Effects of heme oxygenase isozymes on Leydig cells steroidogenesis.

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[*] **BP and CMM must be considered with the same merit**

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ABSTRACT

In the present study we demonstrate the expression of heme oxygenase (HO) isozymes, HO-1 and HO-2, in MA-10 Leydig tumor cells and its effect on steroidogenesis. The well known HO-inducer, hemin, increased both HO-1 and HO-2 protein levels and HO specific activity. Induction of HO by hemin inhibited basal, hCG- and dibutyril cAMP (db-cAMP)-induced steroidogenesis in a reversible way. When we studied the effect of HO isozymes along the steroid synthesis, we found that steroidogenic acute regulatory (StAR) protein levels were decreased and the conversion of cholesterol to pregnenolone was inhibited by hemin treatment, with no changes in the content of cholesterol side-chain cleavage enzyme (P450scc). hCG and db-cAMP also stimulated the expression of HO-1 and HO-2, and HO enzymatic activity in MA-10 cells. Basal and hCG-stimulated testosterone synthesis was also inhibited by hemin in rat normal Leydig cells.

Taken together, these results suggest that: i) at least one of HO products (presumably carbon monoxide), inhibits cholesterol transport to the inner mitochondrial membrane and Leydig cell steroidogenesis by binding to the heme group of the cytochrome P450 enzymes, in a similar way as we described for nitric oxide and ii) hCG stimulation results in the induction of an antioxidant enzymatic system (HO) acting as a cytoprotective mechanism in Leydig cells, as already demonstrated in the adrenal gland.

INTRODUCTION

Steroid synthesis depends on two limiting steps, the first one is at the level of the transport of cholesterol to the inner mitochondrial membrane, a process dependent on the steroidogenic acute regulatory protein (StAR) (Stocco 2001); and the second key step is the conversion of cholesterol to pregnenolone by the cholesterol side chain cleavage enzyme system (Stocco & Clark 1996). In testis, Leydig cells are interstitial cells that synthesize testosterone through P-450 cytochromes dependent monooxygenases. Although steroidogenesis in Leydig cells is primarily under LH control, a number of paracrine/autocrine factors have been suggested to play a role in the modulation of this hormone action (Ascoli*, et al.* 2002; Hales 2002; Mondillo*, et al.* 2005; Stocco*, et al.* 2005; Mondillo*, et al.* 2007). Among them, the inhibitory effect of nitric oxide (NO) on steroid synthesis in Leydig cells had been previously demonstrated in our lab (Del Punta*, et al.* 1996).

Heme oxygenases (HO) catalyze the first and rate-limiting step in the oxidative degradation of heme into three products: carbon monoxide (CO), biliverdin, which is rapidly converted into bilirubin by biliverdin reductase, and free iron, which is sequestered into ferritin (Maines 1997). To date three isoforms have been identified: HO-1, HO-2 and HO-3 (McCoubrey*, et al.* 1997; Maines 2005). While HO-1 (32 kDa) expression can be induced by its own substrate heme, and several other stress stimuli such as heavy metals, LPS, inflammatory mediators and oxidized low density proteins (Otterbein & Choi 2000), the other two isoforms, HO-2 (36 kDa) and HO-3 (33 kDa) are constitutively expressed (McCoubrey*, et al.* 1992; McCoubrey *et al.* 1997). However, recent studies showed that corticosterone, estradiol and a photic signal can induce HO-2 expression in testis (Liu*, et al.* 2000), endothelial cells (Tschugguel*, et al.* 2001), and retina (Sacca*, et al.* 2003), respectively. Both, HO-1 and HO-2 isoforms have been detected in diverse organs, including reproductive ones such as testes (Trakshel & Maines 1988; Ewing & Maines 1995), placenta (Lyall*, et al.* 2000), uterus (Acevedo & Ahmed 1998) and ovary (Alexandreanu & Lawson 2003).

Carbon monoxide is a gaseous second messenger that shares several biological properties with NO; including the activation of guanylate cyclase, signal transduction and gene regulation (Verma*, et al.* 1993; Zhuo*, et al.* 1993). Based on the similarities with NO, recent studies suggested a possible regulatory role for CO on steroid production in ovary and adrenal gland (Alexandreanu & Lawson 2003; Pomeraniec*, et al.* 2004). In addition, Ozawa *et. al.* (2002) reported that CO derived from HO-1 in Leydig cells modulated spermatogenesis and triggered apoptosis of germ cells under stress conditions (Ozawa*, et al.* 2002). Besides, in humans, increased HO-1 expression in Leydig cells improved spermatogenesis in varicocele condition (Shiraishi & Naito 2005), and HO-1-derived CO in testicular Sertoli cells may have a functional role on soluble guanyl cyclase-dependent cGMP production in the seminiferous tubule (Middendorff*, et al.* 2000). Furthermore, numerous studies proposed that the activity of HO system might provide cellular protection against oxidative stress (Stocker 1990; Maines 1997; Niess*, et al.* 1999; Pomeraniec *et al.* 2004; Shiraishi & Naito 2005). Although HO-1 and HO-2 isoforms have been detected in the rat testes, the biochemical mechanisms by which the HO/CO system regulates steroidogenesis have not been investigated so far. So, the aim of this study was to analyze the expression levels of both HO isoforms and the influence of heme oxygenase activity on steroid production in MA-10 Leydig cells.

MATERIALS AND METHODS

Materials

Purified hCG (CR-127, 14; 900 IU/mg) was a gift from the National Hormone and Pituitary Program, National Institute of Diabetes and Digestive and Kidney Diseases (Bethesda, MD). The specific antibody for progesterone and testosterone were a gift from Dr. G.D. Niswender (Animal Reproduction and Biotechnology Lab, Colorado State University, Fort Collins, CO). HO-1 and HO-2 antibodies were from StressGen Biotechnologies Corp. (Victoria, Canada). StAR and cytochrome P450scc (P450scc or CYP11A) antibodies were a gift from Dr. Walter L. Miller (University of California, San Francisco) and Dr. Dale B. Hales (University of Illinois at Chicago), respectively.

Peroxidase-conjugated anti-IgG antibodies were purchased from Amersham Pharmacia. Cell culture supplies and plastic ware were obtained from Gibco-BRL (Gaithersburg, MD) and Corning (Corning, NY), respectively. Hemin (a well known Heme oxygenase-inducer) and db-cAMP (dibutyryl cyclic AMP, the permeable analog of the second messenger) were purchased from Sigma (St. Louis, MO). Collagenase was from Worthington (Freehold, NJ). Other reagents used were of the best grade available and were obtained from commonly used suppliers.

