Role of CREB on heme oxygenase-1 induction in adrenal cells: involvement of the PI3K pathway

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Abstract

In addition to the well-known function of ACTH as the main regulator of adrenal steroidogenesis, we have previously demonstrated its effect on the transcriptional stimulation of HO-1 expression, a component of the cellular antioxidant defense system. In agreement, we hereby demonstrate that, in adrenocortical Y1 cells, HO-1 induction correlates with a significant prevention of the generation of reactive oxygen species induced by H_2O_2/Fe^{2+}. ACTH/cAMP-dependent activation of redox-imbalanced related factors such as NRF2 or NFκB and the participation of MAPKs in this mechanism was, however, discarded based on results with specific inhibitors and reporter plasmids. We suggest the involvement of CREB in HO-1 induction by ACTH/cAMP, as transfection of cells with a dominant-negative isoform of CREB (DN-CREB-M1) decreased, while overexpression of CREB increased HO-1 protein levels. Sequence screening of the murine HO-1 promoter revealed CRE-like sites located at −146 and −37 of the transcription start site and ChIP studies indicated that this region recruits phosphorylated CREB (pCREB) upon cAMP stimulation in Y1 cells. In agreement, H89 (PKA inhibitor) or cotransfection with DN-CREB-M1 prevented the 8Br-cAMP-dependent increase in luciferase activity in cells transfected with pHO-1[−295/+74].LUC. ACTH and cAMP treatment induced the activation of the PI3K/Akt signaling pathway in a PKA-independent mechanism. Inhibition of this pathway prevented the cAMP-dependent increase in HO-1 protein levels and luciferase activity in cells transfected with pHO-1[−295/+74].LUC. Finally, here we show a crosstalk between the cAMP/PKA and PI3K pathways that affects the binding of p-CREB to its cognate element in the murine promoter of the *Hmox1* gene.

Introduction

ACTH is the main regulator of glucocorticoid synthesis in the adrenal cortex where it also affects other biological functions such as differentiation, mitogenesis and apoptosis (Gallo-Payet & Payet 2003). Many of these effects are mediated by ACTH binding to the MC2R receptor and downstream activation of the cAMP/PKA

pathway (Gallo-Payet 2016). In turn, stimulation of PKA activity is involved in the phosphorylation and activation of several transcription factors engaged in the transcriptional stimulation of steroidogenic-related genes. Among them, the cAMP response element binding protein (CREB), the GATA-4 binding protein, the steroidogenic factor 1 (SF1) and the CAAT enhancer binding protein (C/EBP) could be included (Manna *et al.* 2003*a*,*b*, Stocco *et al.* 2005).

Glucocorticoid synthesis in the adrenal cortex is a multistep process where mitochondrial and microsomal cytochrome P450 enzymes, mixed function oxidases, catalyze the hydroxylation of steroids and the reduction of O_2 to water. Even under physiological conditions, ACTH-stimulated steroidogenesis generates oxidative stress, as incomplete reduction in O_2 leads to the production of reactive oxygen species (ROS), lipoperoxides and other oxygenated species (Hanukoglu 2006, Prasad *et al.* 2014). To cope with the potentially damaging effects associated with oxidative stress, adrenal cells activate several antioxidant mechanisms (Chinn *et al.* 2002, Hanukoglu 2006, Battista *et al.* 2009). Among them, we have previously shown that the ACTH-dependent induction of heme oxygenase 1 (HO-1) in adrenocortical cells correlated with the prevention of the generation of lipoperoxides and protein carbonyls triggered by H_2O_2 treatment (Pomeraniec *et al.* 2004).

Heme oxygenases catalyze the rate-limiting step in heme catabolism, the conversion of heme to biliverdin (and then bilirubin) with the concomitant release of iron and CO. In addition to this well-known role in heme catabolism, the activity of the inducible isoform of HO has been recognized as a cellular defense mechanism activated by stressful signals (Elbirt & Bonkovsky 1999, Ryter & Tyrrell 2000, Bach 2002). Heme oxygenase activity has been also involved in the modulation of steroid production. By attenuating the hormone-induced glucocorticoid response, heme oxygenase activity has been recently included among the modulatory systems involved in the fine-tuning regulation of adrenal steroidogenesis (Pomeraniec *et al.* 2004, Grion *et al.* 2007, Astort *et al.* 2009).

The induction of HO-1 expression is primarily controlled at the transcriptional level (Maines 1988, Ryter & Choi 2002, Alam & Cook 2003). Both mouse and human HO-1 promoters are subject to a complex regulation that involves multiple transcription factors, including NRF2 (nuclear factor-erythroid 2-related factor 2), heat-shock factor proteins, NF-κB and members of the AP-1(activating protein-1)

bZIP(basic region/leucine zipper) families, with response elements often embedded in antioxidant response elements (AREs) (Ryter & Choi 2005, Surh *et al.* 2008). Activation of the NRF2 transcription factor, as a consequence of cellular redox imbalance, has been linked to the induction of HO-1 and other cytoprotective genes. In basal conditions, NRF2 is maintained in the cytoplasm by interacting with Klech-like ECH-associated protein 1 (KEAP 1) and targeted to proteosomal degradation by ubiquitin modification. Upon stimulation, NRF2 dissociates from KEAP 1 in the cytosol and translocates to the nucleus where it promotes the induction of antioxidant genes (Kensler *et al.* 2007). Activation of the HO-1 promoter has also been linked to the PKA-dependent phosphorylation of CREB (Lonze & Ginty 2002, Kronke *et al.* 2003, Wright *et al.* 2009). CREB activation has been shown to play a role in regulating ROS detoxification as a direct modulator of antioxidant gene expression (Bedogni *et al.* 2003, Kronke *et al.* 2003).

Although we have previously shown that induction of HO-1 by ACTH involves the activation of the cAMP/PKA pathway (Pomeraniec *et al.* 2004), the underlying molecular mechanism has remained elusive. In the present report, we analyzed the involvement of PKA and CREB activation in the transcriptional stimulation of HO-1 by ACTH, and identified a CREB-responsive binding region in the murine *Hmox1* gene proximal promoter. CREB binding to this region is also affected by activation of the PI3K/AKT pathway.

Materials and methods

Chemicals

The antibody raised against HO-1 was acquired from StressGen Biotechnologies Corp. (Victoria, Canada, and those for pAkt and total Akt were bought from New England Biolabs (Ipswich, MA, USA). CREB and Phospho-CREB (Ser 133) antibodies were from Santa Cruz Biotechnology and Cell Signaling Technology respectively. Actin and peroxidase-conjugated goat antirabbit-IgG antibodies were obtained from Santa Cruz Biotechnology and Bio-Rad, respectively. Fetal calf serum, penicillin, streptomycin, MMVL reverse transcriptase, DNAse I and TAQ polymerase were from Invitrogen (Life Technologies), while 8Br-cAMP was purchased from Sigma. ACTH was obtained from ELEA Laboratories (Buenos Aires, Argentina). All other chemicals were of the highest quality available.

