Vol. 107, No. 2, 1982 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 30, 1982 Pages 721-726

PREPARATION AND CHARACTERIZATION OF A STABLE HALF MET DERIVATIVE OF TYPE 2 DEPLETED RHUS LACCASE: EXOGENOUS LIGAND BINDING TO THE TYPE 3 SITE

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Received June 18, 1982

SUMMARY: We report the preparation and characterization of a stable half met (Cu(II)Cu(I)) type 2 copper depleted derivative of Rhus laccase. Anion binding studies to this mixed valent type 3 protein form indicate no tight binding of anions nor group 1 - group 2 ligand behavior. This suggests that, in contrast to the well-characterized hemocyanins and tyrosinase coupled binuclear sites, exogenous ligands do not appear to bridge the type 3 binuclear copper ions in laccase.

INTRODUCTION

Rhus vernicifera laccase [1] contains one type 1 (blue), one type 2 (normal), and one type 3 (coupled binuclear) copper site which together catalyze the four-electron reduction of dioxygen to water with concomitant oxidation of substrate. In the native enzyme, the types 1 and 2 cupric centers are paramagnetic and EPR detectable, while the binuclear cupric ions are strongly antiferromagnetically coupled and hence EPR non-detectable; this type 3 site reduces with two electrons at the same potential [2].

While all four copper atoms are oxidized in native laccase, we have recently demonstrated [3] that the type 2 copper depleted (T2D) enzyme derivative, after preparation, contains a reduced type 3 binuclear unit in the pres-

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0006-291X/82/140721-06$01.00/0

We report that lung plasma membranes (LPM) from term fetal and maternal rabbit possess high affinity receptors for EGF and propose a direct role of EGF in fetal lung maturation.

MATERIALS AND METHODS:

Pregnant New Zealand White rabbits of known gestation were sacrificed at term (31 days) by intravenous pentobarbital. The fetuses were removed after hysterotomy and killed by cervical dislocation. Lungs from all the fetuses within each of seven litters were pooled in ice cold 0.3 M sucrose - 250 mM Tris-HCl buffer at pH 7.45. No attempts were made to differentiate between male or female pups (mixed groups). Since major differences in the developmental pattern of fetal lung maturation in male and female fetuses have been recently recognized (15-17), in three experiments male or female pups within the same were identified, their lungs pooled and processed separately. Method of sex determination in rabbit pups has been described (18).

Isolation Of Lung Plasma Membrane:

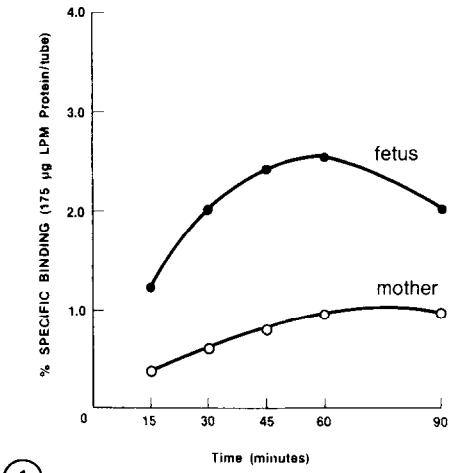
Lung Plasma Membranes were prepared by differential centrifugation as originally described for the isolation of EGF receptor containing membranes from placenta, with minor modifications (19). Briefly, lungs were trimmed of bronchial and connective tissue, cut into small pieces and homogenized in 0.3 M sucrose-250 mM Tris HCl (pH 7.45) buffer (W/V 1:10) in a Dounce homogenizer at 4°C. The homogenate was filtered through two layers of cheese cloth and centrifuged at 600 x g for ten minutes at 4°C. The supernatant was adjusted to 0.1 M NaCl and 0.2 mM $MgSO_4$ and centrifuged at 3000 x g for 20 minutes. Since less than 0.1% specific binding of (125-I) EGF per mg of protein in this pellet was observed in preliminary experiments, this fraction was subsequently discarded. The supernatant was further centrifuged at 48,000 x g for 60 minutes and the pellet was washed in 30 ml of 50 mM Tris HCl, pH 7.45 buffer to remove sucrose. LPM were harvested by centrifugation 48,000 x g for 30 minutes and the membrane pellet resuspended in 50 mM Tris HCl buffer to yield 3 to 5 mg/ml of LPM protein. Using BSA as standards, protein concentrations in the lung homogenate and LPM were quantitated by Lowry's method (20).

5'-Nucleotidase Assay:

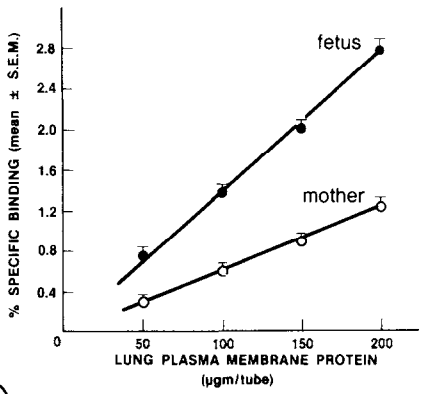
5'-Nucleotidase activity in the lung homogenate and LPM was determined at 30°C by the method of Arkesteijn (21). Assay kits are commercially available from Sigma Chemicals, St. Louis, MO.

