Adrenocorticotropin induces heme oxygenase-1 expression in adrenal cells

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Abstract

Heme oxygenase (HO) catalyzes the first and rate-controlling step of heme catabolism into biliverdin, iron and carbon monoxide. Three isoforms of HO have been identified so far: the inducible HO-1 and the constitutive HO-2 and HO-3. Both HO-1 and HO-2 were expressed in zona fasciculata (ZF) adrenal cells and in a mouse adrenocortical cell line (Y1). HO-1 but not HO-2 expression was upregulated by adrenocorticotropic hormone (ACTH) and accumulation of HO-1 protein correlated with an increase in HO activity in Y1 cells. ACTH induced HO-1 expression in a time- and dose-dependent manner with a maximum after 5 h of treatment and a threshold concentration of 0·1 mIU/ml. Actinomycin D and cycloheximide completely blocked the effect of ACTH on HO-1 mRNA expression whereas mRNA stability was not affected by ACTH. Permeable analogs of cAMP mimicked the effect of ACTH on HO-1 expression and ACTH induction was prevented by the protein kinase A (PKA) inhibitor H89. Steroid production was significantly increased when both HO-1 and HO-2 activities were inhibited by Sn-protoporphyrin IX (SnPPIX). The lipid peroxidation and increase in carbonyl content triggered by hydrogen peroxide was prevented by treatment of Y1 cells with bilirubin and ACTH.

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Introduction

Microsomal heme oxygenase (HO) is the rate-controlling enzyme for heme degradation in mammals (Maines 1988). HO has been classically described as an inducible enzyme (now identified as HO-1) involved in the catabolism of degraded heme-containing proteins, principally hemoglobin but also cytochromes, nitric oxide synthases, guanylate cyclase (GC) and cyclooxygenase. HO activity results in the production of carbon monoxide (CO), free iron, and biliverdin. In the presence of NADPH-cytochrome P450 reductase and NADPH, biliverdin is subsequently converted to bilirubin by biliverdin reductase (Tenhunen *et al.* 1968). A second isoform, HO-2, is constitutively active and is primarily located in the brain and testis (Maines 1997). These two genetically distinct isozymes share about 40% amino acid identities (Cruse & Maines 1988). A third isozyme, HO-3, exhibits only low enzymatic activity and is predicted to play a role in either heme binding or sensing (McCoubrey *et al.* 1997). Gene expression of HO-1 and HO-2 was shown to be highly tissue-dependent. In contrast to the constitutive HO-2, HO-1 is strongly induced by its substrate heme and by numerous stress stimuli such as u.v. light, heavy metals, lipopolysaccharides and heat shock (Keyse & Tyrrell 1989, Gabis *et al.* 1996, Yet *et al.* 1997), and thus, HO-1 gene activation has been considered to be an adaptive cellular defense mechanism against oxidative stress in mammalian cells (Keyse & Tyrrell 1989, Applegate *et al.* 1991, Clark *et al.* 2000, Mantell & Lee 2000, Otterbein & Choi 2000).

Both HO-1 and HO-2 have been detected in human and rat testis (Trakshel & Maines 1988). However, while Sertoli cells represent the major site of HO-1 expression within seminiferous tubules, HO-2 is present in germ cells of rat (Ewing & Maines 1995) and human (Middendorff *et al.* 2000) testis. A direct correlation between HO-1 induction by sodium arsenite in Sertoli cells and cyclic guanosine monophosphate (cGMP) production by a soluble GC was also demonstrated (Middendorff *et al.* 2000). Ozawa *et al.* (2002) suggested that $CdCl_2$-induced HO-1 expression in Leydig cells appears to regulate apoptosis of premeiotic germ cells and thereby modulates spermatogenesis under conditions of stress; in addition, a protective function of spermatogenic cells under conditions of thermal stress was suggested for HO-1 induction (Maines & Ewing 1996). Both HO isoforms are also expressed in human placenta and a functional role in trophoblast invasion and in spiral artery transformation together with the function of CO as a vasodilator in the

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fetoplacental circulation has also been suggested (Lyall *et al*. 2000, Barber *et al*. 2001, Navarra *et al*. 2001). It has also been demonstrated that Cd^{2+} stimulates HO activity in the ovary (Maines & Kutty 1983).

Although HO activity has been assessed in rat adrenal gland (Maines & Trakshel 1992), so far neither the HO isoforms expressed, nor the biochemical mechanisms that regulate adrenal HO activity, have been investigated. The Y1 cell line, derived from murine adrenal cortex, is a useful model for investigating adrenal cells as they behave like normal steroidogenic cells in several aspects including the stimulation of steroid production by adrenocorticotropic hormone (ACTH) in a cAMP-dependent pathway (Schimmer 1979, 1995) and the ACTH-dependent induction of both early and delayed genes (Waterman 1994). In the present work we examined the effect of ACTH on HO expression in both zona fasciculata (ZF) adrenal and Y1 cells. Some of the physiological consequences of HO activity in these cells were also examined.

Materials and Methods

Chemicals

Porcine ACTH (12·5 U/ml) was obtained from Elea Laboratories (Buenos Aires, Argentina). HO-1 and HO-2 antibodies were from StressGen Biotechnologies Corp. (Victoria, Canada). Peroxidase-conjugated anti-IgG antibodies were purchased from Bio-Rad (Hercules, CA, USA). Enhanced chemiluminescence (ECL) reagents came from Amersham Pharmacia Biotech (Little Chalfont, Bucks, UK). All other chemicals were obtained from Sigma-Aldrich (St Louis, MO, USA). Pregnenolone antiserum was kindly provided by Dr A Bélanger (Laval University, Quebec, Canada).

Cell culture and treatments

Murine Y1 adrenocortical tumor cells were generously provided by Dr B Schimmer (University of Toronto, Toronto, Canada). Methods for the culture of Y1 mouse adrenal tumor cells (American Type Culture Collection, Rockville, MD, USA) have been published elsewhere (Schimmer 1979). Cells were maintained at 37 °C in growth medium (HAM F10) containing fetal bovine (2·5%) and horse (12·5%) sera.

For mRNA stability experiments Y1 cells were incubated with ACTH for 5 h and after a medium change, actinomycin D (1 μg/ml) was added. Transcriptional/translational upregulation of HO-1 was determined by treating Y1 cells with either actinomycin D (1 μg/ml) or cycloheximide (1 μg/ml) before ACTH addition. In each case, samples were taken at the indicated times and total RNA and proteins were analyzed by Northern and Western blot respectively. Cell viability was assessed by the trypan blue dye exclusion test as determined by microscopy. No significant difference was observed for any of the treatments.

ZF cell preparation and treatment

ZF cells were isolated using published procedures (Neher & Milani 1978). The cells were suspended in Krebs–Ringer bicarbonate buffer (pH 7·4) containing 10 mM glucose, 0·5% (w/v) bovine albumin and 0·1 mM 3-isobutyl-1-methylxanthine under 95% O_2–5% CO_2 and aliquoted in fractions.

