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Aurelia Walczak-Drzewiecka; ... et. al

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

Aurelia Walczak-Drzewiecka, Marcin Ratajewski, Waldemar Wagner, and Jaroslaw Dastych[2]

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), is 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.**

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Mast cells are hemopoietic cells that reside in a variety of tissues where they function as important components of innate and acquired immunity (1, 2). Mast cells play important roles in multiple physiological and pathological processes, including anaphylaxis and allergic inflammation (2, 3), host defense against bacterial (4) and parasitic infection (5), control of endothelin-mediated toxicity (6), regulation of angiogenesis (7), and remodeling of the extracellular matrix (8). Several pathological conditions are associated with accumulation and activation of mast cells in specific organs and tissues, such as bronchi of asthmatic patients (9, 10), synovial fluids of patients with rheumatic diseases (11), and certain types of solid tumors in cancer patients (12, 13). These tissues are typically exposed to prolonged or intermittent decreases in partial pressure of oxygen (14–16), which implies that the mast cells function under hypoxic conditions. Currently, it is largely unknown how hypoxia can modulate mast cell function and activation, but there are data to support a role of mast cells in the development of hypoxia-induced inflammation (17–19).

A key regulator of cellular response to hypoxia is hypoxia-inducible factor 1 (HIF-1).[3] HIF-1 is critical for adaptation to oxygen deficit and it regulates the expression of genes involved in multiple cellular and physiological processes, including glucose transport, energy metabolism, erythropoiesis, and angiogenesis (20, 21). The HIF-1 nuclear transcription factor is a dimer of Ah receptor nuclear translocator and hypoxia-inducible factor 1α (HIF-1α) proteins. Ah receptor nuclear translocator has a long half-life that is not affected by hypoxic conditions, whereas HIF-1α is extremely unstable due to ubiquitin-dependent proteasomal degradation. The rate of HIF-1α degradation is modulated by oxygen, which is required for the hydroxylation of HIF-1α proline residues and consequently, efficient ubiquitination. Lack of oxygen prevents proline hydroxylation and in turn blocks degradation of the HIF-1α protein. This results in HIF-1α accumulation and the formation of HIF-1 dimers (22). The HIF-1α subunit (basic helix-loop-helix transcription factor (*HIF1A*) gene is constitutively expressed in many cell types, but HIF-1α mRNA accumulation has been documented in monocytes activated with LPS (23).

In this study, we investigated the expression and function of HIF-1α in resting and activated human mast cells. Exposure of human mast cells to ionomycin and substance P leads to up-regulation of *HIF1A* gene expression and this response involves the calcineurin (CaN)-NFAT signaling pathway and a specific NFAT binding site in the distal *HIF1A* promoter. This mechanism results in accumulation of HIF-1α protein in mast cells exposed to hypoxic conditions and activated with ionomycin and substance P, when compared with mast cells exposed to hypoxic conditions without the presence of these two activators.

Materials and Methods

Materials

IMDM, L-glutamine, penicillin-streptomycin, FCS, substance P, wortmannin, mastoparan, compound 48/80, ionomycin, PMA, SB203580, actinomycin D (ActD), bisindoylmaleimide, and protease inhibitors were purchased from Sigma-Aldrich; U0126, HA-1077, KN93, H89, and FK506 were purchased from LC Laboratories; SP600125 and cyclosporin A (CsA) were purchased from Calbiochem; Stem Pro-34 serum-free medium was purchased from Invitrogen; human recombinant stem cell factor was purchased from Immunotools; restriction enzymes and the pUC19 plasmid

Institute of Medical Biology, Polish Academy of Sciences, Lodz, Poland

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[2] Address correspondence and reprint requests to Dr. Jaroslaw Dastych, Institute of Medical Biology Polish Academy of Sciences, Lodowa 106 Strasse, 93-232 Lodz, Poland. E-mail address: jdastych@cbm.pan.pl

[3] Abbreviations used in this paper: HIF-1, hypoxia-inducible transcription factor 1; HIF-1α, hypoxia-inducible transcription factor 1α; HRE, hypoxia-responsive element; *HIF1A*, hypoxia-inducible factor 1, α subunit (basic helix-loop-helix transcription factor) gene; CaN, calcineurin; CsA, cyclosporine A; ActD, actinomycin D; ChIP, chromatin immunoprecipitation.