Cell culture and treatments

The adrenal Y1 cell line (CCL-79, American Type Culture Collection) is an ACTH- and cAMP-responsive mouse adrenocortical tumor cell line established by Yasumura and coworkers (1966). Cells were grown in monolayers in Ham's F10 medium containing heat-inactivated fetal bovine (2.5%) and horse (12.5%) serum, 200U/mL penicillin G and 270µg/mL streptomycin sulfate, in a humidified atmosphere of 5% CO_2 in air at 37°C as described previously (Schimmer 1979). Cells were washed twice with PBS and medium was replaced with serum-free Ham's F10 2h before the initiation of the experiments. Cell viability was determined by trypan blue dye exclusion test.

Determination of intracellular ROS generation

ROS generation was measured using the nonfluorescent probe 2′,7′-dichlorodihydrofluorescein diacetate (H2DCF diacetate, Molecular Probes, Invitrogen) as described previously (Astort *et al.* 2014, Mercau *et al.* 2014). After treatments, cells were washed twice with PBS and then incubated in PBS containing 10µmol/L of 2′,7′-H2DCF for 1h at 37°C. Cells were washed twice with PBS. Upon uptake by the cells and hydrolysis by cellular esterases, H2DCF is rapidly oxidized by ROS to the fluorescent 2′7′-dichlorofluorescein. Fluorescence intensity (excitation 485nm, emission 535nm) was determined in a multimode microplate reader, FLUOstar Omega (BMGLabtech, Ortemberg, Germany). ROS generation levels are expressed in arbitrary units relative to total protein content.

Plasmids

The *HO-1* luciferase reporter plasmid containing the (−4045/+74) fragment from the murine *HO-1* promoter cloned into the basic pGL2 reporter vector [pHO-1(−4045;+74).LUC] was generously provided by Dr M Perrella (Pulmonary and Critical Care Division, Brigham and Women's Hospital, Boston, MA, USA) (Chung *et al.* 2005). A pGL2 reporter plasmid containing only the (−295/+74) fragment from the murine *HO-1* promoter was generated by PCR amplification using the pHO-1(−4045/+74).LUC construct as template [pHO-1(−295;+74).LUC]. The distal enhancer E1 from the murine HO-1 promoter was amplified by PCR (−4045/−3524, 523bp) using the pHO-1(−4045/+74).LUC construct as a template and cloned into a pGL3 promoter plasmid [pHO-1(E1).LUC] (Promega). The identity of reporter plasmids was confirmed by DNA sequencing (MACROGEN, Korea).

Both p3xARE.LUC (pNRF2.LUC) reporter plasmid, pcDNA3.1/V5HisB-mNRF2 (NRF2 FL) (Innamorato *et al.* 2008, McMahon *et al.* 2004) and PGL3-NFκB-LUC (pκB-LUC), containing five NFκB response elements, were kindly provided by Dr O Coso (FCEyN, UBA, CABA, Argentina) (Squarize *et al.* 2006). An expression vector containing the entire wild-type coding region of CREB (RSV-CREB), the dominant-negative isoform (DN-CREB-M1) carrying a mutation in the phosphorylation site S133A obtained from RSV-CREB (Gonzalez & Montminy 1989) and a luciferase reporter for CREB p4xCRE.LUC (pCRE.LUC), were kindly supplied by Dr E Cánepa (FCEyN, UBA, CABA, Argentina) (Giono *et al.* 2001).

Transfection of Y1 cells and luciferase assay

For overexpression assays, 10^5 cells per well were seeded in 24-well plates. Twenty four hours later, 0.5µg of expression plasmids (RSV-CREB or DN-CREB-M1 or pGEX-4T-3 as control) were transiently transfected with Lipofectamine 2000 Transfection Reagent (Invitrogen) according to manufacturer's instructions in serum-free OptiMEM medium; pCMV-βGAL (β-galactosidase expression plasmid) was included as control of transfection. Three hours later, transfection medium was aspirated and replaced with fresh Ham's F10 medium with sera, and the cells were further incubated for additional 24h before any treatment.

For luciferase reporter assays, 10^4 cells were seeded on 96-well plates. One day after, cells were transiently transfected with luciferase 0.2µg of the following reporter plasmids: pHO-1(−4045/+74).LUC or pHO-1(−295;+74).LUC or pHO-1(E1).LUC or pNRF2(ARE).LUC or p4xCRE.LUC), and pCMV-βGAL in Lipofectamine 2000 according to manufacturer's instructions in serum-free OptiMEM medium, and the following day were treated accordingly. In another set of experiments, cells were seeded in 96-well plates and transfected with 0.2µg of total DNA per well in a combination of a reporter plasmid, an expression plasmid (RSV-CREB or DN-CREB-M1 or pGEX-4T-3) and pCMV-βGAL (ratio 8:1:1).

Luciferase activity was determined in cell homogenates using the Steady-Glo Luciferase Assay System (Promega) and values were normalized to β-galactosidase activity. Luminescence was measured in a FLUOstar Omega plate luminometer (BMG LABTECH, Germany).

Immunoblot analysis

Y1 cells were washed twice in PBS and lysed in 20mM Tris–HCl pH 7.4, 250mM NaCl, 1% Triton X-100, 1mM

Na$_3$VO$_4$, 100 mM NaF, 1 mM EGTA, with 1X proteases and phosphatase inhibitors cocktails (Sigma-Aldrich). After electrophoresis, proteins were transferred to polyvinylidinefluoride membranes for 1 h at 15 V in a Bio-Rad Trans-Blot SD system with 25 mM Tris–HCl pH 9.2, 192 mM glycine and 20% methanol. Membranes were blocked in TBS-Tween (50 mM Tris–HCl pH 7.4, 0.15 M NaCl, 0.05% Tween 20) with 5% skimmed milk for 60 min at room temperature and then incubated overnight with the following antisera: HO-1, p-AKT, AKT, p-CREB, CREB or β-ACTIN at 4°C. Specific protein bands were visualized by chemiluminescence (Enhanced Chemiluminescence Reagent, Amersham Pharmacia Biotech,

Little Chalfont, Bucks, UK) in an ImageQuant Imaging System (GE Healthcare) and quantified with the AlphaEase Fluorchem software (V. 4.1.0, Alpha Innotech Corporation, San Jose, CA, USA).