Cellular Culture of MA-10 Leydig Cells

The MA-10 cell line (kindly provided by Mario Ascoli, University of Iowa, Ames, IA) is a clonal strain of Leydig tumor cells that secrete progesterone rather than testosterone as a major steroid. This cell line provides a suitable model system for the study of gonadotropin actions and regulation of differentiated functions of Leydig cells, as they behave like normal steroidogenic cells in several aspects, including the stimulation of steroid production by LH/hCG in a cAMP-dependent pathway. The origin and handling of MA-10 cells has already been described (Ascoli 1981; Pignataro & Ascoli 1990b). Cells were plated in 100-mm Petri dish plates (for immunoblot analysis and heme oxygenase activity) or in 24 x 16-mm well plates (for steroidogenesis experiments) on Day 0 at a density of 3 x 10^6 cells/dish or 1.25 x 10^5 cells/well, and in a total volume of 10 ml or 1 ml of growth medium (Waymouth MB752/1, modified to contain 1.1 g/L $NaHCO_3$, 20 mmol l^{-1} Hepes, 50 mg/ml gentamycin, and 15% (v/v) horse serum, pH 7.4), respectively. The cells were maintained in a humidified atmosphere containing 5% CO_2 and were used on Day 3. At this time, the cell density was approximately 10 x 10^6 cells/dish or 5 x 10^5 cells/well. On this day, the cells were washed with 1 ml of warm serum-free medium supplemented with 1 mg/ml of BSA (assay medium). Incubations were performed in a total volume of 7 ml (for dishes) or 0.5 ml (for wells) assay medium at 37 °C with the corresponding additions as described in each Figure. After 5 h (unless other indicated), media were collected and progesterone (P4) was measured by radioimmunoassay (RIA) (Pignataro & Ascoli 1990a). The intraassay and interassay variations were

8.0% and 14.2%, respectively. Cells were treated as describes below. When hemin was used for experiments, 30 min-pretreatment with the compound was done.

To study the reversibility of the inhibitory effect of the heme oxygenase-inducer on steroid synthesis, MA-10 cells were incubated in the absence or presence of 10 μmol l^{-1} hemin, with or without 0.2 mmol l^{-1} db-cAMP. After 5 h, media were collected for progesterone determination (Day 1). Cells were washed and incubated with hemin-free fresh medium for an additional 24 h. By the end of this incubation period, cells were stimulated with 1 mmol l^{-1} db-cAMP for 5 h (Day 2).

Rat Leydig cell isolation and testosterone production

For all the experiments, Leydig cells were isolated from a pool of 16 testes obtained from 8 adult Sprague-Dawley rats (60 days old, 200-250 g, Charles River descendants, Animal Care Lab, IByME, Buenos Aires) as we previously described (Charreau*, et al.* 1981; Pignataro*, et al.* 1983; Mondillo*, et al.* 2009). Animals were housed in groups in an air-conditioned room, with lights on from 0700-1900 hours. They were given free access to laboratory chow and tap water. Animals were killed by CO_2 asphyxia according to protocols for animal use approved by the institutional animal care and use committee (IBYME-CONICET) that follows NIH guidelines.

The cell pellets were resuspended in M199-0.1% (w/v) BSA and purified by fractionation on a three-layer Percoll density gradient (26%, 34% and 60% (v/v)). The purity of Leydig cells obtained was over 92%, as assessed by histochemical staining for 3β-hydroxysteroid dehydrogenase activity (Payne*, et al.* 1980). Less than 1.7 and 1.2% of the contaminating cells in the Leydig cell preparations were positive macrophages for ED-1 and ED-2 antigens, respectively (Frungieri*, et al.* 2006; Mondillo *et al.* 2009). The remaining cell types had the morphology of either peritubular cells or endothelial cells.

The pellets, enriched in Leydig cells, were gently resuspended in M199 containing 1.2 g/l $NaHCO_3$, 20 mmol l^{-1} Hepes, 0.1 mmol l^{-1} 1-methyl-3-isobutylxanthine (MIX) and 0.5% (w/v) BSA. Incubations were done in plastic tubes in a volume of 500 μl ($5x10^5$ cells/tube), at 34 °C with

shaking, for 5 h. The samples were then centrifuged at 800 *g* for 5 min and the supernatants were used to determine testosterone by RIA (Mondillo *et al.* 2005). The intra-assay and inter-assay variations were 7.3 and 13.2%, respectively.

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MA-10 cell lysates and mitochondria isolation

After collecting the media, MA-10 cells were scrapped and centrifuged at 800 *g* for 10 min. The resulting pellet was resuspended in a medium containing 0.25 mol l^{-1} Sucrose, 0.1 mmol l^{-1} EDTA, 10 mmol l^{-1} Tris-HCl (pH 7.4), with proteases inhibitors (10 mg/ml leupeptine, 5 mmol l^{-1} NaF, 2.5
160 mmol l^{-1} PMSF and 50 mmol l^{-1} sodium orthovanadate), and briefly sonicated. Cell lysates were centrifuged at 800 *g* 10 min to remove cell debris, and the resulting supernatant was further centrifuged at 10000 *g* for 20 min. The pellet obtained was a crude mitochondrial fraction and the supernatant was further centrifuged at 100000 *g* for 1 h, to obtain the microsomal fraction. Protein concentrations were measured using the Bradford assay for total protein.

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Heme oxygenase activity

Heme oxygenase activity was determined in microsomal fractions from MA-10 cells by monitoring the conversion of heme into bilirubin as described (Llesuy & Tomaro 1994). In brief, 200 µl of the reaction mixture (0.2 mg/ml microsomal protein, 0.33 mmol l^{-1} hemin, 0.5 mg/ml rat liver cytosol,
170 0.2 mmol l^{-1} $MgCl_2$, 2 mmol l^{-1} glucose-6-phosphate, 1.62 units/ml glucose-6- phosphate dehydrogenase, 0.5 mmol l^{-1} NADPH, 25 mmol l^{-1} potassium phosphate buffer (pH 7.4)) was incubated at 37°C for 2 h. 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 mmol l^{-1} cm^{-1}).
175 Readings for samples prepared in the absence of cell homogenate (blank) were subtracted from all other values. HO enzyme activity is indicated as nanomol of bilirubin formed per milligram of protein per hour.

Western Blot Analysis and Immunodetection of Proteins

HO isoforms, StAR and P450scc, were determined in microsomal (HO) and mitochondrial (StAR and P450scc) fractions**.** Equal amounts of protein per sample (30-40 μg) were boiled for 5 min in SDS-PAGE loading buffer and then separated in 15% (w/v) polyacrylamide gels. After electrophoresis, proteins were electrotransferred to polyvinylidene difluoride (PVDF) membranes for 30 min at 10 V in a Trans-Blot SD semy-dry electrophoretic transfer cell (Bio-Rad Laboratories Inc.). PVDF membranes were blocked in 5% (w/v) non-fat dry milk in 50 mmol l^{-1} Tris–HCl (pH 7.4), 0.15 mol l^{-1} NaCl, 0.25% (v/v) Tween-20, for 2 h at room temperature (HO and StAR) or overnight at 4°C (P450scc or CYP11A), and then incubated overnight with the corresponding dilution of the primary antibody at 4°C: anti-HO-1 and HO-2, 1/1000; anti-StAR, 1/5000 (Bose*, et al.* 1999); anti-P450scc, 1/2000 (Hales *et al.* 2000, Allen *et al.* 2007). Peroxidase-conjugated secondary antibodies were incubated for 90 min at room temperature. The membranes were washed, and to reveal bound secondary antibody a chemiluminescence enhanced kit and autoradiography were used. The intensity of immunospecific bands was quantified using ImageJ software (NIH, Bethesda, MD) (Stocco & Clark 1996). To normalize samples for protein loading, antibodies against α-actin or cytochrome c were used.

