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REGULATION OF THE BIOSYNTHESIS OF STEROIDOGENIC ENZYMES

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Summary—Recombinant DNA technology can permit study of the regulation of steroid hydroxylase gene expression at three levels. The first of these is cAMP-regulated gene expression. In the adrenal, ACTH, via cAMP, increases the expression of the genes for all of the cytochrome P-450 species involved in the steroid biosynthetic pathway, as well as the iron–sulfur protein, adrenodoxin. This action of cAMP is inhibited by cycloheximide, suggestive of the involvement of a regulatory protein factor in mediating this action of cAMP. The second level is tissue-specific regulation of steroid hydroxylase gene expression. An example of this which we have studied is the expression of cholesterol side-chain cleavage cytochrome P-450 (P-450$_{scc}$) and 17α-hydroxylase cytochrome P-450 (P-450$_{17\alpha}$) in the bovine ovary. P-450$_{scc}$ is expressed at high levels in the corpus luteum but at low levels in follicles, whereas P-450$_{17\alpha}$ is expressed in follicles, but is undetectable in the corpus luteum. The third level is fetal imprinting. A number of the cytochrome P-450 species involving in the steroidogenic pathway are expressed in the fetal adrenal at a time when exposure of the gland to ACTH is very low, suggestive that factor(s) other than pituitary ACTH mediate this expression in fetal life.

INTRODUCTION

The regulation of steroid hydroxylase gene expression is a multifaceted biological phenomenon. Our present knowledge permits evaluation of this process at three distinct levels. The best understood of these is the cAMP-dependent regulation which functions to maintain optimal steroidogenic capacity in the adrenal cortex, ovary and testis throughout life. Tissue-specific regulation of steroid hydroxylase gene expression is the second level of regulation, leading to occurrence of different steroidogenic pathways in different tissues. Also, it is becoming clear that these genes are expressed in fetal life in a cAMP-independent fashion which represents the third level of expression which can be classified as fetal imprinting. It is the purpose of this article to review, in general terms, our present understanding of steroid hydroxylase gene expression at these three levels.

cAMP-DEPENDENT REGULATION

The adrenal cortex manifests two discrete responses to ACTH which can be separated on a temporal basis. The acute response to ACTH occurs rapidly, within seconds or minutes, and results in increased steroidogenesis [1]. This action of ACTH is mediated by cAMP and involves the mobilization of cholesterol from its storage sites (lipid droplets) to the inner mitochondria membrane in the vicinity of cholesterol side-chain cleavage cytochrome P-450 (P-450$_{scc}$). The chronic action of ACTH occurs over a time frame of several hours and is required for the maintenance of optimal steroidogenic capacity in the adrenal cortex [2]. The chronic action of ACTH is also mediated by cAMP and is exerted in large part at the level of steroid hydroxylase gene expression. It is important to note at the outset that the chronic action of ACTH has not been studied independently of the acute action of ACTH. Since both processes are mediated by cAMP, all studies to date on the chronic action of ACTH have been carried out in the presence of the acute response.

Evidence for the chronic action of ACTH was provided initially by studies carried out by Kimura[3] and Purvis *et al.*[4] utilizing hypophysectomized rats. Following hypophysectomy, steroid hydroxylase activities diminished but could be restored by administration of ACTH. Subsequent development of stable adrenocortical cell cultures and antibodies specific for steroid hydroxylases and related enzymes have permitted more detailed investigation of the mechanism by which ACTH regulates steroidogenic capacity. When bovine adrenocortical cells are cultured for 5–6 days, until confluence is achieved, the levels of steroid hydroxylases fall to their constitutive, basal levels [5–7]. Only 17α-hydroxylase (P-450$_{17\alpha}$) is reduced to undetectable levels under these conditions [8]. When such cells are then treated with ACTH (or dibutyryl cAMP), dramatic increases in the activities and protein levels of steroid hydroxylases and related enzymes takes place. Optimal levels of these enzymes are attained between 48 and 96 h after initiation of treatment. In the bovine adrenocortical cell culture system, these increases in steroid hydroxylase levels are between 5- and 10-fold (even greater for P-450$_{17\alpha}$). However, we believe that such large changes in the levels of

these enzymes do not occur *in vivo* and that the cell culture experiments greatly magnify *in vivo* events. Rather, we imagine that cAMP levels are such that they maintain a rather constant level of these enzymes, thereby maintaining optimal steroidogenic capacity in the adrenal cortex.

