

Involvement of hypoxia-inducible factor-1 (HIF-1 α) in IgE-mediated primary human basophil responses

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Basophils play a pivotal role in regulating chronic allergic inflammation as well as angiogenesis. Here, we show for the first time that IgE-mediated activation of primary human basophils results in protein accumulation of the α -subunit of hypoxia-inducible factor 1 α (HIF-1 α), which is differentially regulated compared with signals controlling histamine release. HIF-1 facilitates cellular adaptation to hypoxic conditions such as inflammation and tumour growth by controlling glycolysis, angiogenesis and cell adhesion. ERK and p38 MAPK, but not reactive oxygen species (ROS), ASK1 or PI 3-kinase, were critical for IgE-mediated accumulation of HIF-1 α , although the latter crucially affected degranulation. Abrogating HIF-1 α expression in basophils using siRNA demonstrated that this protein is essential for vascular endothelial growth factor (VEGF) mRNA expression and, consequently, release of VEGF protein. In addition, HIF-1 α protein alters IgE-induced ATP depletion in basophils, thus also supporting the production of the pro-allergic cytokine IL-4.

Key words: Allergology · Molecular immunology · Signal transduction

Introduction

Basophils are comparatively rare granulocytes in the blood, which have multiple functions and migrate into organs affected by allergic inflammation and certain parasitic as well as autoimmune diseases [1]. In recent years there is increasing evidence suggesting that basophils are essential in orchestrating chronic allergic inflammation (reviewed in [2]). Although basophil numbers are low compared with mast cells, they are several orders of magnitude more sensitive to IgE-mediated activation than mast cells and are the main allergic effector cells capable of rapidly generating Th2-type cytokines in humans [3–5]. Furthermore, numerous studies have demonstrated that basophils migrate from the blood into various tissues affected by allergic inflammation (reviewed in [1] and [2]) where they are the principle IL-4-generating cells in developing late-phase responses [6]. By virtue of their ability to release histamine, leukotriene C₄ and Th2-type cytokines (e.g. IL-4) following

allergen-mediated aggregation of high-affinity IgE receptors (Fc ϵ RI), basophils potentially play an important pro-inflammatory and immunomodulatory role in allergy (reviewed in [2]). Furthermore, recent studies clearly implicate basophils in crucially regulating chronic allergic inflammation [7] and Th2-immunity [8]. Additionally, these cells were also found to play an important role in angiogenesis due to their ability to generate vascular endothelial growth factor (VEGF) [9].

The activities of various intracellular signals involved in controlling both the synthesis and the secretion of these different mediator types from basophils lead to considerable demands in ATP generation (required in the phosphorylation of kinases). One possible mechanism that would explain the ability of basophils to contend with an increased demand in ATP, without undergoing cell death, as well as being able to produce VEGF, is due to the actions of hypoxia-inducible factor 1 α (HIF-1 α) [10, 11]. HIF-1 α is the inducible subunit of the heterodimeric HIF-1 transcription complex, which consists of two subunits, HIF-1 α and HIF-1 β . HIF-1 α protein is rapidly accumulated due to post-translational

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stabilisation [11] in cells exposed either to hypoxia or to several types of non-hypoxic stimuli such as inflammatory mediators, cytokines, reactive oxygen (ROS) and nitrogen (RNS) species [10, 11]. In the absence of the above triggers, HIF-1 α protein undergoes rapid ubiquitination followed by proteasomal degradation [10, 11]. This protein plays a pivotal role in mediating angiogenesis, glycolysis and cell adhesion, which are critical for both inflammatory and innate immune responses [10].

Despite the increasing evidence concerning the role of HIF-1 α in innate immune reactions [10] and signalling associated with autoimmune disorders [12], its involvement in basophil-dependent allergic inflammation has not been studied to date.

Here, we report that IgE-mediated activation of purified primary human basophils results in HIF-1 α protein accumulation. ERK and p38 MAPK, but not ROS, apoptosis signal-regulating kinase 1 (ASK1, an upstream kinase of the MAP kinase-dependent apoptotic pathway which may impact on p38 MAPK [13]) or PI 3-kinase, were critical for IgE-mediated accumulation of HIF-1 α , although the latter crucially affected degranulation.

Results

HIF-1 α in IgE-mediated basophil responses

Basophils play a significant role in regulating chronic allergic inflammation as well as angiogenesis [2, 7, 8], responses which are associated with energy consumption and stress. However, there is no evidence concerning the pathways involved in basophil adaptation to stress protecting them against programmed death. Given the above attributes of HIF-1 α , we asked whether HIF-1 α protein accumulation occurs in primary human basophils following Fc ϵ RI aggregation and whether this correlates with pro-inflammatory mediator secretion (exemplified by assessing histamine secretion). Our data show a rapid accumulation of HIF-1 α protein following 30-min stimulation of basophils with anti-IgE (Fig. 1A), which remained stable for at least 2 h. This correlated with an increase in the level of HIF-1 α protein DNA-binding activity and was consistent with the rapid release of histamine from the cells. These data were further supported by an increase in the mRNA level of HIF-1 α as well as VEGF (one of the main target genes of HIF-1 transcription complex) after 2 h of stimulation of basophils with anti-IgE (Fig. 1A). This indicates that the accumulation of HIF-1 α protein, presumably *via* the well-known mechanism of post-translational stabilisation [11] that was observed after 30 min of stimulation with anti-IgE, is supported by an increase in expression of HIF-1 α mRNA at later stages. In contrast, we failed to observe HIF-1 α protein accumulation or increased mRNA expression (and therefore no increase in DNA-binding activity of HIF-1 α protein) in basophils incubated for the same periods in the absence of anti-IgE.

