RESPONSE OF STEROID HORMONE-PRODUCING CELLS ON HORMONAL INFLUENCES

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ABSTRACT. In the present work we demonstrate our summarized data about the influence of various hormones on the morphology and steroidogenic enzyme activity of the steroid hormone producing cells in the rat adrenal cortex and testis, by in vivo and in vitro conditions. The influence of adrenocorticotropic hormone (ACTH), dexamethasone, oxytocyn and progesterone on the adrenal cortex cells and the Leydig cells in testis was carried out. For the purpose we used pregnant Wistar rats and sexually mature male Wistar rats divided into two groups: 1. animals treated with hormones in vivo and 2. control rats injected only with saline solution. Simultaneously, for the in vitro investigation adrenal glands and testes of newborn and mature male Wistar rats were removed under sterile conditions. Fragments of the adrenal cortex and testes were cultivated in diffusion chambers with vitelin membranes for 5 days. In the culture media were placed the corresponding hormones in concentration 1/100 of the dose for adult animals. Cultures with hormone-free medium were used as controls. Adrenal glands and testes of prenatal treated with hormones newborn and mature male Wistar rats and tissue cultures from the in vitro study were removed and examined by routine histological analysis and by enzymehistochemistry. Part of the material was fixed in glutaraldehyde and was embedded in Durcopan for examination with electronic microscope. The changes in the morphology and the activity of enzymes associated with the steroidogenesis in the rat adrenal cortex cells and the Leydig cells and those in the cultures were compared. The results showed different influence of the hormones under study by in vivo and in vitro conditions.

KEY WORDS. adrenal cortex, testis, oxytocin, dexamethasone, progesterone, ACTH
INTRODUCTION
The Leydig cells (LC) in the testis and the adrenal cortex cells (AC) are the main source of testosterone and corticosteroids. Their functional activity is under the control of the luteinizing hormone (LH) and ACTH which acts on steroidogenic enzymes in the LC and AC through stimulated secretion of cAMP. Recently, there is a lot of evidence suggesting that apart from the pituitary gonadotropins other circulating hormones are also involved in the modulating the functional activity of LC in the testis (Hedger et al., 1994). Glucocorticoids, essential for the biological response of different organs and systems, bind to their receptors on LC and exert regulatory effect on key enzymes involved in the steroidogenesis (Weber et al., 2000; Lee et al., 1999). The effect of prolonged dexamethasone administration to pregnant rats on the structure and function of adrenal gland have been investigated especially by stereological and ultrastructural methods but data about its influence on the AC enzyme activity are still insufficient (Hristic M et al., 1997).

The enzyme 3β hydroxysteroid dehydrogenase (Δ^5 3βHSDH) catalyzes an essential step in the biosynthesis of all steroid hormones that requires the reduced form of nicotinamide adenine dinucleotide (NAD) as a cofactor (O’Shaughnessy and Murphy, 1991). Little is known about the effect of glucocorticoids on the Δ^5 3βHSDH activity in rat testis and adrenal cortex in context with the changes in enzymatic activities of NADH2-cytochrome-C-reductase and glucose-6-phosphat dehydrogenase, both enzymes involved in NAD synthesis as a cofactor.

It is now well recognized that neuropeptide oxytocin appears to be both an autocrine and endocrine factor. The intratesticular regulatory role of oxytocin has recently aroused considerable interest. Oxytocin is localized to the testis of several species where it is demonstrated to play an important role in steroidogenesis and seminiferous tubule contractility (Frayne et al., 1996, Harris et al., 1998 and Ivell et al., 1997). Oxytocin and oxytocin receptor are present in the mammalian testis, and both are markedly expressed in the LC (Baghate et al., 1994; Einspanier et Ivell, 1997, Whittington et al., 2001, Yeung et al., 1998). The peptide is also produced locally within the testis where it modulates the steroid metabolism and the functional activity of the reproductive tracts (Ivell et al., 1997, Ungefroren et al., 1994). In vitro studies indicate that mature rat LC is capable of synthesizing and secreting oxytocin in response to stimulation with LH (Frayne et al., 1994). There is a growing body of evidence that oxytocin may act as a gonadal hormone affecting the LC steroidogenic activity (Nicholson et Pickering, 1993). Unfortunately, data about the mechanisms via which oxytocin promotes steroidogenesis in LC are still insufficient.

