Comparison of the degradative fate of monoamine oxidase in endogenous and transplanted mitochondrial outer membrane in rat hepatocytes

Implications for the cytomorphological basis of protein catabolism

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The degradative fate of monoamine oxidase in endogenous and transplanted mitochondrial outer membrane has been compared in rat hepatocyte monolayers. Monoamine oxidase was specifically irreversibly radiolabelled by the suicide inhibitor [^3H]pargyline. Hepatocyte monolayers were cultured in conditions in which rates of protein catabolism like those *in vivo* are maintained [Evans & Mayer (1983) *Biochem. J.* **216**, 151–161]. Incubation of hepatocyte monolayers for 17 h with [^3H]pargyline specifically radiolabels mitochondrial monoamine oxidase, as shown by Percoll-gradient fractionation of broken hepatocytes. Monoamine oxidase is degraded at a similar rate to that observed in liver *in vivo* (t_1 approx. 63 h). The effects of leupeptin, methylamine and colchicine on the degradation of endogenous radiolabelled enzyme has been studied over prolonged culture periods. Culture of hepatocytes for periods of up to 80 h with inhibitors was not cytotoxic, as demonstrated by measurements of several intrinsic biochemical parameters. Leupeptin, methylamine and colchicine inhibit the degradation of endogenous monoamine oxidase by 60, 38 and 18% respectively. Monoamine oxidase in mitochondrial-outer-membrane vesicles introduced into hepatocytes by poly(ethylene glycol)-mediated vesicle–cell transplantation is degraded at a similar rate (t_1 55 h) to the endogenous mitochondrial enzyme. Whereas leupeptin inhibits the degradation of endogenous and transplanted enzyme to a similar extent, methylamine and colchicine inhibit the degradation of transplanted enzyme to a much greater extent (85 and 56% respectively). Fluorescence microscopy (with fluorescein isothiocyanate-conjugated mitochondrial outer membrane) shows that transplanted mitochondrial outer membrane undergoes internalization and translocation to a sided perinuclear site, as observed previously with whole mitochondria [Evans & Mayer (1983) *Biochem. J.* **216**, 151–161]. The effects of the inhibitors on the distribution of transplanted membrane material in the cell and inhibition of proteolysis show the importance of cytomorphology for intracellular protein catabolism.

We have previously shown that mitochondrial-outer-membrane vesicles and mitochondrial preparations can be homologously transplanted into hepatocytes (Evans & Mayer, 1982, 1983) and mitochondrial outer membrane can be heterologously transplanted into Morris hepatoma (HTC) cells (Russell & Mayer, 1983) by poly(ethyl-

ene glycol)-mediated vesicle–cell or organelle–cell fusion.

Transplanted mitochondrial outer membrane is not tolerated in the plasma membrane of hepatocytes, but is removed (within 6 h) and translocated to appear in/on vesicular perinuclear structures before subsequent protein catabolism can begin (Evans & Mayer, 1983). In contrast, in HTC cells, transplanted mitochondrial outer membrane is patched, capped and internalized (within 8 h) into incompletely characterized intracellular vesicular

Abbreviations used: SDS, sodium dodecyl sulphate; PEG, poly(ethylene glycol).

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structures which are not lysosomes (Russell & Mayer, 1983). Transplanted mitochondrial-outer-membrane proteins (including monoamine oxidase) are degraded at rates comparable with those *in vivo* in hepatocytes, but approx. 2.5 times faster in HTC cells.

Percoll-gradient fractionation of hepatocyte homogenates demonstrates that transplanted mitochondrial-outer-membrane proteins are not associated with mitochondria or lysosomes but are located in/on intracellular vesicular structures from which the proteins are donated for destruction, at least in part by a lysosomal mechanism (Evans & Mayer, 1983).

Comparison of the fate of transplanted hepatocyte mitochondrial outer membrane and its incumbent proteins after homologous and heterologous transplantation into hepatocytes and hepatoma cells respectively suggests that an intracellular system of 'self-recognition' may exist which subsequently channels proteins into the intracellular destructive system(s). Preliminary experiments suggested (Evans & Mayer, 1983) that surface-surveillance and translocation of mitochondrial outer membrane in hepatocytes required a functional cytoskeleton.

