Ultrastructural Studies and Metabolic Regulation of Isolated Adrenocortical Carcinoma Cells of Rat

Rameshwar K. Sharma and Ken Hashimoto

SUMMARY

With a trypsin digestion method, an isolated adrenocortical carcinoma cell preparation has been achieved. The electron microscopic examination of tumor cells shows the sparsity of the lipid granules and mitochondria. The cristae of the mitochondria are tubular, in contrast to the vesicular cristae in the normal cells. Other ultrastructural features of these cells are presented.

The adrenocortical carcinoma cells did not respond for corticosteroidogenesis to adrenocorticotropic hormone, cyclic adenosine 3',5'-monophosphate, cyclic inosine 3',5'-monophosphate, cyclic thymidine 3',5'-monophosphate, cyclic uridine 3',5'-monophosphate, cyclic guanosine 3',5'-monophosphate, cyclic cytidine 3',5'-monophosphate, and cyclic N^6-2'-O-dibutyryladenosine 3',5'-monophosphate. Similarly, no response was observed to the varying concentrations of calcium ion. A 7-fold stimulation of corticosteroidogenesis was obtained when the adrenal carcinoma cells were incubated with pregnenolone and progesterone. Adrenocorticotropic hormone was found to inhibit the formation of corticosterone from pregnenolone. Epinephrine, glucagon, insulin, and proinsulin did not stimulate the formation of corticosterone.

INTRODUCTION

Adrenocortical carcinoma 494 (17), a corticosterone-secreting adrenal tumor, has been maintained by transplantation in our laboratories. This tumor lacks the stimulatory effect of ACTH^2 for the formation of corticosterone (10). However, some of the pathways of corticosteroidogenesis after the pregnenolone step are analogous to the steps in the normal adrenal tissue (3). In order to understand the control mechanisms of this adrenal tumor, we have prepared isolated cells of this tissue. This preparation has proven beneficial to the study of abnormalities in steroidogenic control mechanisms in this malignant tissue. A detailed ultrastructural study of adrenal tumor cells has been conducted and compared with normal isolated adrenal cells. Also, several cyclic nucleotides, steroid precursors, and cofactors have been tested for their corticosteroidogenic effect.

MATERIALS AND METHODS

Animals. Adrenocortical carcinoma 494, a spontaneously occurring tumor discovered by Snell and Stewart (17) in Osborne-Mendel rats, has been maintained in our laboratories by i.p. and s.c. transplantation in Sprague-Dawley rats at 3 to 4 weeks of age. Sixty to 80% of the rats receiving implantation successfully developed the tumor. The tumor has been histologically examined periodically and was found to be unchanged (17).

Chemicals. Bovine plasma albumin, obtained from Armour Pharmaceutical Company, Chicago, Ill., was dialyzed for 24 hr against KRB, pH 7.4, adjusted to an albumin concentration of 20% with KRB, and frozen in 20-ml aliquots. For each experiment, 1 vial of albumin was diluted with KRB to a final concentration of 4%. A twice-crystallized preparation of trypsin and the lima bean trypsin inhibitor were purchased from Worthington Biochemical Corporation, Freehold, N.J. ACTH, a United States Pharmacopeia corticotropin standard, was purchased from United States Pharmacopeia. Each vial contained 1.5 i.u. of ACTH and was diluted to give the desired concentration in 0.2 ml of vehicle. The vehicle was composed of 0.5% albumin in 0.9% NaCl adjusted to pH 3.5. cAMP, cGMP, cIMP, cTMP, cUMP, cCMP, and dcAMP were purchased from Sigma Chemical Company, St. Louis, Mo., and Boehringer-Mannheim, New York, N. Y. All the other reagents were reagent grade and were obtained commercially.

Pregnenolone-4,14^C (specific activity, 40 to 50 mCi/mM) and pregnenolone-7,3^H (specific activity, 10 to 15 Ci/mM) were purchased from New England Nuclear, Boston, Mass.

