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Detection of Protein Kinase C Isoenzymes in the Growth of Human Epidermal Keratinocytes by Growth Factors

Eun-Young Joo† and Nam-Woo Kim

Department of Biology, Faculty of Natural Science, Kyungsan University, Kyungsan, Kyungbuk, 712-240, Korea

Abstract: Subconfluent neonatal human epidermal keratinocytes were treated with a concentration 200 ng/ml of human recombinant epidermal growth factor (hrEGF), human recombinant insulin-like growth factor-1 (hrIGF-1), and with a combination of hrEGF and hrIGF-1. Cytoplasmic and membrane-associated proteins were extracted and assayed. Proteins were separated by SDS-PAGE, and subjected to the western blot analysis. In the cytoplasmic fraction, the PKC concentration of keratinocyte treated with hrIGF-1 was higher than the control group, but the concentration of control group was the highest than the others in the membrane fraction. In the cytoplasmic fraction, EGF stimulated PKC-βII, -δ, -θ and also stimulated PKC-α, -βI, -δ, -ζ and -θ in the membrane fraction. IGF-1 stimulated PKC-βI, -ζ and -θ in the cytoplasmic, PKC-α, -βI, -δ, -ζ, -ε and -θ in the membrane. In the cells treated with a combination of EGF and IGF-1, PKC-α, -βI, -ζ and -θ in the cytoplasmic fraction, PKC-α, -δ, -ζ, -ε and -θ in the membrane fraction were stimulated.

Key Words: Protein kinase C isoenzyme, Keratinocyte, hrEGF, hrIGF-1, Western blot analysis

INTRODUCTION

In the skin, the epidermis is a stratified squamous epithelium mainly composed of keratinocytes.[25] The keratinocyte provides mechanical protection, prevents water loss, and forms the first line of immunological defense as well. The immunological function of the keratinocyte appears in pathological conditions-e.g., during wound healing and in allergic and inflammatory reactions. In response to epidermal injury, keratinocytes become 'activated'; i.e., they respond to growth factors and cytokines, become migratory, and can produce components of basement membrane.[15] Keratinocyte behavior can be modulated by diverse factors that can favor either proliferation or differentiation.[18,25,26,40]

Protein kinase C plays a central role in the transduction of a variety of external signals (growth factors, hormones, etc.) at the intracellular level.[21,22,39] Protein kinase C (PKC) belongs to a family of phospholipid-dependent protein serine/threonine kinases activated especially by diacylglycerol.[6,9,21,37] To date, 12 PKC isoenzymes have been identified and classified three groups based on their structure and cofactor regulation.[29,31,32,37] The best characterized and first discovered are the conventional protein kinase Cs (cPKC): α, two alternatively

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†Corresponding author: Eun-Young Joo, Tel: (053) 819-1437, Fax: (053) 819-1271, E-mail: jey@kyungsan.ac.kr
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spliced variants βI, βII, and γ. This class distinguishes itself from the others in that function is regulated by Ca^{2+}. The next well characterized are novel protein kinase Cs (nPKC): δ, ε, η (L), θ, and μ. These isozymes are structurally similar to the cPKCs, except that belong to Ca^{2+}- independent group. The least understood isozymes are atypical protein kinase Cs (aPKC): ζ, ι, and λ.[6,29,33,34,35,37] PKC isoenzymes have different tissue distribution, properties, and function. Studies have linked specific PKC isoenzymes with cell division and proliferation, including PKC-β, -ζ and -α in skin,[6,34] PKC-δ in CHO cells,[39] PKC-βII in erythroleukemia cells,[24] PKC-δ and -ε in fibroblasts,[33] PKC-α, and -ε in smooth muscle cells, PKC-α and -β in T-cells,[14] and PKC-ζ in nuclei of unstimulated nerve cells.[10] Upon activation, certain isoenzymes may translocate from the cytoplasmic to locations other than the plasma membrane, including the nucleus[1] and cytoskeleton.[35]

PKC is known to regulate a variety of cell functions, such as proliferation and differentiation.[1,4,19,22,29,33] The involvement of PKC in skin homeostasis is now established. Indeed, considering that these kinases are a receptor for tumor promoters, phobol ester, it may play an essential role in tumor promotion in human skin. Moreover, a PKC defect seems to be involved in a number of skin diseases, e.g., psoriasis and keloids.[6,22] PKC is crucially involved in the regulation of keratinocyte proliferation but is not only target of anti-proliferative drug action.[11]

