Sources of Extramitochondrial Corticoidogenic Cholesterol in the Adrenal Cortex

Takamasa IWAKI*, Akibumi NOGUCHI and Takehito SEKIMOTO

Department of Pharmacology, and *Department of Experimental Animal Science, The Jikei University School of Medicine, 3-25-8, Nishi-Shinbashi, Minato-ku, Tokyo 105, Japan

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Abstract—Intracellular sources of extramitochondrial corticoidogenic cholesterol in bovine, rat and hamster adrenocortical cells were examined in vitro by comparing the species differences in the effects of various inhibitors on the adrenocorticotropic hormone (ACTH)-induced corticoidogenesis. The inhibitors were ML-236B (3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitor), W-7 (N-(6-aminohexyl)-5-chloro-1-naphthalene sulfonamide; calmodulin inhibitor), dichlorvos (0,0-dimethyl-2,2-dichlorovinyl phosphate; organic phosphorylation inhibitor), chloroquine ((7-chloro-4-4-diethylamino-1-methyl-butylamino) quinoline; lysosomal enzyme inhibitor) and cycloheximide (protein synthesis inhibitor).

During 2 to 3 hr incubation periods, the ACTH-induced corticoidogenesis was not inhibited by ML-236B (100 μM) in the bovine and rat adrenocortical cells. In the hamster adrenocortical cells, ML-236B (100 μM) did not affect the ACTH-induced corticoidogenesis during the initial 1 hr incubation periods; but thereafter, the ACTH-induced corticoidogenesis during the subsequent 2 hr incubation periods was completely blocked by ML-236B. The ACTH-induced corticoidogenesis was inhibited by W-7 (up to 25 μM) in the bovine and rat adrenocortical cells, but this was not the case in the hamster cells. Chloroquine (up to 400 μM) inhibited the ACTH-induced corticoidogenesis in the adrenocortical cells of three different species, but the hamster adrenal cells were much more vulnerable than the bovine and rat cells. The ACTH-induced corticoidogenesis in the adrenocortical cells of three different species were equally inhibited by cycloheximide (up to 1 mM).

It could be deduced from the present data that intracellular sources of corticoidogenic cholesterol during the ACTH-induced corticoidogenesis in vitro are mainly lysosomes and de novo synthesized cholesterol in the hamster cells, and the main sources are lipid droplets and lysosomes in the rat and bovine cells, respectively.

It has been reported that corticoidogenic cholesterol during the ACTH-induced corticoidogenesis in rat adrenocortical cells would be delivered from lipid droplets to the mitochondria, where cytochrome P-450 scc is localized (1), and cytosolic cholesterol ester hydrolase which is activated by cyclic adenosine monophosphate (cyclic AMP) might be involved in the liberation process of free cholesterol from the lipid droplets (2). Rat adrenocortical cells contain a higher concentration of cholesterol esters (34-48 μg/10^6 cells) (3) than bovine cells which contain a very low concentration of cholesterol esters (0.9-1.2 nmol/10^5 cells) (4). The hamster adrenals contain very little free cholesterol (7.5 μg/mg protein) and even less esterified cholesterol (0.9 μg/mg protein) (5). Ultramicroscopic study showed that the rat adrenocortical cell is lipid droplet-rich and lysosome-poor, the bovine adrenocortical cell is lipid droplet-poor and lysosome-poor, and the hamster adrenocortical cell is lipid droplet-deficient and lysosome-rich (6). It has been reported that there are large species differences in adrenocortical HMG-CoA
reductase activity, which is extremely high in the hamster cells and is very weak in the rat cells (5, 7). Intracellular sources of corticoidogenic cholesterol during ACTH-induced corticoidogenesis are supposed to be lipid droplets, lysosomes, mitochondria and de novo synthesized cholesterol. It was reported that ML-236B (specific inhibitor of HMG-CoA reductase) inhibited de novo synthesis of cholesterol in the rat liver (8). W-7 (calmodulin inhibitor) was reported to suppress either the ACTH or cyclic AMP-induced corticoidogenesis by an inhibitory effect on cholesterol ester hydrolysis in the bovine adrenocortical cells (4, 9). Chloroquine is thought to be accumulated specifically into lysosomes to inhibit lysosomal enzymes (10). It has been suggested that the organophosphate insecticides including dichlorvos inhibited adrenal cytosolic cholesterol esterification and hydrolysis in vitro at concentrations that correlate with their inhibition of adrenal steroidogenesis (11). Cycloheximide is an inhibitor of the steroidogenic response to ACTH (1, 12–14). The cycloheximide-sensitive factor in the corticoidogenic responses to ACTH is thought to be the steroidogenic protein (13, 14) which may participate on the translocation of cholesterol from the outer membrane to the inner membrane in the mitochondria (15, 16).