In the studies reported here we have used several agents which disrupt cytomorphological structure and function to examine the subsequent effects on the destruction of a single mitochondrial-outer-membrane enzyme, monoamine oxidase, in transplanted and endogenous mitochondrial outer membrane. Monoamine oxidase was labelled in endogenous and transplanted mitochondrial outer membrane by the suicide inhibitor [³H]pargyline. For fluorescence microscopy, mitochondrial outer membrane was labelled with fluorescein isothiocyanate.

Materials and methods

Materials

Density-gradient marker beads were purchased from Pharmacia Fine Chemicals (Uppsala, Sweden). [¹²⁵I], [¹⁴C]tyramine and [³H]AMP were obtained from Amersham International. Glucose-6-phosphate dehydrogenase (EC 1.1.1.49) assay kit, *M_r* marker kit, leupeptin, methylamine, colchicine, lactoperoxidase, glucose oxidase (type II) and Tricine {*N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine} were obtained from Sigma, Poole, Dorset, U.K. All other reagents were described previously (Evans & Mayer, 1983).

Preparation and culture of hepatocytes

Hepatocytes were prepared and cultured as described previously (Evans & Mayer, 1982).

Iodination of hepatocyte plasma membrane

Hepatocytes were iodinated by the method of Tweto *et al.* (1976). In the study reported here the cells were iodinated in suspension culture rather than in monolayers on plastic dishes (cf. Warren & Doyle, 1981), since the hepatocytes were subsequently cultured on collagen-coated plates. Freshly isolated hepatocytes at a density of 1.2×10^7 cells/ml were washed and resuspended in an equivalent volume of Earle's balanced salt solution (EBSS), pH 7.6 (Tweto *et al.*, 1976), containing 0.1 M-Tricine and 7 mM-glucose (modified ESS).

The iodinations were carried out in stoppered Sterilin Universal containers (volume 30 ml). The cells in several containers were pooled before cell plating. Each Universal vessel contained 1.2 ml of hepatocyte, carrier KI (2.5 nmol/ml), 54 μ l of lactoperoxidase (1 mg/2 ml of modified ESS) and 150 μ Ci of [¹²⁵I] in modified ESS. The reaction was initiated by adding 20 μ l of glucose oxidase (1 mg/2 ml of modified ESS). The Universal containers were incubated with gentle shaking at 25°C for 40 min. The reactions were terminated by the addition of 10 ml of Leibovitz L-15 medium, pH 7.4, containing 8.3 mM-glucose and 25 mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid] and subsequently washed twice in the same medium.

Degradation of hepatocyte plasma-membrane proteins

Hepatocytes were iodinated as described above. Pooled hepatocytes were divided into two portions and pelleted. One cell pellet was treated with 500 μ l of PEG 1500 as in the normal fusion protocol (Evans & Mayer, 1982, 1983); the other pellet was treated with 500 μ l of serum and antibiotic-free L-15 medium supplemented with glucose (8.3 mM) and Hepes (25 mM), pH 7.4. The Universal containers were gently rotated for 90 s and the cells were thrice washed in culture medium as described previously (Evans & Mayer, 1982, 1983). Hepatocytes were plated out at a density of 1.2×10^6 cells in 60 mm-diameter collagen-coated plates. The plating efficiency in these experiments was low, approx. 25%. Poor cell attachment was caused by cell death before plating [viability was estimated by Trypan Blue (0.25%, w/v) uptake].

Labelling of hepatocyte proteins

Hepatocytes were labelled with [³H]leucine for 24 h in leucine-free Leibovitz L-15 medium as described previously (Evans & Mayer, 1982). Two washes (2.5 h) were carried out with culture medium (see below) supplemented with 10 mM-leucine.

Labelling of endogenous monoamine oxidase with [³H]pargyline

Hepatocytes (2.5×10^6) were plated out in 3 ml of culture medium [i.e. Leibovitz L-15 medium, pH 7.4, supplemented with glucose (8.3 mM), Hepes (25 mM), penicillin/streptomycin (100 units/ml), insulin (0.8 μ g/ml), dexamethasone (1 μ M) and 10% (v/v) heat-inactivated (56°C for 30 min) newborn-calf serum]. After 2.5 h the culture medium was replaced by medium supplemented with [³H]-pargyline (36 nM) with or without unlabelled pargyline (100 μ M). The cells were cultured in this medium for 17 h. Two washes (1.5 h) were conducted at 37°C in culture medium containing unlabelled pargyline (100 μ M). After the washes, culture medium was added to the dishes. Addition of culture medium was taken as zero time for degradation measurements (see the legends to the Figures).