RNA isolation and Northern blot analysis

Total RNA was extracted from Y1 cells with the Trizol reagent according to the manufacturer's instructions and subjected to Northern blot analysis as described elsewhere. Blots were hybridized at 42 °C overnight with random primed labeled probes and subsequently washed with 2 × SSPE/0·5% SDS at room temperature (RT) (twice) and 1 × SSPE/0·1% SDS at 65 °C (twice). To correct for differences in RNA loading the filters were stripped and rehybridized with a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probe.

cDNA probes

The rat HO-1, HO-2 and GAPDH cDNA probes were made by RT-PCR on rat adrenal total RNA. Briefly, 1 μg of total RNA was reverse transcribed with random primers. The reverse transcription was performed at 42 °C for 1 h, the reaction was stopped by heating at 95 °C for 5 min. The PCR was performed by adding the PCR mix prepared with specific sense and antisense primers, dNTP, Taq polymerase and reaction buffer. Primers for HO-1 were: forward primer, 5′-ACTTTCAGAAGGGTCAGG TGTCC-3′; reverse primer, 5′-TTGAGCAGGAAGG CGGTCTTAG-3′ (522 bp fragment). Primers for HO-2 were: forward primer, 5′-CCACCACTGCACTTTAC TTC-3′; reverse primer, 5′-GGTCTTCATACTCAGG TCCA-3′ (436 bp fragment). Primers for GAPDH were: forward primer, 5′-TCCCTCAAGATTGTCAGCAA-3′; reverse primer, 5′-AGATCCACAACGGATACA TT-3′ (309 bp fragment). The identity of the amplified products was confirmed by direct sequencing.

Immunoblot analysis

Y1 cells or isolated ZF cells were washed twice in PBS and lysed in 20 mM Tris–HCl (pH 7·4), 250 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton, 10 μg/ml leupeptin, 1 mM phenylmethylsulphonyl fluoride, 2 μg/ml pepstatin A. Samples were boiled for 5 min in SDS–PAGE loading buffer with dithiothreitol and

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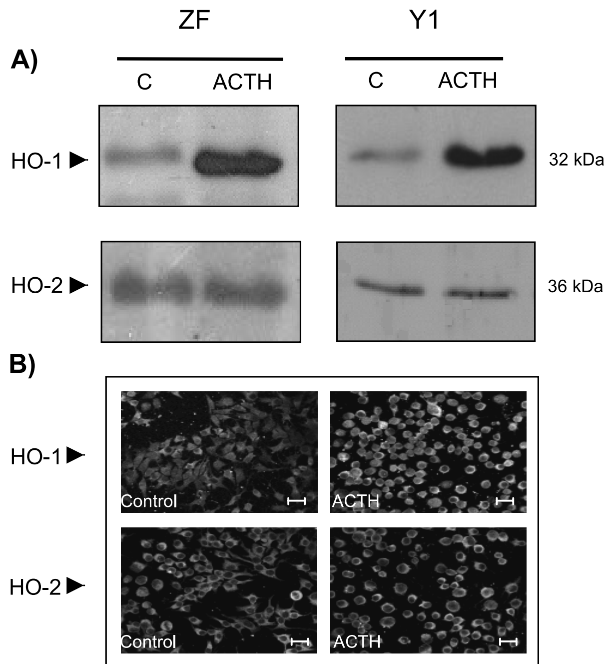

Figure 1 Expression of HO-1 and HO-2 in adrenal cells. (A) Isolated ZF cells (left panel) or Y1 cells (right panel) were incubated for 5 h in the absence or presence of 10 mIU/ml ACTH. Total cell proteins were separated by SDS-PAGE and HO isoforms were detected by immunoblot. (B) Immunofluorescence micrographs showing HO-1 and HO-2 staining in Y1 cells incubated for 5 h in the absence (control, 'C') or presence of 10 mIU/ml ACTH ('ACTH'). Scale bar, 10 µm.

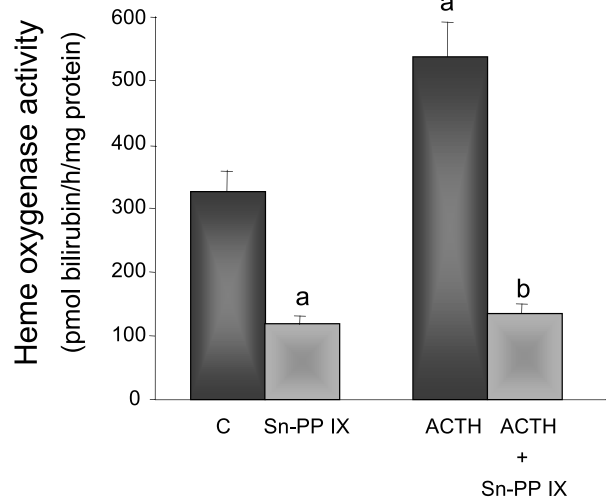

Figure 2 Heme oxygenase activity in Y1 cells. Y1 cells were exposed to 10 mIU/ml ACTH for 6 h and HO activity was determined in the absence or presence of 50 µM Sn-PPIX as reported in the Materials and Methods section. Each bar represents the mean ± S.E.M. of four independent experiments. a, $P<0{\cdot}01$ vs control; b, $P<0{\cdot}01$ vs ACTH without inhibitor; Tukey's test.

electrophoresed on 12% polyacrylamide gels. After electrophoresis, proteins were transferred to polyvinylidenefluoride (PVDF) membranes for 1 h at 15 V in a Bio-Rad Trans-Blot SD system in transfer buffer (48 mM Tris–HCl (pH 9·2), 39 mM glycine and 1·3 mM SDS). PVDF membranes were blocked in TBST buffer (50 mM Tris–HCl (pH 7·4), 0·15 M NaCl and 0·05% Tween 20) and 1% BSA for 60 min at RT and then incubated overnight in a 1:2000 dilution of the respective HO antiserum at 4 °C. Membranes were washed with TBST buffer and then incubated for 2 h with a 1:5000 dilution of a goat anti-rabbit immunoglobulin G (IgG) antibody–horseradish peroxidase conjugate. The filters were washed and the bands were visualized by chemiluminescence (ECL Western Blotting Analysis System, Amersham Pharmacia Biotech).