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were purchased from Fermentas; the plasmid pGL3-basic was obtained from Promega; the pCMV6-XL5 and pCMV6-NFAT expression plasmids were obtained from Origene Technologies; the anti-HIF-1α Ab was purchased from Abcam; an anti-RNA polymerase II (CTD448) Ab was purchased from Upstate; anti-NFATc1 (7A6), anti-actin, and HRP-linked anti-rabbit IgG Abs were purchased from Santa Cruz Biotechnology; SYBR Green PCR master mix and protease inhibitor mixture tablets were purchased from Roche. All other reagents were of the highest grade available. The LAD-2 mast cell line was a gift from Dr. A. Kirschenbaum (National Institutes of Health, Bethesda, MD). The plasmid pSS173 was a gift from Dr. S. Schlatter (Eidgenoessische Technische Hochschule, Zurich, Switzerland).

Mast cell cultures

HMC-1 mast cells (24) were cultured in IMDM supplemented with 10% heat-inactivated FCS, 4 mM L-glutamine, 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 CO_2 incubator.

Mast cell activation under normoxic and hypoxic conditions

HMC-1 and LAD-2 mast cells were suspended in culture medium at 10^6 cells/ml, and aliquots of the cell suspensions were placed in wells of six-well plates. Medium alone, DMSO (carrier), PMA, ionomycin, substance P, mastoparan, C5a, and 48/80 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 CO_2 incubator. 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 mix consisting of 94% N_2, 5% CO_2, and 1% O_2, and the outer chamber was purged with N_2. The O_2 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 N_2 atmosphere.

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 IGEPAL CA-630, 20 mM EDTA, 150 mM NaCl, 1 mM $MgCl_2$, 10 mM tetrasodium pyrophosphate, 100 mM sodium fluoride, 2 mM sodium orthovanadate, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 1 mM PMSF. Lysates were centrifuged at 10,000 × *g* at 4°C for 15 min, and the supernatants were used for SDS-PAGE. Nuclear extracts and cytoplasmic fractions were obtained using a modification of the technique described by Dignam et al. (27). Briefly, cells were lysed by pipeting the cell suspensions in a buffer containing 0.4% IGEPAL CA-630, 10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, and a mixture of protease inhibitors consisting of 1× Roche protease inhibitor mixture and additionally 50 mM ε-amino-*n*-caproic acid, 7 μg/ml pepstatin, 150 μg/ml soybean trypsin inhibitor, 27 mM iodoacetamide, 32 mM benzamidine hydrochloride, 1.7 μg/ml leupeptin, 8.5 μg/ml aprotinin, 10 μM PMSF, and 12.5 μM lactacystin. Lysates were centrifuged at 15,000 × *g* for 3 min. Supernatants containing the cytosolic fraction were used for Western blot analysis, and the pellets were suspended in a buffer containing 20 mM HEPES (pH 7.9) 0.4 M NaCl, 1 mM EDTA, and 10% glycerol; shaken vigorously at 4°C for 2 h; and centrifuged at 15,000 × *g* for 5 min. The resulting supernatants contained nuclear extracts and were used directly in Western blots and EMSA or stored at −70°C. Protein concentrations were determined using the bicinchoninic acid assay (Pierce).

Western blotting

Equal amounts of protein (cell lysate, nuclear extract, or cytoplasmic fraction) were mixed with sample buffer (Invitrogen) containing lithium dodecyl sulfate and DTT, incubated for 10 min at 70°C, and separated on 4–12% Bis-Tris NuPAGE gels (Invitrogen). Proteins were electrotransferred to nitrocellulose and immunoblotted with the anti-HIF-1α Ab followed by an HRP-conjugated secondary Ab. Membranes were stripped and reprobed with an anti-actin Ab followed by a secondary HRP-conjugated Ab. Specific bands were visualized by chemiluminescence using the SuperSignal Chemiluminescent Substrate (Pierce).

