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Biochemical and Immunocytochemical Properties of Peroxisomes and Mitochondria in Bovine Chromaffin Cells

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ABSTRACT. **The biochemical and immunocytochemical properties of peroxisomal and mitochondrial β-oxidation enzymes in bovine adrenal chromaffin cells were investigated. Peroxisomes were detectable by immunofluorescence staining using antibodies against acyl-CoA oxidase, peroxisomal 3-ketoacyl-CoA thiolase and catalase. The mitochondria were abundantly stained with antibody against mitochondrial 3-ketoacyl-CoA thiolase. The biosynthesis and intracellular processing of acyl-CoA oxidase and the peroxisomal 3-ketoacyl-CoA thiolase was slower than that in fibroblasts. The peroxisomal β-oxidation activities shown by [1-^{14}C] lignoceric acid oxidation were slightly lower than those in fibroblasts, whereas the mitochondrial β-oxidation activities shown by [1-^{14}C] palmitic acid oxidation were almost identical to those in fibroblasts. Adrenal chromaffin cells are useful materials for investigating the peroxisomal and mitochondrial metabolism of autonomic neurons and may contribute to the clarification of neuronal dysfunction in peroxisomal and mitochondrial disorders.**

Peroxisomes are ubiquitous organellae present in almost all eukaryotic cells. In humans, peroxisomes are abundant in hepatocytes and renal tubular cells (1). Microperoxisomes are morphologically detectable in fibroblasts (2), neurons (3), intestinal mucosal cells (4) amniotic fluid cells (5) and chorionic villous cells (6). The metabolic functions of peroxisomes, including β-oxidation of very long chain fatty acids, biosynthesis of bile acids, plasmalogen biosynthesis, and degradation of phytanic acid, have been investigated in these tissues and cell lines. The peroxisomal β-oxidation system consists of acyl-CoA oxidase, enoyl-CoA hydratase/L-3-hydroxyacyl-CoA dehydrogenase bifunctional protein (L-bifunctional protein), D-3-hydroxyacyl-CoA dehydratase/D-3-hydroxyacyl-CoA dehydrogenase bifunctional protein (D-bifunctional protein), and 3-ketoacyl-CoA thiolase (7, 8). The results have been applied to the investigations of pathophysiology and diagnosis of Zellweger syndrome, neonatal adrenoleukodystrophy and infantile Refsum disease (9), which are fatal autosomal recessive peroxisome biogenesis diseases with severe psychomotor retardation, visual and hearing impairment, hepatic dysfunction, and dysmorphic features.

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In these disorders, an abnormality of neuronal migration in the central nervous system is the most characteristic finding (10) and is considered to be linked to the severe neurologic symptoms such as hypotonia, psychomotor retardation and convulsions. Biochemical studies revealed the deficiencies of multiple peroxisomal enzymes (9), and immunohistochemical studies using autopsied brain tissues from these patients and control fetuses revealed that peroxisomes may play important roles in the development of neurons and myelinogenesis (11, 12, 13). However, study on the functions of peroxisomes in cultured neuronal or neuroendocrine cells has not been performed. The identification of peroxisomal functions in these cells will provide essential knowledge to elucidate the pathophysiology of neuronal migration.

To determine the fundamental metabolic functions of peroxisomes and mitochondria in neuronal cells, we investigated cultured bovine adrenal chromaffin cells, which play a key role in bovine sympathetic nervous function.

MATERIALS AND METHODS

Isolation and culture of chromaffin cells.

