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18-HYDROXYLATION IN THE Y-1 ADRENAL CELL LINE: RESPONSE TO ACTH AND TO CULTURE CONDITIONS

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Summary—The 18-hydroxylation of deoxycorticosterone in the Y-1 adrenal cell line was studied under various incubation and cell culture conditions and compared to 11β-hydroxylation. Repeated incubation of the substrate increased both 18- and 11β-hydroxylation in the Y-1 cells. Furthermore, both 18- and 11β-hydroxylation were increased with increased serum concentration and prolonged incubation time. While the increase in 11β-hydroxylation seemed to be independent of the type of serum, 18-hydroxylation was much more important in cells cultured in fetal or newborn calf serum supplemented medium than in those cultured in horse serum supplemented medium. As expected, ACTH treatment increased 11β-hydroxylation; however, it decreased 18-hydroxylation. The different regulation of these two hydroxylating pathways by ACTH, point to a heterogeneity of the cytochrome P-450$_{11\beta}$ of the Y-1 cell line.

INTRODUCTION

Since its establishment from a functional mouse adrenocortical tumor [1, 2], the Y-1 cell line has served as a versatile and reliable model for a large number of steroidogenic investigations. Most of the adrenal functions of this cellular system are well known. It possesses the enzymatic activities necessary to transform cholesterol into progesterone and expresses strong 11β-hydroxylase and 20α-reductase activities [3]. However, the Y-1 cell line no longer expresses its original 21-hydroxylase activity [3, 4] and is therefore unable to synthesize corticosterone and aldosterone, the normal corticosteroids secreted by the mouse [5].

Using deoxycorticosterone (DOC), a 21-hydroxylated substrate, we have recently shown the expression of 18-hydroxylase activity in this cell line [6]. The principal compound produced is the 20α-reduced derivative of 18-hydroxycorticosterone. Under the conditions most frequently used for culturing Y-1 cells, 18-hydroxylase activity is generally weak. The present investigation reports cell culture conditions tested in order to increase this activity. The effect of the substrate, the nature and concentration of the serum added to the culture medium, and of ACTH on 18-hydroxylase as well as on 11β-hydroxylase activities, are reported. After comparison of the modulation of these two activities by different factors, it seems possible to suggest that they are catalyzed by different cytochrome P-450s in the Y-1 cell line.

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EXPERIMENTAL

Steroids and reagents

DOC was obtained from Makor chemicals and C_{36} n-alcane from Fluka. Bis(trimethylsilyl)trifluoroacetamide and trimethylchlorosilane were purchased from Supelco and O-methoxyamine hydrochloride from Pierce Chemical Co. All solvents were pure for analysis quality (Merck) or RS HPLC grade (Carlo Erba).

Cell cultures

The Y-1 adrenal cell line (Flow Labs) was routinely grown as described previously [6]. The number of dishes necessary for each experiment was obtained by seeding a cell suspension prepared by trypsination (50 mg/100 ml) of several confluent cell dishes. The medium was renewed every 2 days until the cells reached confluency. For two successive periods of 24 h before the beginning of each experiment, the culture medium was replaced by serum supplemented medium corresponding to that used in the experiment. Experiments were performed with 4.0 ml of Ham's F10 medium supplemented

with the following serum composition: (a) fetal calf serum (FCS) and newborn calf serum (NCS) (1:1, v/v) at various concentrations (1, 5 or 10%), (b) FCS or horse serum (HS) at various concentrations (1, 5, 10 or 20%), and (c) 0.5% FCS completed to 1, 5 or 10% with NCS or HS. The culture flasks which were to receive 5×10^{-9} mol.l^{-1} of ACTH (Choay) during the experiment were treated with the same dose during the 2 days of adaptation to the corresponding serum supplemented medium. Incubations were carried out for 24, 48, and 72 h periods successively, with the substrate added at each incubation period or for the 72 h period only. The steroid precursor, 50, 100, 150, or 200 μg of DOC, corresponding to between 3.3×10^{-5} and 13.2×10^{-5} mol.l^{-1}, was added in 10 μl of ethanol at the beginning of the incubation period.

