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Cisplatin inhibits testosterone synthesis by a mechanism that includes the action of reactive oxygen species (ROS) at the level of P450scc

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ABSTRACT

Cisplatin (Cs) is a chemotherapeutic agent able to generate reactive oxygen species (ROS) which are linked to several side effects of the drug. Even when it is known that Cs produces Leydig cell dysfunction, it is unknown whether this particular side effect is mediated by ROS. The aim of this study was to evaluate the *in vitro* effects of Cs on testosterone production and the participation of ROS in this effect. We demonstrate that Cs promotes the generation of ROS in a time-, and concentration-dependent fashion, not only in mouse testicular interstitial cells but also in MA-10 Leydig cells. Also, Cs inhibits testosterone synthesis in a concentration-dependent fashion (5–50 μM for 4 h) and to a similar extent, in cells exposed to human chorionic gondadotropin hormone (hCG), to an analog of the second messenger cAMP (8Br-cAMP) or to a freely diffusible cholesterol analog (22R-hydroxycholesterol). However, this treatment does not inhibit the conversion of pregnenolone to testosterone. These data suggest that Cs exerts its inhibitory action on testosterone synthesis by an action at the level of P450scc. We also demonstrated that an antioxidant impairs the inhibitory effect of Cs on the conversion of the cholesterol analog into pregnenolone and that Cs does not change the expression level of P450scc mRNA. Therefore, it is concluded that Cs inhibits testosterone synthesis by a mechanism that includes the inhibition of P450scc by ROS.

1. Introduction

Platinum II compounds constitute a class of chemotherapeutic agents widely used in the treatment of cancer. Cisplatin (Cs) was the first member of this class of anti-cancer drugs. Since Cs introduction to clinical oncology [1], hundreds of platinum compounds have been tested, but only a few have been approved and are used in humans [2,3].

Cs is included in numerous chemotherapeutic regimens to treat various types of malignant tumors in ovary, lung, head and neck, bladder, and many other organs and tissues [3–5]. The effectiveness of Cs as an antineoplasic agent is well recognized. However, its full clinical utility is limited by the many side effects that can it cause, mainly nephro and neurotoxicity, as well as ototoxicity and testicular damage [6,7].

As for testicular damage, it has been reported that Cs treatment produces azoospermia [7] and sperm morphology and motility alterations [8,9]. In addition, Cs produces Leydig cell dysfunction mainly through the inhibition of testosterone secretion, an effect that is accompanied by increased FSH and LH plasma levels as observed both in patients and in animal experimental models [10,11]. Although *in vivo* studies suggest that the effect of Cs on steroidogenesis is due to alterations in testosterone biosynthetic pathway, in the luteinizing hormone (LH) receptor function [12,13] and also in Leydig cell viability [14], the molecular mechanism deserves further study.

Steroid biosynthesis begins with the cleavage of the side chain of cholesterol to form pregnenolone (P5). This reaction is catalyzed by the P450scc component of the cholesterol side chain cleavage enzyme system (CSCC) located on the matrix side of the inner mitochondrial membrane. Although this reaction constitutes the rate-limiting enzymatic step in biosynthetic pathway of all steroids, the true rate-limiting step is the delivery of cholesterol to the inner mitochondrial membrane, where is localized the P450scc enzyme [15]. Given that the diffusion of cholesterol across the aqueous space between mitochondrial membranes is extremely slow, cholesterol cannot diffuse to the inner mitochondrial membrane at rates able to maintain the steroid production at physiologically relevant levels. The Steroidogenic Acute Regulatory (StAR) protein is a mitochondrial protein which facilitates cholesterol access to the inner mitochondrial membrane [16]. Steroidogenic hormones regulate steroid synthesis, promoting

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the synthesis and activation of this protein [17–19]. In Leydig cells, LH activates the steroid synthesis through a mechanism that involves an increase in cAMP levels and cAMP-dependent protein kinase (PKA) activation [20] and the PKA-mediated increase of StAR mRNA and protein levels and the activation of StAR protein [17–19]. Within the mitochondria, cholesterol is metabolized to P5 by the action of the cytochrome P450scc and subsequently, P5 is metabolized by the action of 3β-hydroxysteroid dehydrogenase (3β-HSD) to progesterone (P4), which finally renders different steroids through tissue-specific enzymes. Specifically in Leydig cells, the major steroid produced is testosterone. Therefore, the LH receptor and the molecules that comprise its signaling cascade, StAR protein and steroidogenic enzymes are potential targets of drugs causing impaired Leydig cell function such as Cs.

Reactive oxygen species (ROS) are involved in a variety of pathophysiological conditions of the testis [21] and oxidative stress is known to inhibit testicular steroidogenesis. Indeed, StAR expression and P450scc activity are well known targets of H_2O_2-mediated inhibition of steroidogenesis in Leydig cells [22,23].

