Role of oxygen in the regulation of Leydig tumor derived MA-10 cell steroid production: the effect of cobalt chloride

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Abstract

We have earlier shown that cobalt chloride (CoCl₂)-induced hypoxia and second messenger 8-bromoadenosine 3’, 5’-cyclic adenosine monophosphate (8-Br-cAMP) stimulates vascular endothelial growth factor (VEGF) production in Leydig tumor cell derived MA-10 cells. Both stimuli follow common signal transduction pathways including protein kinase A (PK-A), extracellular regulated kinase 1/2 (ERK1/2), and phosphatidyl inositol-3 kinase/akt (PI3-K/Akt) pathways in the stimulation of VEGF by MA-10 cells. In the present study we investigated the role of CoCl₂ and 8-Br-cAMP on steroid production in MA-10 cells. The MA-10 cells were cultured in Waymouth MB 752/1 medium, supplemented with 15% heat inactivated horse serum. Progesterone was estimated by radioimmunoassay (RIA). We report that 8-Br-cAMP stimulated progesterone production by the MA-10 cells whereas CoCl₂ inhibited the same. Also, 8-Br-cAMP stimulated steroidogenic acute regulatory protein (StAR) and cytochrome P450 side-chain cleavage enzyme (P450scc) mRNAs expression. However, CoCl₂ had no effect on StAR mRNA. Cobalt chloride directly inhibited the expression of P450scc mRNA. The decrease in progesterone production could be attributed to three different mechanisms, (1) an increase in production of reactive oxygen species (ROS), (2) an increase in HIF-1α activity, and (3) ultimately a decrease in the level of cytochrome P450 side chain cleavage (CYT P450scc). Hypoxia has an action and mechanism of action similar to that of gonadotropins on VEGF production, whereas they have a contrasting effect on steroidogenesis. This study suggests that hypoxia could be as important as gonadotropins in regulating Leydig cell steroidogenesis.

Abbreviations: 3β-HSD, 3β-hydroxy steroid dehydrogenase; 8-Br-cAMP, 8-bromoadenosine cAMP; 25-OHC, 25-hydroxy cholesterol; CoCl₂, cobalt chloride; CsA, cyclosporin A; CYT P450scc, cytochrome P450 side chain cleavage; ERK1/2, extracellular regulated kinase ½; hCG, human chorionic gonadotropin; HIF-1α, hypoxia inducible factor-1α; IBMX, 3-isobutyl-1-methyl-xanthine; LH, luteinizing hormone; MAPK, mitogen-activated protein kinases; PI3-K/Akt, phosphatidyl inositol-3 kinase/akt; PKA, protein kinase A; PDTC, pyrrolidine dithiocarbamate; ROS, reactive oxygen species; SNAP, S-nitroso-N-acetylpenicilamine; StAR, steroidogenic acute regulatory protein; VEGF, vascular endothelial growth factor.

Introduction

The mammalian testis works in a low oxygen environment [Carlsson et al. 2001; Cross and Silver, 1962; Free et al. 1976; Klotz et al. 1996; Massie et al. 1969; Yu and Cringle 2006] suggesting an important role of oxygen in the regulation of testicular function including steroidogenesis and spermatogenesis. Using a Leydig tumor cell derived MA-10 cell line, we have shown that cobalt chloride (CoCl₂), a hypoxia inducing agent [Maxwell and Salnikow 2004], like luteinizing hormone (LH) and its second messenger cAMP, stimulates vascular endothelial growth factor (VEGF) production [Kumar et al. 2012]. Cobalt chloride stimulates VEGF production sharing common signal transduction pathways with LH/cAMP, which include protein kinase A (PKA), extracellular regulated kinase 1/2 (ERK1/2), and phosphatidyl inositol-3 kinase/akt (PI3-K/Akt) pathways. In addition, both CoCl₂-induced hypoxia as well as cAMP partly mediates their stimulatory effect on VEGF synthesis, through the activation of hypoxia inducible factor-1α (HIF-1α), in MA-10 cells [Kumar et al. 2012].