Steroid extraction and analysis

The extractions were performed using Sep-pak C18 cartridges (Waters Associates) and the extracted steroids were analyzed either by high performance liquid chromatography (HPLC) or by gas chromatography (GC), as described previously [7]. Quantitative measurements were done using DOC acetate (DOCA) as internal standard in HPLC analysis and C_{36} *n*-alkane in GC analysis. The identity of the steroids was verified by gas chromatography-mass spectrometry as described previously [6].

Protein measurement

The method used for protein quantification was that described by Lowry *et al.* [8] using bovine serum albumin (Sigma) as the reference protein.

RESULTS

The amount of 18-hydroxylation was determined by quantification of the following metabolites of DOC: 18-hydroxy-20α-dihydroDOC (18-hydroxy-20α-DHDOC) and 18-hydroxy-20α-dihydrocorticosterone (18-hydroxy-20α-DHB). The amount of 11β-hydroxylation was determined by quantification of the following 11-oxygenated metabolites: corticosterone (B), dehydrocorticosterone (A), 20α-dihydrocorticosterone (20α-DHB), 20α-dihydro-11-dehydrocorticosterone (20α-DHA), and 18-hydroxy-20α-DHB. The 11β,18-dihydroxylated metabolite is counted in both categories.

Effect of the repeated incubation of DOC on its 18- and 11β-hydroxylation

Repeated incubation of DOC for 24, 48, and 72 h increased 18-hydroxylation in comparison to a single 72 h incubation. Table 1 shows the results obtained with different doses of substrate. In fact, 18-hydroxylated metabolites were detected only when the substrate was repeatedly incubated with the cells. While no 18-hydroxylated metabolites were detected with single incubations of substrate, repeated incubation of 50 μg of DOC produced 5.2% (2.6 μg) of 18-hydroxylated metabolites. Repeated incubation of 100 μg of DOC produced 3.1% (3.1 μg) and incubation of 150 μg produced 0.7% (1.1 μg). As shown by this data, 18-hydroxylation was less important with more important concentrations of substrate and seemed to be completely inhibited by 200 μg of DOC, since no 18-hydroxylated metabolites were detected at this dose.

Table 1. Effect of repeated incubations of DOC on its 18- and 11β-hydroxylation by the Y-1 cell line

	DOC (μg/4 ml)							
	50		100		150		200	
18-Hydroxylated metabolites[a]								
24 h	—	ND	—	ND	—	ND	—	ND
48 h	—	4.6	—	1.9	—	ND	—	ND
72 h	ND	5.2	ND	3.1	ND	0.7	ND	ND
11β-Hydroxylated metabolites[a]								
24 h	—	84.7	—	77.5	—	55.9	—	44.6
48 h	—	96.1	—	90.1	—	74.9	—	36.1
72 h	97.4	96.7	92.1	97.1	73.7	95.8	53.3	25.3

Y-1 cells cultured in 5% serum supplemented medium (FCS–NCS; 1:1, v/v) were incubated in 4.0 ml of medium with different concentrations of DOC for 72 h or for 24, 48, and 72 h successively. The 18-hydroxylated metabolites represent the sum of 18-hydroxy-20α-DHDOC and 18-hydroxy-20α-DHB, and the 11β-hydroxylated metabolites the sum of B, A, 20α-DHB, 20α-DHA and 18-hydroxy-20α-DHB.

[a]Metabolites expressed in percent of DOC incubated. Each value is the means of duplicated flasks with two quantifications per flask. The range of variation between quantifications is $< \pm 8\%$.

ND, Metabolites not detected.

—, Change of medium without incubation of substrate.

The stimulation of 11β-hydroxylation by repeated incubation of substrate was also observed (Table 1), but only with the dose of 150 μg of substrate. Repeated incubation of this dose produced 95.8% 11β-hydroxylated metabolites after 72 h compared to 73.2% in the culture with a single incubation. However, repeated incubation of 200 μg of DOC caused a decrease in 11β-hydroxylation, 25.3% in 72 h, in comparison to 53.3% in the culture without repeated incubation. When 50 or 100 μg of DOC were incubated almost all the substrate was exhausted after 48 h of incubation, so that no difference could be seen after 72 h with or without previously repeated incubations.