Other procedures

Preparation of mitochondrial-outer-membrane vesicles, labelling of outer membranes with [³H]-pargyline, fluorescein isothiocyanate labelling and transplantation to hepatocyte target cells were performed as described previously (Evans & Mayer, 1982, 1983). Further experimental details are included in the Figure legends.

Harvesting of hepatocytes

Except for hepatocytes used in Percoll-gradient-fractionation studies, the cells were harvested by washing the monolayer with 1.5 ml of Dulbecco's phosphate-buffered saline, pH 7.4 (Dulbecco & Vogt, 1954), followed by scraping the cells into 500 μ l of the same medium. Any cells remaining on the plates were scraped into a further 500 μ l of phosphate-buffered saline. The cell suspensions and culture medium were frozen at -20°C until processed.

Fluorescence microscopy

This was carried out as described previously (Evans & Mayer, 1983).

Analyses

Tyrosine aminotransferase (EC 2.6.1.5) and lactate dehydrogenase (EC 1.1.1.27) activities were measured as described by Evans (1981). The activities of succinate dehydrogenase (EC 1.3.99.1; Pennington, 1961), acid phosphatase (EC 3.1.3.2; Nelson, 1966), glucose-6-phosphate dehydrogenase (EC 1.1.1.49; Kornberg & Horecker, 1955) and monoamine oxidase (EC 1.4.3.4; Russell *et al.*, 1979) were determined. 5'-Nucleotidase (EC 3.1.3.5; Gentry & Olsson, 1975) was measured with and without its inhibitor, EDTA (20 mM) (Widnell,

1974). This allows correction for the action of non-specific phosphatases. The activity of 5'-nucleotidase was determined by subtracting the apparent activity in the presence of EDTA (20 mM) from that observed in its absence.

Cell suspensions were broken by sonication (two 10 s bursts at an amplitude setting of 6 μ m peak-to-peak in an MSE sonicator). Tubes were cooled in ice. Radioactivity in cell proteins was determined after precipitation with trichloroacetic acid (10%, w/v, final concn.) containing, where appropriate, leucine (5 mM), pargyline (1 mM) or sodium metabisulphite (25 mM) and KI (5 mM). The precipitates were redissolved in 20 mM-NaH₂PO₄, pH 7.4, containing 0.15 M-NaCl and re-precipitated once. ¹²⁵I radioactivity was determined in a Mini-Assay type 620 γ -radiation counter. Precipitates containing ³H were dissolved in formic acid, and radioactivity was measured with FisoFluor as scintillant in a Packard CD 460 liquid-scintillation counter.

Percoll-gradient fractionations

Hepatocytes were harvested by trypsin treatment (Evans & Mayer, 1982) and washed twice in culture medium. Cell pellets were pooled and resuspended in ice-cold sucrose (0.25 M; 1 ml). The cell suspension was sonicated (one 10 s burst) on ice in a MSE sonicator set on automatic at an amplitude setting of 4 μ m peak-to-peak. This procedure has previously been shown to produce maximum cell disruption and minimum organelle damage (Evans & Mayer, 1983). The cell homogenate was layered on to 10 ml of 30% (v/v) Percoll in iso-osmotic sucrose (0.25 M) in 14 ml centrifuge tubes. Centrifugation was for 30 min at 70000 *g* in an MSE Prep-spin 75 centrifuge. Gradient fractions (0.5 ml) were analysed for enzyme activity and ³H label. The density range of the gradient was determined by using density-marker beads.

Analytical polyacrylamide-gel electrophoresis in the presence of SDS

Gels were prepared in silicone-treated glass tubes (0.5 cm $\times$ 14 cm) by a modification of the method of Reisfeld & Small (1966). All gel solutions were supplemented with SDS (0.1%). The running gel (11 cm long) contained 7.5% (w/v) acrylamide and the stacking gel contained 4% (w/v) acrylamide. The electrophoretic buffer was 0.05 M-Tris/HCl (pH 8.5)/0.38 M-glycine/0.1% SDS.

