The Effect of Epidermal Growth Factor on Intracellular Cyclic Nucleotide Levels

by

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Dedication

To Dr. J. B. Moore, Jr. who gave encouragement and willingly sacrificed his time so that I could learn some important aspects of biochemistry.

Ray DuBois

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INTRODUCTION

The purpose of my experimentation is twofold: (1) To isolate Epidermal Growth Factor(EGF) (2) To determine the effect of EGF on intracellular cyclic nucleotide levels.

A host of chemical signals which bind to specific membrane (surface) receptors influence most differentiated cells. A central problem in biochemistry is to explain in detail how such ligand-membrane interactions are trarsduced into intracellular alterations. The signals represent a diverse group of physiological substances and encompass large molecular weight proteins (insulin, nerve growth factor, and glucagon) and small amino acid analogs (epinephrine, dopamine, and serotonin). These compounds attach to the outside of the responsive cell membrane at a specific location called the cell receptor. This binding triggers some intracellular response which causes a transitory metabolic alteration within the cell. The cell responds to the change by drastically altering its growth, transport of metabolites, secretion of pharmacological mediators, or ability to send nerve impulses. Therefore, transducing an extracellular event (binding to receptor) to an intracellular change (e.g., increased glucose uptake by the influence of insulin) must occur through some intermediary process that transmits the outside signal to an inside alteration.

Some of the signals are transmitted through the circulation as the result of hormone release from some endocrine tissue. Others behave as local transmitters between adjacent cells (neurotransmitters). A third type of signal that is now beginning to be explored mechanistically is the group of proteins called growth factors. The three most studied are epidermal growth factor (EGF), nerve growth factor (NGF), and mesenchymal growth factor (MGF). They have been shown to enhance the growth and, more importantly, the differentiation of their responsive cell types.

Epidermal growth factor stimulates the differentiation and growth of epithelial cells. Although the medical applications of EGF are in a very preliminary stage of development, it is currently being studied for its use in burn and wound healing on the skin, psoriasis, and the healing of eye trauma. The mechanisms that EGF utilizes to accomplish this stimulation are not known. Although EGF does not enter the cell, it does cause many intracellular changes. The 'messenger' which transduces the binding of EGF to intracellular alterations may be the cyclic nucleotide system which has been well-documented in other hormone-induced cellular changes (e.g., epinephrine effect on glucose metabolism). Very recent research indicates that nerve growth factor (NGF) may raise intracellular cyclic AMP levels in ganglia. The exact mechanism of transduction for the three mentioned growth factors, in addition to insulin, remains unknown.

The importance of understanding the details by which EGF influences epithelial cells is twofold. Firstly, the delineation of how ligand-membrane interactions alter cellular function is currently one of the most important areas of biochemistry, pharmacology, immunology, and developmental biology. Secondly, EGF research has potential usefulness in several medical problems; the most important is psoriasis, since it afflicts approximately 3% of the population of the USA (5-6 million people). Psoriasis is manifested by the proliferation of epidermal cells that is propagated through an unknown mechanism. The potential for EGF in the treatment of burns and non-perforating eye wounds is also very significant. Therefore, investigating the role of cyclic nucleotides in EGF-stimulation of epithelial cell differentiation and growth will be research not only into a dynamic area of biochemistry but also into the biomedical alleviation of human suffering.

REVIEW OF THE LITERATURE

Epidermal growth factor (EGF) was discovered in 1962 as the result of an observation by Cohen (1) that partially purified extracts of male mouse submaxillary glands when injected daily into newborn mice caused precocious opening of the eyelids and tooth eruption. These observations were made during the course of his studies on characterizing nerve growth factor, another protein isolated from the submaxillary glands of male mice. The EGF protein isolated from the submaxillary glands in 1962 was homogenous by ultracentrifugation with an isoelectric point of 4.2.

Work described in 1963 by Cohen and Elliott (2) revealed EGF stimulated epidermal keratinization of mice and rats as determined by histological examinations. The eyelid, back-skin, and tail were found to have increased keratinization if the mice (or rats) were treated subcutaneously from birth to day-12 with 0.5-4.0ug EGF/gm. body weight. Also, if 12-20 day old mice were treated with 8ug EGF/gm. body weight for 30 days, epidermal sections of the EGF-treated animals showed enhanced cell growth. This is a significant observation since it reveals that mature animals also respond to EGF.

Using cultured embryonic chick dermis, Cohen showed that EGF stimulated the growth of the entire epidermis (3). The thickening noted upon EGF treatment correlated with increased mitosis of basal cells; EGF stimulated the proliferation of basal cells. More mechanistic studies (4-6) demonstrated that EGF enhanced protein and ribonucleic acid synthesis, increased the synthetic activity of ribosomes, and increased the formation of polysomes. EGF, therefore, does stimulate intracellular metabolic processes within the epidermis.

