Alpha-naphthyl acetate esterase activity of Percoll-isolated rat Leydig cells: Electron microscopic and histochemical study

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Several histochemical methods have been used for light microscopic identification of Leydig cells from rat interstitial tissue. In the present paper, Percoll-isolated Leydig cells was studied using a-naphthyl acetate esterase (ANAE) which is an alternative marker enzyme by electron microscope. After isolation of Leydig cells, they were than incubated for non-specific esterases with a-naphthyl acetate as a substrate and hexazotized pararosanilin as coupler for 5 min. at 4°C (pH 6.5). Leydig cells displayed positive ANAE reaction on their plasma membrane only. No activity was seen within the Leydig cell cytoplasm. As a conclusion, ANAE is a reliable marker enzyme for Leydig cells if certain conditions such as pH, temperature and incubation time are used. [Turk J Med Res 1995; 13(6):172-175]

Key Words: Percoll, Testis, Esterase, Leydig cells, Electron microscopy

Although their metabolic function is still osbcure, non-specific esterases are widely distributed in animal and plant tissues. In mammals, esterase activity is found in the duodenum, liver, kidney, uterus, placenta and brain. Speculations have been made that non-specific esterases are present as a complex tissue-specific mixture of various components and each of them has a tissue-specific biological role such as lipid metabolism, protein synthesis, intracellular transport and drug detoxification, and these enzymes also be used as a marker of various cell types. C-esterase (acetylesterase) is one type of non-specific esterases that is more widespread in high vertebrates. The highest activity has been found in testis, kidney and uterus (1-3).

For light microscopical identification of Leydig cells, histochemical 3p-hydroxysteroid dehydrogenase (3p-HSD) activity have been used most frequently. Besides, a-naphthyl acetate esterase (ANAE) can be used alternatively as a simple and reliable method (2,3). When correct incubation time is used, Leydig cells show positive esterase activity. Results indicate that, ANAE is a useful and reliable marker enzyme for isolated Leydig cells in electron microscopy.

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In this study, a-naphthyl acetate esterase activity was studied in percoll-isolated Leydig cells.

MATERIALS AND METHODS

Seven adult male Wistar rats, aged 3 months, were used, modified method described by Rommerts et al was used for Leydig cell isolation (5). They were sacrified by CO_2 inhalation and testes were removed by abdominal incision and carefully decapsulated. Testes were than placed in 50 ml spinner flask containing 2 mg collagenase (Pharmacia Type 3), 5 mg Bovine serum albumine (BSA) in 10 ml Krebs ringer solution (KRB) at pH 7.4. After 20 min. incubation at 36°C in a shaker bath at 120 cycles/min, the resulting supernatants filtered through nylon gauze, centrifuged at 150 g for 10 min and crude cells resuspended in 1 ml phosphate buffered saline (PBS).

Density gradient was prepared as fallows; 22.5 ml Percoll in 2.5 ml KRB and 20 mg BSA in 25 ml KRB were mixed with the aid of peristaltic pump. Crude cell suspension layered on top of the gradient and centrifuged 20 min at 2500 g. Four bands are obtained and Leydig cells collected from the third band from the top. They were then washed twice with PBS (at 4°C) and centrifugated at 1500 g for 5 min at 4°C. Cells were fixed with 1.25% Glutaraldehyte-Collidine solution for 30 min and washed with collidine-succrose. They were then incubated for enzyme reaction using with a-naphthyl acetate as a substrate and hexazotized pararosanilin as coupler for 5 min at 4°C (pH 6.5) (4).