Thawed monolayers of hepatocytes labelled with [³H]pargyline were washed three times in Dulbecco's phosphate-buffered saline, pH 7.4, and scraped into sample buffer containing SDS (3% w/v), 2-mercaptoethanol (1%), EDTA (3 mM), sucrose (0.15 M), Bromophenol Blue (0.005%) and 0.062 M-Tris/HCl, pH 6.8 (Cawthon & Breakefield, 1983). Proteins were solubilized by boiling for

4 min. Samples (75 μ l) (approximately equivalent to 6×10^5 cells) were loaded on to the gels. M_r values were estimated by using a marker kit (10000–70000 M_r).

Electrophoresis was carried out for between 3 and 4 h at 4°C at a constant current of 2.5 mA per tube. After electrophoresis the gels were removed from the tubes by rimming and rinsed with distilled water. The gels were stained for protein with Coomassie Brilliant Blue or analysed for ^3H radioactivity. The latter process entailed slicing the gels into 5 mm pieces, dissolving them by incubating (70°C) them with 500 μ l of H_2O_2 (100%, v/v) in scintillation vials for 12 h. Formic acid (50 μ l) and water (500 μ l) were added along with 10 ml of Fiso-fluor scintillant. Radioactivity was measured with a Packard CD 460 liquid-scintillation counter.

Results

Labelling and degradation of endogenous monoamine oxidase

Fig. 1 contrasts the distribution on Percoll

gradients of trichloroacetic acid-insoluble radioactivity from broken hepatocytes prelabelled by incubating the cells with [^3H]leucine or [^3H]pargyline respectively. [^3H]Leucine labels predominantly cytosolic proteins found at the top of the gradient and coincident with the marker enzyme lactate dehydrogenase (cf. Figs. 1a and 1b). A peak of radioactivity occurs with a modal density of 1.04 g/ml. In this area several organelles are co-distributed, owing to their similar densities [as assessed by enzyme marker measurements (Evans & Mayer, 1983)]. Transplanted mitochondrial outer membrane also occurs at this modal density (Evans & Mayer, 1983). However, Fig. 1(b) shows a small peak of the mitochondrial succinate dehydrogenase at the same modal density. The absence of lactate dehydrogenase at this position (Fig. 1b) suggests that permeabilized, but incompletely disrupted, hepatocytes fractionate at this position on the gradient. A small peak of trichloroacetic acid-insoluble radioactivity (Fig. 1a) is associated with the bulk of the succinate dehydrogenase activity

Fig. 1. Percoll-gradient fractionation of hepatocytes prelabelled with either [^3H]leucine or [^3H]pargyline. Hepatocytes were labelled with [^3H]leucine (a and b) or [^3H]pargyline (c and d) as described in the Materials and methods section. Following the washes (see the text), four plates of cells were treated with trypsin, pooled, and cell extracts (see the Materials and methods section) were fractionated on Percoll gradients. (a) and (c) indicate the distribution of trichloroacetic acid (TCA)-insoluble ^3H label, whereas (b) and (d) show the corresponding succinate dehydrogenase activities. Lactate dehydrogenase activity is also shown in (b).

Fig. 2. Endogenous mitochondrial-inner- and -outer-membrane marker enzymes on Percoll gradients: effect of pargyline on endogenous monoamine oxidase activity. Hepatocytes were incubated in the absence or presence of [3 H]pargyline. Hepatocyte extracts from four plates were fractionated on Percoll gradients 24h after labelling procedures (see the Materials and methods section). Gradients were analysed for (a) monoamine oxidase activity in pargyline-treated cells, (b) monoamine oxidase activity in untreated hepatocytes, and (c) succinate dehydrogenase activity in untreated hepatocytes.

toward the bottom of the gradient (Fig. 1b). The labelling distribution observed in [3 H]pargyline-labelled hepatocytes is totally different from that seen in Fig. 1(a). There is very little radioactivity associated with the cytosolic fraction (cf. Fig. 1c and Fig. 1b; lactate dehydrogenase). Total [3 H]pargyline and trichloroacetic acid-insoluble [3 H]pargyline radioactivities are identical in the cells and have identical distributions, which shows that little, if any, free [3 H]pargyline remains in the cells after the chase period. Two peaks of [3 H]pargyline are seen in the cells (Fig. 1c). The minor one occurs

at the modal density of 1.04g/ml, whereas the major peak is found at a modal density of 1.09g/ml coincident with the mitochondrial marker succinate dehydrogenase (Fig. 1d).