Since Cs is a ROS generator in several system [24,25], it is then possible that Cs-induced Leydig cell dysfunction could be mediated by ROS. Therefore, the purpose of this *in vitro* study was to determine the site(s) of action of Cs on the steroidogenic pathway in Leydig cells and the participation of ROS in Cs-mediated inhibition of steroidogenesis. Our results indicate that exposure of Leydig cells to Cs for a short period of time (4 h) results in reduced testosterone production mainly through a ROS-mediated inhibition of the P450scc enzyme.

2. Material and methods

2.1. Materials

Cisplatin, 8Br-cAMP, 6-hydroxy-2,5,7,8-tetramethylcroman-2-carboxylic acid (Trolox), 2,2′-azobis (2-methylpropionamidine) dihydrochloride (ABAP), 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and 2,7-dichloro-fluorescein diacetate (DCFH-DA), histone type III-S were purchased from Sigma Chemicals (St. Louis, MO, USA). LDH assay kit was obtained from Wiener lab (Rosario, Argentina). Purified human chorionic gonadotropin hormone (hCG) was kindly provided by Dr. Parlow (National Hormone and Pituitary Program, National Institute of Diabetes & Digestive & Kidney Diseases, NIDDK, NIH, Bethesda, MD, USA).

2.2. Mouse interstitial testis cells preparation and MA-Leydig cell culture

Interstitial Leydig cells were obtained from BALB-c adult male mice. Cells were obtained by mechanical dispersion followed by filtration [26] and suspended (10^6 cells/ml) in medium 199 supplemented with 10 μM 3-methylisobutilxanthine and 0.1% bovine serum albumin [27]. At the end of the preparation 85–90% of cells remained viable as assessed by the trypan blue exclusion assay. The MA-10 cell line is a clonal strain of mouse Leydig tumor cells that produces progesterone (P4) rather than testosterone as the major steroid. Cells were generously provided by Dr. Mario Ascoli, University of Iowa, College of Medicine, USA and maintained at 37 °C (5% CO_2) as described [28].

2.3. Cell viability assays

2.3.1. MTT assay

Cell viability was assessed in testicular interstitial cells exposed to Cs using the MTT assay as originally described [29]. Briefly, after exposure for 4 h to Cs, cells were seeded in 96-well microplates at a density of 25,000 cells per well and then incubated for 2 h at 37 °C in the darkness, for the conversion of 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan by the mitochondrial succinate–tetrazolium reductase system. Following incubation, formazan crystals were dissolved by treatment with 5% SDS during 16 h. Color formation was then measured espectrophotometrically (595 nM) in a BioTek Instruments Synergy HT (Winooski, VT, USA) multiplate reader.

2.3.2. LDH assay

Lactate dehydrogenase (LDH) activity released to the cell culture medium was used as a marker of cellular necrosis. Cells were exposed to Cs and LDH activity was determined photometrically at 340 nm by measuring NAD+ levels, which is formed in the LDH-catalyzed reduction of pyruvate (0.6 mM) to lactate in the presence of NADH (0.18 mM), using the LDH-P UV kit (Wiener Lab, Rosario, Argentina) and following manufacturer's instructions.

2.4. Steroids measurement

Testosterone concentration was measured in the incubation media of testicular interstitial cells by radioimmunoassay (RIA) and expressed as ng/ml [30].

2.5. Measurement of intracellular reactive oxygen species (ROS)

Levels of intracellular reactive oxygen species (ROS) produced by MA-10 Leydig cells and mouse interstitial cells were detected fluorometrically using 2,7-dichlorofluorescein diacetate (DCFH-DA). MA-10 cells were subcultured onto 48-well plates and grown to approximately 80% confluence as already described, rinsed with fresh serum-free medium and incubated for 30 min at 37 °C with 10 μM DCFH-DA dissolved in serum-free medium. Mouse interstitial cells were suspended in culture media (1.5×10^6 cells/ml) and loaded with DCFH-DA under the conditions described for MA-10 cells. In all cases, cells were then washed and incubated in Krebs Buffer (120 mM NaCl, 24 mM $NaHCO_3$, 11 mM glucose, 4.7 mM KCl, 2.7 mM $CaCl_2.2H_2O$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 0.5 mM EDTA, pH 7.4) for 30 min at 37 °C prior to Cs exposure and ROS determination. Fluorescence intensity was measured at 485 and 535 nm excitation and emission respectively in a BioTek Instruments Synergy HT (Winooski, VT, USA) multiplate reader. Results were integrated and analyzed using the Gen 5 software (Biotek Instruments, Winooski, VT, USA).