EMSA

The infrared dye-labeled oligonucleotide probe IRD700-W18 (28) representing the sequence 5′-GCCCTACGTGCTGTCTCA-3′ (*EPO* hypoxia-responsive element; HRE) was obtained by annealing two complementary oligonucleotides labeled on their 5′ ends with IRD700. The DNA-binding reaction was conducted by mixing a volume of nuclear extract equivalent to 1.5 μg of protein with a DNA-binding buffer consisting of 5 mM Tris (pH 8.0), 50 mM NaCl, 5 mM DTT, 5 mM $MgCl_2$, 0.5% Igepal, 25% glycerol, and 0.5 μg of salmon testis DNA. The reaction mixture was assembled on ice and followed by incubation for 20 min on ice with 10 fmol of the IRD700-W18 probe. For supershift experiments, 2 μg of rabbit polyclonal anti-HIF-1α Ab were added to reaction mixture 2 h before addition of the probe. The resulting complexes were resolved on 7.5% polyacrylamide gels run in 0.5% Tris-borate EDTA buffer at 160 V for 100 min at 4°C. Gels were scanned with the infrared scanner Odyssey (LI-COR Biosciences) followed by densitometry analysis with the AlphaEaseFC software (Alpha Innotech).

Real-time PCR

Expression of *HIF1A*, aldolase C, fructose biphosphate (*ALDOC*), solute carrier family 2 (facilitated glucose transporter), member 3 (*SLC2A3, GLUT3*), vascular endothelial growth factor A (*VEGFA*), ribosomal protein L13a (*RPL13A*), β actin (*ACTB*), and hydroxymethylbilane synthase (*HMBS*) was quantified by real-time RT-PCR. To this end, HMC-1 and LAD-2 cells were plated at 5 × 10^6 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 instruction. 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′-CTGGAACGGTGAAGGT GACA-3′, antisense 5′-AAGGGACTTCCTGTAACAATGCA-3′; *HMB* (29), sense 5′-GGCAATGCGGCTGCAA-3′, antisense 5′-GGGTAC CCACGCGAATCAC-3′; *RPL13A* (29), sense 5′-CCTGGAGGAGAA GAGGAAAGAGA-3′, antisense 5′-TTGAGGACCTCTGTGTATTTGT CAA-3′; *HIF1A,* sense 5′- GAAAGCGCAAGTCTTCAAAG-3′, antisense 5′-TGGGTAGGAGATGGAGATGC-3′; hHIFexI.1 (30), sense 5′-CAC CTCTGGACTTGCCTTTCCTTC-3′; HIFexI.2 (30), sense 5′-ATGTG CAAGACACTGCATTCTTAG-3′; HIFexI and hHIFexI.2 common (30), antisense 5′-CACCAGCATCCAGAAGTTTCCTCAC-3′; *VEGFA,* sense 5′-TTCATGGATGTCTATCAGCGCA-3′, antisense 5′-CCGCATA ATCTGCATGGTGA-3′; *SLC2A3,* sense 5′-CGTGGCAGGACTTTT GAGGAT-3′, antisense 5′-AGCAGGCTCGATGCTGTTCAT-3′; *ALDOC,* sense 5′-GCGCTGTGTGCTGAAAATCAG-3′, antisense 5′-CCACAATAGGCACAATGCCATT-3′. The value of C_t for each reaction was determined using LightCycler 480 software, and the relative gene expression was calculated using the comparative $\Delta\Delta C_t$ method with the geometric mean of C_t obtained for three housekeeping genes, *RPL13A*, *ACTB*, and *HMBS,* as a reference C_t 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′-AAAGGAAGGGCTT GCTGC-3′ and 5′-GAAGGGATTTCGGTTGCC-3′) and used to PCR amplify an 868-bp (−863 to +5 of *HIF1A*, counting from ATG) fragment. The PCR product was purified and cloned into the pUC19 vector using *Hinc*II and, after sequencing, recloned into a promotorless pGL3-basic vector using *Acc*65I and *Hin*dIII, yielding the plasmid ph *HIF1A* (−863/+5)Luc. To make the phMut *HIF1A* (−863/+5)Luc reporter plasmid with the *HIF1A* promoter carrying mutations in the potential NFAT binding site, an antisense primer (5′-AAACATGCATCATATcTTcaTACTCTTGGT-3′) and sense primer (5′-ACCAAGAGTAtcAAcATATGATGCATGTTT-3′) were used in a PCR reaction with 10 ng of the pUC19 vector containing the *HIF1A* promoter as a template. The sequence of the mutated insert was verified by automated sequencing and then recloned into the pGL3-basic vector using *Acc*65I and *Hin*dIII. Cloning of the plasmid

