Role of AKT-glycogen synthase kinase axis in monocyte activation in human beings with and without type 2 diabetes

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Abstract

Monocyte activation by chemokines is a vital trigger for initiation of atherosclerotic process. Circulating levels of platelet activating factor (PAF), a recognized chemokine, is known to be increased in type 2 diabetes that is linked to accelerated atherosclerosis. To explore the molecular basis we examined the signalling pathways involved in PAF induced monocyte activation. PAF increased migration in monocytes obtained from THP-1 cells, nondiabetic and diabetic subjects. This effect was blocked by AKT inhibition. It did so by phosphorylation of glycogen synthase kinase (GSK)-3βS^9, which was completely blocked by AKT inhibition. Additionally, PAF induced GSK-3β phosphorylation was linked to Rac-1 activation and Rho-A inactivation leading to migration. Paradoxically, inhibition of GSK-3β phosphorylation also augmented monocyte migration in THP-1, ND and diabetic monocytes through phosphorylation of AKT and activation of Rho-A that was independent of GSK. This was validated when (*i*) overexpression of dominant negative mutants of Rho-A reversed GSK inhibitor induced monocyte migration and (*ii*) AKT inhibition blocked GSK inhibitor induced Rho-A activity. Constitutively active ARAP3 (Rho-GAP) appears to have a regulatory role in monocyte activity during GSK inhibition. Finally, inhibition of monocyte GSK-3β activity (by inhibitors and genetic manipulation) led to enhanced migration in diabetes compared to persons without diabetes. We conclude that diabetic monocytes show increased migratory capacity in response to GSK-3β inhibition. GSK inhibitors developed to treat the metabolic complications of diabetes should therefore be used with caution.

Keywords: monocyte ● diabetes ● glycogen synthase kinase

Introduction

In atherosclerosis, chemoattractant activated monocytes, monocyte-derived macrophages and macrophage-derived foam cells contribute to the formation of atherosclerotic plaques. Furthermore, monocytes, once activated, can produce a variety of mediators such as growth factors and angiogenic factors. Diabetes mellitus is a coronary risk equivalent and is linked to accelerated atherosclerosis [1]. The role of these monocytes are highlighted by the finding that human diabetic atherosclerosis is characterized by increased inflammation [2]. Thus far, the search for circulating factors that are likely causal for the accelerated cardiovascular disease in type 2 diabetes (DM) has remained elusive.

Amongst a variety of chemoattractants, recent evidence [3–5] supports the involvement of platelet activating factor (PAF) during all stages of atherosclerosis. Plasma and urinary PAF levels have been shown to be elevated in diabetes [6, 7]. Cellular components of the atherosclerotic process synthesize and respond to PAF and atherosclerotic plaques have been shown to be rich in PAF and PAF-like lipids [3, 8].

Glycogen synthase kinase-3β (GSK-3β) is a crucial signalling molecule involved in glycogen synthesis, cell migration, cell cycle and cellular architectural pathways [9, 10]. Dysregulated GSK-3β signalling is associated with disease states that include DM, Alzheimer's disease and bipolar disorder making it an apt tool for therapeutic intervention [11]. In hepatocytes, insulin signalling results in phosphorylation and consequent inhibition of GSK-3β leading to increased glucose uptake, glycogen synthesis and reduced hepatic glucose production. GSK-3β inhibitors have been shown to reverse abnormalities in hepatic glucose turnover and thus lower plasma glucose levels in several model systems of DM [12–14]. However, the role, if any, of GSK-3β in monocyte migration is currently unknown.

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We suggested, based on preliminary analyses of differential expression of phospho-proteins in monocyte extracts, that GSK-3β plays a prominent role in PAF induced monocyte signalling in human beings and that this effect was enhanced in those with DM. This was especially important because monocyte is the primary organelle that initiates the atherosclerotic process. We provide evidence that inhibition of GSK-3β signalling augments monocyte activity (adhesion, migration and transmigration). Hence GSK inhibitors developed to improve the metabolic abnormalities of DM, or dementia of Alzheimer's or cancer chemotherapy, will need to be used with caution. To establish and understand the role of GSK-3β in monocyte activity, we chose initially to characterize the functionality of these cells in THP-1 cell lines and in individuals without clinical evidence of overt macrovascular disease.

Table 1 Participant characteristics

	ND	DM
	(*n* = 35)	(*n* = 25)
Age (years)	48.1 ± 0.8	51.6 ± 1.2
Sex	15 ♂ 20♀	10 ♂ 15♀
BMI (kg/m^2)	27.1 ± 1.0	28.0 ± 0.6
FPG (mM)	5.0 ± 0.1	8.4 ± 0.3*
HbA1c (%)	5.4 ± 0.1	8.1 ± 0.3*

*$P < 0.05$ *versus* ND; ND: non-diabetic; DM: type 2 diabetes; BMI: body mass index; FPG: fasting plasma glucose.

Experimental procedures

Patient characteristics

Patient characteristics from whom monocytes were isolated are provided in Table 1. Those with DM were either on diet therapy (*n* = 10), metformin alone (*n* = 10) or metformin and sulphonylurea (*n* = 5). None of the individuals were on insulin or thiazolidinediones. Individuals were asked to hold all their medications on the morning of the blood draw.

Human monocyte separation

In compliance with Mayo Institutional Review Board and following informed consent, buffy coat was collected from 100 ml of blood from each participant by graded centrifugation and thoroughly mixed up with HBSS (without Ca^{++} or Mg^{++}) buffer (Mediatech, Inc., Herndon, VA, USA). Then 30 ml HBSS mixed buffy coat was layered upon 15 ml Ficoll-Paque PLUS (Amersham Biosciences, Pittsburgh, PA, USA) in 50 ml tubes, and centrifuged at 1250–1350 rpm for 30 min. (brake in 'off' position). The interface was then remixed with HBSS, re-centrifuged for 10 min. and red blood cell contamination was removed by ACK lysis buffer. The cell pellets were resuspended in RPMI containing 0.1% FBS and the cells counted and passed through the appropriate MACS column (Mini/Midi Macs: Miltenyi Biotec, Auburn, CA, USA) as per protocol. The column was washed thrice with buffer and cells collected immediately. CD14 Ab$^+$ microbeads were used to isolate the monocytes. However, activation of the monocytes does not occur [15] because antibody binding to CD14 does not trigger signal transduction as CD14 lacks a cytoplasmic domain. All experiments on THP-1 and human monocytes were performed in triplicate unless otherwise stated.

Cell culture

THP-1
THP-1 cell line comprises of a myelomonocytic lineage that is widely used to study monocyte signalling in cell culture systems. Cells were obtained from American Type Culture Collection (ATCC) and cultured in RPMI 1640 (Mediatech, Inc.) medium with 2 mM L-Glutamine, 10 mM HEPES and 1.0 mM sodium pyruvate and supplemented with 0.05 mM 2-mercaptoethanol 90% and foetal bovine serum 10%. Cell viability was assessed by trypan blue exclusion in every experiment.

