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Phosphatidylinositol 3-kinase and protein kinase C are required for the inhibition of caspase activity by epidermal growth factor

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Abstract The mechanism by which growth factors exert an anti-apoptotic function on many cell types is not well understood. This issue is addressed in relation to epidermal growth factor (EGF) which inhibits apoptosis induced by staurosporine or wortmannin in an epithelial tumour cell line (CNE-2). The presence of EGF substantially reduced the in vitro Ac-DEVD-AMC hydrolytic activity and almost completely suppressed the intracellular cleavage of poly(ADP-ribose) polymerase in staurosporine- or wortmannin-treated cells. Staurosporine but not wortmannin caused the intracellular proteolytic processing of pro-caspase-3 and this event was transiently inhibited by EGF. Staurosporine-induced apoptosis was not inhibited by EGF in the presence of wortmannin or LY294002. Similarly, EGF failed to inhibit wortmannin-induced apoptosis in the presence of staurosporine, chelerythrine chloride or Gö6850. These results suggest that phosphatidylinositol 3-kinase and protein kinase C play a role in the survival function of EGF but the reduction of cellular caspase activity cannot be satisfactorily explained by a lack of pro-caspase-3 activation.

Key words: Apoptosis; Caspase; Epidermal growth factor; Poly(ADP-ribose) polymerase; Phosphatidylinositol 3-kinase; Protein kinase C

1. Introduction

Apoptosis is the process by which cells are eliminated from a tissue without eliciting an inflammatory response. Apoptosis can be mechanistically divided into two phases: a regulatory and an execution phase [1]. In the regulatory phase, cells receive signals from the extracellular or intracellular environment and through a network of interacting intracellular signalling pathways produce either positive (activating) or negative (inhibitory) regulation of the execution phase. This latter phase is now thought to be responsible for the irreversible stage of apoptosis as characterised by the internucleosomal cleavage of chromosomal DNA [1–3]. The activation of the execution phase involves the activation of proteases known as caspases (cysteinyl aspartate-specific proteinases) which are synthesised as inactive proenzymes [3,4]. Caspases have a general structure consisting of three domains: the NH_2-terminal prodomain, the large and the small subunits. During activation, proteolytic cleavage occurs between the domains, at sites that are preceded by an aspartic acid residue. The prodomain is discarded and the large and small subunits associate together to form the active enzyme [5]. Overexpression of caspases in cells has previously been shown to induce apoptosis [6]. The substrates for the mammalian caspases include poly(ADP-ribose) polymerase (PARP), the lamins, the retinoblastoma protein, the sterol regulatory element binding protein and β-actin, which are degraded at the onset of apoptosis [7–11]. One important target of these caspases is DNA fragmentation factor (DFF). Proteolytic cleavage of DFF releases an active endonuclease that degrades DNA at internucleosomal sites [2].

Apoptosis can be prevented in many instances by growth factors such as insulin-like growth factor, nerve growth factor and granulocyte macrophage colony stimulating factor [12–14]. Although it is highly likely that those growth factors act via the regulatory phase of apoptosis, little is known about the underlying molecular mechanism. There is increasing evidence that phosphatidylinositol 3-kinase (PI 3-kinase) is widely involved in the survival functions of many growth factors [12,13]. Many studies now tend to support a model whereby PI 3-kinase activates an intracellular serine kinase, AKT/PKB, which probably acts through inhibition of the activation of the caspases [15,16]. Recent studies have also documented the involvement of protein kinase C (PKC) isoforms, such as PKCα and the atypical PKCs, in cellular survival [17,18]. Previously, epidermal growth factor (EGF) was shown to be able to suppress apoptosis in a number of cell lines of epithelial origin [19–21]. The survival function of EGF is correlated to the expression of Bcl-x_L which is one of the pro-survival protein of the Bcl-2 family [20,21]. Bcl-x_L is able to bind Apaf1, and hence prevents the formation of a supermolecular complex that is essential for the activation of caspase-9 [22]. It is therefore possible that EGF, through the mediation of Bcl-x_L, negatively regulates the caspase activity within cells, and hence acts as a survival factor. To test this hypothesis, the first step would be to see if EGF treatment of cells resulted in the reduction of caspase activities. We found that EGF could inhibit apoptosis induced by staurosporine or wortmannin in an epithelial tumour cell line and it caused a reduction of caspase activity as well as the intracellular cleavage of PARP. But such reduction in caspase activity is not completely due to a lack of activation of pro-caspase-3. The EGF-stimulated inhibition of caspase activity is inhibited by inhibitors of PI 3-kinase and PKC, suggesting the involvement of these two signalling proteins in this process.