Temporal studies of the regulation of steroid hydroxylase levels in response to ACTH or cAMP treatment revealed that the rates of synthesis of P-450$_{scc}$ [5], P-450$_{17\alpha}$ [21], P-450$_{C21}$ [9], P-450$_{11\beta}$ [7], adrenodoxin reductase [10], adrenodoxin [6] and NADPH–cytochrome P-450 reductase [11] were increased several-fold by such treatments. Furthermore, in each case the rate of synthesis was associated with an increased level of translatable RNA. Among the possible explanations for this action of cAMP, two appeared most plausible. In one case, cAMP could be enhancing the rate of transcription of the genes encoding these enzymes and in the other case it could be increasing the stability of their messenger RNAs.

In order to investigate these possibilities it was necessary to isolate and clone complementary DNA sequences specific for the mRNA species encoding the enzymes. Once this was achieved these cDNA clones could be radiolabeled by nick translation and used as probes to determine the levels of mRNA species specific for these enzymes, following Northern blotting and hybridization of the radiolabeled probe to the RNA present on nitrocellulose filters. When this was done utilizing RNA isolated from bovine adrenocortical cells in culture it was found that both ACTH and cAMP analogues increased the levels of mRNA species specific for P-450$_{scc}$ [12], P-450$_{17\alpha}$ [13], P-450$_{11\beta}$ [14], P-450$_{C21}$ [15], as well as the iron–sulphur protein adrenodoxin [16]. These results are indicative that the actions of ACTH and cAMP are to increase the levels of the mRNA species themselves and are not due to an increase in efficiency of translation.

Having determined that the increase in the synthesis of these enzymes in response to ACTH and cAMP analogues was the result of changes in the levels of mRNA encoding these enzymes, the next step was to establish whether the increase in levels of mRNA was due to an increase in the rate of transcription of the genes specific for these enzymes, or due to an increase in the half-life of the mRNA species, or both of these. In order to answer the first question, nuclei were isolated from bovine adrenocortical cells maintained in the presence or absence of ACTH and the elongation of already initiated messenger RNA species was carried out in the presence of a radiolabeled nucleotide [17]. The RNA was isolated from these incubations and hybridized to the cDNA probes annealed to nitrocellulose filters. To answer the second question, the half-lives of the RNA species were determined following radiolabeling of the intact cells with [^{3}H]uridine and hybridization of this radiolabeled RNA to the cDNA probes. From such studies it was found that ACTH and cAMP analogues increased the transcription of the genes for all of the enzymes studied. On the other hand, ACTH had no effect on the half-lives of the mRNA species except in the case of P-450$_{scc}$ mRNA, where the half-life was increased 4–5-fold by ACTH (unpublished observations).

Following the conclusion that a major action of cAMP on the regulation of steroid hormone biosynthesis was to increase the transcription of the genes encoding these enzymes, it became necessary to ask whether this action was direct or whether it was mediated by other factors, some of which could be proteins. In order to address this issue the effect of cAMP analogues on the increase in mRNA species for these enzymes was determined in cultured adrenocortical cells in the presence or absence of an inhibitor of protein synthesis, namely cycloheximide [13, 17]. It was found that cycloheximide blocks the action of cAMP to increase the levels of mRNA species encoding each of these enzymes, indicative that this action of cAMP was indirect and was mediated by some labile protein factor (Fig. 1). It should be pointed out that this action of cAMP to regulate gene expression in the adrenal cortex is relatively specific for the enzymes directly involved in the steroidogenic pathway, as well as those responsible for ensuring a continuous supply of cholesterol to the steroidogenic apparatus, namely HMGCoA reductase and the LDL receptor. The activities of numerous other enzymes such as those involved in glycolysis, the Krebs acid cycle and the pentose phosphate shunt are not influenced by ACTH in these cells (unpublished observations).

The next step towards understanding of the mechanisms whereby ACTH and cAMP regulate the expression of these genes will be to isolate and characterize these genes, including the sequences in the untranscribed regions, and to isolate and characterize the proteins which bind to these regions. It will then be necessary to work backwards from these proteins to cAMP in order to completely elucidate the mechanism of the chronic action of ACTH.

