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HIF1A-dependent increase in endothelin 2 levels in granulosa cells: role of hypoxia, LH/cAMP, and reactive oxygen species

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Abstract

Hypoxia-inducible factor 1 alpha (HIF1A) and endothelin 2 (EDN2) are transiently expressed during the same time window in the developing corpus luteum (CL). In this study, we sought to investigate the involvement of LH/cAMP, reactive oxygen species (ROS), and a hypoxia-mimetic compound ($CoCl_2$) on HIF1A expression and how it affected EDN2 levels, using transformed human granulosa cells (thGCs) and primary bovine granulosa cells (GCs). $CoCl_2$ elevated HIF1A protein levels in thGCs in a dose-dependent manner. Forskolin alone had no significant effect; however, forskolin and $CoCl_2$ together further induced HIF1A protein and *EDN2* mRNA expression in thGCs. Similarly, in primary GCs, LH with $CoCl_2$ synergistically augmented HIF1A protein levels, which resulted in higher expression of *EDN2* and another well-known hypoxia-inducible gene, *VEGF* (VEGFA). Importantly, LH alone elevated *HIF1A* mRNA but not its protein. The successful knockdown of *HIF1A* in thGCs using siRNA abolished hypoxia-induced EDN2 and also the additive effect of forskolin and $CoCl_2$. We then examined the roles of ROS in thGCs: hydrogen peroxide (20 and 50 μM) elevated HIF1A protein as well as the expression of EDN2, implying that induction of HIF1A protein levels is sufficient to stimulate the expression of EDN2 (and VEGF) in normoxia. A broad-range ROS scavenger, butylated hydroxyanisole, inhibited $CoCl_2$-induced HIF1A protein with a concomitant reduction in the mRNA expression of *EDN2* and *VEGF* in thGCs. The results obtained in this study suggest that HIF1A, induced by various stimuli, is an essential mediator of *EDN2* mRNA expression. The results may also explain the rise in the levels of HIF1A-dependent genes (*EDN2* and *VEGF*) in the developing CL.

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Introduction

Endothelin 2 (EDN2) is a new player in reproductive processes implicated in ovulation and corpus luteum (CL) formation (Palanisamy *et al*. 2006, Klipper *et al*. 2010, Cacioppo *et al*. 2014). Prevention of EDN2 action in rodents using EDN receptor antagonists resulted in unruptured follicles that failed to develop into corpora lutea (CL; Ko *et al*. 2006, Palanisamy *et al*. 2006, Cacioppo *et al*. 2014). Consistent with the contention that EDN2 is essential for CL formation, we reported that *EDN2* mRNA levels were transiently expressed immediately after ovulation, in the early, developing bovine CL. EDN2 in follicles or CL was localized mainly to the luteal steroidogenic cells or luteinized human granulosa cells (GCs; Ko *et al*. 2006, Klipper *et al*. 2010, Imbar *et al*. 2012). GCs collected from patients with polycystic ovary syndrome (PCOS), characterized among other symptoms, by chronic oligo-ovulation or anovulation, had significantly lower *EDN2* mRNA expression when compared with normally ovulating women (Imbar *et al*. 2012). Thus, this study helps in further establishing the roles of EDN2 in follicular rupture, ovulation, and CL formation.

In order to define the regulation of *EDN2* gene expression, we had previously reported that hypoxia and luteinizing hormone (LH) each elevated EDN2 levels in GCs (Klipper *et al*. 2010). In fact, hypoxia (either the mimetic compound, $CoCl_2$, or reduced oxygen) was found to strongly induce EDN2 and VEGF expression in GCs of several species examined thus far (Na *et al*. 2008, Kim *et al*. 2009, Klipper *et al*. 2010). Glucose transporter type 1 (SLC2A1) is yet another known hypoxia-induced gene (Shih & Claffey 1998). Induction of EDN2 expression by LH and hypoxia is physiologically relevant. Hypoxia is an important physiological cue in the developing CL (Nishimura & Okuda 2010) and, together with LH, it orchestrates the formation of CL and the consequent robust angiogenic process. Hypoxia in the CL and also in other fast-growing

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tissues, most notably tumors, induces the expression of a broad genetic program by stabilizing the transcription factors hypoxia-inducible factor 1 alpha (HIF1A) and hypoxia-inducible factor 2 alpha (HIF2A) (Semenza 2007, Brahimi-Horn & Pouyssegur 2009). Under normoxic conditions, HIF1A undergoes O_2-dependent hydroxylation by members of the prolyl hydroxylase domain (PHD) family (for reviews, see Schofield & Ratcliffe (2004) and Kaelin (2005)). Hydroxylation marks HIF1A for ubiquitination and proteasomal degradation (Semenza 2007). When cellular oxygen levels fall, HIF1A hydroxylation is suppressed and HIF1A rapidly accumulates. Consequently, HIF1A is translocated into the nucleus and dimerizes with the constitutively expressed HIF2A subunit. The active HIF1 complex then initiates transcription of target genes by binding to hypoxia-responsive elements located in their promoter or enhancer regions (Hewitson *et al*. 2007), as described for genes such as *VEGF* and *SLC2A1* (Shih & Claffey 1998).

HIF1A can also be hormonally regulated in an oxygen-independent manner. Insulin-like growth factor 1, for instance, augmented the accumulation of HIF1A in normoxia in several cell types (Alam *et al*. 2009, Sartori-Cintra *et al*. 2012, Yu *et al*. 2012). Reactive oxygen species (ROS) is another factor implicated in HIF1A accumulation. Evidence suggests that ROS increases HIF1A by directly inhibiting PHD catalytic activity (Cash *et al*. 2007, Kaelin & Ratcliffe 2008). Finally, reports have also suggested that hypoxia can act independently of the HIF1A transcription factor (Bindra *et al*. 2005, Arany *et al*. 2008, Fujisaka *et al*. 2013).

In this study, we wished to investigate whether: i) hypoxia-induced EDN2 is HIF1A dependent, ii) LH or other cAMP-elevating agents such as forskolin also utilize HIF1A as a tool to induce EDN2, and iii) other potential inducers of HIF1A, such as ROS, would also affect EDN2 levels in GCs. To resolve these questions, we first studied the effects of LH (or cAMP) and a hypoxia-mimetic compound separately or together on HIF1A expression (mRNA and protein) and how it affected EDN2 levels. To critically determine the involvement of HIF1A in *EDN2* mRNA induction, *HIF1A* was knocked down with specific siRNA molecules. The involvement of ROS in these processes on HIF1A accumulation and gene expression in GCs was studied using H_2O_2 and a broad-range ROS scavenger.

