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A LIPID DROPLET-SPECIFIC CAPSULE IS PRESENT IN RAT ADRENAL CELLS: EVIDENCE FROM A MONOCLONAL ANTIBODY

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SUMMARY We have used a monoclonal antibody, A2, to study the structure and function on the lipid droplet capsule in steroidogenic adrenal cells. This antibody reacts with a 160-kD protein found in the rat adrenal cortex. Immunofluorescence microscopy shows a dominant rim pattern, which surrounds individual lipid droplets and is distinct from the filamentous vimentin staining. The boundary of lipid droplets in steroidgenic Leydig cells and 3T3 adipocytes is also immunostained by this antibody. The strong association of the 160-kD protein with the lipid droplet is demonstrated by its resistance to Triton X-100 extraction. Stimulation of steroid secretion by adrenocorticotropin results in the detachment of this protein from the lipid droplet and its movement to the cytosol. These findings suggest that the translocation of this 160-kD protein from lipid droplet surface to cytosol on stimulation might be important in facilitating the binding of cholesterol ester hydrolase to the surface of lipid droplets, as proposed for adipocytes, during lipolytic stimulation.

In adrenocortical cells, most of the cholesterol used in steroid production is stored in the cytoplasmic lipid droplets. Following corticotropin stimulation, cholesterol ester hydrolase catalyzes the release of choletserol from the lipid droplet

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(1). The surface of the droplet might therefore be involved in the binding of the enzyme and in preventing premature fusion of growing lipid droplets. However, little is known about the structure and biochemical nature of the droplet surface.

In 3T3-adipocytes, the vimentin filaments rearranged from a fibrillar network into a cage structure, composed of regularly spaced filaments, which surrounds each individual nascent lipid droplet (2). In contrast, no vimentin cage is found in adrenal Y-1 and bovine fasciculata cells, in which the lipid droplets are randomly attached to vimentin filaments (3,4). In last two cell types, lipid droplets are found, at the electron microscopic level to be surrounded by a layer of electron-dense substance, known as the capsule (4). However, little information is available concerning the nature and function of the coating material.

Recently, during a tissue screening, a monoclonal antibody, A2, raised against the cytoskeleton of xanthophores, was unexpectedly found to immunostain the boundary of the lipid droplet in adrenal cortical cells. The primary objective of this investigation was to use this antibody to demonstrate the presence of the capsule, by immunofluorescence, and also to examine morphological changes of the capsule in response to steroidogenic activation.

MATERIALS AND METHODS

CELL ISOLATION AND CELL CULTURE

Young Wistar rats (either sex), aged 6-8 weeks, were anesthetized with 7% chloral hydrate by intraperitoneal injection. The adrenal glands were trimmed to remove fat, cut into small pieces and incubated in enzyme solution (1.2 mg/ml collagenase, 0.1% bovine serum albumin and 25 mM HEPES in Dulbecco's modified Eagle's medium, pH 7.2) at 34°C for 30 min with gentle shaking to dissociate the cells; the cell suspension was then diluted with Dulbecco's modified Eagle's medium, containing 0.1% bovine serum albumin, and the cells collected by centrifugation (150 g for 5 min). They were then plated on coverslips in 35 mm culture dishes and maintained in medium 199, supplemented with 5% fetal calf serum and 100 IU/ml of penicillin and streptomycin, at 37°C for 3 days. For stimulation of steroidogenic activity, adrenocorticotropin was added at a final concentration of 0.01 I.U/ml, to the culture medium for 2-18 hours.

ANTIBODIES

Monoclonal antibody A2 was produced in our laboratory using a crude preparation of cytoskeletal proteins from goldfish xanthophores as the immunogen. BALB/c mice were immunized by intraperitoneal injection of antigen in adjuvant. Procedures for cell fusion, hybridoma screening and cloning have been previously described (5). The culture supernatants were screened for the presence of immunoglobins by dot assay and immunofluorescence, and antibody A2 (IgM) was selected for a further study using xanthophores. Meanwhile, the antibody was also screened for crossreactivity with other tissues, and was found to give a positive immunoreaction with the cytoplasm of adrenal cortical cells.

WESTERN BLOTTING

The rat adrenal cortex or cultured cells were homogenized in PBS. An equal volume of sample buffer was added and the mixture heated at 90°C for 3 min. Proteins (200 μg per lane) were electrophoresed on 10% SDS polyacrylamide gels and transferred to a nitrocellulose membrane. Strips from the membrane were then blocked with 5% non-fat milk in PBS and incubated in primary antibodies at 4°C overnight. After washes with PBS, containing 0.1% Tween-20, the strips were incubated with peroxidase-conjugated secondary antibodies and positive bands visualized using 4-chloro-1-naphthol as chromogen.