Chromatography. Precoated silica gel plates (Silica Gel HF, Analtech, Inc., Wilmington, Dela.) were used for TLC in the indicated solvent systems. Thin-layer plates were used for the final purification of corticosterone-4,14^C. Chromatographically homogeneous products were further checked for purity and identity by cocrystallization to constant specific activity and constant 3^H:14^C ratio.
Counting. Counting was carried out in the Nuclear-Chicago Mark II automatic liquid scintillation counter. The samples were dissolved in 15 ml of a scintillation solution of toluene containing 4 g of PPO and 100 mg of POPOP per 1000 ml.

Preparation of Isolated Adrenocortical Carcinoma Cells. Isolated adrenocortical carcinoma cells were prepared by the modified method (5, 15) previously described for isolated adrenal cells of rat. Adrenocortical carcinoma tissue weighing 1.30 to 1.40 g was freed of fat and connective tissue. The tumor nodules were halved or quartered depending on the size of the nodules and incubated, respectively, in 40, 30, and 30 ml of KRB (pH 7.4), which contained 0.2% glucose and 0.25% trypsin. The mixture was placed in a beaker and mechanically stirred at the rate of 400 to 450 rpm for 20 min at 37°C for each incubation. After the 3rd incubation, the preparations (100 ml) were pooled, filtered through a double layer of cotton gauze, and centrifuged at 100 × g for 45 min at 4°C, gradually accelerating to the final speed. The cell pellet thus obtained was resuspended in 16 ml of KRB containing 0.20% glucose and 0.40% lima bean trypsin inhibitor for 15 min at 37°C. An additional 32 ml of KRB containing albumin and glucose was added after the incubation, and the cell suspension was recentrifuged, as previously described. The cell pellet was then resuspended in 32 ml of KRB containing albumin and glucose, and 0.80 ml of cell suspension, representing 32.5 to 37.5 mg of adrenal tumor tissue, was used in each tube for incubation experiments. One ml of cell suspension contained 2 to 3 million cells.

Method of Incubation. The method of incubation for ACTH, cyclic nucleotides, nonradioactive steroids, or steroid precursors was that already described (5). Corticosterone was measured fluorometrically (4). The experiments with radioactive pregnenolone were conducted as follows: 3.342 μCi of pregnenolone-4,14C and 100.79 μCi of pregnenolone-7,3H were each preincubated with 25 ml of cell suspension derived from 3 g of tumor, representing 128 million cells at 37°C. ACTH, 4000 microunits, was added to the incubation mixture containing pregnenolone-7,3H, and the incubation was continued for 150 min. The reaction was stopped by the addition of 5 ml of distilled water and 50 ml of methylene chloride into each flask. The contents of both flasks were combined and mixed, and the methylene chloride extract was washed with 2 × 5 ml of 5% sodium hydroxide and then with 2 × 5 ml of distilled water. The methylene chloride extract was dried over sodium sulfate and filtered. The solvent was evaporated to dryness under nitrogen, and corticosterone was isolated from the residue as follows.

Isolation of Corticosterone. The isolation of corticosterone was carried out on the above residue by TLC with benzene:methanol (18:1.5). The radioactive zone corresponding to corticosterone was extracted, diluted with nonradioactive corticosterone (2 mg), and rechromatographed sequentially on TLC plates with n-hexane:ethyl acetate (7:3) and benzene:methanol (18:1.5). The purified radioactive zone of corticosterone was extracted, further diluted with nonradioactive corticosterone (13 mg), and crystallized 3 times from acetone:ligroin until the specific activity and 3H:14C ratio was constant (Table 1). On the basis of the 3H and 14C present in the corticosterone crystallized to constant specific activity and constant ratio, the corticosterone in the total extract contained 8.0116 × 10^4 dpm of 14C (equivalent to the incorporation of 1.0896% of 14C) and 8.8128 × 10^5 (equivalent to the incorporation of 0.4010% 3H).

Preparation of Adrenal Carcinoma Cells for Electron Microscopy. The same tumor used for the biochemical assays was cut into 1-cm mm pieces and fixed with 5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2. After the fixation, the tissue cubes were rinsed overnight in the same buffer and postfixed with 1% osmic acid in 0.1 M cacodylate buffer, pH 7.2, for 30 min. The sample was then dehydrated sequentially with graded concentrations of ethanol followed by propylene oxide. The cell pellets were stained with 1% uranyl acetate for 30 min after dehydration with 50% ethanol. Specimens were embedded in Araldite. Thin sections, 400 to 600 Å, were cut in a Porter-Blum MT-2 ultramicrotome, and stained with lead citrate and 15% uranyl acetate in 50% methanol for 10 min each. Stained sections were observed in a Hitachi HU 11-C electron microscope operated at an accelerated voltage of 100 kV or in an Hitachi HU-12 operated at 125 kV.