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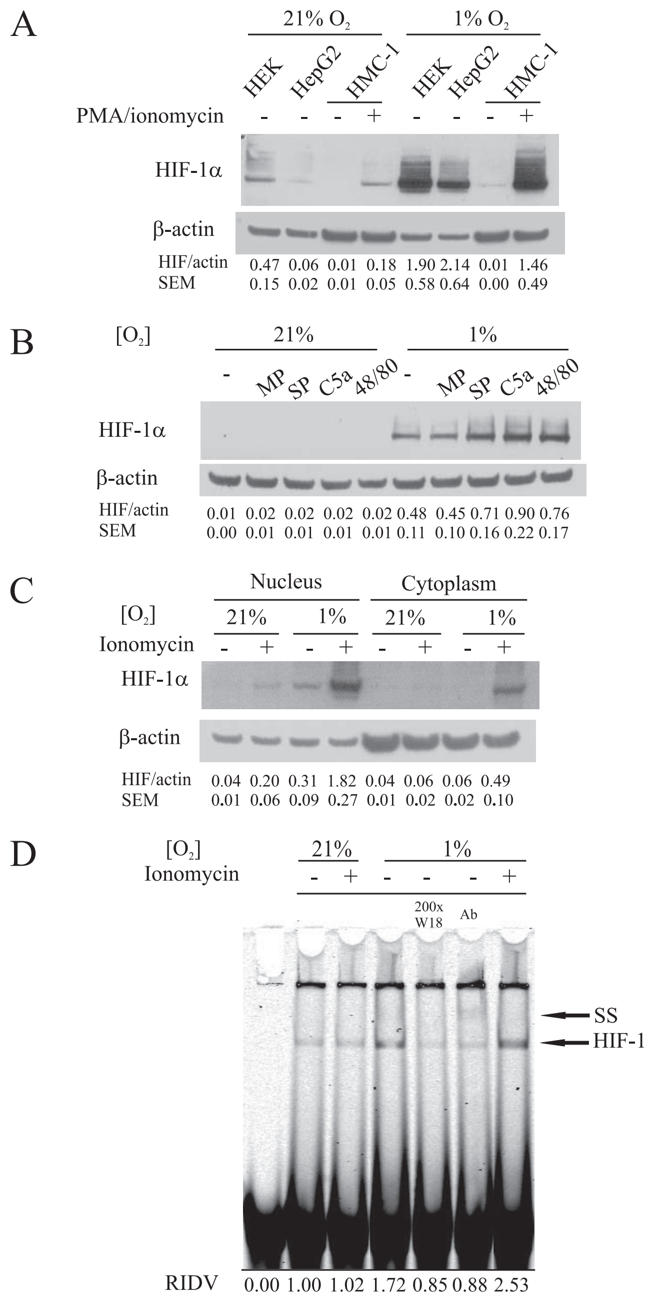

FIGURE 1. Effect of activation and hypoxia on the HIF-1α protein level in mast cells. *A*, HEK, HepG2, and HMC-1 cells were incubated under normoxic (21% O_2) or hypoxic (1% O_2) conditions with or without PMA-ionomycin. The HIF-1α protein level was determined by Western blotting. *B*, HMC-1 cells were 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.