IMMUNOFLUORESCENCE MICROSCOPY

For antibody A2 staining, cells were washed with PBS, then fixed with 0.15% glutaraldehyde in lysis buffer (0.15% Triton X-100, 2 mM $MgCl_2$, 10 mM EGTA, 60 mM PIPES and 25 mM HEPES, pH 6.9) for 5 min. Treatment with $NaBH_4$ (1 mg/ml) in PBS for 30 min was used to block nonspecific binding sites. The cells were then incubated with primary antibodies at 37°C for 1 h, washed with PBS and reacted with FITC-conjugated goat anti-mouse IgG antibody at 37°C for 1 h. The immunostained cells were washed with PBS, mounted using 3% n-propyl gallate and 50% glycerol in PBS and examined with a Leitz epifluorescence microscope.

RESULTS

Using antibody A2, immunoblot analysis revealed the presence of a 160 kD immunoreactive polypeptide in rat adrenal cortex (Fig.1), which accounted for approximately 0.2 % of the total cellular protein.

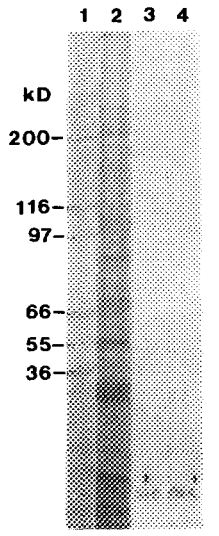

Fig. 1. Characterization of monoclonal antibody A2. 1, Molecular weight standards. 2, Total adrenal gland proteins. Amido black stain. 3, Immunoblot using antibody A2. A 160-kD immunoreactive polypeptide is identified. 4, Control without primary antibody.

Cultured rat adrenal cells contain many lipid droplets of varying size. The vimentin filaments appeared as a complex network in the cytoplasm (Fig. 2A). No vimentin cage structure of the type seen encircling lipid droplets in adipocytes (1) was found in the adrenal cells. When the cells were immunostained with antibody A2, a unique rim, or capsular pattern, was seen at the surface of individual lipid droplets (Fig. 2B). Double labelling experiments showed that this particular staining pattern was not superimposed on that for vimentin filaments (Fig. 2). This capsular staining