In other settings, the immunological activation of myeloid macrophages in a TLR4-dependent manner leads to HIF-1 α protein accumulation *via* a redox-dependent mechanism associated with p38 MAPK phosphorylation, which is activated in an

ASK1-dependent manner [14]. The same mechanism applies to some other types of immunologic stimulation [15] and we therefore tested the activity of ASK1 and production of ROS in basophils exposed to anti-IgE. Surprisingly, however, we observed neither increased ROS production nor activity of ASK1 (Fig. 1A) in these settings. The accumulation of HIF-1 α protein in response to IgE-dependent stimulation of basophils is therefore independent of ROS production and ASK1 activation.

To confirm the specificity of anti-IgE-dependent accumulation of HIF-1 α protein in basophils, we exposed them to 0.0075% anti-IgE and 0.0075% isotype control (goat anti-human IL-6). In contrast to anti-IgE, the isotype control failed to induce either HIF-1 α protein accumulation or histamine release from basophils (Fig. 1B).

Despite a clear time-dependent accumulation of HIF-1 α protein using a sub-optimal concentration of anti-IgE, it is known that supra-optimal IgE-dependent activation of basophils leads to diminished mediator releases compared with optimum concentrations of stimulus [16]. We therefore investigated the dose-dependent effects of IgE-mediated HIF-1 α protein accumulation (stimulated for 2 h) in comparison with histamine release. Intriguingly, although increasing anti-IgE concentrations led to the expected bell-shaped dose-response curve for histamine release, HIF-1 α accumulation was strikingly further enhanced at supra-optimal titres of anti-IgE (Fig. 2). This strongly suggests that IgE-dependent inflammatory mediator secretion in basophils is differentially regulated compared with HIF-1 α protein accumulation.

In addition to the above, no significant increase in ROS production or activity of ASK1 was observed regardless of the anti-IgE concentration employed, confirming that both redox-dependent and ASK1 downstream pathways do not impact IgE-mediated HIF-1 α protein accumulation (Fig. 2). On the other hand, an anti-IgE concentration-dependent decrease in the level of intracellular ATP was clearly seen (Fig. 2). This correlation between decreased ATP levels and increased HIF-1 α accumulation is expected, since HIF-1 α is responsible for transcriptional induction of crucial glycolytic enzymes and glucose transporters protecting the cells against ATP depletion. This is especially important with respect to the dependency of leukocytes on glycolysis in all cases of cellular stress.

To confirm the role of HIF-1 α in the upregulation of VEGF expression, we induced HIF-1 α protein accumulation in basophils by treating them for 2 h with 50 μ M cobalt chloride. Cobalt chloride is a well-known chemical inducer of accumulation of transcriptionally active HIF-1 α protein [17]. As a result, accumulation of HIF-1 α protein correlated with an increase in VEGF mRNA (Fig. 3). Histamine release was not significantly affected by exposure of the cells to cobalt chloride.

IgE-induced histamine release and HIF-1 α activation are controlled by differential mechanisms

Recent evidence suggests that increased activities of PI 3-kinase as well as ERK could lead to the accumulation of HIF-1 α protein under normal oxygen availability. ERK is also known to activate