TISSUE-SPECIFIC EXPRESSION

The second level of regulation of steroidogenic capacity is tissue-specific expression of steroid hydroxylase genes. A well-known example of this type of regulation is the expression of P-450$_{C21}$ and P-450$_{11\beta}$ genes solely in the adrenal cortex and not in other steroidogenic tissues. We have begun to address this issue by comparing the expression of the various steroidogenic genes in the bovine ovary throughout the normal ovarian cycle. By means of Western blot analysis, it has been determined that the levels of expression of P-450$_{scc}$, adrenodoxin, the LDL receptor and HMGCoA reductase are modest in ovaries during the follicular phase of the cycle, but following ovulation the content of each of these enzymes in the developing corpus luteum increases dramatically (Fig. 2; Ref. [18]). The contents of

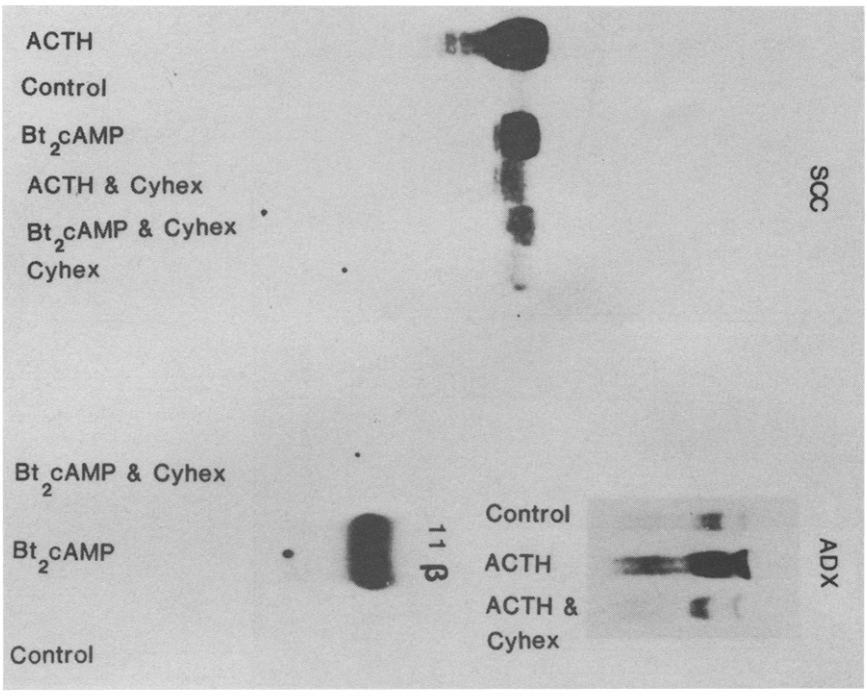

Fig. 1. Inhibition of ACTH- and cAMP-mediated RNA accumulation by cycloheximide (cyhex). Bovine adrenocortical cells were treated with cycloheximide (10 μg/ml) with or without ACTH (1 μM) or dibutyryl cAMP (1 mM) for 24 h. Total RNA was analyzed for mRNA encoding P-450$_{scc}$ (SCC), adrenodoxin (Adx) or P-450$_{11\beta}$ (11β) by Northern blot analysis using cDNA inserts specific for these