Materials and methods

Cell cultures

Transformed human granulosa lutein cells (thGCs; also known as SVOG) were a generous gift from N Auersperg (University of British Columbia, BC, Canada) (Lie *et al*. 1996). Cells were cultured in M-199/MCDB 105 (1:1), containing 10% FCS, 2 mM L-glutamine, 400 ng/ml hydrocortisone, and 50 μg/ml gentamicin sulfate (Lie *et al*. 1996, Kisliouk *et al*. 2003, Klipper *et al*. 2010). Cells from passages 6–22 were used. Cells were grown to 80–90% confluence on Petri dishes, trypsinized with trypsin–EDTA solution, and plated (0.3×106 cells/well) on six-well plates. The following day, cells were incubated with a hypoxia-mimetic compound $CoCl_2$ (25–150 μM) alone or in the presence of 10 μM forskolin in 1% FCS for 3–24 h as indicated. Forskolin, as an inducer of adenylyl cyclase, was used as these cells have lost most of their response to LH/hCG (Lie *et al*. 1996). For experiments examining the effects of ROS, thGCs were incubated with varying doses of H_2O_2 (20, 50, or 100 μM), given as repeated boluses every 15 min in a serum-free medium (Chandel & Schumacker 2000). After 2 h, cells were collected in a sample buffer for protein analysis of HIF1A by western blot or, after 3 h, for RNA analysis of HIF1A-inducible genes by real-time PCR, or thGCs were preincubated with a broad-range ROS scavenger, butylated hydroxyanisole (BHA; 250 μM), for 1 h and then incubated with $CoCl_2$ for 4 or 16 h (for protein isolation and RNA extraction respectively) with or without forskolin in the presence or absence of BHA.

Primary bovine GC culture

Ovaries bearing large follicles (>10 mm in diameter) were collected at a local slaughterhouse as described previously (Meidan *et al*. 1990, Aflalo & Meidan 1993, Mamluk *et al*. 1998). Only follicles containing >4 million viable cells were included in these experiments. GCs were enzymatically dispersed using a combination of collagenase type IA (5000 units), hyaluronidase III (1440 units), and DNase I (390 units) (Meidan *et al*. 1990, Aflalo & Meidan 1993, Mamluk *et al*. 1998), and cultured overnight in DMEM–F12 containing 3% FCS, 2 mM L-glutamine, and penicillin (100 U/ml)/streptomycin (1 mg/ml) solution (Biological Industries, Kibbutz Beit Hemeek, Israel). One day after isolation, bovine GCs (bGCs) were exposed to $CoCl_2$ and/or bLH (100 ng/ml) for various time points as indicated (for RNA extraction and protein isolation respectively) at 1% FCS. Unless otherwise stated, biochemicals were purchased from Sigma, and tissue culture material from Biological Industries.

Cell transfection

ThGCs were trypsinized and plated immediately on six-well plates and cultured for 24 h. Then cells were transfected using Lipofectamine 2000 reagent in 1% FCS, as described previously (Rayhman *et al*. 2008, Klipper *et al*. 2010). Cells were transfected with 50 nM siRNA constructs targeting HIF1A or with scrambled siRNA (the negative control). The *HIF1A* siRNA (si-HIF1A) sequence was sense, CUGAUGACCAGCAACUUGAdTdT and antisense, UCAAGUUGCUGGUCAUCAGdTdT. Scrambled siRNA sequence-negative controls were sense, UUCUCCGAACGUGUCACGUdTdT and antisense, ACGUGACACGUUCGGAGAAdTdT.

RNA isolation and real-time PCR

Total RNA was isolated from tissue and cells using Tri-Fast reagent (Peqlab Biotechnologie GmbH, Erlangen, Germany)

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Table 1 Lists of primers for bovine and human genes.

	Primer sequence (5′–3′)	Accession no.
Bovine genes		
β-actin	F: CGGGCAGGTCATCACCATC	AC_000182
	R: GCCATCTCGTACTCGAAGTCC	
EDN2	F: TTGCCACCTGGACATTATCT	NM_175714
	R: CCTGGCACTGTAGCACTCA	
VEGF	F: CCATGAACTTTCTGCTCTCTTGG	NM_174216
	R: TCCATGAACTCCACCACTTCG	
HIF1A	F: ACTCATCCATGTGACCACG	NM_174339
	R: TAGTTCTCCCCCGGCTAG	
SLC2A1	F: CGCTTCCTGCTCATTAACCG	NM_174602
	R: CCTTCTTCTCCCGCATCAT	
Human genes		
EDN2	F: GCCAGCGTCCTCATCTAT	NM_001956
	R: GCCGTAAGGAGCTGTCTGTTC	
VEGF	F: ATCGAGACCCTGGTGGACA	NM_001025366
	R: CCTCGGCTTGTCACATCTGC	
HIF1A	F: ACTCATCCATGTGACCACG	NM_001530.3
	R: TAGTTCTCCCCCGGCTAG	
SLC2A1	F: CGCTTCCTGCTCATTAACCG	NM_006516.2
	R: CCTTCTTCTCCCGCATCAT	
HPSE	F: CTGAAGGCTGGTGGAGAAGT	NM_006665.5
	R: CTAACCAGACCTTCTTGC	
PTGS2	F: CCCTTCCTCCTGTGCCTGATGA	NM_000963.3
	R: GTGAAGTGCTGGGCAAAGAATG	

F, forward; R, reverse.

according to the manufacturer's instructions. Real-time PCRs were performed using the Mx3000P quantitative PCR system (Stratagene, Garden Grove, CA, USA), using Platinum SYBR Green qPCR SuperMix–UDG (Invitrogen), as described previously (Kisliouk *et al*. 2005, Zalman *et al*. 2012). *ACTB* was used as the reference gene. Dissociation curves after each real-time run confirmed the presence of only one product and the absence of the formation of primer dimers. The threshold cycle number (*C*t) for each tested gene X was used to quantify the relative abundance of the gene; arbitrary units were calculated as $2^{-\Delta Ct}=2^{-(Ct\ \text{target gene X}-Ct\ \text{reference gene})}$. The primer sequences were listed in Table 1.

