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HIF-1α Is Up-Regulated in Activated Mast Cells by a Process That Involves Calcineurin and NFAT

Aurelia Walczak-Drzewiecka, Marcin Ratajewski, Waldemar Wagner, and Jaroslaw Dastych

Mast cells play important roles in many pathological conditions where local hypoxia is observed, including asthma, rheumatic diseases, and certain types of cancer. Here, we investigated how expression of the hypoxia-inducible factor 1, α subunit gene (HIF1A), is regulated in mast cells. The product of HIF1A is hypoxia-inducible factor 1α (HIF-1α), a major nuclear transcription factor modulating gene expression in response to hypoxic conditions. We observed that under hypoxic conditions, exposure of mast cells to ionomycin and substance P resulted in significant up-regulation of HIF1A expression as compared with resting mast cells incubated under identical conditions. The ionomycin-mediated increase in HIF-1α protein levels was sensitive to the transcription inhibitor actinomycin D and to inhibitors of calcineurin, cyclosporin A (CsA), and FK506. The increased HIF-1α protein level was paralleled by a severalfold increase in HIF-1α mRNA that could be also inhibited with actinomycin D and CsA. The HIF1A promoter activity was significantly increased in ionomycin-activated mast cells, and the promoter activity could be inhibited by CsA and FK506. Furthermore, in situ mutagenesis experiments showed that the ionomycin-mediated HIF1A promoter activity depends on a conservative NFAT-binding site. Thus, accumulation of HIF-1α in activated mast cells requires up-regulation of HIF1A gene transcription and depends on the calcineurin-NFAT signaling pathway. The Journal of Immunology, 2008, 181: 1665–1672.
were purchased from Fermentas; the plasmid pG3L3-basic was obtained from Promega; the pCMV6-GLS and pCMV6-NFAT expression plasmids were obtained from Origene Technologies; the anti-HIF-1α Ab was purchased from Abcam; an anti-CD44 antibody (CD44) was purchased from Upstate; antisense-1F4 and actin-1F4 primers were designed using Primer3 software; antisense primer for HIF1A was also purchased from Fermentas; the plasmid pGL3-basic was obtained from Promega; and the infrared dye-labeled oligonucleotide probe IRD700-W18 (28) was synthesized on a custom microarray. The human genomic DNA sequence encoding HIF-1α was obtained from the GenBank database. The PCR primers used were designed using Primer3 software (5).

**Mast cell cultures**

HMC-1 mast cells (24) were cultured in IMDM supplemented with 10% heat-inactivated FCS, 4 mM t-glutamate, and 100 μg/ml penicillin-streptomycin. Cells were cultured at a density of 200,000–400,000 cells/ml, and the cultures were maintained by completely replacing the medium once a week. LAD-2 mast cells (25) were cultured in Stem Pro-34 serum-free medium supplemented with 100 ng/ml human recombinant stem cell factor at density of 300,000–500,000 cells/ml, and the cultures were maintained by hemidepletion of the culture medium once a week. Both mast cell lines were cultured at 37°C in a CO2 incubator.