Heme oxygenase activity

HO activity was determined in microsomal fractions from Y1 cells by monitoring the conversion of heme into bilirubin. In brief, 200 µl of the reaction mixture (0·33 mM hemin as substrate, 0·2 mg/ml microsomal protein, 0·5 mg/ml rat liver cytosol, 0·2 mM $MgCl_2$, 2 mM glucose-6-phosphate, 1·62 units/ml glucose-6-phosphate dehydrogenase, 0·5 mM NADPH, 25 mM potassium phosphate buffer (pH 7·4)) was incubated at 37 °C for 2 h in the presence or absence of 50 µM Sn-protoporphyrin IX (Sn-PPIX). The reaction mixture was extracted with 0·6 ml of chloroform and the bilirubin concentration in the chloroform layer was spectrophotometrically measured by the difference in absorbance between 464 and 530 nm (extinction coefficient 40/mM per cm). Readings for samples prepared in the absence of cell homogenate (blank) were subtracted from all other values. HO enzyme activity is indicated as picomoles of bilirubin formed per milligram of protein per hour.

Immunocytochemistry

Y1 cells grown on poly-L-lysine glass coverslips were washed once with PBS and then fixed overnight at 4 °C with 4% (w/v) paraformaldehyde in PBS. Briefly, sections were rinsed in PBS and incubated with blocking solution (1·5% goat serum in 0·3% Triton-X100 PBS) for 1 h at RT and incubated with rabbit polyclonal antibodies raised against HO-1 (1/100), HO-2 (1/100) or vehicle (control) in a humidified chamber for 24 h at 4 °C. Cy3-conjugated goat anti-rabbit IgG (Molecular Probes, Eugene, OR, USA) was used as a second antibody. After rinsing with PBS, the sections were mounted in FluorSave reagent (Calbiochem, CA, USA), and fluorescence was visualized using a Carl Zeiss 510 confocal laser scanning microscope.

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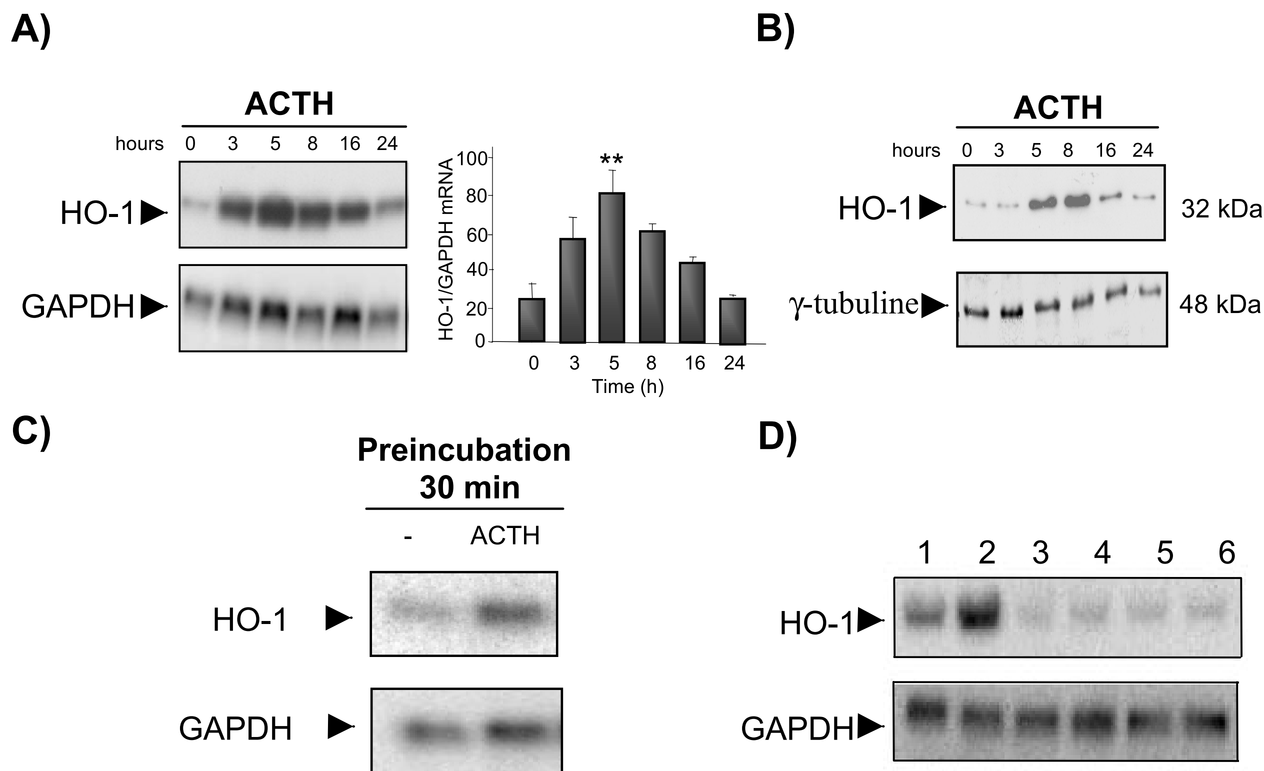

Figure 3 HO-1 mRNA induction in Y1 cells in response to ACTH. (A) Northern blotting analysis showing the induction of HO-1 mRNA expression in Y1 cells after exposure to 10 mIU/ml ACTH for the times indicated. Representative Northern blot of total RNA (24 μg/lane) subsequently probed with cDNAs for HO-1 and GAPDH (left panel). The histogram shows the integrated signals analyzed by densitometry of HO-1 mRNA normalized to GAPDH mRNA for three independent experiments (means ± S.E.M.); $^{**}P<0{\cdot}05$ vs control; Dunnett's test (right panel). (B) Western blot analysis for HO-1 and γ-tubuline of cell proteins (10 μg/lane) from the same experiment as in (A). (C) Northern blot analysis of Y1 cells that were preincubated with ACTH for 30 min and further incubated in fresh medium without ACTH for 5 h. Total RNA was isolated and analyzed as in (A). (D) Effect of steroids on HO-1 mRNA expression in Y1 cells. The cells were incubated without additions (lane 1) or with ACTH (10 mIU/ml, lane 2), 22R-hydroxycholesterol (5 μM, lane 3), pregnenolone (5 μM, lane 4), progesterone (5 μM, lane 5) or corticosterone (5 μM, lane 6) for 5 h and then analyzed as in (A). A representative Northern blot from three separate experiments is shown.

Determination of steroid levels

To assess pregnenolone production the cells were incubated in the presence of cyanoketone (5 μM), an inhibitor of 3β-hydroxy-Δ^5-steroid dehydrogenase, to block further conversion of pregnenolone to progesterone, and the appropriate additions. Pregnenolone levels were determined by RIA.