2. Materials and methods

2.1. Materials

EGF, RPMI 1640 medium, foetal calf serum (FCS), penicillin and

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Abbreviations: EGF, epidermal growth factor; PARP, poly(ADP-ribose) polymerase; PI 3-kinase, phosphatidylinositol 3-kinase; PKC, protein kinase C; Ac-DEVD-AMC, *N*-acetyl-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin

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streptomycin were purchased from Gibco BRL (Life Technologies Inc., New York). Ac-DEVD-AMC (*N*-acetyl-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin) and LY294002 were from BioMol Research Laboratories (Plymouth Meeting, PA). Chelerythrine chloride and Gö6850 were from Calbiochem (La Jolla, CA). Acridine orange, staurosporine, wortmannin and the bicinchoninic acid (BCA) assay kit were from Sigma Chemical (St. Louis, MO). Mouse anti-human caspase-3 antibody, horseradish peroxidase (HRP)-conjugated anti-mouse IgG or anti-rabbit IgG antibody and enhanced chemiluminescence (ECL) kit were from Transduction Laboratories, (Lexington, KY). Rabbit anti-human PARP polyclonal antibody was from Upstate Biotechnology (Lake Placid, NY).

2.2. Culture of CNE-2 cells

The CNE-2 cell line is an epithelial tumour cell line originally isolated and established from a patient with poorly differentiated nasopharyngeal carcinoma [23] and was kindly provided by Professor W.F. Fong of the City University of Hong Kong. The CNE-2 cells grown as a monolayer were maintained in RPMI 1640 medium supplemented with 5% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin under a humidified atmosphere of 5% CO_2/95% O_2 in a 37°C incubator. Prior to experiments, cells were grown to 90% confluence and were quiesced by incubation in serum-free RPMI 1640 medium for 12 h.

2.3. Analysis of apoptosis by transmission electron microscope

After treatment with staurosporine for 6 h, cells were scraped from the culture dishes and pelleted by centrifugation. The cell pellets were washed once with ice-cold phosphate buffered saline (PBS, pH 7.4). The cells were fixed in 0.1 M sodium cacodylate buffer containing 2.5% glutaraldehyde. Ultrathin sections were stained with uranyl acetate and lead citrate as described before [24]. The sections were examined in a Jeol JEM 2000FX transmission electron microscope.

2.4. Quantification of apoptosis by the method of acridine orange staining

Identification of apoptotic cells by acridine orange staining was done essentially as described previously [25]. After treatment of cells with the appropriate stimuli for a certain period of time, cells were trypsinised and collected, followed by washing once with ice-cold PBS. After cells were resuspended and fixed in PBS containing 1% formaldehyde, the cells were stained with acridine orange (100 μg/ml in deionised H_2O) and then examined under a fluorescence microscope. Apoptotic cells were identified as those containing densely fluorescing bodies due to nuclear fragmentation and chromatin condensation. At least 500 cells from each sample were counted and the percentage of apoptotic cells was taken as the extent of apoptosis. Each quantification was done in triplicate.

2.5. Identification of internucleosomal DNA cleavage in apoptotic cells

The internucleosomal cleavage of DNA was analysed as described [24]. Following treatment with the appropriate stimuli, the cells were rinsed with ice-cold PBS and harvested by trypsinisation. The cell pellets were resuspended and incubated in 10 mM Tris-HCl (pH 7.4) containing 25 mM EDTA, 0.5% SDS and 0.1 mg/ml proteinases K at 50°C overnight. The digested cells were extracted for DNA with phenol/chloroform/isoamyl alcohol (25:24:1). The extracted DNA was precipitated and digested in 10 mM Tris (pH 8.0) containing 1 mM EDTA and 10 μg/ml RNase for 1 h at 37°C. 10 μg of DNA per sample was separated by electrophoresis in a 1.8% agarose gel impregnated with ethidium bromide (0.5 μg/ml). The DNA pattern was examined by ultraviolet transillumination.