2.6. RNA extraction and semiquantitative RT-PCR

Total RNA from the different treatment groups was extracted using TriZol reagent following the manufacturer's instructions (Life Technologies, Inc.-BRL, Grand Island, NY). The reverse transcription was done as previously described [31], using 4 μg of total RNA from testicular interstitial cells and generated cDNAs were further amplified by PCR using the following primer pairs: CYP11A1 cDNA, forward, 5′-CTGCCTCCAGACTTCTTTC-3′ and reverse 5′-TTCTTGAAGGGCAGCTTG-3′. For the comparison of the amount of amplified CYP11A1 produced from different RNA samples, ribosomal protein L19 (L19) cDNA was used as an internal standard, using the sense primer, 5′-GAAATCGCCAATGCCAACTC-3′, and the antisense primer, 5′-TCTTAGACCTGCGAGCCTCA-3′. The number of cycles used was optimized for each gene to fall within the linear range of PCR amplification. The reaction conditions were: for CYP11A1, one 5-min cycle at 95 °C followed by 27 cycles consisting of a 30 s step at 95 °C, 20 s at 52 °C and 40 s at 72 °C; for L19, one 5-min cycle at 95 °C followed by 28 cycles consisting of a 30 s step at 95 °C, 30 s at 52 °C and 40 s at 72 °C. PCR products were resolved

on a 1.5% (wt/vol) agarose gel containing 0.5 μg/ml of ethidium bromide to determine the molecular sizes of the CYP11A1 and L19 amplicons. Gel images were acquired with the GelPro analyzer (IPS, North Reading, MA) and the levels of the CYP11A1 and L19 mRNA quantitated using a computer-assisted image analyzer (ImageQuant 5.2). CYP11A1 mRNA abundance was normalized by L19 mRNA as an internal control.

2.7. Protein determination

Protein concentration was determined by the method described by Lowry et al. [32] using BSA as standard.

2.8. PKA determination

PKA activity was determined by measuring the incorporation of [^{32}P] orthophosphate from [^{32}P] γ-ATP into histone type III-S using lysates from MA-10 Leydig cells as source of PKA [33]. MA-10 Leydig cells were treated with or without Cs (50 μM) for 4 h and further treated for 15 min with either 20 ng/ml hCG, 0.5 mM 8Br-cAMP or vehicle (Basal). Following the treatment, MA-10 cell cultures were washed with phosphate-buffered saline, scraped into a buffer containing 25 mM Tris–HCl, pH 7.4, 0.5 mM EDTA, 0.5 mM EGTA, 1 μg/ml leupeptin and 1 μg/ml aprotinine, and sonicated by three 15 s cycles in a Branson 250 sonicator (Branson