RT-PCR

Total RNA was extracted from MA-10 cells with TRIzol reagent (Invitrogen) according to manufacturer's instructions. Before the RT step, RNA was subjected to deoxyribonuclease treatment (deoxyribonuclease 1 amplification grade, Life Technologies Inc.) to eliminate any possible DNA contamination. RT was then performed on total RNA (2 μg). Briefly, cDNA synthesis was carried out using 200 U Moloney murine leukemia virus reverse transcriptase (Promega Corp., Madison, WI), 8 μmol l^{-1} random primers, 0.5 mmol l^{-1} dNTP, and 25 U rRNAsin ribonuclease inhibitor in a total reaction volume of 25 μl. Reverse transcription was performed at

42ºC for 1h and the reaction was stopped by heating the mixture at 95ºC for 5 min. PCR reactions were carried out in a Tpersonal Thermocycler (Biometra Biomedizinische Analytik, Göttingen, Germany) and were performed using 4 µl cDNA and the PCR mix containing specific sense and antisense primers, dNTPs, Taq polymerase (Invitrogen), reaction buffer and PCR grade water.

Primers used for the amplification of StAR cDNA were; Forward: 5´-AAGGATTAAGGCACCAAGCTGTGC-3´; Reverse:5´-CTCTGATGACACCACTCTGCTCCGG-3´ (588bp fragment). 18S RNA was used in the semiquantitative RT-PCR protocol as an internal control to normalize, Forward primer 5´-ACGGAAGGGCACCACCAGGA-3´; Reverse primer: 5´-CACCACCACCCACGGAATCG-3´ (125bp fragment).

PCRs were carried out with a first step at 94°C for 3 min and then the corresponding number of cycles of denaturation at 94°C for 45 s, annealing at 55°C for 30 s, and elongation at 72°C for 90 s plus a final incubation at 72°C for 10 min. Reaction products were electrophoresed on 1.5% (w/v) agarose gels in 40 mmol l^{-1} Tris acetate and 2 mmol l^{-1} EDTA(pH 8) stained with ethidium bromide, photographed, and quantified with FluorChem software 4.1.0. (Alpha Innotech Corporation, Image Processing Solutions, San Leandro, CA).

Statistical analysis

All values are expressed as mean ± SEM of n experiments. Differences between groups were analyzed by factorial one-way ANOVA. When the ANOVA was significant ($p<0.05$) *post hoc* comparisons (Dunnet's or Tuckey's test) were made to determine the statistical levels of difference between groups using GraphPad InStat version 4.0 for Windows (GraphPad Software, San Diego, CA, USA).

RESULTS

Expression of HO isozymes in MA-10 Leydig cells

In order to analyze the expression profile of HO isozymes in these cells, we first studied the basal expression levels of both isoforms and the effect of 10 μmol l^{-1} hemin (a well known HO-inducer) in MA-10 cells. As shown in Figure 1A, both HO-1 and HO-2 isoforms are expressed in MA-10 cells. In addition, hemin treatment resulted in significantly increased levels of both isoforms ($p< 0.001$, Figures 1A and 1B). In agreement with protein expression results, HO-specific activity was increased 3 times by hemin treatment (Figure 1C).

Effect of HO isozymes on MA-10 cells steroidogenesis

Once the expression of HO isozymes in MA-10 cells was demonstrated, the involvement of this enzyme in the modulation of steroid synthesis was analyzed. As shown in Figure 2, preincubation of the cells with 10 μmol l^{-1} hemin for 30 min followed by stimulation with submaximal concentrations of hCG (1 ng/ml) or db-cAMP (0.2 mmol l^{-1}) or maximal concentrations of db-cAMP (1 mmol l^{-1}) for 5 h, resulted in a significant inhibition of progesterone production (60%, 38% and 35% inhibition, respectively, $p< 0.05$). Basal progesterone synthesis was also inhibited in the presence of hemin.

The inhibitory effect of hemin on MA-10 Leydig cell steroidogenesis was not due to a cytotoxic effect of the drug as the percentage of viable cells per well, determined by trypan blue exclusion staining after the incubation, was similar between the hemin-treated cells (95%) and the controls (93%). To confirm that hemin treatment was not causing a permanent effect in the cells, we also examined the reversibility of its effect on steroidogenesis. Table 1 show that the inhibitory effect detected after incubating the cells in the presence of 10 μmol l^{-1} hemin both on basal and db-cAMP stimulated steroid synthesis (35% and 33% inhibition, respectively; Day 1) was completely reversed 24 h after the removal of hemin, as MA-10 cells fully recovered their capacity to respond to a maximal concentration of db-cAMP in Day 2.

Effect of HO isozymes on StAR expression

To further characterize the involvement of HO isozymes on the steroid synthesis, a time course study of hemin action on the stimulation of HO-1 and StAR protein by hCG was performed (Figure 3, upper panel). Cells were preincubated with or without 10 μmol l^{-1} hemin for 30 min. hCG (20 ng/ml) was added, and incubation was continued for 1, 3 or 5 h. Increase of HO-1 and decrease of StAR protein levels by hemin treatment was noted after 3 h. These effects were still evident after 5 h of hCG stimulation (Figure 3A-D; $p<0.05$ vs respective hCG).

To confirm that the effect was due to the action of the second messenger (cAMP), we evaluate hemin treatment on db-cAMP-induced StAR protein expression after 5 h (Figure 3, lower panel). In agreement with the results obtained with hCG stimulation, db-cAMP-dependent HO-1 induction correlates with a marked decrease in StAR protein levels, suggesting that HO stimulation could be affecting cholesterol transport to the inner mitochondrial membrane.

To determine if StAR transcription was also affected, a time course of hemin action on hCG-stimulated StAR mRNA expression was carried out by semiquantitative RT-PCR analysis. In contrast to the effects of hemin on StAR protein levels, Figure 4, shows that hemin treatment results in a marked increase in StAR mRNA levels after 3 hs of hCG-stimulation, with no effects at 1 and 5 h. These results suggest that HO induction could regulate StAR expression by modifying, both, its mRNA and protein levels in MA-10 cells.

Involvement of HO isozymes in the steroidogenic pathway

To evaluate if the activity of HO was affecting any other step in the steroidogenic pathway, we then incubated MA-10 cells in the presence of 22-R-hydroxycholesterol (5 μmol l^{-1}, 22-R-OH), a cholesterol derivative that easily passes through cell membranes, or pregnenolone (5 μmol l^{-1}, P5), with or without hemin.

As shown in Figure 5, the inhibitory effect of hemin was completely prevented by the addition of pregnenolone, indicating that the activity of 3β-hydroxysteroid dehydrogenase was not affected by this treatment. In contrast, a significant inhibition of progesterone synthesis was still observed when

22-R-OH was used as a substrate ($p< 0.05$). These data suggest that at least one inhibitory effect of HO on the stimulated steroidogenic pathway seems to occur at the cholesterol side-chain cleavage (P450scc) step.

Since P450scc catalyzes the conversion of cholesterol to P5, the protein content of this enzyme was evaluated after hemin treatment. Figure 6 show that hemin does not modify P450scc protein expression.