In addition to epidermal growth factor (EGF), transforming growth factor-α (TGF-α) and insulin-like growth factor-1 (IGF-1) are a potent mitogen for keratinocytes.[27] IGF-1 augments the proliferative effects of EGF receptor ligands by increasing EGF receptors[20] and including the production of autocrine EGF receptor ligands, TGF-α and amphiregulin.[38] The IGF-1 receptor is overexpressed in hyperplasic keratinocytes.[13] The IGF-1 signaling pathway has not been completely defined, but protein kinase C may be involved.[12] Activation of PKC leads to feedback inhibition of the IGF-1 receptor.[5] PKC-independent pathways have also been described.[23]

In this report we assayed PKC isoenzymes in neonatal keratinocytes treated with human recombinant EGF, human recombinant IGF-1 and a combination of EGF and IGF-1. Western blot analysis demonstrated that EGF, IGF-1 and a combination of these growth factors stimulated some PKC isoenzymes.

MATERIALS AND METHODS

1. Human Epidermal Keratinocytes Culture and Treatments

Human epidermal keratinocytes were prepared from neonatal foreskins obtained from a local hospital and stored in liquid nitrogen. Passage 2 cells were thawed and plated at tissue culture flasks (1×10^6 cells/150 mm^2 flask) containing a keratinocyte growth medium, (KGM, Clonetics, USA), 10% fetal bovine serum, and 10% Dimethyl sulfoxide (DMSO, Clonetics, USA) as described by Tong, et al.,[36] grown to the cells are 80～90% confluence. For subculturing, the 3 passage cells were trypsinized with 4 ml of trypsin/Ethylenediaminetetraacetic acid (EDTA) solution (Clonetics, USA) containing 0.25 mg/ml trypsin in EDTA, for 5 min at 37℃. The cells were suspended with 8 ml of trypsin neutralizing solution (Clonetics, USA) and centrifuged at 1000 rpm in a Damon IEC centrifuge for 10 min at 4℃. The cells were plated in 100-mm culture dishes with KGM at a density of 5×10^5 cells, and incubated in a CO_2 incubator at 37℃ until 50% confluency. Before the treatment with growth factors, cells were placed in a keratinocyte basal medium (KBM, Clonetics, USA) at 37℃ for 24 hrs. Keratinoctyes deprived of growth factors were incubated in the 10 ml of KBM containing a concentration 200 ng/ml of human recombinant EGF (Sigma, USA), human recombinant IGF-1 (Sigma, USA), a combination of these growth

factors, respectively, at 37℃ for 24 hrs.

2. Protein Extraction

The procedure for preparation of cytosol and particulate extract from keratinocytes was described by Goodell[7] and Gschwendt et al.[8] After treatment with growth factors as above, cells were rinsed 2 times and scraped in Dulbecco's phosphate buffered saline (PBS, Sigma, USA) containing 1 mM phenylmethyl-sulfonyl fluoride (PMSF, Sigma, USA), centrifuged at 2000 rpm in the Damon IEC centrifuge at 4℃ for 20 min. The pellet was resuspended in a extraction buffer containing 20 mM Tris-HCl (pH 7.5), 10 mM 2-mercaptoethanol, 5 mM ethylene glycol-bis [β-aminoethyl ether]-N,N,N',N'-tetraacetic acid (EGTA, Sigma, USA) 5 mM ethylenediaminetetraacetic acid (EDTA, Sigma USA), 5 μg/ml aprotinin, 10 mM pepstatin, 1 mM PMSF, 400 μg/ml soybean trypsin inhibitor and 200 μg/ml leupeptin, and sonicated to break open the cells. The cell homogenates were centrifuged at 15,000 rpm for 15 min in a microcentrifuge (Brand Biofuge 15 R, USA) at 4℃. The supernatant was collected as the soluble (cytoplasmic) protein fraction, and the pellet was washed by adding a buffer (20 mM Tris-HCl, pH 7.5, 10 mM 2-mercaptoethanol, 2 mM EGTA, 2 mM EDTA and 200 μg/ml leupeptin), vortexing and centrifuging at 5,000 rpm for 1 min in the microcentrifuge (Brand Biofuge 15 R, USA) at 4℃ to remove any cytoplasmic proteins adhering to the pellet. The pellet was resuspended in the above extraction buffer containing Triton X-100 and homogenized with a small Dounce homogenizer to obtain the particulate (membrane) protein fraction. The protein concentration was determined using the Bio Rad Protein Assay (Bio-Rad, USA) based on the method of Bradford.[2]