**Preparation of cell lysates, cytosolic fractions, and nuclear extracts**

Cell lysates were produced as previously described (26). Briefly, cells were lysed on ice for 30 min in a buffer containing 50 mM Tris-HCl (pH 8.0), 1% nonionic detergent IGEPLA CA-630, 20 mM EDTA, 150 mM NaCl, 1 mM MgCl2, 10 mM tetrasodium pyrophosphate, 100 mM sodium fluoride, 2 mM sodium orthovanadate, 10 μM leupeptin, 10 μM aprotinin, and 1 mM PMSF. Lysates were centrifuged at 10,000 g for 3 min. Supernatants were added to designated wells at the indicated concentrations. Inhibitors were added at the indicated concentrations 5 min before addition of the activator. In some experiments, ActD (5 μM) was added to the cell suspensions either 5 min before or 1, 2, 3, and 4 h after addition of ionomycin. For incubation under normoxic conditions, the plates were placed in a CO2 incubator. For incubation under hypoxic conditions, the plates were placed in a specialized incubator consisting of an outer chamber and an inner chamber. The inner chamber was continuously purged with 5% CO2 in air for 10 min following addition of activators. For incubation under hypoxic conditions, the plates were placed in a specialized incubator consisting of a small thermostated hermetic inner chamber located inside a hermetic glove box (outer chamber). The inner chamber was continuously purged with a gas mixture consisting of 94% N2, 5% CO2, and 1% O2, and the outer chamber was purged with N2. The O2 concentration in the outer chamber was monitored using an oxygen sensor (Teledyne Analytical Instruments). All manipulations of the cell suspensions, including addition of activators and inhibitors, collection of cells following incubation, and lysis of cell pellets, were performed in the outer chamber under an N2 atmosphere.

**Real-time PCR**

Expression of HIF1A, aldolase C, fructose biphosphatase (ALDO), soluble carrier family 2 (facilitated glucose transporter), member 3 (SLC2A3, GLUT5), vascular endothelial growth factor A (VEGFA), ribosomal protein L13a (RPL13A), β actin (ACTB), and hydroxymethylbilane synthase (ALDOC) was quantified by real-time RT-PCR. HMC-1 and LAD-2 cells were plated at 5 × 105 cells/well in six-well plates and activated in the presence or absence of selected inhibitors, as described in EMSA. Total RNA was extracted using the TRI Reagent (Sigma-Aldrich), according to the manufacturer’s instructions. Next, first-strand cDNAs were synthesized from 5 μg of total RNA using a RevertAid H Minus First Strand cDNA Synthesis Kit (Fermentas) according to the manufacturer’s instructions. The cDNAs were then used as templates (5 μl/reaction) for real-time PCR amplification using a LightCycler 480 (Roche) and the SYBR Green PCR master mix (Roche). All samples were run in duplicates, and PCR amplification consisted of 40 cycles of denaturation at 95°C for 10 s, annealing at 55°C for 10 s, and extension at 72°C for 20 s using the following primers: ACTB (29), sense 5′-CTGGAACGGGAAGGTGACA-3′, antisense 5′-AAGGGAGCTCTTGTTAACAATGC-3′; HMB (29), sense 5′-GCCATGCACAGGGAATC-3′, antisense 5′-GTTTGACAGATGGAT-3′; HIF1A, sense 5′-GAAAGGGCAAGTCTTCAAAG-3′, antisense 5′-TGGGTAGAGGATGATGCCC-3′; ALDOC, sense 5′-GCCCTGCATCTTGCTCCT-3′, antisense 5′-ATGTGGACACACTCATATT-3′; SLC2A3, sense 5′-CTGGTACCGGAGAAGGAGAAGA-3′, antisense 5′-TGGAAGCACTGGTCTCTTTGTTTCA-3′; HIF1A, sense 5′-GAAAGGGCAAGTCTTCAAAG-3′, antisense 5′-TGGGTAGAGGATGATGCCC-3′; ACTB, sense 5′-GCCATGCACAGGGAATC-3′, antisense 5′-GTTTGACAGATGGAT-3′; HMB, sense 5′-GCCATGCACAGGGAATC-3′, antisense 5′-GTTTGACAGATGGAT-3′; HIF1A, sense 5′-GAAAGGGCAAGTCTTCAAAG-3′, antisense 5′-TGGGTAGAGGATGATGCCC-3′; ACTB, sense 5′-GCCATGCACAGGGAATC-3′, antisense 5′-GTTTGACAGATGGAT-3′; HMB, as a reference C, value (29).