Regulation of the expression of HO isozymes by hCG or db-cAMP

As previous results indicated that HO-1 expression was upregulated by ACTH in adrenal cells (Pomeraniec *et al.* 2004); the effect of hCG or db-cAMP on the expression levels of HO isoforms and enzymatic activity in MA-10 cells was studied. Results showed that the addition of 20 ng/ml hCG or 1 mmol l^{-1} db-cAMP (maximally effective concentrations for the stimulation of steroidogenesis) for 5 h, increased both the expression of HO isozymes (Figures 7A and 7B; $p<0.001$) and HO-specific activity (Figure 7C; $p< 0.05$).

Effects of HO regulation on rat Leydig cell testosterone production

Although MA-10 cells are known to preserve most of the characteristic features of Leydig cells, and are a widely used model to study Leydig cell steroidogenesis, they are still tumoral cells that have undergone a series of mutations. It is therefore necessary to validate the cellular model by showing the regulatory effects of HO on normal Leydig cells steroid production. Thus, we studied the effect of hemin on testosterone production in normal rat Leydig cells. Figure 8 shows that hemin significantly reduced basal and hCG-stimulated testosterone production (16% and 20% inhibition, respectively; $p<0.01$)

DISCUSSION

In the present study we demonstrate the presence of both inducible HO-1 (32 kDa) and constitutive HO-2 (36 kDa) isozymes in unstimulated MA-10 cells, a murine cell line derived from Leydig cells. We also show an increase in the expression levels of both HO isoforms and in HO activity by hemin. The expression of HO isozymes in rat Leydig cells had been previously detected, as well as the increase in HO-1 levels in Leydig cells from heat-stressed rats (Ewing & Maines 1995) and $CdCl_2$-treated rats (Ozawa *et al.* 2002).

Previous findings from our group demonstrated an inhibition of Leydig cell steroidogenesis by NO (Del Punta *et al.* 1996), and were further supported by other studies showing that NOS system modulates steroidogenesis (Kostic*, et al.* 1999; Nee Pathak & Lal 2008). Recently, we demonstrated that both, NOS and HO systems are involved in adrenal gland ACTH-dependent steroidogenesis (Pomeraniec *et al.* 2004; Grion*, et al.* 2007). In the past few years, CO has arised as a physiological messenger in the brain and cardiovascular system (Morse & Choi 2002; Maines 2005; Bilban*, et al.* 2008). It is well known that CO and NO share many biological properties. Increasing experimental evidence suggests the existence of a significant 'cross-talk' between HO and NOS systems (Calabrese*, et al.* 2006; Chung*, et al.* 2008). Therefore, based on the similarities of CO with NO, we undertook the study of the effect of HO activity on steroidogenesis in MA-10 cells. The results shown herein demonstrate that a significant inhibition of hormone-induced progesterone production was obtained when HO enzyme activity was increased by hemin treatment, suggesting that a product derived from HO activity may regulate steroidogenesis in MA-10 cells.

As we confirmed that HO system is involved in the regulation of steroid synthesis in MA-10 cells, and previous results showed that CO affects the activity of key enzymes in adrenal steroidogenesis (Pomeraniec *et al.* 2004), it is tempting to suggest that CO locally produced by HO, could be a modulator of testicular steroidogenesis in general, and of the activities of cytochrome P450 enzymes CYP11A (P450scc) and CYP17 (17α-hydroxylase-17,20 lyase), in particular. Regarding this, our experiments with 22-R-OH cholesterol and pregnenolone suggest an inhibitory effect of HO on the activity of cytochrome P450scc, probably by the binding of CO to the heme in

P450scc. This effect was reversible as the removal of hemin from the incubation media led to the full recovery of the cell steroidogenic capacity, in a similar way to that we have observed for NO (Del Punta *et al.* 1996). The inhibitory effect of HO induction was only observed on P450ssc activity but not on P450scc protein expression.

Emerging evidence has suggested a possible role of NO in the inhibition of StAR protein expression in Leydig cells (Diemer *et al.* 2003; Herman & Rivier 2006; Reddy*, et al.* 2006), indicating the existence of at least one additional site of action of NO in the regulation of steroidogenesis (Del Punta *et al.* 1996). In agreement with the results obtained for NO, HO induction by hemin inhibited StAR protein expression, suggesting that CO may also regulate steroidogenesis by modulating cholesterol transport to the inner mitochondrial membrane.

It is known that StAR induction and processing (to the 30 kDa form) is regulated by the levels of mitochondrial ATP and membrane potential, respectively. The ATP levels regulate the exportation of arachidonic acid (AA) from the mitochondria, thus mitochondrial disruption with agents that reduce ATP content inhibits the exportation of AA from the mitochondria, and consequently StAR protein induction (Duarte *et al* 2007). In addition, mitochondrial membrane potential reduction inhibits the import and processing of StAR protein (Allen *et al*. 2006). As CO inhibits cytochrome c oxidase, it affects ATP synthesis and membrane potential (Zuckerbraun *et al* 2007). Therefore, it is possible that CO, by affecting ATP levels and membrane potential, may reduce StAR protein by one or both of these mechanisms.

Noteworthy, contrary to its effect on StAR protein levels, hemin treatment induced an increase in StAR mRNA levels after 3 h of incubation with hCG, declining to normal values at 5 h. In this sense, it should be noted that protein levels do not always temporarily correlate with the levels of its mRNA. In this line, Clark *et. al.* showed that in rodent steroidogenic cells, posttranscriptional mechanisms regulate StAR mRNA stability and degradation, thus altering the efficiency of protein synthesis (Clark, *et al.* 1995).

In this study, we demonstrate that HO induction not only reduces StAR protein levels, but it also affects, at least, some posttranscriptional event leading to an increase in StAR mRNA levels. To this respect, a recent study by Duan *et. al.* showed that silencing of an AURE binding protein, (Zinc finger protein ZFP36L1/TIS11b) resulted in increased StAR mRNA levels while StAR protein induction was markedly reduced in MA-10 cells stimulated with Br-cAMP, (Duan, *et al.* 2009). These interesting findings tempt us to hypothesize that HO expression (or any of its metabolites) could modify StAR mRNA levels by modulating some similar posttranscriptional regulatory mechanism.

The induction of HO isozymes by various hormonal stimuli has been previously demonstrated. In this sense, the induction of HO activity in liver by glucagon, insulin and epinephrine (Bakken*, et al.* 1972) and the expression levels of HO-1 in hepatic and kidney cells by glucagon and angiotensin II, respectively, have been reported (Immenschuh*, et al.* 1998; Haugen*, et al.* 2000). Recently, Pomeraniec *et. al.* (2004) showed that ACTH and 8-Br-cAMP induced HO-1 expression, in adrenal cells, suggesting the involvement of the cAMP/PKA pathway. 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). It has also been shown that HO-2 isozyme, that is constitutively expressed, can be induced by different factors (Liu *et al.* 2000; Tschugguel *et al.* 2001; Sacca *et al.* 2003).