Western blot analyses

Cells were washed with PBS and proteins were extracted by scraping cells in a sample buffer (100 mmol/l Tris–HCl (pH 6.8), 20% (v/v) glycerol, 4% (w/v) SDS, 0.0002% (w/v) bromophenol blue, and 1% (v/v) β-mercaptoethanol). All steps were carried out on ice, and samples were kept frozen until use. The protein samples were separated by 7.5–10% SDS–PAGE under reducing conditions. Proteins were electrically transferred onto a nitrocellulose membrane. After having been blocked for 2 h at 4 °C in TBST (20 mmol/l Tris, 150 mmol/l NaCl, and 0.1% Tween 20; pH 7.6) containing 5% low-fat milk, the membranes were incubated with the following respective primary antibodies overnight at 4 °C: rabbit anti-HIF1A (H-206; Santa Cruz Biotechnology, Inc., diluted 1:500) used to detect human HIF1A, monoclonal anti-HIF1A (Novus Biological (Cambridge, UK), diluted 1:500), used to detect bovine HIF1A, and rabbit anti-bVEGF (kindly provided by D Schams (Germany), diluted 1:1000) and rabbit anti p44/42 total MAPK (diluted 1:50 000; used as the loading control) for 2 h at room temperature. Then, the membranes were washed with TBST (4×5 min) and incubated with secondary antibodies diluted in 1% nonfat dry milk in TBST, peroxidase-conjugated goat anti-rabbit IgG (Jackson Immuno-Research, West Grove, PA, USA), or goat anti-mouse IgG (H+L) (KPL, Baltimore, MD, USA) for 1 h at room temperature and washed as described previously. A chemiluminescent signal was generated with EZ-ECL Chemiluminescence Detection Kit for HRP (Biological Industries), and the membranes were exposed to X-ray films. The films were scanned and analyzed using the Gel-Pro 32 Software (Media Cybernetics, Silver Spring, MD, USA).

Statistical analysis

Statistical analysis was performed by one-way ANOVA followed by the Bonferroni's multiple comparison test (GraphPad Prism Software, version 6.00, San Diego, CA, USA). Results represent the mean±S.E.M. for at least three separate experiments as specified. Significance was defined at a value of $P<0.05$ or lower.

Results

Effects of $CoCl_2$, forskolin, and LH on HIF1A and EDN2 expression

The studies outlined herein have utilized $CoCl_2$ as a hypoxia-mimetic compound. $CoCl_2$ as well as reduced oxygen tension both augmented *EDN2*, *VEGF*, and *SLC2A1* mRNA levels by GCs (Supplementary Figure, see section on supplementary data given at the end of this article). HIF1A protein was determined in cells incubated with various concentrations (25–150 μM) of $CoCl_2$ only or with forskolin (Fig. 1A). Forskolin (used to activate adenylyl cyclase) given alone did not elevate HIF1A protein levels in thGCs, but it additively augmented HIF1A levels that were stimulated by $CoCl_2$ (1.7-fold on average compared with $CoCl_2$ alone, $P<0.04$, $P<0.05$, and $P<0.05$ for 25, 100, and 150 μM $CoCl_2$+forskolin when compared with $CoCl_2$ alone respectively; Fig. 1B). Examination of *EDN2* gene expression revealed a significant additive effect ($P<0.02$) after incubation with forskolin and $CoCl_2$ (Fig. 1C). To examine whether LH produces a similar effect, we employed primary bGCs that express the functional LH/hCG receptors. A day after isolation, bGCs were incubated with varying concentrations of $CoCl_2$ (25–100 μM) with or without LH (100 ng/ml) (Fig. 2). As with thGCs, there was a dose-dependent increase in $CoCl_2$-induced HIF1A protein in bGCs (Fig. 2A and B). LH alone did not elevate the HIF1A protein above the control levels. However, there was a synergistic, dose-dependent effect of LH with $CoCl_2$ on HIF1A protein levels (Fig. 2A and B). For instance, in the presence of LH, HIF1A protein levels were approximately threefold higher when compared with levels induced by 100 μM $CoCl_2$ alone (Fig. 2). The data presented in Fig. 2 also indicate that LH and $CoCl_2$ exert different effects on the protein (Fig. 2B) and mRNA levels (Fig. 2C) of HIF1A.

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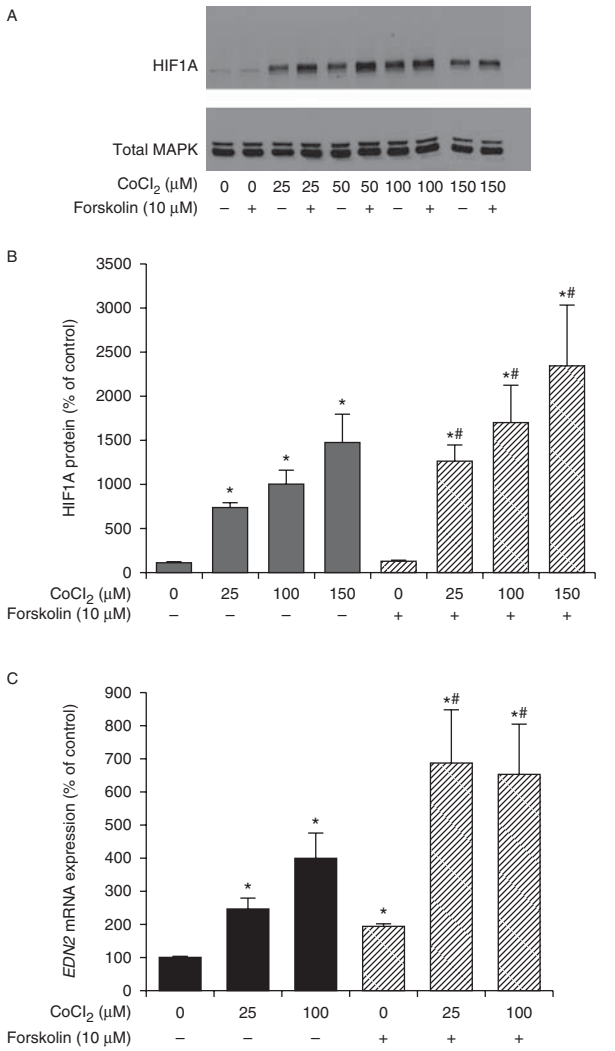

Figure 1 Effect of $CoCl_2$ and forskolin on HIF1A protein and *EDN2* mRNA. ThGCs were cultured with varying concentrations of $CoCl_2$ with or without forskolin (10 μM) for 4 h. (A) Representative western blots of HIF1A protein and total MAPK. (B) Quantification of HIF1A protein using GelPro 32 is depicted as the percentage change from the control (designated 100%); the signal of anti-total MAPK (p44/42) antibody was used to correct for protein loading. (C) Cells were harvested for RNA extraction and the relative *EDN2* mRNA levels were measured by real-time PCR. *Significant differences from their respective controls. #Statistically significant differences between $CoCl_2$ alone and $CoCl_2$ in combination with forskolin ($P<0.05$ for the protein and $P<0.02$ for *EDN2* mRNA). The results (mean ± S.E.M.) were obtained from four independent experiments.

