ELECTRON MICROSCOPIC STUDIES ON THE ADRENAL OF RAT TREATED WITH AMINOGLUTETHIMIDE—WITH SPECIAL REFERENCE TO ACCUMULATION OF CHOLESTEROL IN INTRAMITOCHONDRIAL VACUOLES—

GEN ITOH

1st Department of Pathology, Nagoya University School of Medicine
(Director: Prof. Shooichi Sugiyama)

ABSTRACT

Aminoglutethimide (Elipten, Ciba,) interferes with the biosynthesis of adrenocortical hormones by blocking the conversion of cholesterol to pregnenolone. Male albino rats were fed with the diet containing aminoglutethimide and were killed after various time intervals ranging from 24 hours to 7 days. The adrenal cortices of these animals were studied both by light and electron microscopy. Special effort was made to determine the intracellular localization of free cholesterol by an electron microscopic histochemical method using the digitonin reaction.

The animals killed 24 hours after the initiation of the feeding were found to have small myelin figures in contact with the mitochondrial membrane in fasciculata cells which might reflect compensatory rise of ACTH. The animals fed with the diet for 4 to 7 days were shown to have enlarged adrenal cortex. Light microscopic examination revealed an increase of the lipid globules both in size and number in the zona fasciculata. Ultrastructural studies showed swollen, hypertrophied, bizarre profiles of the mitochondria with the formation of the vacuoles. The electron microscopic histochemical method demonstrated digitonin-cholesterol crystals localized in the vacuoles of the mitochondria, in the perimitochondrial areas and in the lipid globules.

These findings correlate well with the biochemical data which indicate the intracellular site of steroid biosynthesis and lend morphological support to the view that the biosynthesis of pregnenolone from cholesterol takes place within the mitochondria. These results indicate that aminoglutethimide causes accumulation of cholesterol in the vacuoles of the mitochondria.

INTRODUCTION

Aminoglutethimide which was introduced as an anticonvulsant in 1958 was later proved to interfere with the biosynthesis of the adrenocortical hormones by blocking the conversion of cholesterol to pregnenolone. Clinical trials, were performed on the patients suffering from Cushing's syndrome due to

Received for publication February, 25, 1971.
malignant adrenal cortical tumors\(^6\) or hyperfunctioning cortical adenomas\(^9\). Morphological studies\(^9\) on the adrenal glands of these patients treated with aminoglutethimide revealed hypertrophy and vacuole formation of the adrenal cortical cells\(^6\) by light microscopy, and also accumulation of lipid vacuoles bounded by a single limiting membrane in the cytoplasm of the fasciculata cells by electron microscopy\(^8\). In the rat, aminoglutethimide caused accumulation of lipids\(^9\) in the cytoplasm of the fasciculata cells which resulted in destruction of this zone\(^6\). Electron microscopic studies\(^9,10\) demonstrated vacuole formation in the mitochondria of the fasciculata cells. Accumulation of lipids\(^9,11\) and cholesterol\(^2\) was also demonstrated biochemically.

Since the work of Hayano et al.\(^12\) in 1956 which indicated that the mitochondrial fraction was necessary for the biosynthesis of pregnenolone from cholesterol, biochemical works\(^13\)\(^14\)\(^15\) concerning the intracellular localization of steroid biosynthesis followed and confirmed that aminoglutethimide inhibited 20\(\alpha\)-hydroxylation of the cholesterol side chain\(^3\) and desmolase activity\(^8\). These results suggest that aminoglutethimide might cause cholesterol accumulation in the intramitochondrial vacuoles. This speculation has not been proved in any publication in the past. This investigation was designed, therefore, to confirm the morphological changes of the adrenal gland of the rat given aminoglutethimide. Special attention was paid to the ultrastructural histochemical demonstration of cholesterol in the vacuoles of the mitochondria.

**MATERIALS AND METHODS**

Fourteen male albino rats were fasted for 24 hours, although water was allowed ad. libitum. The animals were fed with the standard laboratory diet with 0.6% of aminoglutethimide added. They were killed by decapitation at the same time each afternoon by the following schedule: 4 rats at 24 hours after the initiation of the aminoglutethimide diet, 2 rats at the end of the second day, 4 rats on the fourth day, 4 rats on the seventh day, respectively. A total of four rats were served for the control study.