2.6. Measurement of sub-G_1 DNA content by flow cytometry

CNE-2 cells were treated with an apoptotic stimulus for 24 h. The incubation medium was removed and cells were trypsinised, washed with ice-cold PBS and fixed in ice-cold 70% ethanol for at least 30 min on ice. The fixed cells were stored at −20°C until analysis. Prior to analysis, the cells were rinsed twice with PBS, and stained with a propidium iodide solution (40 μg/ml with 0.1 mg/ml RNase in PBS) at 37°C for 30 min. Analysis was performed by a fluorescence activated cell sorter (Coulter EPICS XL, UK) as described [26]. The percentage of cells in the G_0/G_1, S and G_2/M phases of the cell cycle was calculated from the resultant DNA histogram using Multicycle AV software (Phoenix Flow System, San Diego, CA). Cells in the sub-G_1 peak of the DNA histogram represent those containing a subdiploid amount of DNA and are regarded as apoptotic.

2.7. Detection of caspase-3 and PARP cleavage by Western blotting

The cleavage of caspase-3 and PARP in apoptotic cells was monitored by Western blot analyses essentially as previously described [27]. Cells were first treated with an apoptotic stimulus for various time intervals. At the end of each time interval, the cells were rinsed once with ice-cold PBS and then lysed in 20 mM Tris pH 7.5, 50 mM NaF, 20 mM NaCl, 1 mM EDTA, 2 mM DTT, 0.2 mM PMSF, and 0.5% NP-40 on ice for 20 min. The cells were scraped from the dishes and passed several times through a 26-gauge needle to disperse any large aggregations. After centrifugation at 13 000 rpm at 4°C for 10 min, 50 μg of the cell lysate was separated by electrophoresis under reducing conditions in either a 10% or 12% SDS-polyacrylamide gel. An identical amount of proteins from a Jurkat cell or HeLa cell lysate was run in parallel with the samples to serve as a positive control for the 32 kDa pro-caspase-3 and 116 kDa PARP respectively. After transfer of proteins to nitrocellulose membrane, the membrane was blocked with TBS (10 mM Tris pH 7.6, 100 mM NaCl) containing 0.01% Tween 20 and 5% (w/v) skim milk powder to block non-specific binding. To detect pro-caspase-3, the membrane was first incubated with a mouse anti-human caspase-3 antibody. A HRP-conjugated anti-mouse IgG antibody was used as the second antibody to enable detection of pro-caspase-3 by the ECL method. To detect PARP by the same method, a rabbit anti-human PARP polyclonal antibody was used as the first antibody, and a HRP-conjugated anti-rabbit IgG antibody was used as the second antibody.

2.8. Fluorimetric assay of caspase activity

After treatment with the appropriate stimuli, the cells were washed once with ice-cold PBS and lysed in a lysis buffer containing 20 mM Tris-HCl (pH 7.5), 5 mM DTT, 2 mM EDTA and 0.1% CHAPS. The amount of protein was determined for each lysate using the BCA protein assay kit. 15–20 μl of each of the cell lysates, which had the same protein concentrations, was incubated in a final volume of 100 μl of a reaction buffer containing 50 mM Tris-HCl pH 7.5, 2 mM DTT, 10% glycerol and 100 μM Ac-DEVD-AMC for 30 min at 30°C. Caspase activity was determined with Ac-DEVD-AMC as a specific substrate by liberation of AMC from Ac-DEVD-AMC as described previously [28]. Fluorescence intensity was quantified using the Hitachi 650-60 spectrophotometer at excitation and emission wavelengths of 380 and 460 nm respectively. Enzyme activity is expressed as fluorescent units per mg of protein per min.

2.9. Statistical analysis

Where appropriate, data are mean values of three independent experiments and are expressed as mean ± S.D. Student's *t*-test was used to compare data. A P value of less than 0.05 is regarded as significant.