LH only elevated the mRNA levels of *HIF1A* without affecting its protein concentration. LH and $CoCl_2$ synergized in elevating HIF1A protein levels but not its mRNA levels (Fig. 2B and C). $CoCl_2$ slightly, but significantly, elevated *HIF1A* gene expression (Fig. 2C). Importantly however, HIF1A protein levels were elevated only in the presence of $CoCl_2$ (Fig. 2B).

To examine whether the synergistic effect of LH and $CoCl_2$ on HIF1A induction was transcriptionally active, we exposed cells to these stimuli and examined gene expression. The data presented in Fig. 3 indicate that the combined effect of LH and $CoCl_2$ on HIF1A induction was manifested in the profile of EDN2 and two known hypoxia-induced genes: *VEGF* and *SLC2A1*; approximately twofold increase).

Effects of HIF1A inhibition by siRNA on EDN2 expression

To critically establish the role of HIF1A in EDN2 expression in GCs, we silenced its expression with specific siRNA. Transfection of HIF1A constructs effectively reduced HIF1A protein concentrations induced by $CoCl_2$ approximately to 15% ($P<0.001$) of levels present in cells transfected with scrambled siRNA (Fig. 4A). Specificity of HIF1A silencing was demonstrated by the lack of inhibition in levels of non-hypoxia-dependent

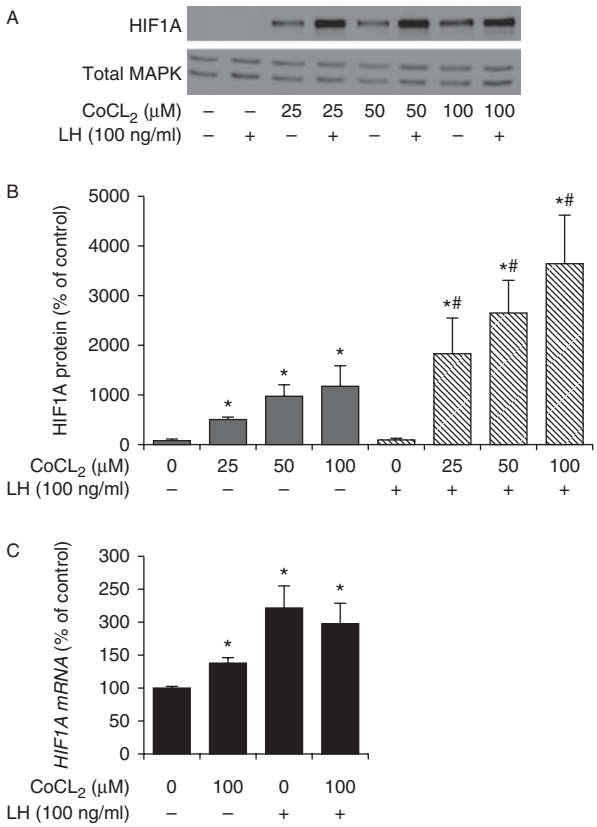

Figure 2 Effect of LH and $CoCl_2$ on mRNA and protein levels of HIF1A in bGCs. Cells were incubated with varying concentrations of $CoCl_2$ with or without LH (100 ng/ml) for 4 h. (A) Representative western blot (left panel) and quantification of HIF1A protein (right panel). (B) Quantification of HIF1A protein using GelPro 32 is depicted as the percentage change from the control (designated 100%); the membranes were also probed with an anti-total MAPK (p44/42) antibody to correct for protein loading. (C) Relative *HIF1A* mRNA levels were measured by real-time PCR. *Significant differences from their respective controls. #Statistically significant differences between $CoCl_2$ alone and $CoCl_2$ in combination with LH ($P<0.01$). The results (mean ± S.E.M.) were obtained from three and five independent experiments for protein and for mRNA levels respectively.

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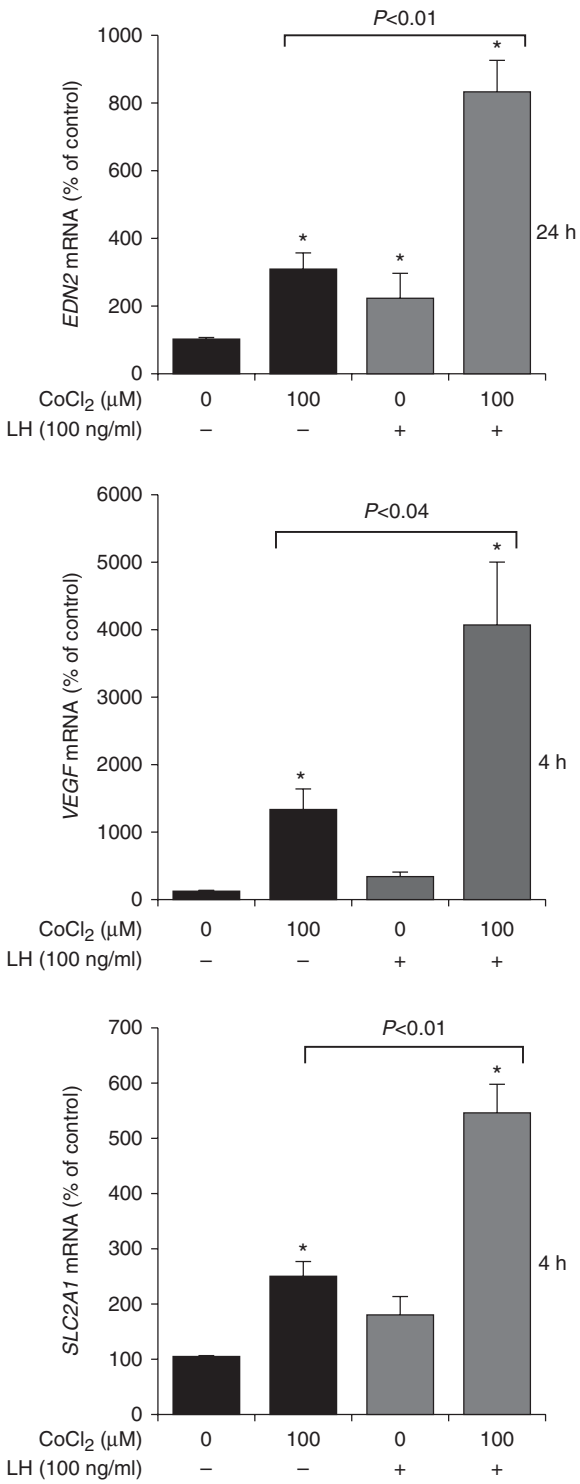