Chromatin immunoprecipitation (ChIP) assay

Chromatin immunoprecipitation assays were performed as described previously (Bertucci et al. 2013, Rocha-Viegas et al. 2014) with chromatin obtained from adrenocortical Y1 cells, using ChIPAb+Phospho-CREB (Ser133) from Millipore (EMD Millipore) and IgG (kch-504-250) from Diagenode (Denville, NJ, USA) as a negative control.
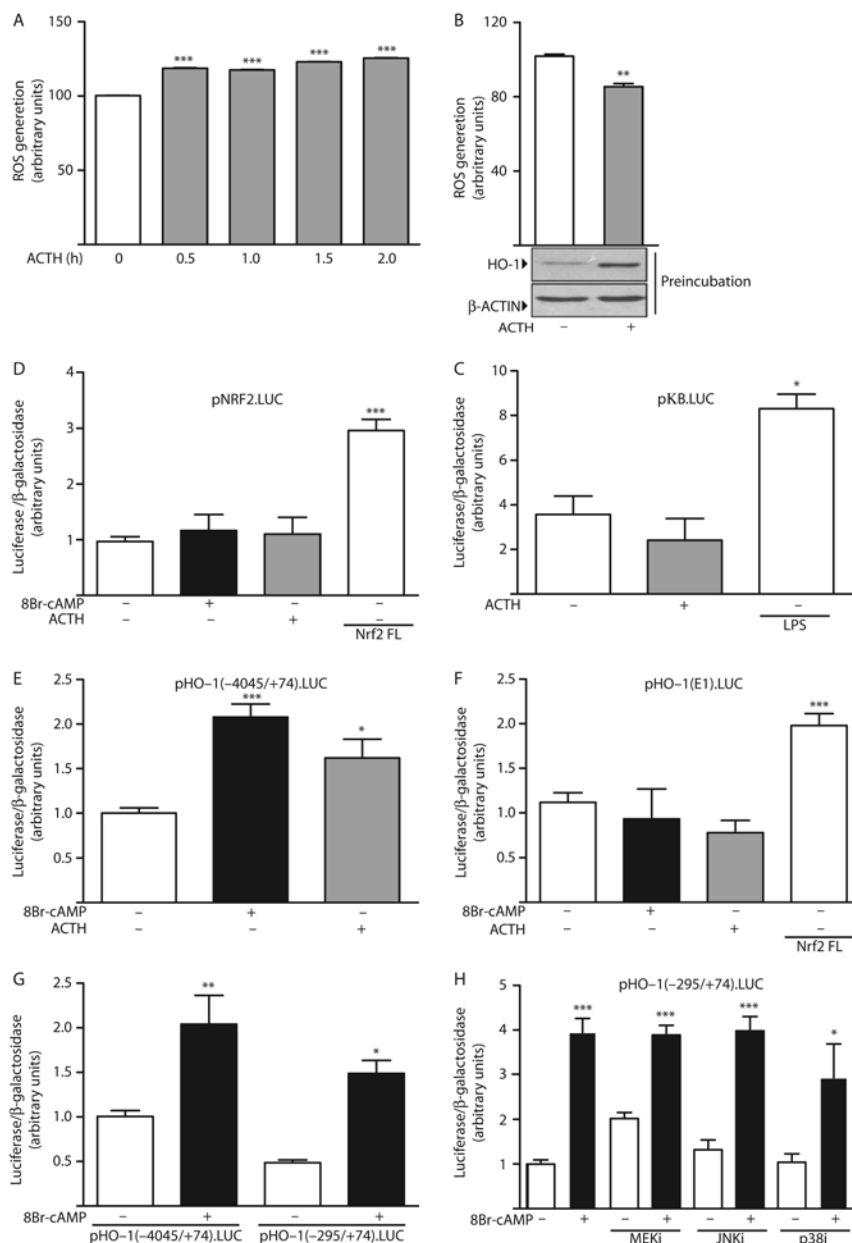
Figure 1
(A) Y1 cells were incubated with 12.5 mIU/mL ACTH for the indicated periods of time and ROS generation was determined as described in Materials and Methods with the oxidant-sensing probe 2′7′dichlorodihydrofluorescein diacetate; (B) Y1 cells were preincubated with or without 12.5 mIU/mL ACTH for 5 h, washed with PBS and further incubated with 100 μM H$_2$O$_2$/80 μM FeSO$_4$ for 1 h. ROS generation was determined as in (A). Below, a representative western blot showing HO-1 and β-ACTIN levels after the 5 h of preincubation in the presence or absence of ACTH; (C) Y1 cells were transfected with the reporter plasmid pNRF2.LUC and stimulated with or without 1 mM 8Br-cAMP or 12.5 mIU/mL ACTH for 24 h. Results from cotransfection experiments with pNRF2.LUC and NRF2 FL are shown as control; (D) Y1 cells were transfected with the reporter plasmid pκB.LUC and stimulated with or without 12.5 mIU/mL ACTH or 10 μg/mL LPS for 24 h; (E) Y1 cells were transfected with a reporter plasmid containing 4045 bp of the murine HO-1 promoter region pHO-1(−4045/+74).LUC and stimulated with or without 1 mM 8Br-cAMP or 12.5 mIU/mL ACTH for 24 h; (F) Y1 cells were transfected with a reporter plasmid containing the E1 enhancer of the murine HO-1 promoter [pHO-1(E1).LUC] and further stimulated with or without 1 mM 8Br-cAMP or 12.5 mIU/mL ACTH for 24 h. Results from cotransfection experiments with pHO-1(E1).LUC and NRF2 FL are shown as control; (G) Y1 cells were transfected with pHO-1(−4045/+74).LUC or with a reporter plasmid containing a proximal promoter fragment of the murine *Hmox1* gene, obtained by PCR performed from the original construct [pHO-1(−295/+74).LUC], and further stimulated with or without 1 mM 8Br-cAMP for 24 h. (H) Y1 cells were transfected with pHO-1(−295/+74).LUC and further incubated with or without 1 mM 8Br-cAMP in the presence or absence of 50 μM PD 98059 (MEKi), 1 μM SP600125 (JNKi) or 5 μM SB203580 (p38i). Data are shown as the ratio of luciferase to β-galactosidase activity determined in lysed cells. Results are indicated as mean ± s.e.m. (n = 3), *P < 0.05, **P < 0.01, ***P < 0.001 vs control, by ANOVA followed by Tukey's test or by Student's t-test.

Quantification of chromatin immunoprecipitation was performed by real-time PCR using DNA Engine OpticonTM System cycler (MJ Research) with the following primers specific for the HO-1 promoter region (−42;+30), forward: 5′-ACGTGACCCGCGTACTTAAA-3′; reverse: 5′-AAACTCTGGAGGCGA CTGG-3′ and for the distal enhancer E1(−3979; −3843), forward: 5′-GCTGGA ATGC TGAGTTGTGAT-3′; reverse: 5′-CTGAGGCTGAGG GAACAGAG-3′. Chromatin immunoprecipitation signal was normalized to input and was expressed as percentage of input (mean±S.E.M., $n=3$).

Statistical analysis

Data are expressed as mean±S.E.M. Statistical analysis was performed by one-way ANOVA followed by Tukey's *post hoc t*-test or Student's *t*-test as considered necessary using GraphPad InStat version 3.06 for Windows (GraphPad Software). Data were considered significantly different when $P<0.05$.