Binding Assay:

(125-I) EGF (New England Nuclear Corporation, Boston, MA. Specific activity 150-200 μCi/μg) was used as the ligand in all experiments. Optimal EGF binding in preliminary experiments was noted at 37°C, pH 7.45 with an incubation time of 60 minutes and 200 μg/tube of BSA (Fig. 1). Binding of (125-I) EGF to LPM was linear from 50 to 200 μg/tube of LPM protein (Fig. 2). Each point in the displacement curve (Fig. 3) was assayed in triplicate using (125-I) EGF (75,000 to 80,000 cpm/tube) in the presence of EGF (Sigma Chemicals, St. Louis, MO, M.W. ~ 6045) at 5×10^{-11} to 1×10^{-8} M concentrations in a final incubation volume of 0.3 ml of 50 mM Tris HCl pH 7.45 containing 175 μg of LPM protein and 200 μg of BSA in 1.5 ml polypropylene tubes (Kew Scientific, Columbus, OH). The binding reaction was terminated by adding 0.8 ml of ice cold Tris HCl buffer and by centrifugation at 12,500 x g for five minutes. The supernatant was aspirated and the amount of radioactivity in the membrane pellet was counted in a Beckman gamma radiation counter. Nonspecific binding was measured as the residual radioactivity in the presence of excess (10^{-7} M) unlabeled EGF and was



(1)



(2)

Fig. 1 - Optimal binding of (125-I) EGF was observed at 60 minutes of incubation.

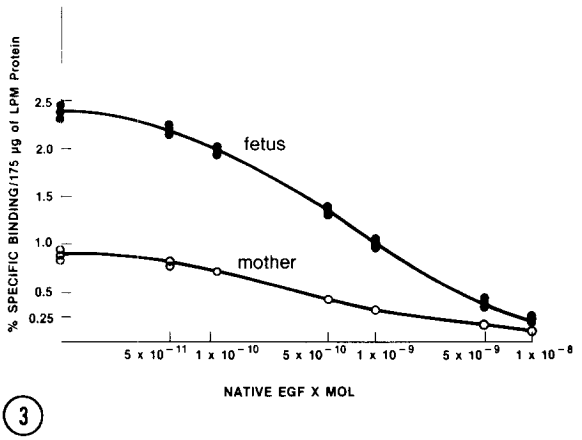
Fig. 2 - Binding of (125-I) EGF to LPM was linear from 50 to 200 µg of protein/tube.

subtracted from all points of the dose response curve to determine percent specific binding. Degradation of (125-I) EGF at the end of incubation (37°C, 60 minutes) was assessed by 50% trichloroacetic acid precipitation and rebinding to fresh LPM. To determine the specificity of (125-I) EGF binding to LPM, incubations in the presence of 10^{-7} M prolactin (ovine and bovine), growth hormone (human and bovine) glucagon or NGF were performed. The number of EGF binding sites and Kd were calculated by Scatchard analysis (22). Statistical significance was determined by Student's "t" test.

RESULTS:

All data are expressed as means ± S.E.M.. Maternal lung had significantly more (65.5 ± 2.3 vs 42.8 ± 1.6 mg) protein per gram of tissue ($p < 0.001$) when compared with the fetal lung. Recovery of protein as LPM in the 48,000 x g pellet, however, was similar in all groups (Table I). Similarly 5'-Nucleotidase activity per mg of protein per minute in the lung homogenate or LPM was significantly higher in the mother compared to the fetus (Table I). The extent of purification, however, was similar in all groups. These results are in agreement with previous observations of Neufeld et al (23).

In all experiments, the percent specific binding of (125-I) EGF to LPM in the mother was significantly less than any of the fetal groups. However no differences within the fetus (mixed, male or female) were noted. Nonspecific binding in all four groups was less than 2.2 ± 0.2% and was similar. Degradation of (125-I) EGF in all groups was less than 1%. All Scatchard plots were linear,



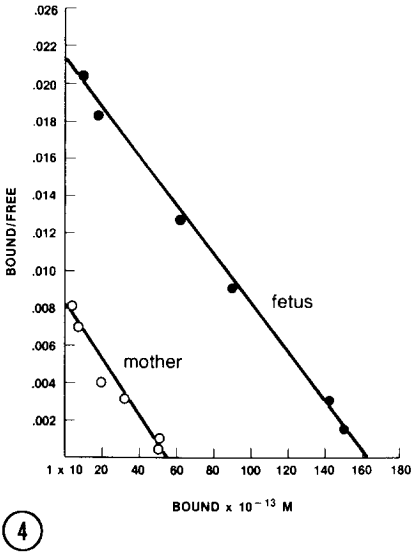


Fig. 3 - Displacement of (125-I) EGF binding to LPM in presence of various concentrations of unlabeled EGF.

