HIGH GLUCOSE-INDUCED CHANGES IN STEROID PRODUCTION IN ADRENAL CELLS

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Abstract

Background: Increased activity of the HPA axis, resulting in enhanced ACTH and serum glucocorticoid levels, has been described in patients with diabetes mellitus and in

and animal models of this disease; however, altered steroid production by adrenocortical cells could result from local changes triggered by increased ROS, induced in turn by chronic hyperglycemia. Experiments were designed 1) to analyze the effects of incubating murine adrenocortical cells in hyperglycemic media on the generation of oxidative stress, on steroid synthesis, and on its modulation by the activity of haeme oxygenase, and 2) to evaluate the effect of antioxidant treatment on these parameters.

Methods: Y1 cells were incubated for 7 days with either normal or high (HG, 30 mmol/l) glucose concentrations, with or without antioxidant treatment. Parameters of oxidative stress and expression levels of haeme oxygenase-1 (HO-1), nitrite levels, L-arginine uptake and progesterone production were determined.

Results: HG augmented ROS and lipoperoxide production, decreasing GSH levels and increasing antioxidant enzymes and HO-1 expression. Basal progesterone production was reduced, while a higher response to ACTH was observed in HG-treated cells. The increase in HO-1 expression and the effects on basal steroid production were abolished by antioxidant treatment. Inhibition of HO activity increased basal and ACTH-stimulated steroid release. Similar results were obtained by HO-1 gene silencing while the opposite effect was observed in Y1 cells overexpressing HO-1.

Conclusions: HG induces oxidative stress and affects steroid production in adrenal cells; the involvement of HO activity in the modulation of steroidogenesis in Y1 cells is postulated.

Abbreviations

ACTH: adrenocorticotropin, HO: haeme oxygenase, Sn-PPIX: tin-protoporphyrin IX, NG: normal glucose (5.5 mmol/l), HG: high glucose (30 mmol/l), LG: L-glucose, ROS: reactive oxygen species, GSH: glutathione, SOD: superoxide dismutase, TBARS:

thiobarbituric acid reactive substances, α-T: α-tocopherol, HPA: hypothalamic-pituitary-adrenal, GSPx: glutathione peroxidase

Key words: haeme oxygenase 1; high glucose; oxidative stress; antioxidants; steroids; nitric oxide; adrenocortical cells

Introduction

Chronic hyperglycemia, a major hallmark of diabetes mellitus, has been linked to the excessive generation of reactive oxygen species in target organs such as the retina and kidney, among others, leading to intracellular biochemical alterations involved in the development of chronic diabetes complications (for review see [1]). Moreover, excessive chronic oxidative stress, *per se*, might affect cellular functions in experimental diabetes models [2], e.g. it has been described that elevated glucose concentrations increase apoptosis by inducing oxidative and nitrosative stress in different cell types [3], and decrease mitochondrial biogenesis by augmenting ROS formation in HepG2 cells [4].

It is well known that oxidative stress induces the expression of antioxidant enzymes such as superoxide dismutases (SOD), catalase, glutathione peroxidase (GSH-Px) and haeme oxygenase-1 (HO-1) [5, 6]. Redox imbalance also induces haeme oxygenase-1 (HO-1) [6], and induction of HO-1 by high glucose concentrations has been demonstrated in many cell types [7-9]. In addition to the antioxidant properties attributed mainly to haeme consumption and biliverdin generation, CO generated during haeme degradation can act as a messenger molecule affecting cellular physiology [10]. Accordingly, we have previously demonstrated the induction of HO-1 by ACTH in zona fasciculata adrenal cells, its role as an antioxidant enzyme, and its involvement in the modulation of adrenal steroidogenesis [11].

Nitric oxide has been also involved in the regulation of steroid biosynthesis in the adrenal gland. We have previously shown an inhibitory effect of NO on basal and ACTH-induced corticosterone production [12, 13]. There is growing interest in the modulation of NOS activity by L-arginine availability, and alterations in this parameter are associated to the diabetic state [14].

