δ12, HIF1α Δ11&12, HIF1α Δ14 and HIF1α Δ11 were produced by deleting the appropriate exons from full length HIF1α. Deletion of the exons was performed using Stratagene's QuickChange II XL Site-Directed Mutagenesis Kit using the HIF1α pcDNA5/FRT plasmid as a template and using a modified protocol (6, 22).

Transfections

HEK 293 cells were transiently transfected with Lipofectamine 2000 (Invitrogen) according to the manufacturer's guidelines.

Immunoblots

Immunoblots were performed as described previously (23, 24) with the

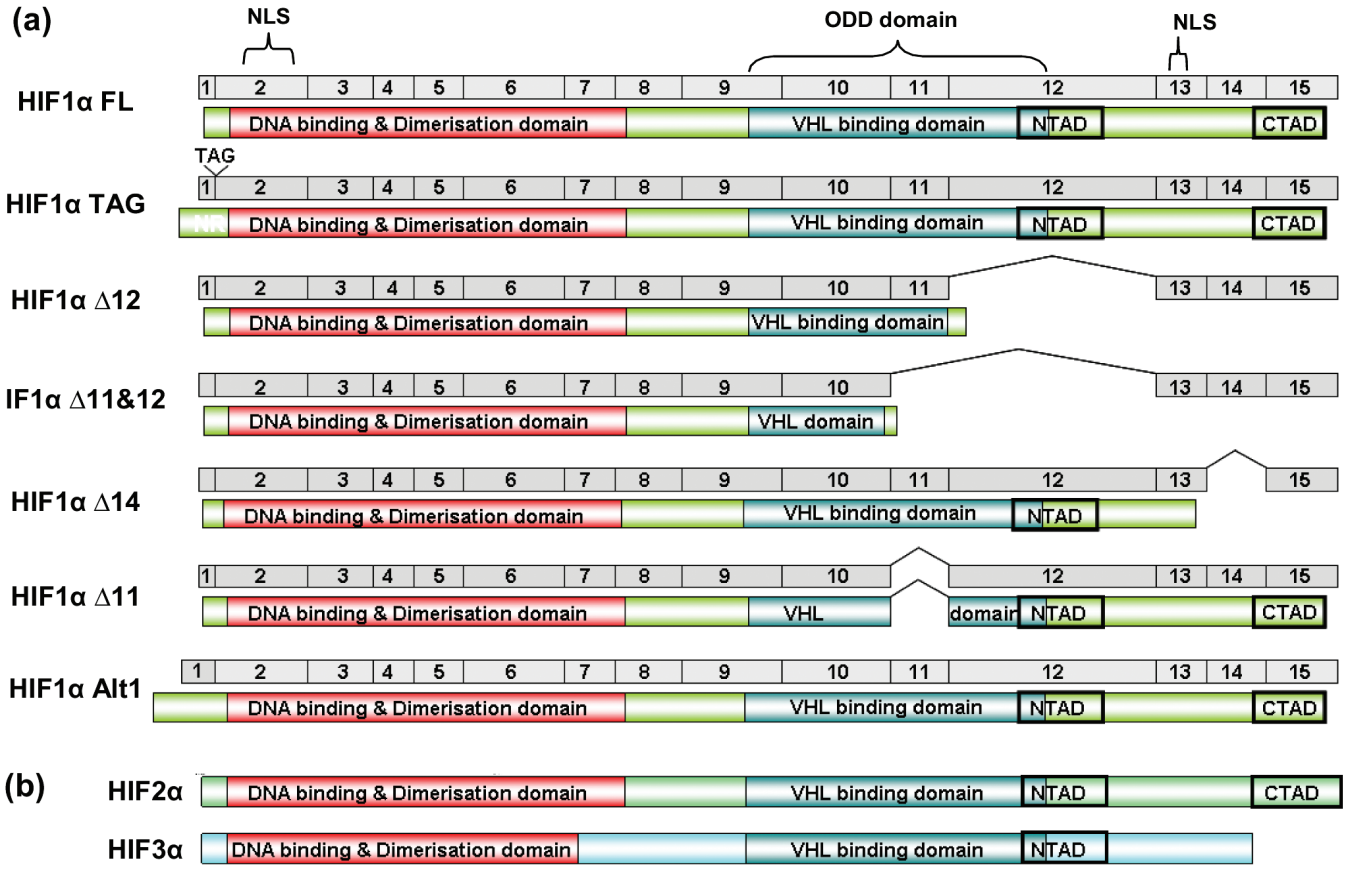

Fig. 1. Schematic showing composition of HIF1α splice variants. (**a**) Transcripts representing full length HIF1α and six HIF1α splice variants are shown in light grey, exons are indicated by numbers. The protein resulting from each transcript is shown below, the position of the DNA binding and dimerisation domain, Oxygen sensitive degradation (ODD) domain/ VHL binding domain and N and C terminal transactivation domains, NTAD and CTAD respectively, are indicated (**b**) Domain structure of HIF1α paralogues, HIF2α and HIF3α with conserved domains aligned with HIF1α.

following changes; proteins were transferred to 0.2-μm PVDF membrane (Bio-Rad Laboratories, Hertfordshire, UK).