3. Results

3.1. EGF promotes the survival of the CNE-2 cells

Initial experiments demonstrated that staurosporine or wortmannin dose-dependently and reproducibly induced apoptosis in CNE-2 cells at or above 20 nM and 4 μM respectively. Examination of the staurosporine-treated cells by electron microscopy revealed cytoplasmic compaction with no structures that was distinctly reminiscent of the nuclei. High-density intracellular bodies due to condensation of chromatin were formed in the drug-treated cells. By contrast, the control cells still retained a well-defined nucleus with no evidence of chromatin condensation (Fig. 1A). The genomic DNA was extracted from drug-treated or control cells and fractionated by agarose electrophoresis. Fig. 1B showed that in both the staurosporine- and wortmannin-treated cells but not in the control cells, fragmentation of DNA characteristic of apoptosis was observed. Analysis of DNA content by flow cytometry revealed a much higher proportion of cells with sub-G_1 DNA content upon treatment with either drugs (Fig. 1C). Thus in the control cells, only 2.2% of the cells had sub-G_1 DNA but

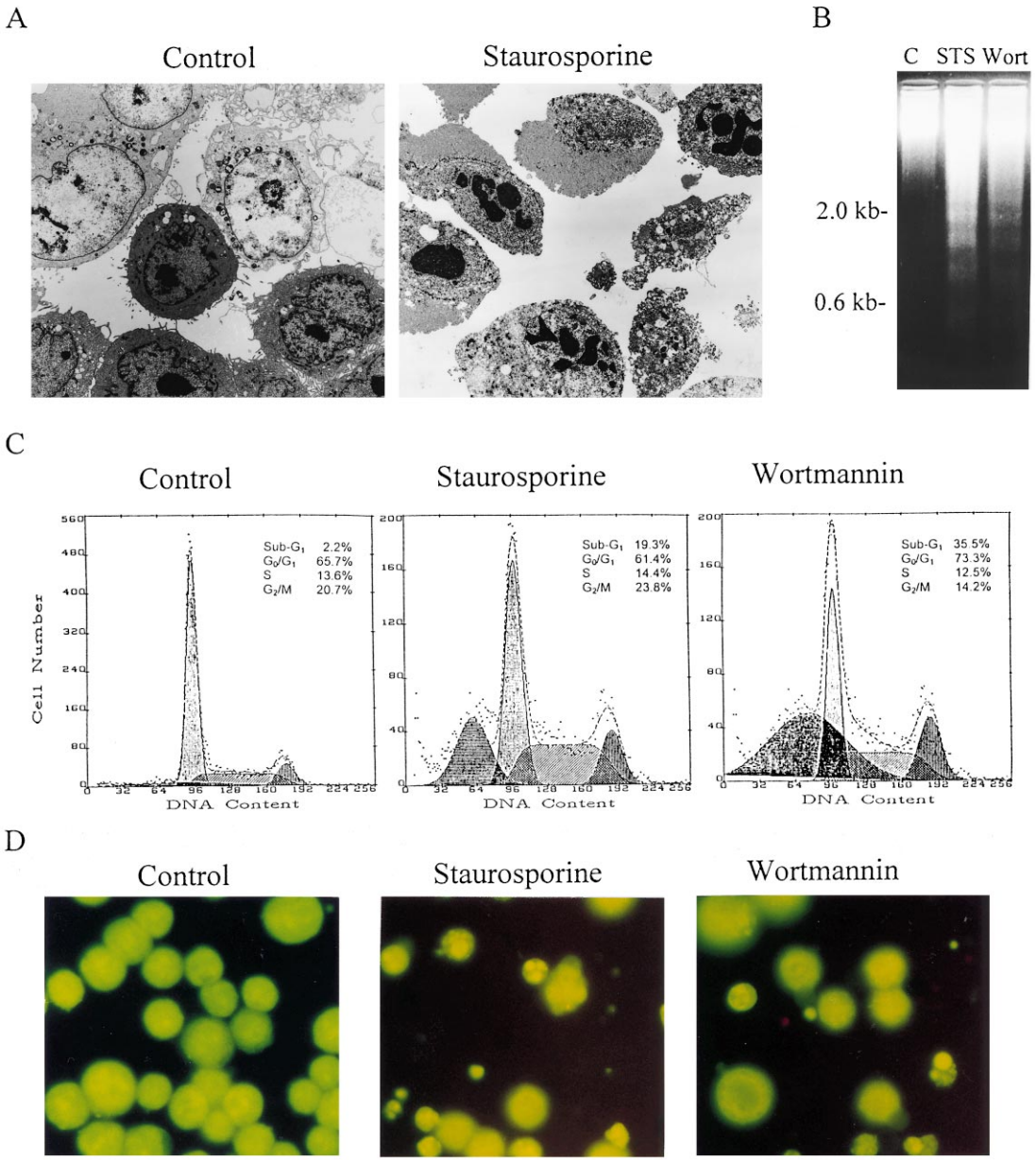

Fig. 1. Induction of apoptosis in CNE-2 cells. A: The ultrastructural morphology of cells examined by electron microscopy after treatment with or without staurosporine (1 μM) for 6 h. Magnification ×1500. B: Examination of internucleosomal cleavage of DNA by agarose gel electrophoresis in cells receiving either no treatment, or treatment with 50 nM staurosporine or 4 μM wortmannin for 12 h. C: The cells were treated with either staurosporine (20 nM) or wortmannin (4 μM) for 24 h. The cells were harvested, fixed and stained with propidium iodide. The stained cells were analyzed for DNA content by flow cytometry. D: After treatment with either staurosporine (50 nM) or wortmannin (4 μM) for 12 h, the cells were harvested, fixed and stained with acridine orange. The stained cells were examined by fluorescence microscopy. Magnification ×400. C: control; STS: staurosporine; Wort: wortmannin.