Increased activity of the hypothalamic-pituitary-adrenal axis, resulting in elevated circulating glucocorticoid levels, has been repeatedly described in patients with diabetes mellitus [15-18], and in streptozotocin-treated rats [19] as well. However, little is known about the local effects of hyperglycemia on the steroidogenic cells of the adrenal cortex. In this sense, two decades ago, Rebuffat et al. demonstrated that STZ-treated rats showed an hypertrophy of the adrenal zona fasciculata [20].

Taking the above data into account, we hypothesized that chronic hyperglycemia increases ROS generation and that the concomitant redox imbalance and/or changes in regulatory mechanisms affect steroid production by adrenocortical cells. Accordingly, the purpose of this study was to analyze the effects of incubating mouse adrenal cells in hyperglycaemic media on oxidative stress and steroid synthesis, as well as on the modulation of the latter by regulatory signals originating in the activation of the HO system. The effect of antioxidant treatment on these parameters was also evaluated.

Materials and Methods

Drugs and chemicals: ACTH was obtained from Laboratorios ELEA (Buenos Aires, Argentina). Antibodies raised against haeme oxygenase and actin were from StressGen Biotechnologies Corp. (Victoria, Canada) and from Santa Cruz Biotechnology (Santa Cruz, CA, USA) respectively; peroxidase-conjugated goat anti rabbit-IgG was from Bio-Rad (Hercules, CA, USA). Progesterone antiserum was kindly provided by Dr A. Bélanger (Laval University, Quebec, Canada). Fetal calf serum, penicillin, and streptomycin were from Invitrogen (Life Technologies, Buenos Aires, Argentina). All other chemicals were of the highest quality available.

Cell cultures and treatments: Y1 cells (generously provided by Dr. B. Schimmer, University of Toronto, Canada) are an ACTH- and cAMP-responsive subclone of the mouse adrenocortical tumor cell line isolated by Yasumura et al [21]. Cells were grown as monolayer in Ham's F10 medium containing heat-inactivated fetal bovine (2.5%) and horse (12.5%) serum, 200 U/ml penicillin G, and 270 μg/ml streptomycin sulphate, in a humidified atmosphere of 5% CO_2 in air at 37°C [22]. Long term high glucose treatments were initiated by replacing the culture medium with fresh Ham's F10 with serum for 7 days with the following additions: D-glucose (none or 24.5 mmol/l, to yield final concentrations of 5.5, NG or 30 mmol/l, HG, respectively), L-glucose as osmotic control (24.5 mmol/l, LG) and α-tocopherol (0.5 mmol/l). In some experiments adrenocortical cells were stimulated during 180 min with ACTH (10 mIU/ml). No differences in cell viability, assessed by the trypan blue dye exclusion test, resulted from any of these treatments.

Primary cultures of rat adrenocortical cells: Preparation of zona fasciculata cells followed published procedures with minor modifications [23]. Briefly, cells were isolated from decapsulated and demedullated adrenals by incubating the tissue in Ham´s

F10 medium with Type I collagenase (0.5 mg/ml, Worthington, NJ, USA) for 20 min at 37 °C. Tissues were disrupted by gentle aspiration with a sterile 10 ml pipette, filtered and centrifuged for 10 min at 200-300 ***g***. The cell pellet was suspended in Ham´s F10 medium (supplemented with 2.5 % FBS, 12.5% horse serum, 100 U/ml penicillin and 100 µg/ml streptomycin) and seeded into 24-well plates at a density of 10^6 cells/well. Cell cultures were incubated at 37°C in a humidified atmosphere of 95% air–5% CO_2. HG treatment began one day after the seeding and continued for another four days.

Determination of intracellular ROS generation: After each treatment, cells were washed twice with PBS and then incubated in PBS containing 10 µmol/l of 2´,7´-dichlorodihydrofluorescein diacetate for 1 hour at 37°C. After that, cells were washed twice and resuspended in PBS. The fluorescent compound 2´, 7´-dichlorofluorescein, generated in the presence of ROS is then detected by determining its fluorescence (excitation 485 nm, emission 535 nm). ROS generation is expressed in arbitrary units relative to total protein content.