The aim of the present study was to examine the mechanism of ACTH action on corticoidogenesis by comparing the species differences in the intracellular sources of corticoidogenic cholesterol during the ACTH-induced corticoidogenesis in rat, bovine and hamster adrenocortical cells.

Materials and Methods

Preparation of cell suspensions: Male and female Wistar-Imamichi rats (200–250 g) and the GN-strain of the Syrian hamster (110–150 g) were bred and maintained in an animal room with a controlled light-dark cycle and room temperature. The hamsters and rats were sacrificed at 11:00 A.M. by decapitation to obtain the adrenals, and bovine adrenal glands were obtained from a Tokyo wholesale meat market.

The adrenocortical cells were dispersed by the trypsin digestion method as described previously (17). The washed cells were resuspended in Ca2+-free Krebs Ringer bicarbonate glucose buffer (pH 7.4) composed of 123.4 mM NaCl, 5.9 mM KCl, 1.2 mM KH2PO4, 25.0 mM NaHCO3, 1.2 mM MgSO4, 0.01 mM EGTA, 11.1 mM glucose, 0.3% bovine serum albumin and 0.2% trypsin inhibitor (Ca2+-free KRBGAT). The cell suspension contained about 5×105 cells/ml.

Incubation: The 0.5 ml (final volume) reaction mixture in Ca2+-free KRBGAT buffer containing about 2×105 cells (hamster and rat) to 3×105 cells (bovine) in the presence or absence of test reagents (ACTH, Ca2+, ML-236B, W-7, chloroquine, dichlorvos and cycloheximide which were dissolved in KRBGAT) was incubated at 37°C under a 95% O2-5% CO2 gas phase for 1 to 3 hr.

Corticoid and cholesterol analysis: The reaction was stopped by putting the tubes in ice-cold water. The whole incubation mixture was extracted with methylenechloride to estimate the corticoid production. Corticoids were determined fluorometrically by the method described by Silber et al. (18) using corticosterone (rat) and cortisol (bovine and hamster) as standards. Reagents in the reaction mixtures did not interfere with the H2SO4-fluorescence reaction. Cellular cholesterol was extracted with a chloroform-methanol mixture (2:1, v/v) and measured by the enzymatic method of Gamble et al. (19).

Chemicals: Trypsin, trypsin inhibitor (from soybean, chromatographically prepared), bovine serum albumin (Fraction V), chloroquine and cycloheximide were purchased from Sigma Chemical Co. Synthetic ACTH [1–24] was a gift from Daiichi Seiyaku Co., Ltd. ML236B was a gift from Sankyo Co., Ltd. W-7 was kindly donated by Prof. Hidaka of the Mie University School of Medicine. Dichlorvos was a gift from Nihon Kagaku Kougyou Co., Ltd. Cholesterol oxidase and cholesterol esterase were obtained from Boehringer Mannheim GmbH. The other chemicals were of analytical grade.

Results

Cholesterol content in hamster, bovine and rat adrenocortical cells: As shown in Table 1.
free and ester cholesterol contents were found to be 2.7±0.10 and 0.1±0.03 nmol/10^5 cells in hamster adrenocortical cells, 1.9±0.23 and 34.0±1.46 nmol/10^5 cells in rat adrenocortical cells and 0.4±0.03 and 0.9±0.05 nmol/10^5 cells in bovine adrenocortical cells respectively.

Effect of ML-236B on the ACTH-induced corticoidogenesis in hamster, bovine and rat adrenocortical cells: The ACTH+Ca^{2+}-induced corticoidogenesis was not significantly inhibited by ML-236B (100 μM) during the 2 to 3 hr incubation periods in the bovine (Fig. 1c) and rat (Fig. 1b) adrenal cells. In the hamster adrenal cells (Fig. 1a), ML-236B (100 μM) did not affect the ACTH+Ca^{2+}-induced corticoidogenesis during the initial 1 hr incubation periods as in the cases of rat and bovine adrenocortical cells; but thereafter, the ACTH+Ca^{2+}-induced corticoidogenesis was completely blocked by ML-236B (100 μM) during subsequent incubation periods as shown in Fig. 1a. The time-course of the ACTH+Ca^{2+}-induced corticoidogenesis in the rat adrenal cells was almost linear during the 3 hr incubation.