Effect of the nature of the serum and its concentration in the culture medium on the 18- and 11β-hydroxylation of DOC

Comparison of HS and FCS. The effect of HS and FCS on the 18-hydroxylation of 50 μg of DOC is illustrated in Table 2. Although not detectable after 24 h of incubation in 1% serum supplemented medium, 18-hydroxylation was detected with all other serum concentrations and incubation periods. The amount of 18-hydroxylated metabolites increased with increasing serum concentration and with increasing incubation time. It became important after 48 h of incubation in 10% FCS supplemented medium (14.3%), and reached the highest value (70.4%) after 72 h of incubation in 20% FCS supplemented medium. These values were almost double the corresponding values in HS supplemented medium, showing higher 18-hydroxylase activity in FCS.

The 11β-hydroxylation of 50 μg of DOC was almost complete (Table 2) after 24 h of incubation in cells cultured in 5, 10, and 20% HS supplemented medium (87.8 to 91.3%) and in 10 and 20% FCS supplemented medium (92.1 and 86.1%, respectively). However, in 5% FCS supplemented medium 11β-hydroxylation was complete only after 48 h (89.1%), showing a slightly less active 11β-hydroxylase in this serum. Although 11β-hydroxylation was not complete in 1% serum supplemented medium, whatever the incubation time and the nature of the serum, it was somewhat more important in HS than in FCS supplemented medium. Table 2 shows that in most cases the amount of 11β-hydroxylated metabolites in μg/mg of protein decreased with increasing incubation time and increasing serum concentration. This decrease is due to the depletion of substrate because of its almost complete conversion and to the increase in cell proliferation with increasing serum concentration.

Comparison of HS and NCS. HS and NCS were compared using 100 μg of DOC as substrate as illustrated in Table 3. Under these culture conditions, 18-hydroxylated metabolites were detected only after 48 h of incubation in 4.5 and 9.5% NCS supplemented medium or in 9.5% HS supplemented medium. 18-Hydroxylase activity increased with increasing serum

Table 2. Effect of various concentrations of HS and FCS on the 18- and 11β-hydroxylation of DOC by the Y-1 cell line

	HS (%)				FCS (%)			
	1	5	10	20	1	5	10	20
18-Hydroxylated metabolites[a]								
24 h	ND	0.7	1.8	1.3	ND	0.9	2.7	5.9
		(0.2)	(0.5)	(0.3)		(0.3)	(0.8)	(1.5)
48 h	0.7	3.7	9.4	15.3	1.7	3.9	14.3	32.4
	(0.2)	(1.0)	(1.9)	(2.9)	(0.5)	(0.9)	(3.4)	(6.6)
72 h	2.0	6.7	21.1	35.9	2.5	4.2	33.8	70.4
	(0.6)	(1.5)	(3.2)	(5.1)	(0.7)	(1.1)	(6.5)	(10.8)
11β-Hydroxylated metabolites[a]								
24 h	80.8	91.1	91.3	87.8	67.5	80.6	92.1	86.1
	(24.1)	(25.8)	(23.6)	(22.0)	(20.3)	(22.8)	(25.1)	(22.3)
48 h	80.4	90.6	91.4	93.1	73.4	89.1	90.6	90.3
	(24.6)	(23.4)	(18.3)	(17.7)	(22.3)	(23.1)	(21.2)	(18.3)
72 h	80.0	94.4	94.6	93.7	75.1	90.9	93.4	82.3
	(25.2)	(21.5)	(14.3)	(13.2)	(22.5)	(20.7)	(17.9)	(12.6)

Y-1 cells cultured in medium supplemented with different concentrations of HS or FCS were incubated with 50 μg of DOC in 4.0 ml of medium for 24, 48 and 72 h successively. The 18-hydroxylated metabolites represent the sum of 18-hydroxy-20α-DHDOC and 18-hydroxy-20α-DHB, and the 11β-hydroxylated metabolites the sum of B, A, 20α-DHB, 20α DHA and 18-hydroxy-20α-DHB.

[a]Metabolites expressed in percent of DOC incubated and in μg/mg of protein (values in parentheses). Each value is the mean of duplicated flasks with two quantifications per flask. The range of variation between quantifications is $< \pm 8\%$.