Measurement of reduced glutathione (GSH): Cells were homogenized in 50 mmol/l pH 7.4 phosphate buffer. Protein and cellular debris were precipitated with 50% trichloroacetic acid and 1 mmol/l EDTA. Dithionitrobenzene (0.25 mg/ml in 0.5 mol/l phosphate buffer) was added to the supernatant and the mixtures were incubated at 37ºC for 15 min. Absorbance was measured at 412 nm. Reduced GSH levels are expressed as nanomoles of GSH per milligram of protein. A standard reduced GSH solution was used for the calibration curve.

Measurement of lipid peroxides as thiobarbituric acid-reactive substances (TBARS): Lipoperoxide content was determined measuring their reactivity with thiobarbituric acid as previously described [24]. Briefly, cells were suspended in 1 ml of PBS with 0,075% SDS and 0.5 ml of 2-thiobarbituric acid (10 mg/ml in 10% sodium

acetate buffer pH 3.5) was added. Then, mixtures were heated in a water bath at 90°C for 60 min and fluorescence was measured (515 nm excitation and 555 nm emission). Values are expressed as nanomoles of TBARS per milligram of protein using malondialdehyde as a standard prepared from 1, 1, 3, 3-tetramethoxypropane.

RNA isolation and RT-PCR: Total RNA was extracted from Y1 cells with TRIzol® reagent (Invitrogen, Buenos Aires, Argentina). RNA (2μg) was pretreated with RNase-free DNase (deoxyribonuclease I, amplification grade; Invitrogen, Buenos Aires, Argentina), heated at 70°C for 10 min, placed on ice for 1 min, and then incubated with a mixture containing 0.5 mmol/l dNTPs mix, 25 ng/μl random primers, 1X first-strand buffer, 25 units of RNAase inhibitor, 200 units of MMLV reverse transcriptase (Promega, Madison, WI, USA), and water to a final volume of 25 μl for 1 h at 42°C. The reaction was stopped by heating at 90°C for 5 min. The reaction mixture was brought to 100 μl with diethylpyrocarbonate-treated water and stored at –70°C. In selected tubes reverse transcriptase was omitted as a contamination control. PCR reactions were carried out in a T personal Thermocycler (Biometra Biomedizinische Analytik, Göttingen, Germany). For the amplification of the gene products 2 μl of cDNA was added to 18 μl of the following reaction mixture: 1X PCR buffer, 1.5 mmol/l $MgCl_2$, 0.2 mmol/l of each dNTP, 500 nmol/l of each specific oligonucleotide primer, and 0.625 U Taq polymerase (Invitrogen, Buenos Aires, Argentina). The sequence for the oligonucleotide primers were as follows: for HO-1: forward: 5'-ACTTTCAGAAGGGTCAGGTGTCC-3' reverse: 5'-TTGAGCAGGAAGGCGGTC TTAG-3'; HO-2: forward 5′-CCACCACTGCACTTTACTTC-3′ reverse: 5′-GGTCTTCATACTCAG GTCCA-3′; 18S: forward: 5′-ACGGAAGGGCACCACCAGGA-3′ reverse: 5′-CACCACCACCCA CGGAATCG-3′; hHO-1: forward: 5′-GAGTGTAAGGACCCATCGGA-3′ reverse: 5′-

GCCAGCAACAAAGTGCAAG-3′ Cu-SOD: forward 5′-GGCGTCATTCACT TCGAGCAGAAG-3′ reverse: 5′-GGCA ATCCCAATCACACCACAAGC-3′; Mn-SOD: forward: 5′-GTTACAACTCAGGTC GCTCTT C-3′ reverse: 5′-GTGCTGCAA TGCTCTACACTAC-3′; GPx: forward: 5′-GCACAGTCCACCGTGTATGCCTTC-3′ reverse 5′-GTTGCTAGGCTGCTTGGACACCAG-3′; Catalase: forward: 5′-ATGGTCTGGGACTTCTGGAGTCTTC-3′ reverse: 5′-GTTTCCTCTCCTCCTCGTT CAACA-3′. 18S rRNA was used in the semiquantitative RT-PCR protocol as a normalizing control. To amplify the genes coding for HO-1 and 18S, reactions 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% agarose gels in 40 mmol/l Tris acetate and 2 mmol/l EDTA pH 8, stained with ethidium bromide, photographed and quantitated by a video documentation system (GelPro Imager; Image Processing Solutions, North Reading, MA).