Results

Taking into consideration that HO-1 belongs to a family of antioxidant enzymes induced by the generation of a cellular redox imbalance and our previous results demonstrating the induction of HO-1 by ACTH in adrenocortical cells, we first evaluated the generation of reactive oxygen species (ROS) upon ACTH treatment. Results from a time course experiment, performed in murine adrenocortical Y1 cells incubated in the presence of ACTH, showed that ROS generation was significantly increased already 30 min after hormone addition and this parameter remained elevated for at least 2 h (Fig. 1A), confirming the ACTH-dependence of ROS generation. Moreover, when cells were preincubated with ACTH during 5 h, in order to induce HO-1 expression, and then treated with H_2O_2/Fe^{2+}, ROS generation was significantly lower compared with cells that were preincubated in the absence of hormone (Fig. 1B). These results prompted us to analyze the involvement of the Nrf2 pathway in the induction
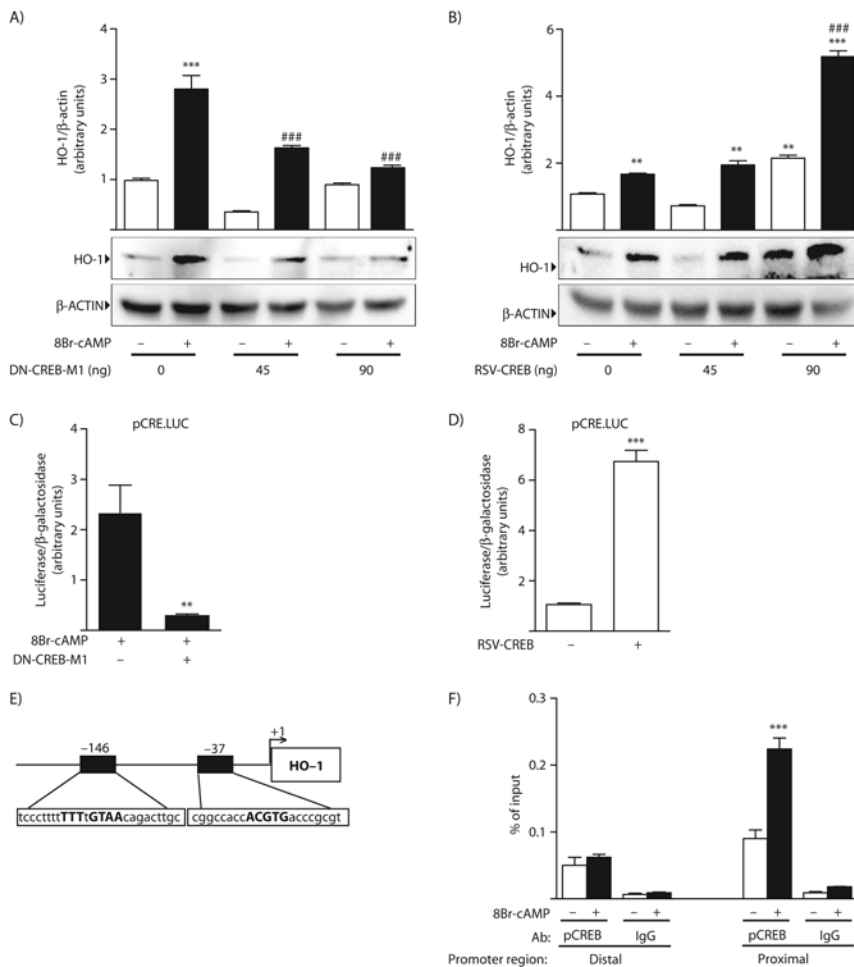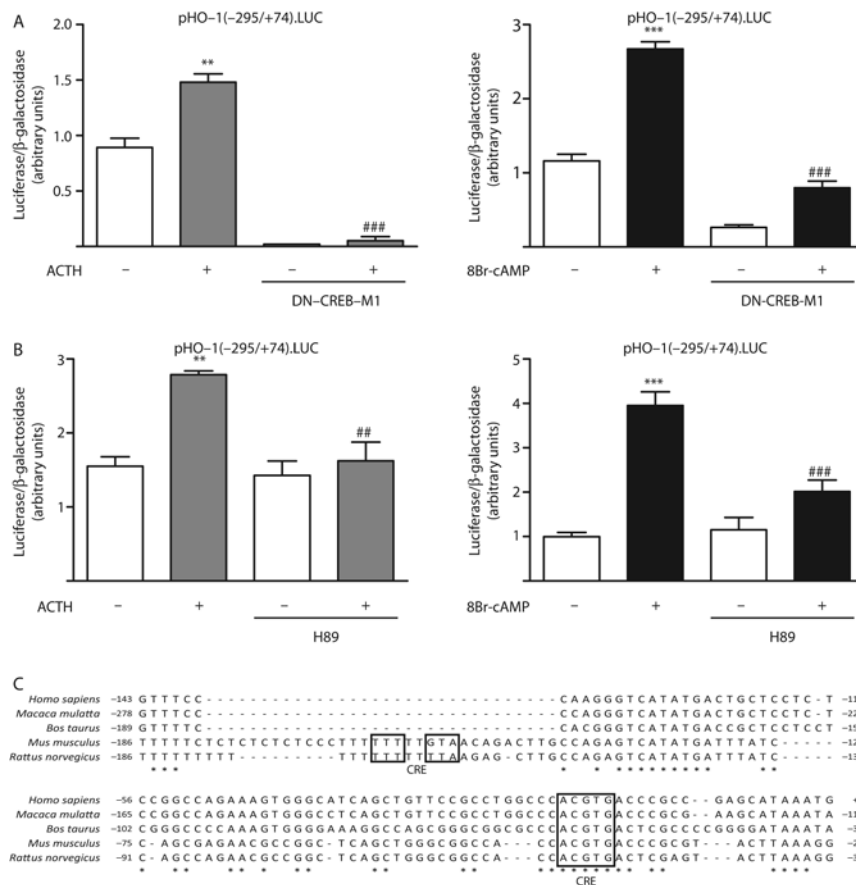

Figure 2
Involvement of CREB in the induction of HO-1 by cAMP. Y1 cells were transfected with increasing amounts of DN-CREB-M1 (A) or RSV-CREB (B) and incubated with or without 1 mM 8Br-cAMP for 5 h. After treatments, protein extracts were prepared and analyzed by western blotting with HO-1 and β-ACTIN antibodies. Densitometry analysis of data is shown as mean (fold induction)±S.E.M., $n=3$, **$P<0.01$, ***$P<0.001$ vs corresponding control and ###$P<0.001$ vs 8Br-cAMP-stimulated cells without DN-CREB-M1 or RSV-CREB, obtained from three independent experiments. Representative images are shown in lower panels. (C) Y1 cells were cotransfected with pCRE.LUC and DN-CREB-M1 and further incubated with 1 mM 8Br-cAMP for 24 h. (D) Luciferase activity was determined in cells cotransfected with pCRE.LUC±RSV-CREB. In C and D, results are shown as mean±S.E.M., $n=3$, **$P<0.01$, ***$P<0.001$ vs corresponding control. (E) Analysis of the −295/+74 region from the mouse HO-1 promoter was performed with the MatInspector software. Textbox detail sequences and relative position of CRE-like binding sites are shown in bold letters. (F) Cells were incubated with or without 1 mM 8Br-cAMP for 2 h and then subjected to ChIP assays with p-CREB antibody. The precipitated DNA fragments were analyzed by qPCR to test the enrichment in sequences corresponding to distal and proximal regions of the murine *Hmox1* gene. Immunoprecipitation with normal rabbit IgG was used as control. Results are shown as mean (% of input)±S.E.M. ($n=3$), ***$P<0.001$ vs respective control, by ANOVA followed by Tukey's test.