It is not possible to determine the distribution of endogenous monoamine oxidase activity in pargyline-treated cells, since, as shown in Fig. 2(a), the enzyme is almost totally inhibited even at 24h after pargyline treatment. The distribution of monoamine oxidase activity in control cells (not treated with pargyline) is shown in Fig. 2(b) and coincides with that of succinate dehydrogenase (Fig. 2c).

Incubation of hepatocytes in the combined presence of [3 H]pargyline (36nM) and unlabelled pargyline (100 μ M) decreased the incorporation of [3 H]pargyline into trichloroacetic acid-insoluble radioactivity by approx. 95%. Polyacrylamide-gel electrophoresis in the presence of SDS of [3 H]pargyline-labelled cells (results not shown) revealed a single peak of radiolabel that was coincident with a peak of radiolabel observed on electrophoresis of [3 H]pargyline-labelled mitochondrial outer membrane. The radiolabelled peak corresponded to a protein subunit with M_r approx. 57000. This corresponds to the subunit of monoamine oxidase (McCauley, 1976; Callingham & Parkinson, 1979).

The effects of leupeptin, methylamine and colchicine on intracellular protein catabolism have been studied. The extended culture periods (up to 80h) in the presence of the inhibitors necessitated the measurement of some intrinsic biochemical processes to distinguish specific perturbations of intracellular protein catabolism from generalized cytotoxic effects. For this purpose the activities of an inducible cytosolic enzyme (glucose-6-phosphate dehydrogenase), a plasma-membrane enzyme (5'-nucleotidase), a constitutive cytosolic enzyme (lactate dehydrogenase) and cell protein content were measured.

Fig. 3 shows that, at the concentrations used, inhibitors have no effect on cellular 5'-nucleotidase (Fig. 3b), lactate dehydrogenase (Fig. 3c) or protein (Fig. 3d) over a 90h period. The induction profile of glucose-6-phosphate dehydrogenase (Fig. 3a) is modified by the inhibitors; the overall increase in enzyme activity is unaffected by methylamine and leupeptin; the increase in enzyme activity is less in the presence of colchicine.

Fig. 4 shows the effect of inhibitors on the degradation of monoamine oxidase labelled endogenously by [3 H]pargyline. Monoamine oxidase is degraded (Fig. 4a) at a similar rate to that measured in rat liver *in vivo* ($t_{1/2}$ 63h; Tipton & Della Corte, 1979; Russell *et al.*, 1980). The degradation of endogenous [3 H]pargyline-labelled monoamine oxidase is dramatically inhibited by methylamine (approx. 38%) and leupeptin (approx. 60%), but less inhibited by colchicine (approx. 18%).

Fig. 3. Biochemical properties of hepatocytes in the presence of proteolytic inhibitors

Hepatocytes were labelled with [^3H]pargyline as described in the Materials and methods section. Zero time in this series of experiments was taken as 2.5 h after cell plating. After 17 h of culture with [^3H]pargyline the cells were extensively washed with non-radioactive pargyline [$100\text{ }\mu\text{M}$]. After 3 h the wash medium was replaced by culture medium alone (●) or medium containing methylamine (10 mM ; ■), leupeptin (0.5 mM ; ○), or colchicine ($2\text{ }\mu\text{M}$; □). Protein (d) and the activities of glucose-6-phosphate dehydrogenase (a), 5'-nucleotidase (b) and lactate dehydrogenase (c) were determined. The values are the means for eight determinations at each time point. For clarity, the s.d. are not shown, but were always less than 10%. When data points coincide within the size of a symbol, the point is indicated by an open triangle (Δ).