Bovine adrenal chromaffin cells were isolated by the retrograde perfusion of the adrenal medullae with collagenase and DNAase I, and purified from other cell types by density gradient centrifugation on Percoll (Pharmacia, Uppsala, Sweden) and by differential plating. The purified cells were kept in sus-

pension on 35 mm plastic dishes at a density of 5×10^5 cells /dish for lignoceric acid oxidation, using Dulbecco's modified Eagle's medium (GIBCO, Grand Island, NY) supplemented with 10% fetal calf serum (FCS), 10 mM HEPES, 20 mM $NaHCO_3$, anti-mitotics (10^{-5} M of 5-fluoro-2-deoxyuridine, uridine and cytosine arabinoside) to inhibit fibroblast growth, and antibiotics (penicillin G, streptomycin and nystatin) (14, 15, 16, 17). The cultures were maintained at 37°C in a humid atmosphere gassed with 5% CO_2 in air. The experiments were performed within 7 days after the culture preparation.

Indirect immunofluorescence staining.

Peroxisomes were immunocytochemically stained as described (18). Chromaffin cells were fixed with 4% paraformaldehyde/0.1 M potassium phosphate buffer (pH 7.4), and stained using antibodies against rat acyl-CoA oxidase, rat peroxisomal 3-ketoacyl-CoA thiolase, or human catalase, which were kind gifts from Prof. T. Hashimoto (Department of Biochemistry, Shinshu University School of Medicine). FITC-conjugated goat anti-rabbit IgG (TAGO, Burlingame, CA) was used as the second antibody.

Lignoceric acid and palmitic acid oxidation.

Peroxisomal and mitochondrial β-oxidation activity was assessed by measuring the degradation products of [1-^{14}C] lignoceric acid or [1-^{14}C] palmitic acid (CEA, Gif-Sur-Yvette, France) added to the medium (19), with slight modifications. The medium was gradually replaced by minimal essential medium (MEM), and the preparation was preincubated with FCS-free MEM. The fatty acid oxidation reaction was initiated by adding 4 nmol of [1-^{14}C] lignoceric acid. After incubating the preparation at 37°C for 1 h, the medium was chilled on ice, deproteinized with 5% bovine serum albumin and 3N perchloric acid, and the acid soluble radioactive degradation products were then extracted with n-hexane and counted in a spectrophotometer.

Continuous labeling and pulse-chase experiments.

The intracellular processing of peroxisomal enzymes was evaluated by continuous labeling and pulse-chase experiments, as described (20). The medium was gradually replaced by MEM, and the preparation was preincubated with methionine-free MEM supplemented with 5% dialyzed FCS for 1 h. Continuous labeling was performed for 24 h using 1.85 MBq of [^{35}S] methionine (ICN Radiochemicals, Costa Mesa, CA). Pulse-labeling was done for 2 h using 7.4 MBq of [^{35}S] methionine followed by 3, 12 and 24 h chases. Immunoprecipitation was performed with the use of antibodies against catalase, acyl-CoA oxidase, L-bifunctional protein and the peroxisomal 3-ketoacyl-CoA thiolase. Fluorography was done using an autoradiography enhancer ENHANCE™ (NEN, Boston, MA) according to the manufacturer's instructions.

RESULTS

Immunofluorescence staining.

The peroxisomes in cultured bovine adrenal chromaffin cells were stained in a granular pattern with the use of anti-acyl-CoA oxidase (Fig. 1A) and anti-peroxisomal 3-ketoacyl-CoA thiolase (Fig. 1B), as was the case with skin fibroblasts (18). The mitochondria