Immunoblot analysis: Y1 cells were washed twice in PBS and lysed in 20 mmol/l Tris–HCl pH 7.4, 250 mmol/l NaCl, 1% Triton X-100 and 1X of protease inhibitor cocktail. Samples were boiled for 5 min in SDS–PAGE loading buffer with 0.1 mol/l dithiothreitol and electrophoresed on 10% polyacrylamide gels. After electrophoresis, proteins were transferred to polyvinylidenefluoride (PVDF) membranes for 1 h at 15 V in a Bio-Rad Trans-Blot SD system with 25 mmol/l Tris–HCl pH 9.2, 192 mmol/l glycine and 20% methanol buffer. PVDF membranes were blocked in TBS-Tween (50 mmol/l Tris–HCl pH 7.4, 0.15 M NaCl, 0.05% Tween 20) with 5% skimmed milk for 60 min at room temperature and then incubated overnight with the following antisera: HO-1 (1:5000), HO-2 (1:10000) or β-actin (1:10000) at 4ºC. Membranes were washed

with TBS-Tween and then incubated for 1 h with a 1:5000 dilution of a goat anti-rabbit immunoglobulin G antibody–horseradish peroxidase conjugate. The membranes were washed and the bands were visualized by chemiluminescence (Enhanced chemiluminescence reagent, Amersham Pharmacia Biotech, Little Chalfont, Bucks, UK).

SiRNA transfection

siRNA was designed to target HO-1 (accession No. NM_010442.2) coding sequence. siRNA sequences 5´-CTGACAGAGGAACACAAAGA-3´ (nucleotides 793–812 of HO-1 cDNA), and 5´-AAGGTAATGCTGCGCTAAAT-3´(scramble siRNA) were synthesized according to published protocols [25]. One day before transfection, Y1 cells (2.5 10^5 cells per well) were grown up to 80% confluence on 24-well plates. Transfection was performed using siRNA (400 ng/ml) in Opti-MEM medium and 2 µl Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) according to the instructions of the manufacturer. Cells were placed in normal culture medium 12 h after transfection and further grown for 48 h. Y1 cells were stimulated with (7.5 mIU/ml) ACTH for 5 h. Progesterone production was measured by RIA, and data are shown as progesterone production (ng/ml) in the incubation medium.

Overexpression of human heme-oxygenase (hHO-1) in Y1 cells: Transient transfection was performed using Lipofectamine 2000 (Invitrogen). Briefly, one day before transfection, 10^5 cells were seeded in 24-well plates in antibiotic-serum free medium. 0,8 µg of DNA (pcDNA3-βgal or hHO-1, kindly provided by Dr E. Vazquez, FCEyN, UBA, Argentina) and 2 µl of Lipofectamine 2000 reagent were diluted in 50 µl of optiMEM separately. After a 5-min incubation, the DNA and the Lipofectamine solutions were mixed and incubated for additional 20 min at room temperature. The DNA-Lipofectamine complex was then added to the cells and incubated at 37°C in a

humidified atmosphere of 95% air–5% CO_2. After 3 h the media was added with 15% serum containing Ham´s F10 medium and the cells were grown for 48 h before initiating the assays.

Nitrite accumulation: Nitrite content in the 24 h incubation medium was determined spectrophotometrically using the Griess reaction, as described previously [12]. Values are expressed as nanomoles of nitrite per milligram of protein.