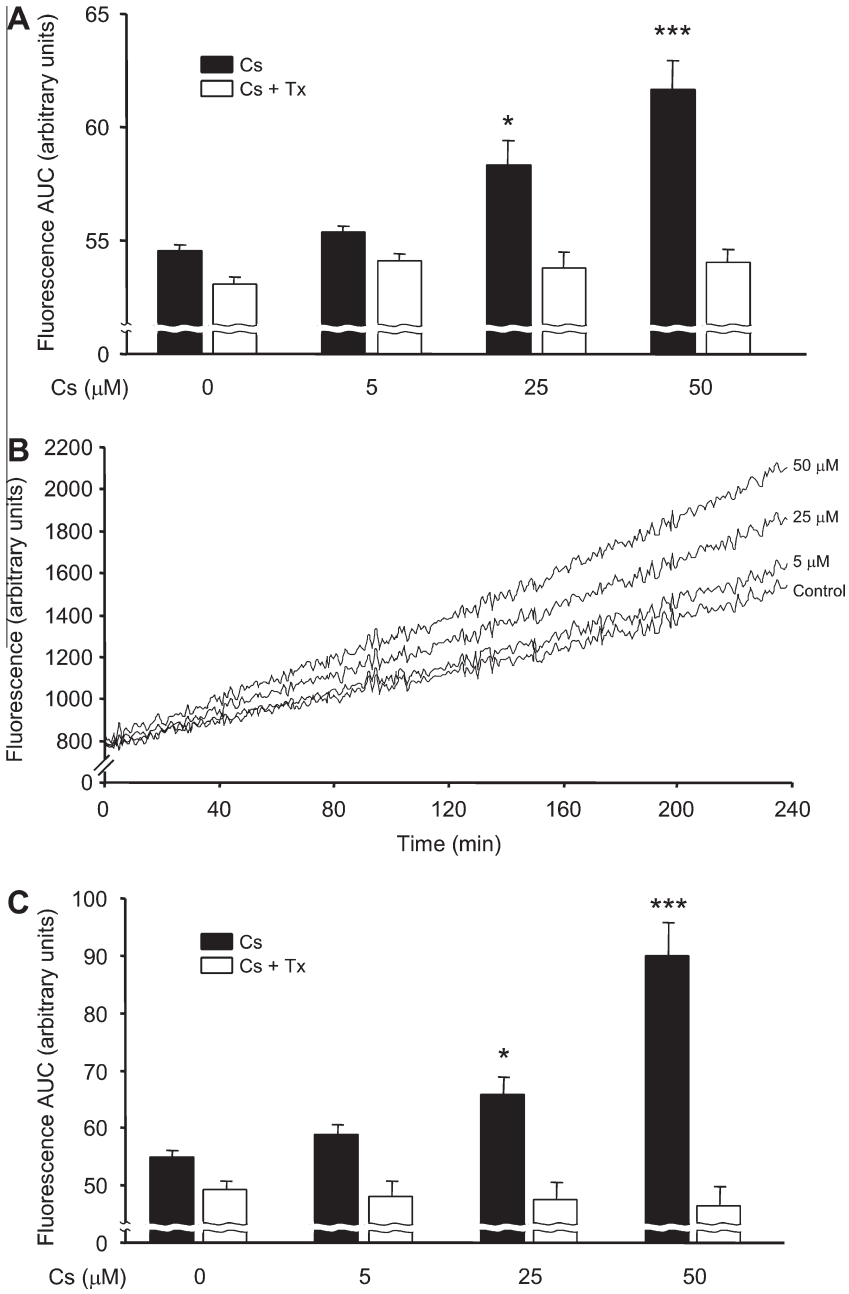

Fig. 1. Effect of Cs on reactive oxygen species (ROS) intracellular levels. Testicular interstitial (A and B) or MA-10 Leydig (C) cells were loaded with dichlorofluorescein as described in Section 2 prior to a 30 min exposure (A and C) or during the indicated times (B) to the indicated concentrations of Cs alone or in combination with 100 μM Trolox (Tx) added 30 min before the drug or to vehicle (A and C), and the levels of intracellular ROS determined fluorometrically. Fluorescence intensity was measured and results are shown as the mean ± SEM integrated area under the curve (AUC) during the first hour of Cs incubation of three independent experiments performed in triplicates (panels A and C) or as fluorescence intensity throughout the measurement of a representative experiment (panel B). In all cases, results are expressed in arbitrary units. $^{*}P < 0.05$; $^{**}P < 0.001$ vs respective controls.

Ultrasonics Corp., Dansbury, CT, USA) at 70 W. This material was used for PKA activity determinations. Cell lisates containing 4 μg of proteins were incubated in a reaction mixture (25 μl total volume) consisting of histone 50 μg, 50 mM Tris–HCl, pH 7.5, 10 mM $MgCl_2$, 200 μM ATP (100 cpm/pmol). The reaction was allowed to proceed for 10 min at 30 °C and was terminated by spotting onto p81 paper (Whatman) and washing in 75 mM orthophosphoric acid.

2.9. Statistical analysis

Results are shown as the mean ± SEM. Statistical significance was evaluated using ANOVA followed by Tukey test. Differences were deemed significant when $P < 0.05$.

3. Results

3.1. ROS generation in cells exposed to Cs

First, we evaluated the levels of intracellular ROS in freshly isolated mouse interstitial cells exposed to different Cs concentrations (5–50 μM). As shown in Fig. 1A, Cs increased intracellular ROS levels in a concentration-dependent manner. ROS levels were significantly elevated in cells exposed to 25 μM or 50 μM Cs ($P < 0.05$ and $P < 0.001$, respectively) during the first hour, an effect that was abolished by addition of the antioxidant Trolox (Tx) added 30 min before the drug. Moreover, Cs increased intracellular ROS levels in a time-dependent fashion throughout the study, up to 4 h (Fig. 1B). Since ROS detected in the interstitial cell preparation could be generated by cellular types other than Leydig cells, we determined ROS accumulation in the mouse Leydig cell line MA-10. Exposure of the cells to Cs for 1 h caused also a concentration-dependent increase of ROS intracellular level in MA-10 Leydig cells that was also blocked by Trolox (Fig. 1C).