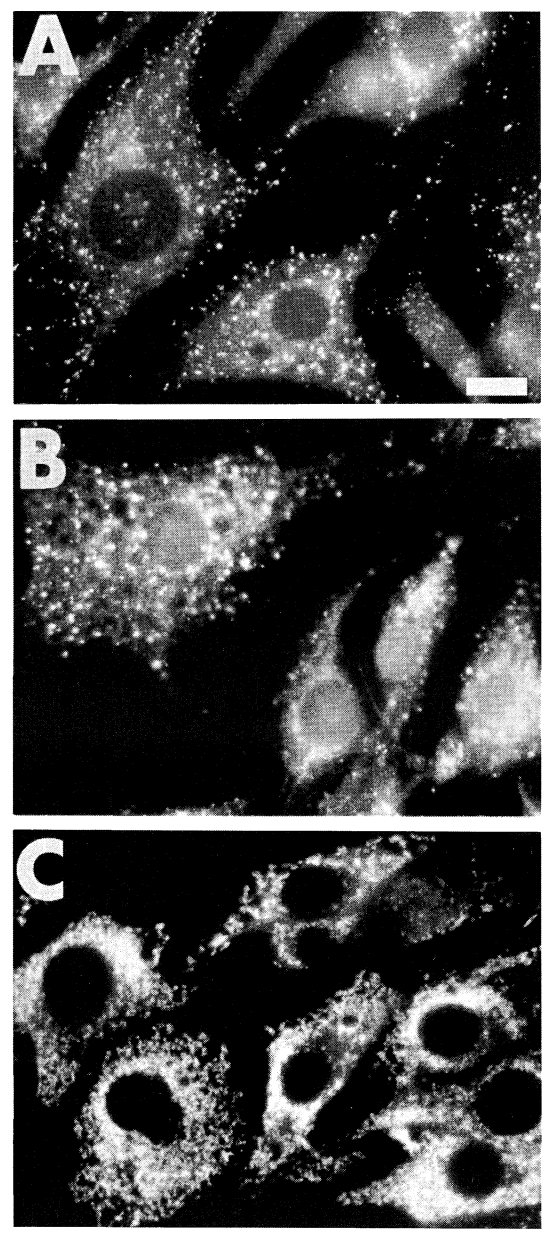

Fig. 1. Immunofluorescence staining of peroxisomes and mitochondria in the bovine adrenal chromaffin cells. A, stained with anti-peroxisomal acyl-CoA oxidase; B, stained with anti-peroxisomal 3-ketoacyl-CoA thiolase; C, stained with anti-mitochondrial 3-ketoacyl-CoA thiolase. Bar = 10 μm.

seemed to be more abundant in the chromaffin cells than in fibroblasts when stained with mitochondrial 3-ketoacyl-CoA thiolase (Fig. 1C), which may relate to the thickness of the chromaffin cells.

Lignoceric acid and palmitic acid oxidation.

Both palmitic acid and lignoceric acid oxidation, which reflect the mitochondrial and peroxisomal β-oxidation activity, respectively, were detectable (Fig. 2). The ^{14}C-labelled acid-soluble degradation products in the medium increased linearly up to 1 h. The mean oxidation rates of [1-^{14}C] lignoceric acid and that of [1-^{14}C] palmitic acid were 138 and 830 pmol/h.mg protein, respectively. The ratio of lignoceric acid oxidation to that of palmitic acid oxidation was 16.6%. The peroxisomal β-oxidation activity, as evaluated by the lignocerate oxidation rate and the ratio of lignocerate oxidation to that of palmitate oxidation, was slightly lower than that in fibroblasts (19), whereas the mitochondrial activity was similar to that observed in fibroblasts.

Continuous labeling and pulse-chase experiments.

The continuous labelling of chromaffin cells showed clear bands of catalase (62 kD), acyl-CoA oxidase (75 kD subunit A, 53 kD subunit B and 22 kD subunit C) and peroxisomal 3-ketoacyl-CoA thiolase (44 kD precursor and 41 kD mature enzyme). L-bifunctional protein was hardly detectable (Fig. 3). The biosynthesis and intracellular processing of acyl-CoA oxidase and the peroxisomal 3-ketoacyl-CoA thiolase was slower than that in fibroblasts (20) (Fig. 4A, B). Subunit B of acyl-CoA oxidase was clearly detected in 1 h-pulse in fibroblasts (20), whereas it was very faint in 2 h-pulse in the chromaffin cells. Precursor of the peroxisomal thiolase disappeared in 6 h-chase in fibroblasts (20), whereas it was detected even in 12 h- or 24 h-chase in the chromaffin cells.