Migration assay using the Boyden chamber

Human monocytes and THP-1 cells were kept in starved media for 2 hrs and overnight, respectively. Staining was done with Calcein-AM (25 μg in 5 μl DMSO/ml media) (Molecular Probes, Carlsbad, CA, USA) at 37°C for 15 min. and washed twice with PBS to remove excess Calcein-AM. Specific inhibitors (AKT–IV and GSK-VIII) (CALBIOCHEM, San Diego, CA, USA) or genetic manipulated cells were added separately and incubated for 0.5 hrs. Cells were then seeded on 5 × 10^5 cells/transwell (FALCON, San Jose, CA, USA) in 300 μl of media and inserted into a 24-well plate containing 750 μl of medium. 100 nM PAF (SIGMA, St Louis, MO, USA) was added and migrated cells were counted in a spectrofluorometer (Spectrafluor, TECAN, San Jose, CA, USA) with Delta Soft 3. Readings were taken at 1 and 2 hrs and normalized to baseline. Data are presented as mean ± S.D. of triplicate/quadruplicates.

Western blot analysis

Primed cells (with or without specific inhibitors for 0.5 hrs) were treated with PAF (100 nM) for 5 min. [16]. The cells were then lysed in radio immuno precipitation assay buffer and protein concentrations measured by BCA reagents. Protein extracts were loaded onto SDS polyacrylamide gels equally and electro-blotted on nitrocellulose (Bio-Rad, Hercules, CA, USA) membranes. After blocking for 1 hr with 5% non-fat dry milk in TBS-T (1% Tween-20), proteins were probed with appropriate antibody (anti-GSK-3βS[9]: Cell Signaling, Carlsbad, CA, USA; GSK-3β: BD Transduction, San Jose, CA, USA; anti-pAKT1/2/3 serine 473: Santa Cruz, Santa Cruz, CA, USA; anti-cdc42, anti-Rac and anti-Rho-A: Upstate, Danvers, MA, USA). After incubation with the primary antibody, the blot was washed 3 times in TBS-T, incubated for an hour with the specific secondary antibody, and developed with Super Signal West chemiluminescence (Pierce, Rockford, IL, USA). For immunoblot analysis, the x-ray bands were scanned and the pixel density measured by using a densitometer and NIH image analysis program.

Rac/cdc42 and Rho-A pull-down assay

Magnesium lysis buffer (MLB) was made by diluting 5× MLB (Upstate) to 1× MLB by adding sterile water containing 10% glycerol. To the 1× MLB

diluted buffer, 10 μg/ml of aprotinin and leupeptin were added. After rinsing with ice cold PBS, appropriate amount of 1× MLB added. The lysates were transferred to microfuge tubes. Protocols as described (Upstate) were followed for cdc42 and Rho-A pull down assay and immunoblotting was performed.

Transfection of dominant negative (DN) mutants in THP-1

The DN mutant of Rac-1 (Rac1–17N) and Rho-A (RhoA-19N) were kindly provided by Margaret M. Chou (University of Pennsylvania) and the DN mutant of AKT1 (K 179M mutation) from Upstate. To prepare retrovirus, 293T cells were seeded on 3×10^6 cells/100 mm plate, 24 hrs prior to transfection. DNA transfection was performed with the effectene[TM] transfection reagent (Qiagen, Valencia, CA, USA), with 2 μg of targeted gene (pMMPs Rac-1 and AKT), 1.5 μg of pMD, MLV gag.pol and 0.5 μg pMD, G (encoding the cDNAs of the proteins that are required for virus packing) [17, 18]. The retrovirus was collected after 48 hrs of transfection by filtering the media with 0.45 μ filter and used immediately or stored at −70°C [19]. For infecting, THP-1 cells were seeded in a mixture containing retrovirus solution in media at 1:1 ratio, together with 10 μg/ml polybrene. The cells were ready for experiment 48 hrs after infection.

Oligonucleotide transfection of human monocytes

GSK antisense (gaggTGGTTCTGGGCCGCCCTacat) and sense control (atgtCAGGGCGCCCAGAACCAcctc) phosphothiotate oligonucleotides were purchased from Mayo Clinic DNA core facility. ARAP3 SiRNA was bought from Santa Cruz Biotechnology. Gene knockdown was performed as described [20]. Freshly isolated human monocytes from blood or THP-1 cells were mixed with human monocyte nucleofector solution to a final concentration of 3×10^6 to 1×10^7 cells/ 100 μl. Highly purified GSK oligonucleotides or ARAP3 SiRNA were added to 100 μl cell suspension and transferred to amaxa certified cuvette which was then inserted into the amaxa cuvette holder. Nucleofection was done by using program Y-01 and cells were cultured in petridishes containing human monocyte nucleofector medium for up to 48 hrs.

Monocyte/THP-1 adhesion assay to HUVEC

The adhesion studies were performed under HUVEC static condition with treated human monocytes or THP-1 cells. Compact monolayer of HUVEC in 24-well plates was kept ready. Human monocytes and THP-1 cells were kept in starving media for 2 hrs and overnight, respectively, and cells stained as described previously. Primed monocytes or THP-1 cells were treated with 100 nM PAF for 5 min. Equal numbers ($4–8 \times 10^4$ cells in 200 μl starving media) of monocytes or THP-1 cells were added onto HUVEC monolayer and incubated at 37°C for 1 hr. The wells were gently washed three times with PBS. Adherent monocytes were measured in a spectrofluorometer (Spectrafluor, TECAN) with Delta Soft 3 software at an excitation wavelength of 485 nm and emission at 530 nm. Fluorescence labelled monocytes and THP-1 cells were photomicrograped by high power field microscope.

Trans-endothelial migration

Thick collagen was prepared as described [21]. HUVEC cells were seeded in 96-well microplate and grown in M199 supplemented media. Trans-endothelial migration of THP-1 cells was performed within a week after a compact HUVEC monolayer on the collagen base was established as described [22]. Media from the HUVEC monolayer was removed and THP-1 cell suspension (100 μl) was added. At 1.5 hrs media containing THP-1 cells were removed and washed with PBS prior to adding 100 μl RPMI media. Photomicrograph was performed by differential phase microscopy.

Kinomics analyses

To determine the post-translational phosphoprotein expression of monocytes before and after PAF exposure, we performed Kinex™ Antibody Microarray (Kinexus: Vancouver, BC, Canada) analysis on monocytes obtained from non-diabetic (ND) persons and DM patients as described before [23]. The Kinex™ Antibody Microarray tracks both total protein expression (with pan-specific antibodies) and phosphorylated protein expression (with phospho-site-specific antibodies). The changes in protein phosphorylation were normalized by total protein levels.