We have previously shown that monoamine oxidase ([^3H]pargyline-labelled) in mitochondrial outer membrane introduced into hepatocytes by PEG-mediated transplantation is degraded with a

$t_{1/2}$ of 55 h (Evans & Mayer, 1983). Transplanted mitochondrial outer membrane is internalized from the cell plasma membrane and must assume a perinuclear distribution before enzyme degrada-

Fig. 4. Degradation of endogenous monoamine oxidase in hepatocyte monolayers: effects of proteolytic inhibitors. Cells were labelled with [3 H]pargyline as described in the Materials and methods section. At zero time the culture medium was replaced by (a) culture medium only, or culture medium containing (b) methylamine (10 mM), (c) leupeptin (0.5 mM), or (d) colchicine (2 μ M). The trichloroacetic acid-insoluble [3 H]pargyline radioactivity is expressed as a percentage ($\pm$ s.d.) of its value at 10 h ($n = 4$). The average d.p.m. per plate ($\pm$ s.d.) at this time was 27483 ± 2980 ($n = 16$).

Fig. 5. Effect of proteolytic inhibitors on the degradation of endogenous and transplanted $[^3\text{H}]$ pargyline-labelled monoamine oxidase

(a) Endogenous monoamine oxidase was labelled with $[^3\text{H}]$ pargyline as described in the Materials and methods section. After pargyline washing, the medium was supplemented appropriately with inhibitors. (b) $[^3\text{H}]$ Pargyline-labelled mitochondrial outer membranes were prepared and fused with hepatocytes as described in the Materials and methods section. Approx. 6×10^5 cells were added to each dish. After 4 h to ensure plating and internalization of the transplanted mitochondrial-outer-membrane material the medium was changed and supplemented appropriately with inhibitors. The values are means $\pm$ S.D. for four determinations at each time point. Results show trichloroacetic acid-soluble radioactivity released into the medium in the presence of: no further additions (●), methylamine (10mM; ■), leupeptin (0.5mM; ○) and colchicine (2μM; □).

tion at this rate can take place (Evans & Mayer, 1983). That mitochondrial-outer-membrane proteins are not degraded at the same rate as target-cell plasma membrane is confirmed by cell-surface $[^{125}\text{I}]$ iodination. The average degradation rate of $[^{125}\text{I}]$ iodinated hepatocyte plasma-membrane proteins is approx. 150h with or without PEG treatment of the cells (results not shown). Leupeptin has a similar inhibitory effect (approx. 50%) on the degradation of monoamine oxidase in both endogenous and transplanted mitochondrial outer membrane (Figs. 5a and 5b). However, the rate of degradation of the enzyme in transplanted outer membrane is inhibited to a greater extent by colchicine (approx. 56%) and methylamine (approx. 85%) compared with the endogenous enzyme (18 and 38% respectively; Fig. 4).

The cytomorphological basis of the different inhibitors was investigated by fluorescence microscopy with fluorescein isothiocyanate-conjugated

mitochondrial outer membranes for the transplantation.

The results are shown in Fig. 6. In the absence of inhibitors the fluorescent mitochondrial-outer-membrane material is translocated (Fig. 6c) to assume a sided compact perinuclear position after 18–28 h (Evans & Mayer, 1983). The fluorescence intensity diminishes as a function of time (Fig. 6f). In the presence of leupeptin the mitochondrial-outer-membrane material is translocated normally (Figs. 6b and 6d). However, the normal processing of the mitochondrial-outer-membrane material is prevented, which results in a progressive movement of fluorescent material away from the nucleus until the whole cytoplasm became full of fluorescent vesicles (Fig. 6h). Colchicine prevents the initial translocation process. In the presence of this microtubule disaggregator, fluorescent material is randomly distributed throughout the cytoplasm (Fig. 6e). Methylamine rapidly causes the forma-

Fig. 6. *Effect of proteolytic inhibitors on the intracellular distribution of transplanted fluorescein isothiocyanate-conjugated mitochondrial outer membranes*

Fluorescein-conjugated mitochondrial outer membranes were transplanted into hepatocytes (see the Materials and methods section). Hepatocytes were plated on collagen-coated coverslips. After 4 h to allow plating and internalization of transplanted membrane, the medium was replaced and where appropriate was supplemented with methylamine (10 mM), leupeptin (0.5 mM) or colchicine (2 μ M). At the times indicated, coverslips were removed from culture dishes aseptically and processed for fluorescence microscopy (see the Materials and methods section). (a) Untreated cells 8 h after plating; (b) cells treated with leupeptin 8 h after plating; (c) untreated cells 28 h after plating; (d) cells treated with leupeptin 28 h after plating; (e) cells treated with methylamine 53 h after plating; (f) cells treated with leupeptin 77 h after plating; (g) cells treated with colchicine 28 h after plating; (h) cells treated with leupeptin 77 h after plating.