DISCUSSION

Neuroectodermal cells in the neural crest migrate to the adrenal medulla and differentiate to the chromaffin cells and sympathetic nerves. Chromaffin cells have the ability to synthesize and secrete catecholamines under the control of presynaptic neurons, and the primary culture method for chromaffin cells is well established (14, 15, 16, 17). However, the metabolic functions of chromaffin cells other than the metabolism of catecholamines have not been well investigated.

Here we examined the biochemical and immunocyto-

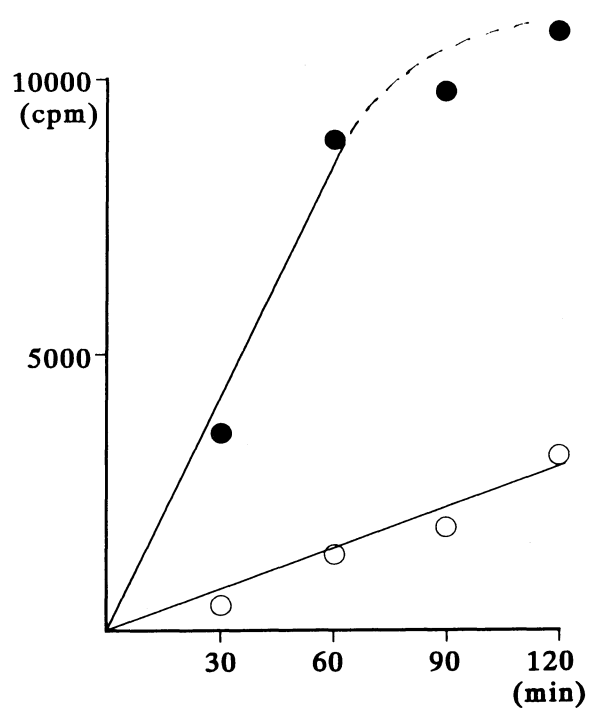

Fig. 2. Lignoceric acid (○) and palmitic acid (●) oxidation in the chromaffin cells. The ^{14}C-labelled acid-soluble degradation products in the medium after incubation for 30 min, 60 min, 90 min and 120 min were measured.

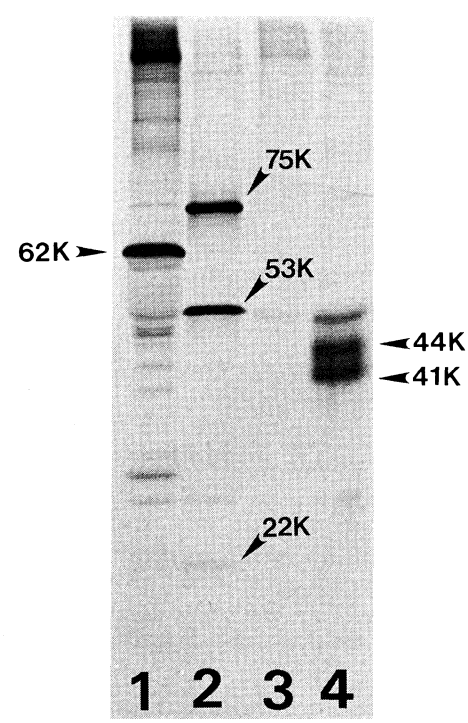

Fig. 3. Continuous labelling of chromaffin cells with [^{35}S]methionine. Lane 1, catalase (62 kD); lane 2, acyl-CoA oxidase (75 kD subunit A, 53 kD subunit B and 22 kD subunit C); lane 3, L-bifunctional protein; lane 4, peroxisomal 3-ketoacyl-CoA thiolase (44 kD precursor and 41 kD mature enzyme). L-bifunctional protein was hardly detectable.