One of the rat adrenal glands was fixed in 3% solution of formol for light microscopic examination. One half of the other side of the adrenal was cut into blocks, fixed in buffered solution of 3% glutaraldehyde for 2 hours, washed for 24 hours, post-fixed with buffered solution of 1% osmic acid, dehydrated in graded alcohol, embedded in epoxy resin and was examined electron microscopically. The other half of the adrenal was fixed, according to Flickinger’s method\(^16\)\(^17\), for 20 hours in buffered solution of 0.1\(\text{N}\) cacodylate containing 2% formaldehyde, 2.5% glutaraldehyde and 0.05% calcium chloride, incubated for 24 hours in buffered solution of cacodylate containing 2% formaldehyde, 2.5% glutaraldehyde, 0.05% calcium chloride and 0.2% digitonin, washed for 24 hours in buffered solution of cacodylate, post-fixed with buffered solution of
RESULTS

Hypertrophy of the adrenal cortex was recognized in the rats sacrificed after 4 and 7 days of aminoglutethimide feeding. The animals belonging to these groups were found to have increased amounts of lipid globules through the semi-thin sections of the epoxy resin embedded blocks.

Electron microscopically, small myelin figures (Fig. 1) having contact with the mitochondrial membrane were frequently found in the fasciculata cells of the rats sacrificed 24 hours after the initiation of the feeding, while these structures were rarely seen in the rats sacrificed 4 and 7 days after the initiation of the aminoglutethimide diet. Dilatation of the vesicular cristae of the mitochondria (Fig. 2) occurred sporadically 2 days after the onset of the diet and increased in degree and frequency as time elapsed, resulting in accumulation of many and large vacuoles in the mitochondria of the fasciculata cells of the rats sacrificed after 4 and 7 days of the feeding (Fig. 3). Lipid globules were of small size at the earlier stage, while they increased in size and number at the later stage. Electron-transparent areas at the periphery of one pole of the lipid globules were frequently observed in the rats fed on the diet for 4 or 7 days.

Electron microscopic examination of the digitonin treated adrenal tissue revealed needle-shaped crystals in the intramitochondrial vacuoles (Figs. 4, 5, 6), in the perimitochondrial areas (endoplasmic reticulum) and in the lipid globules at the periphery of one pole of the lipid globules. The needle-shaped crystals in the intramitochondrial vacuoles were abundant in the cytoplasm of the fasciculata cells of the adrenals in rats 4 and 7 days after the initiation of the aminoglutethimide diet. On the other hand, the needle-shaped crystals were rarely seen in the fasciculata cells of rats 1 and 2 days after the onset of the diet.

The crystals in the lipid globules seemed to be located at the electron-transparent areas of the adrenal specimen not treated with digitonin. The crystals were much more abundant in rats 4 and 7 days after the initiation of the diet than in those 1 and 2 days after the initiation of the diet.

The needle-shaped crystals were assumed to be tubular in structure and were composed of 3 to 7 coaxial lamellae. The innermost and outermost layers of the 3 coaxial lamellae were electron dense while the lamellae inbetween was electron transparent (Fig. 7). They were 400 to 1000 Å in width. The exact length was not calculated. The electron dense and electron transparent lamella
was 30 Å in thickness.

These needle-shaped crystals were entirely lacking in the tissue samples incubated without digitonin in the medium.

DISCUSSION

Aminoglutethimide inhibits conversion of cholesterol to pregnenolone\(^\text{12,3}\) which occurs in the mitochondria\(^\text{12}\) and causes compensatory rise of ACTH production\(^\text{2}\). ACTH produces ultrastructural changes\(^\text{18-24}\) of mitochondria, smooth surfaced endoplasmic reticulum and liposome in the fasciculata cells of the adrenal cortex.

The myelin figures noted in this experiment might reflect a compensatory rise of ACTH. Schwarz \textit{et al.}\(^\text{22}\) observed intramitochondrial vacuoles in fasciculata cells of the rat treated with ACTH. Yoshimura\(^\text{23}\) described large intramitochondrial vesicles which were produced by stimulation of ACTH. The intramitochondrial vacuoles observed in this experiment, however, were much more abundant than those produced by ACTH and also considerably different in appearance. Racela \textit{et al.}\(^\text{10}\) confirmed development of intramitochondrial vacuoles in hypophysectomised rats treated with aminoglutethimide. Taking into consideration these results, it seems reasonable to interpret that the intramitochondrial vacuoles in this experiment arose as a result of the specific action of aminoglutethimide.