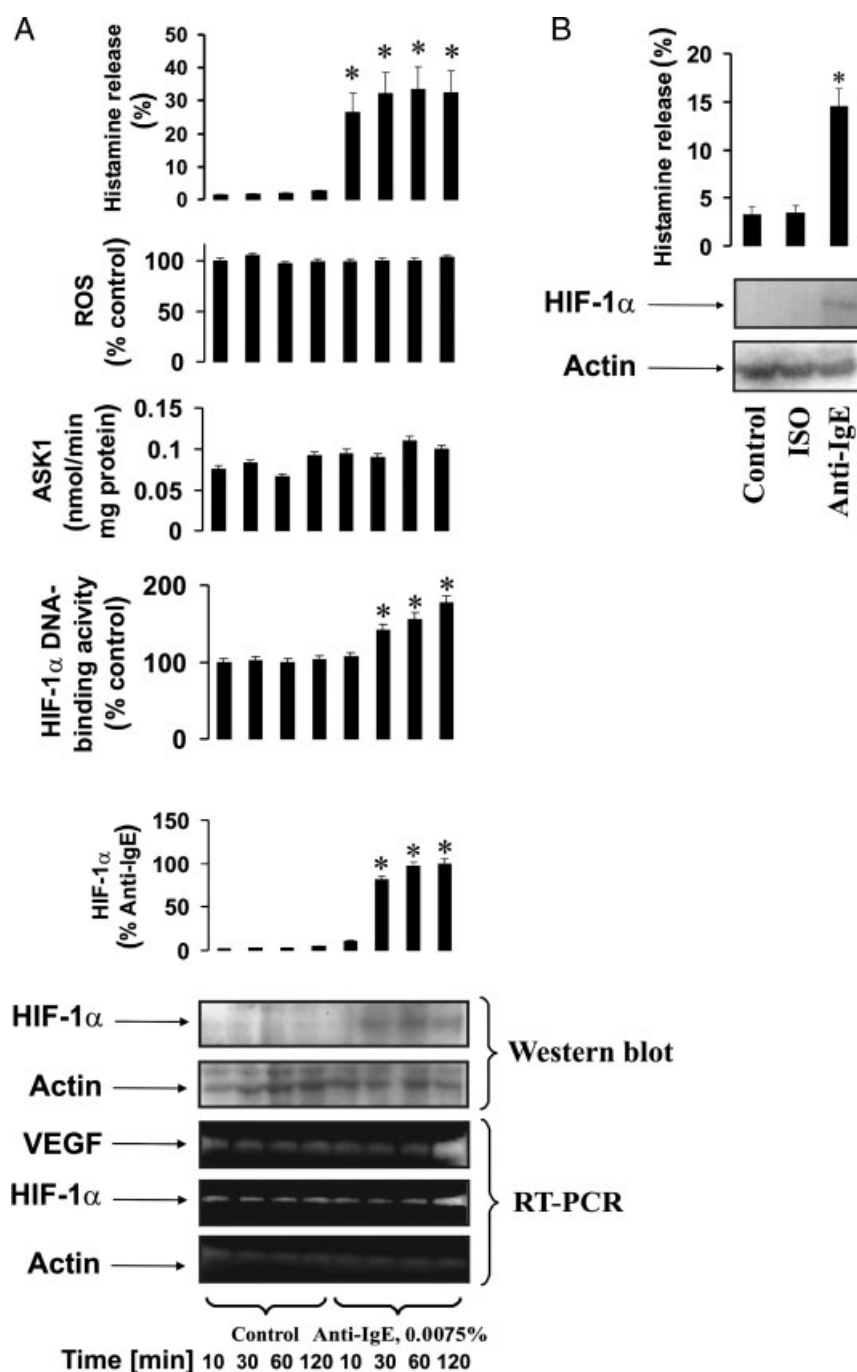


Figure 1. Anti-IgE induces accumulation of HIF-1 α protein and histamine release in primary human basophils. (A) Primary human basophils were exposed to 0.0075% anti-IgE for the indicated periods followed by the detection of HIF-1 α protein accumulation/DNA-binding activity, ROS generation, ASK1 activity and histamine release. “% control”, means that the activity of the process in the control was considered as 100% and normalised against this value in other samples. (B) Primary human basophils were exposed to 0.0075% anti-IgE or 0.0075% goat antibody against human IL-6 for 2 h followed by the detection of HIF-1 α protein accumulation and histamine release. Quantitative data are mean values $\pm$ SD of at least four individual experiments. Western blot and RT-PCR data shown are representative of three experiments. Results of densitometric analysis of the Western blot data are mean values $\pm$ SD, where $n = 3$; * $p < 0.01$ when compared with control as determined by the two-tailed Student's *t*-test.

several isoforms of p38 MAPK that could participate in stabilisation of HIF-1 α [15, 18]. To investigate the role of these pathways in anti-IgE-dependent accumulation of HIF-1 α in basophils, we pre-treated the cells for 15 min with 5 μ M LY294002 (PI 3-kinase inhibitor), 5 μ M SB203580 (p38 MAPK inhibitor) and 10 μ M

PD098059 (an MEK inhibitor which blocks subsequent ERK activation). This was followed by 120 min of exposure of the cells to anti-IgE. Our data clearly show that LY294002 did not affect IgE-dependent accumulation of HIF-1 α protein (Fig. 4). This result suggests that PI 3-kinase and its downstream pathway most

