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EGF and TGFα Modulate Structural and Functional Differentiation of the Mammary Gland From Pregnant Mice In Vitro: Possible Role of the Arachidonic Acid Pathway

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Abstract Epidermal growth factor (EGF) has been suggested to be involved in mammary gland development by mitogenic stimulation of the ductal and alveolar epithelium in virgin mice. The present studies demonstrate that also in late-pregnant mice EGF leads to proliferation of the ductal, ductular, and alveolar epithelium. The mitogenic effect is associated with structural and functional dedifferentiation of alveolar cells as revealed by analysis of morphology, expression of cytosolic and secretory proteins, and fatty acid synthesis. Using a combination of metabolic inhibitors, the dedifferentiating effect of EGF could be blocked while the mitogenic action was not influenced. This finding demonstrates that the signal transduction pathway leading to dedifferentiation and mitosis can be separated, and that the dedifferentiating effect of EGF is independent of its mitogenic properties, but is probably mediated by activation of the arachidonic acid-dependent pathways (cyclo- and lipoxygenase pathways). Release of arachidonic acid from the endogenous phospholipid pool was found to be an early response of the explants to EGF. Accordingly, arachidonic acid itself proved to be capable of inducing epithelial dedifferentiation but failed to stimulate proliferation. TGFα showed qualitatively similar effects as EGF but was generally a stronger agonist. It is suggested that EGF and TGFα also play a role in mammary gland physiology during pregnancy by final developing and maintanance of the lobulo-alveolar structure in the mammary gland and prevention of premature onset of lactation, and that this is mediated through the PLA_2-arachidonic acid signalling cascade.

Key words: explant culture, stimulation of DNA synthesis, inhibition of functional differentiation, endogenous TGFα, arachidonic acid release, phospholipase A_2, metabolic inhibitors

Functional differentiation of mammary gland is characterized by a complex pattern of epithelial synthesis of specific proteins and lipids. The hormonal conditions of this process have been well established [Nandi, 1958; Banerjee and Antoniou, 1985; Borellini and Oka, 1989]. In vivo, serum levels of lactogenic hormones, which increase during pregnancy, are responsible for the formation of the presecretory status. Maintenance of the epithelial cell differentiation probably is due to an interaction between growth stimulatory and inhibitory peptides and hormones. Hormones, such as prolactin, insulin, and cortisol have been shown to induce or stimulate lactogenesis [Vonderhaar, 1987; Borellini and Oka, 1989], whereas progesterone [Haslam, 1987] and TGFβ [Mieth et al., 1990; Robinson et al., 1993; Jhappan et al., 1993] inhibit the expression of milk components.

Beside these substances, EGF has been suggested to play a physiological role in functional differentiation during pregnancy. Plasma EGF concentrations are substantially elevated [Oka et al., 1991], and the EGF receptor level parallels the increase in DNA synthesis in the mammary gland during pregnancy and declines immediately before the onset of lactation [Edery et al., 1985]. Both EGF and TGFα transcripts have been detected in mammary glands of pregnant mice [Snedeker et al., 1991]. There is increasing evidence that EGF plays an important role in the normal growth and development of the

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mouse mammary gland [reviewed in Oka et al., 1991]. The mitogenic action of EGF on mammary epithelial cells results in stimulation of ductal growth and lobulo-alveolar development in vitro [Tonelli and Sorof, 1980] and in vivo [Vonderhaar, 1987; Coleman et al., 1988; Haslam et al., 1993; Plaut et al., 1993]. However, the role of EGF in functional differentiation of the mammary gland is not yet clear. In mammary gland explant cultures from pregnant mice, EGF can either stimulate or inhibit casein expression depending upon the presence of prolactin [Vonderhaar and Nakhasi, 1986]. An inhibition of β-casein synthesis in primary mammary epithelial cells [Taketani and Oka, 1983] and permanent cell cultures [Hynes et al., 1990] after treatment with prolactin and EGF has been reported. In contrast, EGF was shown to be essential for morphological differentiation and reinduction of casein synthesis in regressed glands [Tonelli and Sorof, 1980].

The aim of the present paper was, therefore, to evaluate the role of EGF in functional differentiation during late pregnancy. Because functional differentiation of the mammary gland is a highly complex process, we have used a variety of parameters comprising histological features, expression of milk proteins, and lipid synthesis to study the effects of EGF. In addition, mammary-derived growth inhibitor (MDGI), which has been found to be developmentally expressed and regulated by lactogenic hormones in mammary epithelial cells of explant cultures [Binas et al., 1992], was also investigated, since previous data suggested a close relationship between MDGI and functional differentiation [Kurtz et al., 1990; Grosse et al., 1992]. In the present study, we used organ explant cultures from mammary glands of pregnant mice in which the glandular epithelium can be maintained in a presecretory state when prolactin, insulin, and cortisol are added to the culture media [Lockwood et al., 1966].

In this report, we demonstrate that treatment of mammary gland explant cultures from late-pregnant mice with EGF or TGFα results in epithelial dedifferentiation with respect to the synthesis of milk components and the loss of a presecretory phenotype of the glandular epithelium. In accordance with these data, MDGI expression is significantly reduced. In addition, we performed experiments to elucidate the possible signal transduction mechanism for the dedifferentiating effect of EGF and TGFα, and present results pointing to an involvement of the arachidonic acid pathway.

MATERIALS AND METHODS

Reagents

Ovine prolactin was from Sigma (Deisenhofen, Germany). Hydrocortisone and insulin were purchased from Merck (Darmstadt, Germany), and from Berlin-Chemie (Berlin, Germany), respectively.

Medium 199 was obtained from Gibco (Grand Island, NY) and HEPES-buffered MEM was from Sifin (Berlin, Germany). EGF was prepared as described before [Savage and Cohen, 1972]. TGFα, Na-arachidonate, prostaglandin E_2 (PGE_2), p-bromo-phenacylbromide (pBrphbr), indomethacin, nordihydro-guaretic acid (NDGA), 12-*O*-tetradecanoyl phorbol-13-acetate (TPA), and 1-O-hexadecyl-2-O-methyl glycerol (O-HMG) were from Sigma (Germany). Recombinant melittin was from Peninsula Laboratories (Belmont, CA). U-57, 908 was kindly provided by Dr. D. Morton, The Upjohn Company (Kalamazoo, MI). Radiochemicals (α^{32}P)dATP, (^{3}H)thymidine (TdR), (1-^{14}C)acetate, and (1-^{14}C)arachidonic acid were from Amersham (Amersham, UK). The 0.4 kb cDNA probe in pBR327 [Hennighausen and Sippel, 1982] for whey acidic protein (WAP) mRNA was provided by Dr. N. Hynes. The other mouse cDNA probes for β-actin, β-casein, and MDGI were as described [Binas et al., 1992].