In this work, we have demonstrated that both HO-1 and HO-2 protein levels were increased by hCG or db-cAMP in MA-10 cells, indicating the involvement of cAMP/PKA signal transduction pathway in this mechanism. Our data is in agreement with that of Kutty and Maines (1989) who described a selective induction of HO-1 isozyme in whole testis from hCG-treated rats, *in vivo*. In addition, Liu *et. al.* (2000) presented evidences on the regulation of HO-2 levels in the testis by glucocorticoids; and that developmental and tissue-specific factor(s) determine the generation of transcripts unique to the organ. The apparent exclusive use of rHO-2 by the mature testis is consistent with the possibility that HO-2 may play a role in male reproduction.

As proposed by Stocker in 1990, the induction of HO-1 may play a role in cellular protection against oxidative stress. Many of the protective effects of HO-1 induction have been attributed to two of its enzymatic products, CO (Otterbein & Choi 2000; Maines & Gibbs 2005; Desmard*, et al.* 2007; Bilban *et al.* 2008) or bilirubin (a potent peroxyl radical scavenger) (Clark*, et al.* 2000; Erario*, et al.* 2002). Moreover, HO-1 has been shown to have anti-inflammatory, antiapoptotic and antiproliferative effects in different systems (Morse & Choi 2002; Bilban *et al.* 2008). In steroidogenic tissues, ACTH has been postulated to play a protective role in corticoadrenal cells, as it enhances Mn-SOD activity in ZF adrenal mitochondria (Raza & Vinson 2000), exerts anti-apoptotic effects in primary cultures of bovine adrenocortical cells (Negoescu *et al.* 1995) and in intact adrenal glands cultures (Carsia *et al*. 1998). Besides, ACTH induces HO system which appears to have antioxidant properties in Y1 cells (Pomeraniec *et al*. 2004). In MA-10 cells, we have observed that steroidogenesis inhibition by hemin is much more significant when the steroid production is supported by 22-R-OH cholesterol than when the cells are stimulated using either hCG or db-cAMP. Moreover, given the inhibition of P450scc activity and StAR protein expression, it can be expected that hemin would have a stronger inhibition of hCG or cAMP-induced steroidogenesis. However, this was not the case. Therefore, the induction of HO-1 and HO-2 that we observed in the presence of hCG or db-cAMP, suggests that HO system may be acting as a cytoprotective mechanism, as does in adrenal gland.

Although MA-10 cells are a widely used model to study Leydig cell steroidogenesis and are known to preserve most of the characteristic features of these cells, they are still tumoral cells that have undergone a series of mutations. However, similar results were obtained with rat Leydig cells, ratifying MA-10 cells as a useful experimental model.

In summary, both HO isoforms are present in MA-10 Leydig cells. Moreover, the induction of HO system could modulate steroidogenesis probably by the reversible binding of CO to the heme group of the cytochrome P450scc, as we previously described for NO (Del Punta *et al.* 1996). To our knowledge, this is the first report to show that induction of the HO system inhibits StAR protein

expression, indicating that there are at least two steps in the steroidogenic pathway in which HO system may be acting. In addition, we provide evidence that LH/hCG induces an enzymatic system with known antioxidant properties in Leydig cells and that such mechanism could be functionally relevant to testicular physiology providing the organ with a higher capacity to respond to a wide range of physiological conditions. Then, the HO/CO system may contribute to a fine net of regulation exerted on male reproductive function.

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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Figure Legends

Figure 1. Effect of hemin on HO protein expression and activity in MA-10 Leydig cells. (A) Representative inmunoblot for HO-1 and HO-2 proteins. Cells were incubated with or without 10 μmol l^{-1} hemin for 5.30 hours. Media were collected, microsomal fractions from MA-10 cells were isolated and proteins were analyzed by Western Blot as described in *Materials and Methods*; (B) quantification of HO levels by scanning densitometry, corrected by the corresponding α-actin; (C) HO specific activity was determined in microsomal fractions by monitoring the conversion of heme into bilirubin as described in *Materials and Methods*. Values are expressed as means ± SEM of three independent experiments; * P<0.001 vs. Control

Figure 2. Effect of hemin on progesterone synthesis in MA-10 Leydig cells. Cells were preincubated with or without 10 μmol l^{-1} hemin for 30 min. hCG (1ng/ml) or db-cAMP (0.2 mmol l^{-1} or 1 mmol l^{-1}) were added, and incubation was continued for an additional 5 h. Values are expressed as means ± SEM of three independent experiments each performed with three culture wells per condition; *P<0.05 vs. Control, †P<0.05 vs. 1 ng/ml hCG, #P<0.05 vs. 0.2 mmol l^{-1} db-cAMP, & P<0.05 vs. 1 mmol l^{-1} db-cAMP.

Figure 3: Effect of hemin on HO-1 and StAR protein levels in MA-10 Leydig cells. Time course of hemin effect on hCG-stimulated HO-1 and StAR protein levels (Upper panel). Cells were preincubated with or without 10 μmol l^{-1} hemin for 30 min. hCG (20 ng/ml) was added, and incubation was continued for 1, 3 or 5 h. Media were collected, microsomal and crude mitochondrial fractions from MA-10 cells were isolated and proteins were analyzed by Western Blot as described in *Materials and Methods*. (A) Representative immunoblot for HO-1 protein and the internal control, β-tubulin (55 kDa); (B) quantification of HO-1 levels by scanning densitometry; (C) Representative immunoblot for StAR protein and the internal control cytochrome c (12 kDa); (D) quantification of StAR levels by scanning densitometry. Values are expressed as means ± SEM of three independent experiments, *P< 0.05 vs. respective hCG. Effect of hemin on db-cAMP-stimulated StAR protein levels (lower panel). Cells were preincubated with or without 10 μmol l^{-1} hemin for 30 min. db-cAMP (1 mmol l^{-1}) was added, and incubation was continued for an additional 5 h. Media were

collected, crude mitochondrial fractions from MA-10 cells were isolated and proteins were analyzed by Western Blot as described in *Materials and Methods.* Data were normalized to the 12 kDa internal control cytochrome c. (A) Representative immunoblot for StAR protein; (B) quantification of StAR levels by scanning densitometry. Values are expressed as means ± SEM of three independent experiments, *P< 0.05 vs. db-cAMP.

