Induction of Hypoxia-inducible Factor 1 Activity by Muscarinic Acetylcholine Receptor Signaling*

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Kiichi Hirota‡§, Ryu Fukuda‡, Satoshi Takahuchi‡, Shinae Kizaka-Kondoh**, Takehiko Adachi‡, Kazuhiko Fukudai, and Gregg L. Semenza§

From the ‡Department of Anesthesia, The Tazuke Kofukai Medical Research Institute Kitano Hospital, 2-4-20, Ohigimachi, Kita-ku, Osaka 530-8480, Japan, §Program in Vascular Cell Engineering, The Johns Hopkins University School of Medicine, Baltimore, MD 21205, the Department of Anesthesia, Kyoto University Hospital, Kyoto University, Sakyo-ku, Kyoto 606-8507, Japan, and the **COE Formation for Genomic Analysis of Disease Model Animals with Multiple Genetic Alterations, Kyoto University Graduate School of Medicine, Yoshida-Konocho, Sakyo-ku, Kyoto 606-8507, Japan

Hypoxia-inducible factor-1 (HIF-1) is a master regulator of cellular adaptive responses to hypoxia. Levels of the HIF-1α subunit increase under hypoxic conditions. Exposure of cells to growth factors, prostaglandin, and certain nitric oxide donors also induces HIF-1α expression under non-hypoxic conditions. We demonstrate that muscarinic acetylcholine signals induce HIF-1α expression and transcriptional activity in a receptor subtype-specific manner using HEK293 cells transiently overexpressing each of M1-M4 muscarinic acetylcholine receptors. The muscarinic signaling pathways inhibited HIF-1α hydroxylation and degradation and induced HIF-1α protein synthesis that was confirmed by pulse labeling studies. Muscarinic signal-induced HIF-1α protein and HIF-1-dependent gene expression were blocked by treating cells with inhibitors of phosphatidylinositol 3-kinase, MAP kinase, or tyrosine kinase signaling pathways. Dominant-negative forms of Ras and/or Rac-1 significantly suppressed HIF-1α activation by muscarinic signaling. Signaling via M1- and M3- but not M2- or M4-AchRs promote accumulation and transcriptional activation of HIF-1α. We conclude that muscarinic acetylcholine receptors activate HIF-1α by both stabilization and synthesis of HIF-1α and by inducing the transcriptional activity of HIF-1α.

Hypoxia activates a number of genes that are important in cellular and tissue adaptation to low oxygen conditions (1). These genes include erythropoietin, glucose transporters, glycolytic enzymes, and vascular endothelial growth factor (VEGF).1 The hypoxic expression of these different genes is controlled at the transcriptional level by the ubiquitously expressed transcription factor hypoxia-inducible factor 1 (HIF-1). HIF-1 is a heterodimer composed of a constitutively expressed HIF-1β subunit and an inducibly expressed HIF-1α subunit. The regulation of HIF-1α activity occurs at multiple levels in vivo. Among these, the mechanisms regulating HIF-1α protein expression and transcriptional activity have been most extensively analyzed (4). The von Hippel-Lindau tumor-suppressor protein (VHL) has been identified as the HIF-1α-binding component of a ubiquitin-protein ligase that targets HIF-1α for proteasomal degradation in non-hypoxic cells (5). Under hypoxic conditions, the hydroxylation of specific proline and asparagine residues in HIF-1α is inhibited due to substrate (O2) limitation, resulting in HIF-1α protein stabilization and transcriptional activation (6, 7). The iron chelator deferoxamine (DFX) inhibits the prolyl and asparaginyl hydroxylases, which contain Fe2+ at their catalytic sites, causing HIF-1α stabilization and transcriptional activation under normoxic conditions (6, 8).