Organ Explant Culture

Pregnant (17–18 day) primiparous, Balb/c mice were sacrificed by cervical dislocation, the abdominal and thoracic glands were removed, combined and placed in HEPES-buffered MEM, and sterilely cut into 1–2 mm pieces. Cultivation was performed as described [Binas et al., 1992]. Briefly, the tissue specimens were placed onto the bottom of 25 cm^2 Falcon plastic flasks at a density of about 30 explants per flask. After seeding, MEM was replaced by 1.5 ml of Medium 199 with Earle's salts, gentamicin (50 μg/ml) and prolactin (5 μg/ml), insulin (5 μg/ml), and 1 μg/ml hydrocortisone. Explants were cultured under an atmosphere of 50% O_2/5% CO_2, with daily medium changes. Cultures were terminated by freezing the explants in liquid nitrogen (for RNA and lipid extraction) or fixation in either 4% buffered formalin (for histology and morphometry) or 1% glutaraldehyde in phosphate buffer (for electron microscopy). For thymidine autoradiography, explants were incu-

bated with (^{3}H)TdR (2 mCi/ml, 72 Ci/mmol) in fresh medium for the final 4 h of culture, and fixed thereafter in 4% formalin/0.5% trichloroacetic acid.

Histology and Morphometry

Mammary gland explants were fixed in 4% buffered formalin at 4°C and embedded in paraffin. Sections cut at 4 μm were deparaffinized and stained by hematoxylin/eosin or Toluidine blue. For quantitative evaluation of morphogenesis, histological structures were counted in sections of at least 5 explants at a magnification of 1:150. The following criteria were used: for ducts, an adjacent sheet of connective tissue; for ductules, a linear coat of cubic epithelial cells surrounded by not more than one smooth muscle bundle; for alveoli, a linear coat of epithelial cells with basal membranes and a myoepithelial cell layer, exhibiting more or less numbers of intracellular lipid droplets. Only structures exhibiting lumina were considered. To compare the pattern of different cultures, quotients of the numbers of alveoli showing intraepithelial lipid droplets (more than 50% of cells with droplets) and those devoid of them (less than 50% with droplets) were calculated. At least 500 structures per experimental group were determined.

Electron Microscopy

Mammary gland explants were fixed in 1% glutaraldehyde in 0.1 M phosphate buffer with 0.18 M sucrose for 3 h. Following three washing steps without fixative, and cryoprotection in 2.3 M sucrose overnight, they were frozen in liquid nitrogen. Ultrathin cryosections (40 nm) were cut according to Tokuyasu [1980, 1986] at −115°C using a Reichert-Jung Ultracut E attached with a cryosystem FC4E. Sections were mounted on Formvar-carbon-coated copper grids and washed several times in bidistilled water. They were contrasted and stabilized using a mixture of 0.3% uranylacetate and 2% methylcellulose (25 cps, Sigma, Germany). Electron micrographs were taken with an EM 400T (Philips, The Netherlands) at an acceleration voltage of 80 kV.

Thymidine Autoradiography

Paraffin sections were cut at 4 μm, deparaffinized, incubated with an excess of cold thymidine (0.01% w/v) overnight, and then dried and coated with K8 nuclear research emulsion (ORWO, Germany) at 1:2 dilution at 40°C. After exposure for 4 days at 4°C, sections were developed and counterstained with Neutral Fast red. At least 1,500 nuclei were counted per experimental group. The labeling indices for the epithelial cells were calculated as the percentage of nuclei with more than 5 silver grains per nucleus. Stromal labeling was defined and quantified as labeled fibroblasts related to the absolute number of epithelial cells counted per field.

Northern Blot Hybridization

Total RNA was extracted following the protocol of Chomczynsky and Sacchi [1987]. Fifteen micrograms of RNA from each sample were electrophoresed in 1% agarose containing 6.3% (v/v) formaldehyde in MOPS buffer (20 mM morpholinopropane sulphonic acid, 5 mM Na-acetate, 1 mM EDTA, pH 7.0), stained with ethidium bromide, photographed for evaluation of the ribosomal RNA bands, and finally transferred to Hybond C-extra membranes (Amersham, UK) by capillary blotting. RNA was hybridized in a solution containing 5 × SSPE (1 × SSPE is 180 mM NaCl, 10 mM Na-phosphate, 1 mM EDTA pH 7.7), 5 × Denhardt's solution, 0.5% SDS, and 20 μg/ml DNA to mouse cDNA probes radiolabeled with ^{32}P by random priming. Filters were washed in 0.1 × SSC/0.1% SDS (1 × SSC is 150 mM NaCl, 15 mM Na_3-citrat) at 60°C. The relative intensities of hybridization signals were estimated using a Shimadzu scanning densitometer and normalized to the corresponding β-actin hybridization signal or the amount of 28S ribosomal RNA.

Fatty Acid Synthesis

The rate of synthesis of fatty acids was determined by measuring the amount of incorporation of (1-^{14}C)acetate (5 mCi/ml) over the last 2-h culture period. Lipids were extracted by the method of Bligh and Dyer [1959] with some modifications. Approximately 20 explants were washed twice in MEM, weighed and pulverized in a mortar under liquid nitrogen. Explants were then resuspended in 0.95 ml of a mixture containing $H_2O/CH_3OH/CH_3Cl$ (8:20:10) and sonicated for 3 s with a Sonifier B12 (Branson, MO). The resulting monophasic homogenate was diluted first with 0.25 ml of $CHCl_3$ and second with the same volume of water to yield a biphasic system. For phase separation the mixture was centrifuged for 5 min at 5,000*g*, the lower phase was collected and mixed with 0.7 ml from

the upper phase of a mixture of $H_2O/CH_3OH/CH_3Cl$ (9:10:10). After centrifugation, 0.2 ml of the lower phase was transferred to an ampule and stored under nitrogen at −20°C. Twenty-microliter aliquots of this solution were taken to measure the total incorporation of (^{14}C)-acetate after evaporation of the soluents in a Triton/Toluene-scintillation cocktail. For analysis of milk specific fatty acids, an aliquot of the organic phase was taken for methylation of the fatty acids according to Christie [1992]. Methylesters of fatty acids were separated by TLC on RP18 plates (Merck, Germany) by using a solvent system of acetonitrile/acetic acid/water (60:10:20) under saturating conditions. Positions of the methylated forms of fatty acids with a chain length of C_{10}–C_{18} were estimated by use of corresponding standards (Sigma). Relative radioactivity in the bands was quantified by means of a Bio-Imaging Analyzer (Fuji, Japan). Synthesis of milk specific C_{10} and C_{12} fatty acids [Smith and Stern, 1981] was expressed as the ratio of the added radioactivity in the C_{10} and C_{12} bands and the added radioactivity in the C_{16} and C_{18} bands representing the "basal" fatty acid synthesis. All experimental values have been obtained from quadruplicate determinations.

