The 17 β -oestradiol dehydrogenase of pig endometrial cells is localized in specialized vesicles

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Two monoclonal antibodies against the 17 β -oestradiol dehydrogenase of pig endometrial cells have been used in localization studies with immunogold electron microscopy. The antibodies attach both to a fraction of dehydrogenase-rich cytoplasmic vesicles isolated from homogenates and to vesicles of similar appearance in cells. The vesicles are filled with electron-dense

material. Their tagging intensity indicates a high degree of specialization. Endometrial cells from mature animals contain a host of dehydrogenase vesicles, and cells from prepubertal animals only a few. Functional aspects of the novel organelle are discussed.

INTRODUCTION

The unidirectional 17 β -oestradiol dehydrogenase of target organs is associated with cytoplasmic structures. A confinement of the enzyme to the cytoplasm of uterine luminal and glandular epithelium was suggested by the histochemical studies of Scublinsky et al. (1976). The exact localization of the enzyme, however, was controversially discussed. It was assigned to the microsomal (Pollow et al., 1975) and mitochondrial (Pollow et al., 1976) fractions of homogenates. We (Entenmann et al., 1980) reported an association of enzymic activity with a sub-population of light lysosomes, an unlikely site in view of the pH optimum of the enzyme. Re-investigations with improved cell-biological techniques allowed the collection of a dehydrogenase-rich fraction with very low marker enzyme activities for mitochondria, rough and smooth microsomes, lysosomes, the Golgi apparatus and the plasma membrane (Adamski et al., 1987; Adamski, 1991). The morphological appearance of the fraction resembled that of similar electron-dense vesicles seen in cell sections. Their identity with the isolated vesicles was investigated by employing the post-embedding technique for the attachment of colloidal gold covered with the F(ab')₂ fragments of two monoclonal antibodies against oestradiol dehydrogenase.

EXPERIMENTAL

Antibodies

The monoclonal antibodies F1 and W1 against pig oestradiol dehydrogenase were produced and purified as described by Adamski et al. (1992). Both antibodies are of the IgG₁ subclass and recognize native as well as denatured membrane-bound oestradiol dehydrogenase. The IgGs were digested with pepsin to obtain F(ab')₂ fragments as described by Nisonoff et al. (1960). Particles of 10 nm colloidal gold were prepared as described by Slot and Geutze (1985) and coated with F(ab')₂ fragments as described by Varndell and Polak (1987). BSA (0.5%, w/w) was used as a stabilizer. The F(ab')₂-Au solutions were adjusted to A₅₂₀ 1.7 with 0.5% BSA in 5 mM borate/5 mM NaN₃, pH 9.0, passed through a sterile 0.45 μ m HV Millipore filter and stored at 4 °C.

Electron microscopy

Pig endometrium cells were collected by curettage (Sierralta et al., 1978), fixed in 0.5% glutaraldehyde/4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.2, for 1 h at 4 °C, washed with

phosphate buffer and dehydrated in graded ethanol series for embedding in hydrophilic LR-Gold resin (Sierralta et al., 1992). Sections of 70–80 nm thickness were cut with a Reichert-Jung Ultracut, mounted on pioloform-coated gold grids for post-embedding immunoreaction: preincubation with PBS containing 0.05% Tween 20, 0.5% BSA (PBSTB buffer) and 0.1 M glycine for 30 min at room temperature, three washes with PBSTB, overnight incubation at 4 °C by floating on 20 μ l of F(ab')₂-Au solution. Controls were performed by either preincubations with F(ab')₂ fragments of monoclonal antibodies or by incubations with F(ab')₂-Au adsorbed with purified oestradiol dehydrogenase before immunolabelling. The sections were washed at room temperature with three changes of PBSTB, jet-washed with water and post-fixed with 0.5% OsO₄ in water for 10 min. Counter-staining with saturated uranyl acetate in H₂O for 10 min and in Reynold's lead citrate for 5 s was omitted in sections with low immunogold attachments (controls), to avoid a masking of gold particles by lead crystals. In sections to be observed at low-power magnifications, lead citrate was not used for the same reasons. Samples were viewed in a Philips EM 301 electron microscope.

Subcellular fractionation

Cytoplasmic vesicles rich in oestradiol dehydrogenase activity were isolated from prepubertal pig endometrium cells (Adamski, 1991). Briefly, the homogenates of endometrial cells collected from immature pigs were prepared with a constant-tolerance shearing device (Adamski et al., 1987), centrifuged at 10000 *g*_{av} for 30 min, and the supernatants were subjected to isopycnic centrifugation in 30% Percoll in vertical rotors. Constant-volume sampling (1 ml) proceeded from the bottom of the tube to the meniscus. Markers for endoplasmic reticulum (cytochrome *c* reductase, 7 α -oestrone hydroxylase, oestrone reductase and RNA), Golgi apparatus (thiamin pyrophosphatase, galactosyl-transferase) and plasma membrane (alkaline phosphodiesterase) were found in fractions 32–33, and dense lysosomes in fractions 4–5 (cathepsin D), and were not further processed. Fractions (29 and 30) rich in oestradiol dehydrogenase activity were re-centrifuged on a linear 0.25–2.0 M sucrose density gradient in vertical rotors. Vesicles abundant in oestradiol dehydrogenase equilibrated at 1.18 g/ml (ml 12 of the sucrose gradient). Mitochondrial (isocitrate dehydrogenase), light-lysosomal (acid phosphatase, β -glucosidase) and traces of peroxisomal (peroxidase), endoplasmic-reticulum and Golgi markers were seen in fraction 29.