Statistics

Results are presented as medians ± interquartile range and compared by Mann-Whitney U-tests.

Results

HIF1α transcripts are induced by PBMC activation in normoxia

PBMCs from nine healthy donors were stimulated by phorbol ester (PMA), a strong activating stimulus, and/or plating on plastic, which induces a weaker activation of monocytes by foreign surface recognition, and harvested either four hours post treatment or at time zero (zero spin), with and without the effects of plating on plastic.

Expression of HIF1α was measured by semi-quantitative RT-PCR. As the HIF1α isoforms mainly result from alternative splicing at the 3' end of the transcript, PCR was performed with primers (S1 and A2) designed to PCR exons 10-15 of the HIF1α transcript (Fig. 2a) (13). HIF1α mRNA containing exons 10-15 (HIF1α FL) was detected in PBMCs under basal and stimulated conditions, as expected. A number of smaller PCR products were also detected and confirmed by direct sequencing to be HIF1α splice variants lacking the indicated exons, HIF1α Δ14, HIF1α Δ12 and HIF1α Δ11&12. HIF1α and HIF1α Δ14 showed increased expression when the cells were activated by plating (4hrs plated). However, no further increase was observed in response to PMA (4hrs plated +PMA). HIF1α Δ12 expression increased both in response to activation by plating for 4 hours and PMA treatment. HIF1α Δ11&12 showed similar levels of expression under basal conditions and in response to stimulus, however, this was variable between donors.

To quantify accurately the expression of the various HIF1α isoforms in primary PBMCs from multiple donors we designed Taqman probes which spanned the exon-exon boundaries distinct to each HIF1α isoform and measured relative expression under basal and stimulated conditions (Fig. 2b and suppl Fig. 1). The specificity of these probes for the splice variants was verified using synthetic cDNA constructs. All HIF1α isoforms analysed were found to be co-expressed in PBMCs isolated from the 9 healthy controls. We found a dramatic induction in total HIF1α, HIF1α TAG and HIF1α Δ11 mRNA in all donors in response to plating compared to unstimulated cells (zero spin). However, no further increase in expression was seen with PMA, as observed previously (Fig. 2a and Suppl. Fig. 1). The other four HIF1α splice variants (HIF1α Δ11&Δ12, HIF1α Δ12, HIF1α Δ14 and HIF1α Alt1) showed a heterogenous pattern of expression between