Analysis of Arachidonic Acid Release, Neutral Lipids, and Phospholipids

Explants were labeled for 24 h until equilibrium with (1-^{14}C)arachidonic acid (55 mCi/mMol) at a final concentration of 0.75 mCi/ml in the presence of prolactin, insulin, and hydrocortisone. During this period the medium was supplemented with 8.3 μM fatty acid-free BSA. At the end of the labeling period, 80% of the total added radioactivity was found to be incorporated. Explants were washed with medium and then cultivated under the various hormonal conditions for the indicated time. After termination, explants and medium were separately frozen in liquid nitrogen and further processed for extraction. Frozen explants were pulverized under liquid nitrogen as described above and extracted with a mixture of $CHCl_3/CH_3OH$ (1:1) for 10 min, followed by $CHCl_3/CH_3OH$ (2:1) at 4°C overnight. The upper phases were combined and washed first with $CHCl_3/CH_3OH$ (2:1) and then with 0.2 N KCl (final concentration 20%). After phase separation, the lower phase was collected and concentrated at 60°C under a stream of nitrogen. Lipids were immediately dissolved in $CHCl_3/CH_3OH$ (2:1) and applied to TLC. The following solvent systems were used: for separation of neutral lipids on DC 60 Silica plates (Merck), petrol ether/ether/acidic acid (85:15:1); for separation of phospholipids on LK5 Silica plates (Whatman, Clifton, NJ) $CHCl_3/CH_3OH$/acetone/acetic acid/water (6:2:8:2:1) [Bandyopadhyay et al., 1987; Cybulsky et al., 1989].

Identification and quantification of the corresponding radioactive bands were performed as outlined above. Nonradioactive standards were applied in parallel to identify the positions of free arachidonic acid, mono-, di-, and triglycerides and phospholipids. For neutral lipids the incorporated radioactivity in each band was expressed as relative value normalized to the total radioactivity of the sample. For analysis of (^{14}C)-arachidonate content in the various phospholipids, the total radioactivity of each sample was normalized to the amount of tissue (mg wet weight) used for the extraction procedure.

RESULTS

Mitogenic Effects of EGF and TGFα

Thymidine incorporation into DNA of both the ductular and alveolar epithelium of mammary glands of pregnant mice was low in the presence of prolactin, insulin, and hydrocortisone (PIH medium). Labeling indices ranged from 0.4 to 2.0% in these control cultures. Treatment for 48 h with EGF and TGFα led to a 3- to 9-fold increase which was usually more pronounced in the ductular epithelial cells than in the alveolar epithelial cells (Table I). Likewise, stromal cells showed a 3- to 4-fold enhancement in DNA synthesis in response to EGF or TGFα. In all cases, TGFα was more potent than EGF at equal concentrations.

TABLE I. Different Stimulation of DNA Synthesis in Various Mammary Cell Types by EGF and TGFα*

Growth factor	Labeling index (%)		
	Alveoli	Ducts/ductuli	Stroma
No factor	0.46	0.50	0.40
EGF	1.58	4.33	0.87
TGFα	3.31	4.51	1.86

*Labeling index values were estimated after a 2-day culture period in the presence of prolactin, insulin, hydrocortisone (PIH). Means of two independent experiments are given. For EGF or TGFα 20 ng/ml was added to the PIH medium.

EGF Suppresses Mammary Epithelial Differentiation

Specimens isolated from mouse mammary glands at 17 to 18 d of pregnancy exhibit a highly differentiated phenotype. Histologically, lobules are well developed and alveoli are maintained in a presecretory state (Fig. 1A). The latter are lined by cuboid epithelial cells filled with intracellular lipid droplets of varying size. Addition of EGF to the culture medium led to a dose-dependent effect on epithelial morphology. The alveolar epithelium became flattened, the alveoli occasionally distended, and the intracellular fat droplet content decreased by 80% with EGF (Fig. 1B) as quantified by the fat droplet index (Fig. 2B).

Also on the ultrastructural level the disappearance of intracellular fat droplets of alveolar cells was the most striking result of EGF treatment as compared with the control (Fig. 1C,D). Moreover, a more or less pronounced distortion of the microvilli in the EGF treated sample was evident throughout all sections (Fig. 1D). Other ultrastructural characteristics of the presecretory phenotype of alveolar epithelia did not differ from control explants.

As demonstrated in Figure 2, EGF affected all parameters of functional differentiation. Among the milk protein mRNAs, expression of WAP was most dramatically decreased, whereas the β-casein mRNA level was only slightly reduced after treatment with EGF (Fig. 2A). MDGI expression was similarly sensitive to EGF as WAP. Therefore, mainly the latter parameters were used in the following experiments. The half-maximal effect of EGF on expression of the two proteins was found at 4 ng/ml (data not shown).

Lipid synthesis was determined by measuring the total fatty acid (tFA) production in the epithelium and surrounding adipose tissue of the explants with emphasis on milk-specific, medium-chain fatty acid (MFA) synthesis which is selectively induced in epithelial cells by prolactin [Wang et al., 1972]. Treatment with EGF decreased both tFA and MFA synthesis by nearly 40% as compared to the control (Fig. 2B). Thus, EGF almost completely antagonized the effect of prolactin which stimulated both parameters to about twice the values estimated in the presence of insulin and hydrocortisone alone (data not shown).

TGFα Suppresses Mammary Epithelial Differentiation Similarly to EGF

TGFα was found to be as effective as EGF in depressing all the differentiation parameters determined (Table I). In addition, we observed an EGF dependent stimulation of endogenous TGFα secretion into the culture medium from mammary explants as determined by a commercial rat TGFα radioimmunoassay (Peninsula Laboratories). After a 24-h culture period in the presence of 20 ng/ml of EGF, 2.46 ± 0.82 pg of TGFα per ml medium were found as compared to 1.40 ± 0.47 pg/ml of TGFα in the PIH control medium (n = 5). There was no change in the TGFα concentration in the explant tissues after EGF treatment which was found to range from 200–300 pg of TGFα per mg protein.

