Subcellular Localization of Oestrogen-Induced Uterine Peroxidase

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The distribution of oestrogen-induced peroxidase in the resuspended 8000g pellet of rat uterine homogenates was examined by centrifugation in a sucrose density gradient. Within 10h of treatment with oestradiol, peroxidase activity was found in a region devoid of catalase or urate oxidase (peroxisomal markers) which did not overlap the fractions containing succinate dehydrogenase (mitochondrial marker) or acid phosphatase (lysosomal marker). The induced uterine enzyme was localized in reticular membrane-bound vesicles with isopycnic density of 1.28g/ml from which it could be released by treatment with detergent.

The increase in peroxidase activity in the uterus after treatment of ovariectomized or immature rats with oestrogen was originally attributed to a peroxidase present in tissue eosinophils (Lucas *et al.*, 1955; Klebanoff, 1965). More recent evidence indicates that this enzyme is formed by uterine tissue itself (Brökelmann & Fawcett, 1969; Lyttle & Jellinck, 1972; McNabb & Jellinck, 1974) and its molecular weight and half-life have been determined (McNabb & Jellinck, 1975).

In studies of the subcellular distribution of the oestrogen-induced uterine enzyme, about one-half of the total activity 18h after treatment was found associated with the fraction sedimenting at 11000g, although some activity was also present in the nuclear and microsomal fractions (Lyttle & Jellinck, 1972; Jellinck & Lyttle, 1973). It was therefore decided to examine further the 11000g pellet by sucrose-density-gradient centrifugation and to determine whether peroxidase activity coincided with either that of succinate dehydrogenase (mitochondrial marker) or acid phosphatase (lysosomal marker). The subcellular distribution of uterine peroxidase at various times after treatment with oestradiol was also examined and some additional properties of the enzyme were determined.

Experimental

Materials

Reagents. Oestradiol, succinic acid, uric acid, AMP, *o*-carboxyphenyl phosphate, 2,6-dichlorophenol-indophenol, phenazine methosulphate and Triton X-100 were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Eastman Kodak Co.,

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Rochester, NY, U.S.A., supplied the guaiacol and 2,4-dichlorophenol. [4-¹⁴C]Oestradiol (4mCi/mmol) from Picker Nuclear, Ottawa, Ont., Canada, was shown by chromatography and radioautography to be free of radioactive impurities. It was diluted with carrier to a specific radioactivity of 2–3mCi/mmol and kept at 4°C in the dark as a stock solution in ethanol (1mg/ml). All other chemicals were the purest available commercially.

Animals. Female albino Holtzman rats (Canadian Breeding Laboratories, St. Constant, Quebec, Canada) with free access to food (Purina Labena) and water were used. Oestradiol (20µg/kg) dissolved in sesame oil was given in a single dose to immature (21–28-day-old) rats (50–80g) by subcutaneous injection. Control animals received oil alone.

Methods

Preparation of subcellular fractions. The animals, anaesthetized with CO₂, were killed by cervical dislocation; the uteri (25) were dissected free of adhering fat, blotted and weighed. The tissue was then cut into small pieces and homogenized (6×6 strokes) at 4°C in 0.25M-sucrose in a Potter-Elvehjem homogenizer with a Teflon pestle. The homogenate (10%, w/v) was filtered through two layers of cheesecloth and centrifuged at 600g_{av.} for 10min in a Sorvall RC-5 centrifuge. The supernatant fraction was then centrifuged at 11000g_{av.} for 20min, the pellet washed with 0.25M-sucrose (10ml) followed by centrifugation for 20min at 11000g_{av.} and resuspended in 0.25M-sucrose at a final concentration of 1g wet wt. of the original tissue/ml. A portion (2ml) of this suspension was layered on a linear sucrose gradient and centrifuged at 72000g_{av.} for 90min in a Beckman L5-50 ultracentrifuge with a swing-out bucket rotor (SW 27). Fractions (0.5ml) were collected by upward dis-

placement. The sucrose gradient (36 ml) was prepared in a Buchler Gradient Former and had a concentration range of 27–62% (w/w).