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of HO-1 by ACTH. Thus, transient transfections with an NRF2 reporter, pNRF2.LUC, were performed in Y1 cells. Our results showed that while overexpression of NRF2 (NRF2 FL) significantly increased luciferase activity, neither ACTH nor a permeable analog of cAMP, 8Br-cAMP, treatments were effective (Fig. 1C), suggesting that this transcription factor was not activated by the cAMP pathway. In another set of experiments, we demonstrated that ACTH treatment of Y1 cells did not activate the NFκB pathway (Fig. 1D). Since sequence analysis of the murine HO-1 promoter indicated the presence of an NRF2 consensus binding motif located 4Kbp upstream the transcription start site (TSS), we also performed transient transfections with a reporter plasmid containing 4Kbp of the murine HO-1 promoter (−4045/+74). Results showed that stimulation of these cells with 8Br-cAMP or with ACTH significantly increased luciferase activity (Fig. 1E). However, neither 8Br-cAMP nor ACTH treatments were able to stimulate pHO-1(E1).LUC, a reporter plasmid containing the murine distal enhancer E1 and the Nrf2 consensus site (−4045/−3524, Fig. 1F) further supporting the idea that ACTH-dependent induction of HO-1 is not mediated by NRF2 activation. In addition, stimulation by 8Br-cAMP was conserved in a reporter plasmid containing only the proximal fragment of the HO-1 promoter (−295/+74) (Fig. 1G). 8Br-cAMP-stimulated luciferase activity of pHO-1(−295;+74).LUC was not affected by the addition of specific inhibitors for ERK, JNK or p38 MAPK signaling pathways (Fig. 1H).

It is well known that stimulation of the cAMP/PKA pathway has been associated with the phosphorylation and activation of CREB. Therefore, we sought to analyze the involvement of CREB on the induction of HO-1 in adrenal cells. To that end, we first evaluated the induction of HO-1 by cAMP in Y1 cells transfected with a plasmid expressing a dominant-negative form of CREB (DN-CREB-M1). Our results showed an inhibition of the 8Br-cAMP-induced increase in HO-1 protein levels in cells transfected with increasing levels of DN-CREB-M1 expression vector (Fig. 2A). Conversely, overexpression of CREB in cells transfected with increasing amounts of RSV-CREB significantly upregulated HO-1 protein levels (Fig. 2B). The effect of DN-CREB-M1 or RSV-CREB on the activity of a pCRE.LUC reporter in Y1 cells was also assayed (Fig. 2C and D).

Figure 3

Involvement of CREB in the stimulation of luciferase activity of pHO-1(−295/+74).LUC by ACTH and 8Br-cAMP. (A) Y1 cells were cotransfected with pHO-1(−295/+74).LUC and the expression plasmid DN-CREB-M1 and stimulated with either 12.5 mIU/mL ACTH (left panel) or 1 mM 8Br-cAMP (right panel) for 24 h; (B) Y1 cells transfected with pHO-1(−295/+74).LUC were incubated with or without 10 μM H89 for 30 min and further incubated in the presence or absence of 12.5 mIU/mL ACTH for 24 h (left panel) or 1 mM 8Br-cAMP (right panel). (A and B) Luciferase activity was determined in cell extracts and normalized against β-galactosidase activity. Values are shown as mean ± s.e.m., for three independent experiments. **$P < 0.01$, ***$P < 0.001$ vs respective control, ##$P < 0.01$, ###$P < 0.001$ vs 8Br-cAMP or ACTH by ANOVA followed by Tukey's test. (C) Comparison of the sequences surrounding the +1 promoter region of HO-1 from human (*Homo sapiens*), monkey (*Macaca mulatta*), bovine (*Bos taurus*), mouse (*Mus musculus*) and rat (*Rattus norvergicus*) origin using NCBI blast analysis tool. Enclosed areas correspond to CRE sites; *indicates conserved bases.

In the search of cAMP-response elements (CRE) along 4Kbp of the murine HO-1 promoter, we performed an analysis of transcription factor binding motifs with the MatInspector Release 8.2 software (Genomatix Software Suite, V3.1, Munich, Germany). Results showed in Fig. 2E indicate two different putative CRE binding sites located at −146 and −37.

Therefore, we performed a ChIP analysis in order to assess CREB recruitment to the endogenous gene. Results showed that stimulation of Y1 cells with 8Br-cAMP significantly increased the recruitment of phospho-CREB to the proximal promoter region of the *Hmox1* gene, while no significant binding was observed at the distal enhancer E1 (around 4 Kbp upstream the TSS) (Fig. 2F). These results support the notion that the proximal promoter region is involved in the induction of HO-1 by a cAMP/PKA/CREB-dependent pathway.

In agreement, transfection of Y1 cells with DN-CREB-M1 prevented the increase in luciferase activity of pHO-1 (−295/+74).LUC induced by ACTH or 8Br-cAMP (Fig. 3A). Similar results were obtained when the cells were incubated in the presence of the PKA inhibitor, H89 (Fig. 3B). A sequence comparison of the proximal promoter region of the murine *Hmox1* gene with the human, monkey, bovine and rat sequences indicates that the −37 CRE site is highly conserved among species, while a significant homology was observed in the −146 CRE site between mouse and rat promoter regions (Fig. 3C).