Table 1. Cholesterol content in the adrenocortical cells

<table>
<thead>
<tr>
<th>Species</th>
<th>Free cholesterol</th>
<th>Cholesterol ester</th>
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<tbody>
<tr>
<td>Hamster</td>
<td>2.7±0.10</td>
<td>0.1±0.03*</td>
</tr>
<tr>
<td>Rat</td>
<td>1.9±0.23</td>
<td>34.0±1.46†</td>
</tr>
<tr>
<td>Bovine</td>
<td>0.4±0.03*†</td>
<td>0.9±0.06*†</td>
</tr>
</tbody>
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The adrenocortical cells were dispersed by trypsin digestion as described under "Materials and Methods." Each value shown is the mean±S.E. for nine separate experiments. *Significantly different from rats at P<0.001 †Significantly different from hamsters at P<0.001

Fig. 1. Effect of ML-236B on the ACTH-induced corticoidogenesis in the hamster (a), rat (b) and bovine (c) adrenocortical cells. Incubation was performed at 37°C for 2 to 3 hr in the presence of 1 nM ACTH [1-24], 1.2 mM Ca^{2+} without (●) and with 100 μM ML-236B (■). Each value represents the mean±S.E. of triplicate determinations using 1.1×10^5 (hamster), 0.7×10^5 (rat) and 5.8×10^5 (bovine) cells/tube, respectively.
periods (Fig. 1b), and it was hyperbolic during the 2 hr incubation periods in the bovine adrenal cells (Fig. 1c). However, it was very interesting to find that the time course was biphasic in the hamster adrenal cells: it was hyperbolic during the initial 1 hr incubation periods, but thereafter, it showed a parabolic nature as indicated in Fig. 1a.

Effect of W-7 on the ACTH-induced corticoidogenesis in hamster, bovine and rat adrenocortical cells: The ACTH+Ca²⁺-induced corticoidogenesis was not affected by W-7 in the hamster adrenal cells at concentrations up to 25 μM (Fig. 2a), but the ACTH+Ca²⁺-induced corticoidogenesis in the rat and bovine adrenal cells was markedly inhibited by W-7 (up to 25 μM) as indicated in Fig. 2 (b and c).

Effect of chloroquine on the ACTH-induced corticoidogenesis in hamster, bovine and rat adrenocortical cells: Chloroquine inhibited the ACTH+Ca²⁺-induced corticoidogenesis in the hamster, rat and bovine adrenal cells significantly during the 1 hr incubation periods as shown in Fig. 3 (a, b and c). Fifty % inhibitory doses of chloroquine on the ACTH+Ca²⁺-induced corticoidogenesis in the hamster, bovine and rat cells were 15, 40 and 50 μM, respectively. The hamster cells were much more sensitive to chloroquine than the bovine and rat adrenal cells.

Effect of dichlorvos on the ACTH-induced corticoidogenesis in hamster, rat and bovine adrenocortical cells: Dichlorvos inhibited slightly the ACTH+Ca²⁺-induced corticoidogenesis in the hamster and bovine adrenal cells (Fig. 4 a and c), but inhibited it markedly in the rat adrenal cells as indicated in Fig. 4b at concentrations up to 200 μM.

Effect of cycloheximide on the ACTH-induced corticoidogenesis in hamster, rat and bovine adrenocortical cells: Cycloheximide (up to 1 mM) equally inhibited the ACTH+Ca²⁺-induced corticoidogenesis in the hamster, rat and bovine adrenal cells during the 1 hr incubation periods as indicated in Fig. 5 (a, b and c). In the hamster adrenal cells, cycloheximide similarly inhibited the ACTH+Ca²⁺-induced corticoidogenesis during the 1 hr and 3 hr incubation periods as shown in Fig. 5a.

Discussion

Tanaka et al. reported that bovine adre-
Fig. 3. Effect of chloroquine on the ACTH-induced corticoidogenesis in the hamster (a), rat (b) and bovine (c) adrenocortical cells. Incubation was performed as for Fig. 2 except that 0–400 μM of chloroquine was used. Each value represents the mean ± S.E. of triplicate determinations using 2.3×10⁵ (hamster), 1.2×10⁵ (rat) and 0.9×10⁵ (bovine) cells/tube, respectively.