ND, Metabolites not detected.

Table 3. Effect of various concentrations of HS and NCS on the 18- and 11β-hydroxylation of DOC by the Y-1 cell line

	HS (%)			NCS (%)		
	0.5	4.5	9.5	0.5	4.5	9.5
18-Hydroxylated metabolites[a]						
24 h	ND	ND	ND	ND	ND	ND
48 h	ND	ND	3.2 (1.2)	ND	2.2 (1.3)	10.4 (4.3)
72 h	ND	ND	7.9 (3.2)	ND	3.3 (2.0)	15.9 (6.6)
11β-Hydroxylated metabolites[a]						
24 h	62.3 (48.0)	84.5 (46.1)	83.6 (31.3)	69.1 (53.1)	91.7 (50.0)	92.9 (34.8)
48 h	72.7 (57.4)	93.6 (53.0)	93.7 (36.5)	59.0 (46.6)	95.7 (54.2)	96.2 (37.5)
72 h	79.0 (65.8)	91.2 (54.3)	97.1 (40.1)	58.2 (48.5)	95.5 (56.9)	96.9 (40.0)

Y-1 cells cultured in medium supplemented with 0.5% FCS and 0.5, 4.5, and 9.5% HS or NCS were incubated with 100 μg of DOC in 4.0 ml of medium for 24, 48 and 72 h successively. The 18-hydroxylated metabolites represent the sum of 18-hydroxy-20α-DHDOC and 18-hydroxy-20α-DHB, and the 11β-hydroxylated metabolites the sum of B, A, 20α-DHB, 20α DHA and 18-hydroxy-20α-DHB.

[a]Metabolites expressed in percent of DOC incubated and in μg/mg of protein (values in parentheses). Each value is the mean of duplicated flasks with two quantifications per flask. The range of variation between quantifications is $< \pm 8\%$.

ND, Metabolites not detected.

concentration and increasing incubation time. It was favored in NCS, where it reached 10.4 and 15.9% after 48 and 72 h, respectively, compared to 3.2 and 7.9% in HS supplemented medium.

Almost complete 11β-hydroxylation of DOC occurred after 24 h of incubation in cells cultured in 4.5 and 9.5% NCS supplemented medium (91.7 and 92.9%, respectively) but only after 48 h in 4.5 and 9.5% HS supplemented medium (93.6 and 93.7%, respectively), showing a slightly more active 11β-hydroxylase in NCS. However, in 0.5% HS supplemented medium, 11β-hydroxylation was slightly more important than in 0.5% NCS supplemented medium after 48 and 72 h of incubation. In either case, as with FCS and HS (Table 2), 11β-hydroxylation was not complete in 1% serum supplemented medium even after 72 h of incubation. Although the amount of substrate was increased to 100 μg, it was depleted in the incubations with 5 and 10% serum supplemented medium.

Effect of ACTH on the 18- and 11β-hydroxylation of DOC

Table 4, shows the results of experiments using 1, 5, and 10% FCS–NCS (1:1, v/v) supplemented medium with or without ACTH and with 100 μg of DOC as substrate. In control cells, when 18-hydroxylation was detected, it increased with increasing serum concentration and incubation time. However, 18-hydroxylation was markedly decreased by ACTH treatment of the cells. In 5% serum supplemented medium control cells produced 3.5% 18-hydroxylated metabolites after 48 h of incubation and 6.7% after 72 h, but there were no 18-hydroxylated metabolites detected in ACTH treated cells even after 72 h of incubation. 18-Hydroxylation was also decreased by ACTH in 10% serum supplemented medium, 2.7% 18-hydroxylated metabolites in cells treated with ACTH after 48 h of incubation compared to 8.2% in control cells, and 3.4% after 72 h compared to 16.1%.

In these experiments the substrate was not depleted by 11β-hydroxylation until the 72 h incubation. In control cells 11β-hydroxylation also increased with increasing serum concentration and increasing incubation time. As expected, treatment of Y-1 cells with ACTH increased 11β-hydroxylation under all conditions studied, that is, whatever the percentage of serum or the incubation time.