Ara C Antagonizes the Mitogenic Effect of EGF and TGFα

In order to test whether the mitogenic action of both growth factors was a prerequisite for their dedifferentiating effects, we used cytosine arabinoside (ara C) to block DNA synthesis in the explants. As shown in Table II, stimulation of epithelial DNA synthesis induced by EGF or TGFα was completely blocked by 15 μg/ml of ara C. In contrast, the dedifferentiating effects of the growth factors, resulting in a reduction in WAP and MDGI mRNA expression, MFA synthesis, and fat droplet index, persisted in the ara C-treated groups. Thus, ara C was able to uncouple the mitogenic and dedifferentiating effects of EGF and TGFα. However, besides blockade of the DNA synthesis, a significant decrease of WAP expression and of the fat droplet index in the control cultures was noted (Table II).

Arachidonic Acid Substitutes for EGF in Suppressing Epithelial Differentiation

Because ara C acts at a very late step of the signal transduction pathway, we were interested to look at earlier stages of the signaling cascade for a possible discrimination between the mitogenic and the differentiation affecting activities of EGF and TGFα. Candidates for an early involvement in the EGF/TGFα initiated events are cytosolic phospholipase A_2 (PLA_2) and its metabolic product arachidonic acid [Spaargaren et al., 1992; Kast et al., 1993]. Therefore, we tested whether arachidonic acid could simulate the EGF dependent effects in the explant cultures. A 48-h treatment with arachi-

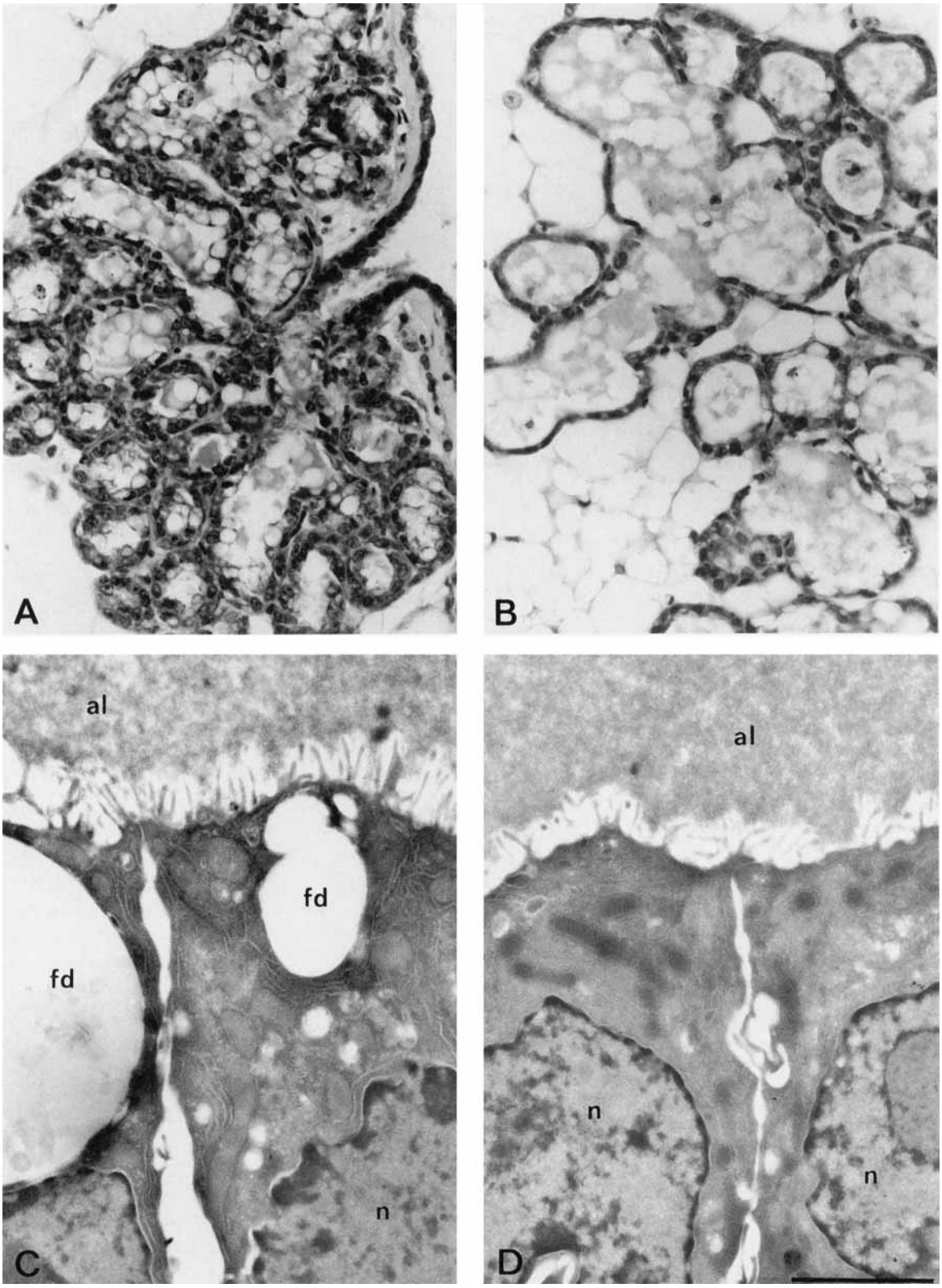

Fig. 1. Effect of EGF on the histology of mammary gland explants of late-pregnant mice. Explants were cultured for 2 days in presence of prolactin, insulin, and hydrocortisone with (B,D) or without addition of 100 ng/ml of EGF (A,C). **A,B:** 5-μm paraffin sections stained with hematoxylin/eosin. **C,D:** 40-nm ultrathin cryosections. Note dense intracellular lipid droplets (appearing as vacuoles after paraffin embedding) in the cuboid epithelial cells in a control explant (A). The alveolar epithelium is flattened and mainly free of intracellular lipid droplets in the explants treated with EGF (B). Magnification, ×250. This is confirmed at the electron microscopic level. Fat droplets (fd) are abundant in control cultures (C), but have disappeared after EGF treatment (D). al, alveolar lumen; n, nucleus. Magnification ×12,000; bar = 2 μm.