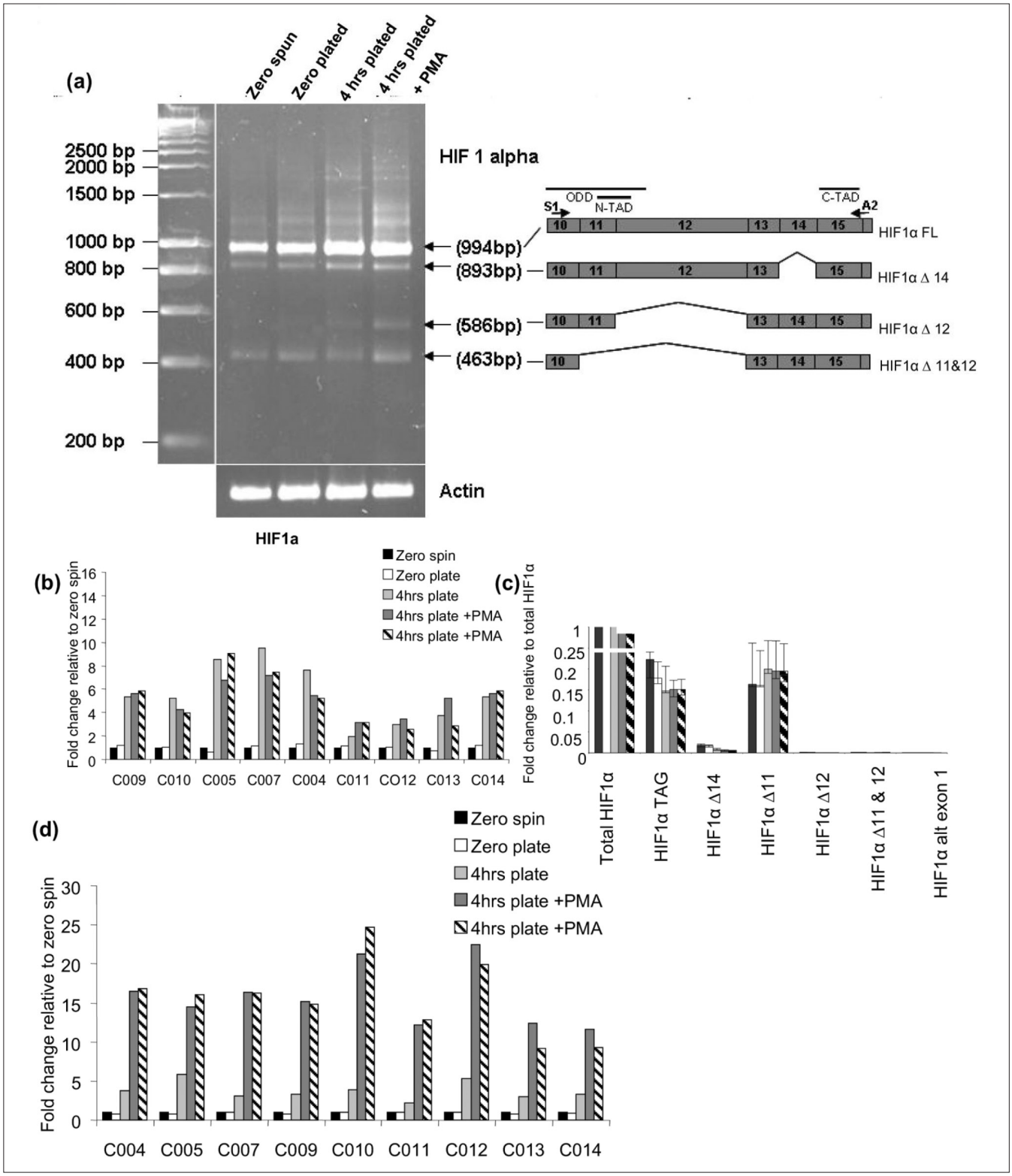

Fig. 2. HIF1α is upregulated in activated PBMCs. PBMCs were isolated from healthy donors and activated by either plating on plastic for 4hrs (4hrs plated) or plating in combination with PMA treatment for 4hrs (4hrs plated + PMA). As controls, PBMCs were harvested at time zero with and without the effects of plating (zero plate and zero spun respectively). (**a**) Semi-quantitative RT-PCR was performed with primers (S1 and A2) designed to amplify exons 10 to15 of the HIF1α transcript. Four PCR products were identified (molecular size indicated), excised and subject to gel extraction and sequencing. HIF1α splice variants identified by sequencing are shown schematically adjacent to the corresponding PCR products. The position of oxygen sensitive degradation domain (ODD) and transactivation domains CTAD and NTAD are indicated. (**b**) Real time RT-PCR was performed with Taqman probes designed to detect HIF1α. Results are presented relative to mRNA levels in cells harvested at zero without plating (zero spun). Each healthy donor is represented by C009-C014 (**c**) Same as (**b**) but results are presented relative to mRNA levels of total HIF1α for each treatment. (**d**) Same as (**b**) but instead Taqman probes designed to detect HIF2α were used.