However, hypoxia induced by a low oxygen environment has been shown to inhibit steroidogenesis in several steroidogenic tissues from different species including human adrenal cells [Panesar et al. 2003; Raff and Bruder 2006], monkey corpus luteum cells [Tesone et al. 2005], non-luteinized monkey granulosa cells [Martinez-Chequer et al. 2003], swine granulosa cells [Basini et al. 2004], and bovine luteal cells [Nishimura et al. 2006]. Cobalt chloride-induced hypoxia also inhibited steroidogenesis in non-luteinized
monkey granulosa cells [Martinez-Chequer et al. 2003]. In contrast, Hwang et al. [2007] have reported that hypoxia enhanced the stimulatory effect of human chorionic gonadotropin (hCG) on testosterone release in the mouse Leydig cell derived TM3 cell line. The present study therefore was undertaken to study the effects of CoCl₂ induced hypoxia on steroidogenesis in MA-10 cells and to identify the associated mechanism of action.

Normal Leydig cells in vivo comprise a very low percentage of total cells of the testis. Therefore, to obtain large quantities of highly enriched and viable Leydig cells requires sacrificing a large number of mice. Also, a multi-step procedure that involves filtration with nylon mesh combined with centrifugation and Percoll density gradient sedimentation yields only 95% enriched Leydig cells [Salva et al. 2001]. To avoid such problems we used the MA-10 cell line, a well studied line, derived from mouse Leydig cells. The MA-10 cells have been shown to have LH receptors and behave physiologically like Leydig cells in terms of steroid and VEGF production in response to LH/cAMP [Anand et al. 2003; Ascoli 1981; Schwarzenbach et al. 2004]. However, MA-10 cells lack 17α hydroxylase activity so the end product in steroidogenesis is progesterone and not androgens [Ascoli 1981].

Results

Effect of CoCl₂ on progesterone production

Time course studies of progesterone production for 48 hours were performed with hCG in combination with 3-isobutyl-1-methylxanthine (IBMX), a cyclic nucleotide phosphodiesterase inhibitor. The progesterone levels peaked at 24 hours (data not shown). Therefore subsequent experiments were performed for 24 hours. Progesterone production was stimulated 5-fold with hCG and 11.5-fold with 8-bromoadenosine cAMP (8-Br-cAMP). Cobalt chloride decreased basal, hCG, and 8-Br-cAMP stimulated progesterone production by 15%, 77%, and 75%, respectively (Figure 1). Cells treated with 25-hydroxy cholesterol (25-OHC) increased progesterone production in a dose (1.5 μg ml⁻¹ to 15 μg ml⁻¹) dependent manner. Doses higher than 15 μg ml⁻¹ of 25-OHC progressively decreased cell viability and consequently progesterone production (data not shown). Therefore, 15 μg ml⁻¹ of 25-OHC was selected as the preferred dose for subsequent experiments. Cells treated with 15 μg ml⁻¹ of 25-OHC increased progesterone production by 3 fold. Cobalt chloride decreased the 25-OHC-induced progesterone production by 45% (Figure 2A). Cells treated with pregnenolone resulted in a 13-fold increase in the level of progesterone. Cobalt chloride had no significant effect in regulating pregnenolone induced progesterone production (Figure 2B).

Effects of CoCl₂ on StAR and P450scc mRNA expressions

Reverse transcription polymerase chain reaction (RT-PCR) analysis showed that the MA-10 cells have almost no expression of steroidogenic acute regulatory protein (StAR) mRNA under basal conditions. However, stimulation by 8-Br-cAMP significantly (p<0.05) increased StAR mRNA expression, but CoCl₂ co-treatment failed to show any inhibitory response (Figure 3A). In comparison, 8-Br-cAMP stimulated the mRNA levels of cytochrome P450 side chain cleavage (P450scc) enzyme by 30%. Cobalt chloride inhibited basal P450scc mRNA levels by 30% and 8-Br-cAMP stimulated P450scc levels by 28% (Figure 3B).
Effect of inhibitors on progesterone productions