this value increased to 19.3% and 35.5% for the staurosporine- and wortmannin-treated cells respectively. The apoptotic cells could easily be distinguished from the normal cells by staining with acridine orange. Upon treatment with either drug, many cells shrank considerably due to cytoplasmic condensation, with the formation of densely fluorescing bodies which might be a result of chromosomal condensation and fragmentation (Fig. 1D).

To see if EGF would counteract the apoptotic effect of staurosporine and wortmannin, CNE-2 cells were incubated in the presence of EGF for 15 min prior to treatment with either staurosporine or wortmannin for 12 h. The cells were harvested and stained with acridine orange and the percentage of cells displaying typical features of apoptosis was determined. Significant reduction of staurosporine- and wortmannin-induced apoptosis could already be seen in cells pretreated with 10 ng/ml EGF. At an EGF dosage of 100 ng/ml, the percentage of cells with apoptotic features decreased by approximately 50% and 60% upon staurosporine and wortmannin treatment respectively (Fig. 2).

3.2. The effect of EGF on the activity of caspase

The enzyme activity of caspase was then measured in cells treated with staurosporine or wortmannin to see if the reduc-

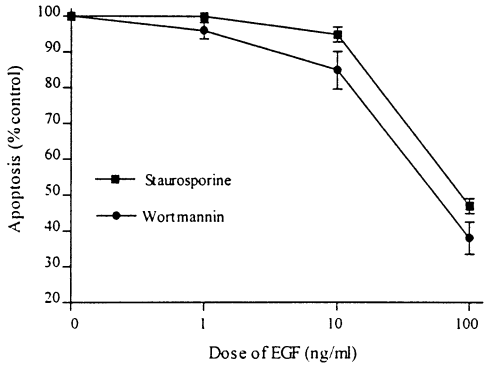

Fig. 2. Anti-apoptotic effects of EGF in CNE-2 cells. Cells were incubated with EGF at 0, 1, 10 or 100 ng/ml for 15 min followed by addition of 50 nM staurosporine or 4 μM wortmannin for 12 h. The cells were stained with acridine orange and the apoptotic cells were identified and counted as explained in Section 2. The reduction of apoptosis was calculated as a percentage of the control, i.e. cells treated with staurosporine or wortmannin only. The percentages are expressed as mean ± S.D. from three independent experiments.