3. Detection of PKC Isoenzymes by Western Blot Analysis

Fractions of partially purified soluble and particulate PKC protein obtained above were subjected to 8% SDS-polyacrylamide gel electrophoresis (1.5 M Tris-HCl, pH 8.8) in a Mini-ProteinII electrophoresis (Bio-Rad, USA), then transferred to nitrocellulose membranes by electro-blotting using a Genie electrophoretic blotter (Idea Scientific, USA). The nitrocellulose membranes were blocked using a blocking buffer (Tris-buffered saline, pH 7.4, containing 0.1% Tween 20 and 1% non-fat dried milk) for 30 minutes, and incubated with a 1:200 dilution of polyclonal PKC isoenzyme-specific antibodies, rabbit polyclonal IgG (Santa Cruz Biotechnology, USA) for 2 hours at room temperature. Immunoreactive protein bands were detected using a 1:30,000 dilution of a alkaline phosphatase-conjugated secondary antibody, goat anti-rabbit IgG (Sigma, USA) which give a color reaction when incubated with the chromogenic substrate, a mixture of NBT (nitro

Table 1. Concentrations of total PKC protein of human epidermal keratinocytes in response to growth factors

	Total PKC protein concentration (μg/μl)	
	Cytoplasmic fraction	Membrane fraction
control	1.20	2.30
EGF	1.36	2.01
IGF-1	1.55	2.06
EGF + IGF-1	1.44	2.24

The assay procedure was described in the experimental methods (PKC: protein kinase C, EGF: epidermal growth factor, IGF-1: insulin-like growth factor, EGF + IGF-1: combination of epidermal growth factor and Insulin-like growth factor)

blue tetrazolium) and BCIP (5-bromo-4-chloro-3-indolyl phosphate-toluidinium salt).

RESULTS

To determine the amount of total PKC in human epidermal keratinocytes could be affected by stimulating human recombinant EGF (hrEGF), human recombinant IGF-1 (hrIGF-1) and a combination of these growth factors for 24 hours, the concentrations of total PKC were monitored by measuring a color change of the dye occurs when it binds to the protein, according to the method of Bradford.[2)]

Table 1 shows the concentrations of cytoplasmic and membrane PKC proteins extracted from control (unstimulated) and growth factor-treated cells (stimulated). The protein concentrations from cytoplasmic fractions in stimulated cells were higher than in unstimulated cells, that of IGF-1 showed the highest concentration. In membrane fractions, in contrast the concentrations of stimulated cells were lower than that of unstimulated cells, which showed the highest concentration. All of protein concentrations from membrane fractions were much higher than those of cytoplasmic fractions.

In order to detect PKC isoenzymes from untreated and growth factor-treated keratinocytes, partially purified extracts from untreated keratinocytes cultured in KBM and treated cells cultured in KBM with growth factors were separated by 8% SDS-polyacrylamide gel electrophoresis, blotted onto nitrocellulose membrane and probed for the presence of PKC isoenzymes using polyclonal PKC isoenzyme-specific antibodies, PKC-α, -βI, -βII, -δ, -ε, -θ and -ζ.

Fig. 1 shows that the result of Western blot analysis of PKC isoenzymes in cytoplasmic fractions extracted from keratinocytes treated for 24 hours with 200 ng/ml hrEGF, 200 ng/ml hrIGF-1 and a combination of 200 ng/ml each of hrEGF and hrIGF-1. The results in Fig. 1 show that exposure to the hrEGF for 24 hrs