Table I. *Expression of HRE-regulated genes in HMC-1 mast cells*[a]

Gene	Fold Increase[b]		
	Normoxia-ionomycin	Hypoxia	Hypoxia-ionomycin
VEGFA	1.82 ± 0.59	3.84 ± 0.76*[c]	4.07 ± 0.24*
ALDOC	1.30 ± 0.09	4.36 ± 0.56*	7.08 ± 1.30*§
SLC2A3 (*GLUT3*)	1.33 ± 0.05*	5.26 ± 0.50*	9.17 ± 1.17*§

[a] 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*.

[b] 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 $\Delta\Delta C_t$ method. Each value represents mean ± SEM ($n = 6$).

[c] *, significantly different ($p < 0.05$) compared with resting under normoxic conditions; §, significantly different ($p < 0.05$) compared with resting under hypoxic conditions.

phABCC6(−1313/+72)Luc used as a negative control was previously described (31).

Transient transfection and promoter activity assays

For transfection, mast cells were suspended in a hypo-osmolar electroporation buffer (Eppendorf) at a density of 3.5×10^6 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. HepG2 hepatocytes were cotransfected with combination of one of the expression plasmids pCMV6-XL5 (empty control vector) and pCMV6-NFAT, and one of the reporter plasmids ph*HIF1A* (−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 pSS173, 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 GAAGGGCTTGCTGC-847 and downstream primer −701-GGCAAC CGAAATCCCTTC-684. The relative abundance of specific sequences in immunoprecipitated DNA was determined using the $\Delta\Delta C_t$ method with C_t 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

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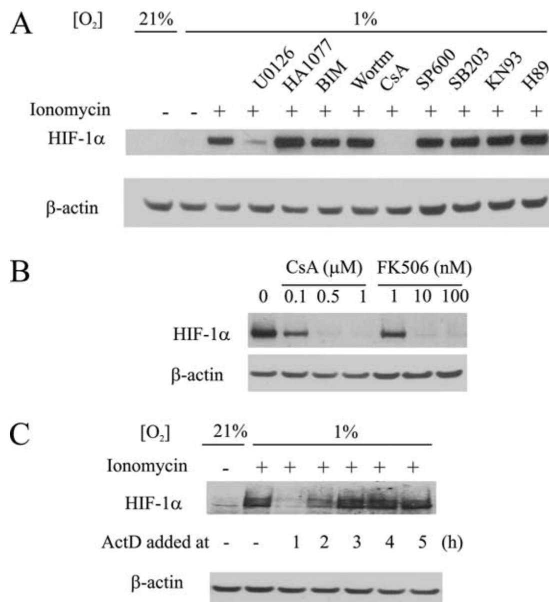

FIGURE 2. Effect of pharmacological inhibitors on ionomycin- and hypoxia-mediated HIF-1α protein accumulation in mast cells. *A*, HMC-1 cells were incubated under normoxic (21% O_2) and hypoxic (1% O_2) conditions with or without ionomycin and with or without the inhibitors 10 μM U0126, 30 μM HA1077, 10 μM bisindoylmaleimide (BIM), 1 μM CsA, 100 nM wortmannin (Wortm), 10 μM SP600125 (SP600), 1 μM SB203580 (SB203), 10 μM KN93 (KN93), and 5 μM H89 (H89). After 5 h, the cells were lysed and analyzed for HIF-1α protein expression by Western blotting. *B*, HMC-1 cells were incubated under hypoxic conditions with ionomycin alone (0) or ionomycin and the indicated concentrations of CsA and FK506. After 5 h of incubation, the cells were lysed, and the HIF-1α protein levels were measured by Western blotting. *C*, HMC-1 cells were incubated for 5 h under normoxic (21% O_2) or hypoxic (1% O_2) conditions with or without ionomycin. The transcription inhibitor ActD was added at the indicated time. Cells were collected by centrifugation, lysed, and analyzed for HIF-1α protein levels by Western blotting. The results are represent three independent experiments.