Determination of enzymic activities. Uterine peroxidase activity was determined by using guaiacol as substrate (Himmelhoch *et al.*, 1969), acid phosphatase activity with *o*-carboxyphenyl phosphate as substrate (Worthington Biochemical Corp., 1972, pp. 75–76), and succinate dehydrogenase activity as outlined by Reid *et al.* (1975). A Gilford modification of the Beckman DU spectrophotometer was used to determine E_{300} , E_{470} and E_{600} at 25°C and appropriate blanks were subtracted from the observed values. The peroxidase and phosphatase activities of fractions from the gradient were

measured in the presence of Triton X-100 (0.3%, v/v). Catalase activity was determined by the method of Sinha (1972), urate oxidase as outlined by Worthington Biochemical Corp. (1972, pp. 37–38), and 5'-nucleotidase activity by the rate of release of P_i from AMP (Aronson & Touster, 1974). All these assays were carried out in both the presence and the absence of the detergent after preincubation for 3 min. The ability of fractions to convert [4- 14 C]oestradiol into water-soluble products was determined as described previously (Lyttle & Jellinck, 1972). The concentration of sucrose was measured with an Abbé refractometer and the E_{280} profile of the gradient was monitored with an Isco UA-5 flow-through monitor. Protein was determined by the method of

Fig. 1. Sucrose-density-gradient analysis of the resuspended 11000g pellet of rat uteri

Oestradiol (20 μ g/kg body wt.) was injected subcutaneously 18 h before the animals were killed. A suspension (2 ml) of the 11000g pellet in 0.25 M-sucrose was layered on a linear sucrose gradient (27–62%, w/w) and centrifuged at 72000 $g_{av.}$ for 90 min. The fractions were monitored for peroxidase (E_{470} , Δ), succinate dehydrogenase (E_{600} , $\square$) and acid phosphatase (E_{300} , $\bullet$) activities. Protein was monitored at E_{280} ($\circ$) and the sucrose concentration (Δ) was determined with an Abbé refractometer.

Fig. 2. Sucrose-density-gradient analysis of the resuspended 11000g pellet of rat uteri 1 h after treatment with oestrogen

Oestradiol was injected 1 h before the animals were killed. Other conditions and methods of assay were as in Fig. 1.

Fig. 3. Sucrose-density-gradient analysis of the resuspended 11 000g pellet of rat uteri 10 and 36 h after treatment with oestrogen

Oestradiol was injected 10 h (a) or 36 h (b) before the animals were killed. Fractions were assayed for peroxidase (Δ), and the protein concentration ($\circ$) and sucrose concentration ($\blacktriangle$) were determined. Other conditions were as in Fig. 1.

Fig. 4. Subcellular distribution of peroxidase in rat uteri as measured by the ability of fractions of the resuspended 11 000g pellet to convert [^{14}C]oestradiol into water-soluble products

The treatment of animals and the sucrose-gradient analysis were as described in Fig. 1. A portion (1 ml) from five combined fractions was assayed. The concentration of protein (mg/ml) in these fractions is indicated by the numerals in parentheses.

Lowry *et al.* (1951) and RNA by the orcinol method as outlined by Campbell & Sargent (1967), with bovine serum albumin and yeast RNA (Sigma) as standards.

Results

Subcellular distribution

The distribution of peroxidase in the 'mitochondrial-lysosomal' fraction of rat uteri relative to that of two marker enzymes after centrifugation in a sucrose density gradient for 90 min is shown in Fig. 1. Peroxidase activity appeared in a narrow band which did not overlap the fractions containing succinate dehydrogenase or acid phosphatase and which sedimented more rapidly than the subcellular marker enzymes. It coincided with an E_{280} peak. These results were obtained from animals that had

been given oestradiol ($20\text{ }\mu\text{g/kg}$ body wt.) 18 h before being killed.

No protein peak or peroxidase activity was found in the corresponding fractions 1 h after the subcutaneous injection of oestradiol (Fig. 2).

The distribution of peroxidase 10 and 36 h after treatment with oestrogen is given in Fig. 3. Peroxidase activity again appeared as a relatively sharp peak at both these times, but the lower E_{280} after 36 h of treatment may not be significant because the amount of protein varied in different uterine preparations. However, peroxidase activity was always separate from that of succinate dehydrogenase and acid phosphatase.

Fractions were also tested for their ability to inactivate [$4\text{-}^{14}\text{C}$]oestradiol to water-soluble products (Fig. 4). In these experiments five fractions from the gradient were combined and 1.0 ml samples incubated with the radioactive oestrogen. Maximum activity coincided with the peak of induced peroxidase activity as measured by the guaiacol assay.