**HIF1A promoter and reporter plasmid constructs**

For cloning of the 5′-flanking region of the human HIF1A gene, two primers were designed using Primer3 software (5′-AAAGGGAAAGGGCTGCTGC-3′ and 5′-AAAGGGATCTTGCCGTC-3′) and used to make the phMut HIF1A plasmid, and after sequencing, recombined into a pLUC vector containing the HIF1A promoter as a template. The sequence of the mutated insert was verified by automated sequencing and then recloned into the plgbasic-3 vector using Acc65I and HindIII. The phMut HIF1A plasmid was linearized by EcoRI digestion and then recloned into the plgbasic vector using Acc65I and HindIII. Cloning of the plasmid.
of HIF-1α protein level in mast cells. A, HEK, HepG2, and HMC-1 cells were incubated under normoxic (21% O2) or hypoxic (1% O2) conditions with or without PMA-ionomycin. The HIF-1α protein level was determined by Western blotting. B, HMC-1 cells were incubated under normoxia or hypoxia and activated with 1 μM mastoparan (MP), 10 μM substance P (SP), 100 nM C5a, and 10 μg/ml compound 48/80 (48/80). After 5 h, the cells were lysed, and the HIF-1α protein level was analyzed by Western blotting. C, HMC-1 cells were incubated with or without ionomycin under normoxia or hypoxia. Nuclear and cytoplasmic fractions were analyzed for HIF-1α protein level by Western blotting. Immunoblot band intensity was evaluated by densitometric analysis and is expressed as the ratio of HIF-1α protein to β-actin protein (mean ± SEM) from three independent experiments. D, HMC-1 cells were preincubated for 16 h with I or without ionomycin, then incubated for additional 4 h under normoxic (N) or hypoxic (H) conditions and analyzed for HRE DNA-binding proteins by EMSA using an IRD700-labeled W18 oligonucleotide. The relative integrated density values (RIDV) were normalized to a band representing resting mast cells incubated under normoxic conditions. The results represent three independent experiments.

![Image](http://www.jimmunol.org/)

**FIGURE 1.** Effect of activation and hypoxia on the HIF-1α protein level in mast cells. A. HEK, HepG2, and HMC-1 cells were incubated under normoxic (21% O2) or hypoxic (1% O2) conditions with or without PMA-ionomycin. The HIF-1α protein level was determined by Western blotting. B. HMC-1 cells were incubated under normoxic or hypoxic conditions with or without PMA-ionomycin and activated with 1 μM mastoparan (MP), 10 μM substance P (SP), 100 nM C5a, and 10 μg/ml compound 48/80 (48/80). After 5 h, the cells were lysed, and the HIF-1α protein level was analyzed by Western blotting. C. HMC-1 cells were incubated with or without ionomycin under normoxic or hypoxic conditions. Nuclear and cytoplasmic fractions were analyzed for HIF-1α protein level by Western blotting. Immunoblot band intensity was evaluated by densitometric analysis and is expressed as the ratio of HIF-1α protein to β-actin protein (mean ± SEM) from three independent experiments. D. HMC-1 cells were preincubated for 16 h with I or without ionomycin, then incubated for additional 4 h under normoxic (N) or hypoxic (H) conditions and analyzed for HRE DNA-binding proteins by EMSA using an IRD700-labeled W18 oligonucleotide. The relative integrated density values (RIDV) were normalized to a band representing resting mast cells incubated under normoxic conditions. The results represent three independent experiments.

**Table 1. Expression of HRE-regulated genes in HMC-1 mast cells**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Normoxia-ionomycin</th>
<th>Hypoxia-ionomycin</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEGFA</td>
<td>1.82 ± 0.59</td>
<td>3.84 ± 0.76**</td>
</tr>
<tr>
<td>ALDOC</td>
<td>1.30 ± 0.09</td>
<td>4.36 ± 0.56*</td>
</tr>
<tr>
<td>SLC2A3</td>
<td>1.33 ± 0.05*</td>
<td>5.26 ± 0.50*</td>
</tr>
</tbody>
</table>

*HMC-1 mast cells were preincubated for 16 h with or without ionomycin and then incubated for an additional 4 h under normoxic or hypoxic conditions as indicated. Levels of VEGFA, ALDOC, and SLC2A3 mRNA were determined by real-time PCR as described in Materials and Methods. A data are expressed as fold increase in mRNA level normalized to the level detected in HMC-1 mast cells under normoxia and calculated by the comparative ΔΔCt method. Each value represents mean ± SEM (n = 6).