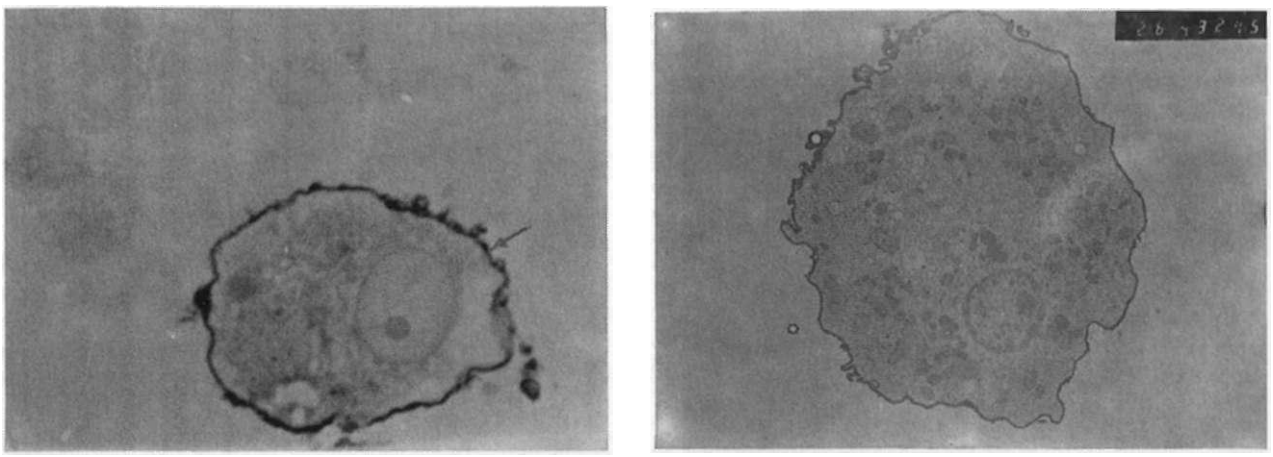

Figure 1a. One jjm cut semi-thin section of isolated Leydig cell (incubation with substrate). Positive ANAE reaction is seen on the plasma membrane only (—). X320

After centrifugation at 2000 g at 4°C, cells were washed and centrifugated (4000 g/3 min) and post fixed with Os04. The resultant cells were embedded in Epon. Ultrathin sections were observed weakly stained with uranyl acetate for low contrast and they were then examined using a Jeol 100 electron microscope.

RESULTS

In this study, a-napthyl acetate esterase activity was studied in Percoll-isolated Leydig cells. All methods particularly isolation and incubation procedure we used worked well. When interstitial cells were fractionated using Percoll gradient, 4 bands were obtained. Band-I was composed of damaged cells, band-II contained germ cells, band-III contained Leydig cells and band IV consisted almost exclusively of red blood cells. Leydig cells collected from band-III were easily recognized due to their characteristic organelles, i.e., prominent nucleus, smooth endoplasmic reticulum and mitochondria by electron microscopy. To observe the enzyme reaction, certain incubation time, pH and intact Leydig cells are needed. When examined semi-thin Araldite sections, Leydig cells displayed a continuous and dense ANAE reaction product on their plasma membrane after 5-min incubation at pH 6.5 (4°C) and this reaction product was confirmed by electron microscopy. No significant variation was seen in density and/or thickness of the reaction from cell to cell. However, weaker reaction was detected when incubation time is reduced. No reaction for ANAE was observed in Leydig cell cytoplasms and/or organelles (Figure 1a-b, 2). ANAE activity was negative when substrate was omitted and these were used as control group (Figure 3a-b).

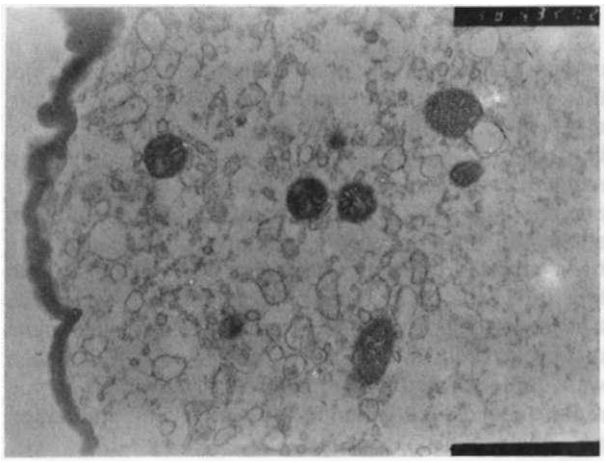

Figure 2. Higher magnification of Leydig cell. Smooth endoplasmic reticulum and mitochondria show no reaction (incubation with substrate (-»). ElectronmicrographX8800

DISCUSSION

The aim of this preliminary study was to demonstrate a-Naphthyl acetate esterase (ANAE) activity by electron microscopy in Percoll-isolated Leydig cells. It is suggested that Percoll gradients possess several advantages over other isolation methods e.g; it very effective, easy to prepare, and can be stored prior to use. Besides it exerts very low osmotic pressure. During preparation, cell membranes must be well preserved. Damage of the cell membranes may result in damage of the enzyme. Therefore low osmotic pressure needed during the isolation procedure (4-6). Certain other conditions such as temperature and especially pH values may play very important roles to detect the enzyme. Optimum pH for non-specific esterases is between 5 and 8. ANAE is a very active enzyme therefore incubation time is important factor. When correct incubation time is used, ANAE is a specific marker enzyme for Leydig cells and can be estimated with a simple histochemical assay. In the