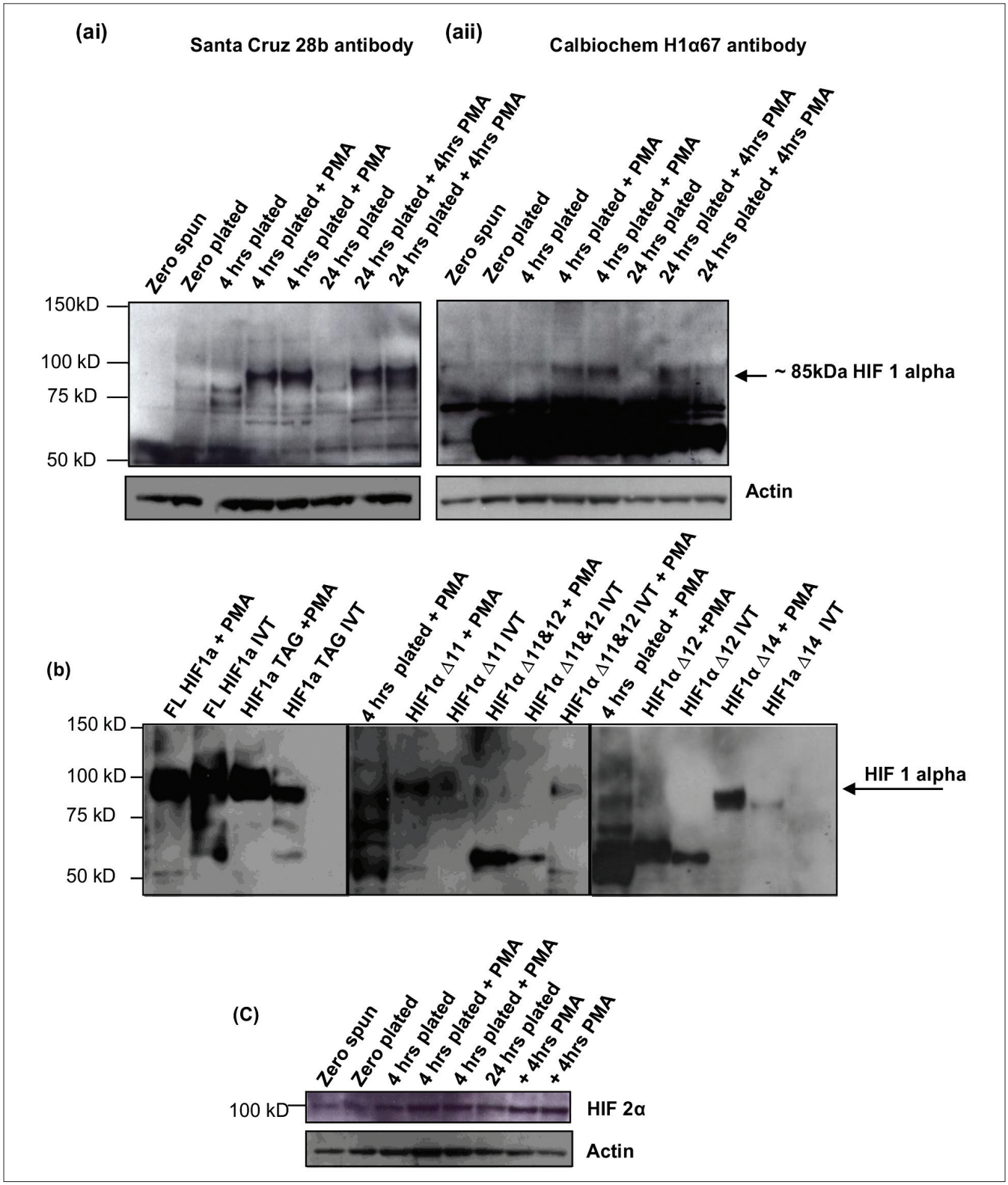

Fig. 3. HIF1α isoforms are stabilised in response to PMA treatment (**a**) PBMCs were isolated from healthy donors & activated by either plating on plastic or plating in combination with PMA treatment. Cells were lysed 4hrs and 24hrs post treatment as well as at time zero with and without the effects of plating (zero plate and zero spun respectively). Immunoblot analysis was performed with two different anti-HIF1α antibodies and also anti-actin antibody as loading control (i) 28b-Santa Cruz, (ii) H1α67 Calbiochem.
(**b**) HEK cells transfected with the indicated HIF1 constructs were harvested, and the cell lysates resolved by SDS-PAGE, and immunoblotted as in (ai). In addition, *in-vitro* transcribed, and translated proteins corresponding to the same isoforms were run in adjacent lanes (IVT). The PBMC lysate after 4hours plating and PMA was run in two lanes for direct comparison, and the non-transfected HEK cell treated with PMA lysate was run in the lane indicated +PMA.
(**c**) Same as (**a**) but instead immunoblots were performed with anti-HIF2α antibodies.

	01	02	03	04	05	06	07	08	09	10	11	12
A	ADM **44.95**	AGPAT2 **-14.79**	AGTPBP1 **-1.26**	ANGPTL4 **2.34**	ARD1A **-1.14**	ARNT2 **46.21**	BAX **1.13**	BHLHE40 **3.36**	BIRC5 **-1.16**	CA1 **-1.39**	CASP1 **2.22**	CAT **-5.04**
B	CDC42 **1.26**	CHGA **-1.13**	COL1A1 **9.61**	CREBBP **-1.58**	CSTB **11.42**	CYGB **8.83**	DAPK3 **-1.2**	DCTN2 **-1.09**	DR1 **2.11**	ECE1 **2.23**	EEF1A1 **1.23**	ENO1 **3.1**
C	EP300 **1.16**	EPAS1 **14.82**	EPO **1.15**	PRPF40A **1.54**	GNA11 **-2.03**	GPI **1.1**	GPX1 **-1.3**	HBB **1.07**	HIF1A **5.53**	HIF1AN **1.45**	HIF3A **1.15**	HK2 **9.79**
D	HMOX1 **4.79**	KAT5 **1.31**	HYOU1 **1.35**	IGF2 **1.73**	IGFBP1 **1.15**	IL1A **16.89**	IL6 **5.41**	IL6ST **1.24**	IQGAP1 **1.23**	KHSRP **1.7**	KIT **-4.18**	LCT **7.42**
E	LEP **2.28**	MAN2B1 **-1.32**	MOCS3 **1.56**	MT3 **-1.85**	MYBL2 **-1.78**	NOS2 **1.15**	NOTCH1 **1.66**	NPY **3.92**	NUDT2 **-1.26**	PDIA2 **2.22**	PEA15 **4.68**	IPCEF1 **-3.8**
F	PLAU **93.75**	PLOD3 **-2.09**	PPARA **-2.96**	PPP2CB **3.13**	PRKAA1 **1.16**	PSMB3 **1.45**	PTX3 **3.06**	RARA **2.27**	RPL28 **-1.06**	RPL32 **1.5**	RPS2 **1.04**	RPS7 **1.27**
G	SAE1 **-1.08**	SLC2A1 **-2.68**	SLC2A4 **1.15**	SNRNP70 **-1.1**	SPTBN1 **-1.09**	SSSCA1 **1.57**	SUMO2 **1.12**	TH **1.15**	TST **-2.42**	TUBA4A **3.32**	UCP2 **-18.07**	VEGFA **8.44**