3.2. Effect of Cs on testosterone production in mouse testicular interstitial cells

We next investigated Cs site(s) of action on testosterone synthesis. For this purpose, freshly isolated testicular interstitial cells were exposed to different concentrations of Cs (5–50 μM) for 4 h in the presence or absence of hCG (a hormone able to activate the LH receptor and frequently used instead of LH), and testosterone production was determined in the culture media by RIA. As shown in Fig. 2, out of the different Cs concentrations assayed, only 50 μM significantly reduced testosterone production in both hCG-stimulated and non-stimulated cells. We also tested the effect of 50 μM Cs on cell integrity following 4 h of incubation. Cs did not affect cell viability, as indicated by mitochondrial reduction of MTT, nor did it cause cellular necrosis, as indicated by the levels of lactate dehydrogenase (LDH) detected in the culture media (Fig. 3A and B). We thus analyzed the effect of 50 μM Cs on testosterone production triggered by different steroidogenic stimuli or sustained by different substrates, in order to identify Cs site of action in steroidogenesis. Cells were incubated for 4 h with either hCG, a membrane-permeable analog of cAMP (8Br-cAMP), a freely diffusible cholesterol analog 22R-hydroxycholesterol (22R-HC) or pregnenolone (P5). Cs showed no inhibitory effect on P5-supported testosterone synthesis, while it was effective in reducing hCG-, 8Br-cAMP- or 22R-HC-induced testosterone synthesis to a similar extent (Fig. 4). We also tested the effect of Cs on P450scc mRNA by measuring its levels by semi-quantitative RT-PCR. As shown in Fig. 5, Cs did not modify P450 mRNA levels after 4 h of incubation. Moreover, Cs was ineffective to inhibit P5-supported testosterone synthesis even after 6 h of incubation, whereas it reduced

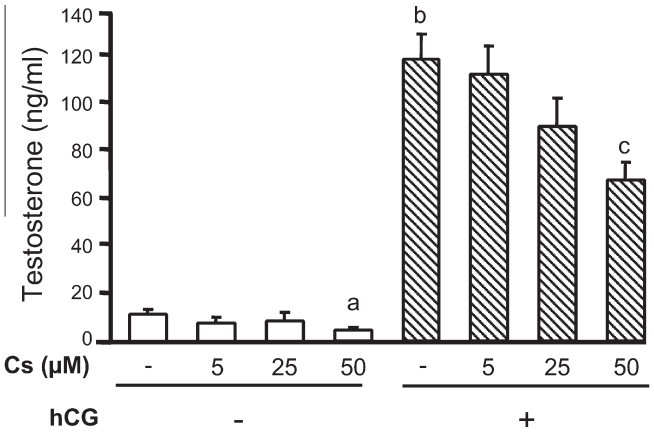

Fig. 2. Effect of Cs on hCG-stimulated testosterone production by mouse testicular interstitial cells. Testicular interstitial cells were incubated with increasing concentration of Cs or vehicle (−) for 4 h in the absence or the presence of 10 ng/ml hCG. Testosterone produced was quantified in the culture media by RIA. Data represent the mean ± SEM of three independent experiments performed in triplicates. (a) $P < 0.01$ and (b) $P < 0.001$ vs unstimulated cells incubated without Cs; (c) $P < 0.01$ vs stimulated cells incubated without Cs.

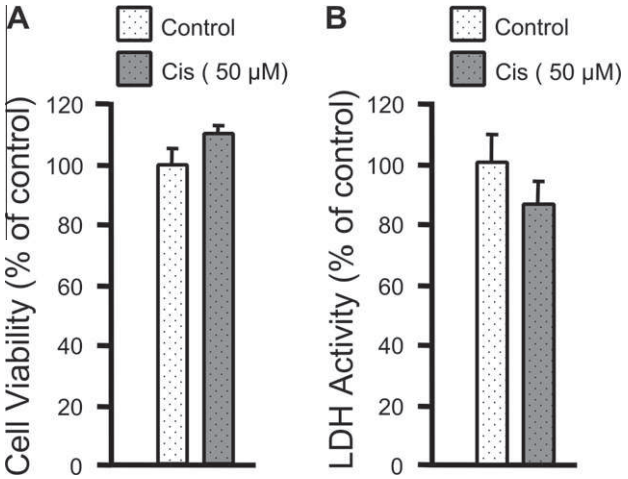

Fig. 3. Cytotoxic effect of Cs on testicular interstitial cells. Testicular interstitial cells were exposed or not (Control) to 50 μM Cs for 4 h and cell viability and cellular necrosis evaluated by means of the MTT assay (Panel A) and lactate dehydrogenase (LDH) released to the culture medium (Panel B), respectively. Results represent percentage of control and are expressed as the mean ± SEM of three independent experiments performed in triplicates. *$P < 0.05$ vs control.

22R-HC-induced testosterone synthesis in this condition (data non show).

3.3. Role of ROS on Cs effect on testicular steroidogenesis

The effect of Cs on 22R-HC-supported testosterone production suggests that at least a site of action of Cs is the cytochrome P450scc. Thus, we investigated the possible role of ROS on Cs-mediated inhibition of testosterone production in cells exposed to 22R-HC. As shown in Fig. 6, Cs inhibitory effect on testosterone production was abolished by preincubation of the cells with Trolox. Accordingly, the ROS-generating compound ABAP mimicked the inhibitory effect of Cs on testosterone synthesis and Trolox overcame the effect of ABAP (Fig. 6).

