EFFECT OF FREEZING ON CYTOCHROME C OXIDASE CYTOCHEMISTRY IN CELLS IN MONOLAYER CULTURE

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Our objective was to evaluate a method that incorporates a freezing and thawing process to detect cytochrome c oxidase in normal mitochondria in myogenic cell lines in monolayer culture, without the loss of enzyme activity. We used electron microscopy to detect and investigate cytochrome c oxidase activity. When cell preparations were subjected to a freezing and thawing process after being fixed, 91.4% of mitochondria were positively stained. No positive staining was obtained in cells treated by conventional procedures without the freezing process. This method facilitates the positive staining of mitochondria and should be useful in analyzing pathological conditions of mitochondria in cultured cells.

Cytochrome c oxidase (COX) is an enzyme involved in the electron transport system in mitochondria. The ultrastructural cytochemical localization of COX activity in mitochondria was first attempted in animal tissues in 1967 (14). The basic method now in use was established by Seligman and his coworkers in 1968 (13), and since then, many reports of electron microscopy studies of COX have appeared (1, 2, 5, 8, 11, 15). Electron microscopy of tissue samples provides satisfactory results, but the use of monolayer cell cultures sometimes results in uneven staining or a low yield of positively stained mitochondria (3, 6).

The establishment of the improved methods of cytochemical staining in monolayer cell cultures is needed in the study of mitochondrial myopathy, in which the expression of COX activity is affected in a varied degree. To detect abnormal mitochondria in cultured cells from patients, measures must be taken to avoid artificial loss of the enzyme activity.

Our objective was to evaluate a method that incorporates a freezing and thawing process to detect cytochrome c oxidase in all normal mitochondria in myogenic cell lines in monolayer culture, without the artificial loss of enzyme activity. We also evaluated the effects of the addition of dimethyl sulfoxide (DMSO) (7) to the fixation medium and the incubation medium.

MATERIALS AND METHODS

Cell culture

The human myogenic cell line used was established by transfecting a replication of origin-defective Simian virus 40 DNA (15) into a primary muscle culture. This cell line expresses a normal level of COX activity, as measured by the spectrophotometric method (15). The cells were cultured in a Ham F12 medium (Nissui Pharmaceutical Co., Ltd., Japan) containing 10% fetal bovine serum, penicillin, streptomycin, and glutamine at 37°C in an atmosphere of 5% CO2 and 95% air, as previously described (14).

Enzyme histochemistry by electron microscopy

Cells were cultured in polystyrene dishes in a semiconfluent concentration and fixed by being exposed to 2% glutaraldehyde in a 0.1 M cacodylate buffer (pH 7.4) at 4°C for 10 min or 30 min. The cultured cells were thoroughly washed with a 0.1 M cacodylate buffer (pH 7.2). After the remaining solution was completely aspirated, half of the dishes were frozen in...
liquid nitrogen and then thawed completely by being left at 23.5°C; the other half of the dishes were similarly processed but were not frozen. Dishes were incubated in a reaction medium containing 0.1 M phosphate buffer (pH 7.4), 0.5 mg/ml 3,3’-diaminobenzidine tetrahydrochloride (DAB), 0.1 mg/ml catalase, and 1 mg/ml cytochrome c at 37°C for 1 hr or at 4°C for 22 hr. For electron microscopical histochemistry studies, the cells were rinsed with a 0.1 M cacodylate buffer, pH 7.2, for 30 min, postfixed with Caulfield’s solution (1% Osmium tetroxide) at 4°C for 60 min, dehydrated in a series of graded ethanol solutions and embedded in epoxy resin (Quetol-812, Nissin EM Co., Tokyo). Ultrathin sections were made with an LKB Ultrotome III (LKB Co., Stockholm), stained with uranyl acetate and Reynolds’ solution, and examined under a Hitachi HU-12A or Hitachi H-7000 electron microscope (Hitachi Co., Ltd., Tokyo).

Some dishes were fixed by being exposed to a solution contained 2% glutaraldehyde and 0.5% DMSO, they were then frozen and thawed, and then incubated in the reaction medium containing 0.5% DMSO.

Cytochemical controls for enzyme activity were established by sequentially altering the reaction medium after the freezing and thawing process, as follows: 1) a COX inhibitor, sodium azide (10 mM), was added to the reaction medium, 2) DAB was omitted, and 3) cytochrome c was omitted.

RESULTS

In samples treated by the conventional histochemical procedure without freezing and thawing, the reaction products were scarcely discernible in the mitochondria of the cultured cell after 2% glutaraldehyde fixation for 30 min (Fig. 1). When cells were frozen in liquid nitrogen after being fixed in 2% glutaraldehyde for 30 min, the reaction products were clearly visible in the intracisternal spaces and intermembrane spaces of almost all mitochondria (Fig. 2a). However, in a few mitochondria, only partial reaction products were detected in the intracisternal spaces, and a few mitochondria showed no reaction products.