Physiological stimuli other than hypoxia can also induce HIF-1α activation, and the transcription of hypoxia-inducible genes (9–14). Signaling via the HER2/neu or IGF-1 receptor-tyrosine kinase induces HIF-1α expression by an oxygen-independent mechanism. HER2/neu activation increases the rate of HIF-1α protein synthesis via phosphatidylinositol 3-kinase (PI3K) and the downstream serine-threonine kinases AKT (protein kinase B) and FRAP (FKBP12 rapamycin-associated protein), which contain mTOR (mammalian target of rapamycin) homologues (10–12). IGF-1-induced HIF-1α synthesis is dependent upon both the PI3K and MAP kinase (MAPK) pathways (13). The effect of HER2/neu signaling on HIF-1α protein translation is dependent upon the presence of the 5′-untranslated region of HIF-1α mRNA. In addition to growth factors, prostaglandin E2, thrombin, angiotensin II, and 5-hydroxytryptamine induce HIF-1α activation (11, 14). Notably, cellular receptors for these agents are heterotrimeric guanine nucleotide binding (G) protein-coupled receptors (GPCR). Moreover, a constitutively active GPCR encoded by the Kaposi’s sarcoma-associated herpes virus/human herpes virus 8 is reported to induce HIF-1α activation in a MAPK-dependent manner (15).

In this study, we demonstrate that muscarinic acetylcholine receptor (mAchR)-mediated signals induce HIF-1α activation in a receptor-subtype specific manner using HEK293 cells transfected with various types of mAchR. Signaling via M1- and M3- but not M2- or M4-AchRs promote accumulation and transcriptional activation of HIF-1α. We also provide evidence that the activation is dependent upon the expression of HIF-1α and tyrosine kinase, MAPK, and PI3K activity.

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† To whom correspondence should be addressed: Dept. of Anesthesia, The Tazuke Kofukai Medical Research Institute Kitano Hospital, 2-4-20, Ohigimachi, Kita-ku, Osaka 530-8480, Japan.

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Activation of HIF-1 by Muscarinic Signaling

**Fig. 1. Effect of carbachol on HIF-1α levels in muscarinic acetylcholine receptor-expressing HEK293 cells.** A, HEK293 cells were transiently transfected with an empty vector (EV) or expression plasmids encoding M1-M4-muscarinic acetylcholine receptors (500 ng) and treated with 100 μM carbachol (CCH) for 20 min. Cells were harvested, and lysates were subjected to immunoblot assay (IB) using an Ab that recognize the phosphorylated forms of p42/p44 MAPK. B, transfected cells expressing mACHR were treated with CCH or DFX (lane 2) for 4 h. Cell lysates were subjected to immunoblot assay using anti-HIF-1α or anti-HIF-1β Abs. C, M1-expressing cells were treated with CCH (100 μM) in the presence or absence of atropine (atr) (5 μM) (C), with 100 mM to 100 μM CCH (D), with CCH for 1–8 h (E) or with CCH, DFX, or CCH and atr (F), and analyzed for HIF-1α expression by immunoblot assay using anti-HIF-1α Ab.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Reagents—**HEK293 cells and human dopaminergic neuroblastoma SK-N-SH cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. DFX was obtained from Sigma. Carbachol (CCH), cycloheximide (CHX), genestein, LY294002, PD98059, SB203580 and GF109203X were obtained from Calbiochem (San Diego, CA).