In 1969, Turkington (7) reported that EGF was a potent regulator of mammary cell proliferation. Using pregnant mice as the source of the mammary cells, he showed that EGF in-

creased the rate of DNA synthesis in-vitro 3-4 fold with a maximal response at $8x10^{-9}$M EGF. Turkington also found that, rather than altering the rate of DNA replication, EGF increased the number of cells engaged in DNA synthesis. Hollenberg (8) demonstrated that EGF stimulated thymidine incorporation into contact-inhibited rabbit lens epithelial cells in culture. Half-maximal stimulation was noted at $6x10^{-10}$M. In these studies insulin was also shown to bind to lens cells, but the stimulation of thymidine incorporation was only 1/10 that of EGF. Hence, using epithelial cells, transduction of the EGF binding event to an intracellular effect was much more efficient than that of insulin.

EGF had very similar effects in mammary explants from non-pregnant mice (7). The proliferating cells, when incubated with EGF and insulin, assumed an ordered arrangement around an alveolar lumen, and, after the addition of prolactin, the cells elicited a secretory appearance with full alveolar development. Further work (9) demonstrated that EGF also stimulated the proliferation of a mouse (C_3H) mammary carcinoma. Again, the effective concentration ($8x10^{-10}$M) in vitro is in the concentration range found in vivo in plasma indicating the relative specificity of the EGF protein.

Cohen (10) reported that EGF stimualted the induction of ornithine decarboxylase in chick embryo epidermis in vitro and in vivo in the mouse. The de novo synthesis of ornithine decarboxylase was EGF dose dependent, had a maximal (40x) induction at 4 hr. post treatment, and was enzyme specific in that the activity of other decarboxylases, dehydrogenases, and phosphatases was not enhanced. The marked but transient stimulation of an enzyme thought to be the rate limiting step in polyamine synthesis (11) is an important finding in regard to cell growth. Polyamines have been implicated in many and diverse biological processes such as the stimulation of DNA and RNA synthesis (12), stabilization of ribosomes and mem-

branes (13), and inhibition of RNA degradation (14). Therefore, their importance in rapidly dividing cells is significant, and the mechanisms which initiate induction of this enzyme are pertinent areas for research.

The chemical and physical structural studies of EGF have revealed a high and low molecular weight form of the protein (15). In crude homogenates, the EGF activity was found almost entirely in a high molecular weight complex (MW 74000). This high molecular weight form can be dissociated reversibly into EGF (MW 6400) and an EGF-binding protein (MW 29000) displaying arginyl-esterase activity. Epidermal growth factor (MW 6400) is a polypeptide chain containing 53 amino acids and 3 disulfide bonds; its primary sequence has been determined (16). The binding protein (MW 29000) displaying arginyl-esterase activity does not modulate the biological activity of EGF *in vivo* or *in vitro* and binds to EGF to form larger molecular weight complex (MW 74000) with a stoichiometry of EGF_2. binding $protein_2$ (17).

Epidermal growth factor is synthesized and stored in the male mouse submaxillary gland (18). Immunofluorescent staining techniques reveal EGF is stored in male glands but not in female glands. Testosterone (a male sex hormone) treatment, however, induces the female glands to stain for EGF in a similar manner to male glands (19).

In the mouse, EGF is normally found in plasma in the range of 1-5ng/ml, in saliva and urine at 1000ng/ml, and in milk at 200-400ng/ml (20). The androgen stimulation of EGF synthesis and storage (3-fold increase) does not cause increased levels of EGF in the circulation (plasma). Administration of α-adrenergic agonists (e.g., phenylephrine), however, does cause a dose-related increase in plasma EGF. As EGF levels in the plasma increase over a 100x, EGF levels in the submaxillary gland decrease. However, animals without submaxillary glands maintain plasma levels of EGF (1-5ng/ml)

months after excision of the glands indicating another location for synthesis of EGF within the mouse.

The characteristics of the binding of radiolabeled EGF to membranes were similar to those for insulin binding to fat cells (21). Cuatrecases showed that EGF binding to rat liver membranes and human placental membranes was specific, saturable, and of high affinity. No competition for sites occurred when other hormones (including insulin and glucagon) were used to displace EGF. Further research by Cuatrecases revealed that EGF binding to human fibroblasts caused a stimulation of thymidine incorporation and α-aminoisobutyrate uptake (8). Therefore, one aspect of EGF binding to receptors on the cell surface is to alter membrane permeability in stimulating the growth of the cells.