tion of extremely large vesicles seen by optical microscopy (result not shown) as previously reported (Solheim & Seglen, 1983). However, fluorescent material is excluded from these structures (Fig. 6g). Vacuolation prevents the translocation of fluorescent mitochondrial-outer-membrane material from assuming a perinuclear orientation.

Discussion

Labelling of monoamine oxidase by [³H]pargyline in hepatocytes

In the present study the degradation of [³H]pargyline-labelled monoamine oxidase in endogenous and transplanted mitochondrial outer membrane has been compared in hepatocyte monolayers. Pargyline is a specific irreversible inhibitor of monoamine oxidase (Chuang *et al.*, 1974). [³H]Pargyline has been used routinely to derivatize monoamine oxidase in mitochondrial preparations (Callingham & Parkinson, 1979; Cawthon & Breakefield, 1983). We have used the inhibitor to label monoamine oxidase in intact hepatocytes. In isolated mitochondrial preparations, pargyline at a concentration of 1 μ M specifically labels monoamine oxidase, whereas at higher concentrations (100 μ M) non-specific labelling of mitochondrial-outer-membrane 'sites' may occur (McCauley, 1976). For this reason we chose to label endogenous monoamine oxidase by incubating hepatocytes for an extended time (17 h) in the presence of a low concentration of [³H]pargyline (36 nM). Radiolabelling of mitochondrial monoamine oxidase by [³H]pargyline is shown by Percoll fractionation of hepatocyte extracts in Fig. 1.

The predominant radiolabelling of mitochondria (coincident with succinate dehydrogenase marker enzyme) by [³H]pargyline is markedly contrasted with the predominant labelling of extra-mitochondrial proteins with [³H]leucine.

The distribution of succinate dehydrogenase on Percoll gradients of extracts of mildly sonicated cells reproducibly has a bimodal distribution. Mild sonication, like electroporabilization (Gordon & Seglen, 1982) and homogenization techniques (Stanley *et al.*, 1980), creates a proportion of permeabilized, but incompletely broken, cells, characterized in our studies by a lack of lactate dehydrogenase and the presence of succinate dehydrogenase (Figs. 1b and 1d), and monoamine oxidase (Fig. 2b) activities. These permeabilized cells fractionate as buoyant structures on Percoll gradients compared with intact mitochondria (Figs. 1 and 2).

Inhibitors and the degradation of endogenous and transplanted monoamine oxidase

We have previously demonstrated that proteins in transplanted mitochondrial outer membrane are degraded at rates comparable with those *in vivo* in

hepatocyte monolayers (Evans & Mayer, 1982, 1983), but only after internalization and translocation to a perinuclear site (Evans & Mayer, 1983). Incubent proteins are destroyed at rates comparable with those *in vivo*, in spite of being miscompartmentalized in the cell (Evans & Mayer, 1983; see below).

In the present study we have used inhibitors to examine these internalization, translocation and degradation events for a single enzyme, monoamine oxidase, in transplanted mitochondrial outer membrane and have compared the effects of the inhibitors on the destruction of monoamine oxidase in endogenous mitochondrial outer membrane. The long half-life of the enzyme ($t_{1/2}$ approx. 60 h) necessitates the culture of hepatocytes for prolonged periods (up to 80 h) with the inhibitors. The results in Fig. 3 show that hepatocytes can be cultured with leupeptin, methylamine or colchicine for prolonged periods with minimal effects on intrinsic biochemical processes. Therefore the inhibitors do not exert general cytotoxic effects on hepatocytes over prolonged periods at the concentrations employed in the present studies.