The influence of the oxytocin on the steroid activity of the AC by in vivo and in vitro experimental models is a subject of different studies (Malendowicz L, 1993, Legros J-J, 1995). The immunohistochemical localization of oxytocin is established in the adrenal cortex of various species. As immune staining a highest degree of intensity is observed in the AC of the zona glomeruloza (Nessey S, 1987, Hawthorn J, 1987). In spite of this, there are extremely scarce data about the effect of the neuropeptide oxytocin on the activity of key steroidogenic enzymes in the AC. 
The progesterone is one of the female sexual hormones produced by the cells of the corpus luteum. It has an influence not only on the sexual organs but also on the function of a lot of other organs. Progesterone receptors have been found in a lot of cells (Raza FS, 2001). Braley LM proves that chronic progesterone administration apparently does not directly influence aldosterone secretion, but rather acts indirectly to increase aldosterone by mechanisms similar to sodium restriction. (Braley LM, 1996).

The present study aimed at demonstrating the in vivo effect of ACTH, dexamethasone, progesterone and oxytocin on the activity of key enzymes involved in steroid hormones biosynthesis in rat LC and AC and to establish whether comparable effects occurred under in vitro conditions.

MATERIAL AND METHODS

We studied the influence of adrenocorticotropic hormone (ACTH), dexamethasone (synthetic glucoctocoid hormone), oxytocin (synthetic analog of nonapeptide oxytocin in magnocellular neurons of the hypothalamus) and progesterone (hormone of the corpus luteum) on AC and LC. For the purpose we used pregnant female Wistar rats (n=12) and sexually mature male Wistar rats (n=16) divided into two main groups:

I group – influence in vivo

1. Injection of ACTH by 0.05E/kg. s.c. on the 17th and 19th day of the pregnancy
2. Injection of Dexamethasone by 0.4 mg i.p on the 13th and 14th day of the pregnancy
3. Injection of Progesterone by 10 mg s.c. on the 15th, 17th and 19th day of the pregnancy
4. Injection of Oxytocin of mature male Wistar rats, as follows:
   A) short-term effect of oxytocin- single injection of 0.25IU/100g s.c.
   B) long-term effect- after a 10-day period of injections of 0.25IU/100g. s.c. per day.

As a control for each group pregnant and male Wistar rats injected with saline solution on corresponding terms were used.

II group – influence in vitro

For the in vitro investigation adrenal glands and testes of newborn (n=10) and mature male Wistar rats (n=4) were removed under sterile conditions. Fragments of the adrenal cortex and testes were cultivated in diffusion chambers with vitelin membranes according to Georgiev & Berbenkova, 1977 for 5 days. In the nutrition media (medium 199) were placed the hormones under study, divided into 5 groups:

1. Cultures with media 199 and ACTH – 0.2 E/100ml media
2. Cultures with media 199 and Dexamethasone – 0.004 µg/ml media
3. Cultures with media 199 and Progesterone – 0.05 µkg/1 ml media
4. Cultures with media 199 and Oxytocin – 0.005IE/ml media
5. Controls – cultures with hormone-free medium

Adrenal glands and testes of newborn (n=20) and mature male Wistar rats (n=12) from the I group and tissue cultures from the II group were removed and examined.
by routine histological analysis and by enzymehistochemistry. Fresh cryostat sections (6 µm thick) were stained with hematoxylin-eosin for routine morphological analysis, Sudan III-hematoxylin for lipids and histochemical reactions for the enzymes NADH2- cytochrome-C-reductase, 3β hydroxysteroid dehydrogenase (Δ53βHSDH) and glucose-6-phosphat dehydrogenase. Part of the material was fixed in glutaraldehyde and was embedded in Durcopan for examination with electronic microscope.

RESULTS AND DISCUSSION
The morphological and structural changes in the AC and the LC were followed after treatment in vivo and in vitro. The basic criteria we used to describe this changes are the following:

1. **Morphological** – the microscopic changes in the size, structure and vitality of the examined cells, the quantity and disposition of the lipids

2. **Enzyme-histochemical** - the activity of the enzymes NADH2 cytochrome-C-reductase, Δ53βHSDH and glucose-6-phosphat dehydrogenase which are associated with the processes of steroidogenesis in the adrenal cortex and testis.