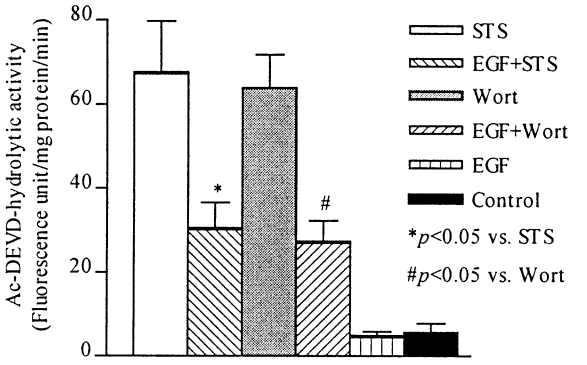

Fig. 3. The effect of EGF on the enzyme activity of caspase(s). Cells were treated with 50 nM staurosporine or 4 μM wortmannin in the absence or presence of 100 ng/ml EGF for 12 h. The enzyme activity of caspases in the cell lysate was assayed with Ac-DEVD-AMC as a substrate. The values are expressed as mean ± S.D. from three different experiments. STS: staurosporine; EGF: epidermal growth factor; Wort: wortmannin.

tion of apoptosis was associated with a similar reduction of caspase activity. Cells were treated with either 50 nM staurosporine or 4 μM wortmannin alone or in the presence of 100 ng/ml EGF for 12 h. The caspase activity was then measured in the cell lysates using Ac-DEVD-AMC as a substrate. The caspase activity in cells treated with both staurosporine and EGF was only 50% of that found in cells treated with staurosporine alone. A similar percentage of reduction of caspase activity was seen in cells treated with both wortmannin and EGF, in comparison to cells treated with wortmannin only (Fig. 3).

The effect of diminished caspase enzyme activity on the intracellular degradation of PARP, which is one of the commonest proteins found to be cleaved during apoptosis, was then examined by monitoring the time course of proteolytic cleavage of PARP. In cells treated with staurosporine alone for 6 h, Western analysis showed that the 116 kDa PARP was cleaved to produce the 85 kDa fragment which continued to accumulate over the next 6 h. By the end of the 24 h incubation period, almost all the 116 kDa PARP was converted to the 85 kDa form (Fig. 4A). In the presence of both EGF and staurosporine, there was very little cleavage of the 116 kDa PARP to form the 85 kDa fragment after 6 and even up to 12 h of incubation. In cells treated with wortmannin alone, a decrease in the 116 kDa form of PARP and a simultaneous appearance of an 85 kDa fragment of PARP were detected after 6 h of incubation. Unlike staurosporine, there was no further accumulation of the 85 kDa PARP fragment over the rest of the 24 h incubation period. The abundance of the 85 kDa form of PARP was substantially reduced in the presence of both EGF and wortmannin (Fig. 4B).

The cleavage of PARP, which is a substrate for caspase-3 in the apoptotic cells, suggests that this caspase might be activated in these cells. The activation of pro-caspase-3 can be monitored by following its proteolytic cleavage which is manifested as the disappearance of the 32 kDa form [27,29]. The time course of change in the 32 kDa form of this caspase was therefore monitored by Western analysis. After 6 h of treatment, the protein level of the 32 kDa pro-caspase-3 decreased markedly in cells treated with staurosporine alone while most of the 32 kDa pro-caspase-3 still remained in the EGF/staurosporine-treated cells. However, this inhibition of pro-caspase-3 processing was only transient since after 12 h of incubation, almost all of the 32 kDa caspase had also disappeared in the EGF/staurosporine-treated cells, to the same extent as