Statistical analysis

ANOVA was used to determine differences in rates of monocyte migration among THP-1, ND and DM monocytes. Effects of PAF and the effects of modulators on monocyte activation within each group were assessed by Student's t-test. A P-value of <0.05 was considered significant.

Results

Role of AKT on PAF induced monocyte activation

PAF effects on monocyte migration (Fig. 1A)
To examine the effects of PAF on monocyte migration, we performed migration assays on THP-1 cells and monocytes derived from ND individuals. We observed increased ($P < 0.01$) monocyte migration following exposure to PAF at 1 and 2 hrs in both THP-1 and ND monocytes when compared to the corresponding time-points in control experiment. PAF induced migration was higher ($P < 0.05$) in ND than THP-1 cells at both times. We did not observe any time-dependent changes in monocyte migration without PAF stimulation.

Monocyte migration through AKT pathway in PAF activated monocytes (Fig. 1B and C)
We found reversal of PAF induced migration after AKT-IV (AKT inhibitor) exposure in both THP-1 and ND monocytes (Fig. 1B).
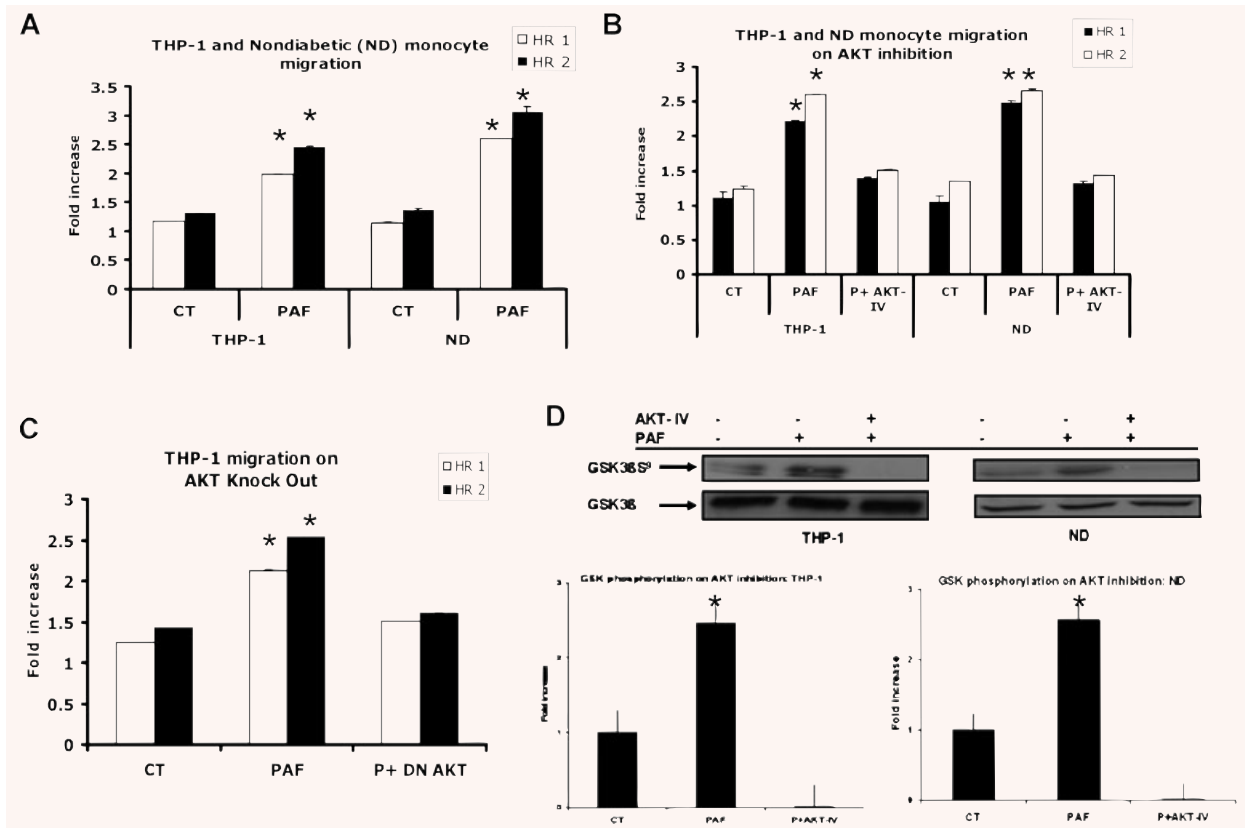
Fig. 1 (**A**) Shows fold changes in monocyte migration responses to PAF in THP-1 cells and ND monocytes at 1 hr (open box) and 2 hrs (closed box). *$P < 0.01$ *versus* corresponding times in control (CT). ($n = 6$ ND individuals). (**B**) Shows effects of AKT inhibition by AKT-IV on PAF induced monocyte migration on THP-1 and ND monocytes ($n = 6$ ND individuals). *$P < 0.01$ *versus* corresponding times in control (CT). (**C**) Shows effect of DN mutant of AKT on PAF activated THP-1 monocyte migration. *$P < 0.02$ *versus* controls and DN-AKT. (**D**) Shows immunoblot analysis of AKT-IV inhibition on PAF induced phosphorylation of GSK-3βS^9 in THP-1 cells and in ND individuals. The corresponding image analyses are also shown ($n = 6$ ND individuals) in the lower panels.

We then tested DN mutants of AKT in THP-1 cells and found that over expression of DN AKT mutant blocked monocyte migration on PAF stimulation (Fig. 1C) thus confirming the vital role of AKT on monocyte migration.

AKT-GSK axis involvement in PAF induced monocyte signalling (Fig. 1D)

To assess the signalling cascades downstream of AKT, *viz.* GSK-3β activity, we observed that phosphorylation of GSK-3β at serine 9 was up-regulated (2.3–2.5 fold) following PAF exposure in both THP-1 and ND monocytes (Fig. 1D). Following exposure to AKT-IV, GSK-3β phosphorylation was completely inhibited in both cells. These results show that PAF phosphorylates GSK-3β at serine 9 in monocytes confirming that AKT–GSK-3β axis is a critical intermediary pathway. The cells were viable after 48 hrs of PAF and AKT-IV exposures, as determined by cell morphology and trypan blue exclusion assays.