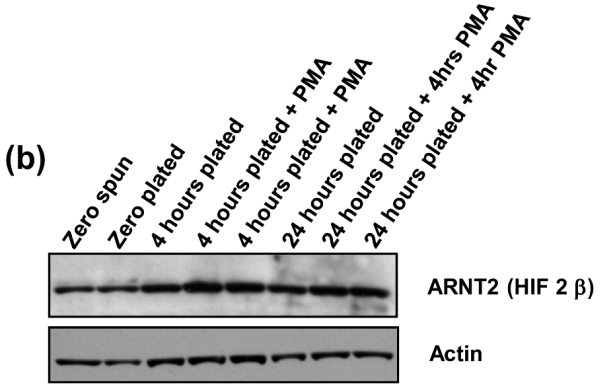

Fig. 4. HIF1α target genes are upregulated in response to PMA treatment in PBMCs.
(**a**) PBMCs were isolated from healthy donors and activated by plating on plastic with PMA treatment for 4hrs (4hrs plated + PMA) or harvested at zero time point after plating on plastic (zero plate). Total RNA was isolated, cDNA prepared and used as a template for a PCR array with primers targeted to a panel of hypoxia inducible genes. Results are presented in grid format. Highlighted genes were verified using independent qRT-PCR analysis. EPAS1=HIF2α.
(**b**) PBMCs were isolated from a healthy donor and activated by plating on plastic or combined PMA treatment for 4hrs and harvested 4hrs or 24hrs post treatment or at zero with and without the effects of plating. Whole-cell lysates were prepared and subject to immunoblot analysis with anti-ARNT2 (HIF2β) antibodies and anti-actin antibodies.

individuals; with PBMCs isolated from some donors showing no response to activation by plating or PMA (Suppl. Fig. 1).
The abundance of each HIF1α isoform mRNA was also measured relative to total HIF1α, by comparing against total HIF1a for each cell culture condition (Fig. 2c). The abundance of the two HIF1α paralogs, HIF2α and HIF3α were also measured in PBMCs isolated from the same nine healthy donors. HIF2α expression showed an approximately 5 fold increase in response to plating alone but in contrast to HIF1α showed an additional 10 to 20-fold increase in response to PMA treatment (Fig. 2d). HIF3α mRNA on the other hand was undetectable in PBMCs under all experimental conditions.

HIF1α protein isoforms are stabilised by PBMC activation even in normoxia
HIF1α Δ11, HIF1α Δ12 and HIF1α Δ11&12 lack part of the ODD domain required to interact with VHL and so may evade degradation by the proteasome. We isolated PBMCs from additional healthy donors and measured HIF1α protein by immunoblot using anti-HIF1α antibodies (Fig. 3a). Appearance of a ~85kD HIF1α immunoreactive protein was observed in response to PMA treatment. This was confirmed as HIF1α protein by immunoblot with a second anti-HIF1α antibody, raised to a different epitope (H1α67, Calbiochem). As full length HIF1α has a predicted molecular weight of 93kDa this anti-HIF1α immunoreactive protein may either represent full-length HIF1α, or may result from stabilisation of one of the splice variants.
To aid identification of the HIF protein species candidate HIF splice isoforms were expressed either in HEK cells, or *in-vitro*, using a reticulocyte lysate system. Expression of HIF1Δ12 resulted in a much smaller protein, and so the 85kD band observed in PBMCs could not result from a transcript lacking exon 12. However, expression of HIF1α Δ11 resulted in a protein of similar size, but this was consistently seen to migrate more slowly through the gel, suggesting either that it was too large to explain the 85kD protein seen in PBMCs, or that the protein was subject to post-translational modification. HIF1Δ14 generated an immunoreactive band of identical size to that seen in PBMCs. When overexpressed in HEK cells the protein migrated as a broad band, compared to the sharp, narrow band seen in the *in vitro* translated lane. This may reflect post-translational modification in the HEK cells. As an additional control HEK cells were treated with phorbol ester alone, which resulted in a faint band on the gel, showing slower migration than either the HIFΔ14, or the immunoreactive band seen in PBMCs (Fig. 3b). Attempts to silence HIF gene expression by using viral delivery of shRNA constructs in PBMCs were unsuccessful, due to a combination of difficulty infecting the cells, and also the very rapid induction of protein expression (4 hours culture post-harvest).
HIF2α protein was present under basal conditions and, in contrast to HIF1α,