3.4. Effect of Cs on hCG-mediated PKA activation

It is known that Cs produces a marked decrease in the binding capacity of the LH receptor [13]. Thus, we analyzed the LH receptor

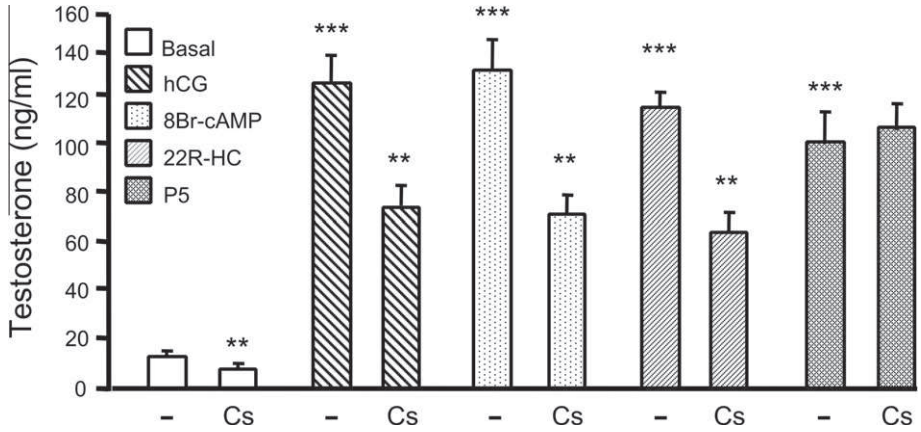

Fig. 4. Site of action of Cs on testosterone biosynthesis in mouse testicular interstitial cells. Testicular interstitial cells were incubated in the absence (-) or the presence of 50 μM Cs for 4 h under basal conditions (Basal) or in the presence of 10 ng/ml hCG (hCG), 100 μM 8Br-cAMP (cAMP), 5 μM 22R-hydroxycholesterol (22R-HC) or 5 μM pregnenolone (P5) and testosterone produced was quantified. Data represent the mean ± SEM of three independent experiments performed in triplicates. $^{***}P < 0.001$ vs cells incubated under basal conditions without Cs; $^{**}P < 0.01$ vs cells incubated in the absence of Cs and under basal conditions or with the same stimulus or steroidogenic substrate.

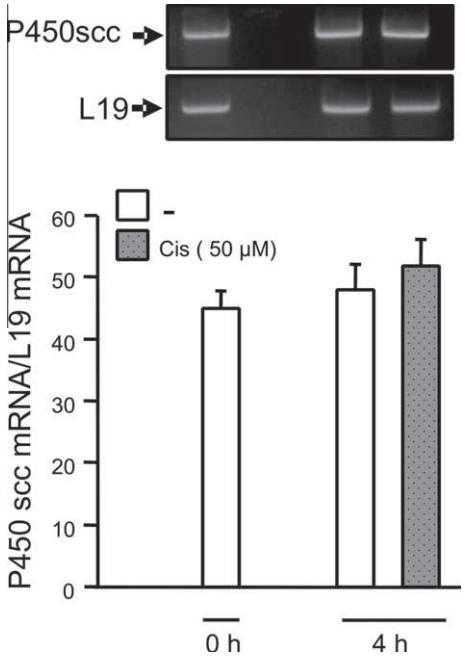

Fig. 5. Analysis of P450scc messenger levels in cells exposed to Cs. Testicular interstitial cells were incubated in the absence (-) or the presence of 50 μM Cs (4 h) or processed immediately after their preparation (0 h). Total RNA was isolated and subjected to RT using specific primers for P450scc and L19. Obtained cDNA fragments were amplified by semi quantitative PCR and RT-PCR products resolved in ethidium bromide-stained agarose gels. The figure shows a representative image (upper panel). Integrated optical density of each band was quantitated by densitometry and P450scc mRNA values normalized against the loading control and expressed as the mean ± SEM arbitrary units of three independent experiments (lower panel).

function in cultured Leydig cells exposed to Cs. The effect at the receptor level was indirectly evaluated through the determination of PKA activity in samples obtained from MA-10 Leydig cells exposed to 50 μM Cs for 4. Cells were exposed or not (control) during 4 h to Cs in the presence or absence of Trolox, and then incubated with hCG or 8Br-cAMP or without stimuli (basal) for 15 min. The effect of Cs on PKA activity present in the corresponding cell lysates is shown in Table 1. PKA activity in control or stimulated cells (either with hCG or 8Br-cAMP) was not modified by pretreatment with Cs for 4 h. These results show that Cs does not interfere with hCG-induced PKA activation, suggesting that LH receptor activation is not a target of Cs. Moreover, these results agree with the observed inhibition, to a similar extent, of testosterone synthesis in both hCG- and 8Br-cAMP-stimulated cells.