Role of GSK on PAF induced monocyte activation

GSK-3β inhibitor-VIII (GSK-VIII) increases PAF stimulated monocyte migration (Fig. 2A)

Because GSK inhibitors are currently being developed for treatment of hyperglycaemia in diabetes and for dementia of Alzheimer's, we sought to determine the role of GSK inhibition on monocyte activation. To do so, we initially utilized GSK inhibitor-VIII. We observed down-regulation of GSK-3 β phosphorylation by GSK-VIII in a dose-dependent manner (Fig. 2A, upper panel). However, to our surprise, GSK inhibition enhanced PAF induced migration at 1 hr in THP-1 and ND monocytes ($P < 0.01$) when compared to PAF alone. But, there was no additional enhancement in monocyte migration by GSK-3β inhibitor use at 2 hrs (Fig. 2A, lower panel). The paradoxical effect of GSK inhibition on PAF induced monocyte migration required further experiments to understand the mechanism by which this occurred.
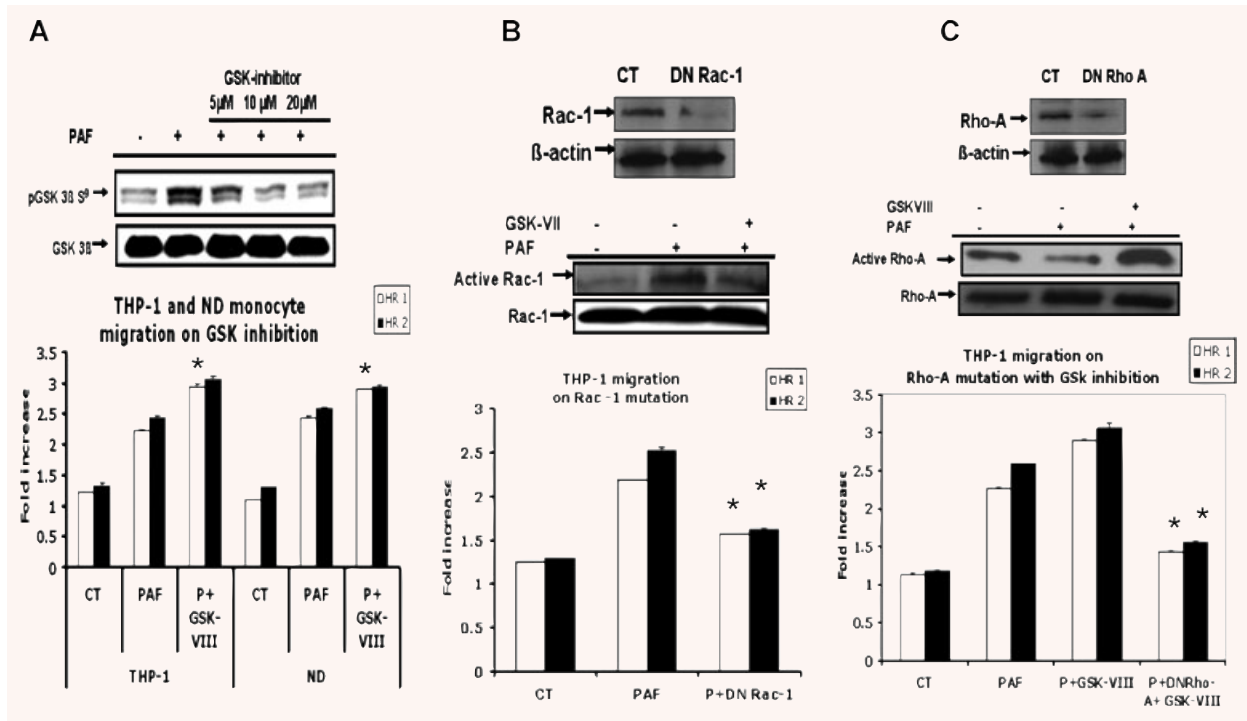
Fig. 2 (**A**) Shows dose-dependent effects of GSK-VIII on GSK serine 9 phosphorylation in THP-1 cells (upper panel). Shows effects of GSK inhibition by GSK-VIII on PAF activated monocyte migration in THP-1 and ND monocytes (lower panel). *$P < 0.01$ *versus* corresponding time in PAF treated cells. ($n = 6$ ND individuals). (**B**) Shows effect of GSK-VIII on PAF induced Rac-1 activation and monocyte migration in THP-1 cells with and without DN Rac-1 transfection. *$P < 0.01$ *versus* controls and DN Rac-1 mutant. (**C**) Shows effect of GSK-VIII on PAF induced Rho-A inactivation and monocyte migration in THP-1 cells with and without DN Rho-A transfection. *$P < 0.01$ *versus* DN Rho-A mutant.

Furthermore, because the signalling pathways appeared to be similar in both THP-1 and ND monocytes, we elected to perform some of the subsequent experiments in THP-1 cells. Several of the experiments described subsequently required large samples of monocyte and monocyte extracts that was not possible to obtain safely from individuals. Additionally, monocytes isolated from a single participant were not enough to conduct all of the experiments outlined below, hence restricting our sample sizes ($n = 6$) for some experiments. However, a few of the experiments were performed pooling monocytes obtained from batches of three ND or DM individuals at a time.

### Involvement of small GTPases on PAF activated monocyte migration (Fig. 2B and C)

We next examined activities of small GTPases involved in cytoskeletal functions *viz.* Rac-1, Rho-A and cdc42 on GSK inhibition. To do so, PAF stimulated THP-1 cells were examined pre- and post-GSK inhibition. PAF increased Rac-1 activity (Fig. 2B, upper panel) as previously reported for other chemokines [24] and this effect was reversed by GSK inhibition. To further validate the role of Rac-1 on PAF induced monocyte migration, we utilized DN mutants of Rac-1 (Fig. 2B, lower panel) and observed inhibition of

monocyte migration on PAF exposure. Conversely, Rho-A activity decreased on PAF treatment but reversed by GSK inhibition (Fig. 2C, upper panel). However, DN mutant over expression of Rho-A abolished GSK-VIII treated PAF induced monocyte migration in THP-1 cells (Fig. 2C, lower panel). No toxicity was observed 48 hrs after treatment with PAF and/or GSK-VIII. These data demonstrate that PAF activation on THP-1 cells up-regulates Rac-1 but down-regulates Rho-A. However, we failed to observe any changes in cdc42 activity with PAF (data not shown). These results suggest a prominent role of small GTPases on PAF induced monocyte migration.

Interplay of AKT-GSK axis on monocyte activation

GSK inhibition increases phosphorylation of AKT and migration in PAF stimulated monocytes (Fig. 3A–C)