**Transient transfection and promoter activity assays**

For transfection, mast cells were suspended in a hypo-osmolar electroporation buffer (Eppendorf) at a density of 3.5 × 10⁶ cells/ml, mixed with DNA to a final concentration of 25 μg/ml and a final volume of 0.8 ml, and then electroporated in cuvets with a 4-mm gap width using a Multiporator (Eppendorf) to apply two pulses of 720 V and 100 μs. Hsp27-haptenocytes were cotransfected with combination of one of the expression plasmids pCMV6-XL5 (empty control vector) and pCMV6-NFAT1, and one of the reporter plasmids phHIF1A (−863/+5)Luc, phABCC6(−1313/+72)Luc, and pGL3-basic using the Exgene 500 transfection reagent as previously described (31). Twenty-four hours after transfection, cells were treated with 1 μM ionomycin and/or CsA or FK506 to final concentration of 1 μM and 100 nM, respectively. Cells were harvested and lysed 48 h after transfection. The luciferase activity of the cell lysates was determined in a Fluoroskan Ascent FL luminometric plate reader (Labsystems) using a commercial luciferase substrate (BD Biosciences). Additionally, all cells were transfected with the plasmid pS173, which encodes a secreted alkaline phosphatase under the control of a constitutive CMV-derived promoter, as an internal control for transfection efficiency. The level of alkaline phosphatase activity was determined spectrophotometrically.

**Chromatin immunoprecipitation (ChIP)**

ChIP was performed using the EZ-Chip kit from Upstate according to the manufacturer’s protocol. Briefly, DNA fragments immunoprecipitated with anti-RNA polymerase and anti-NFATc1 Abs were analyzed for enrichment with specific sequences by real-time PCR performed as described above, using primers for the GAPDH promoter (part of the EZ-Chip kit) and primers complementary to the HIF1A promoter (sequence positions relative to the translation start site): upstream primer −864-AAAG GAAAGGGCTTGCTGC-847 and downstream primer −701-GGCAAC CGAAATCCCTTC-684. The relative abundance of specific sequences in immunoprecipitated DNA was determined using the ΔΔCt method with C, obtained for total extracted DNA (Input DNA) as a reference value.

**Bioinformatics**

For the in silico sequence analysis, the following web servers were used: Biology Workbench (Computational Biology Group at the National Center for Supercomputing Applications at the University of San Diego, San Diego, CA); MatInspector (32); and Transfac (33).

**Statistical analysis**

Data are expressed as means ± SEM unless otherwise stated. The statistical significance of the observed differences was tested by two-way repeated measures ANOVA followed by the Holm-Sidak test using SigmaStat 2.03 software (SPSS).