DISCUSSION

The regulation of 18- and 11β-hydroxylase activities in the Y-1 cell line by the substrate, the nature and composition of the serum as well as by ACTH was compared. However, only under particular conditions could both activities be measured correctly. In effect, as shown by the results, 11β-hydroxylation was almost always complete after 24 h of incubation in cells cultured in medium supplemented with at

Table 4. Effect of ACTH on the 18- and 11β-hydroxylation of DOC by the Y-1 cell line in medium supplemented with different concentrations of serum

	Control			ACTH		
FCS/NCS (%):	1	5	10	1	5	10
18-Hydroxylated metabolites[a]						
24 h	ND	ND	ND	ND	ND	ND
48 h	ND	3.5 (1.6)	8.2 (2.7)	ND	ND	2.7 (1.2)
72 h	ND	6.7 (3.1)	16.1 (5.3)	ND	ND	3.4 (1.5)
11β-Hydroxylated metabolites[a]						
24 h	11.9 (10.9)	24.6 (10.7)	48.5 (18.4)	30.6 (31.7)	80.9 (37.9)	85.5 (35.6)
48 h	11.2 (11.2)	39.7 (17.8)	70.8 (23.6)	29.4 (33.9)	90.9 (47.1)	96.1 (39.8)
72 h	14.6 (16.1)	76.7 (35.9)	90.2 (29.7)	26.6 (37.4)	96.1 (53.7)	93.8 (41.9)

Y-1 cells cultured in medium supplemented with different concentrations of FCS and NCS (1:1, v/v) were incubated with 100 μg of DOC in 4.0 ml of medium for 24, 48 and 72 h successively. ACTH treatment consisted of 5×10^{-9} mol.l^{-1} added at the beginning of each incubation period. The 18-hydroxylated metabolites represent the sum of 18-hydroxy-20α-DHDOC and 18-hydroxy-20α-DHB, and the 11β-hydroxylated metabolites the sum of B, A, 20α-DHB, 20α-DHA and 18-hydroxy-20α-DHB.

[a]Metabolites expressed in percent of DOC incubated and in μg/mg of protein (values in parentheses). Each value is the mean of duplicated flasks with two quantifications per flask. The range of variation between quantifications is $< \pm 8\%$.

ND, Metabolites not detected.

least 5% of serum. If the amount of serum in the medium or the incubation time were lowered, 11β-hydroxylation could be measured but 18-hydroxylation was not detectable. An increase in substrate to greater than 100 μg in order to avoid depletion, was not favorable for hydroxylation. For these reasons all experiments were done using several concentrations of serum and incubation times with 50 or 100 μg of substrate.

The repeated incubation of DOC stimulates both 18- and 11β-hydroxylation. The stimulation of 18-hydroxylation is quite evident since this activity was not detectable without repeated incubation of the substrate. The increase in 11β-hydroxylation is seen most clearly with 150 μg of substrate due to the depletion of substrate at lower doses. Therefore, in this cell line, there is stimulation of the activity of cytochrome $P\text{-}450_{11\beta}$ by its substrate, as is generally the case of enzymes belonging to the family of *P*-450 proteins. The hepatic cytochrome *P*-450 system, involving many different *P*-450 enzymes, is characterized by its inductibility by a variety of endogenous and exogenous substrates. A substrate-dependent post-translational stabilization of *P*-450 enzymes, by ligands which appear to protect the enzyme from degradation or inactivation, has been shown to occur *in vitro* [9, 10] as *in vivo* [11]. Another steroidogenic enzyme, cytochrome $P\text{-}450_{scc}$, is also induced by its substrate, cholesterol [12].

The decrease in hydroxylation caused by repeated incubation of 200 μg of DOC could be due to the intercalation of the substrate into the membrane. The stability of the membrane could be thus modified, also altering the topology of the membrane and eventually cell viability. Serum seems to counter this effect; cells grown in serum-free medium lose almost completely their hydroxylating activities after repeated incubation of much lower amounts of substrate (results not shown).

Both 18- and 11β-hydroxylation increase with increasing serum concentration and increasing incubation time. However, while 11β-hydroxylation is stimulated similarly by all three sera, with a slightly higher stimulation at 1% by HS, 18-hydroxylation is much stronger in cells cultured in FCS and NCS. A combination of factors is probably responsible for stimulating differently the two hydroxylating activities. One of them could be the influence of the different constitutive lipids of the sera on the activity of membrane enzymes.