We have shown that AKT inhibition decreases monocyte migration (Fig. 1B and C) and phosphorylation of GSK-3βS^9 (Fig. 1D). In contrast, inhibition of GSK phosphorylation by GSK-VIII paradoxically stimulates monocyte migration (Fig. 2A). To elucidate this further, we examined AKT phosphorylation of THP-1 monocytes after inhibition of GSK followed by PAF treatment. We demonstrate
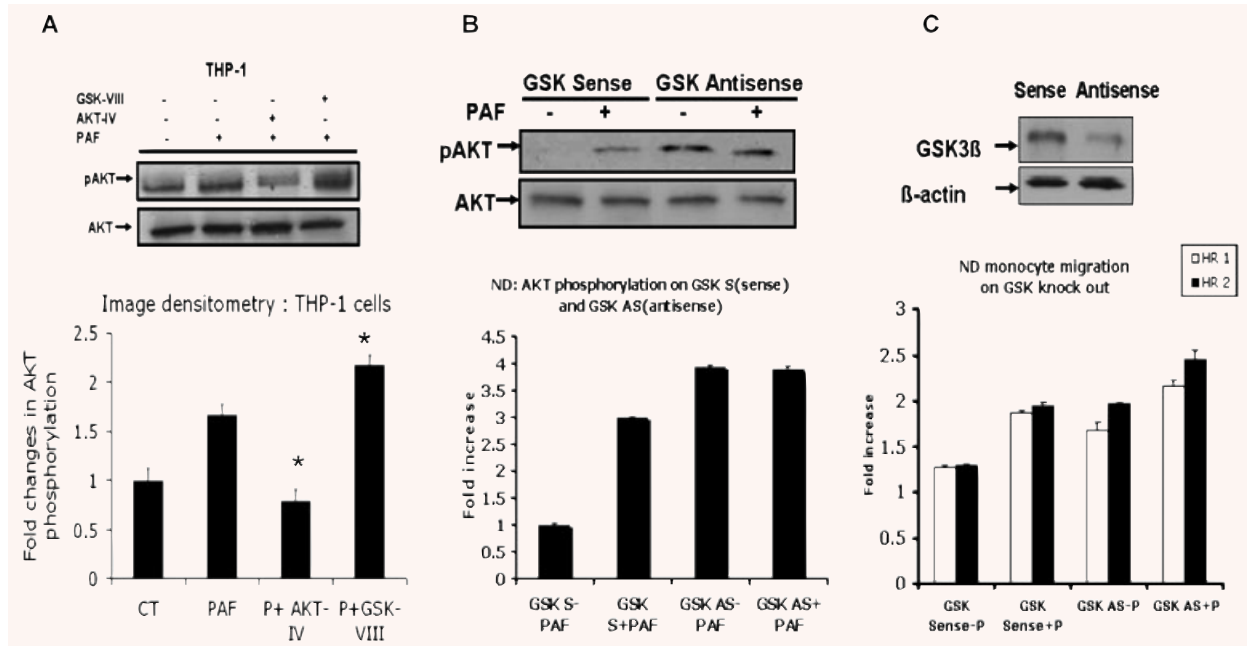
Fig. 3 (A) Shows effect of GSK-VIII and AKT-IV on PAF activated monocyte expressions for phospho-AKT in THP-1 cells. *$P < 0.01$ *versus* PAF stimulated AKT phosphorylation. (**B**) Shows effects of GSK sense and antisense on AKT phosphorylation with and without PAF in ND monocytes. (**C**) Shows effects of GSK sense and antisense on monocyte migration with and without PAF in ND monocytes at 1 (open bars) and 2 hrs (closed bars) (*n* = 6 ND individuals).

that GSK inhibition by GSK-VIII (Fig. 3A, upper panel) enhance AKT phosphorylation that was further quantified by image analyses (Fig. 3A, lower panel). A similar phenomenon was observed upon sense-antisense GSK oligonucleotide expression in ND monocytes following PAF stimulation (Fig. 3B). A likely explanation for this intriguing observation could be that GSK inhibition, somehow promotes cell signalling through phosphorylation of both AKT and active Rho-A (as validated in Fig. 2C), thereby augmenting migration. Additionally, we observed increased monocyte migration on GSK antisense transfection with and without PAF stimulation in ND monocytes (Fig. 3C). The mechanism by which this occurs requires further investigation.

Unraveling of GSK inhibitory pathway

Role of ARAP3 on Rho-A activity by GSK inhibition (Fig. 4A)
To determine the molecular signalling involved following GSK inhibition we tested Rho-A activity by ELISA after knocking down ARAP3 (Rho-GAP) in ND human monocyte. In the control SiRNA experiment we observed that PAF down-regulates Rho-A activity which was reversed upon GSK inhibition (as in Fig. 2C). In contrast, we observed enhanced Rho-A activity on ARAP3 knockout at baseline (*i.e.* without PAF), but following PAF exposure Rho-A activity was reduced that was partially reversed on GSK inhibition.

### Role of AKT on GSK inhibitor induced Rho-A activity (Fig. 4B)
We observed that AKT inhibition further down-regulates PAF treated Rho-A activity in ND human monocyte. However, despite GSK inhibition, there was no increase in Rho A activity in PAF treated monocytes that were pre-exposed to AKT inhibitor. This demonstrates the modulatory role of AKT on GSK-Rho A signalling pathway.

Role of GSK on PAF induced transendothelial migration of monocytes

Effect of GSK knockout on transendothelial monocyte migration (Fig. 5)
On testing the role of GSK on PAF induced transendothelial migration of monocytes obtained from THP-1 cells, we found enhanced transendothelial migration of GSK$^{-/-}$ monocytes at baseline (without PAF) that was further augmented following PAF exposure when compared to GSK$^{+/+}$ monocytes (Fig. 5, upper panel) and this was confirmed on quantification (Fig. 5, lower panel). These

Fig. 4 (**A**) Shows effects of ARAP3 knock-out on PAF and GSK-VIII induced Rho-A activity in THP-1 cells. (**B**) Shows effects of AKT inhibition on PAF (±GSK-VIII) induced Rho-A activity in THP-1 cells.
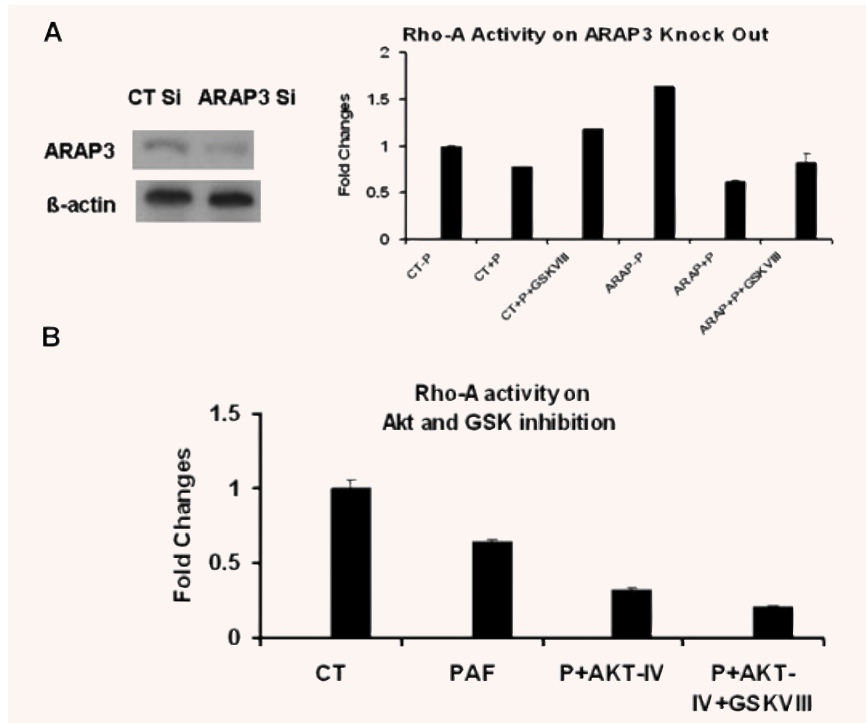
observations confirmed our earlier findings on monocyte migration assays (please see Figs 2C and 3C).