Cyclosporin (CSA) A hypoxia inducible factor-1α (HIF-1α) inhibitor alone did not produce any significant change either in basal progesterone production or hCG stimulated production of progesterone. However, in the presence of CoCl₂, it partially reversed the inhibitory action of CoCl₂ on basal and hCG stimulated progesterone production (Figure 4A). H-89 (protein kinase A (PKA) inhibitor), U0126 (MEK1/2 inhibitor), and LY294002 (PI3-K inhibitor) significantly decreased 8-Br-cAMP stimulated progesterone production by 60%, 72%, and 50%, respectively (Figure 4B). These inhibitors had no significant effect on the basal and CoCl₂ treated progesterone production (Figure 4C).

Effect of CoCl₂ on ROS and nitrogen reactive species production

Cobalt chloride treatment of MA-10 cells induced a rise in reactive oxygen species (ROS) by 44%. In comparison, 8-Br-cAMP did not alter the basal levels of ROS in these cells but supported the CoCl₂ stimulated rise (Figure 5A). Pyrrolidine dithiocarbama (PDTC), a potent antioxidant agent decreased ROS production under basal, 8-Br-cAMP, and CoCl₂ induced conditions (Figure 6A). PDTC had no significant effect on basal and hCG stimulated progesterone production in MA-10 cells. However, PDTC partially reversed the action of CoCl₂ on basal and hCG induced progesterone production (Figure 6B). One method for indirect determination of NO involves the spectrophotometric measurement of its stable decomposition products, nitrate and nitrite radicals. MA-10 cells were treated with S-nitroso-N-acetylpenicilamine (SNAP, 0.01 mM), CoCl₂, or 8-Br-cAMP for 24 hours. There was a robust increase in nitrite as well as nitrate in response to the NO releasing agent SNAP. However, both CoCl₂ and 8-Br-cAMP
failed to elicit any change in the production of nitrogen reactive species by MA-10 cells (Figure 5B).

Discussion

In our earlier study we showed that both CoCl₂ and 8-Br-cAMP stimulate VEGF production in Leydig cell derived MA-10 tumor cell line via cAMP downstream pathways that include PK-A, mitogen-activated protein kinases (MAPK)/extracellular regulated kinase (ERK1/2), and PI3K/Akt pathways [Kumar et al. 2012]. In contrast, the current study reports that both CoCl₂ and 8-Br-cAMP have divergent actions on progesterone production. Cobalt chloride–induced hypoxia decreased basal hCG- and 8-Br-cAMP stimulated progesterone production. Inhibitors of the PK-A, MAPK/ERK1/2, and PI3K/Akt pathways decreased 8-Br-cAMP stimulated progesterone production, but they had no effect on CoCl₂ induced inhibition of progesterone production. These observations suggest that CoCl₂ affects steroid synthesis by inhibiting a step(s) distal to the target of these inhibitors. The rate limiting step in steroidogenesis is the translocation of cholesterol from the outer mitochondrial membrane to the inner mitochondrial membrane by the StAR protein. However, 25-OHC, a lipid soluble side-chain oxygenated sterol, readily diffuses across mitochondrial membranes [Jefcoate et al. 1974; Meaney et al. 2002]. Presumably, the diffusion of polar cholesterol is independent of the StAR protein; however, 25-OHC has also been shown to stimulate the synthesis of the StAR protein to further promote steroidogenesis in MA10 cells [King et al. 2004]. In our studies, 25-OHC stimulated progesterone production by 3 fold in MA-10 cells. This stimulation of progesterone synthesis was inhibited by CoCl₂.

Exogenous pregnenolone also stimulated progesterone production in the MA-10 cells. Stimulation of progesterone production was not abolished by CoCl₂ suggesting that CoCl₂ has possibly no effect on 3β-hydroxysteroid dehydrogenase (3β-HSD activity). Therefore, it seems that CoCl₂ probably inhibits progesterone production at a site proximal to the step catalyzed by 3β-HSD, namely either the StAR protein or cytochrome P450 side chain cleavage (CYT P450scc). The expression of Star mRNA in the MA-10 cells was stimulated by 8-Br-cAMP. But CoCl₂ did not alter Star mRNA expression in response to 8-Br-cAMP. The data suggests that the inhibitory action of CoCl₂ did not involve StAR mRNA.