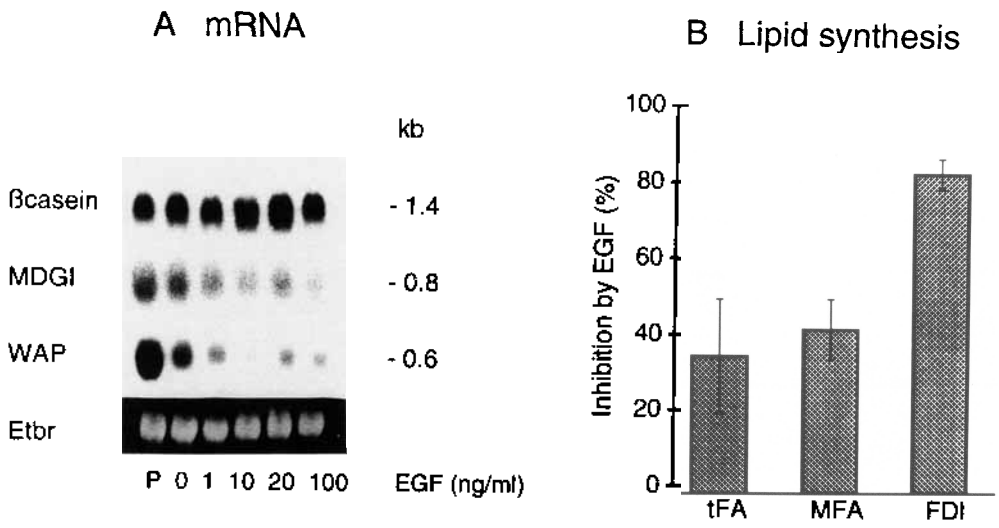

Fig. 2. Inhibitory EGF effect on parameters of functional differentiation. Explants were cultured for 2 days in PIH medium in the absence or presence of EGF at the indicated concentrations. **A:** A typical Northern blot with 15 μg of total RNA per lane is shown. P, control sample from non-cultivated tissue. The same blot was consecutively analysed with specific mouse ^{32}P-cDNA probes for the various transcripts. To demonstrate equal loading of the agarose gel the ethidium bromide staining of the 28-S ribosomal RNA bands is shown. **B:** Inhibition of (^{14}C)-acetate incorporation into the fraction of total fatty acids (tFA) or milk-specific fatty acids (MFA) and the prevention of intraepithelial fat droplet accumulation (FDI) by EGF. Inhibition by 100 ng/ml EGF was expressed as percent of the corresponding PIH control. Columns represent mean values ± sd obtained from 6 independent experiments.

TABLE II. Effect of Cytosine Arabinoside on EGF and TGFα Dependent Inhibition of Differentiation Parameters and Stimulation of DNA Synthesis*

Culture conditions	mRNA level		Synthesis of ^{14}C-milk FAs	Fat droplet index	Labeling index
	WAP	MDGI			
PIH	100	100	100	100	100
PIH + EGF	37 ± 7	42 ± 7	83 ± 12	34 ± 15	180 ± 26
PIH + TGFα	38 ± 7	45 ± 3	69 ± 4	16 ± 4	220 ± 29
PIH + ara C	50 ± 7	87 ± 43	97 ± 2	62 ± 24	<0.1
PIH + ara C + EGF	31 ± 1	38 ± 18	65 ± 4	27 ± 5	<0.1
PIH + ara C + TGFα	nd	53 ± 3	nd	22 ± 6	<0.1

*Explants were cultivated for 2 days in the presence of prolactin, insulin, and hydrocortisone (PIH). EGF and TGFα were added at a concentration of 20 ng/ml. Ara C was present at 15 μg/ml (nd, not determined). Values represent the mean ± sd of at least three independent experiments (<, less than). All values given in percent of control (PIH).

donate led to a concentration dependent suppression of WAP and MDGI mRNA, MFA synthesis, and fat droplet index (Fig. 3). The response of the explants to 25 μM arachidonate was equal to the effect of 20 ng/ml of EGF. However, arachidonate was ineffective to stimulate (^{3}H)-thymidine incorporation into mammary gland epithelium. Next, we investigated the effects of inhibitors of PLA_2 activity as pBrphBr [Irvine, 1982] or of the arachidonic acid metabolic pathways as indomethacin and NDGA [Needleman et al., 1986] in the presence of EGF. As shown in Figure 3, the addition of all three substances antagonized the dedifferentiating effect of EGF. However, the inhibitors were effective only in combination, whereas addition of each inhibitor alone or the combination of indomethacin and NDGA were ineffective. Reversal of the EGF effect was complete in the case of MDGI expression and fat droplet accumulation, whereas tMFA synthesis was restored to only about 80%. In contrast, the inhibitors failed to affect the mitogenic response to EGF.

The described effects of the three metabolic inhibitors suggested that PLA_2 activity as well as lipoxygenase and cyclooxygenase activity should be involved in the EGF induced responses. This was further substantiated by the