The digitonin reaction which was initiated by Windaus in 1910\(^\text{26}\) for estimation of free cholesterol, was applied for histochemistry by Brunswick in 1922\(^\text{27}\) and was further employed for the determination of 3\(\beta\)-hydroxysteroids by Clarenburg \textit{et al.} in 1966\(^\text{28}\). Ökrös\(^\text{29}\) and Lévy \textit{et al.}\(^\text{30}\), independently in 1968 and in 1967, were the first who applied this method to electron microscopic histochemistry. Ökrös demonstrated the needle-shaped crystals of digitonin-cholesterol complex in the normal rat adrenal tissue treated with digitonin. According to his description, the digitonin-cholesterol crystals were made up of electron transparent and electron dense coaxial lamellae; the crystals varied between 6000 and 7000 Å in length and 400 to 1000 Å in width; the electron dense and electron transparent lamellae of the crystals were 20 to 30 Å in thickness; the number of the lamellae was 1 to 9. Scallen \textit{et al.}\(^\text{17}\) recently demonstrated the needle-shaped crystals of digitonin-cholesterol complex in the murine liver treated with digitonin and proved that as much as 99\% of the unesterified cholesterol was preserved by this procedure. The needle-shaped crystals observed in this experiment had entirely the same appearance as those described by Ökrös and Scallen \textit{et al.} Digitonin reacts not only with free cholesterol but also with 3\(\beta\)-hydroxysteroids\(^\text{25}\). Whether or not the crystals of the digitonin-cholesterol complex are different in structure from those of digitonin-3\(\beta\)-hydroxysteroids is not known. The latter, however, is scanty in
amount. Consequently it is concluded that the needle-shaped crystals represent the digitonin-cholesterol complex.

It is generally accepted that cholesterol, the precursor of steroid hormones, comes mainly from serum free cholesterol in the rat adrenals. Moses et al.\(^3\) recently demonstrated by electron microscopic autoradiographs and biochemical analysis that tritiated cholesterol injected intravenously, though only 9% of the tritiated cholesterol given, accumulated in the mitochondria. The needle-shaped crystals at the perimitochondrial areas observed in this experiment might, therefore, indicate accumulation of cholesterol of extra-adrenal origin.

Ökrös\(^2\) observed in 1967 digitonin-cholesterol crystals localized in lipid globules in normal rat adrenal tissue treated with digitonin by electron microscopy. Moses et al.\(^3\) confirmed that 70 to 80% of tritiated cholesterol injected intravenously are distributed within lipid globules in the adrenal of normal rats. In this experiment digitonin-cholesterol crystals were observed in lipid globules. This means that aminogluthethimide causes accumulation of cholesterol in lipid globules.

Moses et al.\(^3\) postulate that cellular mechanisms exist which cause mobilization of cholesterol from the lipid globules to the mitochondria. Further studies are needed as to this point.

ACKNOWLEDGEMENT

I express my sincere gratitude to former Prof. M. Miyakawa for his cordial consideration and to Prof. S. Sugiyama and Prof. H. Tauchi for their king advice in this experiment. I am indebted to Dr. T. Hirano for his careful review of the manuscript and to my colleagues for their friendly encouragement and criticism throughout this work. Thanks are also due to my technical officials for their kind technical helps.

This is a doctoral thesis judged by Nagoya University inquiry council of medical degree.

REFERENCES


19) Symington, T., In Functional pathology of the human adrenal gland, Edited by symington, T. E and S Livingstone Ltd.


CHOLESTEROL IN INTRAMITOCHONDRIAL VACUOLES

65, 110, 1910.


EXPLANATION OF FIGURES

MT. ; mitochondria
LG. ; lipid globule
VAC. ; vacuole
CRYS. ; crystal of digitonin-cholesterol complex

FIG. 1. The fasciculata cell of the rat sacrificed 24 hours after the aminogluthethimide feeding. Note the myelin figures having contact with the mitochondrial membrane.

FIG. 2. The fasciculata cell of the rat 2 days after the initiation of the aminogluthethimide diet. Note sporadic dilatation of vesicular cristae of the mitochondria.

FIG. 3. The fasciculata cell of the rat 4 days after the initiation of the diet. Note the enlargement of the mitochondria and accumulation of large vacuoles in the mitochondria. Those vacuoles contain fragments of membrane like materials.

FIG. 4. The fasciculata cell of the rat 4 days after the initiation of the diet. Needle-shaped crystals of digitonin-cholesterol complex are found in the vacuoles of the enlarged mitochondria and in the perimitochondrial endoplasmic reticulum. (the adrenal tissue with digitonin treatment)

FIG. 5. The fasciculata cell of the rat 7 days after the initiation of the diet. The needle-shaped crystals are also found in the intramitochondrial vacuoles and in the perimitochondrial endoplasmic reticulum. (the adrenal tissue treated with digitonin)

FIG. 6. The fasciculata cell of the rat 7 days after the initiation of the diet. Note the degenerated mitochondria containing large vacuoles. The needle-shaped crystals are also found in the vacuoles. The lipid globule is found to contain the crystals at its periphery. (the adrenal tissue treated with digitonin)

FIG. 7. Digitonin-cholesterol crystal is assumed to be tubular in structure composed of a total of three coaxial lamellae. The innermost and outermost layers are electron dense while the lamella in-between is electron transparent. (the adrenal tissue treated with digitonin)