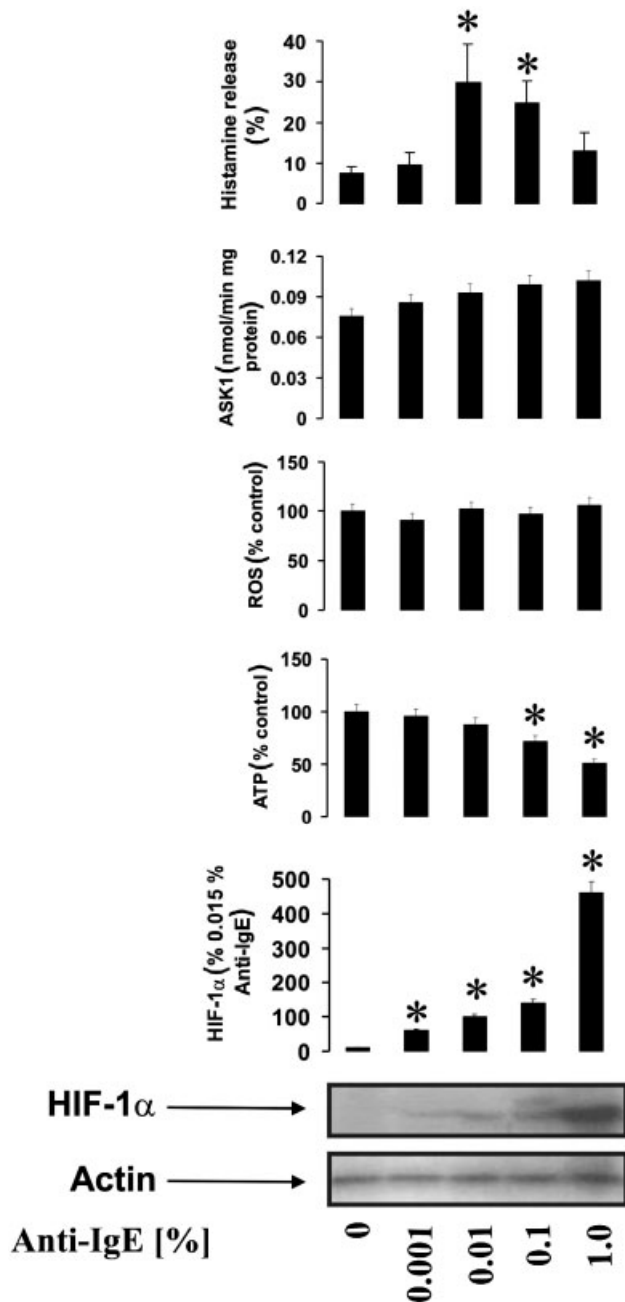


Figure 2. Concentration-dependent effects of IgE-mediated HIF-1 α accumulation in human basophils correlate with decreased levels of intracellular ATP. Basophils were stimulated for 2 h with 0.001, 0.01, 0.1 and 1% anti-IgE followed by the detection of HIF-1 α protein, ROS production, ASK1 kinase activity, intracellular ATP and histamine release, as described in *Materials and methods*. Quantitative data are mean values+SD of at least four individual experiments. Western blot data shown are representative of three experiments. Results of densitometric analysis of the Western blot data are mean values+SD, where $n = 3$; * $p < 0.01$ when compared with control as determined by the two-tailed Student's *t*-test.

likely do not participate in IgE-dependent accumulation of HIF-1 α protein, in stark contrast to histamine release. Conversely, pre-treatment of the cells with SB203580 inhibited accumulation of the protein (Fig. 4), suggesting that p38 MAPK is involved in

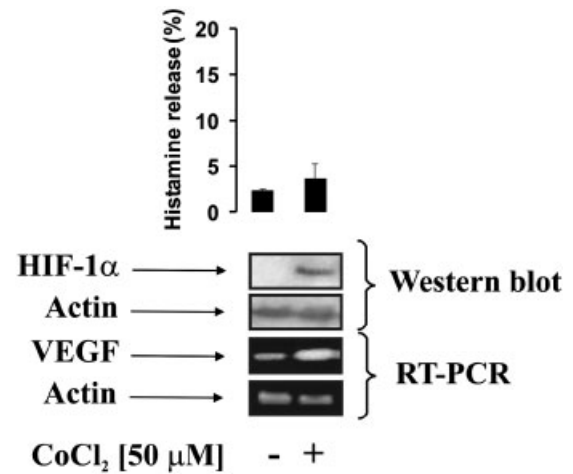


Figure 3. HIF-1 α accumulation leads to an increase in VEGF expression in primary human basophils. Primary human basophils were exposed to 50 μ M cobalt chloride for 2 h followed by Western blot analysis of HIF-1 α accumulation and RT-PCR analysis of VEGF expression. Western blot and RT-PCR data shown are representative of three experiments. Quantitative data are mean values+SD of at least four individual experiments.

HIF-1 α accumulation. Furthermore, we could demonstrate that HIF-1 α is activated in an ASK1-independent manner since the kinase activity of ASK-1 did not undergo any significant changes upon stimulation of basophils with anti-IgE. Pre-treatment of basophils with PD098059 completely attenuated anti-IgE-dependent accumulation of HIF-1 α protein (Fig. 4), suggesting a crucial role of ERK for the process. The concentrations of the inhibitors used were selected based on the data available in the literature and our own experiments, in which we have found that the concentrations used are functional in human basophils as well as myeloid macrophages and do not affect the viability of the cells as analysed by the MTS test (data not shown).

HIF-1 α protein is critical for IgE-induced expression of VEGF and IL-4

To investigate the role of HIF-1 α protein in the pro-allergic and inflammatory functions of basophils, we transfected the cells with HIF-1 α siRNA as described in the *Materials and Methods*. Normal 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP)-treated basophils and those transfected with siRNA were exposed to 0.0075% anti-IgE for 2 h. The successful knockdown was confirmed by the detection of HIF-1 α mRNA (RT-PCR) and protein accumulation (Western analysis). In addition, we studied the mRNA levels of VEGF and IL-4. We observed that siRNA successfully attenuated both expression and accumulation of HIF-1 α in basophils. DOTAP-treated non-transfected basophils responded to stimulation with anti-IgE, giving rise to increases in both VEGF and IL-4 mRNA. This increase was not observed in HIF-1 α knockdown cells (Fig. 5A).