L-Arginine uptake assay: Unidirectional transport of L-[2,3,4-^{3}H]arginine was measured in Y1 cells. The cells were plated at a density of 2 x 10^5 cells per well (1.000 cells/mm^2) in 12-well trays. After the treatments, the cells were washed twice with 1 ml buffer A (2 mmol/l KCl, 2.5 mmol/l $CaCl_2$, 1 mmol/l $MgCl_2$, 5.6 mmol/l D-glucose, 140 mmol/l choline chloride and 20 mmol/l HEPES, pH 7.4) and uptake was measured by adding 500 µl of buffer A containing L-[2,3,4-^{3}H]arginine monohydrochloride (50 µmol/l, AE: 38,5 Ci/mmol, final concentration 1 µCi/ml) to each well. L-arginine influx was measured over 2 min at 37°C. Incubations were terminated by rinsing the cells three times with 1 ml buffer A. Cells were disrupted by adding 300 µl ethanol per well, and radioactivity was quantified in the ethanolic wash by liquid scintillation counting.

Steroid production Progesterone and corticosterone levels in the incubation media were determined by RIA using specific antibodies [26].

cAMP levels: Cells were incubated in fresh media containing 0.5 mmol/l 3-isobutyl-1-methyl xanthine with or without 10 mIU/ml ACTH. After 3 hours of incubation, cAMP levels were determined by RIA in the incubation media [27].

Statistical analysis: Numerical results (presented as mean ± SEM) were tested for normality using the Kolmogorov-Smirnoff test. Statistical analysis was performed by one-way ANOVA followed by Tukey's *post hoc* test using GraphPad InStat version

3.06 for Windows (GraphPad Software, San Diego CA), or using Student´s t test for non-paired data, as considered necessary. A p value < 0.05 was considered significant.

Results

Figure 1A shows that Y1 cells incubated in media containing 30 mmol/l D-glucose (HG) for 7 days produced significantly less progesterone than those incubated in media with 5.5 mM D-glucose (NG). In the presence of HG, however, ACTH stimulation of Y1 cells resulted in a significantly higher steroid output as compared to NG conditions (Figure 1B). Similar results were obtained when primary cultures of rat adrenal cells were incubated in HG (Figure 1C and D). None of the above mentioned parameters showed significant changes when the cells were incubated in media containing L-glucose 24.5 mmol/l plus D-glucose 5.5 mmol/l (LG) as an osmotic control.

No significant changes in basal or ACTH-stimulated cAMP production resulted from HG treatment (data not shown).

Different parameters of oxidative stress were analyzed in Y1 cells incubated in media containing HG for 7 days. As shown in Figure 2A, significantly higher levels of ROS were detected in this condition, as compared to cells incubated in media with NG. These cells also exhibited higher levels of lipid peroxidation (Figure 2B) and lower reduced glutathione levels (Figure 2C).

Treatment of Y1 cells with HG for 7 days increased the mRNA levels or Mn-superoxide dismutase (Mn-SOD), Cu-superoxide dismutase (Cu-SOD), catalase and glutathione peroxidase (GPx) (Figure 3A). Moreover, this treatment also increased HO-1 mRNA and protein levels (Figures 3B and 3C). HO-2 expression levels were not affected by this treatment (data not shown).

The involvement of HO activity in the modulation of steroid production in HG-treated cells was evaluated using Sn-PPIX, a competitive inhibitor of HO activity. The addition of Sn-PPIX to the incubation media resulted in a higher steroid output by Y1

cells, both in basal and ACTH-stimulated conditions (Figure 4A). In another set of experiments, ACTH-dependent steroid output raised after gene silencing using a HO-1 directed siRNA (Figure 4B), and overexpression of HO-1 resulted in a significant inhibition of steroid production (Figure 4C).

Antioxidant treatment prevented the effects of HG on HO-1 protein levels (Figure 5A) and on basal steroid production (Figure 5B), but failed to modify the exaggerated response to ACTH induced by HG in Y1 cells (Figure 5C).

Nitrite levels, measured in the incubation media of Y1 cells in order to assess the effects of HG on NOS activity, were significantly decreased by this treatment (Figure 6A). A reduced nitrite production could result from changes in the activity of L-arginine transport systems. In this respect, our results show that L-arginine uptake into Y1 cells was inhibited by HG treatment (Figure 6B).