**RESULTS**

Ultrastructural Characteristics of the Adrenocortical Carcinoma Cells. The tumor cells were round to oval and uniform in size, measuring about 15 to 20 μm (Figs. 1 and 2A). They were solidly packed together and connected with interdigitation of numerous short villi projecting from cell wall (Figs. 1 and 2A). Desmosome-like junctional devices were occasionally seen (Figs. 1 and 2A). They were not necessarily present between each pair of cells. No cell was covered with a basal lamina. Lipid granules, if present, were not numerous, usually only 1 or 2 per cell. Their sizes varied from very small (0.5 μm) to very large (4 to 5 μm) in diameter (Figs. 1 and 2A). About one-half of the tumor cells did not contain lipid granules at all (Figs. 1 and 3). Occasionally, dense, whorled, membranous bodies, as described in the fasciculata cells of the normal and stimulated rat adrenal cortex (12), appeared to form in the cytoplasm (Fig. 2C). The majority of the lipid granules were not surrounded with smooth-surfaced endoplasmic reticulum and even lacked delimiting membrane (Figs. 1 and 2C).

Smooth-surfaced endoplasmic reticulum and Golgi apparatus were fairly well developed in most tumor cells, and numerous small vesicles derived from these organelles were seen (Fig. 2D). Stacks of smooth endoplasmic reticulum-forming bundles were found in the cytoplasm (Figs. 1 and 2D). They were free from mitochondria and lipid granules.
(Figs. 1 and 2D). Mitochondria were round to oval, and an average of 15 to 20 were seen in a section through the middle of the cell (Fig. 1). Large bizarrely shaped mitochondria, as seen in normal rat adrenal cortex (12), were absent. Mitochondrial cristae were thin and tubular, and no vesicular cristae were observed. Mitochondrial matrix (sap) filling the spaces between the cristae was electron light (Figs. 1, 2B, and 3). Many mitochondria contained dense, myelinated bodies (Figs. 1 and 2B); this feature may be due to fixation artifact. Most mitochondria were surrounded with only a few to several layers of smooth-surfaced endoplasmic reticulum (Figs. 2B and 3).

Rough-surfaced endoplasmic reticulum was present in various amounts, and polysomes were abundant (Fig. 2C). Glycogen particles were scarce. No lipofuscin pigment granules were observed. The nucleus had a very thin rim of heterochromatin, and 1 to 3 nucleoli were present. Neither nuclear nor cytoplasmic structures suggestive of viral inclusions were seen.

Effect of Pregnenolone and Progesterone on Corticosteroidogenesis. For a study of the formation of corticosterone from pregnenolone and progesterone in isolated adrenocortical carcinoma cells, these 2 substrates were used in concentrations ranging from 0.0032 to 0.316 mM. The formation of corticosterone was linear up to the concentration of 0.0632 mM for pregnenolone (Chart 1). Pregnenolone and progesterone at 0.0632 mM concentrations were converted to the same amount of corticosterone (Table 2).

20α-Hydroxycholesterol and deoxycorticosterone were found to interfere (R. K. Sharma, unpublished observations) with the fluorometric determination of corticosterone (4). Therefore, to determine the effect of ACTH on the conversion of pregnenolone to corticosterone and also to ascertain the ability of the tumor cells to form corticosterone, pregnenolone-4-14C without ACTH and pregnenolone-7-3H with ACTH were incubated with tumor cells. The 2 incubates were mixed, and corticosterone was isolated and purified by chromatography and recrystallized to constant specific activity and 3H :14C ratio. As can be seen (Table 1) from the results, although the tumor did produce corticosterone, ACTH inhibited this formation, since the starting 3H :14C ratio of 30.16 in pregnenolone was reduced to a ratio of 10.88 of the isolated corticosterone. The alternative possibility that the removal of tritium may be biosynthetic, however, cannot be eliminated at this time.