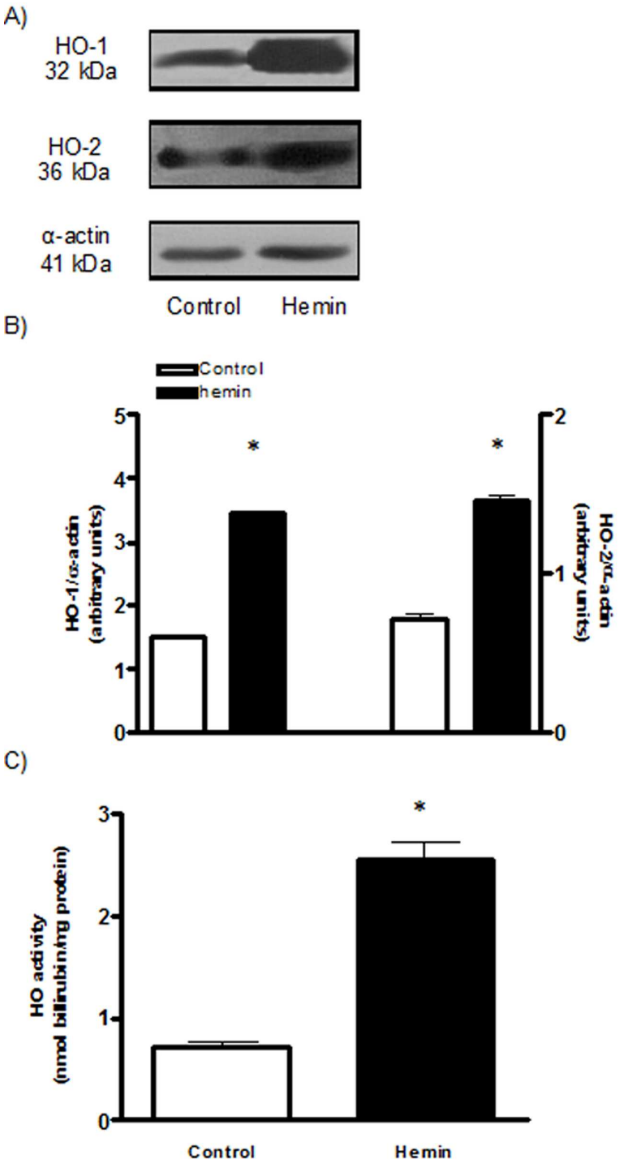
Figure 4: Effect of hemin on StAR mRNA levels in MA-10 Leydig cells. Cells were preincubated with or without 10 μmol l^{-1} hemin for 30 min. hCG (20 ng/ml) was added, and incubation was continued for 1, 3 or 5 h. RNA from MA-10 cells were isolated, and StAR expression levels were analyzed with RT-PCR as described in *Materials and Methods*. (A) Representative RT-PCR for StAR mRNA; (B) quantification of RT-PCR products of StAR. The histogram shows the densitometric analysis of the relative abundance of StAR mRNA normalized with the corresponding 18S.Values are expressed as means ± SEM of three independent experiments, *P< 0.001 vs. respective hCG.

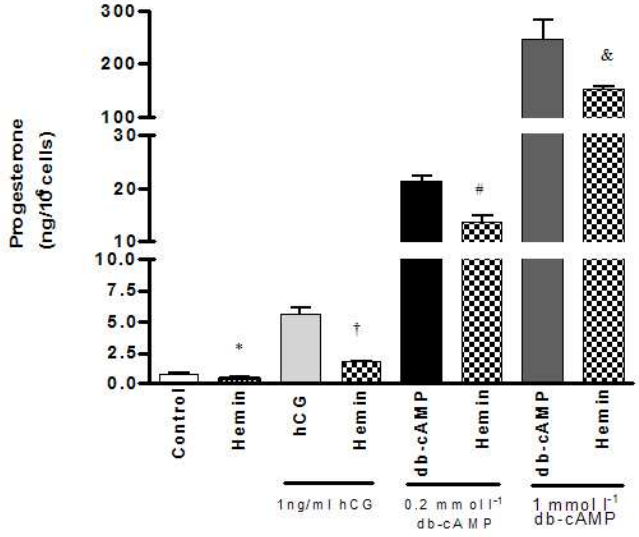
Figure 5. Effect of hemin on progesterone synthesis stimulated by 22R-hydroxycholesterol (22-R-OH) and pregnenolone (P5) in MA-10 Leydig cells. Cells were preincubated with or without 10 μmol l^{-1} hemin for 30 min. 22-R-OH (5 μmol l^{-1}) or P5 (5 μmol l^{-1}) were added, and incubation was continued for an additional 5 h. Values are expressed as means ± SEM of three independent experiments each performed with three culture wells per condition. *P<0.001 vs. 22-R-OH.

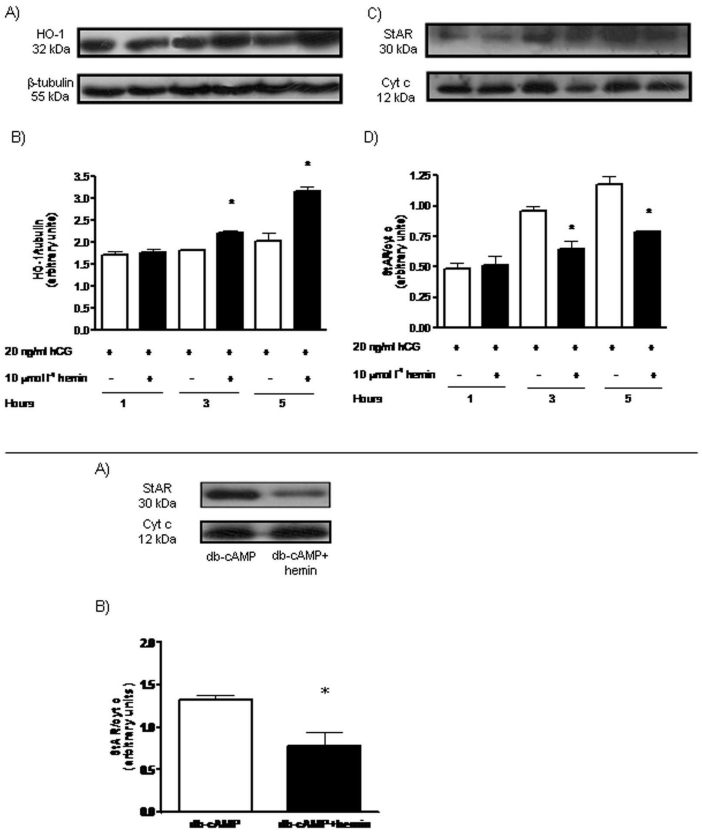
Figure 6. Effect of hemin treatment on cytochrome P450scc protein levels in MA-10 Leydig cells. Cells were preincubated with or without 10 μmol l^{-1} hemin for 30 min. db-cAMP (1 mmol l^{-1}) was added, and incubation was continued for an additional 5 h. Media were collected, crude mitochondrial fractions from MA-10 cells were isolated and proteins were analyzed by Western Blot as described in *Materials and Methods.* Data were normalized to the 12 kDa internal control cytochrome c. (A) a representative immunoblot for P450scc protein; (B) quantification of protein levels by scanning densitometry. Values are expressed as means ± SEM of three independent experiments.

Figure 7. Effect of db-AMPc and hCG treatments on HO protein expression and activity in MA-10 Leydig cells. (A) Representative immunoblot for HO-1 and HO-2 proteins. Cells were incubated with 1 mmol l^{-1} db-AMPc or 20 ng/ml hCG for 5 h. Media were collected, microsomal fractions from MA-10 cells were isolated and proteins were analyzed by Western blot as described in *Materials and Methods*; (B) quantification of HO levels by scanning densitometry, corrected by corresponding α-actin; (c) HO specific activity was determined in microsomal fractions by monitoring the conversion of heme into bilirubin as described in M*aterials and Methods*. Values are expressed as means ± SEM of three independent experiments.*P<0.001 vs Control, **P< 0.05 vs. Control

Figure 8: Effect of hemin on testosterone production in normal rat Leydig cells. Purified Leydig cells were preincubated for 30 min at 34 °C with or without 10 μmol l^{-1} hemin. hCG (1ng/ml) was added, and incubation was continued for 5 h. Values are expressed as means ± SEM of three different experiments each performed with triplicate incubations per condition; *P<0.01 vs. Control, † P<0.01 vs. hCG 1 ng/ml.