Figure 1 Immunogold labelling of prepubertal and mature pig endometrial cells

(a) Section of prepubertal cell exposed to W1 F(ab')₂-gold (arrow points at reactive organelle). (b) Section of mature cell exposed to F1 F(ab')₂-gold. (c) Section of mature cell exposed to W1 F(ab')₂-gold; a continuity between a vesicular membrane and the endoplasmic reticulum is marked by an arrow. (d) Control, section of mature cell, preincubated with F1 F(ab')₂ before exposure to W1 F(ab')₂-gold. (a–c) were post-fixed with OsO₄ and contrasted with uranyl acetate. (d) was post-fixed with OsO₄ only to facilitate the detection of a single gold particle (arrow). Bar represents 200 nm.

Figure 2 Alignments of oestradiol dehydrogenase organelle with nuclear membrane and nuclear pore

Sections of mature pig endometrial epithelium incubated with W1 F(ab')₂-gold. (a) Post-fixation with OsO₄ only; (b) post-fixation with OsO₄ and contrasting with uranyl acetate. Bar represents 200 nm.

All materials used, the measurements of marker enzymes and auxiliary techniques have been described in detail elsewhere (Entenmann et al., 1980; Adamski et al., 1987, 1989, 1992; Thole et al., 1991; Sierralta et al., 1992).

RESULTS

Attachments of colloidal gold coated with F(ab')₂ fragments of the monoclonal antibodies were restricted to the cytoplasmic areas of cell sections. The particles associated in groups with vesicles of 120–200 nm diameter and moderately dense matrices (Figure 1). Mitochondria, the endoplasmic reticulum, the Golgi apparatus, the plasma membrane and primary lysosomes remained untagged. The vesicles were surrounded by membranes which became visible at high magnification only after contrasting with uranyl acetate. In some instances continuities between the

membranes of organelles and of the endoplasmic reticulum were observed (Figure 1c). The numbers of vesicles marked and their tagging intensities were independent of the antibodies used (Figures 1b and 1c). Cells from mature animals (Figures 1b and 1c) contained more immunoreactive vesicles than cells from prepubertal pigs (Figure 1a). A predominant localization of the organelles in the cytoplasm was not observed; however,

Table 2 Characterization of vesicles rich in oestradiol dehydrogenase activity

Marker	Activity or amount in homogenate (per mg of protein)	Enrichment (fold) over homogenate	
		Percoll (ml 29–30)	Sucrose (ml 12)
Oestradiol dehydrogenase	1 μ -unit	8.5	19.9
Cytochrome <i>c</i> reductase	390 μ -units	2.7	0.9
7 α -Oestrone hydroxylase	0.2 μ -unit	2.9	0.8
Oestrone reductase	0.02 μ -unit	2.5	1.5
RNA	25.9 μ g	2.4	0.9
Thiamin pyrophosphatase	190 m-units	1.1	1.2
Galactosyltransferase	21 μ -units	1.8	0.9
Isocitrate dehydrogenase	40 m-units	3.9	1.9
Alkaline phosphodiesterase	335 μ -units	1.9	0.5
Acid phosphatase	10 μ -units	3.7	2.0
β -Glucosidase	1.4 m-units	5.1	1.4
Cathepsin D	9 m-units	0.5	0.2
Peroxidase	1.8 μ -units	0.9	0.2

Table 1 Tagging intensities and frequencies of occurrence of dehydrogenase vesicles in prepubertal and mature endometrial cells

Gold particles were counted over 50 cells; values are given as means $\pm$ S.E.M. Controls: preincubations with F(ab')₂ of antibodies before exposure of the same antibodies to F(ab')₂-gold.

Tissue	No. of gold particles/ μ m ² of vesicles	No. of vesicles/ μ m ² of cell
Prepubertal	468 $\pm$ 43	0.09 $\pm$ 0.001
Prepubertal control	9 $\pm$ 2	0.005 $\pm$ 0.002
Mature	484 $\pm$ 55	0.47 $\pm$ 0.045
Mature control	8 $\pm$ 2	0.004 $\pm$ 0.003

Figure 3 Immunostaining of oestradiol dehydrogenase vesicles enriched from homogenates of prepubertal endometrial cells

Fraction (ml) 12 of the sucrose gradient was used. (a) Section treated with W1 F(ab')₂-gold; (b) section preincubated with F(ab')₂ of W1 before exposure to W1 F(ab')₂-gold (control). Post-fixation with OsO₄ was done in (b) only for facilitating the detection of a single gold particle (arrow); additional contrasting with uranyl acetate was done in (a). Bar represents 200 nm.

alignments with the nuclear envelope occurred frequently (Figure 2).