mast cells, we activated HMC-1 mast cells with PMA and ionomycin under standard (21% O_2) and hypoxic (1% O_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. 1*A*, 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 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. 1*B*). 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. 1*C*, 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. 1*D*, 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

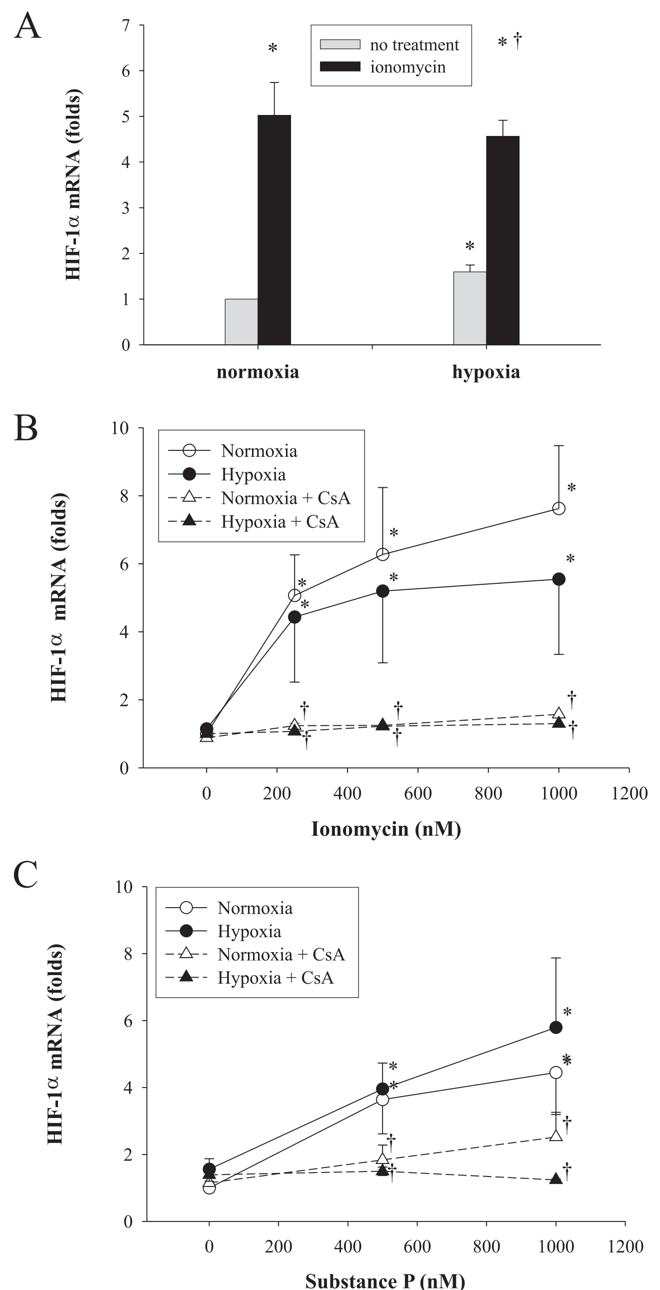

FIGURE 3. Effect of activation and hypoxia on *HIF1A* expression in mast cells. HMC-1 (*A*) and LAD2 (*B* and *C*) mast cells were incubated with or without ionomycin (*A* and *B*) and with or without the indicated concentration of substance P (*C*) and with or without 1 μM CsA (*C*) under normoxic or hypoxic conditions for 4 h. Cells were collected by centrifugation, and the HIF-1α mRNA levels were determined by real-time PCR as described in *Materials and Methods*. Each point represents mean ± SEM for at least three independent experiments. ∗. Statistically significant ($p < 0.05$) difference as compared with resting cells under normoxic conditions; †, statistically significant ($p < 0.05$) difference as compared with substance P (without CsA).