**Plasmid Constructs—**Expression vectors for porcine M1-M4 AChRs were described previously (16, 17). Expression vectors pGAL4/HIF-1α (531–826), pGAL4/HIF-1α (531–575), pGAL4/HIF-1α (786–826) and pGAL4/HIF-1α (786–826) were described previously (8). Plasmid p2.1 contains a 68-bp hypoxia response element (HRE) from the ENO1 gene inserted upstream of an SV40 promoter in the luciferase reporter plasmid pGL2-Promoter (Promega) and p2.4 contains a 3-bp mutation in the HRE (18). Plasmid pVEGF-KpnI contains nucleotides −2274 to +379 of the VEGF gene inserted into luciferase reporter pGL2-Basic (Promega) (19). The reporter pG5E1bLuc contains five copies of a GAL4 binding site upstream of a TATA sequence and firefly luciferase coding sequences. The expression plasmid pCR3.1-HA-FIH-1 and a plasmid encoding a dominant negative form of HIF-1α (pcEP4-HIF-1α N234A) were described previously (20, 21). The expression plasmid pCH-NLS-HIF-1α (548–660) LacZ was described elsewhere (22). Plasmids encoding constitutively activated from of heterotrimeric G protein α-subunits pcDNA3-Gα12 Q226L, pcDNA3-Gα12 Q226L, and pcDNA3-Gα13 Q226L were kindly provided by Dr. Manabu Negishi (Kyoto University, Japan) (23). Plasmids encoding bovine Gβ1 and Gγ2 are made from Gβ1 and pcDNA3.1 (+) and Gγ2 and pcDNA3.1 (+), respectively (24). Plasmids encoding a dominant negative form of Ras (RasNN) or Rac1 (Rac1NN) were generous gifts from Dr. Kaikobad Irani (Johns Hopkins University, Baltimore, MD) (25) and Dr. Kozo Kaido (Nagoya University, Nagoya, Japan) (26), respectively. A dominant negative form of MEK1, MEK1(A) and a dominant negative form of MEK5, MEK5(A) were from Dr. Eisuke Nishida (Kyoto University) (27, 28).

**Hypoxic Treatment—**Tissue culture dishes were transferred to a modular incubator chamber (Billups-Rothenberg, Del Mar, CA) which was flushed with 1% O2, 5% CO2, 94% N2, sealed, and placed at 37 °C (29).

**Immunoblot Assays—**Whole cell lysates were prepared by incubating cells for 30 min in cold radioimmune precipitation assay (RIPA) buffer containing 2 mM dithiothreitol, 1 mM NaVO3, and Complete protease inhibitor™ (Roche Applied Science) (29). Samples were centrifuged at 10,000 × g to pellet cell debris. For HIF-1α and HIF-1β, 100-μg aliquots were fractionated by 7.5% SDS-polyacrylamide gel electrophoresis and subjected to immunoblot assay using mouse monoclonal antibodies against HIF-1α or HIF-1β (H1a67 and H1b294; Novus Biologicals, Littleton, CO) at 1:1000 dilution. Signal was developed using the ECL reagent (Amersham Biosciences). For analysis of phosphorylated proteins, HEK293 cells were treated with CCH and 50-μg aliquots were analyzed using specific antibodies (1:1000 dilution) (Cell Signaling
Technology, Beverly, MA). Signal was developed using the ECL reagent (Amersham Biosciences).

Inhibitor Treatments—PD98059, SB203580, LY294002, GF109203X, or genistein was added 1 h before exposure to CCH or 1% O2. CHX was added to the medium of HEK293 cells that were treated with CCH, or DFX for 4 h, and whole cell extracts were prepared at 15, 30, and 60 min (12, 13).

RNA Blot Hybridization—Total RNA was extracted from HEK293 cells using TRIzol reagent (Invitrogen) 24 h after CCH stimulation and 48 h after transfection of expression plasmid encoding AchR. 10-μg aliquots of RNA were fractionated by electrophoresis in 1.5% agarose, 2.2 M formaldehyde gels, transferred to Hybond N membranes (Amersham Biosciences), and hybridized with a 32P-labeled human HIF-1 or VEGF cDNA probe as described previously (13).