The results in Fig. 4 show that leupeptin and methylamine inhibit dramatically the degradation of endogenous monoamine oxidase (60 and 38% respectively), whereas colchicine treatment causes relatively slight inhibition (18%) of enzyme degradation. Fig. 5 contrasts the effects of the inhibitors on monoamine oxidase in endogenous and transplanted mitochondrial outer membrane as assessed by the appearance of trichloroacetic acid-soluble radiolabel in the medium. Leupeptin inhibits the degradation of endogenous and transplanted enzyme to a similar extent. In contrast, methylamine and colchicine inhibit the degradation of transplanted enzyme to a much greater extent (85 and 56% respectively) than the endogenous enzyme.

The differential effects of methylamine and colchicine on the rates of degradation of transplanted and endogenous enzyme can be explained by consideration of their effects on the cytomorphological location of transplanted mitochondrial-outer-membrane material (Fig. 6).

The cytomorphological location of the transplanted material was determined by introducing fluorescein isothiocyanate-conjugated mitochondrial outer membranes into hepatocytes. This permitted the effect of inhibitors on the cellular distribution of the fluorescent mitochondrial-outer-membrane material to be determined. Leupeptin prevented the loss of the mitochondrial-outer-membrane fluorescence (cf. Figs. 6f and 6h), but did not prevent translocation of fluorescent material to the perinuclear site (cf. Figs. 6c and Figs. 6b,d). After prolonged leupeptin treatment, fluorescent

mitochondrial-outer-membrane material moves away from the nucleus to become distributed in large vesicles throughout the cell (Fig. 6h). This is consistent with leupeptin blocking degradation of macromolecules at the secondary-lysosome or autophagosome level (Kovacs *et al.*, 1982; Furuno *et al.*, 1982; Kominami *et al.*, 1983). Colchicine (Fig. 6e) prevents the initial translocation of fluorescent material to a perinuclear site. Cytochalasin B has no effect on the translocation process (P. J. Evans & R. J. Mayer, unpublished work). This suggests that microtubules, rather than microfilaments, are involved in the translocation event. The greater inhibition of the degradation of transplanted monoamine oxidase, as opposed to the endogenous enzyme, by colchicine indicates that microtubule-dependent membrane translocation is of fundamental importance in the degradation of the transplanted mitochondrial-outer-membrane enzyme. There is abundant evidence that mitochondria are associated with microtubules in cells (Heggeness *et al.*, 1978; Ball & Singer, 1982; Bernier-Valentin & Rousset, 1982) and intermediate filaments (Summerhayes *et al.*, 1983). Colchicine has been reported preferentially to inhibit the degradation of mitochondria in muscle tissue (Crie *et al.*, 1983), and in view of the inhibition (18%) of the degradation of endogenous monoamine oxidase observed in the present work, it is tempting to suggest that cytoskeleton-dependent organelle translocation may be a normal component of endogenous mitochondrial destructive processes.

The mode of action of methylamine is equivocal, since the inhibitor can act either on lysosomes (Solheim & Seglen, 1983) or the Golgi complex (Clementi *et al.*, 1983). The present study shows that, although vacuoles (lysosomes?) are formed in the presence of methylamine, the fluorescent mitochondrial-outer-membrane material is excluded from these structures (Fig. 6g). The greater inhibition of the degradation of monoamine oxidase in transplanted mitochondrial outer membrane compared with endogenous mitochondrial outer membrane (Fig. 5) may be a direct consequence of vacuolation preventing translocation of transplanted material to assume a perinuclear orientation.

Transplanted mitochondrial outer membrane is recognized by some cellular surface surveillance system, internalized/translocated in vesicular form by a cytoskeleton-dependent mechanism to some perinuclear site [showing sidedness reminiscent of cellular Golgi apparatus (Couchman & Rees, 1982; Pastan & Willingham, 1981)], from where incumbent mitochondrial-outer-membrane proteins are delivered into the cellular protein-destructive apparatus.

The fate of mitochondrial-outer-membrane proteins in hepatocytes appears less similar to the fate of protein ligands internalized by receptor mediated endocytosis (Pastan & Willingham, 1981; Hubbard, 1982) than to the fate of proteins internalized by adsorptive endocytosis. In the former case, protein ligands are internalized and degraded very rapidly (e.g. Berg & Tolleshaug, 1980), whereas in the latter case internalized proteins may be degraded quickly or slowly, dependent on the physicochemical properties of the proteins (Davies *et al.*, 1981; Stenseth *et al.*, 1983).

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