Time Course of Steroidogenesis. The measurement of corticosterone production in the presence of pregnenolone (0.0632 mM) was done in isolated adrenal tumor cells from 0 to 120 min (Chart 2). The formation of corticosterone was found to be linear with time. No lag period was found.

Effect of ACTH and Cyclic Nucleotides on Corticosteroidogenesis. The studies on the adrenal tumor slices (10) have shown that ACTH or cAMP does not stimulate corticosteroidogenesis in this tissue. In view of the fact that other cyclic nucleotides such as cIMP, cGMP, and cCMP in normal adrenal cells and cCMP in monolayer culture of the mouse adrenal tumor cells (6) stimulate steroidogenesis, the following cyclic nucleotides were therefore tested for their ability to stimulate corticosteroidogenesis: cAMP (10.0 mM), cGMP (1.0 mM), cAMP (10.0 mM), cGMP (1.0 mM), cIMP (20.0 mM), cTMP (20.0 mM), cUMP (20.0 mM), and cCMP (20.0 mM). All were found to have no activity. Even when the systems were fortified with either ATP- or NADPH-generating system, no corticosteroidogenic response was obtained (Table 3).

Effect of Steroid Precursors on Corticosteroidogenesis. Squalene and mevalonic acid, corticosteroid precursors before the rate-limiting step in the formation of corticosteroids, were tested for corticosteroidogenesis with and without ACTH or cAMP. None of these precursors stimulated corticosteroidogenesis.

Effect of Calcium Concentration on Corticosteroidogenesis. Since calcium ion has been implicated in the mechanism of action of ACTH (1, 11), corticosteroidogenesis in isolated adrenocortical carcinoma cells was tested with varying concentrations of calcium ranging from 2.2 to 20 mM, with and without ACTH and cAMP. Contrary to that reported for the normal isolated adrenal cells (15), calcium was found to have no stimulatory response upon corticosteroidogenesis in tumor cells.

Effect of Various Hormones on Corticosteroidogenesis. The adrenal tumor has been found to be stimulated for its adenyl cyclase activity by several other hormones in addition to ACTH (16). This is in contrast to the studies conducted with the normal adrenal homogenate, where the adenyl cyclase

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Table 2

<table>
<thead>
<tr>
<th>Additions</th>
<th>µg/2,000,000 - 3,000,000 cells</th>
<th>µg/0.5 mg of protein cells</th>
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<tbody>
<tr>
<td>Baseline*</td>
<td>0.0482 ± 0.009</td>
<td>0.1176</td>
</tr>
<tr>
<td>Pregnenolone, 0.0632 mM</td>
<td>0.3394 ± 0.0681</td>
<td>0.8270</td>
</tr>
<tr>
<td>Progesterone, 0.0632 mM</td>
<td>0.3400 ± 0.0672</td>
<td>0.8301</td>
</tr>
</tbody>
</table>

* Average of 8 observations. Values are expressed as ± S.E.

b Baseline value has been subtracted from the experimental results.

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Chart 1. Conditions of the experiment were the same as in Table 2.
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Table 4

Effects of various hormones on isolated adrenal tumor cells in the formation of corticosterone

Conditions of the experiment similar to the experiment in Table 2.

<table>
<thead>
<tr>
<th>Additions</th>
<th>Concentration</th>
<th>Corticosterone (µg/2 hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ACTH</td>
<td>100 microunits/ml</td>
<td>0.023</td>
</tr>
<tr>
<td>Pregnenolone</td>
<td>0.0632 mM</td>
<td>0.332</td>
</tr>
<tr>
<td>Epinephrine</td>
<td>1 µg/ml</td>
<td>0.014</td>
</tr>
<tr>
<td>Insulin</td>
<td>100 microunits/ml</td>
<td>0.000</td>
</tr>
<tr>
<td>Proinsulin</td>
<td>100 microunits/ml</td>
<td>0.002</td>
</tr>
<tr>
<td>Glucagon</td>
<td>0.4 µg/ml</td>
<td>0.000</td>
</tr>
</tbody>
</table>

a Average of 3 observations. Baseline value has been subtracted.