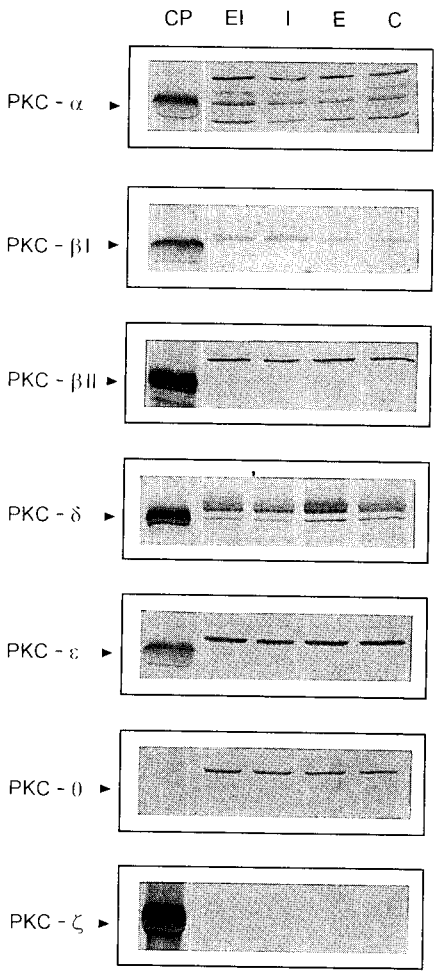

Fig. 1. Western blot analysis of PKC isoenzymes in cytoplasmic fractions extracted from human epidermal keratinocytes treated for 24 hours with 200 ng/ml hrEGF (lane E), hrIGF-1 (lane I) and a combination of 200 ng/ml each of hrEGF and hrIGF-1 (lane EI). Control proteins (lane C) from keratino-cytes cultured in KBM. Control peptide (lane CP) of PKC-α, -βI, -βII, -δ, -ε, -ζ and -θ isoenzymes.

caused an increase in the cytoplasmic PKC-βII, -δ, -ε and -θ and no changes in the cytoplasmic PKC-α, -βI, -ε, and -ζ, that exposure to the hrIGF stimulated the cytoplasmic PKC-βI, -θ and -ζ and no changes in the others, that incubation of keratinocytes with the combination of hrEGF and hrIGF induced an increase in the cytoplasmic PKC-α, -βI, -θ and -ζ, while no changes in the other isoenzymes.

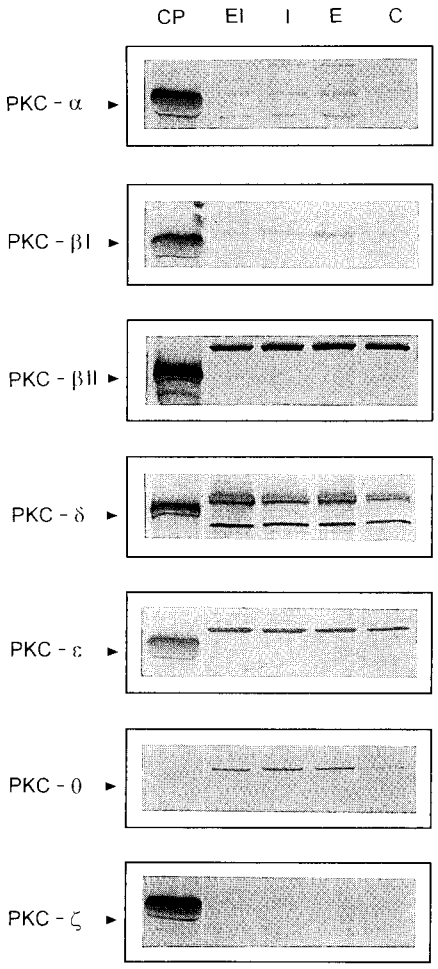

Fig. 2. Western blot analysis of PKC isoenzymes in membrane fractions extracted from human epidermal keratinocytes incubated for 24 hours with 200 ng/ml hrEGF (lane E), hrIGF-1 (lane I) and a combination of 200 ng/ml each of hrEGF and hrIGF-1 (lane IE). Control proteins (lane C) from keratinocytes cultured in KBM. Control peptide (lane CP) of PKC-α, -βI, -βII, -δ, -ε, -ζ and -θ isoenzymes.

Fig. 2 shows that the result of Western blot analysis of PKC isoenzymes in membrane fractions extracted from keratinocytes incubated for 24 hours with 200 ng/ml hrEGF, 200 ng/ml hrIGF-1 and a combination of 200 ng/ml each of hrEGF and hrIGF-1. Treatment of keratinocytes with hrEGF and hrIGF for 24 hrs induced an increase in the most part of membrane PKC isoenzymes except PKC-βII. Incubation of keratinocytes with the combination of hrEGF and hrIGF stimulated in the membrane PKC-α, -δ, -ε, -θ and -ζ, while there were no changes in PKC-βI, -βII.