**Results**

A previous report showed that HIF-1α is expressed in mast cells (18). To further explore the regulation of HIF-1α expression in...
mast cells, we activated HMC-1 mast cells with PMA and ionomycin under standard (21% O\textsubscript{2}) and hypoxic (1% O\textsubscript{2}) conditions and determined the HIF-1α protein levels by Western blot analysis. HepG2 and HEK cells were included as controls for the hypoxia effect. As seen in Fig. 1A, both hypoxia and PMA-ionomycin resulted in an increase in the level of HIF-1α protein in the HMC-1 mast cells. However, in mast cells the HIF-1α protein level induced by hypoxia was much lower than in the HepG2 and HEK control cells. In contrast to the effect of hypoxia on resting mast cells, a combination of hypoxia and PMA-ionomycin resulted in HIF-1α protein levels comparable to those observed in hypoxic HepG2 and HEK control cells. In additional control experiments, we compared the effects of PMA-ionomycin, ionomycin, and PMA alone. Ionomycin alone could increase HIF-1α expression similar to PMA and ionomycin combined and was a more potent activator than PMA (data not shown). To extend these observations to other activators of human mast cells, HMC-1 mast cells were incubated in the presence or absence of the mast cell activators mastoparan (1 μM), C5a (100 nM), and substance P (10 μM) under normoxic and hypoxic conditions. C5a and substance P resulted in significant increases in the amount of HIF-1α under hypoxic conditions (Fig. 1B). To further analyze the role of HIF-1α expression in HMC-1 mast cells, we investigated the level of HIF-1α in nuclear extracts after ionomycin treatment. As seen in Fig. 1C, both hypoxia and ionomycin resulted in increased levels of nuclear HIF-1α in the HMC-1 cells compared with the control cells (resting cells incubated under normoxic conditions). The amount of nuclear HIF-1α in cells incubated with ionomycin under hypoxic conditions was significantly higher than those observed with hypoxia or ionomycin alone. Next, the HIF-1 DNA binding activity of nuclear extracts from activated HMC-1 mast cells was determined by EMSA. As seen in Fig. 1D, the slowly migrating DNA-protein complexes were observed in resting cells, but treatment with ionomycin, hypoxia, or a combination of both resulted in a visible increase in the
amount of DNA-protein complexes. The strongest effect was observed when cells were treated with a combination of ionomycin and hypoxia. The specificity of the bands was confirmed by competition with an excess of unlabeled oligonucleotides (Fig. 1D), and a control reaction was performed with an oligonucleotide mutated in the HRE motif (data not shown). Next, we analyzed the effect of hypoxia and ionomycin on the expression of selected HIF-1-regulated genes. HMC-1 cells were incubated under normoxic or hypoxic conditions, with or without ionomycin and lysed, and the mRNA was isolated and transcribed to cDNA. The relative expression of VEGFA, ALDOC, and SLC2A3 was determined by real-time PCR. As expected, hypoxia resulted in a significant increase in the expression of HRE-regulated genes compared with normoxia (Table I). The combination of hypoxia and ionomycin resulted in significantly higher SLC2A3 and ALDOC expression levels (Table I) than ionomycin and hypoxia alone. Although expression of SLC2A3 was already increased after 4 h of incubation with ionomycin under normoxic conditions (data not shown), the increased ALDOC expression was observed only under hypoxic conditions and required longer incubations with ionomycin (Table I). VEGF expression was not modulated by ionomycin under any tested conditions. In additional series of experiments, CsA (1 μM) inhibited ionomycin-induced SLAC3 expression in LAD-2 (87%) and HMC-1 (40%) mast cells. Thus, exposure to ionomycin resulted in up-regulation of HIF1A expression, accumulation of the HIF-1α protein and increased expression of some of the HIF-1-regulated genes in mast cells under hypoxic conditions.