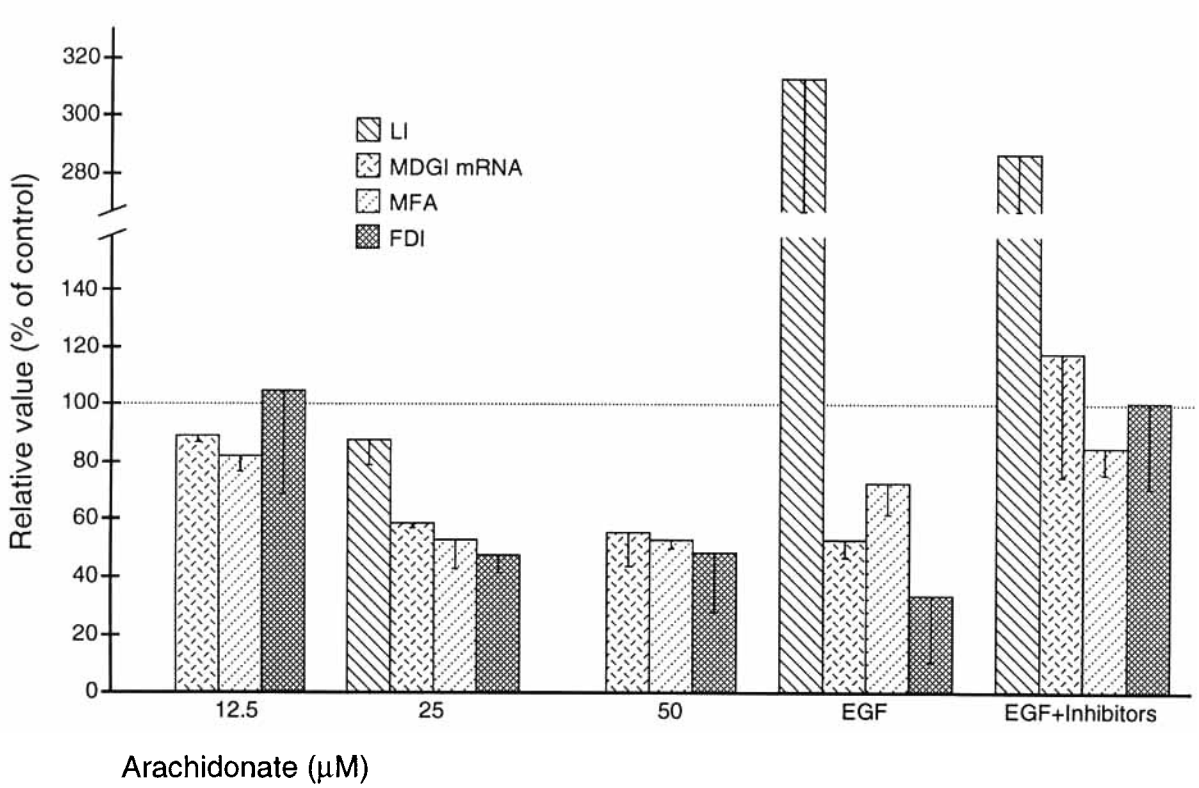

Fig. 3. Effects of arachidonic acid and its metabolic inhibitors on parameters of functional differentiation. Experimental values are expressed as percentage of the corresponding control values obtained after 2-day culture in PIH medium. Mean values ± sd from 4 independent experiments are given. EGF, if present, was added at 20 ng/ml. The inhibitors pBrphBr, indomethacin, and NDGA were added at 10 μM in combination.

finding that recombinant melittin, an activator of the endogenous PLA_2, or prostaglandin E_2, a metabolite of the cyclooxygenase pathway, was able to suppress functional differentiation similar to EGF (Table III).

EGF and TGFα Induce Release of Endogenous Arachidonic Acid

To demonstrate direct involvement of the arachidonic acid pathway in the action of EGF and TGFα, we ascertained whether changes in endogenous tissue arachidonic acid level occurred after growth factor treatment. After presaturation of the explants with (^{14}C)-arachidonic acid, 70% of the total radioactivity incorporated was found in the triglyceride and phospholipid pools, whereas only 4.2% was detectable as free arachidonic acid (Table IV). Treatment with EGF and TGFα induced a slight but significant increase from 4.2 to 5.6 and 7.0% of total radioactivity, corresponding to an elevation of endogenous free arachidonic acid by 33 and 66%, respectively. In parallel, a concomitant slight decrease of the arachidonate content in the phospholipid pool was observed (Table IV). No significant release of arachidonic acid into culture medium was found. This could be due to the presence of a high number of adipocytes in the explants which represent an additional reservoir of fatty acid uptake and storage with high capacity.

TABLE III. Melittin and Prostaglandin E_2 Substitute for EGF in Inhibition of Differentiation Parameters*

	Relative values, % of control	
Culture conditions	MDGI mRNA	FDI
PIH	100	100
PIH + EGF	42 ± 10	43 ± 19
PIH + 1 μM melittin	59 ± 13	29 ± 3
PIH + 10 μM prostaglandin E_2	62 ± 8	43 ± 16

*Explants were cultured for 2 days in PIH medium. EGF was present at 20 ng/ml. Melittin was present in the culture medium only during the first 2 h of the culture period. Values represent the mean ± sd of three independent experiments.

To identify the phospholipid source of arachidonic acid mobilization by EGF and TGFα as demonstrated in Table IV, we determined the

TABLE IV. Release of Free ^{14}C-arachidonic Acid by EGF or TGFα†

Conditions	% of total ^{14}C-arachidonate		
	Arachidonic acid	Triglycerides	Phospholipids
Zero time	4.0 ± 0.5	34.4 ± 3.8	43.4 ± 4.3
20 min PIH	4.2 ± 0.4	31.1 ± 2.8	37.0 ± 4.2
20 min PIH + EGF	5.6 ± 1.0*	28.6 ± 4.7	31.6 ± 4.3
20 min PIH + TGFα	7.0 ± 1.1*	34.2 ± 0.5	32.3 ± 5.6

†EGF or TGFα were present at 20 ng/ml. Values represent the mean ± sd of four independent experiments.
*Values that are statistically different from the control value ($P < 0.05$, Student's t-test).

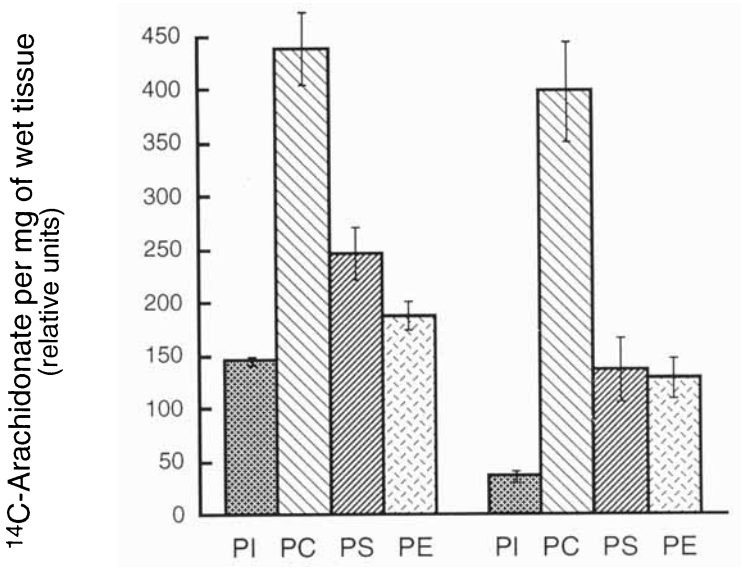

Fig. 4. Release of (^{14}C)arachidonic acid from phospholipids. Explant cultures were pre-equilibrated with (^{14}C)arachidonic acid for 24 h in PIH medium. After withdrawing the labeling medium incubation in the absence (PIH) or presence of 20 ng/ml of EGF, EPIH was performed for 20 min. The (^{14}C)arachidonate content in each individual phospholipid class was analysed by thin layer chromatography, quantified by means of a Bio-Imaging Analyzer (counts as "relative units"), and normalized to the amount of tissue used for the extraction procedure. (PI, phosphatidylinositol; PC, phosphatidylcholine; PS, phosphatidylserine; PE, phosphatidylethanolamine). Means ± sd of 3 independent experiments are given.