Yasumura *et al.* [2] reported that the Y-1 cell line kept at a high HS concentration retained its steroidogenic activity indefinitely. Since then, this cell line has been routinely grown in culture medium containing at least 15% of HS. Indeed, cells kept in HS supplemented medium grow in a more regular fashion than those kept in FCS or NCS supplemented medium. But, since FCS assures the best cell proliferation and NCS

favors cell attachment, and both are stimulators of cytochrome $P\text{-}450_{11\beta}$ activity, a mixture of both sera at equal proportions seems to be the most appropriate culturing conditions for studying this enzyme's activity.

The prolonged stimulation of 11β-hydroxylase activity in the Y-1 cell line by ACTH was reported by Kowal in 1969 [13]. Other authors have studied the acute steroidogenic response of these cells to ACTH [14–16]. Since it is known that a single cytochrome $P\text{-}450_{11\beta}$ catalyzes both 11β- and 18-hydroxylation, ACTH was expected to have a similar effect on both activities in the Y-1 cell line. Our results show that although ACTH increases 11β-hydroxylation, it decreases 18-hydroxylation in this cell system. Two possible hypothesis exist: either one enzyme, cytochrome $P\text{-}450_{11\beta}$, exists within the Y-1 cells and catalyzes both 11β- and 18-hydroxylation. In that case, ACTH would regulate each hydroxylation by a different mechanism maybe by creating a different environment which favors 11β-hydroxylation [17]. The other hypothesis would be the existence of two forms of cytochrome $P\text{-}450_{11\beta}$ regulated differently by ACTH. Two forms have been purified from mouse mitochondria, $P\text{-}450_{11\beta}$ and $P\text{-}450_{aldo}$ [18]; two forms have also been shown to exist in the rat, the bovine and the human [19–22]. The transcripts of the genes of both 11β-hydroxylase and aldosterone synthase have been detected in Y-1 cells [18]. In the mouse, there is little or no 18-hydroxylation of DOC, only 18-hydroxycorticosterone and aldosterone are produced [5]. Similarly, in the Y-1 cell line, the major 18-hydroxylated steroid produced is 18-hydroxy-20α-DHB [6]. It is possible that in this cell line, cytochrome $P\text{-}450_{11\beta}$ responding strongly to ACTH, would catalyze principally the 11β-hydroxylation of DOC and cytochrome $P\text{-}450_{aldo}$, less responsive to ACTH, would catalyze the formation of 18-hydroxy-20α-DHB and probably 20α-dihydroaldosterone from DOC. This hypothesis is under further investigation.