DISCUSSION

With a trypsin digestion method, an isolated adrenocortical carcinoma cell preparation has been achieved. Such a preparation is remarkably homogeneous and gives a 7-fold increase in corticosteroidogenesis when pregnenolone or progesterone are used as precursors. It has a low basal value of corticosterone (0.038 µg/3 million cells), and therefore should be suitable for the study of the direct mechanism of ACTH or cyclic nucleotide action on the isolated target tumor tissue.

The electron microscopic studies have shown that the most striking difference between the tumor cell and the cells of the normal adrenal cortex of the rat is the sparsity of lipid granules and mitochondria. In the normal rat, adrenal cortical cells contain at least 50 mitochondria and 25 lipid granules, except for the reticularis cells, which contain only 5 lipid granules (12). A great variation of lipid granules is abnormal since, in the normal rat adrenal cells, large granules measuring greater than 4 µm were not reported (12). Mitochondria with a few thin cristae are not found in any of the 3 zones of the normal rat adrenal cortex (12). A large number of myelinated dense bodies were not described in the mitochondria of normal rat adrenal cortex. Stacks of smooth endoplasmic reticulum without mitochondrial or lipid granule association are not known in the normal cortex. Although lysosomes are present in all 3 zones of the normal rat adrenal cortex (7), autophagocytosis of its own cellular organelles, such as mitochondria, has not been found.

From the above, it is difficult to associate these tumor cells with any 1 zone of the normal rat adrenal cortex. The origin of the tumor, therefore, is not clear.

Rather clear mitochondrial matrix and poor development of cristae may indicate that the enzymes involved in the conversion of acetate to steroids are deficient. Formation of dense myelinated dense bodies in many mitochondria may suggest further deterioration of their function. A poor development of the husks of smooth-surfaced endoplasmic reticulum enveloping lipid granules and mitochondria may indicate that exchange of intermediate products of steroidogenesis between these organelles is not easily carried out. This agrees with the biochemical data presented that certain steps of steroidogenesis in this tumor are not active.

Normal adrenal cells are stimulated to produce corticosterone by cAMP, dcAMP, cIMP, and cGMP for steroidogenesis (5, 8). Since the adrenal tumor cells do not respond to ACTH or cAMP for steroidogenesis, the question was raised as to whether the tumor had developed other cyclase systems which may show specificity to other steroid-stimulating cyclic nucleotides. However, no response to cGMP, cIMP, cUMP, cTMP, and cCMP was found. Recent reports (16) indicate that the adrenal tumor is stimulated for its adenyl cyclase activity by other hormones such as epinephrine, norepinephrine, and thyroid-stimulating hormones as well as by ACTH. However, the lack of activity of...
any of the cyclic nucleotides in stimulating corticosteroidogenesis does not imply that such nucleotides are not present in the tumor. For instance, if the defect in the tumor lies in its inability to displace the protein kinase from the repressor protein, still no steroidogenesis will be observed. In fact, recent results (16) do suggest that, in spite of the high levels of cAMP in the adrenal tumor, no steroidogenesis is obtained.

When the incubation experiment was done with radioactive pregnenolone (Table 1), the incorporation of radioactivity into corticosterone was noted; however, ACTH apparently inhibited this formation. These results are in marked contrast to those found in the normal adrenal (2), where ACTH is found to stimulate the formation of corticosterone. cAMP also stimulates the 11β-hydroxylation in rat adrenal (13, 14). From this observation, it is possible that adrenal tumor has developed certain distinct biochemical characteristics which may give quite the opposite physiological response to that found in the normal adrenal.

Of interest was the slight but significant stimulation of the tumor cells by cholesterol (0.0316 µg of corticosterone per 2 million cells). No further stimulation was obtained when cholesterol was fortified with either ACTH or dcAMP. Electron microscopic studies show the sparsity of the lipid globules and mitochondria in tumor cells. One of the causes of there being no corticosteroidogenic effect to ACTH might be the diminished amount of mitochondria in the tumor cell. The limiting step from cholesterol to pregnenolone takes place in the mitochondria. Insufficiency of the mitochondrial bodies coupled with the lack of the substrate (cholesterol) could contribute to very little conversion of cholesterol to pregnenolone and therefore to corticosterone. No further stimulation by ACTH in the presence of added cholesterol was noted. This could be due to the fact that the tumor is already maximally stimulated by endogenous cAMP.