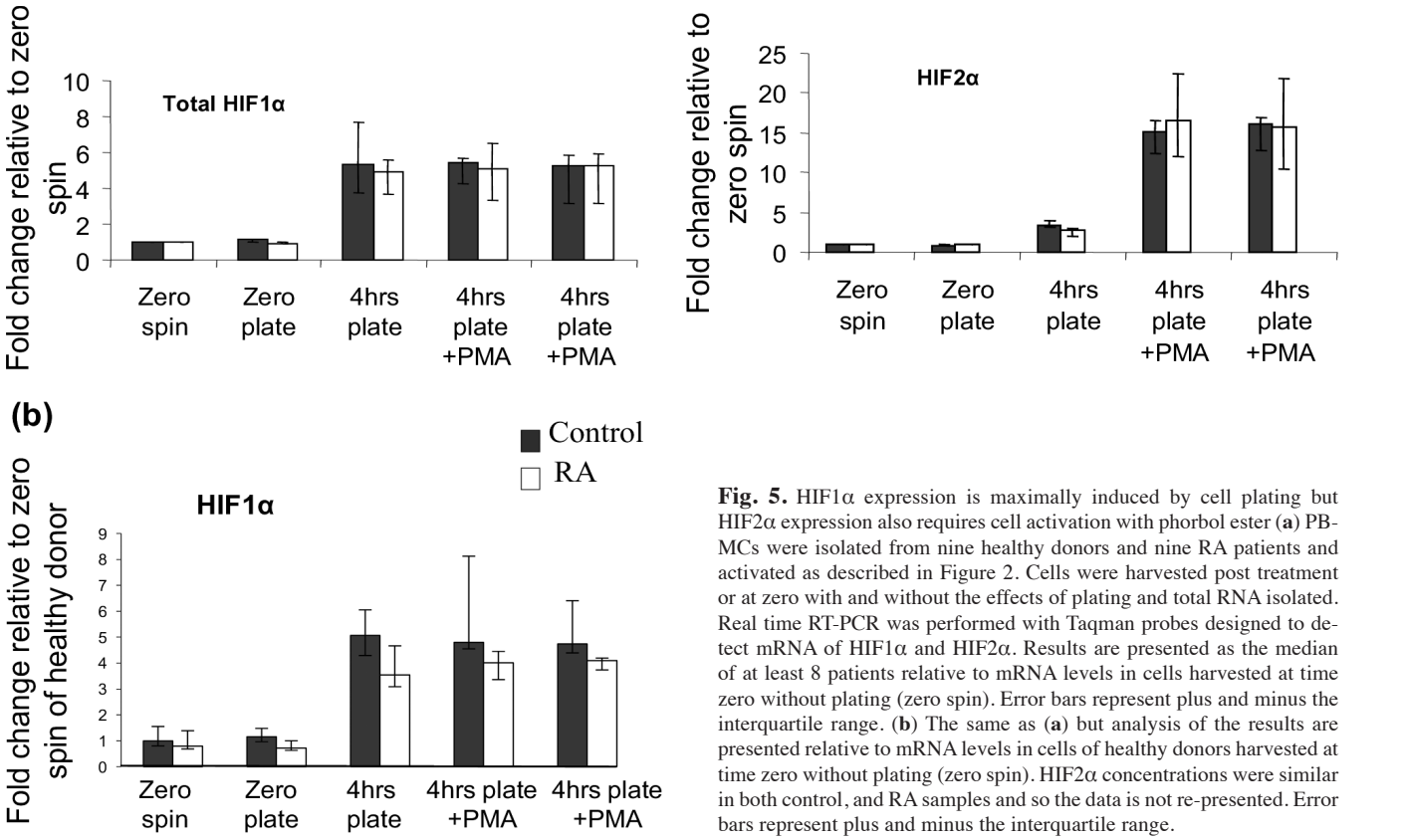

Fig. 5. HIF1α expression is maximally induced by cell plating but HIF2α expression also requires cell activation with phorbol ester (**a**) PBMCs were isolated from nine healthy donors and nine RA patients and activated as described in Figure 2. Cells were harvested post treatment or at zero with and without the effects of plating and total RNA isolated. Real time RT-PCR was performed with Taqman probes designed to detect mRNA of HIF1α and HIF2α. Results are presented as the median of at least 8 patients relative to mRNA levels in cells harvested at time zero without plating (zero spin). Error bars represent plus and minus the interquartile range. (**b**) The same as (**a**) but analysis of the results are presented relative to mRNA levels in cells of healthy donors harvested at time zero without plating (zero spin). HIF2α concentrations were similar in both control, and RA samples and so the data is not re-presented. Error bars represent plus and minus the interquartile range.

there is no increase in HIF2α protein in response to PBMC activation (Fig. 3c).

HIF1α splice variants do not differ significantly between RA cases and controls

In chronic inflammatory diseases such as RA disease-specific changes in the signalling pathways may lead to a disease-specific cellular phenotype. Therefore, the expression, splicing, and regulation of HIF1α was compared between patients with active RA, and healthy controls. There were no significant differences in the regulation of HIF1α (Fig. 5a), the various HIF1α splice variants (Suppl. Fig. 2a), or HIF2α in patients with RA (Fig. 5a), or the absolute concentrations of the various transcripts (Fig. 5b and Suppl. Fig. 2b).

Basal expression of the HIF1α target gene adrenomedullin is increased in RA

Although the expression of HIF1α transcripts did not differ between RA and control PBMCs we sought differences in the expression, or regulation of characterised HIF1α target genes, which may reflect differences in protein stability too subtle to detect by immunoblot. Initially, we used SupraArray screening to identify HIF1 α target genes in PBMCs from a healthy donor (see Supplementary information: Methods, Results and Fig. 4). From this analysis we selected a core set of target genes for to examine in the RA cases compared to controls. We observed differences in the expression of the HIF1α target gene adrenomedullin (ADM) in cells isolated from RA patients compared with cells isolated from healthy donors but not in the other HIF1α target genes measured (Fig. 6a and Suppl. Fig. 3).

In healthy donors ADM mRNA levels increase in response to activation by plating with a further increase in response to combined plating and PMA treatment, relative to un-stimulated cells (zero spun) (Fig. 6). However, the increase in ADM mRNA in response to plating and PMA treatment in cells from RA patients was significantly reduced compared with controls. Absolute ADM mRNA concentrations levels are higher in PBMCs isolated from RA patients compared with control cells but after stimulation these differences disappear (Fig. 6b).

As the differences in ADM expression were not accompanied by differences in HIF1α transcript expression, or by changes in the other HIF, and inflammatory target genes measured we attempted to discover which pathway was responsible. A series of kinase inhibitor studies revealed that p38 kinase inhibition significantly inhibited ADM induction seen in response to cell plating, whereas inhibition of ERK, PI3kinase, JNK, or IKK2 had no effect (Fig. 6c).