Characterization of the peroxidase fraction

Peroxidase in the resuspended 11 000g pellet obtained from rat uteri was found in the same fractions after centrifugation at 72 000g in a sucrose gradient for 16 h as after 90 min, showing that it had reached its isopycnic density (1.28 g/ml) in sucrose at 4°C . Peroxidase activity in these combined fractions was increased considerably after incubation at 37°C with 0.03% (v/v) Triton X-100, reaching a maximum after 5 min. No increase in activity was produced by various concentrations (0.01–0.1%, v/v) of this detergent added to peroxidase that had been partially purified as described previously (McNabb & Jellinck, 1975). Some inhibition was observed with Triton X-100 concentrations above 0.5% (v/v).

No detectable catalase or urate oxidase activities were found in the fractions containing peroxidase,

but small amounts of 5'-nucleotidase were present and catalysed the release of P_i from AMP at the rate of $0.015 \mu\text{mol/min}$ per mg of protein. The ratio RNA/protein in the combined fractions showing peroxidase activity was 0.11 (w/w).

Discussion

These experiments demonstrate that much of the peroxidase activity induced in rat uteri by oestradiol is present in a region of the resuspended 11000g pellet which sediments more rapidly than either mitochondria (succinate dehydrogenase marker) or lysosomes (acid phosphatase marker) in a sucrose density gradient. The absence of catalase and urate oxidase in the fraction containing peroxidase rules out peroxisomes (de Duve, 1969), but the presence of RNA indicates that some endoplasmic-reticular membranes were present. These findings were confirmed by electron microscopy (preliminary studies by Dr. K. Brasch) which showed a high content of membrane-bond vesicles. The very low concentration of 5'-nucleotidase indicates a lack of any significant contamination of the preparations by plasma membranes. The gradual increase in peroxidase activity produced by Triton X-100, reaching a maximum at 5 min, suggests a sequestering of this enzyme inside vesicles or an association with membranes from which it is then released by the detergent. These results therefore support the findings of Anderson *et al.* (1975), who showed the cytochemical localization of peroxidase 12–24 h after treatment with oestrogen in the cisternae of the granular endoplasmic reticulum as well as in the nuclear envelope and some regions of the Golgi apparatus of the rat uterus. They also support the results of Hosoya *et al.* (1971) on the localization of peroxidase in thyroid cells. These workers found that the distribution pattern of peroxidase in sucrose fractions was completely different from that of cytochrome oxidase, acid phosphatase and 5'-nucleotidase. The fractions that showed strong peroxidase activity were always accompanied by vesicles or fragments derived from rough-surfaced endoplasmic reticulum rather than from other cell organelles.

In our studies, peroxidase activity could not be detected in the resuspended 11000g pellet fraction 1 h after the injection of oestradiol, but it had reached a high value by 10 h and was still present after 36 h of treatment. These results therefore give no indication about whether any subcellular redistribution of peroxidase occurs from the rough endoplasmic reticulum, where presumably it is synthesized, into secretory granules as suggested by Anderson *et al.* (1975). Further studies at times between 1 and 10 h after injection of oestradiol are required to resolve this problem. However, it was shown cytochemically

that, in maturing bone-marrow eosinophils of mice, peroxidase gradually disappeared from the endoplasmic reticulum and Golgi apparatus and accumulated in granules (Rogovine *et al.*, 1975).

These results add to the growing list of evidence (Brökelmann & Fawcett, 1969; Jellinck & Lyttle, 1973; McNabb & Jellinck, 1974; Churg & Anderson, 1974; Anderson *et al.*, 1975) that uterine peroxidase is produced *in situ* and is distinct from the enzyme brought to the uterus by eosinophils as a result of treatment with oestrogen. Churg & Anderson (1974) have shown that the epithelium of the immature uterus was devoid of peroxidase activity and that not all cells of the uterine epithelium synthesized and stored peroxidase after treatment with oestrogen. More recently, a good correlation has been obtained between the quantity of [^3H]oestradiol specifically bound to the nuclear pellet of rat uteri and the amount of peroxidase induced by physiological doses of oestradiol (McNabb & Jellinck, 1976). However, the question about the physiological role of this induced enzyme, which appears to be a specific marker for tissues displaying growth dependency on oestrogen (Anderson *et al.*, 1975), remains unanswered.

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