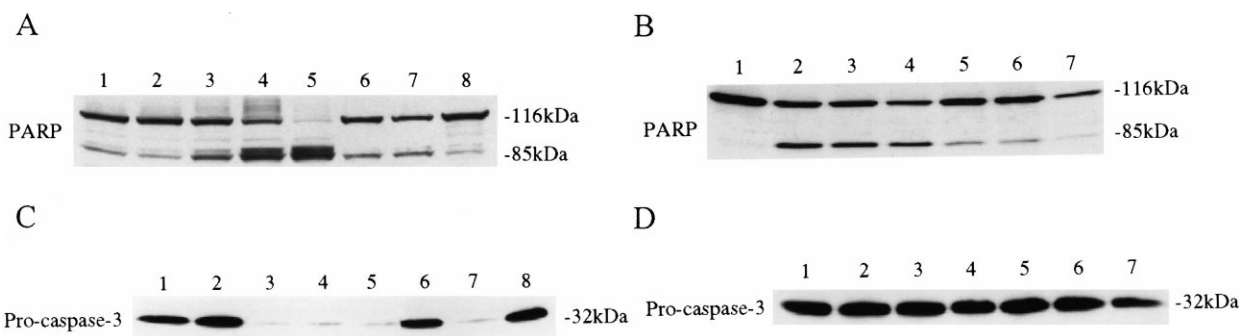

Fig. 4. The cleavage of PARP and pro-caspase-3 in the absence or presence of EGF. Cells were treated with 50 nM staurosporine or 4 μM wortmannin alone, or in the presence of 100 ng/ml EGF for various time intervals as indicated. At the end of incubation, the cell lysate was analysed for the proteolytic cleavage by Western blotting. A and B: Cleavage of PARP in staurosporine- or wortmannin-treated cells and its inhibition by EGF. C and D: Cleavage of pro-caspase-3 in the absence or presence of EGF. A and C: lane 1: zero time control; lanes 2–5: cells treated with staurosporine for 3, 6, 12, 24 h respectively; lanes 6 and 7: cells treated with EGF and staurosporine for 6 and 12 h respectively; lane 8: cells treated with EGF for 12 h. B and D: lane 1: zero time control; lanes 2–4: cells treated with wortmannin for 6, 12, 24 h respectively; lanes 5–7: cells treated with EGF and wortmannin for 6, 12, 24 h respectively.

in cells treated with staurosporine alone (Fig. 4C). Rather unexpectedly, the protein level of the 32 kDa pro-caspase-3 in cells treated with wortmannin alone or with wortmannin and EGF together did not decrease significantly throughout the entire incubation period, suggesting that the proteolytic processing of pro-caspase-3 did not occur in wortmannin-induced apoptosis (Fig. 4D).

3.3. Effects of PI 3-kinase inhibitors on the ability of EGF to prevent staurosporine-induced apoptosis

To identify the intracellular signalling molecules that might mediate the survival effect of EGF on apoptosis, we first investigated the effect of specific inhibitors of PI 3-kinase on EGF's ability to inhibit apoptosis induced by staurosporine. We used a relatively low concentration (100 nM) of wortmannin, which has been shown to fully inhibit the activity of PI 3-kinase in a wide variety of cell types [13,30]. The reduction of staurosporine-induced apoptosis by EGF in the absence and presence of PI 3-kinase inhibitors was measured by the acridine orange assay (Fig. 5A) as well as cleavage of PARP (Fig. 5B). Wortmannin at 100 nM did not induce apoptotic morphological changes and cleavage of PARP in CNE-2 cells. As expected, staurosporine induced a significant percentage of cells with apoptotic morphology and cleavage of PARP and this was inhibited by EGF. However, this survival effect of EGF was not seen in the presence of wortmannin. The percentage of apoptosis and the cleavage of PARP were approximately the same as in cells treated with staurosporine alone. Another specific PI 3-kinase inhibitor, LY294002 [31], at 5 μM has the same effect of wortmannin to inhibit the survival action of EGF. LY294002 by itself also did not induce any apoptosis and cleavage of PARP.