Interplay of AKT-GSK axis on monocyte activation in type 2 diabetes

Determination of differential phosphoprotein expression in PAF activated monocyte (Fig. 6A)

In order to identify and compare changes in functional phospho-protein expression in monocytes obtained from both DM and ND individuals, we performed kinomics analyses on monocyte extracts (±PAF). We observed increased expression of several post-translationally modified phosphoproteins after PAF activation in both ND and DM monocytes. Whereas PAF activation increased GSK-3α/β Ser9/21 phosphorylation moderately in ND (2.4 fold; data not shown), it dramatically increased (8.6 fold) GSK-3α/β Ser9/21 phosphorylation in DM individuals. Based on this result, we decided to focus our attention on better defining the role of GSK on PAF activated monocyte signalling and functions in DM individuals. This was especially so because GSK has been known to play a role in cell motility besides possessing a regulatory role in hepatic glucose metabolism in DM [13, 25].

AKT-GSK crosstalk in PAF induced DM monocyte signalling (Fig. 6B)

DM monocytes show increased GSK phosphorylation on PAF stimulation thus confirming the kinomics data. This effect was totally abolished by AKT inhibition and also down-regulated by GSK inhibition. Image densitometry analysis is confirmatory.

Effect of GSK inhibition on PAF induced diabetic monocyte migration (Fig. 6C)

We found enhanced monocyte migration following GSK inhibition in DM individuals at both 1 and 2 hrs when compared to ND monocytes.

Effect of GSK antisense on AKT phosphorylation and monocyte activation in DM monocytes (Fig. 6D)

As observed in THP-1 and ND monocytes, we found augmented AKT phosphorylation and migration in DM monocytes following transfection with GSK antisense oligonucleotide both with and without PAF exposure.

Discussion

Our results clearly reveal that GSK-3β inhibition in human monocytes enhances their ability to migrate and transmigrate across endothelial surfaces thereby increasing potential for atherogenesis. This effect appears to be augmented in monocytes obtained from individuals with DM. Hence, GSK inhibitors currently being developed for treating hyperglycaemia of diabetes
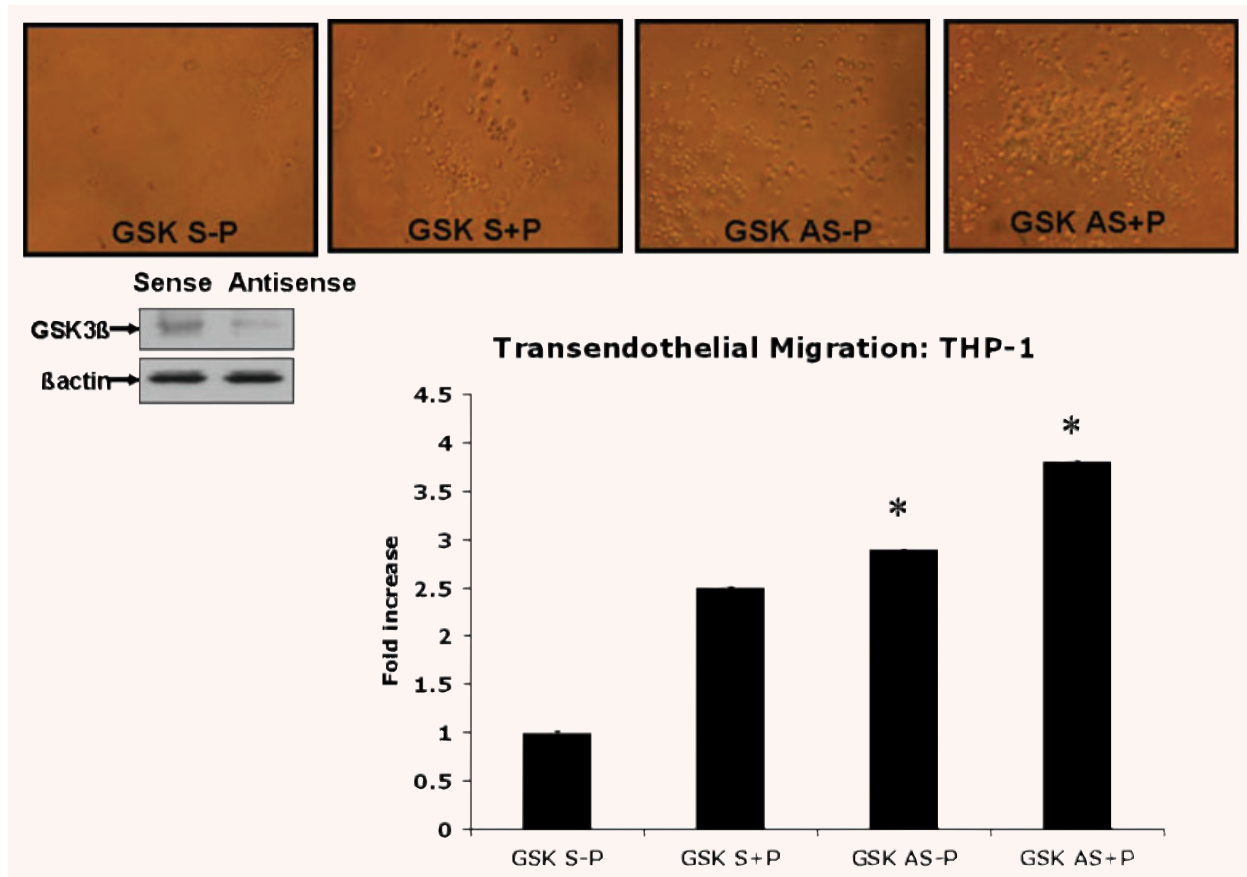
Fig. 5 Shows trans-endothelial migration of THP-1 cells with and without GSK antisense and with and without PAF. *$P < 0.01$ *versus* corresponding effects with and without PAF, respectively.

or dementia of Alzheimer's will need to be used with caution and deserve further study.