Application of EGF research to human and veterinary medicine is in a very preliminary stage of development. The finding that EGF stimulates <u>in vivo</u> cell proliferation of the adult rabbit's corneal epithelium (22) is an important beginning for EGF application to humans. Research with rabbits demonstrated that EGF significantly enhanced the healing of nonperforating wounds in the corneal epithelium (EGF caused the healing time, as determined by histology, to be reduced by 50%). Using human corneas, Cohen (23) described the EGF stimulation of human epithelial cells. This important discovery that mouse EGF caused a direct growth-stimulating effect on human fetal cells initiated his search for human EGF. Indeed, Cohen (24) recently has isolated and characterized human EGF. Isolating the protein from the urine of pregnant women, Cohen has used this human EGF to stimulate the growth of human foreskin fibroblasts and to cause precocious eyelid opening and tooth eruption in newborn mice. Very recently, Moore et al (25) have used EGF to develop anti-psoriasis screening test for the discovery of effective psoriatic drugs. Cyclic nucleotides have been

implicated in psoriasis (26) and a current approach to psoriatic patients is to apply topically agents which will elevate cyclic AMP levels. Further research into the relationships of EGF and cyclic nucleotides is needed to clarify the mehanisms employed by EGF to stimulate cell growth.

RECORD OF STUDY

The first semester of research involved familiarization with cAMP assy and isolation of epidermal growth factor. A simple and sensitive assay for adenosine 3':5'-cyclic monophosphate(cAMP) has been developed that is based on competition for protein binding of the nucleotide, presumably to a cAMP-dependent protein kinase. The protein--nucleotide complex is adsorbed on a cellulose ester filter. Assay conditions are such that a binding constant approaching 10^{-9}M is obtained and the assay is thus sensitive to 0.05-0.10 picomoles of cAMP (27). This assay was developed by Alfred G. Gilman of the National Institute of Health in Bethesda, Maryland. The purification procedure for EGF was developed by Stanly Cohen who is now doing research at Vanderbilt University in Nashville, Tennessee. The method involves excising the glands from male mice(25-30g), freezing immediately in liquid nitrogen(glands kept at 20C until use), homogenizing in distilled water, and treating the supernatant overnight with streptomycin sulfate (pH 6.8-7.1). The supernatant is then treated with ammonium sulfate(100g/178ml), and the precipitate, recovered by centrifugation, is resuspended in water, and dialyzed extensively against distilled water. The solution is then placed in a boiling water bath for 5 minutes, cooled, and centrifuged. The supernatant contains EGF. Column chromatography is used to purify the EGF protein. The first step is to place the EGF(in distilled water) solution on a CM-cellulose column (2mM sodium acetate pH 5.6). The column is washed with distilled water; EGF, under these conditions is not absorbed. A second CM-cellulose column is used to absorb the EGF protein. The eluent from the first column is acidified to pH 4.2, poured onto the second column, and EGF is eluted with a hyperbolic salt gradient. After dialysis against distilled water for 5-6 hours, the

solution is concentrated by lyophilization for fractionation on Sephadex G-75. The EGF fraction from gel filtration is further purified by eluting on a DEAE-cellulose column (2mM sodium acetate buffer, pH 4.2) using a pH gradient to elute EGF.

The second semester was spent determining the effect of epidermal growth factor on intracellular cAMP levels. I used an in vitro system in which the back skin from newborn rats(2 days old) was incubated in physiological buffer for 3 minutes at 37C with varying concentrations of EGF. The tissue was then extracted for cAMP. The results of the cAMP assays for experiments I & II, which were duplicates, can be seen below.

Experiment I.

Sample	EGF(ug)	cAMP(p.mol./mg wet tissue)
1	348	0.115
2	162	0.052
3	35	0.045
4	23	0.027
5	None	0.015
6	None	0.011

Experiment II.

1	348	0.078
2	162	0.052
3	35	0.057
4	23	0.085
5	None	0.106

CONCLUSION

The results of the duplicate experiments are not identical and this brings up the question of how to interpret the data. The first experiment indicates that there really is a change of cAMP levels upon the addition of EGF. However, in the second experiment the levels remained virtually the same. This contradictory result is almost impossible to explain without conducting further experiments.

I suggest another experiment be done exactly as these two were. The results of such an experiment would seem to indicate what actually is happening. An *in vivo* study would be extremely helpful because the *in vivo* system shows definite changes(i.e., precocious opening of eyelids and tooth eruption) upon injection of EGF subcutaneously. The *in vitro* study could have been affected by a variance of cell response. In other words, there might have been no change in some of the test cases simply because a failure of the cells to respond due to shock or injury.

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