**Fig. 3. Effect of muscarinic signaling on HRE-dependent gene expression.** A, HEK293 cells were transfected with pTK-RL (10 ng), encoding Renilla luciferase, and HRE reporter p2.1 (150 ng), encoding a muscarinic AchR or EV. After 6 h, cells were treated or not treated with CCH (100 μM) or DFX (100 μM) for 16 h and then harvested for luciferase assays. The ratio of firefly:Renilla luciferase activity was determined and normalized to the value obtained from untreated cells transfected with empty vector to obtain the relative luciferase activity (RLA). B, HEK293 cells were transfected with pTK-RL, p2.1, and either M1-AchR (upper) or M3-AchR expression vector. After 6 h, cells were treated with the indicated doses of CCH for 16 h and then harvested for luciferase assays. C, HEK293 cells were co-transfected with M1-AchR, p2.1 or mutant HRE reporter p2.4, pTK-RL, and expression vector encoding either no protein (EV) or a dominant negative form of HIF-1α (DN). After 6 h, cells were treated with 100 μM CCH alone or plus 5 μM atropine for 16 h and then harvested for luciferase assays. D, HEK293 cells were transfected with pTK-RL, VEGF promoter reporter pVEGF-KpnI, and either M1- or M2-AchR plasmid. After 6 h, cells were treated with 100 μM CCH alone or plus 5 μM atropine for 16 h and then harvested for luciferase assays.

**Fig. 4. Involvement of G proteins in HIF-1α accumulation.** A, HEK293 cells were transfected with plasmid encoding a constitutively activated form of Goq, Go12, or Go13. After 24 h, cells were harvested, and lysates were subjected to immunoblot assay using anti-HIF-1α Ab. B and C, HEK293 cells expressing M1-AchR were treated with CCH alone or plus pertussis toxin (PTX) (B), or treated with isoproterenol (ISO; lane 2) or CCH (lane 3) (C) and analyzed for HIF-1α protein expression. D, HEK293 cells expressing M1-AchR were transfected with pTK-RL, HRE reporter p2.1, and expression vector encoding a constitutively activated form of Goq, Go12, Go13, or Gβγ. Transfected cells were incubated for 24 h, harvested, and lysates were subjected to luciferase assays.
Anti-HIF-1 min, and whole cell lysates were subject to immunoblot assay using pulse label experiment, expression of HIF-1 \( /H9251 \) lysates and immunoprecipitation of HIF-1 and the cells were incubated for 40 min prior to preparation of cell lysates. For each experiment, at least two independent transfections (Promega). The ratio of firefly to \( \text{Renilla} \) was determined using the Dual-Luciferase Reporter Assay System. After treatment, the cells were harvested and the luciferase activity was determined by PAGE and autoradiography. One-fifth of the input VHL protein was also analyzed.

In Vitro HIF-1α-VHL Interaction Assay—\(^{35}\)S-Methionine-labeled VHL protein was synthesized in vitro and glutathione S-transferase (GST)-HIF-1α (429–608) fusion protein was expressed in \( E. \ coli \) as described previously (20). HEK293 cells expressing M1- or M2-AchR were treated with CCH or DFX for 4 h prior to lysate preparation. GST-HIF-1α (429–608) was immunoprecipitated with 10 \( \mu \)l of the GST-HIF-1α (429–608) preincubation and VHL in vitro translation reactions were mixed in 150 \( \mu \)l of NETN buffer (150 mM NaCl, 0.5 mM EDTA, 20 mM Tris-HCl (pH 8.0), 0.5% (v/v) Nonidet P-40). After 90 min at 4 °C, 20 \( \mu \)l of glutathione-Sepharose-4B (Amersham Biosciences) was added. After 30 min of mixing on a rotator, beads were washed three times with NETN buffer. Proteins were eluted in 2 × SDS sample buffer, fractionated by SDS-PAGE, and detected by autoradiography.