Measurement of lipid peroxides as thiobarbituric acid-reactive substances (TBARS)

Y1 cells were incubated with or without 10 mIU/ml ACTH for 5 h or with no additions for 4 h and with 1 μM bilirubin for 1 h. The medium was removed and the cells were further incubated for 1 h in 140 mM NaCl, 5 mM KCl, 2·5 mM $CaCl_2$, 1 mM $MgCl_2$ in 10 mM HEPES (pH 7·4) containing 80 μM H_2O_2. The content of lipid peroxides formed in Y1 cells was determined by the reactivity of malondialdehyde (MDA), an end product of lipid peroxidation, with thiobarbituric acid (TBA) as described previously (Yagi 1976). Briefly, cells were suspended in 2 ml of 0·05 M SDS and 0·5 × PBS and 1 ml 0·1 M HCl, 0·15 ml 10% phosphotungstic and 0·5 ml of 0·7% 2-thiobarbituric acid were added to each tube. The samples were heated for 60 min in a boiling water bath and, after cooling, 2·5 ml of butanol was added. The fluorescence of the butanol layer was measured at 515 nm excitation and 555 nm emission. The values are expressed as nanomoles of TBARS per milligram of protein using a malondialdehyde standard prepared from 1,1,3,3-tetramethoxypropane (Esterbauer *et al.* 1982).

Measurement of carbonyl production

Protein carbonyl content was determined by the method based on 2,4-dinitrophenylhydrazine (Reznick & Packer 1994). After the appropriate treatments Y1 cells were suspended in 50 mM phosphate buffer (pH 7·4). The

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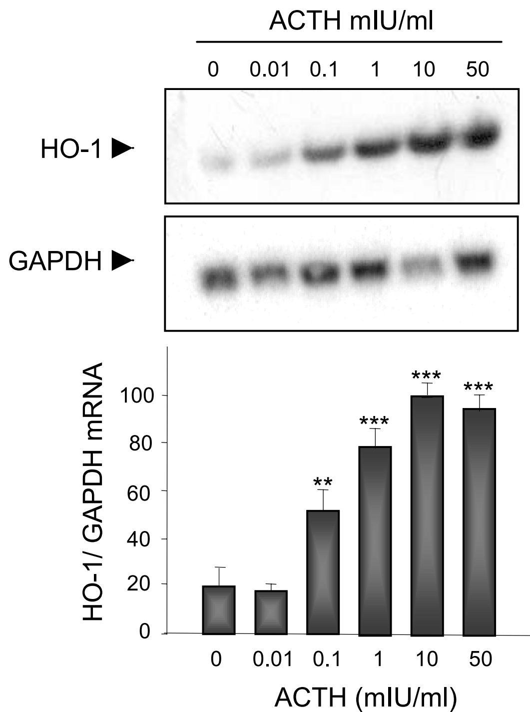

Figure 4 Dose–response effects of ACTH on HO-1 mRNA expression in Y1 cells. Cells were incubated for 5 h in the presence of increasing concentrations of ACTH (0·01–50 mIU/ml). Analysis by Northern blotting was performed essentially as described in the legend of Fig. 3. Values are means ± S.E.M. of three independent experiments. ***P*<0·05; ****P*<0·01 vs without ACTH; Dunnett's test.

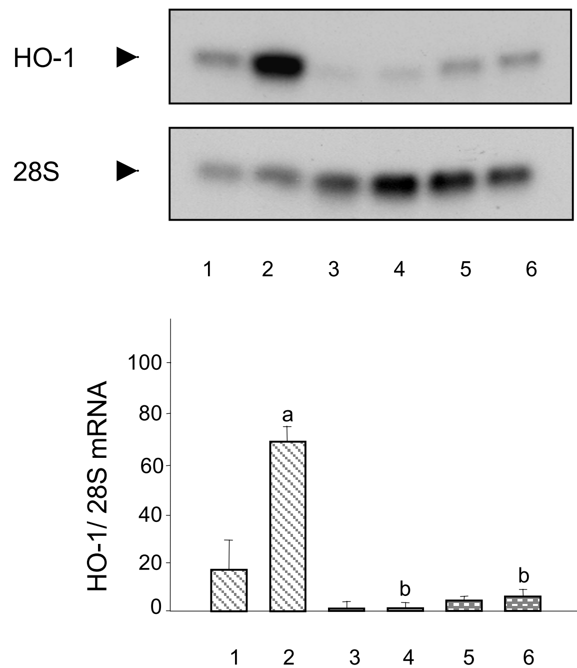

Figure 5 Effect of actinomycin D or cycloheximide on ACTH-dependent induction of HO-1 mRNA in Y1 cells. The cells were incubated for 30 min without additions (lanes 1 and 2), with actinomycin D (1 µg/ml, lanes 3 and 4) or with cycloheximide (1 µg/ml, lanes 5 and 6); cells were further incubated for 5 h in the absence (lanes 1, 3 and 5) or presence of ACTH (10 mIU/ml, lanes 2, 4 and 6). Total RNA was analyzed by Northern blot as indicated in the legend of Fig. 3. The densitometric evaluation of HO-1 mRNA normalized to 28S RNA, from three independent experiments (means ± S.E.M.) is shown below. a, *P*<0·01 vs 1 (control); b, *P*<0·01 vs 2 (ACTH); Tukey's test.

carbonyl content was determined by incubating Y1 cell suspensions with 1 ml 10 mM dinitrophenylhydrazine in 2·5 M HCl for 1 h in the dark. After the incubation period the proteins were precipitated with 20% tricarboxylic acid (TCA), the pellet was washed once with 10% TCA and three times with ethanol:ethyl acetate (1:1) and then redissolved by incubation in 1 ml of 6 M guanidine hydrochloride at 37 °C for 10 min. Carbonyl content was quantitated by its absorbance at 360 nm. The values are expressed as nanomoles per milligram of total protein (extinction coefficient 22/M per cm).

Statistical analysis

Statistical analysis of results was performed by one-way analysis of variance (ANOVA) followed by Dunnett's or Tukey's test.

Results

The presence of HO-1 and HO-2 in adrenal cells was examined by Western blot analysis. As shown in Fig. 1, both HO isoforms (HO-1 and HO-2) were detected in isolated ZF rat adrenal cells. When the cells were incubated in the presence of ACTH (10 mIU/ml), HO-1 but not HO-2 levels were increased (Fig. 1A, left panel). As in ZF cells, both HO-1 and HO-2 were detected in Y1 cells. Also in this case, ACTH increased the levels of HO-1 but not of HO-2 (Fig. 1A, right panel). Immunocytochemical staining for both HO-1 and HO-2 proteins in control and ACTH-treated Y1 cells is shown in Fig. 1B. An increase in HO-1-like immunoreactivity was observed in the presence of ACTH.