Discussion

The main findings of our study include the following: first, steroid production by murine adrenocortical Y1 cells (basal and ACTH-stimulated) is altered by a high glucose (HG) environment. Second, oxidative stress generated by this treatment triggers the induction of different antioxidant enzymes, including HO-1. Third, and as previously shown by our group, in addition to its well known antioxidant function, HO activity is involved in the modulation of basal and stimulated steroid production. Changes in medium osmolarity were not responsible for the observed effects of HG, since these parameters were not altered in cells exposed to high concentrations of L-glucose.

In spite of their tumoral origin, Y1 cells behave like normal steroidogenic cells in many aspects, including the stimulation of steroid production by ACTH [22, 28]; as far as the effects of HG on steroid production are concerned, our results obtained in primary rat adrenal cell cultures support the use of this cell line as a model of an adrenocortical cell system.

HG treatment significantly inhibited basal steroid output in Y1 cells. Similarly, significant decreases in progesterone release by trophoblastic cells [29], and in oestradiol and progesterone production by rat primary granulosa cells [30] have been described after exposing these cells to elevated glucose concentrations. Thus, acting by still unknown mechanisms, HG seems to affect hormone biosynthetic pathways in several steroidogenic tissues. As to the effects of prolonged hyperglycemia (e.g., resulting from inducing diabetes in rats by means of STZ treatment) on steroid production, a reduced basal secretion of aldosterone and corticosterone was observed in isolated glomerulosa cells obtained from these animals [31], while a lowering in the

plasma corticosterone levels was detected in STZ-treated rats in which the HPA axis had been interrupted [20].

Incubation of Y1 cells in the presence of HG for 7 days resulted in oxidative stress, increasing ROS generation and lipid peroxidation, and decreasing reduced GSH levels. In fact, it has been suggested that depletion of reduced GSH, considered a main antioxidant molecule, predisposes cells to oxidant-induced damage [32]. Similar results were obtained in mesangial and renal tubular epithelium cells, in retinal endothelial cells and pericytes, and in intestinal epithelial cells [33-35] exposed to HG environment.

We propose that, in Y1 cells, oxidative stress generated by HG treatment negatively affects basal steroid production, as antioxidant treatment prevented this effect. In this sense, the inhibition of steroid production by hydrogen peroxide or a superoxide generator in Y1 cells [36] supports this hypothesis.

Oxidative stress originated by HG treatment triggered the stimulation of the cellular antioxidant defense system. Our results show that incubation of Y1 adrenal cells in HG increases the mRNA levels of SOD isozymes, catalase and GSH-Px. A wide variety of changes in the levels of antioxidant enzymes have been described in other cell types (such as vascular smooth muscle cells, retinal epithelial cells, among others) under HG treatment [32, 37].

The activity of HO and, in particular, the induction of HO-1 have been associated with the cytoprotective response against oxidative stress in different cellular systems [38-41]. More specifically, a decrease in several oxidative stress parameters has been detected after the induction of HO-1 by ACTH in Y1 cells [11]. Present experiments show that incubation of Y1 cells in HG medium also results in increased HO-1 but not HO-2 levels. The fact that antioxidant treatment prevents the increase in

HO-1 levels strongly suggests a causative relation between oxidative stress and HO-1 induction, as demonstrated previously [7, 42].

Interestingly, ACTH stimulation of HG-treated cells resulted in higher levels of steroid production (compared to ACTH + NG treatment). The mechanism involved deserves further investigation, although it seems likely that this effect is exerted downstream cAMP generation, as no significant differences in cAMP levels were detected in HG treated cells. We have previously demonstrated that steroid production is negatively modulated by NO [13]; accordingly, a decrease in NO generation exerted by HG in Y1 cells could contribute to a higher ACTH-dependent steroid output. In this sense, reduced L-arginine uptake and lower nitrite levels were detected in HG treated cells. Other authors, as well, have demonstrated an inhibitory effect of HG treatment on NOS activity [43, 44]. According to our results, the effects of HG on ACTH-stimulated steroid production were not prevented by antioxidant treatment, suggesting that oxidative stress-dependent and -independent mechanisms are triggered by HG treatment of Y1 cells. In agreement to our results, Trachtman and coworkers showed that the reduction in NOS activity induced by HG could not be prevented by α-tocopherol treatment[44].