As several antioxidant and protective effects have been associated with the stimulation of the PI3K/AKT pathway in different systems, we analyzed the involvement of this
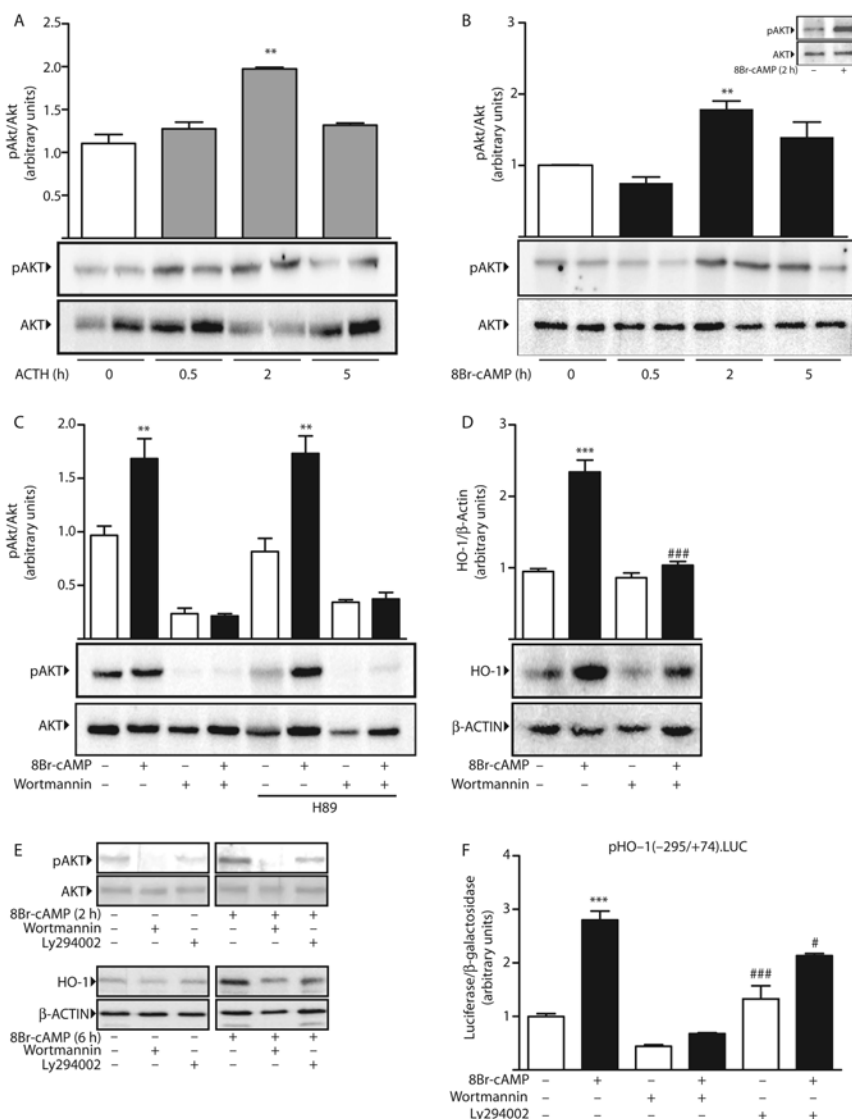
Figure 4

Activation of the PI3K pathway in adrenocortical cells. Y1 cells were incubated in the presence of 12.5 mIU/mL ACTH (A) or 1 mM 8Br-cAMP (B) for different periods of time; (C) Y1 cells were preincubated in the presence of 0.25 μM wortmannin and/or 10 μM H89 for 30 min and further incubated with or without 1 mM 8Br-cAMP for 2 h. AC protein extracts were prepared after treatments and analyzed by western blotting with p-AKT and AKT antibodies. Densitometry analysis of data is shown as fold induction (mean ± s.e.m.), obtained from three independent experiments. Representative images are shown in lower panels. A representative western blot showing pAKT and AKT levels in control and 8Br-cAMP-stimulated Y1 cells 2 h after the initiation of the treatment is shown as an inset in Fig. 4B. (D) Y1 cells were incubated with 0.25 μM wortmannin for 30 min and further treated with or without 1 mM 8Br-cAMP for 6 h. Protein extracts were prepared and analyzed by western blotting with HO-1 and β-ACTIN antibodies. Representative images are shown in lower panels. (E) Y1 cells were preincubated with 0.25 μM wortmannin or 10 μM LY 294002 for 30 min and further incubated with or without 1 mM 8Br-cAMP for 2 h or 6 h. Protein extracts were analyzed by western blotting with pAKT and AKT antibodies (2 h) and HO-1 and β-ACTIN antibodies (6 h). Representative images from three independent experiments are shown. (F) Y1 cells transfected with pHO-1(−295/+74).LUC were preincubated with 0.25 μM wortmannin or 10 μM LY294002 for 30 min and further treated with or without 1 mM 8Br-cAMP for 24 h. Luciferase activity was determined in cell extracts and normalized against β-galactosidase activity. Values are shown as fold induction (mean ± s.e.m.) for three independent experiments, **$P < 0.01$, ***$P < 0.001$ vs control and #$P < 0.05$, ###$P < 0.001$ vs 8Br-cAMP, by ANOVA followed by Tukey's test.
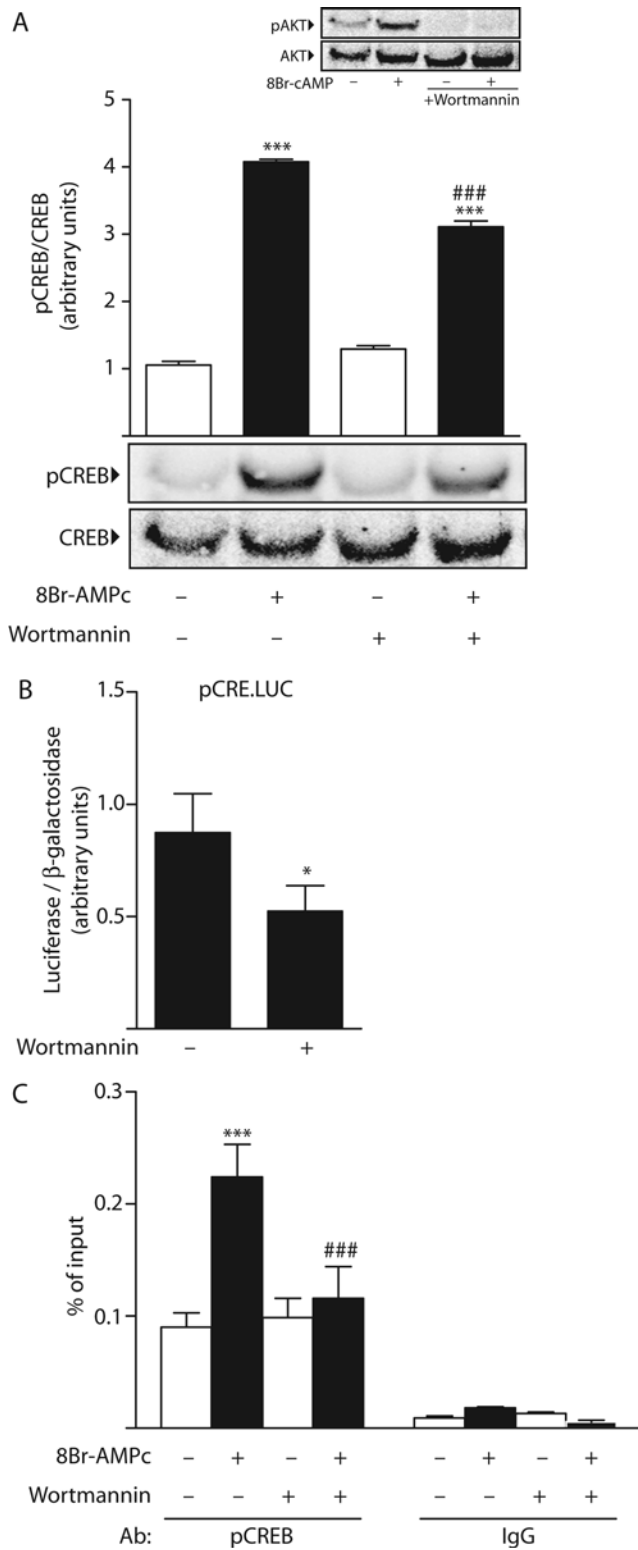
Figure 5