Discussion

In the current study we have comprehensively examined the HIF signalling cascade in circulating myeloid cells in both healthy volunteers, and those with active RA. These studies show expression of multiple HIF isoforms, activated by both foreign surface contact, and maximal cell activation with phorbol ester treatment. Importantly, there were differences in the response to cellular activation, with HIF1α isoforms in general showing near maxi-

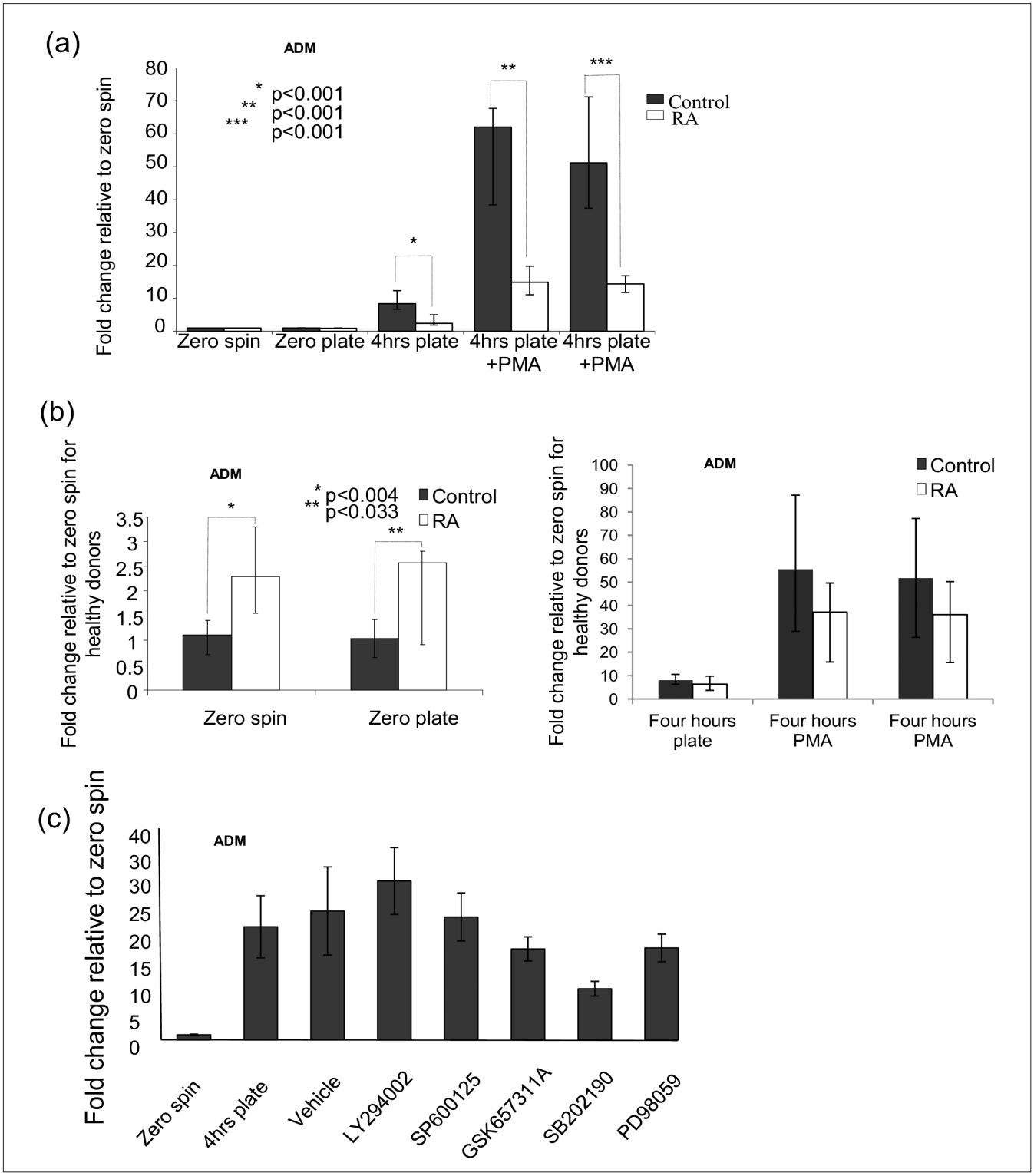

Fig. 6. Adrenomedullin expression is dramatically different in RA patients relative to controls and is partly p38 dependent (**a**) PBMCs were isolated from 15 healthy donors and 14 RA patients (DAS>4) and activated with PMA treatment and /or plating, cells harvested as indicated and total RNA isolated. Real time RT PCR was performed with Taqman probes designed to detect ADM. Results are presented as the median relative to mRNA levels in cells harvested at zero without plating (zero spin). Error bars represent plus and minus the interquartile range. (**b**) The same as (**a**) but analysis of the ADM Taqman results are presented relative to the ADM mRNA level in cells harvested at zero in healthy donors. Error bars represent plus and minus the interquartile range. (**c**) Adrenomedullin expression in activated PBMCs is partly P38 dependent. PBMCs were isolated from healthy donors and incubated with the indicated kinase inhibitors: PI3 kinase inhibitor (LY294002), ERK1/2 inhibitor (PD98059), p38 inhibitor (SB202190), JNK inhibitor (SP600125) or IKK2 inhibitor (GSK657311A) with plating for 4 hours. Cells were harvested and total RNA isolated. Real time RT PCR was performed with Taqman probes designed to detect ADM mRNA. Results are presented as the average of 3 experiments expressed relative to mRNA levels in cells harvested at zero without plating (zero spin). Error bars represent SEM.