RESULTS

Muscarinic Acetylcholine Receptors Induce HIF-1 Activity in a Receptor Subtype-specific Manner under Non-hypoxic Conditions—Because HEK293 cells express only low levels of mAchRs (33), it is possible to examine the effect of individual mAChRs signaling on HIF-1 activation by overexpression of each mAChR. HEK293 cells were transfected with expression plasmid encoding the M1-, M2-, M3-, or M4-AchR and stimulated by the addition of the cholinergic agonist CCH. As shown in Fig. 1, A, p42/44MAPKs were activated in response to treatment with CCH (100 \( \mu \)M) (lanes 4, 6, 8, and 10). CCH simulation also induced transcripational activation of Elk-1 in mAchR-expressing HEK293 cells (data not shown). Together, these data demonstrate that each mAChR subtype was functionally expressed in HEK293 cells. Furthermore, the levels of
ERK1/2 phosphorylation in response to CCH stimulation were similar, suggesting that the different mAchRs were expressed at comparable levels.

Using this system we investigated impact of mAchR stimulation on activation of HIF-1. 100 μM CCH increased HIF-1α protein levels in M1- or M3-AchR- but not in M2- or M4-AchR-expressing HEK293 cells (Fig. 1B). In contrast, HIF-1β protein levels were not affected by the binding of CCH to mAchR in HEK293 cells. The induction of HIF-1α expression was inhibited by atropine, demonstrating that the effect is receptor agonist-specific (Fig. 1C). The M1- or M3-AchR signal induced the accumulation of HIF-1α in a CCH dose-dependent manner to 100 nM (Fig. 1D). In M1-transfected HEK293 cells exposed to CCH (100 μM), HIF-1α protein levels peaked at 4 h (Fig. 1E).

We next investigated involvement of mAchR in HIF-1α protein accumulation in SK-N-SH human neuroblastoma cells, which endogenously express M3-AchR. Treatment with CCH (100 μM) induced accumulation HIF-1α protein in 4 h and this accumulation was inhibited by treatment with atropine (Fig. 1F). Thus mAchR-mediated signals induced HIF-1α protein accumulation under non-hypoxic conditions.

M1- or M3-AchR Stimulation Activates HIF-1-dependent Gene Expression—We examined the impact of mAchR system on HIF-1-dependent gene expression. As shown in Fig. 2, VEGF gene expression was induced in M1-AchR-expressing HEK293 cells exposed to CCH (100 μM) or to the iron chelator DFX. The expression of HIF-1α mRNA was not affected by any treatment.

Next, HEK293 cells were transfected with reporter plasmid p2.1, which contains a HIF-1-dependent HRE, or p2.4, which contains a mutated, non-functional HRE. Stimulation of M1- or M3-AchR with CCH induced HRE-dependent expression comparably to 100 μM DFX treatment (Fig. 3A). The activation was CCH dose-dependent (Fig. 3B). In contrast, the reporter p2.4 was not activated by the treatment. Expression of a dominant negative form of HIF-1α markedly reduced p2.1 reporter gene expression, demonstrating that gene activation was both HRE- and HIF-1-specific (Fig. 3C). CCH treatment of cells expressing M1-AchR also induced expression of the pVEGF-KpnI reporter, which contains promoter sequences encompassing nucleotides −2274 to +797 relative to the transcription start site of the VEGF gene. In contrast, CCH had no effect on VEGF promoter activity in cells transfected with expression vector M2-AchR or empty vector (Fig. 3D).

Involvement of G Proteins in M1- or M3-AchR Stimulation—Because mAchRs are G protein-coupled receptors, we examined involvement of G proteins in the activation of HIF-1. Because M1- or M3-AchR mainly couples with Goq protein in HEK293 cells (33, 34), we first examined involvement of Goq in the process. Expression of a constitutively activated form of Goq (Goqα-αQ209L) induced the accumulation of HIF-1α protein (Fig. 4A, lane 3) in HEK293 cells. Expression of Goqα-αQ229L or Goqα-αQ226L also induced the accumulation of HIF-1α (lanes 4 and 5). M1-AchR-mediated HIF-1α accumulation was not affected by treatment with pertussis toxin, which blocks interaction of Goq α GTPase with receptors (Fig. 4B). Stimulation of the endogenous β-adrenergic receptor with the β-adrenergic agonist isoproterenol did not induce HIF-1α protein accumulation, suggesting that Goqα-mediated signaling did not contribute to HIF-1α accumulation (Fig. 4C) (35). Expression of a constitutively activated form of Goqα−β, Goqα−γ, Goqα−α, or Gγy-induced HRE-dependent reporter gene expression in HEK293 cells (Fig. 4D).