Treatment with CoCl₂ resulted in a significant decrease in P450scc mRNA expression in both basal and 8-Br-cAMP treated cells. Nishimura et al. [2006] also demonstrated in bovine luteal cells a decline in the levels of P450scc mRNA in

Figure 5. The effect of cobalt chloride (CoCl₂) on basal and 8-bromoadenosine cAMP (8-Br-cAMP) stimulated reactive oxygen species (ROS) production by MA-10 cells and the production of nitrite ions by MA-10 cells upon treatment with CoCl₂, 8-Br-cAMP, and S-nitroso-N-acetylpenicilamine (SNAP). (A) Effect of CoCl₂ on basal and 8-Br-cAMP stimulated ROS production by MA-10 cells. Bars represent mean ± SEM of four experiments. Each experiment was performed in quadruplicate. ***Indicate p<0.001 between a and c; **Indicate p<0.01 between b and d. Panel (B) Production of nitrite ions by MA-10 cells upon treatment with CoCl₂, 8-Br-cAMP, and SNAP. Bars represent mean ± SEM of two experiments. Each experiment was done in quadruplicate. ***Indicate p<0.001 between SNAP and control values.

Figure 6. The effect of pyrrolidine dithiocarbamate (PDTC) on reactive oxygen species (ROS) production under basal, cobalt chloride (CoCl₂), and 8-bromoadenosine cAMP (8-Br-cAMP) stimulated conditions by MA-10 cells and the effect of PDTC on human chorionic gonadotropin (hCG) stimulated and CoCl₂ treated progesterone production by MA-10 cells. (A) Effect of PDTC on ROS production under basal, CoCl₂ and 8-Br-cAMP stimulated conditions by MA-10 cells. Bars represent mean ± SEM of three experiments. Each experiment was performed in quadruplicate. ***Indicate p<0.001 between a and e; b and f; c and g; d and h. Panel (B) Effect of PDTC on hCG stimulated and CoCl₂ treated progesterone production by MA-10 cells. Bars represent mean ± SEM of three experiments. Each experiment was done in quadruplicate. **Indicate p<0.01 between c and g; d and h. IBMX: 3-isobutyl-1-methylxanthine.
response to hypoxia. This group, too, did not find any effect on StAR mRNA/protein, and 3β-HSD mRNA expression/activity. The presence of cobalt along with heme leads to the formation of cobalt protoporphyrin owing to the competition of cobalt with ferrochelatase [Sinclair et al. 1979]. Sinclair et al. [1982] suggested that the formation of the cobalt protoporphyrin occurs at the expense of heme, leading to a decrease in CYT P450. Cobalt has also been shown to substitute for ferrous iron in the center of the porphyrin ring of heme of hemoglobin. The resultant cobalt hemoglobin has lower affinity to bind with oxygen [Yonetani et al. 1974]. These observations suggest that cobalt can also inactivate the pre-synthesized heme.

We showed that CoCl2-induced hypoxia and 8-Br-cAMP increased HIF-1α protein activity in MA-10 cells without any change in its mRNA levels. Also, the rise in HIF-1α protein activity was inhibited by CsA, a stimulant of prolyl hydroxylase activity [Kumar et al. 2012]. Similarly, D’Angelo et al. [2003] showed that CsA destabilized HIF-1α induced by hypoxia in C6 glioma cells. In our present study we report that CsA partially reversed the inhibitory action of CoCl2 on basal and hCG stimulated progesterone production, suggesting a possible role of HIF-1α in the regulation of progesterone production by CoCl2.