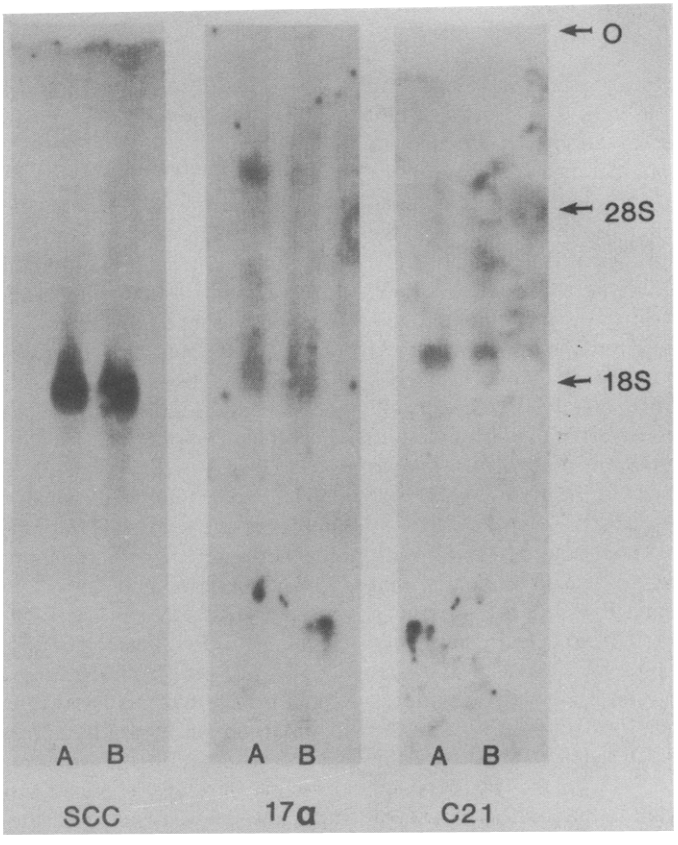

Fig. 3. Expression of steroid hydroxylase RNA in normal and anencephalic human fetal adrenals. Total RNA (50 μg) from normal fetal adrenals (Lanes B) and anencephalic adrenals (Lanes A) was size-fractionated on an agarose–formamide gel and subjected to Northern analysis using cDNA inserts specific for cytochromes P-450$_{scc}$ (SCC), P-450$_{17\alpha}$ (17α) and P-450$_{C21}$ (C21).

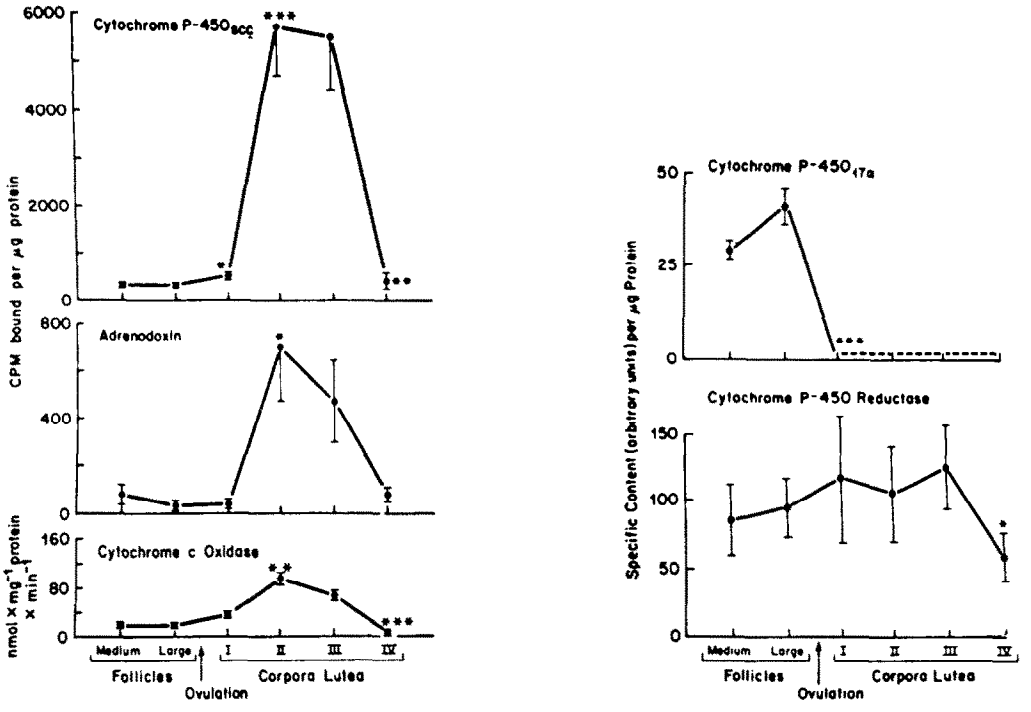

Fig. 2. Specific contents of cytochrome P-450$_{scc}$ and adrenodoxin, and the specific activity of cytochrome oxidase (left panel), and the specific contents of cytochrome P-450$_{17\alpha}$ and NADPH-cytochrome P-450 reductase (right panel) in follicles and corpora lutea at sequential stages of development throughout the bovine ovarian cycle. Corpora lutea were staged as early (I), early-mid (II), late-mid (III) and late (IV) according to the criteria of Ireland *et al.*: *J. anim. Sci.* **49** (1979) 1261–1268.

mRNA species specific for these enzymes increase in corresponding fashion as judged by Northern blot analysis (unpublished observations). Maximal levels are obtained at the midpoint of the luteal stage of the cycle, but following the onset of luteolysis, there is an equally precipitous decline in the levels of both the enzymes themselves and their mRNA species. The changes in the levels of these enzymes parallel corresponding changes in the secretion of progesterone by the ovary throughout the cycle. In marked contrast are the levels of P-450$_{17\alpha}$ and its mRNA species which are readily detectable in follicles, but decrease to undetectable levels following ovulation, and remain undetectable in the corpus luteum throughout the luteal phase of the cycle (Fig. 2; Ref. [18]).