M1-AchR Stimulation Stabilizes β-Galactosidase Fused to the O2-dependent Degradation Domain of HIF-1α—We examined the impact of M1-AchR-mediated signaling on the stability of HIF-1α protein by transfecting cells with expression vector pCH-NLS-HIF1α-(548–603)-LacZ, which encodes a fusion protein consisting of β-gal and the HIF-1α sequences encompassing the hydroxylation site at Pro-564. The levels of this protein, which can be monitored by measuring β-gal activity, are negatively regulated by O2-dependent hydroxylation, ubiquitination, and proteasomal degradation (22). HEK293 cells transfected with M1-AchR and pCH-NLS-HIF1α-(548–603)-LacZ were treated with CCH, DFX, or IGF-1 and lysates were analyzed for β-gal activity. As shown in Fig. 5A, CCH (100 μM) stimulation induced β-gal activity although to a lesser extent than DFX. In contrast, IGF-1 stimulation did not significantly induce β-gal activity. These data suggest that M1-AchR signaling results in stabilization of HIF-1α protein.

To further investigate whether mAChR signaling affected HIF-1α protein half-life, mAChR-expressing HEK293 cells were treated with CCH, IGF-1, or DFX for 4 h to induce HIF-1α expression, CHX was added to block ongoing protein synthesis, and cell lysates were prepared for immunoblot assay (Fig. 5B). In the presence of CHX, the half-life of HIF-1α was more than 60 min in DFX-treated cells, ~30 min in CCH-treated cells, and less than 15 min in IGF-1-treated cells. These results indicate that M1-AchR stimulation induced accumulation of HIF-1α by increasing protein half-life.

To analyze the rate of HIF-1α synthesis, serum-starved
HEK293 cells were pretreated with CCH or IGF-1 for 30 min and then pulse-labeled with [35S]Met-Cys for 40 min, followed by immunoprecipitation of HIF-1α/H9251 (Fig. 5C). In contrast to control serum-starved cells, 35S-labeled HIF-1α/H9251 was clearly increased in CCH-treated cells as well as IGF-1-treated cells (Fig. 5C, upper). Expression of HIF-1α/H9251 mRNA was not affected during treatment (lower). Thus, both the cycloheximide and metabolic labeling experiments provide evidence for increased synthesis of HIF-1α in response to M1-mediated signal.

**AchR Signaling Inhibits the Interaction of HIF-1α/H9251 and VHL**—Incubation of a GST-HIF-1α-(429–608) fusion protein with control lysate from untreated cells resulted in prolyl hydroxylation of HIF-1α and interaction with VHL (Fig. 6, lane 1). Lysate from DFX-treated 293 cells did not promote the interaction of GST-HIF-1α with VHL (lane 2). Lysate from CCH-treated M1-AchR-expressing cells was less effective in promoting the interaction (lane 5) than lysate from M1-AchR expressing cells without CCH treatment (lane 4). In contrast, lysates from M2-AchR-expressing cells promoted the interaction of GST-HIF-1α with VHL regardless of whether they were exposed to CCH or not (lanes 6 and 7).