4. Discussion

Testicular dysfunction is a common side effect associated with Cs and other Pt II compounds (PtCs) treatment. Indeed, several investigators have described a reduction in testosterone plasma levels both in patients under treatment with these drugs and in experimental animal models [34,14]. However, little is known on the mechanism involved in the cytotoxic effect of PtCs in Leydig cells. Given that oxidative stress and free radicals have been implicated in the development of numerous side effects of PtCs, we have analyzed whether ROS generation induced by Cs and the disruption of steroidogenesis by this drug are certainly linked. Our study demonstrates that Cs strongly inhibits testosterone production through a mechanism that involves ROS-mediated P450scc inhibition in testicular interstitial cells.

Although MA-10 Leydig cells are widely used to study the acute regulation of steroid production [28,35,36], their major steroid output is not testosterone. Therefore, we used here a preparation of testicular interstitial cells for studying the site of action of Cs on testosterone synthesis. On the other hand, the molecular events triggered by Cs in Leydig cells were studied using the MA-10 Leydig cell line as experimental model.

One of the molecular events analyzed was ROS production. We have demonstrated that a concentration of 50 μM of Cs, which significantly increases ROS levels in MA-10 Leydig and mouse interstitial cells, is enough to inhibit testosterone synthesis. This concentration is used in several *in vitro* studies and it also approximately represents the maximal plasma levels obtained following standard Cs administration regimens [37]. It should be noted that 25 μM Cs significantly increased ROS levels as early as 30 min. This concentration also reduced testosterone synthesis but without reaching statistical significance (Figs. 1 and 2). It is possible that the intracellular antioxidant mechanisms and the intrinsic properties of the P450scc enzyme might determine that only high ROS concentrations are required to significantly inhibit testosterone synthesis.

As already mentioned, in basal conditions the diffusion of cholesterol across the aqueous space between mitochondrial

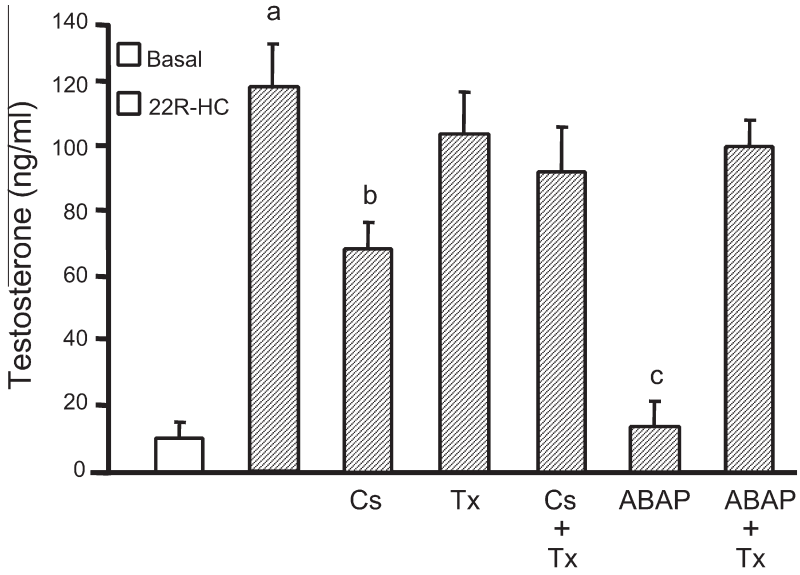

Fig. 6. Role of ROS on the effect Cs on 22R-HC – supported steroidogenesis in mouse testicular interstitial cells. Cells were incubated in the absence (Basal) or in the presence of 5 μM 22R-hydroxycholesterol (22R-HC) alone or in combination with Cs 50 μM (Cs) or Cs plus 100 μM Trolox (Cs + Tx) added 30 min before the drug. The effect of 100 nM ABAP alone (ABAP) or in the presence of Trolox (ABAP + Tx) on 22R-HC-supported steroidogenesis was also evaluated. Testosterone accumulated during 4 h in the culture media was quantified by RIA in the culture medium. Data represent the mean ± SEM of two independent experiments performed in triplicates. (a) $P < 0.001$ vs cells incubated under basal conditions; (b) $P < 0.01$ and (c) $P < 0.001$ vs cells incubated with 22R-HC alone.