3. **Ultramicroscopical** - the changes in the organelles, connected with the steroidogenesis – smooth endoplasmatic reticulum (SER), mitochondria and size, type and number of the lipid droplets

**Testis**

There are several lines of evidence indicating the bidirectional mechanism of action of neuropeptide oxytocin on LC by in vivo and in vitro conditions- stimulatory on basal testosterone accumulation during a short-term exposure and inhibitory after a long-term administration (Frayne et al., 1994, Nicholson et al., 1991, Tahri-Joutei et Pointis, 1988). In the present study we establish that in vivo short-term oxytocin administration in rats results in increasing activity of Δ53βHSDH, which is one of most relevant markers for LC steroidogenic capacity. Related enzymes NADH2-cytochrome-C-reductase and glucose-6-phosphat dehydrogenase are similarly affected. Our findings are consistent with previous data indicating a stimulatory effect of oxytocin on the basal testosterone production in vitro and/or serum testosterone concentration during short-term exposure (Frayne et Nicholson, 1995, Gerendai et Csernus, 1995, Tahri-Joutei et Pointis, 1988). In the present work we found an increasing Δ53βHSDH enzyme activity in the LC following prolonged in vivo oxytocin treatment, with lower staining intensity compared to the group of single injected rats and in vitro study closely supports this finding. Pieces of testicular parenchyma are used to evaluate the effect of oxytocin on rat LC steroidogenesis. By the routine light microscopical analysis, a preserved vitality of the testicular fragments under in vitro conditions was observed. Staining with H-E showed abundant interstitial LC with their specific morphological features- polygonal shape, eosinophilic cytoplasm and euchromatic round eccentric nuclei (not shown). Oxytocin added to culture medium induce rise in 3β HSD enzyme activity in the LC compared to the control cultures without hormone supplementation. Our results are in contrast to previously described reduction in testicular and plasma testosterone levels
following long-term *in vivo* and *in vitro* oxytocin administration (Nicholson et al., 1991, Tahri-Joutei et Pointis, 1988). A possible explanation of this discrepancy is that the lower limits of testosterone production by LC after the long-lasting action of oxytocin is associated with the increase of 5α-reductase activity which converts testosterone to dihydrotestosterone, thereby decreasing the testosterone level (Nicholson et Jenkin, 1995). The results obtained allow suggesting that the low measured levels of testosterone concentration do not exclude a stimulating effect of oxytocin on key steroidogenic enzymes in the LC and are a step towards elucidating the mechanisms of this action. The changes in the histochemical staining for NADH2-cytochrome-C-reductase and glucose-6-phosphat dehydrogenase revealed the same pattern as for 3βHSD which suggest a close relationship between this enzyme and NAD as an important cofactor involved in the steroidogenic pathway (Table 1.)

The substrate specificity and the mode of action of glucocorticoids on steroidogenesis in LC have been an object of extensive *in vivo* and *in vitro* investigations (Page et al., 2001; Gow et al., 2001; Koeva et Popova 1997; Koeva et Popova, 2002). Previous data demonstrated that treatment with cortisol suppresses the plasma testosterone level in sexually mature rats and guinea pigs (Page et al., 2001; Fenske et al., 1997). In cultivated LC glucocorticoids through specific receptors reduce the production of cAMP and inhibit the activity of the key steroidogenic enzymes (Lee et al., 1999, Hales et Payne, 1989; Welsh et al., 1982). Histological analysis in our study showed that prenatal administration of dexamethasone caused a significant decrease of the $\Delta^5\beta$HSDH activity in rat LC compared to the control animals. The enzymehistochemical reaction for NADH2-cytochrome-C-reductase and glucose-6-phosphat dehydrogenase showed the same degree of low intensity as for $\Delta^5\beta$HSDH enzyme activity (Table 1.)