Although ACTH is a key regulator of adrenal steroidogenesis, multiple experimental evidences indicate that different molecules are able to modulate steroid production in autocrine/paracrine ways [11, 13]. In fact, we have previously suggested that CO, resulting from HO activity, modulates basal and ACTH-stimulated steroid synthesis [11, 45]. Present results lead us to hypothesize that steroid production by Y1 cells is negatively regulated by HO activity, as steroid output was significantly increased after inhibiting HO activity (both by pharmacologic blockade and gene silencing). Correspondingly, overexpression of hHO-1 in Y1 cells resulted in decreased

steroid production, further supporting this hypothesis. The quantitatively lesser response obtained using this molecular approach could be attributed to a low (~20%) efficiency of transfection, and/or to the fact that HO-2 activity is also involved in the modulation of steroid production in these cells.

The causative mechanisms by which elevated glucose concentrations increase HO-1 levels in Y1 cells remain to be elucidated. Glucose-induced increases in HO-1 expression have been described in endothelial cells [9, 46], as well as in pancreatic islets [41]. However, and in contrast to the latter studies, our results indicate that in Y1 cells the cAMP signaling pathway might not be involved in the upregulation of HO-1 by HG, as this treatment did not modify cAMP levels. At present, studies are in progress in our laboratory using promoter analysis techniques to evaluate the effect of elevated glucose concentrations on HO-1 gene expression.

As to the link between HO activation and decreased steroid production, it has been hypothesized that haeme moieties in CYP steroidogenic enzymes could be a substrate for HO activity; alternatively this effect might be attributed to the interaction of CO with the haeme group of these enzymes [47, 48]. In this sense, inhibition of cytochrome P450 by CO has been demonstrated [47]. Moreover, HO products could inhibit gene transcription, as was demonstrated for bilirubin [49].

Elevated circulating glucocorticoid levels have been described in patients with type 1 and type 2 diabetes mellitus [15-18], and in STZ-diabetic rats [19]. In reference to this subject, Chan et al. suggested that while hyperactivation of the HPA axis in both human and experimental diabetes could be mainly attributed to an increase in central drive arising from a decrease in negative feedback sensitivity, impaired HPA responsiveness to stress would involve a decrease in sensitivity of the adrenal cortex [19]. Our experiments show that incubation in HG of either Y1 cells or primary cultures

of rat adrenal cells does not result in a decreased response to ACTH, suggesting that hyperglycemia alone does not account for this effect, and that additional regulatory mechanisms should be taken into account.

In conclusion, present results demonstrate the involvement of HO activity in the modulation of glucocorticoid production by adrenal cells incubated with HG. The incubation of adrenocortical Y1 cells in the presence of high glucose concentrations modifies basal and ACTH-stimulated steroid production, and triggers oxidative stress resulting in the activation of antioxidant defense mechanisms. Results obtained by means of the pharmacologic and molecular manipulation of the HO system lead us to propose HO activity both as an antioxidant enzyme and as a regulator of adrenal steroidogenesis as well. Negative modulation of adrenal steroidogenesis by HO activation could contribute to restrain ACTH-driven glucocorticoid hypersecretion in poorly controlled patients with diabetes mellitus.

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Legends for the figures

Figure 1: Effect of HG on basal and ACTH-dependent steroid production. Y1 cells were incubated in Ham's F10 media containing 5.5 mmol/l D-glucose (NG), 30 mmol/l D-glucose (HG) or 5.5 mmol/l D-glucose plus 24.5 mmol/l L-glucose (LG) for 7 days. The medium was renewed and the cells were further incubated with no additions (A) or in the presence of 10 mIU/ml ACTH for 3 hours (B). Primary cultures of rat zona fasciculata cells, obtained as described in Materials and Methods, were incubated for 4 days in NG, LG or HG. After renewing the medium, cells were further incubated with no additions (C) or with 10 mIU/ml ACTH for 2 hours (D). Steroid production was assessed by RIA. Data represent mean ± S

EM, n=6. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. NG by ANOVA followed by Tukey´s *post hoc* test.