Table 1
Effect of Cs on hCG-, or 8BrcAMP-induced PKA activity on MA-10 Leydig cells. MA-10 Leydig cells were exposed to Cs during 4 h in the absence or presence of Trolox (Tx) added 30 min before the drug and further treated for 15 min with either 20 ng/ml hCG, 0.5 mM 8Br-cAMP or to vehicle (Basal) and PKA activity was determined as described in Section 2. Results are expressed as pmol Pi transferred/mg protein ± SEM of three independent experiments performed in quadruplicates.

	PKA activity (pmol Pi transferred/mg protein)		
Treatment	Control	Cs	Cs + Tx
Basal	1.6 ± 0.2	1.4 ± 0.3	1.8 ± 0.3
hCG	17.6 ± 0.9***	15.3 ± 1.4***	16.6 ± 1.3***
8Br-cAMP	23.4 ± 1.8***	21.6 ± 2.0***	20.8 ± 1.8***

*** $P < 0.001$ vs corresponding basal.

membranes is extremely slow. Therefore, in non-stimulated cells the rate of steroid synthesis is very low when cholesterol is used as substrate. However, when steroidogenic cells are exposed to water-soluble cholesterol analogs which can freely diffuse to the inner mitochondrial membrane such as 22R-HC, maximal steroid production can be achieved even in the absence of hormonal stimulation. Our results show that Cs inhibits hCG-, and 8Br-cAMP-stimulated as well as 22R-HC-supported steroid production to a similar extent, but that it fails to inhibit P5-induced steroidogenesis. The mechanism of action of hCG implies several different events including the reaction catalyzed by P450scc, whereas 22R-HC bypasses those steps to target P450scc directly. It follows then from our results that the events upstream the access of cholesterol to the active site of P450scc are not affected by Cs, which demonstrates that P450scc is the main target of Cs in our experimental conditions. This argument is sustained by the fact that Cs does not prevent PKA activation. Given that oxidative stress is known to inhibit ovarian and testicular steroidogenesis [38,39], we wondered whether the effect of Cs on testosterone synthesis in testicular interstitial cells involves ROS generation. In fact, we demonstrate here that Cs exerts an acute effect on steroidogenesis by reducing P450scc activity in a ROS-dependent manner. An effect of Cs on CYP450scc has been reported earlier [12,13]. Our study extends that observation by providing an insight into the mechanism of action of the drug and on the role of ROS in this process.

A decrease in the level of hepatic microsomal CYP2C11 and CYP3A2 and of specific P450 isozymes in a rat model of Cs-induced acute renal failure has been reported [40]. This study shows significant changes in enzyme levels after one or more days of drug administration and, accordingly, it also reports a reduction in serum testosterone levels after 4 days of treatment, a result similar to those discussed above [12,13]. In our experiments, the inhibition of testosterone production by Cs due to the down-regulation of P450scc levels seems unlikely since P450scc shows a low turnover rate, and the decrease in testosterone production is detected after 4 h or less (2 h, data not shown) of Cs treatment. Moreover, Cs did not affect P450scc mRNA levels, supporting the notion that it affects P450scc enzyme activity by a ROS-mediated mechanism.

Our study demonstrates that StAR protein is not a target for Cs. Would Cs, apart from its inhibitory effect on P450scc, affect StAR induction then a higher inhibitory effect on hCG-, or 8Br-cAMP-stimulated testosterone synthesis over 22R-HC sustained steroidogenesis is to be expected. This is not the case in our study, since the extent of inhibition on testicular steroidogenesis produced by Cs using a freely diffusible substrate, 22R-HC, is similar to the effect registered in hCG- or 8Br-cAMP-stimulated cells. Therefore, we conclude that Cs does not inhibit StAR protein expression under our experimental conditions. Tsai et al. reported that H_2O_2 inhibits basal and hCG-induced testosterone release from primary Leydig cells by inhibiting P450scc activity and also by reducing StAR expression [23]. In MA-10 cells, H_2O_2 also inhibits cAMP-stimulated steroidogenesis by reducing StAR protein induction [22]. It should be noted that the studies of Diemer and Tsai show a reduction in StAR protein levels with concentrations of H_2O_2 higher than 250 μM; exposure of the cells to Cs 25 or 50 μM, as we have used, is unlikely to release such a high amount of H_2O_2. Thus, a comparison between Tsai's [23] and Diemer's [22] results and ours, showing that the target of Cs-induced ROS in the steroidogenic pathway is localized downstream of StAR action, should be done with caution.

In summary, we report here that Cs generates ROS, which in turn reduce P450scc activity leading to the inhibition of testosterone production. Thus, our *in vitro* study shows the relevance of ROS in the mechanism involved in a well recognized side effect of Cs, that is, the reduction of serum testosterone levels.

Conflict of interest

The authors declare that there are no conflicts of interest.

Acknowledgments

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