In conclusion, our results demonstrate rise in the steroidogenic enzyme activity in rat LC following *in vivo* and *in vitro* exposures with oxytocin and suggest that neuropeptide oxytocin may act as a local modulator of the testicular functions. Histochemical findings following prenatal administration of dexamethasone in rats demonstrate the suppressive effect of glucocorticoids on LC steroidogenic activity.
Table 1.*

<table>
<thead>
<tr>
<th>rat Leydig Cells</th>
<th>∆(^\text{3})β hydroxysteroid dehydrogenase</th>
<th>NADH(_2) cytochrome-C-reductase</th>
<th>glucose-6-phosphat dehydrogenase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxytocin</td>
<td></td>
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</tr>
<tr>
<td><em>In vivo</em> short-term effect</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td><em>In vivo</em> long-term effect</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td><em>In vitro</em></td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Dexamethasone</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

*compared to the control groups

+++ Strong intensity
++ Moderate intensity
+ Low intensity

Adrenal gland

After treatment with ACTH the AC show signs of strengthened synthesis. The enzyme activity of the cells is significantly increased and this is particularly characteristic for the enzyme ∆\(^\text{5}\)3βHSDH. The lipid droplets in zona fasciculata become less and decrease in quantity. The mitochondria are with dense matrix and tubule-vesicular cristae. Enlarged cisterns of the smooth endoplasmic reticulum are observed. The cultures treated with ACTH survive better and an expressed tendency of differentiation of the cells, especially in the adrenal cortex cultures is observed. The activity of the both studied enzymes connected with the steroidogenesis is increased than that of the control cultures. *(Table. 2)* By electronic microscope is observed an enlargement of the smooth endoplasmic reticulum. The mitochondria are numerous with dense matrix and tubulo-vesicular cristae. ACTH reacts with a specific hormone receptor of the adrenal cell plasma membrane, thereby stimulating adenylate cyclase activity. The resulting rise in cylic adenosine monophosphate (cAMP) increases the synthesis of pregnenolone and adrenocortical hormones *(Jonson, G., E., 1986)*

The effect of dexamethasone on the studied tissues in the newborn rats is based on their influence on the carbohydrates, fats and proteins. Significant changes of the AC are not observed. The quantity of the lipids in the three zones of the adrenal cortex is slightly increased. A change in the activity of ∆\(^\text{5}\)3βHSDH and NADH\(_2\)-diaphorase - enzymes involved in the steroidogenesis is not observed. More significant are the changes on ultramicroscopic level - increase of the smooth endoplasmatic reticulum, increase of the number and size of the lipid droplets and the size of the mitochondria. A preserved or decreased activity is observed in the cultures with dexamethasone. The NADH\(_2\)-diaphorase activity is slightly decreased in the adrenal and proliferated cells. The activity of ∆\(^\text{5}\)3βHSDH is decreased in the zona fasciculate cells. These changes testify for some decrease of the functional activity. *(Table. 2)* Other authors *(Bakker, JM. and al, 1995)* report these changes. The influence of dexamethasone on the cortex cells is on the principle of “feedback”
mechanism. The high level of synthetic glicocorticosteroids in the blood suppresses the ACTH secretion. This leads to a decrease of the synthesis of steroid hormones from the cortical cells (Johnson, GE., 1986). The dexamethasone effect during embryogenesis concerns mainly the differentiation and the process of synthesis of the adrenal cells (Black V. a. G. Russo, 1980; Bakker G. a. E. Schmidt, 1995; Hausman G., 1992). Probably it is not very strong, may be because it is not direct, but via the maternal adrenal gland and placenta.

The continuous treatment of the pregnant rats with high doses of progesterone decreases the enzyme activity of the $\Delta^5\beta$HSDH especially in the cells of zona fasciculata and zona reticularis. A decrease of the quantity of the mitochondria with tubular-vesicular cristae, increase of the lipid droplets and enlargement of the smooth endoplasmic reticulum is stated by means of electronic microscopy. Myelin-like figures appear. A suppression of the reactivity of cells is observed in the cultures with progesterone. The enzyme activity of the $\Delta^5\beta$HSDH is decreased in the cortex cells and is absent in the proliferated ones. Destructive changes in the mitochondria, enlarged smooth endoplasmic reticulum and many liposomes are observed (Table. 2). These changes show that high doses of progesterone have depressive effect on the steroid genesis. In the cultures this effect is better emphasized. (Petrova, E., 2001)

The progesterone appears a precursor to one of the intermediate stages of the steroid hormone synthesis. Additionally entered as an exogenic product, it includes in the following stages, where the normal level of the last secretion products is increased. This effects in reverse oppression of the steroidogenesis (Pelletier G, 2001).