We know from our previous studies that HIF-1 α is involved in TLR4-, TLR7- and TRP-induced production of pro-inflammatory

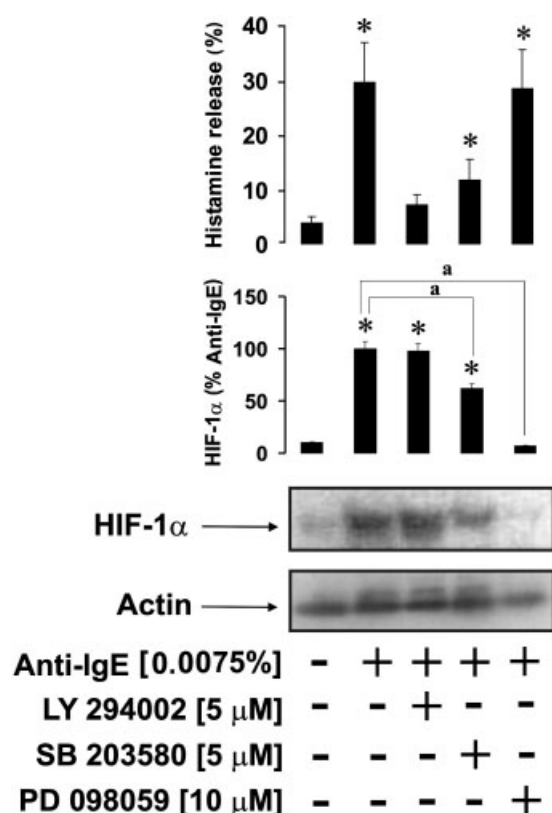


Figure 4. IgE-mediated accumulation of HIF-1 α and histamine release in primary human basophils is differentially regulated. Cells were pre-treated for 15 min with the indicated inhibitors (concentrations are shown in the figure) followed by 120 min of exposure to 0.0075% anti-IgE. Accumulation of HIF-1 α protein and histamine release were then analysed as described in *Materials and methods*. Quantitative data are mean values $\pm$ SD of at least four individual experiments. Western blot data are representative of three experiments. Results of densitometric analysis of the Western blot data are mean values $\pm$ SD, where $n = 3$; * ($p < 0.01$) when compared with control; ^a ($p < 0.01$) when indicated values compared as determined by the two-tailed Student's *t*-test.

cytokines in human myeloid cells via the protection of the cells against depletion of ATP [19, 20]. To investigate the actual function of HIF-1 α in the production of VEGF and IL-4, we analysed both mRNA levels and cytokine synthesis in normal DOTAP-treated basophils and those transfected with HIF-1 α -specific siRNA exposed for 2 h to 0.0075% anti-IgE in the presence or absence of 100 μ M extracellular ATP. We observed that HIF-1 α -knockdown basophils failed to respond to stimulation with anti-IgE in terms of VEGF and IL-4 expression/production. This correlated with a significant depletion of the intracellular ATP (Fig. 5B). In the presence of extracellular ATP, however, which subsequently increased the intracellular ATP levels, expression of IL-4 mRNA and release of this cytokine was restored to the level observed in non-transfected basophils following anti-IgE stimulation (Fig. 5B). However, addition of ATP did not affect the expression/release of VEGF in cells transfected with HIF-1 α siRNA (Fig. 5B). These results are similar to those obtained in the cases of TLR-mediated inflammatory reactions.

Discussion

HIF-1 α protein is critical for the expression of around 40 genes associated with cellular adaptation to pathological reactions associated with decreased oxygen availability or oxidative/nitrosative stress [11]. In addition, this protein is involved in regulation of energy metabolism in leukocytes and their adhesion [11, 18, 21]. Since allergic responses are associated with both increased oxygen consumption and ATP-dependent signalling, one could hypothesise that HIF-1 α protein is involved in molecular events that take place in basophils during pro-allergic reactions. Our data have demonstrated that HIF-1 α protein is indeed expressed and accumulated in primary human basophils in response to IgE-dependent stimulation in both a concentration and a time-dependent manner.

Recent studies demonstrated the involvement of HIF-1 α protein in innate immune responses induced via membrane-associated TLR. In those cases, accumulation of HIF-1 α protein was mediated by increased NADPH oxidase-dependent generation of ROS [14, 15, 19] and also by p38-dependent phosphorylation. It is interesting to note that p38 MAPK activation was dependent on ASK1, which was selectively required for this process [14, 22]. However, in our current investigation we observed no increase in ROS generation upon stimulation of basophils with anti-IgE (Figs. 1 and 2), independently of the concentration employed. Based on these data, one could suggest that ROS are not involved in IgE-mediated accumulation of HIF-1 α protein in primary human basophils. This intriguing conclusion may imply that phosphorylation of the protein could be the primary strategy employed in HIF-1 α accumulation in basophils during pro-allergic reactions. Since ASK1 is selectively required for membrane-associated TLR4-dependent activation of p38 MAPK, we analysed the activity of this enzyme in basophils exposed to different concentrations of anti-IgE for different periods of time (Figs. 1 and 2). However, it was clear that no concentration or time-dependent activation of ASK1 took place. Therefore, ASK1 and its downstream cascade are unlikely to play any role in HIF-1 α accumulation.