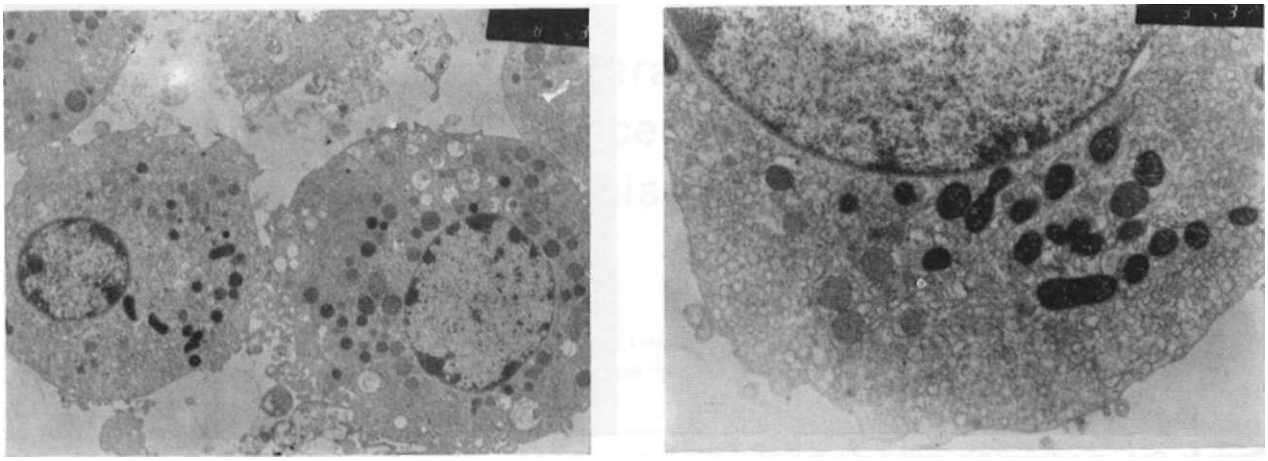

Figure 3. Controls (incubation without substrate), a) Leydig cell show no ANAE reaction (—). ElectronmicrographX2600
b) Higher magnification of Leydig cell. Negative ANAE reaction. ElectronmicrographX8300 (X is given in its original magnification)

demonstration of non-specific esterases the choice of the substrate is also important. a-Naphthyl acetate esterase is generally used as a standart substrate for light microscopic investigation (2,3). The incubation medium is unstable and hexazotized pararosaniline is decomposed after 15-20 min and therefore medium should be used immediately after preparation. It has been reported that this enzyme is much higher in mature rat Leydig cells than in immatures so ANAE can also be of help in studies concerning the development of Leydig cells. In this study, positive ANAE reaction was demonstrated on ultrastructurally well-preserved mature Leydig cell membranes after the incubation procedure mentioned above.

When incubation is used without substrate, Leydig cells were always show negative ANAE reaction on the plasma membrane of the cell. Although mature Leydig cells show esterase activity on their plasma membrane, we suggest that different procedures particularly electrophoretic methods are needed to prepare pure isoenzyme of esterase in Leydig cells. On the other hand, it has been pointed out that non-specific esterase can be used as a marker for macrophages when incubation is continued for 15-20 min (2). Then, macrophages can be identified by their phagocytic activity but not by staining for esterase activity. However, Leydig cells show strong esterase reaction but not phagocytic activity after 5 min staining procedure. The presence of esterase activity on the plasma membrane has also been reported for Kurloff cells of spleen which have been reported to have natural killer activity. Besides, monocytes and basophils show positive reaction on their lysosomes and endoplasmic reticulum. The significance of the presence of ANAE in these cells is still uncertain, but it has been suggested that the esterase activity might be related to their cytotoxic properties (8,9,11).

In general, authors can only speculate about their function but it is assume that non-specific esterases may be involved in drug detoxication, protein synthesis, intracellular transport and lipid metabolism (7-11). Our conclusion, ANAE can be used as a marker enzyme for Leydig cells but today we are unable to explain the exact functional role of esterase in Leydig cells and we can only speculate that this enzyme might be related to plasma membrane receptors, lipid metabolism and/or androgen synthesis of the cells.

Further biochemical, electrophoretic and immunoelectron microscopic methods are needed for more detailed information about functional importance and/or preparation of pure isoenzyme of esterase in Leydig cells.

Percoll'le izole edilmiş sıçan testisi Leydig hücrelerinde a-Naftil Asetat Esteraz Enzim Aktivitesi: elektron mikroskopik ve histokimyasal çalışma

Sıçan testisi Leydig hücrelerinin ışık mikroskopu düzeyinde gösterilmesinde çeşitli histokimyasal yöntemler geliştirilmiştir. Bu çalışmada Percoll gradienti kullanarak dokudan izole edilmiş Leydig hücrelerindeki ANAE aktivitesi elektron mikroskopunda incelendi. Leydig hücrelerindeki enzim aktivitesinin ortaya çıkarmak için alfa-naftil esteraz substrat, hekzasotize pararosanilin bağlayıcı olarak kullanıldı ve enkübasyon +4°C'de pH 6.5'da yapıldı. Yapılan incelemede Leydig hücre plazma membranında pozitif ANAE reaksiyonu saptandı. Elektron mikroskopik düzeydeki bu enzim çalışmasında ortamın ısısı, enkübasyon süresi ve

pH'nın çok iyi ayarlanması gerektiği görüldü. Sonuç olarak, belirli koşullarda ANAE'ın Leydig hücreleri için güvenilir bir işaretleyici enzim olduğu kanısına varıldı.

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