Primary Leydig cells treated with H2O2 resulted in a decrease in StAR and 3β-HSD protein levels [Allen et al. 2004]. Similarly, Diemer et al. [2003] showed that H2O2 decreased progesterone production in MA-10 cells. The inhibition was due to a decrease in StAR protein levels without any change in their mRNA levels or P450scc protein levels. In the present study, CoCl2 induced hypoxia increased ROS levels which could subsequently lead to a reduction in steroid production. PDTC, a potent anti-oxidant, has been shown to enhance the degradation of ROS such as H2O2 [Schreck et al. 1991]. It decreased ROS production under hypoxic conditions. Here, use of PDTC partially abolishes the inhibitory action of CoCl2 on progesterone production suggesting that the inhibitory action of CoCl2 is partly mediated by ROS. However, ROS induced inhibition of steroid production could be camouflaged because of direct inhibition of CYT P450scc by CoCl2. Nitric oxide, a highly reactive inorganic free radical gas has been shown to inhibit steroidogenesis in granulose - luteal cells and MA-10 cells [Punta et al. 1996; Van Vooris et al. 1994]. In the present study, there was no change in nitrogen species in response to CoCl2 or 8-Br-cAMP.

The low level of oxygen in the testes seems to be as important as gonadotrophins in the regulation of Leydig cell function. Hypoxia has an action and mechanism of action similar to that of gonadotrophins on VEGF production, whereas they have divergent effect on steroidogenesis. This inhibition of steroidogenesis by hypoxia seems to be necessary to regulate spermatogenesis and other androgen dependent effects of gonadotropins. In conclusion, hypoxia, particularly induced by CoCl2, lowers progesterone production in Leydig cell-derived MA-10 cells. This decrease in progesterone production could be attributed to three different mechanisms: (1) an increase in ROS production, (2) an increase in HIF-1α activity, and (3) ultimately a decrease in the level of CYT P450scc.

Materials and Methods
Waymouth MB 752/1 medium, horse serum, CoCl2, 8-Br-cAMP, 25-hydroxycholesterol (25-OHC), H-89 (N-[2-(p-bromocinnamylamino)ethyl]-5-isouquinolinesulfonamide dihydrochloride), U0126 [1,4-Diamo-2,3-dicyano-1,4-bis-(a-aminophenylmercapto) butadiene] monoethanolate, LY294002 [2-(4-Morpholino) -8-phenyl-1(4H)-benzopyran-4-one hydrochloride], cyclosporin A (CsA), gentamicin solution and specific primers were obtained from Sigma Aldrich (St. Louis, MO, USA). Trypsin and ethylenediaminetetraacetic acid (EDTA) were obtained from Life Technologies Pvt. Ltd. (Carlsbad, CA, USA). RNA isolation kit was obtained from Qiagen (Hilden, Germany). RETROscript kit was obtained from Ambion (Austin, TX, USA). Taq DNA polymerase from Genel (Bangalore, India), ethanol was purchased from Merck (Whitehouse Station, NJ, USA). 1,2,6,7-3H progesterone was obtained from Amersham Biosciences (Piscataway, NJ, USA). Progesterone polyclonal antibody was provided by Hormone Research Foundation (India). All other chemicals and reagents, unless specified, were obtained from Sigma Aldrich (USA).

Cell culture
MA-10 cells were cultured in T-75 flask in Waymouth MB 752/1 medium, supplemented with 15% heat inactivated horse serum. Cells were incubated at 37°C in 5% CO2 in a humidified chamber and grown until 75% confluency. The cells were trypsinised using a 0.25% trypsin-EDTA solution. A total of 105 cells ml-1 per well were plated in medium containing serum in 24/48-well plates for 17h. Thereafter, the medium was replaced with fresh serum-free medium (SFM). The cells were pre-incubated without or with 100 μM CoCl2 for 6h. The dose of CoCl2 was selected based on previous studies [Kumar et al. 2012]. Cells were further incubated with one of the following reagents: 1 mM 8-Br-cAMP (a cAMP analogue), 0.5 IU hCG + 0.5 mM IBMX, 25-OHC and 20 μM pregnenolone.