Using pharmacological inhibitors, we have investigated the role of other pathways in IgE-dependent accumulation of HIF-1 α . Based on the recent reports concerning accumulation of this protein in human leukocytes [15], we investigated the role of ERK and p38 MAPK (which could also be activated by upstream kinases different from ASK1). In addition, since PI 3-kinase could act as a potential mediator of HIF-1 α accumulation, we considered it as one of the possible candidates. However, our studies clearly demonstrated that, whereas HIF-1 α accumulation depends on ERK and p38 MAP kinase, it does not depend on PI 3-kinase (Fig. 4). ERK and p38 MAPK were recently found to critically regulate non-hypoxic accumulation of HIF-1 α protein, especially in human leukocytes (mostly in macrophages), which become dependent on glycolysis upon immunologic activation that induces activity of numerous ATP-consuming kinase cascades [19, 21, 23]. It was reported that HIF-1 α is a target for p38 MAPK and ERK and is directly phosphorylated by these

kinases, which supports the stabilisation of the protein [14, 24, 25]. ERK was also found to support the interaction of HIF-1 α with its co-activator proteins, increasing its transcriptional activity [25]. Under pathophysiological conditions this process could be associated with increased cellular influx into inflamed tissues and associated with the concomitant decrease in oxygen availability [21]. Therefore, accumulation of HIF-1 α protein could be considered a pathological reaction of leukocytes to the stress associated with decreased oxygen availability or immunologic activation.

The results showing changes in accumulation of HIF-1 α protein upon pre-treatment of basophils with pharmacological kinase inhibitors before exposure to anti-IgE were inconsistent with those obtained with histamine release. Pre-treatment of basophils with LY294002 attenuated anti-IgE-induced histamine secretion. A decrease in anti-IgE-induced histamine release was also observed upon pre-treatment of basophils with SB203580 but not with PD098059 (Fig. 4). This suggests involvement of PI 3-kinase and p38 MAPK in IgE-dependent histamine release. However, the results show that isoforms of ERK inhibited by PD098059 are not involved in the release of histamine induced by anti-IgE. These data actually indicate that p38 MAPK could be activated in basophils in some way, which does not depend on either ASK1 (as confirmed by our data – Figs. 1 and 2) or the isoforms of ERK inhibited by PD098059. Additionally, one could argue that different IgE-dependent signalling mechanisms are involved in the accumulation of HIF-1 α protein and histamine release.

The experiments with HIF-1 α knockdown basophils have demonstrated that this protein is critical for the expression and accumulation of the major angiogenic factor VEGF, and IL-4 – one of the key immunomodulatory cytokines that promote allergic inflammation. Further studies demonstrated that the presence of extracellular ATP was able to restore both IgE-induced expression and accumulation of IL-4 to the levels observed with non-transfected basophils. In contrast, the addition of extracellular ATP did not impact the expression or release of VEGF in HIF-1 α knockdown basophils exposed to anti-IgE. These data suggest, on the one hand, that HIF-1 α protein is indirectly involved in the pro-allergic expression of IL-4 in basophils *via* protection against ATP depletion. On the other hand, it has been known for many years that VEGF is a HIF-1 target gene. In this case, HIF-1 α most likely directly influences pro-allergic expression of VEGF as the specific transcription factor. These data are consistent with the observations reported for inflammatory reactions induced by membrane-associated TLR4 (which recognises LPS of Gram-negative bacteria) and endosomal TLR7 and TLR8 (which recognise viral single-stranded RNA). In both cases HIF-1 α promotes the expression of VEGF and supports pro-inflammatory cytokine synthesis (IL-6, TNF- α) by protecting the effector cells against ATP depletion associated with TLR-mediated stress [19, 20, 26]. The extracellular application of ATP may also impact on purinergic receptors present on basophils [27], thus affecting cytokine production. However, the crosstalk between the ATP-activated purinergic receptors and the expres-

sion/release of pro-inflammatory cytokines has not been studied in these cells. Despite this uncertainty, our data clearly demonstrate that HIF-1 α -dependent support of the ATP level during IgE-induced stress is critical for IL-4 production. Our observations show that HIF-1 α protein is a major contributor not only to the pro-allergic functions of basophils but also to the TLR-mediated innate immune reactions. However, this study also highlights that different immune receptors employ differential signalling mechanisms to activate this protein.

Overall, our observations in this study show a clear role of HIF-1 α in supporting IgE-mediated inflammatory responses of basophils (*e.g.* by regulating the expression/release of the pro-inflammatory cytokine IL-4 and pro-angiogenic cytokine VEGF). These attributes serve as further evidence to support the notion that basophils contribute to allergic inflammatory events [1–9] and that HIF-1 α maintains the ability of basophils to sustain their level of activity over long periods. Despite their small number, basophils have recently been shown to be major regulators of IgE-mediated allergic responses [7, 8]. Therefore, it is vital to understand the signalling mechanisms that govern basophil activation and mediator release. Our study shows for the first time that HIF-1 α protein is involved in IgE-induced responses of primary human basophils, responses which are considered central to allergic reactions in general (reviewed in [2]). In summary, our findings indicate that HIF-1 α facilitates the adaptation of basophils to IgE-mediated stress and promotes VEGF expression, and that these processes are differentially regulated compared with degranulation and histamine release.

