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ULTRASTRUCTURAL IMMUNOCYTOCHEMISTRY OF A LEYDIG CELL ENZIME AND A SPERM TAIL PROTEIN USING ANTIGEN RETRIEVAL BY MICROWAVE IRRADIATION

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RESUM

Presentem aquí un protocol simple i ràpid per a la localització de diversos antígens en mostres biològiques incloses en resines acríliques per electromicroscòpia i immunoprecipitació amb or. S'han utilitzat cèl·lules de Leydig de rates adultes separades per gradients de densitat i espermatozoides humans per estudiar la localització subcel·lular d'antígens específics i analitzar la influència de l'exposició a la irradiació per microones sobre la immunoreactivitat valorada per marcatge d'or immunoprecipitat sobre material fixat per formaldehid i inclòs en LR White. Es varen utilitzar seccions ultrafines muntades sobre suports de níquel i immerses en tampó citrat, exposant-les a la irradiació per microones (a màxima potència: 800 W) durant breus períodes de temps. La microscòpia immunoelectrònica es va fer utilitzant anticossos primaris contra l'enzim esteroidogènic (3-beta-hidroxiesteroid-deshidrogenasa, 3-beta-HSD) del reticle endoplasmàtic lliss i una proteïna del citosquelet de l'embolcall fibrós de l'espermatozoide (AKAP4). L'anticòs secundari utilitzat fou una immunoglobulina anticonill conjugada amb or (partícules de 15 nm). Les seccions control es varen incubar en sèrum no immune. Les seccions varen ser breument exposades a tetraòxid d'osmi i a acetat d'uranil o foren analitzades, senzillament, sense cap tipus de tinció. El marcatge es va expressar per densitat de partícules d'or per μm^2. El soroll de fons fou negligible. La localització de la 3-beta-HSD fou específica en el reticle endoplasmàtic lliss de les cèl·lules de Leydig, mentre que els mitocondris i les gotes lipídiques foren negatives. La proteïna AKAP4, principal component de l'embolcall fibrós de la cua de l'espermatozoide i clàssicament localitzada en les fibres d'aquest embolcall fibrós, i en altres components de la cua de l'espermatozoide, varen ser negatius. Després de l'exposició de les seccions a les microones, la densitat de partícules d'or en el reticle endoplasmàtic lliss de les cèl·lules de Leydig i en l'embolcall fibrós dels espermatozoides havia augmentat tres vegades en relació amb els mateixos teixits no exposats a les microones, sense comprometre la localització específica de la immunotinció. Aquest mètode permet un augment significatiu de la sensibilitat i de la densitat del marcatge sense modificar les concentracions d'anticòs, l'especificitat o la

tinció no específica o de soroll de fons.

Paraules clau: immunomicroscòpia electrònica, tinció amb or, cèl·lula de Leydig, espermatozoides, irradiació amb microones.

SUMMARY

We report here a simple and fast protocol for enhanced immunogold electronmicroscopic localization of various antigens in biological materials embedded in acrylic resins. Adult rat Leydig cells separated by density gradients and washed human spermatozoa were used to study the subcellular localization of specific antigens and to test the influence of exposure to microwave irradiation on the immunoreactivity assessed by immunogold labeling on formaldehyde fixed, LR White embedded material. Ultrathin sections mounted on nickel grids and immersed in citrate buffer were exposed for brief periods to microwave irradiation at full power (800 W). Immunoelectron microscopy was achieved using primary antibodies raised against a smooth endoplasmic reticulum steroidogenic enzyme (3β Hidroxysteroid Dehydrogenase, 3β-HSD) and a cytoskeletal protein of the sperm fibrous sheath (AKAP4). The secondary antibody was a gold conjugated (15 nm particles) anti-rabbit immunoglobulin. Control sections were incubated in non immune serum. Sections were briefly exposed to osmium tetraoxide and uranyl acetate or were observed without any further staining. Labeling was expressed as gold particle density per μm^2. Background labeling was negligible. 3β-HSD specifically localized to the smooth endoplasmic reticulum of Leydig cells, while mitochondria and lipid droplets were negative. AKAP4, the main protein component of the fibrous sheath of the sperm tail typically localized to the fibers of the fibrous sheath, while other components of the sperm tail were negative. After on section exposure to microwaves, gold density over the Smooth Endoplasmic Reticulum of Leydig cells and the fibrous sheath of spermatozoa increased by three fold over the same tissues not pretreated by microwaves without compromising the specific localization of the immunolabeling. This method allows for a significant increase of sensitivity and labeling density without modifying antibody concentrations, specificity or background staining.