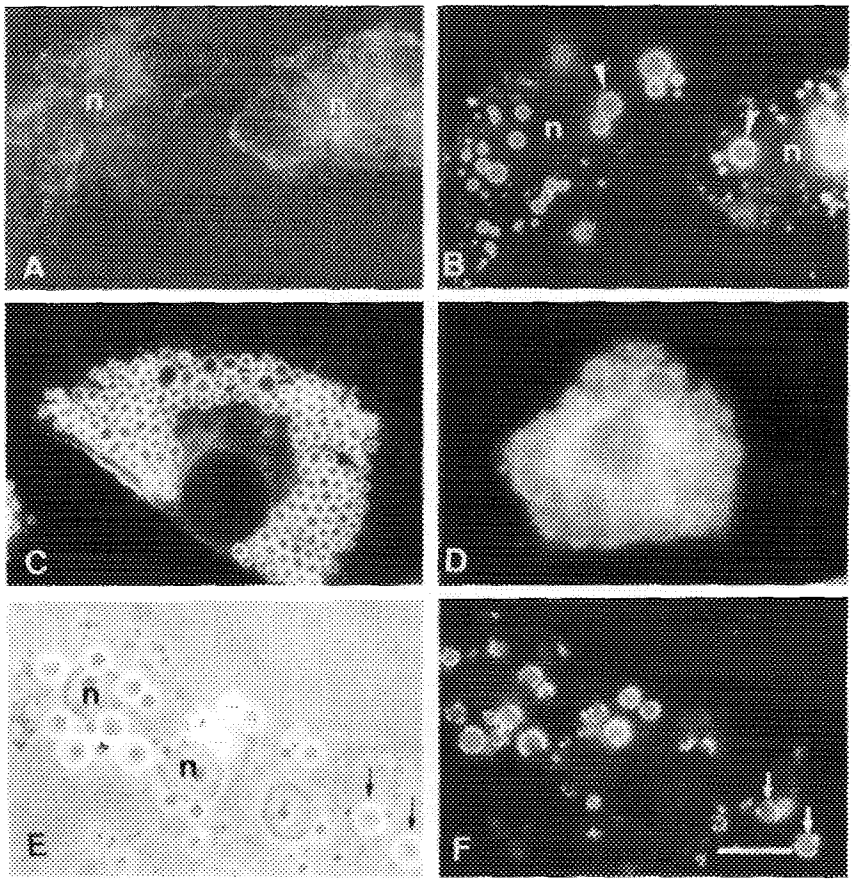

Fig. 2. *Immunofluorescence staining of adrenal cells, adipocyets and Leydig cells. A and B, Adrenal cells doubly immunostained with anti-vimentin (A) and antibody A2 (B). Staining of a continuous capsule is seen encircling each droplet (arrowheads). Antibody A2 also stains the capsule of lipid droplets in hamster Leydig cells (C) and 3T3 adipocytes after lipid conversion (D). E and F, Adrenal cells extracted with 0.5% Triton X-100. E is the phase pair of F. This treatment results in a dotted staining pattern of the capsule. Arrows indicate the isolated lipid droplets. n, nucleus. Bar, 10 μm.*

on lipid droplets is also seen in hamster Leydig cells (Fig. 2C) and 3T3 adipocyets (Fig. 2D) with the antibody A2.

When extracted with 0.5% Triton X-100, the lipid droplets in the cytoplasm became enlarged and the antibody A2 staining became a discontinuous pattern (Fig. 2 E anf F), possibly due to uneven distribution of the 160 kD protein over a larger surface area or to a partial removal of this protein by detergent. In some isolated lipid globules released from vigorously-extracted cells, staining was retained (Fig. 2E and F), suggesting a firm association of the 160 kD protein with the lipid droplet.

In order to study the function of the capsule, we challenged the cultured cells with adrenocorticotropin to activate steroidogenesis. In control cells, bright fluorescent staining was found exclusively on the capsule of lipid droplets (Fig. 3 A and B). As shown in a time-course study, 14 h after stimulation, the lipid droplets became smaller and irregular in shape and the intensity of fluorescent staining of the capsule was significantly reduced (Fig. 3 C-H). A discontinuous capsular staining was also noted in Figs. 3E and G. In many cases, fluorescence was no longer localized on the lipid droplets, especially on the smaller ones (Fig. 3 G and H), but now appeared as a diffuse staining in the cytosol. In immunoblots prepared from control and stimulated cells, no change in the intensity of 160 kD protein band was detected (Fig. 4).

DISCUSSION

For years, little attention has been paid to the composition and function of the proteins in the lipid fraction. Recently, perilipin A and B have been found closely associated with the periphery of lipid droplets in freshly-prepared and 3T3- adipocytes (6.7) and perilipin A and C in adrenal Y-1 cells and MA-10 Leydig cells by Western and Northern blot analyses (8,9). In view of the hyperphosphorylation response to hormonal stimulation, it has been proposed that the perilipins might have a role in the packaging or mobilization of lipids across the lipid-cytosol interface in adipocytes (7).

In the present study, we provide direct evidence for the presence of a capsular protein on lipid droplets in rat adrenal cells. This protein, detected by antibody A2, is a 160 kD polypeptide which relocates from the capsule to the cytosol upon hormone stimulation. The capsule may also contain proteins other than the 160 kD protein; if so, it will be necessary to determine whether all capsular proteins are involved in this redistribution.

The question then arises as to how and why this process occurrs during steroidogenesis. A strong association of the 160 kD protein with lipid droplets was