In experiments with CsA, cells were pre-treated with CsA for 2h, before incubation without or with 100 μM CoCl2. After the specified incubation period, serum-free medium (SFM) was removed and stored at -20°C until assayed for progesterone. All the treatments were done at least in three replicates. Each experiment was performed twice or thrice to check the reproducibility.

Progesterone production
Progesterone was estimated by Radioimmunoassay (RIA, Panesar et al. 2003). Progesterone concentration in each sample was measured in duplicate. The antibody used for RIA was highly specific and showed no cross reactivity for pregnenolone as well as testosterone.

Semi quantitative reverse transcriptase PCR
Total RNA from MA-10 cells was isolated according to the manufacturer’s instructions using RNeasy mini kit from Qiagen (Hilden, Germany). RNA (2 μg) from each isolate was reverse transcribed in oligo (dT)-primed reverse transcription reactions with reverse transcriptase RETROSCRIPT kit provided by Ambion according to the manufacturer’s
instructions. A 1 μl aliquot of the reverse-transcribed cDNAs was then used to amplify mRNA sequences coding for StAR and P450scc. L19 and S15 were taken as internal controls. The sequence of the primers used is provided in Table 1. The polymerase chain reaction was carried out for 31 cycles with annealing temperatures 60°C and 58.5°C for StAR and P450scc, respectively. The amplified fragments were size-fractionated in a 1.5% (wt/v) agarose gel and visualized by staining with ethidium bromide (0.2 ng/ml). The amplified bands were quantified by Life Science software from UVP (Upland, CA, USA).

**Determination of ROS and nitrogen reactive species**

A total of 2 × 10⁵ cells ml⁻¹ per well were plated in medium containing serum in 24-well plates for 17 h. Thereafter, the medium was replaced with fresh SFM. The cells were pre-incubated without or with pyrrolidine dithiocarbamate (PDTC) for 1.5 h. Fluorescence intensity was determined with the help of Hitachi F 4500 spectrofluorometer with excitation wavelength 488 nm and emission wavelength 522 nm. The values were expressed in terms of arbitrary units (AU). Nitrogen reactive species were determined with the Total NO determination kit obtained from R&D systems (Minneapolis, USA).

**Statistical analysis**

The data were analyzed by using SPSS 15.0 package (SPSS Inc, Chicago, IL, USA). The results were analyzed by paired student’s t-test or Kruskal Wallis test as and when required. Kruskal Wallis test was followed by Man-Whitney test after adjusting p values. p values <0.05 were considered significant.

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**Declaration of interest**

This work was partly funded by the Department of Science and Technology, Government of India. The authors state no conflict of interest.

**Author contributions**

Conceived the work and co-authored the manuscript: AK; Performed the work as part of her PhD thesis: LR; Performed some confirmatory experiments, tabulated the data, performed the statistics, and helped in the preparation of the manuscript: BD.

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**Table 1. Primer sequences used in the study.**

| Primer                      | Primer sequence               | Product size  |
|-----------------------------|-------------------------------|---------------|
| StAR [(Manna et al., 2006)] | Sense: 5’GACCTTTGAAAGGCTCAGGAAGAC 3’ | 980bp         |
|                            | Antisense: 5’TAGCTGAAGATGGACAGACTTGTC 3’ |               |
| P450scc [(Kimoto et al., 2010)] | Sense: 5’CAACATCAGAGATGCTGGCAGG 3’ | 559bp         |
|                            | Antisense: 5’CTCAGGCATCAGAAGTGAGGTTGAA 3’ |               |
| L19 [(Manna et al., 2006)]  | Sense: 5’GAAATCGCAATGCAACTC 3’ | 405bp         |
|                            | Antisense: 5’TCTTAGGACCTGGAGCTCTCA 3’ |               |
| S15 small ribosomal subunit protein | Sense: 5’TTCGCCAAGTTCACCTACC 3’ | 361bp         |
| (Provided in RETROscript kit Ambion*) | Antisense: 5’CGGGCGCGCATGCTTTAGC 3’ |               |

*Ambion, Austin, TX, USA.*

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