The influence of the oxytocin on the different tissues depends on the way of applying and on the used methods. By the acute experiment practically no changes are observed in the structure and ultrastructure of the AC. A slight increase of the enzyme activity, especially for $\Delta^5\beta$HSDH is established. Our findings correspond to those quantitative changes of the secreted corticosteron described by other authors (Stachowiak et al. 1995). By the prolonged treatment with oxytocin are observed expressive morphological and histochemical changes in AC. The size of the cells is decreased and the distances between them are increased. Blood sinuses are enlarged. The sizes of the zones are decreased, especially those of zona fasciculata. The enzyme activity is decreased mainly for $\Delta^5\beta$HSDH. The enzymes glucolose-6-phosphat dehydrogenase, NADH$_2$ taking part indirectly in the steroidogenesis in cells are with lower activity. The cells in single sectors of zona fasciculata are with preserved normal enzyme activity for NADH$_2$. A great quantity of lipid droplets and vacuolated smooth endoplasmic reticulum are observed by electron microscope. The mitochondria are with light matrix and reduced quantity of the tubule-vesicular cristae. (Table. 2) The obtained results correspond with the established stimulating effect (in vivo and in vitro) of the oxytocin on the adrenocortical steroid production, namely the aldosterone secretion. (Stachowiak A.et al.1995, Hinson J,1987). The increased activity of key steroidogenic enzymes after in vivo and in vitro treatment with oxytocin is identified also in other steroid producing cells, as LC in testis and granulosa cells in ovary. (11-13). The decrease of the enzyme activity for $\Delta^5\beta$HSDH established by us predominantly in the zona fasciculata and zona glomeruloza cells, is
correlated with the low level of basic corticosterone secretion after a prolonged in vitro treatment with oxytocin (Stachowiak et al. 1995)

Our results show that the effect of the oxytocin on the cortex cells depends on the experimental model as well as on the prolongation of treatment. By single injection the oxytocin has direct stimulating effect and by continuous treatment the effect is inhibitory. This is probably due to the intracellular interference of the oxytocin and of the ACTH secreted in the organism.

Table 2

<table>
<thead>
<tr>
<th>REACTION</th>
<th>ADRENAL CORTEX</th>
<th>NADH₂ – diaphorase</th>
<th>Δ³βHSDH</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>In vivo control</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>intact</td>
<td>z. glomerulosa</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>z. fasciculata</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td></td>
<td>z. reticularis</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td><em>In vivo ACTH</em></td>
<td>z. glomerulosa</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>z. fasciculata</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td></td>
<td>z. reticularis</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td><em>In vivo dexamethasone</em></td>
<td>z. glomerulosa</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>z. fasciculata</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>z. reticularis</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>In vivo progesterone</em></td>
<td>z. glomerulosa</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>z. fasciculata</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>z. reticularis</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>In vivo oxytocine</em></td>
<td>z. glomerulosa</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>long-term effect</td>
<td>z. fasciculata</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>z. reticularis</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>CULTURI – IN VITRO control</td>
<td>Cells in the fragment</td>
<td>+++</td>
<td>+++ in the periphery</td>
</tr>
<tr>
<td></td>
<td>Cells proliferate</td>
<td>+++</td>
<td>0</td>
</tr>
<tr>
<td>CULTURI – IN VITRO ACTH</td>
<td>Cells in the fragment</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td></td>
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</tr>
<tr>
<td></td>
<td>Cells proliferate</td>
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<td>0</td>
</tr>
</tbody>
</table>

+++ Strong intensity
++ Moderate intensity
+ Low intensity
The present results show that the effect of the action of the studied hormones appears in different ways according to the experimental conditions (in vivo and in vitro). By in vivo experiments the influence of the hormones is combined with the overall reaction of the organism. By in vitro experiments the hormones have direct influence on the cells.

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