Fig. 4 - Representative Scatchard plot from maternal and fetal experiment.

indicating one order of binding sites. A representative plot from one such experiment is shown in Fig. 4. The number of EGF binding sites per mg of LPM protein in the mother were significantly lower than any of the fetal groups, however the K_d of all four groups were similar (Table I). Prolactin, growth hormone, glucagon or NGF were found not to displace binding of (125-I) EGF to LPM at anytime.

DISCUSSION:

EGF has been shown to exert a powerful mitogenic effect in a variety of developing organs such as the mammary gland, smooth muscle, uterus and adrenal gland among several species including the rat, rabbit and human (12,24). In vivo administration of EGF in the fetal rabbit or sheep induced accelerated maturation of the lung and protected the treated fetus from development of hyaline membrane disease (11,12). Pulmonary histopathology, e.g. cell differentiation and appearance of a complement of Type II pneumocytes (12), and pressure-volume relationships (11) (physiologic index of surfactant activity) suggest increased production of pulmonary surfactant after EGF treatment. Demonstration of high affinity receptors for EGF in the LPM from fetal as well as maternal rabbit in this study

Table I - Amount of protein, 5'-Nucleotidase activity and EGF receptor sites in maternal and fetal rabbit lung.

	Lung Homogenate Protein (mg) per gm of lung	% Recovery of LPM Protein	5'-Nucleotidase activity (units/mg of protein/minute)			% Specific Binding per 175 ugm of LPM protein	Number of receptor sites per mg of LPM protein X 10^{-10}	K_d X 10^9
			Homogenate	LPM	Purification			
1 Mixed Fetuses (n = 7)	42.8 ± 1.6	11.0 ± 0.9	.033 ± .002	.077 ± .006	2.3 ± .01	2.25 ± 0.122	250 ± 24	2.47 ± .24
2 Male Fetuses (n = 3)	43.7 ± 0.2	10.7 ± 1.2	.034 ± .002	.098 ± .006	2.8 ± .15	2.25 ± 0.042	270 ± 30	2.67 ± .47
3 Female Fetuses (n = 3)	43.6 ± 1.2	9.7 ± 0.9	.038 ± .001	.096 ± .007	2.5 ± .20	2.34 ± 0.124	206 ± 10	2.13 ± .90
4 Mother (n = 5)	65.5 ± 2.3	11.1 ± 0.5	.098 ± .007	.177 ± .010	2.4 ± .13	1.08 ± 0.087	44 ± 11	2.10 ± .39
Statistical Significance (1 vs 4)	p < .001	N.S.	< .001	< .001	N.S.	p < .001	< .001	N.S.

further supports a proposed role of EGF in fetal lung maturation and suggests a direct rather than an indirect effect. Although the occurrence of EGF receptors in the pulmonary fibroblasts and Type II pneumocytes from adult rat lung has been demonstrated (14), EGF did not stimulate phosphatidylcholine labelling in Type II cells in tissue culture (14). It is possible that EGF stimulates surfactant production by Type II cells only during the fetal or neonatal period since this is the phase of active growth. Alternatively EGF may stimulate hyperplasia and/or hypertrophy of Type II cells as has been shown in fetal sheep (12) and thereby increase the number of surfactant producing cells.

Since LPM rather than isolated cells were utilized in this study, location of EGF receptors within different fetal pulmonary cells remains unknown. The number of EGF binding sites per mg of LPM protein was higher in the fetus when compared to the mother, which is compatible with a higher density of EGF receptors per fetal cell, and a more potent biological effect of EGF during this period of development. Alternatively the observed differences in receptor density in maternal and fetal LPM in this study may reflect other variables. Maternal lung

homogenate had significantly higher protein per gram of wet lung when compared to the fetus. Although percent recovery as LPM protein and degree of purification as judged by 5'-Nucleotidase activity was similar in all groups, LPM fraction utilized in this study is a crude preparation. The presence of other proteins might have interfered with or diluted (125-I) EGF binding in maternal LPM. Resolution of the above mentioned hypotheses is possible using fetal pulmonary Type II cells and fibroblasts in vitro cultures.

No differences in the density of EGF receptors in male or female fetuses at term within the same litter were noted. Whether there is a difference in the development of pulmonary EGF receptor density during early gestation in male or female fetuses, with no apparent distinction at term, as has been shown with pulmonary surfactant production in fetal rabbit (17) remains unknown. Future investigations should examine these possibilities in further establishing the proposed role of EGF in fetal pulmonary maturation.

ACKNOWLEDGMENT

I thank Dr. William J. Longmore, Ph.D. for reviewing this manuscript and Ms. Diane Klima for splendid secretarial assistance.

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