Fig. 4. Effect of dichlorvos on the ACTH-induced corticoidogenesis in the hamster (a), rat (b) and bovine (c) adrenocortical cells. Incubation was performed as in Fig. 2 except that 0–200 μM of dichlorvos was used. Each value represents the mean of duplicate (hamster and rat) and the mean ± S.E. of triplicate (bovine) determinations using 1.8×10⁵ (hamster), 1.6×10⁵ (rat) and 3.9×10⁵ cells/tube, respectively.
nocortical mitochondria contained 35 to 40 nmoles of cholesterol/mg protein, of which 15 to 20 nmoles could be used for the corticoidogenic cholesterol and the remainder must be a structural constituent and was not efficiently utilized as a substrate for the mitochondrial steroidogenesis (20). They have suggested that corticoidogenic cholesterol must be supplied from an extramitochondrial pool to the mitochondria to maintain the suitable level of corticoidogenesis in the adrenal, because about 0.6 nmole cholesterol/mg mitochondrial protein/min was utilized for steroidogenesis in bovine adrenocortical mitochondria. It could be assumed from the data shown in Fig. 1 (b and c) that the rat and bovine adrenal cells must contain sufficient corticoidogenic cholesterol, which might be derived from lipid droplets and lysosomes, in order to maintain the ACTH-induced corticoidogenesis without de novo synthesis of cholesterol at least during the 2 hr (bovine) or 3 hr (rat) incubation periods. The data shown in Fig. 1a suggest that lysosomal cholesterol might be mostly consumed for the corticoidogenesis during the initial 1 hr incubation periods, and thereafter, de novo synthesized cholesterol might be utilized for the ACTH-induced corticoidogenesis in the hamster adrenal cells. The Ca²⁺-calmodulin system may play a regulatory role in the supply of free cholesterol to the mitochondria during the corticoidogenic responses to ACTH in bovine adrenocortical cells (4). As shown in Fig. 2, W-7 did not inhibit the ACTH-induced corticoidogenesis during the initial 1 hr incubation periods in the hamster adrenal cells. Thus, the Ca²⁺-calmodulin system may not be involved in liberation of cholesterol from lysosomes. The Ca²⁺-calmodulin system might be involved in the cholesterol liberation step from lipid droplets, because W-7 inhibited markedly the ACTH-induced corticoidogenesis during the initial 1 hr incubation periods in the rat and bovine adrenal cells. Chloroquine is thought to be accumulated into lysosomes to inhibit lysosomal enzymes (8). Nakamura et al. have reported that the size of the lysosomes

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**Fig. 5.** Effect of cycloheximide on the ACTH-induced corticoidogenesis in the hamster (a), rat (b) and bovine (c) adrenocortical cells. Incubation was performed at 37°C for 1 hr (○) and 3 hr (●) in the presence of 1 nM ACTH [1-24], 1.2 mM Ca²⁺ and 0-1000 μM of cycloheximide. Each value represents the mean±S.E. of triplicate (hamster, rat) or the mean of duplicate (bovine) determinations using 0.8x10⁵ (hamster), 1.4x10⁵ (rat) and 1.4x10⁵ (bovine) cells/tube, respectively.
increased in adrenocortical cells after in vivo treatment of rats with chloroquine, and even broken mitochondrial fragments were seen in the lysosomes (21). The inhibitory effect of chloroquine on the ACTH-induced corticoidogenesis in adrenal cells might be due to relative specificity of chloroquine accumulation into the lysosomes. It could be assumed from the higher sensitivity of the hamster cells to chloroquine, as shown in Fig. 3, that corticoidogenic cholesterol could be liberated largely from a lysosomal pool in the hamster cell. Dichlorvos was thought to inhibit either the ACTH or cyclic AMP-induced corticoidogenesis via direct inhibition of cholesterol ester hydrolase or cyclic AMP-dependent protein kinase in isolated rat adrenal cells (11). Although it can not be answered at present why the bovine adrenal cells (lipid droplet-poor and lysosome-poor type) were not so sensitive to dichlorvos (Fig. 4c), the data shown in Fig. 4b suggest that some phosphorylation steps might be involved during the liberation of cholesterol from the lipid droplets in the rat adrenal cells (lipid droplet-rich and lysosome-poor type), because the rat cells were extremely sensitive to dichlorvos. The precise mechanisms of the inhibitory effect of dichlorvos on the corticoidogenic response is not known at present. It could be deduced from the present data that extramitochondrial corticoidogenesis might be derived from lipid droplets and lysosomes in the rat and bovine cells, and lysosomal and de novo synthetized cholesterol would be utilized as corticoidogenic cholesterol during the corticoidogenic responses to ACTH in adrenocortical cells.

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