Figure 3 Effect of $CoCl_2$ and LH on the expression of hypoxia-induced genes in bGCs. Cells were cultured with $CoCl_2$ (100 μM) with or without LH for 4–24 h. At the end of the incubation period, the cells were harvested for RNA extraction and *EDN2*, *VEGF*, and *SLC2A1* mRNA levels were measured by real-time PCR. *Significant differences from their respective controls. The horizontal bar denotes the statistical difference between $CoCl_2$ alone and $CoCl_2$ in combination with LH. The results (mean ± S.E.M.) were obtained from four independent experiments.

genes such as heparanase (*HPSE*) and prostaglandin-endoperoxide synthase 2 (*PTGS2*) (Fig. 4B). To examine the effect of HIF1A silencing on gene expression, cells were transfected with either scrambled siRNA or HIF1A-specific siRNA and, 48 h later, were exposed to $CoCl_2$ for an overnight incubation. The results, presented in Fig. 5, demonstrate that HIF-silenced and $CoCl_2$-treated cells exhibited significantly lower expression of EDN2 as well as of VEGF and SLC2A1. This was evident for both 25 and 100 μM $CoCl_2$. HIF1A silencing also successfully reduced VEGF protein present in cells transfected with scrambled siRNA (Fig. 5). Importantly, as can be observed from Table 2, *HIF1A* knockdown not only reduced the effect of a hypoxia-mimetic compound but also abolished the additive effect of forskolin and $CoCl_2$ on *EDN2* mRNA levels. HIF1A silencing lowered EDN2 levels induced by forskolin alone, but this was not statistically significant (Table 2).

Effects of ROS on HIF1A and EDN2 expression

The involvement of ROS was studied using H_2O_2 and a broad-range ROS scavenger (BHA). The results presented in Fig. 6 A and B indicate that H_2O_2 augmented the levels of HIF1A protein by approximately fourfold when compared with the control. The magnitude of stimulation by 20 or 50 μM was similar, suggesting that 20 μM of H_2O_2 already attained the maximal stimulatory effect. H_2O_2 also significantly elevated the expression of *EDN2* and *VEGF* mRNAs (Fig. 6); actually the effect of H_2O_2 on *EDN2* mRNA was higher than that of forskolin (Fig. 6). To further investigate the role of ROS, we examined the effects of BHA, ROS scavenger. The presence of BHA significantly reduced HIF1A protein levels elevated by hypoxia (2.5 times, on an average, less compared with $CoCl_2$ alone; $P<0.05$; Fig. 7). Notably, BHA not only reduced the levels of HIF1A and the genes induced by $CoCl_2$ but also eliminated the combined stimulatory effect of $CoCl_2$ and forskolin on HIF1A protein and *EDN2* and *VEGF* mRNA levels (Figs 7 and 8).

Discussion

There was a close relationship between the profiles of HIF1A protein and EDN2 expression in the various experiments utilizing primary GCs and thGCs described in this study. Stimulation of HIF1A by either the hypoxia-mimetic compound alone or in combination with cAMP-elevating agents (forskolin and LH), or yet by H_2O_2, resulted in higher *EDN2* mRNA expression (Fig. 9). Moreover, the synergistic effect of LH and $CoCl_2$ on HIF1A induction was also manifested in the profile of EDN2. In accordance, inhibition of HIF1A by siRNA or by an ROS scavenger reduced EDN2 levels. Thus, the findings reported in this study imply that HIF1A is a critical mediator of EDN2 expression in GCs. The profile

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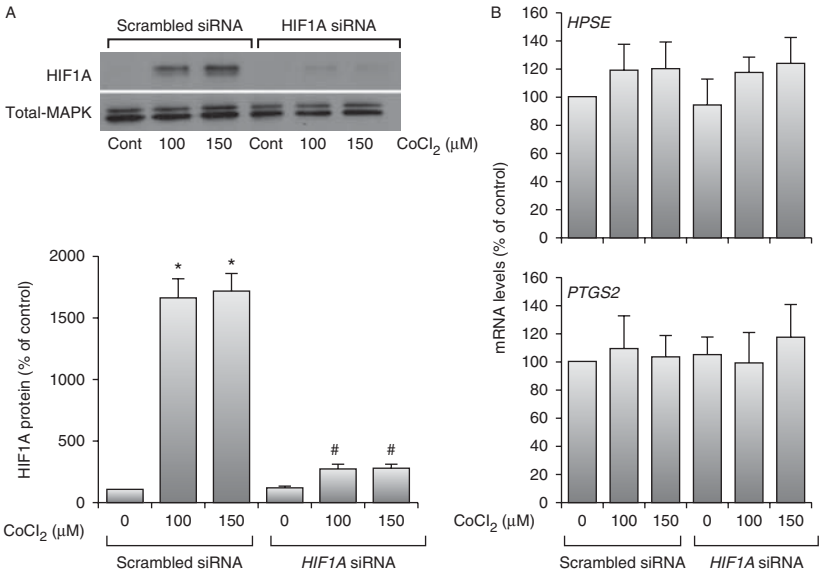

Figure 4 HIF1A silencing. ThGCs were transfected with 50 nM of scrambled siRNA (negative control) or a *HIF1A* siRNA-specific sequence. Forty-eight hours after transfection, the cells were treated with 100 or 150 μM $CoCl_2$ for 4 h. (A, upper panel) Representative western blot of HIF1A protein in silenced vs non-silenced thGCs. (A, lower panel) Quantification of HIF1A protein levels (percentage change from the negative control designated 100%). (B) At the end of the incubation period, the cells were harvested for RNA extraction and the mRNA levels of *HPSE* and *PTGS2* were measured by real-time PCR. *Significant differences from their respective controls. #Statistically significant differences ($P<0.001$) between cells treated with *HIF1A* and scrambled siRNA. The results (mean±S.E.M.) were obtained from three independent experiments.

of EDN2 expression closely resembled that of VEGF and SLC2A1 – two hypoxia-induced genes that are known to be activated by HIF1A, further portraying *EDN2* as a bona fide hypoxia-dependent gene.