A)
HO-1
32 kDa
HO-2
36 kDa
α-actin
41 kDa
Control
Hemin
B)
Control
hemin
HO-1/α-actin (arbitrary units)
HO-2/α-actin (arbitrary units)
*
*
C)
HO activity (nmol bilirubin/mg protein)
*
Control
Hemin


Progesterone (ng/10⁶ cells)
300
200
100
30
20
10
10.0
7.5
5.0
2.5
0.0
*
†
#
&
Control
Hemin
hCG
Hemin
db-cAMP
Hemin
db-cAMP
Hemin
1ng/ml hCG
0.2 mmol l⁻¹ db-cAMP
1 mmol l⁻¹ db-cAMP



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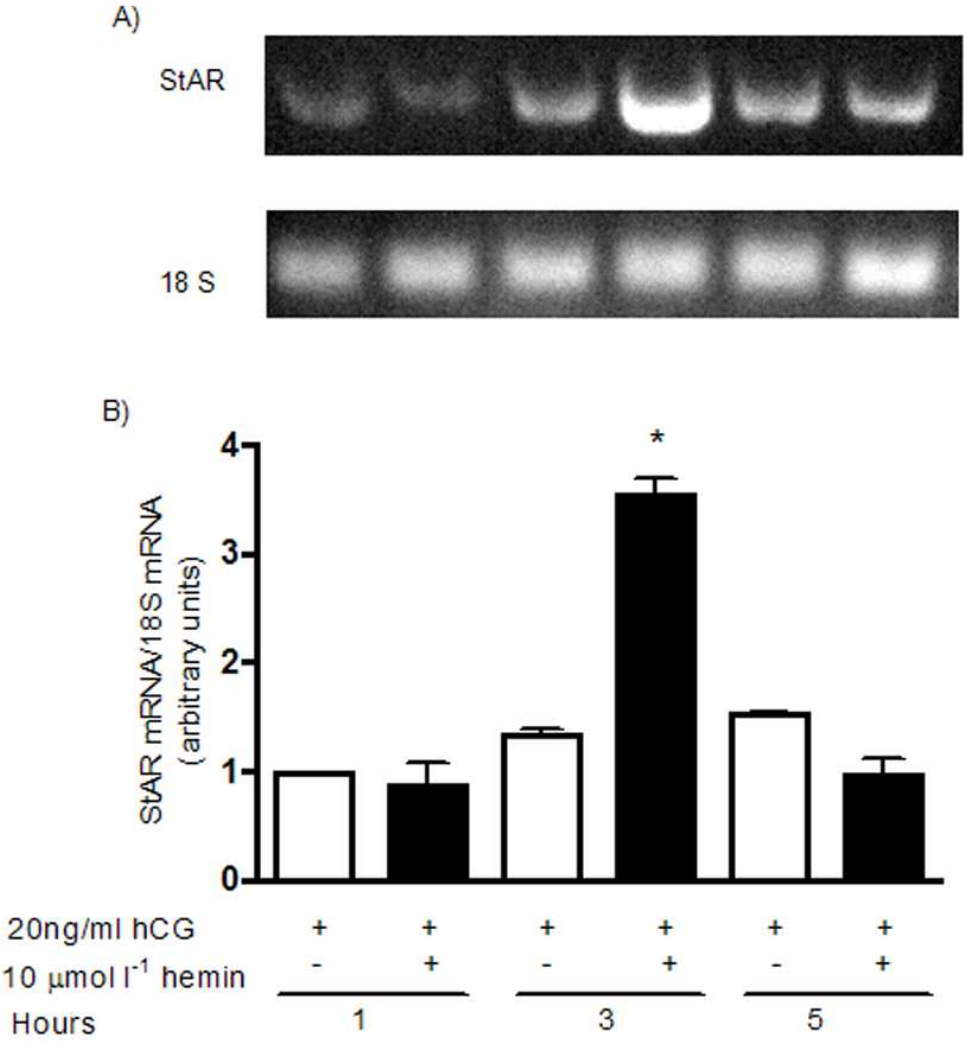
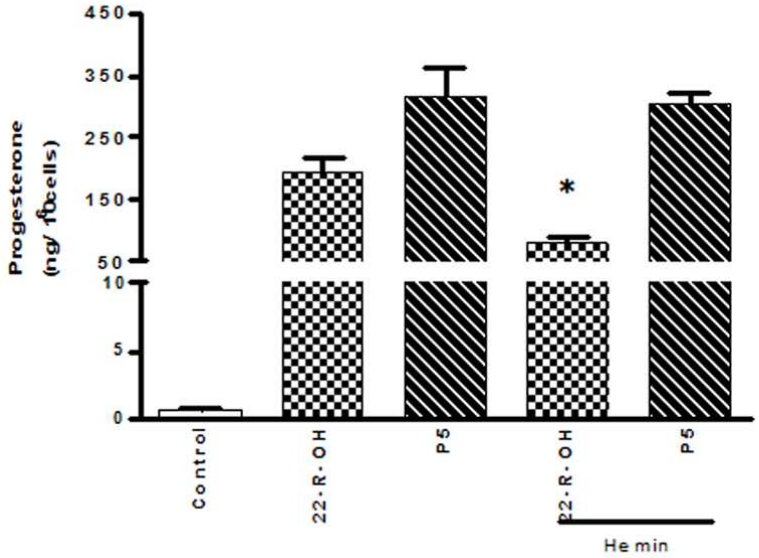
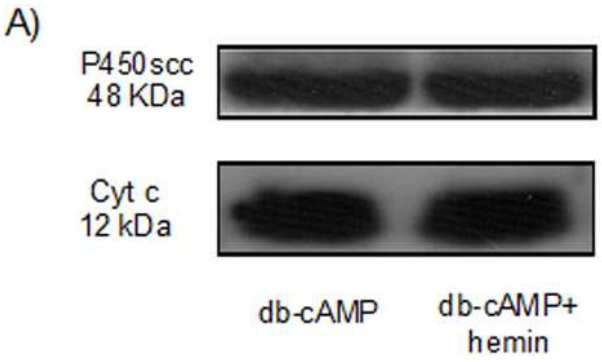
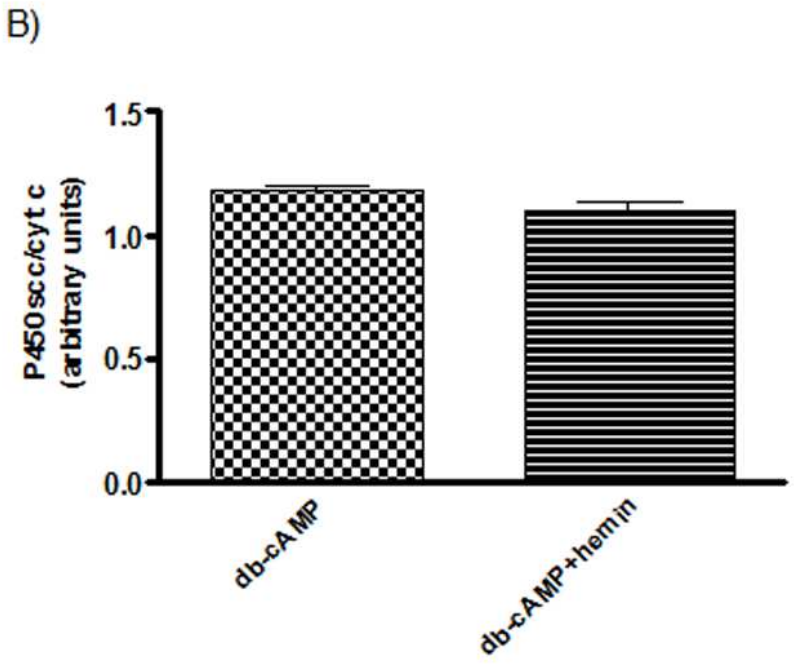
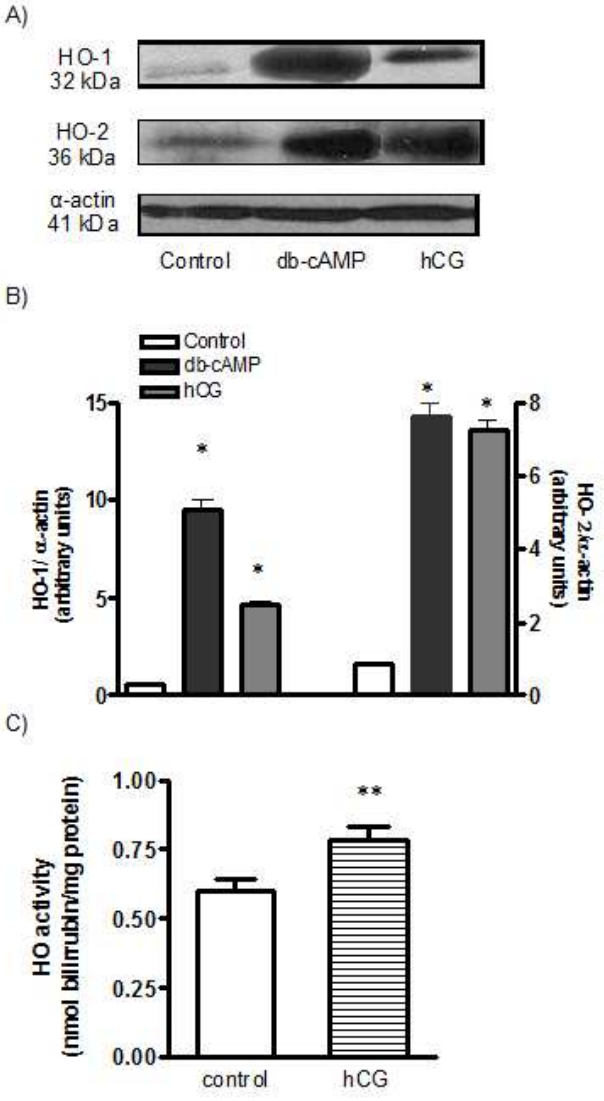