ACTH significantly stimulated HO-specific activity in Y1 cells, as shown in Fig. 2. This enzymatic activity was completely abolished by the HO-inhibitor, Sn-PPIX, both in the absence or presence of ACTH (Fig. 2). The time course of ACTH-stimulated HO-1 mRNA expression in Y1 cells was examined. HO-1 mRNA levels increased up to 5 h and slowly declined thereafter (Fig. 3A). HO-1 mRNA levels remained unchanged when Y1 cells were incubated for up to 24 h in the absence of ACTH (data not shown). Figure 3B shows that HO-1 expression were also increased by ACTH after 5 h and declined to basal levels at 24 h. The effect of ACTH was evident both

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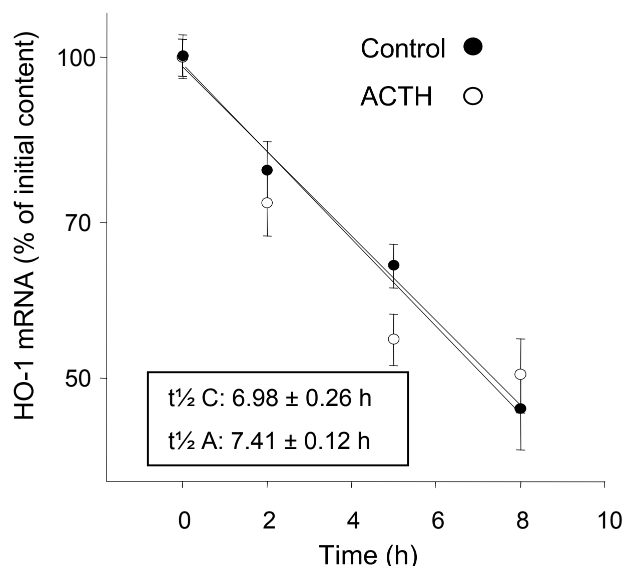

Figure 6 Effect of ACTH on the rate of degradation of HO-1 mRNA in Y1 cells. Cells were cultured in the absence (●) or in the presence (○) of 10 mIU/ml ACTH for 5 h. Cell culture was continued with actinomycin D (1 µg/ml). Total RNA was isolated at the times indicated, and levels of HO-1 mRNA and 28S RNA were determined by Northern blot analysis. Data are represented by a semilog plot of normalized individual points from three independent experiments (means ± S.E.M.). t½, half-lives are calculated from the slope of the corresponding curves.

in the absence or presence of serum (data not shown). In another set of experiments, Y1 cells were incubated in the presence of ACTH for 30 min, the medium was then replaced by fresh medium (without ACTH) and the cells were further incubated for 5 h. Results showed that ACTH enhanced HO-1 mRNA levels when it was present for only a 30-min period (Fig. 3C). The effect of ACTH on HO-1 mRNA levels in Y1 cells could not be reproduced by 22R-hydroxycholesterol (a permeable analog of cholesterol, 5 µM), pregnenolone (5 µM), progesterone (5 µM) or corticosterone (5 µM) (Fig. 3D). Figure 4 shows that ACTH increased HO-1 mRNA levels in a dose-dependent manner. This increase was already detectable at ACTH concentrations of 0·1 mIU/ml ($\cong 3 \times 10^{-10}$ M).

In order to get an insight into the mechanism of ACTH-dependent HO-1 mRNA induction, Y1 cells were treated with actinomycin D or cycloheximide before the addition of ACTH. As shown in Fig. 5, both inhibitors abolished the effect of ACTH. The HO-1 mRNA half-life did not differ between control and ACTH-treated cells (Fig. 6).

The time- and dose-dependency of the effect of a permeable analog of cAMP (8-Br-cAMP) is shown in Fig. 7. This nucleotide increased HO-1 mRNA levels from a concentration of 500 µM (Fig. 7A, left panel), this effect being already evident at 5 h. Similar results were obtained with dibutyryl cAMP (data not shown). To further investigate the signal transduction mechanism triggered by ACTH, the involvement of protein kinase A (PKA) and protein kinase C (PKC) was studied. A PKA inhibitor, H89 (*N*-[2-((*p*-bromocinnamyl)amino)ethyl]-5-isoquinolinesulfonamide, HCl; 10 µM), significantly inhibited HO-1 induction by ACTH (Fig. 7B, left panel). A higher dose of H-89 (20 µM) had no further effect on HO-1 induction by ACTH (data not shown). As shown in Fig. 7B, H89 also inhibited the increase in HO-1 mRNA levels induced by 8-Br-cAMP. In this case, the magnitude of the inhibition (60%) was higher than that observed in the presence of ACTH (45%). H89 (10 µM) treatment resulted in a 45–55% inhibition of 8-Br-cAMP- or ACTH-stimulated steroid production by Y1 cells (data not shown).

Although a phorbol ester (phorbol 12,13-dibutyrate (PDBu)) increased HO-1 mRNA levels, the effect of ACTH was not modified by the presence of two PKC inhibitors (Ro318220 and Ro320432; 3-[1-[3-(amidinothio)propyl-1H-indol-3-yl]-3-(1-methyl-1H-indol-3-yl)maleimide, methane sulfonate and 2-{8-[dimethylamino)methyl]-6,7,8,9-tetrahydropyrido[1,2-a]indol-3-yl]-3-(1-methyl indol-3-yl)maleimide, hydrochloride) (Fig. 8A and B respectively). The effect of HO activity on steroid production in Y1 cells was examined. Sn-PPIX significantly increased pregnenolone levels in control, 22R-hydroxycholesterol- and ACTH-stimulated cells (Table 1). Cytochrome P450 side-chain cleavage (Cyt P450 scc) protein levels were not affected by Sn-PPIX (data not shown).

TBARS levels and carbonyl contents (two indices of oxidative stress) were assessed in Y1 cells. Although ACTH treatment *per se* had no effect on these parameters, it significantly reduced lipid peroxidation and protein oxidation triggered by hydrogen peroxide. Bilirubin exerted a similar effect (Fig. 9).

Discussion

In the present work we demonstrated the presence of both HO-1 and HO-2 in Y1 cells as well as in isolated rat adrenal ZF cells. The coexistence of these HO isoforms has been demonstrated in other endocrine tissues such as human and rat testis and in human placenta (Trakshel & Maines 1988, Ewing & Maines 1995, Barber *et al.* 2001).

To our knowledge, the only previous report on HO in the adrenal gland was the assessment of its activity in the rat gland by Maines & Trakshel (1992). However, the identification of the isoforms was not previously provided. In the present report the coexistence of both isoforms was demonstrated by both Western and Northern blots, as well as by immunocytochemistry. In particular, immunocytochemical staining of Y1 cells supports a microsomal distribution of these enzymes that correlated with previous