These observations are supported by immunofluorescence studies which indicate that whereas both P-450$_{scc}$ and P-450$_{17\alpha}$ are present in cells of the theca interna of the follicle, in the corpus luteum only P-450$_{scc}$ is present, whereas P-450$_{17\alpha}$ is undetectable. This is also consistent with the patterns of androgen and estrogen formation throughout the ovarian cycle, and indicates that in the ovary the synthesis of P-450$_{scc}$ and P-450$_{17\alpha}$ may be regulated in opposite directions while in the zona fasciculata of the adrenal cortex, they appear always to be regulated in the same direction. Thus, tissue-specific factors are present in the ovary which regulate expression of these genes in a differential fashion at different times throughout the ovarian cycle [19, 20].

cAMP-INDEPENDENT REGULATION

The third level of regulation of steroidogenic capacity relates to the expression of these genes in fetal life in a trophic hormone-independent fashion. This concept stems from studies in which a comparison was made of the levels of the steroidogenic enzymes and their mRNA species in normal human fetal adrenal tissue and in adrenal tissue from anencephalic fetuses. Such fetuses have a non-functional hypothalamic–pituitary axis, the plasma levels of ACTH are low [21], and the levels of adenylate cyclase in the fetal adrenal are extremely low and are unresponsive to ACTH (unpublished observations). Furthermore, these adrenals have lower levels of LDL receptors and lower levels of HMGCoA reductase [22]. Nonetheless, it was found that the levels of mRNA specific for P-450$_{scc}$, P-450$_{17\alpha}$, P-450$_{C21}$, P-450$_{11\beta}$ and adrenodoxin as determined by Northern blot analysis were similar to those in normal human fetal adrenal tissue (Fig. 3; unpublished observations). Furthermore, the levels of the proteins were also similar to those in normal fetal adrenals. These results indicate that the expression of steroid hydroxylase genes occurs at the normal fetal level in anencephalic adrenals despite the chronic lack of stimulation by ACTH.

To investigate this issue further it was necessary to study the ontogeny of the expression of these genes in fetal adrenal throughout gestation. Such a study is being performed utilizing fetal sheep adrenals. In the fetal sheep adrenal, cortisol secretion commences around day 120 due to an increase in ACTH secretion by the fetal pituitary. Prior to that time the fetal adrenal is quiescent. However, when we determined the levels of these steroidogenic enzymes in adrenals of fetal sheep of various gestational ages, once again it was observed that the levels in adrenals of fetuses prior to 120 days gestation were similar to those following 120 days gestation with the exception of P-450$_{17\alpha}$, the levels of which were lower in younger fetuses, and increased following the rise in fetal plasma ACTH levels towards the end of gestation (unpublished observations).

We conclude from these studies that, in both the human and the sheep during early fetal life, factors other than pituitary ACTH maintain the expression of these steroidogenic genes at a high level, and that during maturation of the developmental process, this function is taken over by ACTH (cAMP) itself.

CONCLUSIONS

In summary, we hypothesize that at least three classes of proteins regulate steroid hydroxylase gene expression. One group of proteins is required for cAMP-dependent regulation, and their action is controlled by levels of peptide hormones from the anterior pituitary. A second group of proteins is required for tissue-specific regulation of steroid hydroxylase gene expression. One subset of tissue specific proteins exerts an all-or-none action leading to expression of P-450$_{C21}$ and P-450$_{11\beta}$ exclusively in the adrenal cortex. Another subset of tissue-specific proteins regulates P-450$_{17\alpha}$ expression vs P-450$_{scc}$ expression, depending on the physiological requirements for products of these enzymes. The third group of proteins regulates steroid hydroxylase gene expression in a cAMP-independent fashion leading to fetal imprinting of expression of these genes. Studies designed to identify and characterize these proteins and their interaction with steroid hydroxylase genes will prove very interesting.

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