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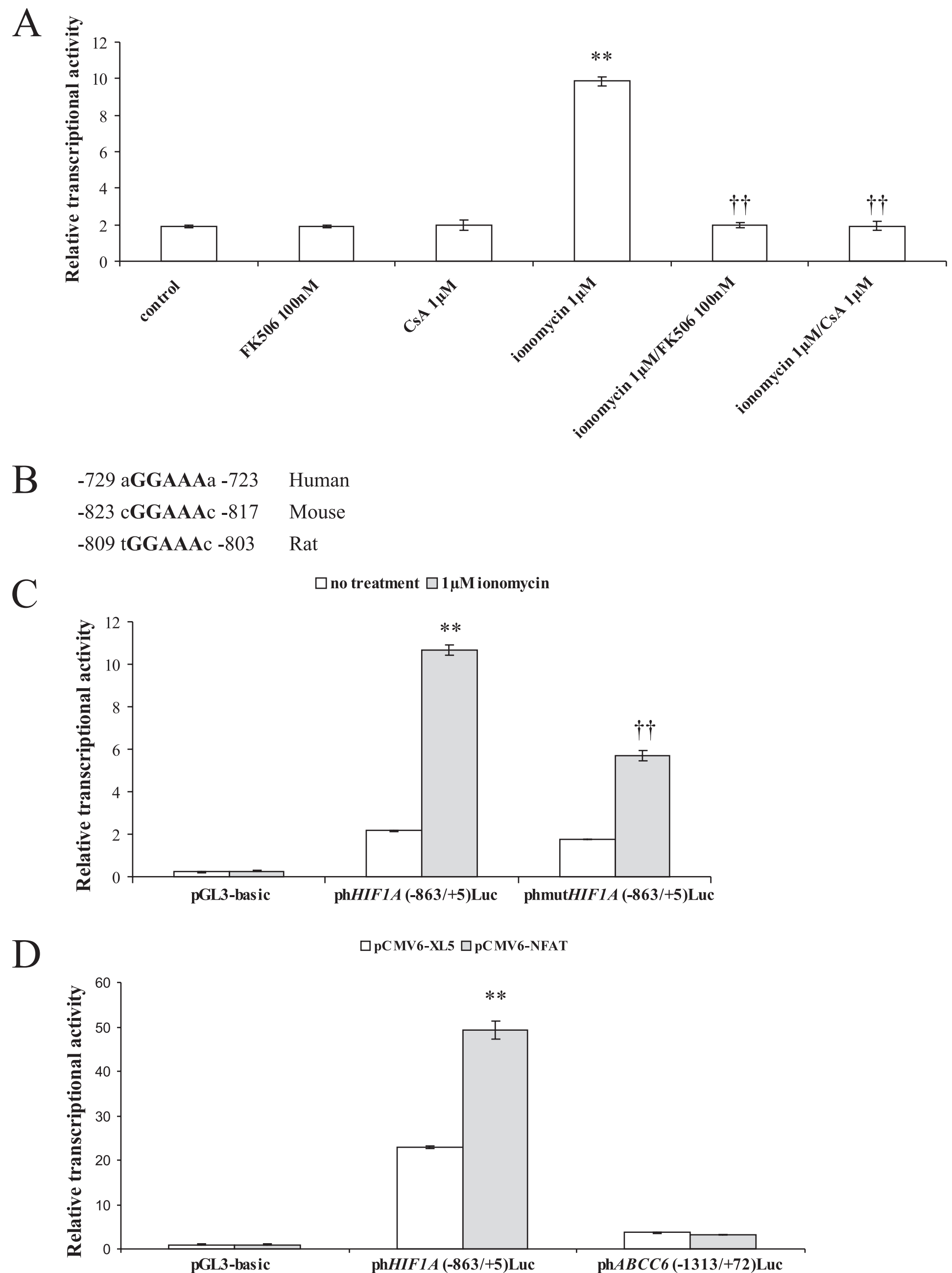