DISCUSSION

The involvement of PKC in cell growth and differentiation is now well established.[4,22] The interaction between growth factor signaling pathways and PKC are very important to modulate cellular functions and can vary from cell type to cell type.[21] Therefore we have tested the response of PKC isoenzymes in human epidermal keratinocyte for hrEGF, hrIGF-1 and a combination of high concentration of these growth factors.

EGF, a small (53 amino acids) polypeptide, are major growth factor involved in skin cell interactions,[21] directly stimulates epidermal growth and differentiation.[3,16,17] IGF-1 stimulates proliferation of keratinocytes in vitro through synergistic interaction with epidermal growth factor and fibroblast growth factor-like hormones.[13] The concentrations of total PKC protein from cytoplasmic fractions in growth factor treated cells were higher than in the control, that of IGF-1 showed the highest activity. In contrast, at membrane fractions, the concentrations of treated cells were lower than that of untreated cells, which showed the highest value. All of protein concentrations from membrane fractions were much higher than those of cytoplasmic fractions. These results indicate that growth factors are capable of activating PKC in cytoplasmic fractions of keratinocytes, especially IGF-1 give rise to the highest concentration, whereas in the membrane fraction these factors cause a decrease of protein concentration.

Here we show that growth factors acting through receptors with intrinsic tyrosine kinase activity, namely hrEGF and hrIGF-1,[28] induce an increment of some PKC isoenzymes in the human epidermal keratinocytes. Western blot

analysis of keratinocytes treated for one day with growth factors showed that hrEGF stimulated PKC-βII, -δ and -θ in the cytoplasmic fraction, PKC-α, -βI, -δ, -ζ and -θ in the membrane fraction, that hrIGF-1 stimulated PKC-βI, -ζ and -θ in the cytoplasmic, PKC-α, -βI, -δ, -ζ, -ε and -θ in the membrane, that in the cells treated with a combination of EGF and IGF-1, PKC-α, -βI, -ζ and -θ in the cytoplasmic fraction, PKC-α, -δ, -ζ, -ε and -θ in the membrane fraction were stimulated. These results suggest that single mitogens and a combination of high concentration of growth factors stimulate several PKC isoenzymes.

In the present work, we observed that human epidermal keratinocytes contain different PKC isoenzymes that have distinct properties concerning for example their regulation and their substrates. Moreover, in human keratinocytes, PKC isoenzymes participate in a different manner to total PKC protein concentration.[22,25] For example, some PKC isoenzymes might negatively control EGF effects on keratinocyte growth,[21] while others might transduce EGF effects. Consequently, it is possible that the various intracellular effects of EGF, IGF-1 and a combination of these growth factors.

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=국문초록=

Growth Factor를 처리한 피부상피세포로부터 Protein Kinase C Isoenzyme의 검출

경산대학교 자연과학부 생물학과

주 은 영† · 김 남 우

Protein Kinase C는 세포의 신호전달계에 관여하는 중요한 조절효소로서 여러 가지 세포의 분화와 증식과도 밀접한 관련이 있다. 신생아의 포피 keratinocyte를 농도 200 ng/ml의 human recombinant epidermal growth factor (hrEGF)와 human recombinant insulin-like growth factor-1 (hrIGF-1) 그리고 hrEGF와 hrIGF-1의 혼합액을 각각 첨가하여 24시간 배양한 후 세포질과 세포막의 PKC 단백질을 추출하여 그 농도를 측정하고, Western blot analysis를 이용하여 각 growth factor들의 PKC isoenzyme에 대한 영향을 분석하였다. 세포질의 총 PKC 단백질의 농도는 hrIGF-1을 처리한 keratinocyte에서 가장 높았으며, 세포막에서는 대조군의 단백질 농도가 가장 높게 나타났다. EGF를 처리한 keratinocyte의 세포질에서는 PKC-βII, -δ, -θ가 막성분에서는 PKC-α, -βI, -δ, -ζ, -θ가 증가하였다. IGF-1을 처리한 군의 세포질성분에는 PKC-βI, -ζ, -θ, 막성분에서는 PKC-α, -βI, -δ, -ζ, -ε, -θ가 증가하였다. EGF와 IGF-1의 혼합처리군에서는, PKC-α, -βI, -ζ, -θ이 세포질에서, PKC-α, -δ, -ζ, -ε, -θ은 세포막에서 증가하였다.

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†별책 요청 저자