Thus, we observed three patterns of staining in the mitochondria: 1) positive reaction products of COX in all intracisternal spaces and intermembrane spaces, 2) a mosaic pattern of partially positive and negative cristal spaces, and 3) no visible reaction products. (Fig. 2b)

Even when the fixation time was shortened to 10 min, which was expected to enhance the detection of COX reaction product in mitochondria, specimens

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Fig. 1. Cytochrome c oxidase activity was detected in the mitochondria of a cultured myogenic cell fixed in 2% glutaraldehyde for 30 min and incubated for 1 hr. Most mitochondria showed negative reactions. ×3,000 Bar=1 μm.
Figs. 2a, b. Cytochrome c oxidase activity in mitochondria of a cultured myogenic cell that was frozen and thawed after being fixed for 30 min with 2% glutaraldehyde, and then incubated or 1 hr. 2a. Most mitochondria showed positive reactions in the cristal space and intermembrane spaces. ×4,000 Bar=1 μm. 2b. High magnification of a portion of 2a. Occasionally, a mosaic pattern showing partial activity in the cristal space was seen in some mitochondria (arrow). A few mitochondria with negative activity, which lacked reaction products in all sites, were also seen (arrowhead). ×4,000 Bar=1 μm.
Fig. 3. Cytochrome c oxidase activity in a specimen fixed for 10 min with 2% glutaraldehyde without freezing and incubated for 1 hr with the incubation medium. No electron-dense reaction products were observed. ×3,000 Bar=1 μm.

Fig. 4. Cytochrome c oxidase activity in a specimen fixed for 10 min with 2% glutaraldehyde, frozen and thawed, and then incubated for 1 hr in the incubation medium. COX reaction products were visible in the cristal space and intermembrane spaces. The localization of enzyme activity in the mitochondria was the same as that seen in fig. 2. However, the number of mitochondria with positive activity increased with the short fixation time. ×3,500 Bar=1 μm.
FIG. 5. Cytochrome c oxidase activity in a cultured myogenic cell fixed in 2% glutaraldehyde for 10 min and incubated for 22 hr at 4°C, without freezing. No mitochondria were stained in this section. ×2,000  Bar=1 μm.

FIG. 6. Cytochrome c oxidase activity in a cultured myogenic cell fixed for 10 min with freezing process and incubated for 22 hr at 4°C. Freezing and thawing drastically increased the reaction product. Prolongation of incubation time also enhanced the staining pattern. ×3,000  Bar=1 μm.
treated without freezing and thawing process showed no enzyme activity (Fig. 3). However, in specimens processed by the freezing and thawing method, strong positive activity was apparent in the mitochondria of the cells when the fixation time was shortened to 10 min, although some structural damage was observed. (Fig. 4). A mean 91.39±7.60% of mitochondria per cell were positively stained, 7.86±6.66% were partially stained, and 0.71±1.71% were negatively stained. Because of poor fixation and ice crystal damage, it did not seem to be worthwhile to examine the ultrastructure at higher magnification in cells fixed for only 10 min and then treated by freezing and thawing.

When the cells were incubated for up to 22 hr at 4°C without freezing, only a low percentage of mitochondria demonstrated positive enzyme activity (Fig. 5). In the cells incubated for 22 hr at 4°C after freezing and thawing, positive staining was clearly observed in the cisternal and intermembrane spaces of mitochondria (Fig. 6).

When cells were fixed with 2% glutaraldehyde mixed with 0.5% DMSO, frozen and thawed, and then incubated in the DMSO medium, positive staining was clearly detectable. However, the addition of DMSO resulted in less enhancement of the staining pattern (Fig. 7).

When cells were incubated in medium containing 10 mM sodium azide, no enzyme activity was detected (Fig. 8). No positive mitochondrial staining was seen when DAB or the substrate (cytochrome c) was omitted from the incubation medium (Figs. 9, 10).

**DISCUSSION**

When the pathological conditions of the muscle electron transport system are evaluated, it is important to determine whether the COX activity in the mitochondria in cells is uniformly normal, partly defective, or completely defective. Ultracytochemical detection of enzyme activities in mitochondria has been the strategy of choice for measuring COX activity since the basic method was established by Seligman and his associates (13).

Studies have attempted to identify the cause of uneven staining (3, 6). Seligman and his associates attributed differences in degree of COX localization between fresh tissues and tissues fixed with formaldehyde, to the inability of the reagents to penetrate the inner membrane (13). Spector et al. (16) reported that localization of COX in the cochlear hair cell mitochondria was similar in fresh tissues and formaldehyde prefixed tissues, although the reaction was spottier in the fresh tissues. They suggested that the heterogeneous pattern of mitochondrial staining was related to the osmolarity of the incubation media, solubility of the enzymes, and pH of the medium, and not to the method of fixation (16). Reith and his associates also observed heterogeneity of mitochondrial COX staining in 40-μm sections of rat livers and suggested that the differences in DAB-COX staining
of mitochondria in fixed and unfixed tissues were related to either varying COX enzyme activity or to a differential loss in COX (12).