**mAchR Signaling Stimulates the Transcriptional Activity of HIF-1α**—Two independent transactivation domains have been localized to amino acid residues 531–575 (TAD-N) and 786–826 (TAD-C) of HIF-1α/H9251 (8, 20). Because it has been shown that steady-state levels of the fusion proteins containing GAL4 DNA-binding domain and HIF-1α TAD are similar under hypoxic and non-hypoxic condition, these GAL4-HIF-1α fusion proteins can be utilized to analyze HIF-1α TAD activity independent of any effects on protein expression. Activity of TAD-N is hypoxia-induced whereas TAD-C is constitutively active. M1- or M3-AchR stimulation enhanced gene expression mediated by GAL4-HIF-1α-(531–826), which contains both TAD-N and TAD-C (Fig. 7). GAL4-HIF-1α-(531–575)-mediated gene expression is also stimulated by M1- or M3-AchR, indicating that TAD-N function is regulated by mAchR signaling. The activity of GAL4-HIF-1α-(531–826) is stimulated by M1- or M3-AchR, indicating that TAD-N function is regulated by mAchR signaling.
(786–826) was not affected by mAChR stimulation.

Effect of Kinase Inhibitors on AchR-mediated HIF-1 Activation—To investigate further the molecular mechanisms whereby muscarinic receptors activate HIF-1, M1-AchR-expressing HEK293 cells were stimulated by CCH under treatment with LY294002, PD98059, SB203580, GF109203X, or genistein which are selective pharmacologic inhibitors of PI3K, MEK, p38 MAPK, PKC, and tyrosine kinase activity, respectively. As shown in Fig. 5A, genistein almost completely inhibited HIF-1α accumulation induced by CCH (lane 7) similar to the effect of atropine (lane 8). GF109203X (lane 6) partially inhibited HIF-1α accumulation (lane 6), whereas treatment with LY294002 (lane 3), PD98059 (lane 4), or SB203580 (lane 5) had little or no effect.

Next, we investigated the effect of the inhibitors on HRE-dependent gene expression using the HRE-dependent p2 reporter (Fig. 8B). Genistein or PD98059 almost completely suppressed the gene expression by CCH. GF109203X had a partial inhibitory effect. In contrast, treatment with LY294002 or SB203580 did not have any inhibitory effect. The transcriptional activity of HIF-1α was examined using pGAL4/HIF-1α-(531–826) and pG5ElbLuc (Fig. 8C). PD98059 or GF109203X almost completely suppressed HIF-1α TAD activation induced by CCH. In contrast, treatment with genistein, LY294002, or SB203580 did not block TAD function induced by CCH.

Previously, we demonstrated that small G protein Ras and Rac1 are involved in hypoxia-induced HIF-1 activation process (29). We next examined involvement of Ras and Rac1 in the CCH-induced HIF-1 activation (Fig. 8D). Expression of dominant negative forms of Ras (RasN17) or Rac1 (Rac1N17) inhibited CCH-induced HRE-dependent gene expression in M1-AchR-expressing HEK293 cells. Expression of a dominant negative form of MEK1 or MEK5 also inhibited CCH-induced gene expression (Fig. 8E), which is consistent with the observed effects of PD98059 in Fig. 8B.

DISCUSSION

Molecular cloning studies have revealed the existence of five distinct muscarinic receptor subtypes referred to as M1-M5 (33). The M1-M5 receptors are members of the GPCR superfamily. Although even numbered receptors (M2 and M4) are selectively coupled to G proteins of the Gaq/G12 family, the odd-numbered receptors (M1, M3, and M5) are preferentially linked to Gai/G13 proteins (16, 17). Our results demonstrate that muscarinic acetylcholine receptor-mediated signals induce HIF-1 activation under non-hypoxic conditions in a receptor subtype-specific manner. Only odd numbered receptors induced HIF-1α protein expression and transcriptional activity in a receptor-ligand-dependent manner in HEK293 cells. Expression of a constitutively activated form of Ga, Gaiα, G12α, or Gβγ is sufficient to induce accumulation of HIF-1α and HIF-1-mediated transcription (Fig. 4D). The selective effect of M1- or M3- versus M2- or M4-AchR signaling does not appear to be related to the level of receptor expression based on the ERK activation (Fig. 1A).