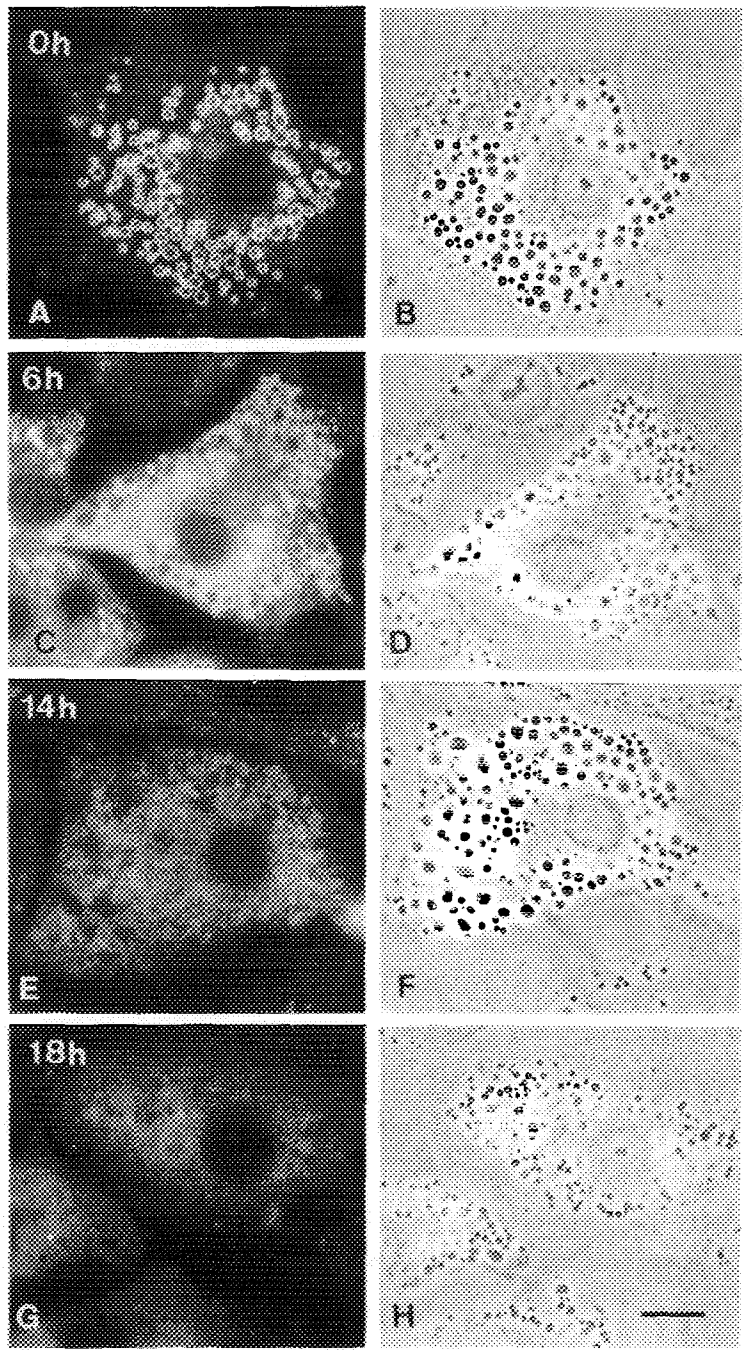

Fig. 3. Effect of adrenocorticotropin application on the immunoreactivity of the capsule . A and B, control. C and D, E and F, G and H present 6 h, 14 h and 18 h after stimulation. B , D , F and H are the phase images of A, C, E and G (stained by antibody A2), respectively. Both large and small lipid droplets are surrounded by an intact layer of highly fluorescent capsule in the control cells (A and B). 14 to 18 h following hormonal stimulation, the staining intensity of the capsules is gradually reduced. Bar, 10 μm.

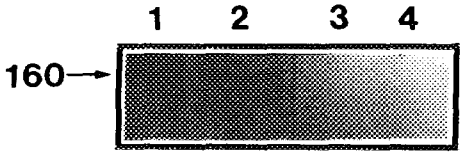

Fig. 4. Effect of ACTH on immunoblotting of adrenal cells. 1, Immunoblot of control cells. 2, immunoblot of stimulated cells. The reaction intensity of 160-kD protein band is equivalent to that of the control. 3 and 4, controls without primary antibody.

demonstrated by the resistance to Triton X-100 extraction. On the basis of the relatively equal amount of 160 kD protein present in cells before and after stimulation, it is possible that hormonal stimulation activates certain kinases and thus changes the conformation of the 160 kD protein, e.g., phosphorylation or dephosphorylation, resulting in its detachment from the lipid droplets; however, this remains to be verified. A hormone-sensitive lipase (84 kD), also known as cholesterol ester hydrolase found in the adrenal cortex (10), has been shown to be phosphorylated and translocated from the cytosol to the lipid droplets in freshly-prepared and 3T3-adipocytes aupon lipolytic stimulation (11,12). Dissociation of the 160 kD protein from lipid droplets might expose cholesterol ester to the direct action of the lipase, thus enhancing the process of cholesterol ester hydrolysis. The presence of capsular proteins in unstimulated cells would prevent the access of lipase to the cholesterol-storage lipid droplets.

We believe that the use of this particular probe, antibody A2, will lead to a better understanding of the molecular basis of the mobilization of cholesterol from lipid droplets.

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