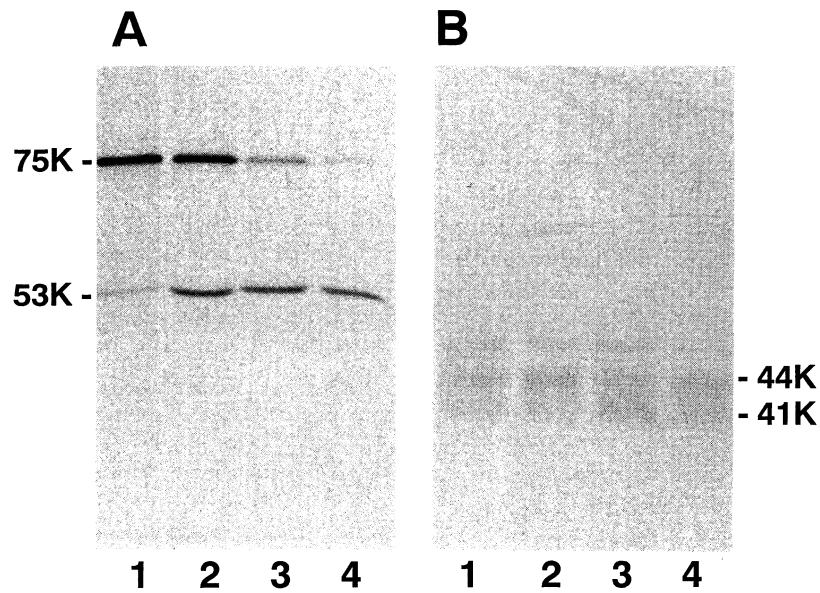

Fig. 4. Biosynthesis and intracellular processing of acyl-CoA oxidase and peroxisomal 3-ketoacyl-CoA thiolase. A, acyl-CoA oxidase; B, peroxisomal 3-ketoacyl-CoA thiolase; lane 1, pulse labelling for 2 h; lane 2, chase for 3 h; lane 3, chase for 12 h; lane 4, chase for 24 h.

chemical properties of peroxisomes and mitochondria in cultured bovine adrenal chromaffin cells as a model of neurons, since there is no data available on the metabolic functions of peroxisomes and mitochondria in cultured neural cells. Peroxisomes are considered to be essential for neurons, based on the finding that patients with an inborn error of peroxisome biogenesis such as Zellweger syndrome and neonatal adrenoleukodystrophy manifest severe neurologic dysfunction and a typical neuronal migration disorder (9, 10). Patients with peroxisomal biogenesis disorders also manifest adrenocortical dysfunction.

In our present experiments, the presence of peroxisomes was shown by immunofluorescence staining, peroxisomal enzymes were synthesized, and the peroxisomal β-oxidation activity was detected. The peroxisomal β-oxidation activity was slightly lower than that reported in fibroblasts (19, 21). L-bifunctional protein was hardly detectable as was the case with fibroblasts (20). Recent study revealed that D-bifunctional protein plays essential roles in the β-oxidation of very long chain fatty acids, intermediates of bile acids and branched chain fatty acids (8). D-bifunctional protein is present in both hepatic and extra-hepatic tissues, whereas L-bifunctional protein seems to be present predominantly in the liver and plays a role in the degradation of very long chain fatty acids (8). D-bifunctional protein may be a key enzyme in the chromaffin cells as in the case of fibroblasts. Another characteristic finding is that the processing of the acyl-CoA oxidase and the peroxisomal 3-ketoacyl-CoA thiolase is slower than that in fibroblasts (20). It remains to be clarified whether this phenomenon is due to the difference of culture condition or due to the difference of nature of these two cells.

The peroxisomal β-oxidation system has several important roles, including degradation of very long chain fatty acids, xenocompounds, eicosanoids, and the biosynthesis of bile acids. Although we did not obtain direct evidence that peroxisomes and mitochondria play essential roles in the chromaffin cells, the evidence suggests that peroxisomes may have an important role in autonomic nervous cells. This study also clarified the presence of abundant mitochondria in chromaffin cells, suggesting that mitochondria may supply energy for the active metabolism of catecholamines.

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