Figure 2: Oxidative stress is generated by HG treatment of Y1 cells. Y1 cells were incubated in Ham's F10 medium with NG, LG or HG for 7 days. ROS generation (A) TBARS levels (B) and reduced GSH levels (C) were determined as described in Material and Methods. Values represent mean ± SEM, n=6. $^{**}p < 0.01$, $^{***}p < 0.001$ vs. NG by ANOVA followed by Tukey´s *post hoc* test.

Figure 3: Effect of HG treatment on endogenous antioxidant mechanisms. Y1 cells were incubated in Ham's F10 medium with NG, LG or HG for 7 days. Semiquantitative RT-PCR was performed on 2 µg total RNA as described in Materials and Methods to detect both isoforms of superoxide dismutase (Cu-SOD and Mn-SOD), catalase and GPx (A) and HO-1 (B). The expression levels of 18S rRNA were determined as internal

controls. A representative western blot of HO-1 and Actin proteins obtained from Y1 cells subjected to NG, HG and LG for 7 days is shown (C).

Figure 4: Effect of HO activity on steroid production by Y1 cells incubated in HG. *Panel A:* Cells were incubated in Ham's F10 medium with NG or HG for 7 days. Thereafter the medium was replaced with fresh medium with or without 30 μmol/l Sn-PPIX and incubated for another 3 hours with no additions or with 10 mIU/ml ACTH. *Panel B*: Cells were transfected with HO-1 or control (CTRL) siRNAs and incubated in medium for 48 h. Thereafter the medium was replaced with fresh medium alone or with 10 mIU/ml ACTH and incubation was continued for another 5 h. Total cell proteins were isolated and analyzed by western blotting (10 μg/lane), as described in Materials and Methods, to detect HO-1 and Actin signals. *Panel C:* Y1 cells were transfected with the expression plasmid pCDNA3 (hHO-1 or β-Gal) and grown for 48 h as described in Materials and Methods. The medium was renewed and the cells were further incubated with no additions or with 10mUI/ml ACTH for 3 hours. Semiquantitative RT-PCR was performed on 2 μg total RNA to detect hHO-1 mRNA and 18S rRNA as an internal control. Steroid concentration was determined in the incubation media by RIA. Data are mean ± SEM (n=6). ^{a} $p < 0.01$ and ^{b} $p < 0.001$ vs. NG; ^{c} $p < 0.05$ and ^{d} $p < 0.001$ vs HG, ^{e} $p < 0.001$ vs NG-SnPPIX (ANOVA followed by Tukey´s *post hoc* test); * $p < 0.05$, *** $p < 0.001$ vs. CTRL siRNA or β-Gal by Student´s t test.

Figure 5: Effect of antioxidant treatment on HO-1 expression levels and steroid production by Y1 cells incubated in HG. Cells were incubated in Ham's F10 medium with NG, LG or HG with or without 100 μmol/l α-tocopherol (α-T) for 7 days. A

representative western blot for HO-1 and Actin proteins (10 μg/lane) is shown. The densitometric analysis shows the relative abundance of the HO-1 protein compared to Actin (A). After 7 days, the medium was replaced with fresh medium with no additions or with 10 mIU/ml ACTH and incubations were continued for 2 hours (B). Steroid concentration was determined in the incubation media by RIA. Data represent mean ± SEM, n=6. $^{\blacklozenge}$ $p < 0.05$ vs NG; ** $p < 0.01$ and *** $p < 0.01$ vs. LG; $^{\#\#\#}$ $p<0.001$ vs LG-α-T by ANOVA followed by Tukey´s *post hoc* test.

Figure 6: Effect of HG treatment on nitric oxide synthase activity. Y1 cells were incubated in Ham's F10 medium with NG, LG or HG for 7 days. The medium was replaced with fresh medium and the incubations were continued for 5 h. Nitrite levels in the incubation medium were determined with the Griess reactive (A) and L-[2,3,4-^{3}H]arginine transport was determined as described in Materials and Methods (B). Values represent mean ± SEM, n=6. * $p<0.05$ and ** $p<0.01$ vs. NG by ANOVA followed by Tukey´s *post hoc* test.