A)
StAR
18 S
B)
StAR mRNA/18S mRNA
(arbitrary units)
0
1
2
3
4
*
20ng/ml hCG
10 µmol l-1 hemin
Hours
+ + + + + +
- + - + - +
1
3
5


Progesterone
(ng/ 10^6 cells)
450
350
250
150
50
10
5
0
*
Control
22-R-OH
P5
22-R-OH
P5
Hemin


A)
P450scc
48 KDa
Cyt c
12 kDa
db-cAMP
db-cAMP+
hemin


B)
P450scc/cyt c
(arbitrary units)
1.5
1.0
0.5
0.0
db-cAMP
db-cAMP+hemin


A)
HO-1
32 kDa
HO-2
36 kDa
α-actin
41 kDa
Control
db-cAMP
hCG
B)
Control
db-cAMP
hCG
HO-1/ α-actin
(arbitrary units)
HO-2/α-actin
(arbitrary units)
15
10
5
0
8
6
4
2
0
*
C)
HO activity
(nmol bilirrubin/mg protein)
1.00
0.75
0.50
0.25
0.00
**
control
hCG

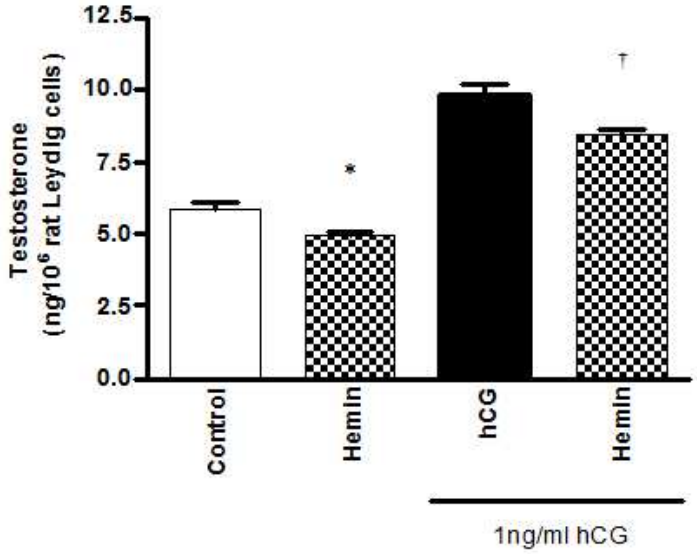

Testosterone
(ng/10^6 rat Leydig cells)
12.5
10.0
7.5
5.0
2.5
0.0
*
†
Control
Hemin
hCG
Hemin
1ng/ml hCG

Table 1. Recovery of MA-10 Leydig cell steroidogenesis after 10 μmol l^{-1} hemin treatment

Day 1		Day 2	
Treatment	P4 (ng/ 10^6 cells)	Treatment	P4 (ng/ 10^6 cells)
Control	0.75 ± 0.11	1 mmol l^{-1} db-cAMP	499 ± 23
Hemin	0.49 ± 0.07*	1 mmol l^{-1} db-cAMP	413 ± 30
0.2 mmol l^{-1} db-cAMP	26.7 ± 2.9	1 mmol l^{-1} db-cAMP	466 ± 28
0.2 mmol l^{-1} db-cAMP + Hemin	17.8 ± 1.3**	1 mmol l^{-1} db-cAMP	540 ± 20

MA-10 Leydig cells were preincubated with or without 10 μmol l^{-1} hemin for 30 min. db-cAMP (0.2 mmol l^{-1}) was added and incubation was continued for 5 h. Media were collected for progesterone determination (Day 1). Cells were washed and reincubated with hemin-free fresh medium for an additional 24 h. At the end of the incubation, cells were stimulated with db-cAMP (1 mmol l^{-1}) for 5 h (Day 2).

Data represent mean ± SEM of three independent experiments each performed with two culture wells per condition; *P< 0.05 vs. Control, **P<0.05 vs. 0.2 mmol l^{-1} db-cAMP.