HIF-1α protein expression level is determined by the balance between protein synthesis and degradation (12, 13). CCH stimulation of M1-expressing HEK293 cells increased the half-life of HIF-1α protein compared with that in IGF-1-treated cells although the effect was less than that induced by DFX, which inhibits HIF-1α prolyl hydroxylases (Fig. 5B). CCH also induced stabilization of HIF-1α-(548–603)-β-gal fusion protein in HEK293 cells expressing M1-AchR (Fig. 5A). Moreover, lysate of M1-expressing HEK293 cells stimulated by CCH was less effective than lysate from unstimulated cells in promoting the interaction between HIF-1α and VHL. Together, these results suggest that M1-AchR signaling regulates HIF-1α hydroxylation, ubiquitination, and/or proteasomal degradation. However, the effect of mAChR signaling on HIF-1α accumulation is not explained fully by the stabilization mechanism, because the effect on the half-life of HIF-1α is much less than that induced by DFX (Fig. 5B). We have reported that certain growth factors such as IGF-1 (12, 13), prostaglandin E2 (14), and the nitric oxide donor NOC18 increase the rate of HIF-1α protein synthesis rather than increasing the stability of HIF-1α. The pulse-labeling data (Fig. 5C) indicate that mAChR stimulation also increases the rate of HIF-1α synthesis. The dual effects on both synthesis and stability account for the high levels of HIF-1α induced by mAChR signaling.

Src family kinases are also activated by Gaq/G12-coupled receptors via the proline-rich tyrosine kinase 2 (PYK2) (36). v-Src and RasV12 signaling in RCC4 and 786–0 cells has been shown to stabilize HIF-1α by inhibiting hydroxylation of Pro-564 (37). The effects of dominant negative forms of Ras and Rac1 (Fig. 8D) suggest that similar mechanisms may be involved in AchR-mediated HIF-1 activation. We demonstrate that the activation of HIF-1 in response to muscarinic signaling is suppressed by the PKC inhibitor GF109203X or the tyrosine kinase inhibitor genestein. (Fig. 8). M1 and M3 muscarinic receptors stimulate phospholipase Cβ, which hydrolyzes phosphatidylinositol 4,5-bisphosphate to generate the second messengers diacylglycerol and inositol 1,4,5-triphosphate (34) causing PKC activation. In fact, genestein or GF109203X inhibits M1-AchR-mediated HIF-1α accumulation. Taken together, the pathway may lead to HIF-1α accumulation (Fig. 9). Regulation of HIF-1 activity involves changes in both the protein expression and transcriptional activity of HIF-1α (8, 20, 29). Our data analyzing transactivation mediated by Gal4-HIF-1α-TAD fusion proteins demonstrate that muscarinic receptor signaling also induces HIF-1α TAD activity under non-hypoxic conditions. A major determinant of TAD function is the interaction between HIF-1α and the coactivators p300/CBP, which regulated by O2-dependent hydroxylation of Asn-803 by FIH-1 (7, 20). TAD activity is also regulated by a
MAPK-dependent mechanism (29, 38) that enhances recruitment of p300/CAF-1 (39). The MEK inhibitor PD98059, which is a selective pharmacologic inhibitor of MEK1 and MEK5, blocked muscarinic signal-induced HIF-1, suggesting a link between muscarinic signaling, MEK/ERK, and HIF-1α. The data presented in Fig. 8E suggest that MEK1 and MEK5 cooperatively play a critical role in this process. The signal transduction pathway leading from muscarinic receptor activation to HIF-1α TAD activation is also sensitive to the PKC inhibitor GF109203X.

The M1-M4 receptors are widely expressed throughout the central and peripheral nervous systems (34). The M1 receptor is found in greatest abundance in the cortex and hippocampus where it constitutes 40–50% of the total mAChR. Activation of HIF-1 may provide a mechanism to increase glucose uptake and/or perfusion in response to increased neuronal activation to HIF-1 transduction pathway leading from muscarinic receptor activation. Further studies are required to determine whether HIF-1 activation by muscarinic receptor signaling may play an important role in regulating cell survival in neurodegenerative disorders.

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