Transendothelial monocyte migration is a vital initial step towards the development of atherosclerosis. This leads to differentiation of these monocytes into macrophages, formation of foam cells and release of inflammatory cytokines causing disruption of vascular integrity and plaque formation [26]. The current data highlight the cross-talk between crucial signalling molecules, *viz.* AKT and GSK-3β on monocyte migration in individuals with and without DM. We have demonstrated that (*i*) PAF stimulated monocyte activity is mediated through AKT-GSK axis; (*ii*) PAF stimulates monocyte migration and transendothelial migration through enhancement of downstream Rac-1 activity and inhibition of Rho-A activity; (*iii*) inhibition of GSK-3β phosphorylation leads to paradoxical stimulation of AKT phosphorylation that in turn stimulates monocyte migration *via* a GSK independent pathway; (*iv*) DM enhances these processes causing augmented monocyte migration on inhibition of GSK-3β phosphorylation. Figure 7 shows putative signaling pathway. Furthermore, we did not rely

solely on AKT and GSK inhibitors but also confirmed the findings through use of genetic manipulations of these kinases and small GTPases on monocytes.

PAF and atherogenesis are closely linked. Elevated levels of plasma and urinary PAF can be found in several disease states known to be associated with atherosclerosis including diabetes [3, 7]. A majority of cells involved in atherosclerotic plaque formation (*e.g.* endothelial cells, monocytes) can synthesize and respond to PAF. We therefore decided to examine the downstream signalling pathways involved in monocyte migration, especially GSK, following PAF stimulation.

GSK3 is a serine–threonine kinase. Several kinases including AKT, can phosphorylate GSK. It is noteworthy that each kinase likely affects only a specific pool of the GSK-3 protein depending on the subcellular distribution and cell specificity. Furthermore, serine phosphorylation inhibits GSK-3 activity [12]. Insulin is the classic ligand that inhibits GSK-3 through activation of AKT [27], stimulating glycogen synthesis in liver and muscle cells, enhancing glucose uptake and lowering hepatic glucose production,
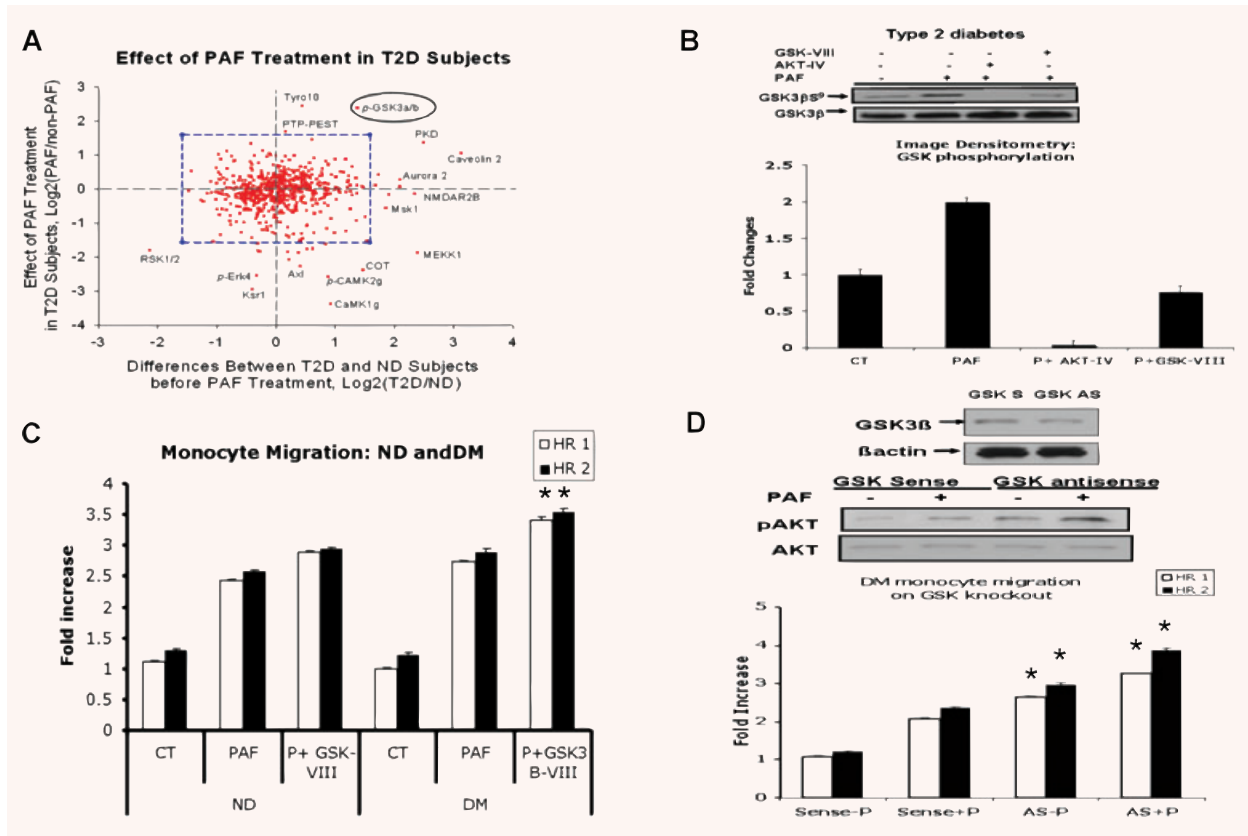
Fig. 6 (**A**) Shows differential phosphoprotein expression of kinases in monocyte extracts after PAF exposure in DM compared to ND individuals. Box indicates limit of 3-fold changes. Top right and bottom left quadrants highlight kinases of interest that are either up- or down-regulated following PAF activation in DM compared to ND individuals. (n = 2 ND and DM individuals). (**B**) Shows effects of GSK-VIII and AKT-IV on GSK serine 9 phosphory-lation in DM monocytes (n = 6 DM individuals). (**C**) Shows PAF($\pm$GSK-VIII) effects on monocyte migration in ND and DM monocytes (n = 6 each of ND and DM individuals). *$P < 0.01$ *versus* corresponding time-points in ND individuals with PAF + GSK-VIII. (**D**) Shows PAF induced migration of DM monocytes with and without GSK antisense. *$P < 0.01$ *versus* corresponding effects with and without PAF, respectively.

hence fasting hyperglycaemia. GSK-3 is also involved in maintenance of cellular motility in neuronal tissues, epithelial cells and ker-atinocytes [11]. However, to our knowledge, there have been no prior studies investigating the role of GSK-3 on monocyte activity and migration.

Our data reveal that PAF stimulation results in migration of THP-1, ND and DM monocytes through phosphorylation of AKT and GSK-3β leading to Rac-1 activation. These finding seems to be in contrast to a recent report [28] in THP-1 macrophages that showed no change in Rac-1 activity on PAF stimulation but is in concordance to a prior report that showed stimulation of Rac-1 upon PAF exposure in neutrophils [29]. These conflicting reports may represent cell specific responses to PAF.

It is noteworthy that our data suggest an intriguing interaction between GSK and AKT in that GSK-3β inhibitor further stimulated PAF induced migration in THP-1, ND and DM monocytes by reduc-ing Rac-1 and enhancing Rho-A activities. These effects were con-

firmed by genetic mutations of the GTPases. Interestingly, we observed that AKT phosphorylation was significantly elevated on GSK inhibition. The molecular mechanism by which this occurs needs to be evaluated. Additionally, it appears paradoxical that both phosphorylation and de-phosphorylation of GSK augments monocyte migration. Precedence of similar phenomenon has been described in src signalling [30] pathways.