Keywords: immunoelectronmicroscopy, gold labeling, Leydig cell, spermatozoa, microwave irradiation.

INTRODUCTION

Antigen retrieval techniques by microwaves have been extensively used to enhance the immunoreactivity of a wide range of paraffin embedded normal and pathologic tissues (Cuevas *et al*., 1994). This technique has allowed the use of higher primary antibody dilutions leading to an increased labeling specificity, and has been applied to the immunolocalization of antigens previously demonstrable only on unfixed sections. Increased antigen immunoreactivity has also been achieved with microwave heating of freshly frozen cryostat sections or fixed vibratome tissue slices in pre-embeddment protocols (DeHart *et al*., 1996; Stone *et al*., 1999). More recently, these techniques have been extended to on section immunoelectron microscopy of tissues embedded in Epoxy resins or metacrylates (Xiao *et al*., 1996; Brorson *et al*., 1998; Leong *et al*., 1998). We report here a simple and fast protocol for enhanced immunogold electronmicroscopic localization of various antigens in biological materials embedded in acrylic resins. We have applied this

protocol to the study of the subcellular localization of 3β-HSD, a steroidogenic enzyme of Leydig cells and of AKAP4 the main protein component of the fibrous sheath of human spermatozoa.

MATERIALS AND METHODS

The materials used for immunolocalization were pellets of spermatozoa from infertile patients suffering from a hyperplastic disorder of the fibrous sheath (Chemes *et al*., 1998), and rat testicular Leydig cells. Spermatozoa were pelleted in a table centrifuge after dilution of semen (1:5) in 0.1 M phosphate buffer, pH 7.4. Leydig cells were collected from testes of normal adult rats by sequential digestion with collagenase, filtration and purification in discontinuous Percoll density gradients. Pellets of spermatozoa or Leydig cells were fixed for 1 hour at 4° C in 5 % phosphate buffered formaldehyde (0.1 M, pH 7.4), rinsed in buffer, dehydrated in an increasing series of ethanol, infiltrated in LR-White Resin, medium grade (London Resin Co, Ltd, Reading, England) and polymerized at 60° C for 24 hours. Thin sections displaying pale gold to silver interference colors were mounted on 300 mesh nickel grids and dried at room temperature. On section ultrastructural immunocytochemical localization was performed with or without pre treatment with microwaves. The grids were hydrated by flotation on distilled water drops. Antigen retrieval was attempted by immersing the grids in 10 mM Na citrate buffer pH 6.0 and subjecting them to 1 minute of microwave irradiation at 800 watts. The grids were subsequently washed and incubated for 30 minutes at room temperature in Blocking Buffer (tris buffered saline: TBS 225 nM, pH 7.5 + 10% normal goat serum) and then floated on drops of primary antibody on a humid chamber overnight at 4° C. Primary antibodies were a rabbit antibody against AKAP4 (the main protein constituent of the sperm fibrous sheath, Carrera *et al*., 1994) at a 1:100 dilution and a rabbit antibody against human placental 3β-Hydroxysteroid Dehydrogenase (3β-HSD, a smooth endoplasmic reticulum steroidogenic enzyme. Oxygene, Dallas, 1:100). After three washes in TBS the grids were incubated for 1 hour at 4° C with Blocking Buffer containing 15 nm colloidal gold labeled anti-rabbit IgG (Pelco International, Redding, Ca) at 1:25 or 1:50 dilutions, and rinsed three times in TBS. Grids were subsequently counterstained with 1% Osmium Tetraoxide followed by 1:1 aqueous Uranyl acetate: absolute acetone or were left without any further staining. Specimens were studied and photographed in a Zeiss EM109 transmission Electron Microscope (EM, Zeiss, Oberkochen, Germany). Negative controls were processed identically replacing the primary antibody by equivalent dilutions of non-immune rabbit serum.

For quantification of gold particle density, EM pictures of similar magnification were used. Gold particles were counted in square areas and the number of particles per area was divided by the corresponding surface. Density was expressed as number of particles/μm^2.