Next, we addressed which signaling pathways were responsible for the ionomycin-mediated accumulation of HIF-1α in HMC-1 cells. In a screening experiment, HMC-1 cells were exposed to ionomycin under hypoxic conditions in the absence or presence of the following inhibitors: 10 μM concentrations of the MAPK kinase inhibitor U0126, 30 μM concentrations of the calmodulin-independent kinase inhibitor HA1077, 10 μM concentrations of the
protein kinase C inhibitor bisindoylmaleimide, 1 μM concentrations of the CaN inhibitor CsA, 100 nM PI3K inhibitor wortmannin, 10 μM concentrations of the c-Jun kinase inhibitor SP600125, 1 μM concentration of the p38 kinase inhibitor SB203580, 10 μM concentrations of the kinase inhibitor KN93 and 5 μM concentrations of the protein kinase A inhibitor H89. As seen in Fig. 2A, the inhibitors of CaN (CsA) and to lesser degree MAPK kinase (U0126) significantly blocked the up-regulation of HIF1A expression in the HMC-1 cells incubated with ionomycin under hypoxic conditions. High concentrations of CsA can interfere with the mechanism that regulate degradation of HIF-1α (34). Therefore, we determined the minimal CsA concentration required for inhibition of HIF-1α protein accumulation and tested whether another CaN inhibitor, FK506, had a similar effect. HMC-1 mast cells were activated with ionomycin under hypoxic conditions in the absence (control) or presence of increasing concentrations of CsA or FK506. Both inhibitors resulted in a dose-dependent decrease in the level of HIF-1α (Fig. 2B). An inhibitory effect was achieved with 100 nM CsA and 1 nM FK506, and higher concentrations (0.5 μM CsA and 10 nM FK506) resulted in complete inhibition of HIF1A expression. Next, we verified the effect of the transcription inhibitor ActD on HIF1A expression in HMC-1 mast cells activated with ionomycin under hypoxic conditions. As seen in Fig. 2C, 5 μM ActD completely abolished the up-regulation of HIF1A observed in activated mast cells after 5 h of ionomycin exposure. ActD significantly (56%) reduced the amount of HIF-1α protein when added 1 h after the addition of ionomycin. This inhibitory effect of ActD was not observed when the inhibitor was added 2 or 3 h after the addition of ionomycin. This suggests that transcription occurring after ionomycin activation is necessary for the accumulation of HIF-1α. Therefore, we investigated the effect of ionomycin and hypoxia on HIF-1α mRNA in HMC-1 mast cells. In a series of experiments, HMC-1 mast cells were activated with ionomycin under normoxic and hypoxic conditions for 4 h, and the HIF-1α mRNA were levels analyzed by real-time PCR. Ionomycin exposure resulted in a significant 5-fold increase in the HIF-1α mRNA level under normal and hypoxic conditions (Fig. 3A). In additional series of experiments, we investigated the effect of thapsigargin (1 μM) on the level of HIF-1α mRNA in HMC-1 mast cells under normoxic conditions and observed 13.2 ± 0.22-fold increase of the level of HIF-1α mRNA (n = 3, statistically significant at p < 0.05). Selective up-regulation of a HIF-1α mRNA variant, from an alternative transcription initiation site within exon I, has been reported in activated lymphocytes (30). Therefore, we tested whether one or both HIF1A splice variants were up-regulated in activated mast cells. Using isoform specific primers, only the HIF-1α mRNA originating from the primary transcription initiation site was detected in resting and ionomycin-activated HMC-1 mast cells (data not shown). Next, we verified that up-regulation of HIF1A expression by ionomycin treatment was not limited to HMC-1 cells but could be observed in other mast cell lines. As illustrated in Fig. 3B, human LAD-2 mast cells showed a dose-dependent increase in HIF-1α mRNA in response to ionomycin treatment. The CsA and U0126 inhibitors blocked the ionomycin-mediated accumulation of HIF-1α mRNA in response to ionomycin treatment. CsA and U0126 inhibited the ionomycin-mediated up-regulation of HIF1A transcription. CsA almost completely blocked the increased HIF1A transcription induced by ionomycin (Fig. 3B). A weaker inhibitory effect was also observed with U0126 (data not shown). Next, endogenous activators were tested, and incubation of HMC-1 (data not shown) and LAD-2 (Fig. 3C) human mast cells with substance P under normoxic or hypoxic conditions resulted in significant increases in the HIF-1α mRNA levels. The increased expression was sensitive to CsA (Fig. 3C). The ionomycin- and substance P-induced increase in HIF-1α mRNA is consistent with transcriptional up-regulation of the HIF1A gene. To test this hypothesis, a reporter plasmid was made containing the promoter region of the HIF1A gene. Human mast cells were transiently transfected with this constructs, activated with ionomycin, and assayed for promoter activity (Fig. 4A). A 5-fold increased activity was observed and the increase could be inhibited by CsA and FK506 treatment (Fig. 4A). Further analysis of the HIF1A promoter identified a consensus NFAT site at position −728 bp that is evolutionarily conserved (Fig. 4B). To verify that this NFAT-binding motif was required for CaN-dependent up-regulation of the promoter activity, a reporter plasmid containing a 3-bp mutation in the NFAT binding site was made and tested in a series of promoter activity assays (Fig. 4C). Resultant data showed decrease in ionomycin-mediated promoter activity (up to 50%) similar to that observed in other promoter following the same 3-bp substitution of the single NFAT binding site (35). Thus, the activity of the mutated promoter could not be induced efficiently by ionomycin consistent with the −863 to +5 region of the HIF1A gene being ionomycin- and NFAT-dependent in human mast cells. To further verify the role of NFAT in regulation of HIF1A promoter HepG2 cells were cotransfected with the reporter plasmids pHIF1A (−863/+5)Luc and the NFATC4 expression plasmid pCMV6-NFAT. As seen in Fig. 4D overexpression of NFATC4 resulted in 2.3-fold increase in the promoter activity of HIF1A but not the control promoter (ABC6) which does not contain NFAT binding site consistent with NFAT being capable to up-regulate HIF1A promoter activity ChIP analysis was used to further document the role of the NFAT motif in the regulation of HIF1A gene expression. First, HMC-1 cells were incubated without (control) and with ionomycin for 4 h, collected, and lysed with a non-ionic detergent. Next, chromatin was isolated, fragmented by sonication, and subjected to immunoprecipitation with control, anti-polymerase, and anti-NFAT Abs. The immunoprecipitates were analyzed for the presence of selected HIF1A promoter sequences by PCR (Fig. 5). Ionomycin treatment resulted in a several fold increase in the amount of promoter sequences in DNA immunoprecipitated with the anti-NFAT Ab. The ionomycin treatment also resulted in a 2-fold increase in the amount of promoter sequence DNA immunoprecipitated with the anti-polymerase Ab