FIGURE 4. Effect of ionomycin, FK506, CsA, and overexpression of NFAT on the activity of the human *HIF1A* promoter. *A* and *C*, HMC-1 cells were cotransfected with ph*HIF1α*(−863/+5)Luc (*A* and *C*) or promotorless pGL3-basic (*C*) or phMut*HIF1A*(−863/+5)Luc (*C*), and pCMV-SEAP (*A* and *C*) plasmids. After transfection, the cells were incubated in medium alone, with 1 μM ionomycin and with a combination of 1 μM ionomycin and 1 μM CsA or 1 μM ionomycin and 100 nM FK506 under normoxic conditions for 48 h. *D*, HepG2 cells were cotransfected with ph*HIF1α*(−863/+5)Luc or ph*ABCC6*(−1313/+72)Luc (as negative control) or promotorless pGL3-basic and pCMV-SEAP. Cells were also transfected (*D*) with an expression vector for human NFAT- (pCMV6-NFAT) or with the empty vector (pCMV-XL5). The cells were collected by centrifugation and lysed, and the luciferase and secreted alkaline phosphatase activities were determined. The level of luciferase activity was normalized to the secreted alkaline phosphatase activity. *B*, The sequences of putative NFAT binding sites in distal fragments of human, mouse, and rat 5′upstream regions of the *HIF1A* gene were identified using MatInspector software. **, Statistically significant ($p < 0.001$) difference compared with resting cells; ††, statistically significant ($p < 0.001$) difference compared with ionomycin-activated cells.

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. 1*D*), 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-dependent kinase inhibitor HA1077, 10 μM concentrations of the

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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. 2*A*, 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. 2*B*). 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. 2*C*, 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. 3*A*). 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. 3*B*, 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 protein, and it was therefore tested whether these two inhibitors would interfere with ionomycin-mediated up-regulation of *HIF1A* transcription. CsA almost completely blocked the increased *HIF1A* transcription induced by ionomycin (Fig. 3*B*). 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. 3*C*) 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. 3*C*). 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. 4*A*). A 5-fold increased activity was observed and the increase could be inhibited by CsA and FK506 treatment (Fig. 4*A*). Further analysis of the *HIF1A* promoter identified a consensus NFAT site at position −728 bp that is evolutionarily conserved (Fig. 4*B*). 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. 4*C*). 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 ph*HIF1A* (−863/+5)Luc and the NFATC4 expression plasmid pCMV6-NFAT. As seen in Fig. 4*D* overexpression of NFATC4 resulted in 2.3-fold increase in the promoter activity of *HIF1A* but not the control promoter (*ABCC6*) that 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 severalfold 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

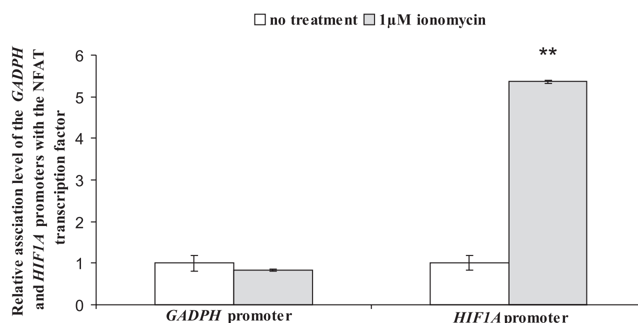

FIGURE 5. Relative in vivo association of the NFAT1 transcription factor with the human *GADPH* and *HIF1A* promoter regions. HMC-1 mast cells were incubated in medium alone and with 1 μM ionomycin under normoxic conditions for 4 h and collected by centrifugation. Cells were lysed, and chromatin was 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.

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(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. 1*A*) and other mast cell activators (Fig. 1*B*) 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 de novo 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 Pro^{564} 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. 2*B*), 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. 3*C*). The HIF-1α mRNA level increased in response to both ionomycin and the endogenous activator substance P, in a CsA sensitive way (Fig. 3*D*). 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. 4*A*). 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 NFκB site (23, 42) might be also engaged in this process as the mutation of the single consensus NFAT site did not abolished 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. 4*D*) 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 pathological 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.

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