RESULTS

Two kinds of biological materials were used to test the influence of exposure to microwave irradiation on the immunoreactivity assessed by immunogold labeling. Primary antibodies raised against 3β-HSD and a cytoskeletal protein of the sperm fibrous sheath (AKAP4) were used in these experiments. 3β-HSD localization was attempted in purified rat Leydig cells and AKAP4 localization in pelleted human spermatozoa. In both cases, ultrastructural immunocytochemical localization was first tried in tissues not subjected to microwaves.

Figure 1A depicts the ultrastructure of a normal adult rat Leydig cell obtained af-

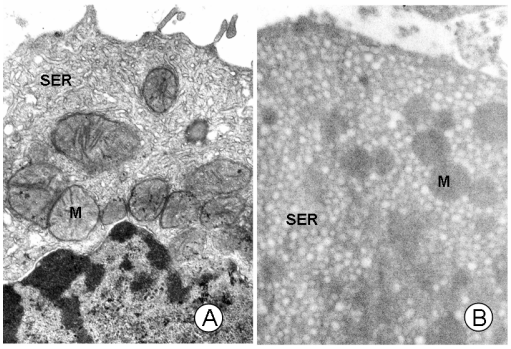

FIGURE 1. A: normal rat Leydig cell, fixed in glutaraldehyde-osmium, embedded in Epon Araldite and stained with lead citrate-uranyl acetate. Note normal ultrastructural characteristics with abundant smooth endoplasmic reticulum (SER) and steroidogenic type mitochondria (M). B: negative control subjected to microwave irradiation in citrate buffer and normal rabbit serum instead of primary antibody. The smooth endoplasmic reticulum is seen in the form of spherical vesicles (SER) and mitochondria (M) as dense round structures. Note absence of gold particles.

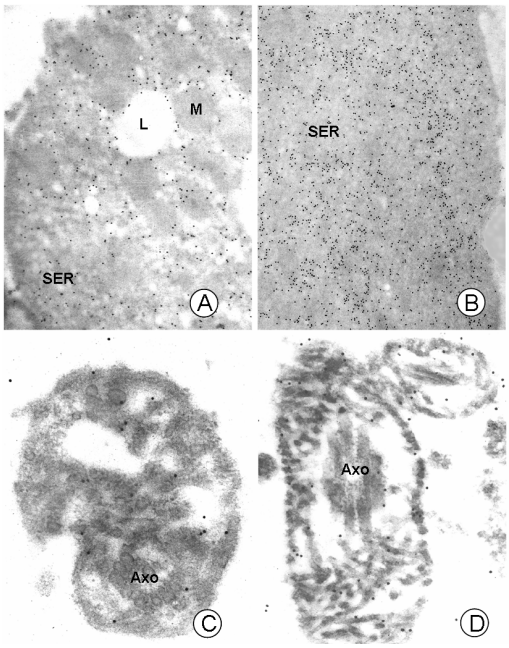

FIGURE 2. A: No antigen retrieval, anti 3β-HSD antibody (1:100 dilution). Coloidal gold particle density up to 22 particles/μm^2 over the smooth endoplasmic reticulum (SER). Mitochondria (M) and lipid droplets (L) are negative for gold labeling. B: Antigen retrieval (microwaves and citrate buffer). Same dilution of 3β-HSD antibody. Note very significant increase in labeling density over A (up to 127 particles/μm^2). C, D: tails of human spermatozoa treated with anti AKAP4 at 1:100 dilution. The gold label is over the hyperplastic fibers of the fibrous sheath. The axoneme (Axo) is negative and the background labeling very low. C: No antigen retrieval, D microwave irradiation in citrate buffer. The labeling density is markedly increased in D over C. Coloidal gold (15 nm particles, 1:25 dilution) was used as secondary antibody in all cases.