**FIGURE 5.** Relative in vivo association of the NFAT1 transcription factor with the human GADPH and HIF1A promoter regions. HMC-1 mast cells were incubated in medium alone and with 1 μM ionomycin under normoxic conditions for 4 h and collected by centrifugation. Cells were lysed, and chromatin was isolated as described in Materials and Methods. Chromatin was fragmented by sonication, and the fragments were immunoprecipitated with anti-NFATc1 Abs and quantified by real-time PCR using primers specific to the GADPH and HIF1A promoters. The data were normalized to the total input of DNA used before immunoprecipitation. **,** Statistically significant (p < 0.001) difference as compared with cells cultured in medium alone.
(data not shown). Thus, NFAT preferentially associates with HIF1A promoter sequences in activated human mast cells.

Discussion
Based on the location of mast cells and their involvement in different inflammatory reactions, it is expected that mast cell activation would frequently occur in vivo during insufficient oxygen supply. Here, we investigated the effect of mast cell activation on expression of the nuclear transcription factor HIF-1α, which is a central component of the major hypoxia-sensing mechanism in eukaryotic cells. Human mast cells were activated with the calcium ionophore ionomycin (Fig. 1A) and other mast cell activators (Fig. 1B) under hypoxic conditions. The cells showed significantly up-regulated HIF1A expression far exceeding the expression levels observed in mast cells exposed to hypoxic conditions or activated under normoxic conditions. Ionomycin was previously reported to increase trans activation of HIF-1 in HepG2 cells, but not the level of the HIF-1α protein (36, 37). Ionomycin-induced HIF-1α reached its physiological targets, because the activated mast cells had increased levels of HIF-1α and HRE-binding protein in their nuclei (Fig. 1, C and D). These observations are consistent with ionomycin enhancing the expression level of the HIF-1-regulated genes SLC2A3 (GLUT3) and ALDOC in mast cells exposed to hypoxia (Table I). The increase in the HIF-1α protein level was sensitive to the transcription inhibitor ActD (Fig. 2, A and C); this indicates that the novel transcription of HIF1A gene is responsible for the increased protein level and that the ionomycin effect involves transcriptional regulation. Using pharmacological inhibitors, the MAPK signaling cascade and the CaN-NFAT pathway were identified as possible mediators of the effect of ionomycin on HIF1A expression (Fig. 2, A and B). The CaN-NFAT pathway has not been implicated in regulation of HIF-1, although CsA was reported to decrease the HIF-1α protein level by activating enzymatic hydroxylation of the Pro404 residue in HIF-1α (34). However, the accumulation of HIF-1α observed in ionomycin-activated mast cells exposed to hypoxia was similarly sensitive to FK506 (Fig. 2B), which does not interfere with hypoxia-mediated HIF-1α protein accumulation (38). These observations support the hypothesis that CaN is involved in ionomycin-mediated transcriptional up-regulation of HIF1A expression in activated mast cells. This mechanism is consistent with the observation that ionomycin significantly increased the HIF-1α mRNA level in mast cells (Fig. 2, A and B) by a process sensitive to ActD (data not shown) and CsA (Fig. 3C). The HIF-1α mRNA level increased in response to both ionomycin and the endogenous activator substance P, in a CaSsA sensitive way (Fig. 3D). The capability of substance P to up-regulate HIF1A expression is particularly interesting because substance P and its receptor were both up-regulated under hypoxic conditions in lungs (39), and this up-regulation was linked to the development of pulmonary edema, an inflammatory lung condition that potentially involves mast cell-derived mediators (40). The sensitivity of HIF-1α mRNA up-regulation in mast cells to CsA might be important for understanding the mechanisms underlying the beneficial effects of this drug observed in animal model of hypoxia-induced pulmonary hypertension (41).

The hypothesis that CaN-NFAT mediates the increased HIF1A expression is strongly supported by the HIF1A promoter activity observed in the activated mast cells (Fig. 4A). The role of NFAT is further supported by the observation that the ionomycin-mediated promoter activity depends in part on a conserved NFAT binding motif in the promoter sequence (Fig. 4, B and C). Additional promoter sequences capable of NFAT binding such as NFXb site (23, 42) might be engaged in this process as the mutation of the single consensus NFAT site did not abolish completely ionomycin-mediated promoter activity. The role of NFAT in this process is supported by observation that expression of NFAT protein up-regulates HIF1A promoter activity in HepG2 cells (Fig. 4D) and by the ChIP data that document a preferential association of NFAT with HIF1A promoter sequences after mast cell activation (Fig. 5). The increased HIF1A gene expression, mediated by CaN-NFAT, is consistent with the small increase in the HIF-1α level observed in mast cells activated with ionomycin under normoxic conditions and the substantial accumulation of HIF-1α in activated mast cells under hypoxic conditions (Fig. 1, A and B). Under hypoxic conditions, the effect may arise from a combination of transcriptional up-regulation and posttranslational up-regulation of HIF-1α, mediated by oxygen-sensitive proline hydroxylases.

To our knowledge, this is the first observation of NFAT- and CaN-dependent transcriptional regulation of HIF1A expression. CaN is a central signal molecule that regulates the expression of multiple cytokines in immune cells (43, 44). In mast cells, CaN controls the expression of IL-4, IL-5, TNF, and MIP-1α (45). Understanding the role of CaN and NFAT signaling in the regulation of HIF-1α accumulation is critical in understanding how mast cells function in pathologic conditions such as asthma (9, 10, 14), rheumatoid diseases (11, 15), keloid formation (19), and tumorigenesis (12, 13, 16).

Disclosures
The authors have no financial conflict of interest.

References