Homogenates of mature cells contain 4.5 ± 0.7 μ -units of oestradiol dehydrogenase activity/mg of protein compared with 0.8 ± 0.2 μ -unit/mg measured in homogenates of prepubertal cells. This is in good agreement with the numbers of tagged organelles counted in sections of cells. However, the tagging intensities of organelles in mature and prepubertal cells are identical (Table 1).

The high frequency of dehydrogenase-containing vesicles in mature cells should make them the preferable source for isolation from homogenates. This is prohibited by the pronounced glycosaminoglycan contents of adult endometrium, which spoil the Percoll-gradient separation. The efficacy of the two-step Percoll/sucrose-gradient technique for the enrichment of oestradiol dehydrogenase activity from homogenates of immature endometrium is reviewed in Table 2. It is paralleled by the high frequency of immunolabelling in sections of the particles harvested from ml 12 of the final sucrose gradient (Figure 3). Their morphology is indistinguishable from that of the vesicles seen in cell sections (Figure 1). A comparison of immuno-tagged sections of a mature cell and of an isolated organelle fraction from prepubertal cells at higher magnification is shown in Figure 4. The similarity of the structures proves the reality *in vivo* of the enzyme vesicles and their lack of impairment by the isolation procedure. The vesicles, both *in situ* and in the isolated fraction, possess single membranes, as observed by negative staining with uranyl acetate. The enhancement with lead citrate does not reveal any particles on these membranes, in

contrast with the rough endoplasmic reticulum visible in the same sections (Figure 4c).

DISCUSSION

The immunogold technique with monospecific polyclonal antibodies and with monoclonal antibodies of high avidity is very reliable, provided that F_c-mediated attachments can be excluded. We coated the colloidal gold with the F(ab')₂ fragments of the antibodies F1 and W1 as a safeguard. The technical quality of our results is essentially comparable with that of the high-resolution assignment of the cytochrome P-450 side-chain-cleavage enzyme to mitochondrial cristae by Farkash et al. (1986), and with the localization of phenobarbital-inducible P-450 enzymes in tubular parts of the endoplasmic reticulum by Marti et al. (1990). The post-embedding immunoreaction with LR Gold sections is superior to pre-embedding immunoreactions. This advantage is at the expense of a limited applicability of contrasting by heavy metals. Uranyl acetate tends to mask the occasional gold particles in controls, and lead citrate totally obscures gold particles at low magnification. The simultaneous detection of immunogold particles and structural details such as membranes therefore demanded cautious compromises.

Our results are at variance with the recently published immunolocalization of membrane-bound oestradiol dehydrogenase in human endometrium, for which Mäentausta et al. (1991) employed antibodies raised against the soluble oestradiol oxidoreductase from human placenta. Those authors saw a sparse attachment of immunogold to membranes 'unrelated to

Figure 4 Comparison of isolated vesicles and vesicles *in situ*

(a) Section of mature cell; (b) and (c) sections of vesicles enriched from homogenates of prepubertal endometrial cells; arrow points at co-isolated fragment of rough endoplasmic reticulum. All sections were exposed to F1 F(ab')₂-gold, and contrasted with uranyl acetate and lead citrate; bar represents 200 nm.

the endoplasmic reticulum, or other distinct cell organelles, such as mitochondria and nuclei'. Our antibodies do not react with the soluble enzyme from human placenta, the amino acid sequence of which has a low similarity to that of the structure-associated dehydrogenase from pig endometrium (Leenders, 1991).

The groups of gold particles attaching to the cross-sections of the vesicles and the good correlation between the number of tagged organelles in the isolated fraction and its enzymic activity indicate a high, if not exclusive, enzyme content. The cytoplasmic structures participating in the formation of the organelles are as yet unknown. An involvement of the plasma membrane via an endocytotic mechanism is unlikely, because a tagging of plasma membranes was not observed, not even in enzyme-rich postpubertal cells. The 17β -oestradiol dehydrogenase-containing organelles could represent dilated cisternae budding off the endoplasmic reticulum. Remaining connections with the

endoplasmic reticulum could be ruptured during the tissue homogenization, which would explain the lack of markers for the smooth endoplasmic reticulum in the fraction of isolated vesicles.

The provoked maturation of prepubertal cells by pulse exposure to oestradiol (or a progestagen?) may be a good model for studying the induction and the packaging of the enzyme. Time-course experiments after a hormonal pulse can also answer the questions of whether the enzyme attenuates the action of incoming oestradiol (Gurpide and Marks, 1981), or oxidizes it after nuclear passage (Jungblut et al., 1979), or both. Each task would gain from a directed mobility of the organelles. Irrespective of either possibility, the observation of vesicles specialized in oestradiol dehydrogenation introduces a new compartment to the subcellular organization of steroid metabolism.

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