ter enzyme digestion and density gradient separation. The main characteristics of this cell are the abundance of smooth endoplasmic reticulum (SER) and steroidogenic type mitochondria in the cytoplasm. The very good preservation observed was achieved after glutaraldehyde-osmium fixation and embeddment in Epon-Araldite resins for routine electronmicroscopic evaluation. Figure 1B depicts a negative control immunocytochemistry (non immune serum) of a Leydig cell similarly isolated. In this case fixation and embeddment were performed as indicated in material and methods to facilitate ultraestructural immunocytochemistry with coloidal gold labeling. Due to brief fixation and staining, cytological preservation and ultrastructural detail are poorer that that observed in figure 1A, particularly at the level of the SER that shows dilatation of the flat cisternae. This negative control shows extremely low gold particle density. When anti 3β-HSD antibodies were used on normal rat Leydig cells there was an intense labeling over the SER. The intensity of label varied from cell to cell. When microwave irradiation was omitted the maximal intensity of labeling ranged between 23 and 36 gold particles/μm^2 (mean 29.5 ± 9.1, see figure 2A). A mean three-fold increment was observed after microwave treatment, with maximal values ranging between 72 and 127 gold particles/μm^2 (mean 99.5 ± 38.9, see figure 2B). The gold label specifically localized over the SER and not over mitochondria or lipid droplets and this specific pattern was not modified by microwave treatment.

When human spermatozoa from infertile

patients suffering from Dysplasia of the Fibrous Sheath (Chemes *et al*., 1998) were immunolabelled with anti AKAP4 antibodies the intensity of label was also variable. Maximal labeling intensity in basal conditions (no microwave irradiation) ranged between 10 and 17 gold particles/μm^2 over the disorganized fibrous sheaths of sperm tails (mean 13.5 ± 4.9, see figure 2C). This density increased to values between 24 and 56 gold particles/μm^2 when the samples were subjected to microwave irradiation under citrate buffer (mean 40 ± 22.6, see figure 2D). This also represented a mean three fold increment over the basal condition. The gold label was specifically localized over the fibers of the Fibrous Sheath, but not over the axoneme. Background labeling was neligible as was that observed in control sections (normal rabbit serum instead of primary antibody, not shown).

DISCUSSION

Microwave irradiation has been successfully introduced to accelerate tissue fixation and increase immunoreactivity of numerous tissue antigens on unfixed tissue, vibratome sections of fixed material and paraffin blocks (Cuevas *et al*., 1994; DeHart *et al*., 1996; Leong and Sormunen 1998; Stone *et al*., 1999). More recently, the same techniques have been applied to epoxy and metacrylate embedded materials where the use of postembedding on-section immunoelectron microscopy has demonstrated good potential (Brorson and Strom, 1998). Similar results were achieved by the use of other methods of tissue heating such as autoclaving or pressurized boiling (Xiao *et al*., 1996)

The introduction of colloidal gold particles of sharp definition and varying sizes in ultrastructural immunocytochemistry has made possible the precise subcellular localization of tissue antigens without compromising subcellular detail. Provided that the primary antibody is of good quality, the concentration and accessibility of antigens in the tissue under study is probably the primary determinant of labeling density in immunogold electron microscopy. In addition to these factors, various modifications in the protocol utilized can lead to stronger tissue labeling. Such a result can be achieved by increasing the concentration of the primary antibody or the gold labeled secondary antibody, but beyond certain concentrations this approach may result in loss of specificity and/or unwanted background. The method here presented allows for a significant increase of labeling density without modifying antibody concentrations. This is due to enhanced immunoreactivity by microwave irradiation that "unmasks" tissue antigens concealed by aldehyde fixation-induced protein cross-linking, making them more accessible to the primary antibody. A short 1-minute exposure in a family type microwave oven resulted in a dramatic three-fold increment of particle density with no detectable changes in specificity and very low levels of background labeling. Similar results were obtained by Brorson and Nguyen (2001) in autoclaved epoxy sections heated to 135° C which yielded very important increases in labeling density. This technique is particularly important in cases of low or negative labeling. The same group (Brorson *et al*., 1999) has indicated that the increase of labeling due to heating LR White embedded tissues only operates for paraformaldehyde fixed material. This can be extended to formalin-fixed tissues, as is the case for human spermatozoa and rat Leydig cells in the present report. The subcellular localization observed in the present study confirms the specificity of the label, over the cisternae of the SER in the cytoplasm of Leydig cells in the case of 3βHSD and overlying the fibers of the fibrous sheath with anti AKAP4 antibodies.

The protocol utilized by us was successful in two different biological materials (of rat and human origin) treated with antibod-

ies raised against human or mouse antigens, indicating that it can be utilized in different situations. Moreover, this postembedding on-section procedure is extremely simple and can be successfully used for the localization of antigens adversely affected by fixation and or embedding.

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