Of further interest was the slight steroidogenesis from 0.024 µg of corticosterone to 0.048 µg of corticosterone during 2 hr of incubation without the stimulation of adrenal tumor cells with ACTH. If this is an ACTH-producing tumor remains to be ascertained.

Since a role of calcium ion as a mediator of ACTH action on adrenal protein synthesis has been proposed (1, 11), the incubation experiments of adrenal tumor cells with ACTH and dcAMP with varying concentrations of calcium ion were conducted. However, no stimulation of corticosteroidogenesis was observed even when the system was fortified with varying concentrations of calcium ion.

Of considerable interest was the recent report (16) that the adenyl cyclase receptor of the rat adrenocortical carcinoma had lost specificity in its singular response to ACTH. This conclusion was based on the observation that adenyl cyclase activity of the tumor homogenate was stimulated not only by ACTH but also by several other hormones, whereas normal rat adrenal homogenates responded only to ACTH (16). Therefore, epinephrine, glucagon, insulin, and proinsulin were tested for their efficiency in promoting corticosteroidogenesis in isolated adrenocortical carcinoma cells, but no stimulation of corticosteroidogenesis was obtained. These results suggest that the steps of steroidogenesis in the adrenal tumor are impaired after the adenyl cyclase step.

Although normal adrenal cells could show changes similar to those of the adrenal tumor cells in response to various nontumorous hyperplasias, the present studies suggest that the transformation of the normal adrenal cell to the tumor cell is accompanied by a characteristic change in its ultrastructural composition. Such a transformation may involve reduction in enzyme activity. In addition, a modified ACTH-sensitive adenyl cyclase system may be present. If the latter possibility is true, it suggests that a considerable modification of the genetic expression in the cancer tissue has taken place. A remarkable difference in the phosphodiesterase activities of tumor and normal adrenal tissue has been observed earlier (R. K. Sharma, unpublished observations). Nevertheless, that the tumor may have developed a derepressed system resulting in the production of ACTH and thus may not respond to exogenous ACTH is an attractive possibility.

ACKNOWLEDGMENTS

We thank Dr. R. L. Ney, Department of Medicine, University of North Carolina, for the supply of tumor rats and Dr. James Brush for stimulating discussions during the course of this investigation. We gratefully acknowledge the help of Dr. Charles Irving in initiating this project and the excellent assistance of Mrs. Margaret Cirtain. We also thank Dr. H. Bernhardt for histological examination of the adrenal tumor.

REFERENCES

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Fig. 1. Tumor cells contain varying numbers of mitochondria, which contain several thin, tubular cristae, an electron-light matrix, and myelinated dense bodies (*). Stacks of smooth endoplasmic reticulum (arrows) are not associated with either lipid granules (L) or mitochondria. X 8,187. Inset, high magnification of desmosome-like junctional devices. X 62,500.
Fig. 2. A, low-magnification scanning view of the tumor mass. Individual tumor cells are round to oval and interdigitated with peripheral villi (v). Only 1 desmosome is seen (arrow). Nuclei have a thin rim of heterochromatin and a few nucleoli (n). Two large lipid granules (L) are observed. X 4,250. B, myelinated dense bodies are found within several mitochondria (*). N, nucleus. X 29,938. C, dense, whorled membranous body screwed into a lipid granule (L). This lipid granule is not surrounded with either a delimiting membrane or smooth endoplasmic reticulum. P, polysome. X 61,125. In D, stacks of parallel arrays of smooth endoplasmic reticulum (*) are present in the cytoplasm without connection to lipid granule or mitochondria. G, part of Golgi's apparatus. X 61,125.
Fig. 3. A number of lysosomes are present (*). Some of these contain half-degenerated mitochondria (arrows) of the origins of the cells themselves (also see inset), thus classing themselves with autophagosomes. Mitochondria contain sparse cristae of the elongated tubular variety. Spaces between cristae are almost empty. These mitochondria are surrounded with only 1 or 2 layers of smooth endoplasmic reticulum (Er).
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