Materials and methods

Materials

Anti-human IgE, RPMI-1640 medium, fetal calf serum and supplements, DOTAP transfection reagent, enhanced avian HS RT-PCR kit, GenEluteTM mammalian total RNA miniprep kit, and all pharmacological kinase/phosphatase inhibitors, HIF-1 α -specific siRNA were purchased from Sigma (Suffolk, UK). MaxisorpTM microtitre plates were obtained from Nunc (Roskilde, Denmark). The ELISA-based assay kit for the detection of VEGF and isotype control antibody was bought from R&D Systems (Abingdon, UK). IL-4 ELISA kits were purchased from Biolegend (Cambridge BioScience, UK). Mouse monoclonal antibody to HIF-1 α and mouse monoclonal antibody to β -actin as well as rabbit polyclonal HRP-labelled antibody to mouse IgG were from Abcam (Cambridge, UK). All other chemicals were commercially available and of the highest grade of purity.

Basophil purification

Human basophils were obtained from buffy coats acquired from healthy blood donors undergoing routine blood donation

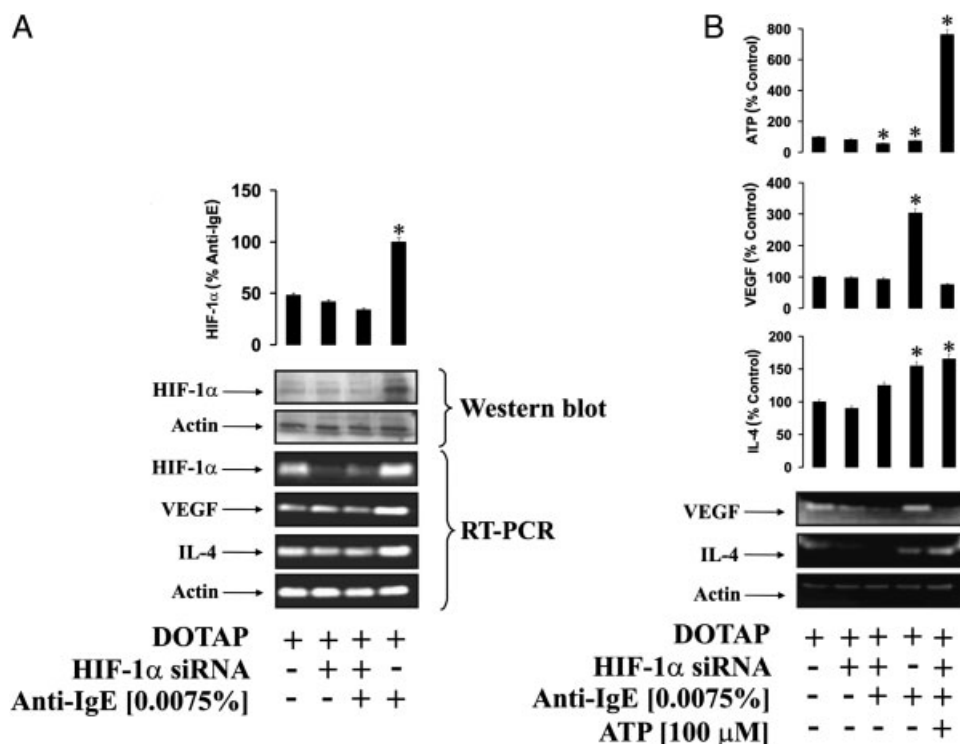


Figure 5. Involvement of HIF-1 α protein in IgE-mediated expression of VEGF and IL-4 in primary human basophils. (A) Normal and HIF-1 α knockdown primary human basophils were exposed to 0.0075% anti-IgE for 2 h. All samples contained DOTAP to control for the impact of the transfection reagent. Levels of HIF-1 α , VEGF, IL-4 and actin mRNA were analysed by RT-PCR as described in the *Materials and Methods*. Accumulation of HIF-1 α protein was detected by Western blot. (B) Normal and HIF-1 α knockdown primary human basophils were exposed to 0.0075% anti-IgE for 2 h in the absence or presence of 100 μ M extracellular ATP. All samples contained DOTAP to control for the impact of the transfection reagent. Levels of VEGF, IL-4 and actin mRNA were analysed by RT-PCR. Release of VEGF and IL-4 was detected using ELISA assays. The level of intracellular ATP was analysed by luminometric assay. Levels of VEGF, IL-4 and ATP assayed were expressed as % control (the amounts of VEGF/IL-4/ATP in non-transfected DOTAP-treated basophils were set as 100%). Quantitative data are mean values $\pm$ SD of at least three individual experiments. Western blot and RT-PCR data shown are representative of three experiments. Results of densitometric analysis of the Western blot data are mean values $\pm$ SD, where $n = 3$; * $p < 0.01$ when compared with control as determined by the two-tailed Student's t -test.

following ethical approval. Basophils were purified by Ficoll density centrifugation followed by negative selection using magnetic cell sorting [28]. Basophils ($94 \pm 2\%$ pure, as determined by alcian blue staining with viability of $>95\%$) were incubated for 15 min at 37°C in HEPES-buffered Tyrode's solution and treated as indicated. Following stimulation, histamine release analysis and cell lysis were performed as described previously [28].

Transfer of HIF-1 α siRNA to primary human basophils

siRNA specific to HIF-1 α was obtained from Sigma. Transfection into basophils was performed using DOTAP transfection reagent [19, 20] according to the manufacturer's protocol.