Noda investigated localization of COX activity in the osteoclasts of hard tissue using electron microscopy and found that active osteoclasts contained numerous mitochondria with reaction products of the enzyme in both the inner membrane and intermembrane spaces. Only a few mitochondria with reaction products only in the inner membrane of the cristae, including the intracristal spaces, or with no reaction products, were observed (11). Adequate penetration of constituent chemicals into tissue cells is necessary to avoid false-negative and false-positive results in histochemical studies. Mayahara and Ogawa observed false-negative and false-positive reactions in the deeper part of tissue blocks in enzyme histochemistry studies (9). The absence of detectable COX reaction products in mitochondria may be related to difficulty of constituent chemicals in the incubation medium to penetrate tissues. Biological membranes may prevent DAB (mol. wt. 360.1), cytochrome C (mol. wt. 12,384), and catalase (mol. wt. 240,000) from freely passing into cells or cell organelles during a short incubation period. To reach the mitochondria, chemicals have to penetrate three biological membranes; the plasma membrane, the outer mitochondrial membrane, and the inner mitochondrial membrane.

The penetration of antibody into cell organelles is one of the most difficult problems to be overcome in immunohistochemistry, especially in the case of mitochondrial enzymes, because the molecular size of the antibody together with its marker is too large to allow it to readily penetrate the biological membranes (10). In lectin histochemistry, the tissues have to be treated with saponin to open the pores on the biological membranes to enable detection of such inner cell organelles as the Golgi apparatus (17).

Moreover, unlike other tissue sections conventionally used in histochemistry, the cultured muscle cell has an intact plasma membrane over the entire cell surface, which may act as a barrier to the penetration of chemicals in the incubation medium. In cells in
monolayer culture, electron microscopic cytochemistry of mitochondria often results in an uneven staining for enzyme activity. Inuma and his associates reported that 84.19% of the mitochondria was positively stained for COX activity in normal control cultured skin fibroblasts, 14.48% of mitochondria showed equivocal staining, and 1.20% showed no staining (6). When Douglas and his associates attempted to localize COX activity in mitochondria of human diploid cells in vitro (3), they found that electron-opaque reaction products were not observed in the mitochondrial cristae after an incubation period of up to 70 min. After 80 min of incubation, a faint reaction product was visible and after 2-hr incubation, a reaction product was distinctly evident in the cristae. Douglas et al. suggested that the need for an extended incubation period may have been related to the presence of plasma membranes. Plasma membranes are damaged when tissue samples are surgically removed from an animal and cut into 10 to 50 μm thick sections. This tissue-chopping process produces discontinuities in the plasma membrane that facilitate the diffusion of reagents, such as DAB, catalase, or cytochrome c. Because cells in monolayer culture are fixed and incubated in their culture vessels, it is less likely that the plasma membrane will be broken. Therefore, an extended period of incubation is required to compensate for the diminished rate of reagent diffusion (3).

In our study, the additional freezing and thawing process following fixation increased the detection rate of positive enzyme activity. Detection of COX activity in the mitochondria was reduced in specimens treated without the freezing process. The effects of the freezing and thawing process on the plasma membrane appeared to facilitate the penetration of DAB, catalase, and cytochrome c. No morphological changes in the biological membrane of the cell organelles were detected after freezing and thawing, presumably because of the cytoprotectant effect of sucrose in a fixation medium and a buffer (4). The prolonged incubation time (4°C, 22 hr) also increased the percentage of positive activity of mitochondria because of enhanced reagent penetration. Makita and Sandborn reported that the heterogeneity in reaction product in the intracristal space of mitochondria of muscle cells was diminished when DMSO was added to the incubation medium for the staining of succinate dehydrogenase activity (7). In this study, the addition of DMSO to the fixation and incubation media in association with the freezing and thawing process did not significantly enhance COX activity.

Our observations suggest that the heterogeneity of COX activity in mitochondria observed in electron microscopic enzyme cytochemistry studies are related to technical problems, such as limited permeability of the medium. Freezing cells after fixation improved detection of COX in most mitochondria, and should be a useful method in studies of pathological conditions of mitochondrial enzymes because of the greatly diminished artificial loss of enzyme activity in normal mitochondria, compared with conventional methods. Because the incubation medium of COX consists of rather large molecules, this method may also be useful in the study of the heterogeneity of other enzymes in normal mitochondria.

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REFERENCES