mal induction just with foreign surface contact, whereas HIF2α required additional activation with phorbol ester. We detected the expression of multiple HIF1α splice variants and their molecular identity was confirmed by sequencing. HIF1αTAG and HIF1αΔ11 were the most abundant splice variants at mRNA level. No differences in HIF1α isoform expression was seen in the active RA cases compared to the healthy controls.

To investigate if the induction of HIF1α transcripts by activation of PBMCs resulted in a detectable increase in HIF1α protein cells were prepared as above, and lysates analysed by immunoblot using two different antibodies. Both antibodies detected a massive induction of protein expression, but only in cells subject to both plating and phorbol ester activation. Both antibodies detected a single, but broad, immunoreactive band at approximately 85kD. This protein is slightly smaller than that predicted to result from translation of full-length HIF1α, but no larger bands were observed. Attempts to purify and sequence the protein were unsuccessful, but the predicted migration of HIF1αΔ11, and HIF1αΔ14 are just below 100kD, and in transient transfection studies over expressed HIF1αΔ11 migrated at a similar position on the gel, with the migration of HIF1αΔ14 showing near identical migration. Therefore, we propose that the primary protein product of the HIF1α gene in activated PBMCs is either the HIF1αΔ11 isoform, or, the HIF1αΔ14 isoform, although a contribution by other splice variants with disrupted VHL binding domains is also possible (13, 19, 25). HIF1α protein stabilisation required additional cellular activation, mimicked here by phorbol ester. Loss of exon 14 introduces a frame shift to the cDNA, and so a premature stop codon. This splice variant does not have a disrupted oxygen dependent degradation domain and so the stability of its protein product remains unclear (19). To be certain of the molecular identity of the induced HIF1α protein in human PBMCs would require protein purification and sequencing, or the application of isoform specific antibodies.

We next analysed a limited panel of genes, which we had identified as HIF1α targets in PBMCs from a healthy individuals (Suppl. Fig. 3), to test differential activity of the HIF1α signalling cascade in RA, by comparison with cells from healthy donors. When the resulting data was analysed by fold change over the zero plated cells from each donor we observed a striking difference in the expression profile of adrenomedullin. Whereas the expression, and regulation of hexokinase, interleukin 6, VEGF, and interleukin 1α did not differ significantly between patients and controls there was an apparent failure of adrenomedullin induction in patients with RA. This may have resulted from differences in the basal expression of adrenomedullin, so resulting in reduced capacity for further induction. Therefore, the data was also analysed by normalising all qRT-PCR values against the zero-spin of the control cells. This analysis revealed that adrenomedullin expression was significantly higher in freshly isolated PBMCs from RA patients, with a resulting impaired induction resulting in similar concentrations of transcript after cellular activation between the two groups. This induction of adrenomedullin expression cannot be explained by altered HIF1α expression, nor were there concordant differences seen in basal expression of the other HIF target genes. Adrenomedullin is subject to regulation by multiple signalling pathways in addition to being a target for HIF1α, and the clear conclusion from our studies is that differential activity of one of these is responsible for the basal induction of adrenomedullin expression. Such potential adrenomedullin inducing factors include retinoic acid and low density lipoprotein (26).

The dramatic augmentation in adrenomedullin gene expression seen on plating suggests activation of a specific signalling kinase. To determine the identity of this pathway a series of inhibitor studies were undertaken. The p38SAPK inhibitor nearly abolished the observed induction, confirming a key role in PBMC adrenomedullin regulation. Previous studies have suggested a role for ERK, and NFκB in adrenomedullin expression, but reliance on transformed cell lines may explain the differences observed between our analysis, and the earlier reports (25). Our data suggest that there is a primary defect in adrenomedullin gene regulation that is not a direct consequence of HIF1 action.

In addition to its potent role in angiogenesis (27) adrenomedullin acts as an endogenous immunomodulatory factor, with pre-dominant anti-inflammatory effects. It decreases IL1-β induced rheumatoid arthritis synovial fibroblast (RASF) proliferation, MMPs, COX-2 and PGE2 expression (28). Adrenomedullin significantly reduces the incidence and severity of experimental models of inflammatory arthritis via a reduction in Th1-driven inflammatory responses and induction of $CD4^+CD25^+$ regulatory T cells (29, 30).

In summary, we describe expression of HIF1α and HIF1α splice variants in primary human PBMCs. These did not vary between active RA patients compared with controls. However, additional analysis revealed specific dysregulation of adrenomedullin expression in PBMCs from patients with RA, which could not be explained by changes in HIFα, MAP kinase, or NFκB signalling. This dysregulated expression of a potent anti-inflammatory and angiogenic modulator may contribute to the development of synovial hyperplasia and to the chronicity of inflammation characteristic of RA.

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