EGF effect on the arachidonic acid content of the individual phospholipid groups (Fig. 4). Under control conditions, incorporation of (^{14}C)-arachidonate was highest in the phosphatidylcholine fraction. Radioactivity in the phospholipids of EGF treated explants followed the same pattern but was found to be reduced by 6 to 75% in all four phospholipid groups (Fig. 4). This decline in the absolute amount of radioactivity incorporated corresponded to the decrease of relative radioactivity in the phospholipid pool as shown in Table IV. The maximal effect of EGF was found on the level of (^{14}C)-phosphatidylinositol, which was reduced by about 75%. Significant reduction of incorporated radioactivity was also determined for (^{14}C)-phosphatidylserine (−31%) and (^{14}C)-phosphatidylethanolamine (−29%) (Fig. 4). Because PLA_2 preferentially utilizes phosphatidylcholine and phosphatidylethanolamine as substrates, probably the last one might function as the source of arachidonic acid release by PLA_2 in our culture system. PLA_2 can also hydrolyze phosphatidylinositol [Holtzman, 1992], but more frequently this phospholipid is cleaved by the phospholipase C (PLC) isoenzymes, which can also be stimulated by EGF or TGFα [Hernandez-Sotomayor and Carpenter, 1992].

Modulators of 1,2-Diacylglycerol Lipase (DAG Lipase) or Protein Kinase C (PKC) Do Not Simulate or Counteract the EGF Effects

The generation of DAG from phosphatidylinositol as a consequence of PLC activation can lead

to an alternative mechanism of arachidonate release than that by PLA_2: the sequential degradation of the generated DAG by DAG lipase and monoacylglycerol lipase which finally liberates the free fatty acid.

To test this possibility, we made use of the potent and selective DAG lipase inhibitor U57,908 [Yang et al., 1991]. The concentrations employed had been shown to be highly effective in other culture systems [Yang et al., 1991]. As demonstrated in Table V, the inhibitor failed to counteract the EGF induced changes in our explant culture. But a decrease of both MDGI mRNA level and DNA synthesis in the PIH control sample treated with 50 μM U57,908 alone was noted (Table V). The lack of effect of the DAG lipase inhibitor in presence of EGF excludes an involvement of this enzymatic pathway in the EGF induced changes.

The release of arachidonic acid and the liberation of DAG as a consequence of phosphatidylinositol degradation by PLC could result in the activation of PKC, a further key enzyme of the intracellular signalling pathway and candidate of EGF dependent activation [Fabbro et al., 1991]. Therefore, we tested in further experiments the effects of TPA as an activator and of O-HMG as a specific inhibitor of PKC [Bandyopadhyay et al., 1993].

With both modulators no evidence for an involvement of PKC in the EGF induced responses was found: (1) The PKC activator TPA failed to simulate both the mitogenic and dedifferentiating effects elicited by EGF (Table V); (2) O-HMG was unable to counteract the EGF induced decrease of the MDGI mRNA level and stimulation of DNA synthesis (Table V). Nevertheless, the decrease of thymidine incorporation in the control sample treated with O-HMG in the PIH medium suggests some role of PKC isoenzymes in the "basal" PIH-dependent DNA synthesis in the absence of EGF. Alternatively, both O-HMG and TPA may have non-specific effects in the explant culture, which could be responsible for the inhibitory effects of the drugs in the PIH control assays.

TABLE V. Modulators of PKC or DAG Lipase Activity Do Not Simulate or Counteract EGF Effects*

Culture conditions	Relative values, % of control	
	MDGI mRNA	LI
PIH	100	100
PIH + EGF	31 ± 6	186 ± 8
PIH + 50 μM U57,908	22 ± 8	48 ± 13
PIH + EGF + 50 μM U57,908	24 ± 0	215 ± 33
PIH + 10 ng/ml TPA	86 ± 13	84 ± 17
PIH + 25 μM O-HMG	117 ± 17	48 ± 36
PIH + EGF + 25 μM O-HMG	37 ± 1	181 ± 25

*Explants were cultured for 2 days in PIH medium. EGF was added at a concentration of 20 ng/ml. Values represent the mean ± sd of two experiments.

DISCUSSION

Previous studies have mainly focussed on EGF induced growth and development of the virgin mammary gland [Banerjee and Antoniou, 1985; Tonelli and Sorof, 1980; Vonderhaar, 1987; Coleman et al., 1988; Haslam et al., 1993]. However, observations concerning the mitogenic activity of EGF and the inhibitory effects of EGF on milk production as demonstrated in the present experiments support the suggestion that EGF also performs a physiological role in the mammary gland of pregnant mice. As indicated by (^{3}H)-TdR incorporation, EGF affects DNA synthesis of ductular epithelial cells more strongly than that of alveolar epithelial cells. This effect was found to be closely associated with suppression of processes such as WAP mRNA, MDGI mRNA, tFA, and MFA synthesis. In parallel, accumulation of milk fat droplets in the alveolar epithelium—which reflects the result of a complex cellular process including fatty acid uptake and synthesis, triglyceride production and intracellular transport—as markedly reduced. This effect could correspond to an in vivo function of EGF during mid or late pregnancy in stimulating the final processing and maintenance of the lobulo-alveolar gland structure associated with prevention of premature milk overproduction. In this respect, EGF is a physiological antagonist of MDGI at the cellular level. In mammary epithelial cells from mammary explant or from whole organ cultures, MDGI has been shown to be expressed during hormonally induced differentiation [Binas et al., 1992] [Yang et al., in press]. Recently, it has been demonstrated that MDGI when added to whole mammary gland organ cultures of virgin mice, induces growth suppression, stimulates differentiation, and counteracts the proliferative effects of EGF (Yang et al., manuscript submitted).

TGFα is also a potent mitogen for mammary epithelial cells and binds to the same receptor as EGF [Bates et al., 1990]. In our experiments, TGFα showed qualitatively similar effects as EGF but was generally a stronger agonist. Since EGF was found to stimulate the release of TGFα in the mammary explant cultures, it cannot be excluded that the effects of EGF are mediated in part through the induction of endogenous TGFα. This would be in accord with the suggestion that TGFα and not EGF is the predominant growth factor operating locally in the mammary gland [Vonderhaar, 1988]. In this context the higher sensitivity of the alveolar epithelial cells to the mitogenic stimulus of TGFα is of special interest. Whereas the underlying mechanism of this effect is not clear it could be related to the finding that transgenic mice overexpressing TGFα in their mammary glands show earlier alveolar development compared with nontransgenic controls and develop alveolar hyperplasia and adenocarcinomas [Halter et al., 1992].