The studies outlined herein have utilized $CoCl_2$ as a hypoxia-mimetic agent, though, $CoCl_2$ may not mimic all the effects of hypoxia (reduced oxygen tension); it induces HIF1A protein and also mimics many of its physiological effects (Chandel & Schumacker 2000). Critically, Supplementary Figure demonstrates that $CoCl_2$ and reduced oxygen tension both significantly augmented *EDN2*, *VEGF*, and *SLC2A1* mRNA levels by GCs.

Hypoxic conditions exist in the newly formed CL, because the angiogenic process lags behind the intense luteal cell proliferation. Many studies utilizing various animal species have demonstrated the accumulation, nuclear localization, and activity of HIF1 proteins following ovulation and CL development (Boonyaprakob *et al*. 2005, Duncan *et al*. 2008). For example, HIF1A was detected in the early luteal stage in the cow, but no expression could be found in later stages of the cycle (Nishimura & Okuda 2010, Jiang *et al*. 2011). Similarly, in human CL, nuclear HIF1A protein in granulosa lutein cells was highest during luteal formation and was absent from fully functional CL

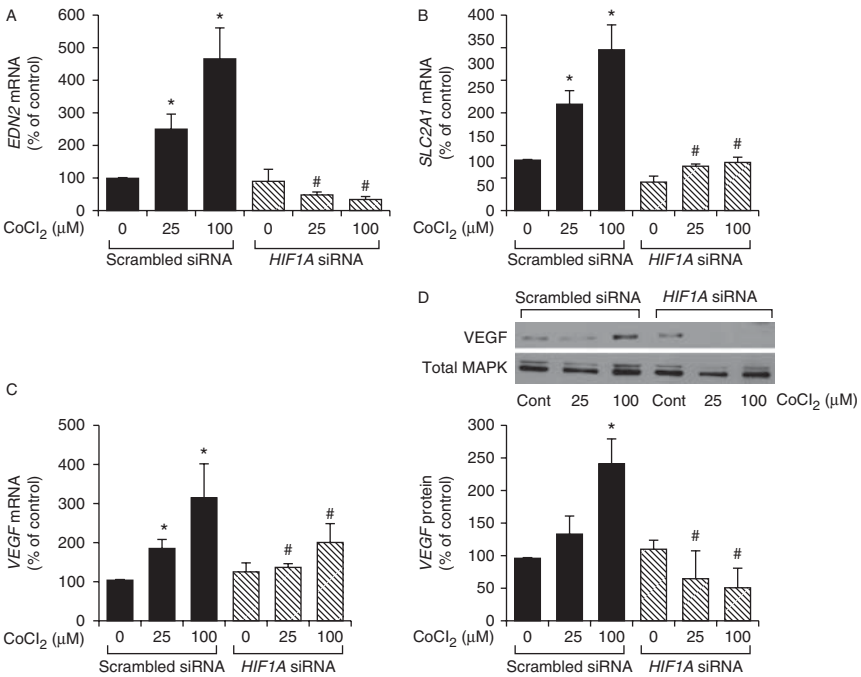

Figure 5 Effect of HIF1A silencing on EDN2, VEGF, and SLC2A1 expression. Forty-eight hours after transfection, thGCs were incubated overnight with 25 or 100 μM $CoCl_2$. At the end of the incubation period, the cells were harvested for RNA extraction and *EDN2* (A), *SLC2A1* (B), and *VEGF* (C) mRNA levels were measured by real-time PCR. Cells were also harvested for VEGF protein (D) determination using a specific antibody (representative blot – upper panel and quantification lower panel). *Significant differences from their respective controls. #Statistically significant differences ($P<0.001$ for SLC2A1 and EDN2; $P<0.05$ for VEGF mRNA and protein) between cells treated with *HIF1A* and scrambled siRNA. The results (mean±S.E.M.) were obtained from three independent experiments.

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Table 2 Effect of HIF1A silencing on *EDN2* mRNA levels by $CoCl_2$ and forskolin.

Treatment	Scrambled siRNA	*HIF1A* siRNA
Control	100	94±20
Forskolin (10 μM)	213±55	140±32, NS
CoCl2 (25 μM)	268±46	73±21*
$CoCl_2$+forskolin	475±98	110±39*

ThGCs were transfected with siRNA molecules as indicated in the legend to Fig. 5. *EDN2* mRNA levels were determined by quantitative real-time PCR. The results (mean±S.E.M.) were obtained from three independent experiments. *Significant differences between scrambled and *HIF1A* siRNA. NS, non significant.

(van den Driesche *et al*. 2008). Hypoxia is therefore an important physiological cue in the developing CL. It is well established that hypoxia plays an important role in the up-regulation of VEGF that occurs at this stage and is responsible for the angiogenic process in early CL (Tesone *et al*. 2005, van den Driesche *et al*. 2008, Nishimura & Okuda 2010, Meidan *et al*. 2013). The current study shows that the hypoxia-mimetic compound elevated HIF1A protein and *EDN2* mRNA levels, whereas HIF1A silencing (employed herein for the first time in GCs) reduced EDN2 expression and ablated its response to hypoxia. This therefore suggests that HIF1A in the developing CL may be responsible for high *EDN2* mRNA levels present at this stage (Klipper *et al*. 2010). In addition to hypoxia, EDN2 and VEGF were induced in cells treated with forskolin or LH/hCG on their own, as demonstrated in quite a few previous studies (Klipper *et al*. 2010, Zhang *et al*. 2012, this study). This increase appears to be independent of HIF1A, because HIF1A protein was not induced by the cAMP-elevating agents and HIF1A silencing did not inhibit forskolin-induced EDN2 expression. But more than each stimuli alone, we show herein that there was a synergism between LH and the hypoxia-mimetic compound in inducing the levels of HIF1A and EDN2 (Fig. 9). Indeed, the highest *EDN2* mRNA levels were observed in early bovine CL (~30 h after ovulation) when both LH and hypoxia occur (Klipper *et al*. 2010). We show that the combined effect is the result of HIF1A being induced transcriptionally and post-transcriptionally, first enhanced transcription by LH, which cannot be manifested in higher HIF1A protein levels unless the protein is stabilized under hypoxic conditions (Fig. 9). This issue was not clearly resolved in earlier studies that have not measured the separate and combined effects of hypoxia and cAMP in the mRNA and protein levels.