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REFERENCES

1. Buonassisi V., Sato G. and Cohen A. I.: Hormone-producing cultures of adrenal and pituitary tumor origin. *Proc. Natn. Acad. Sci. U.S.A.* **48** (1962) 1184–1190.
2. Yasumura Y., Buonassisi V. and Sato G.: Clonal analysis of differentiated function in animal cell cultures. I. Possible correlated maintenance of differentiated function and the diploid karyotype. *Cancer Res.* **26** (1966) 529–535.
3. Pierson R. W.: Metabolism of steroid hormones in adrenal cortex tumor cultures. *Endocrinology* **81** (1967) 693–707.
4. Kowal J. and Fiedler R.: Adrenal cells in tissue culture. I. Assay of steroid products; steroidogenic responses to peptide hormones. *Archs Biochem. Biophys.* **128** (1968) 406–421.
5. Raman P. B., Ertell R. J. and Ungar F.: Conversion of progesterone-4-C^{14} to 18-hydroxycorticosterone and aldosterone by mouse adrenals *in vitro*. *Endocrinology* **74** (1964) 865–869.
6. Ramirez L. C., Es-souni M. and Bournot P.: 18-Hydroxylase activity in the Y-1 adrenal cell line. *Molec Cell Endocr.* **66** (1989) 83–91.
7. Ramirez L. C., Millot C. and Maume B. F.: Sample purification using a C_{18}-bonded reversed-phase cartridge for the quantitative analysis of corticosteroids in adrenal cell cultures by high-performance liquid chromatography or gas chromatography-mass spectrometry. *J. Chromat.* **229** (1982) 267–281.
8. Lowry O. H., Rosenbrough N. J., Farr A. L. and Randall R. J.: Protein measurement with the folin phenol reagent. *J. Biol. Chem.* **193** (1951) 265–275.
9. Eliasson E., Johansson I. and Ingelman-Sundberg M.: Ligand-dependent maintenance of ethanol-inducible cytochrome *P*-450 in primary rat hepatocyte cell cultures. *Biochem. Biophys. Res. Commun.* **150** (1988) 436–443.
10. Paine A. J.: The maintenance of cytochrome *P*-450 in rat hepatocyte culture: some applications of liver cell culture to the study of drug metabolism, toxicity and induction of the *P*-450 system. *Chem. Biol. Interactions* **74** (1990) 1–31.
11. Song B. J., Veech R. L., Park S. S., Gelboin H. V. and Gonzalez F. J.: Induction of rat hepatic *N*-nitrosodimethylamine demethylase by acetone is due to protein stabilization. *J. Biol. Chem.* **264** (1989) 3568–3572.
12. Simpson E. R., Boggaram V., Funkeinstein B. and Waterman M. R.: Lipids in the regulation of hormone-responsive cells. *Biochem. Soc. Trans.* **13** (1985) 55–58.
13. Kowal J.: Adrenal cells in tissue culture. III. Effect of adrenocorticotropin and 3′,5′-cyclic adenosine monophosphate on 11β-hydroxylase and other steroidogenic enzymes. *Biochemistry* **8** (1969) 1821–1831.
14. Asano K. and Harding B. W.: Biosynthesis of adrenodoxin in mouse adrenal tumor cells. *Endocrinology* **99** (1976) 977–987.
15. Dazort A., Gallet D. and Saez J. M.: ACTH-dependent stimulation of a specific peptide in adrenocortical cells in culture. *Biochem. Biophys. Res. Commun.* **76** (1977) 1238–1246.
16. Morera A. M. and Saez J. M.: Mechanisms implicated in ACTH-induced steroidogenic and DNA synthesis refractoriness of adrenal mouse cell line (Y-1). *J. Steroid Biochem.* **12** (1980) 245–251.
17. Hall P. F., Yanagibashi K. and Kobayashi Y.: Synthesis of aldosterone by mitochondria and homogenous 11β-hydroxylase from beef and pig. *Endocrine Res.* **17** (1991) 135–149.
18. Domalik L. J., Chaplin D. D., Kirkman M. S., Wu R. C., Liu W., Howard T. A., Seldin M. F. and Parker K. L.: Different isoenzymes of mouse 11β-hydroxylase produce mineralocorticoids and glucocorticoids. *Molec. Endocr.* **5** (1991) 1853–1861.
19. Ogishima T., Mitani F. and Ishimura Y.: Isolation of aldosterone synthase cytochrome *P*-450 from zona glomerulosa mitochondria of rat adrenal cortex. *J. Biol. Chem.* **264** (1989) 10935–10938.

20. Lauber M., Sugano S., Ohnishi T., Okamoto M. and Müller J.: Aldosterone biosynthesis and cytochrome $P\text{-}450_{11\beta}$: evidence for two different forms of the enzyme in rats. *J. Steroid Biochem.* **26** (1987) 693–698.
21. Ogishima T., Mitani F. and Ishimura Y.: Isolation of two distinct cytochromes $P\text{-}450_{11\beta}$ with aldosterone synthase activity from bovine adrenal mitochondria. *J. Biochem.* **105** (1989) 497–499.
22. Kawamoto T., Mitsuuchi Y., Ohnishi T., Ichikawa Y., Yokoyama Y., Sumimoto H., Toda K., Miyahara I., Kuribayashi I., Nakao K., Hosoda K., Yamamoto Y., Imura H. and Shizuta Y.: Cloning and expression of a cDNA for human cytochrome $P\text{-}450_{aldo}$ as related to primary aldosteronism. *Biochem. Biophys. Res. Commun.* **173** (1990) 309–316.