Detection of HIF-1 α protein

HIF-1 α was determined by Western blot analysis, as described previously [14]. Briefly, cells were incubated for the times indicated, washed twice with ice-cold PBS and lysed in 200 μ L of

lysis buffer (50 mM Tris-HCl, 5 mM EDTA, 150 mM NaCl, 0.5% Nonidet-40, 1 mM PMSF, pH 8.0). After centrifugation (13 000 rpm, 15 min) the protein content in the supernatants was analysed (the same procedures applied to Western blot analysis of other proteins). Finally, 100 μ g of protein was added to the same volume of $2 \times$ sample buffer (125 mM Tris-HCl, 2% SDS, 10% glycerine, 1 mM DTT, 0.002% bromophenol blue, pH 6.9) and boiled for 5 min. Proteins were resolved on 7.5% SDS-polyacrylamide gels and blotted to nitrocellulose membranes. Molecular weights were calibrated in proportion to the running distance of rainbow markers. Transblots were washed twice with TBS (50 mM Tris-HCl, 140 mM NaCl, pH 7.3) containing 0.1% Tween 20 before blocking unspecific binding with TBS containing 5% skim milk for 1 h. The HIF-1 α (1:1000 in TBS plus 5% milk) antibody was added and incubated for 60 min at room temperature. Afterwards, nitrocellulose membranes were washed five times for 15 min with TBS containing 0.1% Tween 20. For protein detection, blots were incubated with goat secondary antibodies conjugated with HRP (1:1000 in TBS plus 5% milk) for 60 min, followed by ECL detection. Blots were stripped and re-probed with a rabbit monoclonal antibody against β -actin to confirm equal protein

loading. Briefly, membranes were washed five times with TBS (50 mM Tris-HCl, 140 mM NaCl, pH 7.3) containing 0.1% Tween 20 then incubated for 1 h at room temperature with monoclonal antibody directed against β -actin followed by ECL detection. The Western blot data for HIF-1 α protein were subjected to densitometric analysis and were normalised against respective actin bands.

Determination of HIF-1 DNA-binding activity

HIF-1 DNA-binding activity was measured with modifications as described previously [14]. Briefly, a 96-well maxisorpTM microtitre plate was coated with streptavidin and blocked with BSA. In total 2 pmol/well biotinylated 2HRE-containing oligonucleotide were immobilised by 1 h incubation at room temperature. Plates were then washed five times with TBST buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween-20) followed by 1 h incubation with 20 μ L/well of cell lysate at room temperature. Following further washing, mouse anti-HIF-1 α antibody (1:1000 in TBS plus 2% BSA) was added. After 1 h of incubation at room temperature, plates were washed five times with TBST buffer and incubated with 1:1000 HRP-labelled rabbit anti-mouse IgG in TBST buffer for 1 h. After extensive washing with TBST, the bound secondary antibody was detected by the peroxidase reaction (ortho-phenylenediamine/H₂O₂, Kem-En-Tek Diagnostics, Copenhagen, Denmark). Reactions were quenched after 10 min with an equal volume of 1 M H₂SO₄ and colour development was analysed in a microplate reader (492 nm). DNA-binding activity of HIF-1 was calculated as a percentage of the value obtained when analysing lysates of non-treated cells (control).

Measurement of HIF-1 α , VEGF and IL-4 mRNA by RT-PCR

Total RNA was isolated using GenEluteTM mammalian total RNA miniprep kit, followed by HIF-1 α mRNA RT-PCR [19, 20]. Primer selection was as follows: HIF-1 α , 5'-CTCAAAGTCGGACAG-CCTCA-3', 5'-CCCTGCAGTAGGTTTCTGCT-3' [19, 20]; VEGF, 5'-GTATAAGTCCTGGAGCGT-3', 5'-CTCGGAGGGAGTCCCAAA-3' [20]; IL-4, 5'-CTCATTTTCCCTCGGTTTCA-3', 5'-GTCCTTCT-CATGGTGGCTGT-3' [29]; actin, 5'-TGACGGGGTCACCCACAC TGTGCCCATCTA-3', 5'-CTAGAAGCATTTGCGGTCGACGATGGA GGG-3' [19, 20]. The amplification program was as follows: 95°C, 30 s; 56°C, 30 s; 72°C, 1 min; 20 cycles; 72°C, 10 min. Products were separated on 2% agarose gels and visualised with ethidium bromide.

Measurement of VEGF and IL-4 production

Production of VEGF and IL-4 by primary human basophils was analysed by ELISA according to the manufacturer's protocols.

Measurement of ASK1 kinase activity and detection of ROS generation

The kinase activity of ASK1 was assessed by means of immunoprecipitation followed by analysis of phosphorylation of exogenous substrate, myelin basic protein, [14]. Nox-dependent ROS generation was analysed as described earlier [14, 30].

Histamine release

Basophil histamine releases were assessed using spectrofluorometric autoanalysis as described previously [16]. Percentage histamine releases were calculated from the total histamine content in the sum of lysed cell pellet and supernatant vials.

Detection of intracellular ATP

This was analysed using a luminometric kit (Sigma) according to the manufacturer's protocol.

Statistical analysis

Each experiment was performed at least three times and statistical analysis was performed using the two-tailed Student's *t*-test.

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Abbreviation: ASK-1: apoptosis signal-regulating kinase 1 · DOTAP: 1,2-dioleoyl-3-trimethylammonium-propane · HIF-1 α : hypoxia-inducible factor 1 α · RNS: reactive nitrogen species · VEGF: vascular endothelial growth factor

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