Exposure of GCs to the hypoxia-mimetic compound also enhances the levels of HIF1A and HIF2A proteins (Kim *et al*. 2009). Although HIF1A and HIF2A have many overlapping functions, recent studies have illustrated distinct roles for each α-isoform in both normal physiology and disease (Keith *et al*. 2012). Delineation of a potential role for HIF2A in ovarian function will require further research.

PCOS is one of the most common endocrine metabolic disorders in women of reproductive age (Asuncion *et al*. 2000). It is characterized by anovulation, oligomenorrhea or amenorrhea, hyperandrogenism, and obesity and is related to insulin (Legro *et al*. 2004). Recently, we have reported that significantly lower levels of EDN2 were present in GCs derived from women with PCOS, compared with normally ovulating women (Imbar *et al*. 2012). Their response in culture to luteotropic agents was also different from that in the control group (Imbar *et al*. 2012), further demonstrating the importance of LH/hCG in EDN2 induction. The involvement of hypoxic conditions in the abnormal responses of GCs derived from women with PCOS is yet to be investigated.

There is increasing evidence that ROS, particularly H_2O_2, also operate as signaling molecules to mediate various responses (Rhee 2006, Forman *et al*. 2010). It may seem paradoxical that cells respond to low O_2 levels by elevating ROS formation, yet there is ample evidence that ischemia, chemical hypoxia, or low oxygen tension can induce ROS in some or most tissues (Guzy *et al*. 2005, Guzy & Schumacker 2006, Walshe & D'Amore 2008). ROS can modulate HIF1A activity by directly inhibiting the PHD catalytic activity (Chandel *et al*. 1998, Cash *et al*. 2007, Kaelin & Ratcliffe 2008).

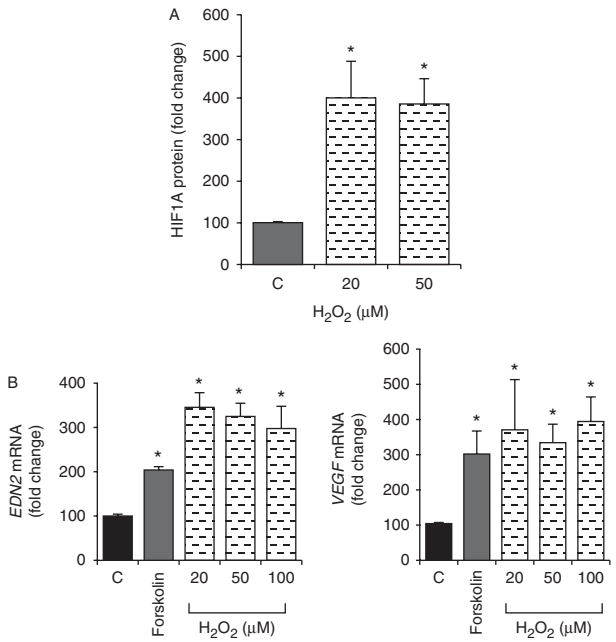

Figure 6 Effect of H_2O_2 on HIF1A protein and gene expression. (A) ThGCs were incubated with H_2O_2 for 2 h and harvested for protein extraction. Quantification of HIF1A protein western blot was carried out as described in the legend to Fig. 1. (B) Cells were incubated with H_2O_2 or forskolin for 3 h, then were harvested for RNA extraction, and *EDN2* and *VEGF* mRNA levels were measured by real-time PCR. Results are expressed as mean±S.E.M. for four independent experiments. *Significant differences from their respective controls.

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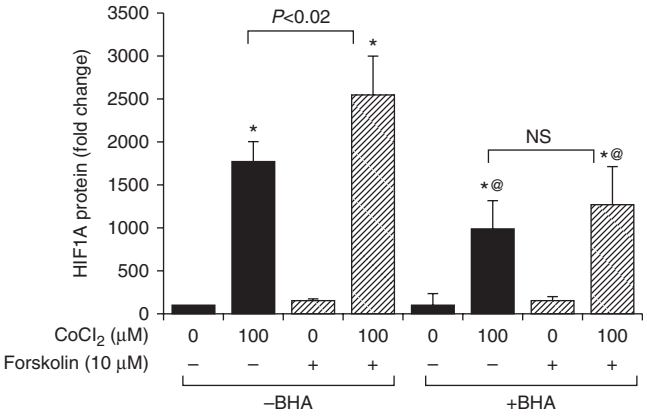

Figure 7 Effect of ROS scavengers on HIF1A protein levels. ThGCs were incubated with $CoCl_2$ (100 μM), forskolin (10 μM), or the two treatments together in the presence or absence of BHA (250 μM) for 4 h. The incubation period was preceded by preincubation for 1 h (with BHA or a control medium). Western blot and quantification of HIF1A protein were carried out as described in the legend to Fig. 1, values are relative to the control (100%). Results are expressed as mean ± S.E.M. for four independent experiments. *Significant differences from respective controls (without $CoCl_2$). @Statistically significant differences between cells incubated with the same treatments with and without BHA. Horizontal bar represents the statistical difference between $CoCl_2$ alone and $CoCl_2$ in combination with forskolin.