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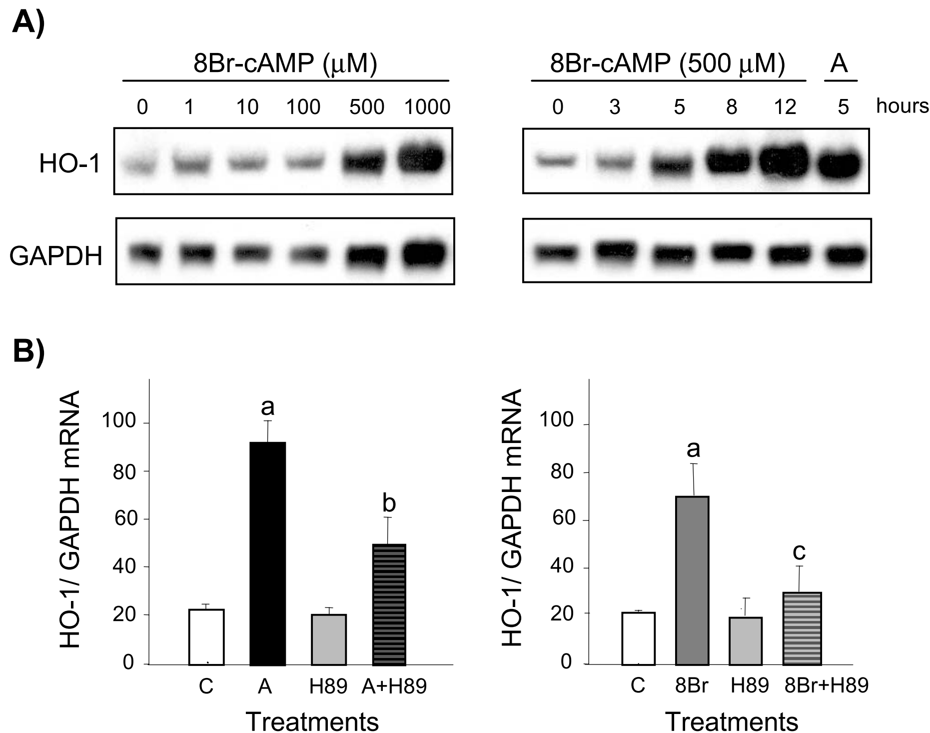

Figure 7 Signal transduction pathways in ACTH-dependent HO-1 induction in Y1 cells. (A) Dose-dependency (left panel) and time course (right panel) of HO-1 mRNA expression in Y1 cells treated with 8-Br-cAMP. Cell cultures were incubated for 5 h in the presence of increasing concentrations of 8-Br-cAMP (1–1000 μM) or in the absence or presence of 500 μM 8-Br-cAMP or 10 mIU/ml ACTH (A) for the times indicated. Total RNA was subjected to Northern blot analysis, probed with the cDNA of HO-1 and subsequently probed with the cDNA of GAPDH. (B) Effect of a cell-permeable inhibitor of PKA activity on ACTH- and 8-Br-cAMP-dependent induction of HO-1 expression in Y1 cells. Cells were incubated in the presence or absence of 10 μM H89 for 30 min and were further incubated without or with 10 mIU/ml ACTH ('A') (left) or 500 μM 8-Br-cAMP ('8-Br') (right) for 5 h. Total RNA was obtained and analyzed by Northern blot as described. Autoradiograms were quantitated, and values represent the relative HO-1 mRNA levels from three independent experiments (means ± S.E.M.). a, $P<0{\cdot}01$ vs control; b, $P<0{\cdot}01$ vs ACTH; c, $P<0{\cdot}01$ vs 8-Br-cAMP; Tukey's test.

reports in other cell types (Maines 1988). The present results also indicate that HO-1 but not HO-2 levels could be enhanced by the tropic hormone ACTH with a concomitant increase in HO enzymatic activity.

Although Y1 cells behave like normal steroidogenic cells in many aspects including the stimulation of steroid production by ACTH (Schimmer 1979, 1995), it is noteworthy that these cells derive from an adrenocortical tumor, so care must be taken when extrapolating results to normal steroidogenic cells. However, as far as HO expression is concerned, similar results were obtained in Y1 cells as compared with isolated ZF cells. Thus, for HO activity, these results further support the use of this cell line as a model of an adrenocortical cell system.

The induction of HO-1 by various hormonal stimuli has been previously demonstrated. It has been reported that hepatic HO activity is induced *in vivo* by glucagon, insulin and epinephrine (Bakken *et al.* 1972), while human chorionic gonadotropin increases this parameter in rat testis (Kutty & Maines 1989). Moreover, glucagon and angiotensin II induce HO-1 expression in hepatic and kidney cells respectively (Immenschuh *et al.* 1998, Haugen *et al.* 2000).

Our results showed that a significant increase in HO-1 mRNA and protein levels was observed in Y1 cells after 5 h of incubation in the presence of ACTH. However, the presence of the hormone during the first 30 min-period of incubation was enough to increase HO-1 mRNA levels. This time frame, as well as the effective dose of the hormone, are compatible with the occurrence of endogenous ACTH *in vivo* (Lu *et al.* 1983, Atkinson & Waddel 1997), supporting the physiological relevance of this effect. Although the influence of glucocorticoids on heme oxygenase activity has been demonstrated in other tissues (Deramaudt *et al.* 1999, Liu *et al.* 2000) the increase in HO-1 mRNA levels induced by ACTH in adrenal cells

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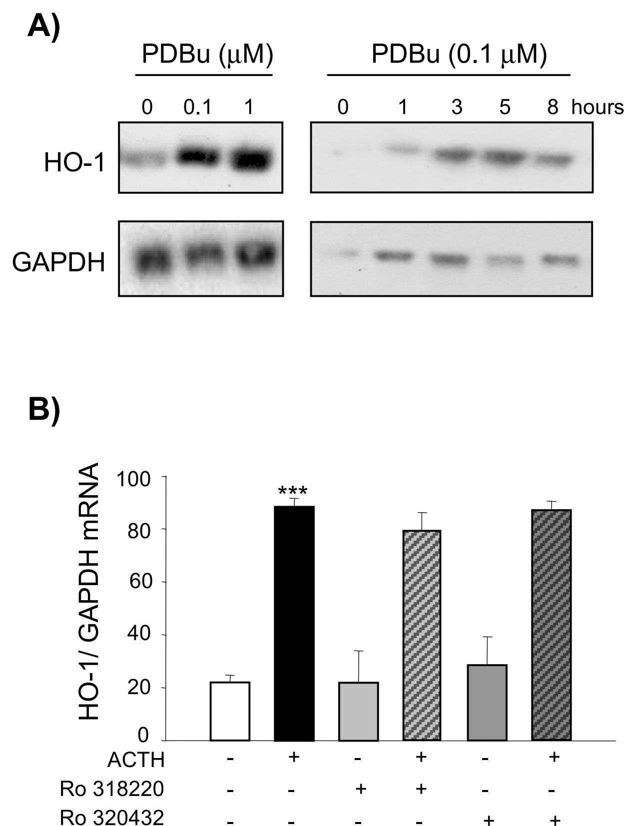

Figure 8 Phorbol esters induce HO-1 expression in Y1 cells. (A) Dose-dependency (left panel) and time course (right panel) of HO-1 mRNA expression in Y1 cells treated with the phorbol ester PDBu. Cell cultures were incubated for 5 h in the absence or presence of 0·1 or 1 μM PDBu or in the absence or presence of 0·1 μM PDBu for the times indicated. Total RNA was subjected to Northern blot analysis, probed with the cDNA of HO-1 and subsequently probed with the cDNA of GAPDH. (B) Effect of cell-permeable inhibitors of PKC activity on ACTH-dependent induction of HO-1 expression in Y1 cells. Cells were incubated in the presence or absence of 5 μM Ro 318220 or Ro 320432 for 30 min and were further incubated with or without 10 mIU/ml ACTH for 5 h. Total RNA was obtained and analyzed by Northern blot as described. Autoradiograms were quantitated, and values represent the relative HO-1 mRNA levels from three independent experiments (means ± S.E.M.). ***$P<0·01$ vs control; Tukey's test.

was not secondary to the increase in the steroidogenic activity caused by the hormone; this is demonstrated by the fact that the effect of ACTH on HO-1 expression could not be reproduced by 22R-hydroxycholesterol, pregnenolone, progesterone or corticosterone.