Modulation of CREB activation and binding to the HO-1 promoter by the PI3K/AKT pathway. (A) Y1 cells were treated with 0.25 μM wortmannin for 30 min and further incubated with or without 1 mM 8Br-cAMP for 2 h. (A) Protein extracts were prepared and analyzed by western blot with

pathway in the induction of HO-1 by cAMP in Y1 cells. Figure 4 shows a time course of AKT phosphorylation by ACTH (Fig. 4A) and 8Br-cAMP (Fig. 4B) indicating the activation of this signaling pathway. As shown in Fig. 4C, this effect was inhibited by the PI3K inhibitor, wortmannin, but not by H89, ruling out the involvement of PKA on Akt activation. As for the expression of HO-1, wortmannin treatment also inhibited HO-1 induction by 8Br-cAMP, as determined by western blotting (Fig. 4D). Accordingly, the PI3K inhibitor, LY204002, also prevented the increase in the pAKT/AKT ratio and in the expression levels of HO-1 triggered by 8Br-cAMP (Fig. 4E). Luciferase activity of the reporter plasmid containing the proximal fragment of the HO-1 promoter pHO-1(−295/+74).LUC was also inhibited by wortmannin and LY294002 (Fig. 4F). These results suggest the establishment of a crosstalk between the cAMP and PI3K pathways affecting the stimulation of HO-1 transcription.

In order to test this hypothesis, we first incubated Y1 cells with 8Br-cAMP in the presence of wortmannin and assessed CREB activation by determining Ser133-CREB phosphorylation. Results presented in Fig. 5A indicated that wortmannin slightly but significantly prevented the increase in pCREB/CREB ratio triggered by 8Br-cAMP. This treatment also prevented the 8BrcAMP-dependent increase in the pAKT/AKT ratio (insert) and the stimulation of luciferase activity in cells transfected with pCRE.LUC, further supporting the crosstalk hypothesis (Fig. 5B).

When ChIP analysis was performed to assess the effect of wortmannin on the binding of pCREB to the proximal promoter of HO-1, we observed that the PI3K inhibitor completely blocked the 8Br-cAMP-dependent increase in pCREB binding to the proximal promoter region, suggesting that an active PI3K signaling pathway

p-CREB and CREB antibodies. Densitometry analysis of data is shown as fold induction (mean ± s.e.m.), obtained from three independent experiments, ***$P < 0.001$ vs control and ###$P < 0.001$ vs 8Br-cAMP, by ANOVA followed by Tukey's test. Representative images are shown in the lower panel, Inset: A representative western blot analysis with anti p-AKT and AKT is shown. (B) Y1 cells were transfected with pCRE.LUC, preincubated with 0.25 μM wortmannin for 30 min and further incubated with 1 mM 8Br-cAMP for 24 h. Luciferase activity was determined in cell extracts and normalized against β-galactosidase activity. Values are shown as fold induction (mean ± s.e.m.) for three independent experiments. *$P < 0.05$ vs control by Student's t-test. (C) Cells were treated as in (A) and then subjected to ChIP assays with p-CREB antibody. The precipitated DNA fragments were analyzed by qPCR to test the enrichment in the sequences corresponding to the proximal CRE sites (−156; −37) from murine HO-1 promoter. Immunoprecipitation with normal rabbit IgG was used as control. Results are shown as mean ± s.e.m. ($n = 3$), ***$P < 0.001$ vs corresponding control and ###$P < 0.001$ vs 8Br-cAMP, by ANOVA followed by Tukey's test.

is necessary for the proper recruitment of pCREB to this region (Fig. 5C).

Discussion

In this study, we report that the transcriptional stimulation of the *Hmox1* gene by ACTH/cAMP relies on the phosphorylation of CREB and its binding to a functional CRE localized within the proximal promoter region of HO-1. We also suggest the involvement of the PI3K pathway in the recruitment of pCREB to the HO-1 promoter.

Induction of HO-1 has been widely recognized as an antioxidant response upon exposure to stressful stimuli. In adrenal cells, HO activity has also been included in the category of autocrine/paracrine modulators of steroidogenesis (Bhasker *et al.* 1989, Bohm *et al.* 1993, Pomeraniec *et al.* 2004, Spiga *et al.* 2011). As HO-1 expression is induced in adrenocortical cells 5–8 h after ACTH addition (Pomeraniec *et al.* 2004), we hypothesize that induction of HO-1 by ACTH in adrenocortical cells could serve two purposes: (a) protects the cells from the deleterious effects associated with an increased generation of ROS and (b) inhibits steroidogenesis by a mechanism involving heme oxygenase activity (CO generation, heme catabolism etc.), thus allowing the cells to avoid an unregulated steroidogenic response. In fact, we have previously demonstrated that inhibition of HO-1 expression or activity results in significantly higher steroid levels in both Y1 cells and rats (Pomeraniec *et al.* 2004, Grion *et al.* 2007).

ACTH is the main regulator of glucocorticoid synthesis in adrenal cells, a process that, even under physiological conditions, releases reactive oxygen species by leaking incompletely reduced products of O_2 (Battista *et al.* 2009). Accordingly, we have previously demonstrated an increase in ROS generation in adrenocortical Y1 cells incubated with ACTH. The role of HO-1 as an antioxidant enzyme is suggested, as ROS generation induced by H_2O_2 was significantly lower in cells where HO-1 was induced by pretreatment with ACTH. Although the involvement of NRF2 in HO-1 induction in adrenocortical cells stimulated with NO-donors has been previously demonstrated (Astort *et al.* 2014), several evidences led us to discard its participation in the induction of HO-1 by ACTH/cAMP. First, neither ACTH nor 8Br-cAMP treatment stimulated luciferase activity of an NRF2 reporter plasmid, and although 8Br-cAMP was able to stimulate luciferase activity in Y1 cells transfected with a reporter plasmid containing 4 Kbp of the HO-1 promoter of the murine gene (including the E1 enhancer containing an ARE element),

stimulation was conserved in a reporter plasmid carrying only 300 bp of the proximal promoter that lacks the ARE. Finally, 8Br-cAMP treatment failed to induce luciferase activity of a reporter plasmid containing only the E1 region.

Several effects of ACTH in adrenal cells are mediated by the activation of the cAMP/PKA pathway (Kimura 1986, Schimmer 1995, Gallo-Payet & Payet 2003, Sabban *et al.* 2004, Gallo-Payet 2016). As stated previously, among them we have demonstrated the participation of the cAMP/PKA pathway in the transcriptional stimulation of HO-1 by ACTH (Pomeraniec *et al.* 2004). Similar results were obtained in hepatic cells, where glucagon treatment led to the induction of the *Hmox1* gene through a cAMP/PKA-dependent mechanism (Immenschuh *et al.* 1998*b*).