Ara C was able to block EGF or TGFα induced mitogenesis in the mammary explant cultures of late pregnant mice but failed to affect concomitant dedifferentiation. This finding confirms previous data obtained with primary mammary epithelial cell culture and explant cultures obtained from mid-pregnant mice [Owens et al., 1973; Smith and Vonderhaar, 1981; Taketani and Oka, 1983] and extends them to other differentiation parameters. It should be mentioned, that ara C at higher doses may also evoke a slight decrease of total RNA synthesis (P. Langen, personal communication), as observed in our experiments. Ara C treatment leads to uncoupling of mitosis and dedifferentiation. However, since ara C acts at a very late stage by blocking the DNA synthesizing machinery, this compound fails to discriminate between a possible common or different signal mechanism(s) for the two EGF/TGFα-induced cellular processes. Therefore, the role of arachidonic acid and/or its metabolites as candidates involved in EGF/TGFα signal transduction were studied [Skouteris and McMenamin, 1992; Spaargaren et al., 1992; Peppelenbosch et al., 1992; Kast et al., 1993].

Evidence for the existence of distinct intracellular signalling pathways for the mitotic and dedifferentiating effects in mammary gland epithelial cells is based on the following data: (1) Arachidonic acid is released endogenously in response to EGF/TGFα. When added to the culture medium, the fatty acid substitutes effectively for EGF in inducing dedifferentiation but fails to excert mitogenic stimulation. (2) The dedifferentiating activity of EGF can be blocked by substances abolishing arachidonic acid generation and metabolism such as inhibitors of PLA_2 activity and inhibitors of the cyclooxygenase and lipoxygenase pathways without affecting the mitogenic activity of this peptide. To completely neutralize the dedifferentiating action of EGF a combination of inhibitors for PLA_2, cyclooxygenase, and lipoxygenase activity was needed. This means, that both the release of arachidonic acid and the formation of its metabolites must be hindered. The involvement of PLA_2 activity and the arachidonate metabolism in the EGF induced responses is further supported by the findings that activation of the endogenous PLA_2 by melittin or treatment of the explants with PGE_2 can simulate the inhibitory EGF effect on parameters of functional differentiation.

The main sources for arachidonic acid release from membrane phospholipids by lipase catalyzed hydrolysis are phosphatidylcholine, phosphatidylethanolamine, and phosphatidylinositol [Holtzman, 1992]. Accordingly, we found significant breakdown of the corresponding (^{14}C)-arachidonoyl-derivatives of the last two phospholipids in response to EGF. The degradation of membrane lipids probably involves different lipolytic enzymes. Phosphatidylethanolamine is preferentially utilized by PLA_2 to directly yield free arachidonic acid. Phosphatidylinositol can be cleaved to yield arachidonic acid by the sequential actions of PLC, DAG lipase, and monoacylglycerol lipase [Irvine, 1982; Yang et al., 1991]. Because the inhibition of DAG lipase activity by the potent and selective drug U57,908 [Yang et al., 1991] did not counteract the EGF induced changes, this metabolic pathway should not be involved in EGF signal transduction leading to dedifferentiation and increased DNA synthesis in the explant culture.

Since arachidonic acid and DAG are known to activate PKC, this key enzyme was a further candidate suggested to be involved in mediating the described EGF/TGFα action. Various PKC isoenzymes are expressed and functionally active during pregnancy in the mouse or rat mammary gland [Caulfield and Bolander, 1986; Connor and Clegg, 1993], or in a mouse mammary epithelial cell line [Marte et al., 1994]. TPA as an activator and O-HMG as a selective inhibitor of PKC activity [Bandyopadhyay et al., 1993]

were tested, but neither could TPA simulate the EGF effects, nor O-HMG block them. These findings are in contrast to data reported previously by other authors [Taketani and Oka, 1983; Bandyopadhyay et al., 1993], which suggest an involvement of PKC in the mitogenic and dedifferentiating response of mammary epithelial cells in primary culture to EGF. The reason for the failure of TPA to elicit a mitogenic stimulus in our explant culture similar to that found after TPA treatment of the primary cells [Taketani and Oka, 1983; Bandyopadhyay et al., 1993] is not clear. Possibly, the higher differentiation stage of the epithelial cells maintained in the explant cultures accompanied by a downregulation of various PKC isoenzymes under conditions of functional differentiation as described previously [Caulfield and Bolander, 1986] might be responsible for the contradictory results. On the other hand, in a very recent report the PKCα isoenzyme was shown to be positively involved in mammary differentiation, measured by β-casein induction in HC-11 cells [Marte et al., 1994]. Taken together, these first preliminary results with the various modulators of DAG lipase or PKC activity suggest a mechanism of EGF action which is independent of these enzymes.

In this paper, the main attention was focussed on the effects of EGF on cellular synthetic activities. EGF is well known, however, to influence cell-to-cell interactions and cell shape of normal epithelia, too. (1) Following ligand-EGF receptor interaction, the growth factor is capable of loosening cell adherence by phosphorylation of β-catenin and, in this way, affecting the E-cadherin-catenin complex [Behrens et al., 1993]; (2) EGF leads to actin remodeling followed by the rounding up of epithelial cells and functional disturbance of myoepithelial cells [Koyasu et al., 1988; Den Hartigh et al., 1992]. Both events should result in distension of alveoli as observed in our experiments after EGF treatment of the mammary gland explants. The arachidonate pathway is involved at least in the second process [Peppelenbosch et al., 1993]. Thus, EGF induced actin remodeling based on this pathway could contribute to structural dedifferentiation of mammary epithelium.

Taken together, our findings suggest a key role for arachidonic acid and its metabolic derivatives in mediating the inhibitory effect of EGF and TGFα on the structural and functional differentiation of the mammary epithelium in the explant culture system. The capability of uncoupling EGF-induced DNA synthesis and dedifferentiation by PLA_2, cyclooxygenase, and lipoxygenase inhibitors suggests for the first time that the EGF activated signaling cascade can be separated into one way, leading to inhibition of differentiation and alternative or additional pathways, resulting in the mitogenic stimulus at least in highly differentiated mammary epithelial cells. Further studies are needed to demonstrate directly the activation of PLA_2 and the synthesis of arachidonate metabolites in response to EGF.

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