The results obtained in the presence of actinomycin D indicated that the effect of ACTH required *de novo* RNA synthesis and that HO-1 mRNA stability was not affected by the hormone. Accordingly, the stimulation of HO-1 expression by most, if not all, inductors is controlled primarily at the transcriptional level (Shibahara *et al.* 1987, Alam & Smith 1989). In addition, *de novo* protein synthesis appears to be a requisite for ACTH-dependent increase in HO-1 mRNA levels suggesting that protein(s) with a short half-life participate(s) in this pathway.

The classical mechanism of action of ACTH involves its binding to specific G-protein-coupled surface receptors in the adrenal cells and activation of a cAMP-dependent protein kinase that in turn acutely induces secretion of steroid hormones (Simpson & Waterman 1983). A delayed response implicates transcriptional activation of steroidogenic genes such as the gene for steroidogenic acute regulatory protein (StAR) (Stocco 2000) and the gene for cholesterol side-chain cleavage P450 (CYP11A) (Simpson & Waterman 1988). Two lines of experimental evidence indicated the involvement of the cAMP/PKA pathway in HO-1 induction by ACTH: (i) two cAMP analogs reproduced the effect of the hormone; (ii) a PKA inhibitor (H89) partially reverted its effect. Transcriptional activation of the HO-1 gene via the PKA signaling pathway has been demonstrated in rat hepatocyte cultures and vascular smooth muscle (Durante *et al.* 1997, Immenschuh *et al.* 1998). In addition, inhibition of dephosphorylation of phospho-CREB with a concomitant increase in cAMP-dependent gene expression, has been involved in HO-1 induction by okadaic acid, a serine threonine pyrophosphate inhibitor (Hagiwara *et al.* 1992, Immenschuh *et al.* 2000).

H89 inhibited HO-1 induction by 8-Br-cAMP more effectively than it inhibited induction by ACTH, suggesting that HO-1 induction by ACTH may involve other signaling pathways in addition to PKA-dependent mechanisms. In this sense, other secondary pathways, including PKC, Ca^{2+}–calmodulin-dependent protein kinase and the phosphoinositol pathway have also been involved in the effect of ACTH (Vilgrain *et al.* 1984, Bird *et al.* 1990, Papadopoulos *et al.* 1990). Since two inhibitors of PKC activity did not block the effect of ACTH on HO-1 induction, the participation of the PKC pathway in this mechanism seems unlikely.

In the last few years, the specificity of many widely used protein kinase inhibitors has been re-examined. These studies have demonstrated that the most commonly used inhibitors (H89 among them) exert an inhibitory effect on a number of protein kinases besides those previously reported (Davies *et al.* 2000). Although the inhibitory effect of H89 on ACTH and 8-Br-cAMP-stimulated steroid production indicates the inhibition of PKA activity in Y1 cells, the participation of other protein kinases in ACTH-dependent HO-1 induction cannot be formally ruled out. The involvement of signal transduction pathways other than PKA and PKC in ACTH-dependent HO-1 induction in Y1 cells is currently under investigation. In this sense, upregulation of HO-1 expression by different mechanisms has been demonstrated. Studies on the effect of cobalt, cadmium, arsenite and NO have shown the involvement of mitogen-activated kinases (ERK and P38) and of tyrosine phosphorylation of key

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Table 1 Effect of Sn-PPIX on pregnenolone production by Y1 cells

Pretreatment	Treatment	Pregnenolone (ng/mg protein)
None	None	1·51 ± 0·19[a]
None	Cyanoketone (5 μM)	6·20 ± 0·80
None	Cyanoketone (5 μM)+Sn-PPIX (50 μM)	18·74 ± 1·6[b]
None	Cyanoketone (5 μM)+22-R hydroxycholesterol (5 μM)	145·2 ± 2·4
ACTH	ACTH (10 mIU/ml)	19·99 ± 2·4[c]
ACTH	Cyanoketone (5 μM)+ACTH (10 mIU/ml)	72·53 ± 5·5[f]
ACTH	Cyanoketone (5 μM)+ACTH (10 mIU/ml)+Sn-PPIX (50 μM)	95·26 ± 5·2[d]
ACTH	Cyanoketone (5 μM)+ACTH (10 mIU/ml)+22R-hydroxycholesterol (5 μM)	157·7 ± 5·4
ACTH	Cyanoketone (5 μM)+ACTH (10 mIU/ml)+22R-hydroxycholesterol (5 μM)+Sn-PPIX (50 μM)	190·8 ± 7·0[e]

Y1 cells were preincubated for 5 h in the presence or absence of 10 mIU/ml ACTH, the medium was changed and the cells were further incubated for 2 h with the indicated additions. Pregnenolone levels were assessed by RIA. Each value represents the mean ± S.E.M. of three independent experiments, performed in quadruplicate.
[a]$P<0·01$ and [b]$P<0·001$ vs cyanoketone. [c]$P<0·001$ and [d]$P<0·05$ vs cyanoketone+ACTH. [e]$P<0·01$ vs cyanoketone+ACTH+22-R hydroxycholesterol.
[f]$P<0·001$ vs cyanoketone+22-R hydroxycholesterol. Tukey's test.

proteins in this mechanism (Elbirt *et al.* 1998, Shan *et al.* 1999, Alam *et al.* 2000, Chen & Maines 2000). A role for PI3K activation was demonstrated for the hepatocyte growth factor effect (Tacchini *et al.* 2001). It is increasingly apparent that the signal transduction pathway utilized to induce HO-1 expression depends on the inductor studied and on the cell type- and/or species-specific differences in the regulatory mechanisms.