The Rho subfamily belongs to the larger Ras family of small GTPases that play an important role in cell morphology, motility and migration [31]. Regulation of Rho activity is governed by G-protein coupled receptors that include PAF-Receptor. Intermediary molecules that also play a role in regulation of Rho activity include GTPase activating proteins (GAP) and Rho-spe-cific guanine nucleotide exchange factors [32]. The questions however remain as to the mechanism by which GSK influences the activities of the small GTPases [33]. As Rho-A is a small GTPase whereas AKT is a kinase, it is very unlikely that AKT directly
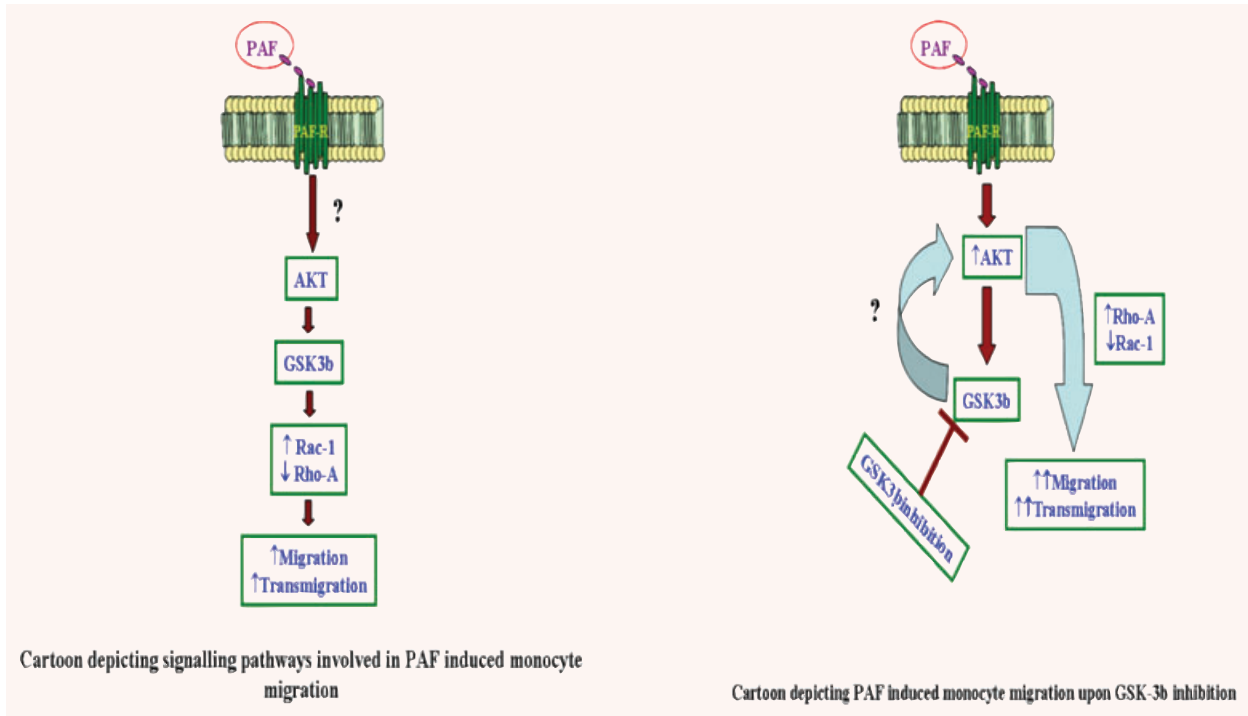
Cartoon depicting signalling pathways involved in PAF induced monocyte migration

Cartoon depicting PAF induced monocyte migration upon GSK-3b inhibition

Fig. 7 Shows cartoon of putative signalling pathways on PAF induced monocyte activation of the left panel and in the presence of GSK inhibition on the right panel.

activates Rho-A. This was confirmed when we failed to detect any direct association between phospho-AKT and active Rho-A on immunoprecipitation.

ARAP3 is PI3K dependent GAP protein, abundant in leucocytes, that couples with PI3K and thus regulates Rho-A activity [34]. As we have shown, PAF stimulation up-regulates AKT/GSK phosphorylation (Figs 1D, 3B, 6B and D). Because both AKT and ARAP3 are substrates of PI3K, it is conceivable that active ARAP3 GAP regulates Rho-A activity. As shown in Fig. 4(A), PAF down-regulates Rho-A activity that is reversed by GSK inhibition and this effect is significantly dampened on ARAP3 knockout in monocytes thereby linking ARAP 3 as a modulatory molecule in monocyte activation. We also observed Rho-A up-regulation (Fig. 4A) upon ARAP3 knock down at baseline (*i.e.* without PAF) thus implying a constitutively active ARAP3 in monocytes. As anticipated, AKT inhibition overrides all PAF and GSK effects on Rho-A activity (Fig. 4B) and consequent migration (Fig. 1B and C).

AKT, a serine–threonine kinase, plays a diverse role in cell migration. Current data suggest a prominent role of AKT on PAF induced monocyte migration through activation of small GTPases akin to a recent report [35]. However, AKT has potentially complex and mutually antagonistic effects on angiogenesis and atherogenesis [36]. It is therefore critical that tissue specific and function specific substrates of AKT are targeted for therapeutic interven-

tions and not AKT itself because that could potentially affect a multitude of intracellular processes.

In this study we have limited our attention to the effects on monocyte functions to a single chemoattractant PAF. *In vivo*, multiple other chemoattractants influence cellular activities. To characterize the effects of other chemokines on monocyte functions was beyond the scope of our experiments and as such would have made our experiments and interpretations unduly complicated. However, we recognize that the interplay of various chemoattractants on monocyte activities including differentiation into macrophages, merit future study. Furthermore, *in vivo* animal studies, including experiments on animal models of diabetes will need to be performed to confirm our *in vitro* observations and to explore the role of hyperglycaemia on monocyte functions. We are currently conducting such experiments.

To conclude, our current data highlight the important role played by GSK-3β in association with small molecule GTPases in monocyte migration in ND and DM human beings. Furthermore, monocytes from DM individuals revealed enhanced and prolonged PAF induced migration following GSK inhibition. This effect maybe a limiting factor in the development of GSK inhibitors for treating the metabolic complications of DM as well as their use in Alzheimer's dementia and as an adjuvant for cancer chemotherapy. Additional studies are needed to evaluate these regulatory

pathways in individuals with overt and established vascular disease both with and without DM. Further exploration of the subcellular mediators of atherosclerosis could also potentially lead to identification of specific molecular targets for drug development not only for therapy but also prevention of vascular disease in predisposed individuals.

Acknowledgements

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