Phosphorylation of CREB on Ser133 by PKA regulates its transcriptional activity and promotes the recruitment of the co-activator protein CBP (CREB-binding protein)/p300 linking the CRE/CREB complex to components of the basal transcriptional machinery. It was therefore a suitable candidate as a mediator for the ACTH effect at the transcriptional level. Moreover, CREB phosphorylation and binding to CREs has been involved in the induction of HO-1 by different stimuli (Kronke *et al.* 2003, Wright *et al.* 2009, Park *et al.* 2013, Barnett *et al.* 2015). In our experimental system, targeting CREB expression with a dominant-negative isoform of CREB (DN-CREB-M1) decreased, while overexpressing CREB (RSV-CREB) increased HO-1 protein levels in 8Br-cAMP-stimulated Y1 cells, suggesting the involvement of CREB in the ACTH/cAMP-mediated induction of HO-1.

Involvement of CRE sites in the induction of HO-1 has been linked to the stimulation of cGMP/PKG, to the inhibition of protein phosphatases 1 and 2A and to the overexpression of biliverdin reductase via ATF-2 (Immenschuh *et al.* 1998*a*, 2000, Kravets *et al.* 2004). In rat hepatocytes, cGMP/PKG-dependent induction of HO-1 involves a CRE/AP-1 site located −665/−654 upstream the TSS (Immenschuh *et al.* 1998*a*), while in human endothelial cells, a distal promoter fragment (between 3.8 and 4.9 kb upstream the TSS) containing a CRE site was responsible for the PKA-mediated induction of HO-1 by oxidized phospholipids (Kronke *et al.* 2003). This CRE site has been also shown to act in concert with a proximal E-box located within the proximal promoter of the human *Hmox1* gene (Wright *et al.* 2009).

Analysis of 4 kb from the murine HO-1 promoter showed the presence of only two putative CREB binding sites located at −146 and −37 upstream the TSS.
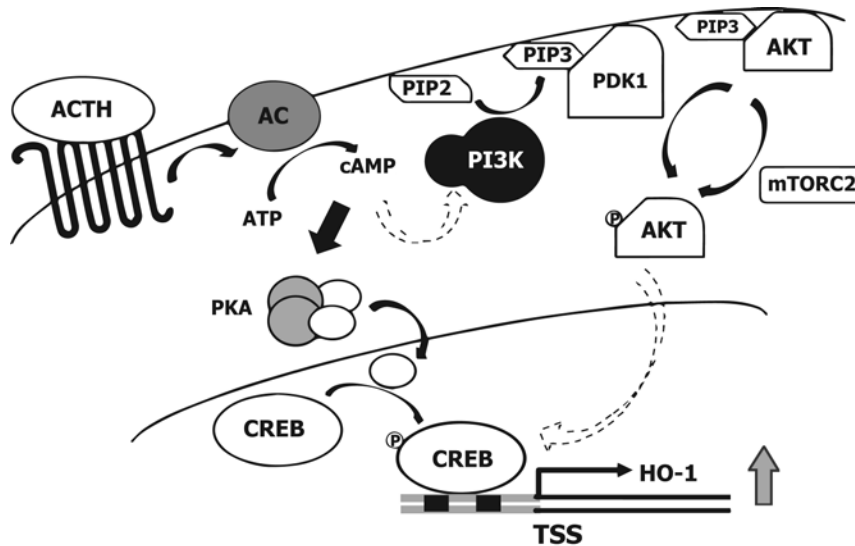
Figure 6

Signaling pathways involved in ACTH-induced HO-1 expression in murine adrenocortical cells. Transcriptional stimulation of HO-1 expression by ACTH is mediated by its binding to a membrane G-protein-coupled receptor (Melanocortin type 2 receptor), the consequent generation of cAMP and the activation of PKA. This, in turn, triggers the phosphorylation and binding of CREB to the proximal promoter region of the murine *Hmox1* gene. In addition, stimulation of the PI3K pathway by cAMP, by a yet unknown mechanism, results in the modulation of the binding of pCREB to its cognate binding site in the proximal promoter region.

These CRE-like sites present a high homology with those in the proximal promoter of the rat *Hmox1* gene, while the −37 site is conserved in all the species analyzed. In particular, this last site contains the Ebox sequence reported by Wright and coworkers in the human HO-1 promoter (Wright *et al*. 2009).

Our results showed that this proximal promoter region of the murine *Hmox1* gene recruited phospho-CREB in cAMP-stimulated Y1 cells, as determined by ChIP analysis. In agreement, both pharmacological (PKA inhibitor H89) and molecular (DN-CREB-M1) inhibition of CREB activation resulted in a significant decrease in the activity of the pHO-1(−295/+74).LUC reporter plasmid. These results further support the involvement of this proximal promoter region in the PKA-mediated induction of *Hmox1* gene. This is, to our knowledge, the first report on the induction of HO-1 mediated by CREB recruitment within the proximal promoter region of the murine *Hmox1* gene.

It is well known that activation of PI3K/AKT signaling functions as a cytoprotective mechanism against oxidative stress (Mo *et al*. 2012, Ryu *et al*. 2014, Yang *et al*. 2013). In this sense, our results demonstrated that, in addition to stimulating *Hmox1* gene expression, treatment of Y1 cells with either ACTH or 8Br-cAMP also leads to an increase in Akt phosphorylation levels. As this effect was prevented by the addition of specific PI3K inhibitors (Bain *et al*. 2007), but not by H89, we ruled out the involvement of PKA on AKT phosphorylation. As an alternative mechanism, Baviera and coworkers described an Epac-dependent PI3K activation by cAMP in rat skeletal muscle (Baviera *et al*. 2010).

Our results also indicated that the PI3K signaling pathway could modulate the transcriptional stimulation of the *Hmox1* gene triggered by cAMP, as wortmannin and LY 294002 treatments inhibited HO-1 induction and the activation of the pHO-1(−295/+74).LUC reporter induced by 8Br-cAMP. Based on these results, we hypothesized that PI3K activation could affect the phosphorylation status of CREB and/or the binding of p-CREB to the HO-1 promoter. Although PI3K inhibition, only slightly (but significantly) decreased CREB phosphorylation by 8Br-cAMP, this pathway appears to play a significant role in the recruitment of pCREB to the HO-1 promoter, suggesting that protein kinases activated downstream of PI3K (such as AKT, PKCξ, RSK, SGK, p70S6K etc.) could modulate the binding of CREB to the HO-1 promoter. We do not discard the possibility that activation of the PI3K pathway affects the binding of CREB co-activators, thus influencing HO-1 transcription. In this sense, an increase in the recruitment of CBP to NRF2 or NFκB upon activation of the PI3K pathway has been previously demonstrated (Tang *et al*. 2007, Sakamoto *et al*. 2009).

In summary, binding of ACTH to the MC2R induces steroidogenesis and triggers the transcriptional stimulation of *Hmox1* gene. In the present work, we demonstrate that HO-1 induction involves the classical cAMP/PKA pathway that leads to the phosphorylation and activation of CREB and to p-CREB recruitment to a proximal region of theHO-1 promoter. Our results also show that HO-1 induction is modulated by a functional crosstalk between the cAMP/PKA pathway and the PI3K-dependent cascade (Fig. 6).

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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