In the past few years, CO has been implicated as a physiological messenger in the brain and cardiovascular system (as has nitric oxide (NO). Moreover, increasing experimental evidence suggests the existence of a significant 'cross-talk' between CO and NO systems: (i) both mediators share common molecular targets; (ii) NO induces the expression of HO-1; (iii) CO may regulate nitric oxide synthase (NOS) activity by interacting with its heme group. We have previously shown that NOS is involved in the regulation of both rat adrenal zona fasciculata (Cymeryng *et al.* 1998, 1999) and the physiology of Y1 cells (Cymeryng *et al.* 2002). Therefore we investigated the effect of HO activity on steroidogenesis in Y1 cells. Our results show that the HO inhibitor Sn-PPIX significantly increased steroid production both in control and stimulated Y1 cells, indicating that CO, or another product yielded by the reaction catalyzed by HO, may regulate steroidogenesis. Our results also suggest that basal steroid production is more effectively modulated by HO activity. It has been shown that metalloporphyrins also inhibit soluble guanylate cyclase and NOS activity *in vitro* (Grundemar & Ny 1997). However, this appears not to be the case in these cells as adrenal steroidogenesis is not regulated by cGMP (Cymeryng *et al.* 1999) and endothelial NOS activity (the only isoform detected in Y1 cells) was not inhibited by 50 μM SnPPIX (Zakhary *et al.* 1996, Cymeryng *et al.* 2002). In addition, present results indicate that cytochrome P450 scc is a putative physiological target for at least one of the products generated by HO activity. Therefore, as inhibition of cytochrome P450 by CO has been demonstrated (Trela *et al.* 1988), it is tempting to speculate that CO locally synthesized by HO, could be a modulator of adrenal steroidogenesis in general, and of the activity of cytochrome P450 scc in particular.

Another explanation for the results described above involves heme from cytochromes P450 acting as a substrate for HO activity. This is probably not the case in our system as only mitochondrial cytochrome P450 scc is involved in pregnenolone production in Y1 cells and cytochrome P450 scc protein levels were not affected by SnPPIX treatment. Nonetheless, an effect of HO activity on the stability of microsomal cytochrome P450 enzymes catalyzing subsequent steps of steroid synthesis in ZF adrenal cells cannot be ruled out. Veltman & Maines (1986) suggested that microsomal cytochrome P450 enzymes are a suitable substrate for HO activity induced by mercury in adrenal cells.

Even under physiological conditions, mitochondria in adrenocortical cells are a major source of free radicals which can cause critical damage (Hanukoglu *et al.* 1993). In order to deal with such oxidative stress, the adrenal cortex has abundant non-enzymatic antioxidants such as ascorbic acid and vitamin E (Hornsby & Crivello 1983). The adrenal cortex also expresses enzymes such as manganese superoxide dismutases (Mn-SOD) and glutathione peroxidase (GPx) to detoxify superoxide. Chronic administration of ACTH upregulates Mn-SOD and decreases GPx in rat adrenal capsules (Suwa *et al.* 2000). This imbalance could provoke the accumulation of H_2O_2, thus leading to the generation of the toxic noxious hydroxyl radicals. However, after chronic ACTH treatment the steroidogenic functions in the ZF are preserved leading to the speculation that the ZF may be equipped with more effective antioxidant systems than the zona glomerulosa.

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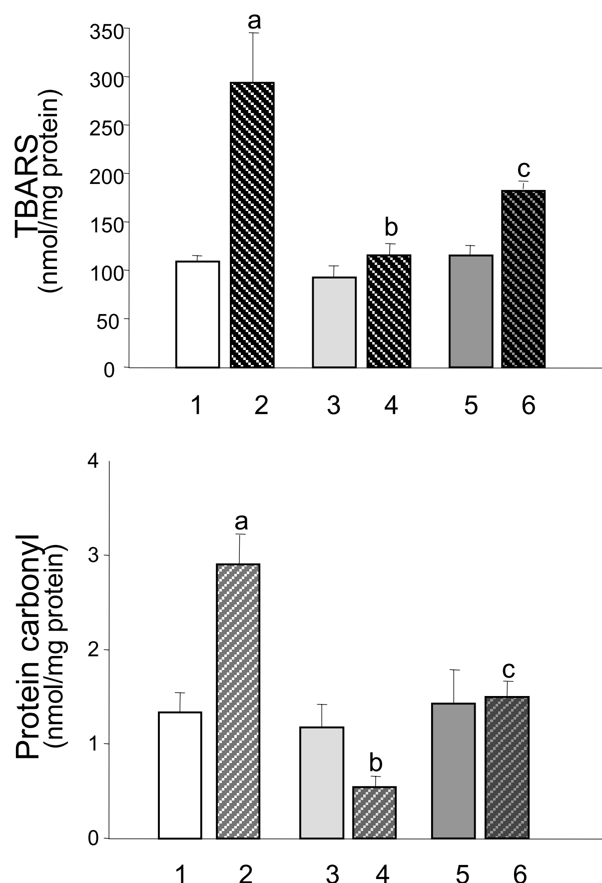

Figure 9 Indices of oxidative stress in Y1 cells. Cells were incubated with no additions (1 and 2) or with 10 mIU/ml ACTH for 5 h (3 and 4) or with no additions for 4 h and with 1 µM bilirubin for 1 h (5 and 6). The medium was removed and the cells were further incubated for 1 h with 80 µM H_2O_2 (2, 4 and 6). TBARS levels and carbonyl contents were determined as described in the Materials and Methods section. Values represent the means ± S.E.M. from three independent experiments. a, $P<0{\cdot}01$ vs control; b, $P<0{\cdot}001$ vs H_2O_2 and c, $P<0{\cdot}01$ vs H_2O_2; Tukey's test.

ACTH also enhances the mitochondrial Mn-SOD activity in ZF adrenal mitochondria (Raza & Vinson 2000). It has been suggested that ACTH plays a protective role in adrenal cells. Results have shown that ACTH exerts an anti-apoptotic effect in primary cultures of bovine adrenocortical cells (Negoescu *et al.* 1995), in hypophysectomized rats (Ceccatelli *et al.* 1995) and in intact adrenal glands in cultures (Carsia *et al.* 1998).

Several lines of evidence indicate that the induction of HO-1 may play a role in cellular protection against oxidative stress (Keyse & Tyrrell 1989, Applegate *et al.* 1991, Llesuy & Tomaro 1994, Vile *et al.* 1994, Clark *et al.* 2000, Mantell & Lee 2000, Otterbein & Choi 2000). This protective mechanism may involve the degradation of the pro-oxidant heme (Balla *et al.* 1993), production of bilirubin (a potent peroxyl radical scavenger) (Stocker *et al.* 1987, Llesuy & Tomaro 1994, Clark *et al.* 2000), iron extrusion from cells (Eisenstein *et al.* 1991, Vile & Tyrrell 1993) or production of CO (Otterbein *et al.* 2000, Thom *et al.* 2000). Our results support the hypothesis that in adrenal cells HO-1 induction is involved in the protective role of ACTH against oxygen toxicity. Whether this protection is achieved by the production of bilirubin, CO or by a combination of these factors is still not known.

The role of ACTH as a pleiotropic regulator in the ZF of the adrenal gland has been widely documented. In this study we provide evidence that ACTH induces an enzymatic system with antioxidant properties in Y1 cells and that such a mechanism is functionally relevant to adrenal physiology providing the gland with a higher capacity to respond to a wide range of physiological conditions.

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