Substantial data link LH and ROS; for instance, LH elevated ROS levels in ovarian follicles (Yacobi *et al.* 2007) and hydrogen peroxide up-regulated the expression of ovulatory genes, mimicking the effect of LH (Shkolnik *et al.* 2011). Furthermore, LH-induced expansion of cumulus mass could be inhibited by the ROS scavenger, BHA (Shkolnik *et al.* 2011). However, these studies have not explored the involvement of HIF1A in ROS action. Our data are the first to provide evidence, suggesting that ROS actions in ovarian cells are mediated by HIF1A (Fig. 9). We found that H_2O_2 increased the levels of HIF1A protein in normoxic conditions. Notably, H_2O_2 significantly elevated *EDN2* and *VEGF* mRNA levels confirming the ability of H_2O_2 to mimic LH actions as proposed for rat GCs (Shkolnik *et al.* 2011). In this study, the involvement of ROS in stabilizing HIF1A protein was also observed in cells treated with BHA. In thGCs, BHA significantly reduced the levels of HIF1A protein induced by $CoCl_2$. The combined stimulatory effect of forskolin and $CoCl_2$ on HIF1A protein was also significantly decreased by the presence of BHA. Notably, the expression levels of HIF1A-dependent genes (*EDN2* and *VEGF*) were markedly reduced by BHA, emphasizing the importance of HIF1A protein for transactivation of these genes. Our findings are in agreement with those of Chandel *et al.* (1998). These authors reported that Ebselen (a synthetic glutathione peroxidase mimetic that degrades H_2O_2 to H_2O) abolished mRNA expression of erythropoietin (a model protein expressed upon hypoxia), *VEGF*, and glycolytic enzymes during low oxygen tension and $CoCl_2$ exposure (Chandel *et al.* 1998). The results of this study and those cited earlier indicate that ROS, most probably H_2O_2, contributes to the induction of gene expression by hypoxia (Fig. 9). These data also indicate that the mechanism underlying the synergism between LH and hypoxia may also result from the direct effects on HIF1A (mRNA and protein respectively), but may involve ROS as well. LH and hypoxia can each generate ROS production and their combined effect can result in a stronger inhibition of PHD activity, elevating consequently higher HIF1A protein levels. It should be noted that although both low oxygen levels and $CoCl_2$ can generate ROS (most probably H_2O_2) (Chandel *et al.* 1998, Grasselli *et al.* 2005), $CoCl_2$ acts via a mitochondria-independent mechanism (Chandel *et al.* 1998).

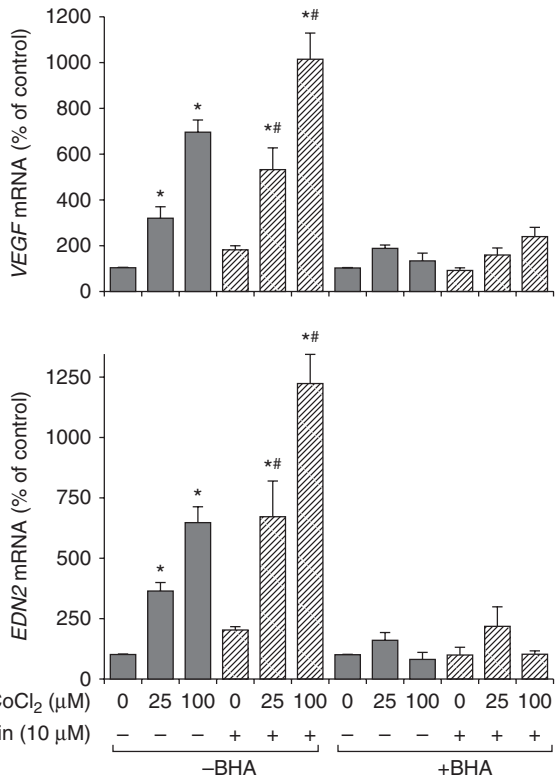

Figure 8 Effect of BHA on expression of EDN2 and VEGF. ThGCs were preincubated with BHA (250 μM) for 1 h and then incubated with $CoCl_2$ (25 or 100 μM) with or without forskolin (10 μM) in the presence or absence of BHA (250 μM). After overnight incubation, RNA was extracted and gene expression was determined by real-time PCR. The results (mean ± S.E.M.) were obtained from four independent experiments. *Significant differences from their respective controls (without $CoCl_2$). #Statistically significant differences between $CoCl_2$ alone and $CoCl_2$ in combination with forskolin for VEGF ($P<0.06$ and $P<0.05$ for 25 and 100 μM respectively), and for EDN2 ($P<0.01$ and $P<0.02$ for 25 and 100 μM between $CoCl_2$ alone and $CoCl_2$ in combination with forskolin respectively).

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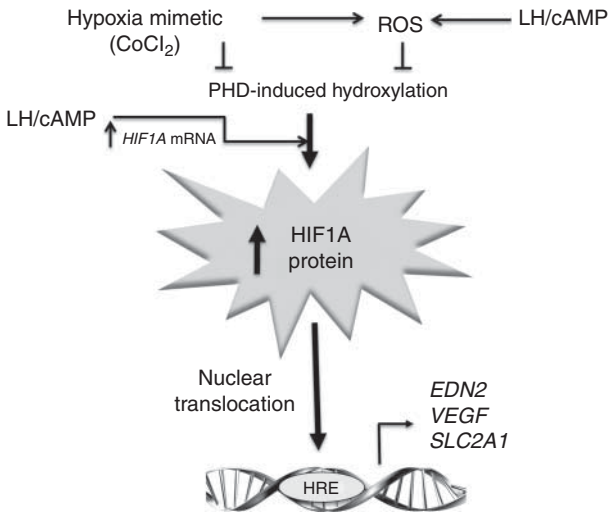

Figure 9 Schema summarizing HIF1A protein accumulation induced by a hypoxia-mimetic compound ($CoCl_2$), LH/cAMP, and ROS (H_2O_2) in GCs. $CoCl_2$ and ROS inhibit PHD-dependent hydroxylation of HIF1A, thereby stabilizing its protein levels (Chandel *et al.* 1998, Cash *et al.* 2007, Semenza 2007, Kaelin & Ratcliffe 2008). LH and forskolin stimulate *HIF1A* mRNA further elevating HIF1A protein levels in the presence of a hypoxia-mimetic compound. In addition, ROS production is enhanced by $CoCl_2$ (Chandel & Schumacker 2000, Grasselli *et al.* 2005) and LH (Yacobi *et al.* 2007). Stabilized HIF1A protein then translocates into the nucleus, where it binds hypoxia-responsive elements (HREs) in the 5′ ends of hypoxia-dependent genes such as *EDN2*, *VEGF*, and *SLC2A1*.

In conclusion, the data presented in this study indicate that HIF1A is an essential mediator of LH/cAMP, hypoxia, and ROS in their induction of EDN2 expression (Fig. 9). These findings are physiologically significant as they suggest a possible mechanism for optimal up-regulation of EDN2 and VEGF during ovulation and CL formation.

Supplementary data

This is linked to the online version of the paper at http://dx.doi.org/10.1530/REP-14-0409.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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