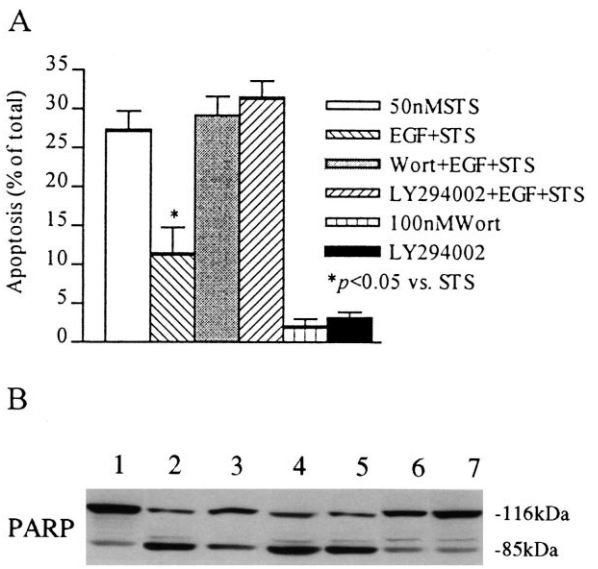

Fig. 5. Effects of PI 3-kinase inhibitors on the survival action of EGF. After treatment with 100 nM wortmannin or 5 μM LY294002, cells were incubated with 100 ng/ml EGF and 50 nM staurosporine for 12 h. The percentage of apoptosis was determined by acridine orange staining (A) and the cleavage of PARP was determined by Western blot analysis (B). B: lane 1: untreated cells; lane 2: 50 nM staurosporine; lane 3: EGF+50 nM staurosporine; lane 4: 100 nM wortmannin+EGF +50 nM staurosporine; lane 5: 5 μM LY294002+EGF+50 nM staurosporine; lane 6: 100 nM wortmannin; lane 7: 5 μM LY294002.

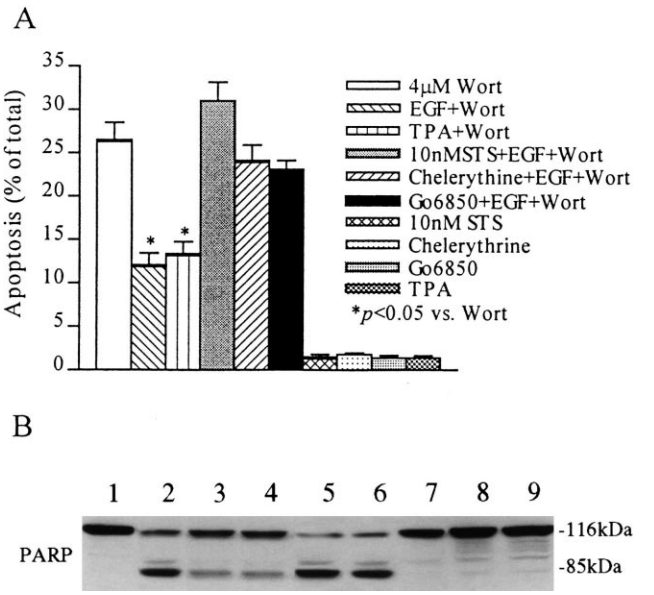

Fig. 6. Role of PKC in the anti-apoptotic function of EGF. To examine the effect of PKC inhibition, cells were treated with 10 nM staurosporine or 2 μM chelerythrine chloride or 100 nM Gö6850 for 15 min, cells were incubated with 100 ng/ml EGF and 4 μM wortmannin for 12 h. To examine the effect of PKC activation, cells were incubated with 100 nM TPA alone or in combination with 4 μM wortmannin for 12 h. The percentage of apoptosis was determined by acridine orange staining (A) and the cleavage of PARP was determined by Western blot analysis (B). B: lane 1: untreated cells; lane 2: 4 μM wortmannin; lane 3: EGF+4 μM wortmannin; lane 4: TPA+4 μM wortmannin; lane 5: 10 nM staurosporine+EGF+4 μM wortmannin; lane 6: 2 μM chelerythrine chloride+EGF+4 μM wortmannin; lane 7: 10 nM staurosporine; lane 8: 2 μM chelerythrine chloride; lane 9: 100 nM TPA.

3.4. Effects of PKC inhibitors on the ability of EGF to prevent wortmannin-induced apoptosis

The involvement of PKC in the survival action of EGF was also investigated in cells induced to undergo apoptosis by wortmannin. The reduction of wortmannin-induced apoptosis by EGF in the absence or presence of PKC inhibitors was again measured by the acridine orange assay (Fig. 6A) and also by monitoring of PARP cleavage (Fig. 6B). On its own, wortmannin induced a substantial percentage of cells with apoptotic morphology and cleavage of PARP to form the 85 kDa fragment. However, in the presence of EGF, the percentage of cells with apoptotic morphology and the cleavage of PARP were markedly reduced. This effect of EGF was mimicked by the PKC activator TPA. To see if inhibition of PKC would abolish the survival effect of EGF, we first used a low concentration of staurosporine (10 nM) on its own so that no apoptosis was induced. In the presence of staurosporine (10 nM) and EGF, the percentage of wortmannin-induced apoptosis and cleavage of PARP was not reduced when compared to cells treated with wortmannin alone. To confirm this result, two other PKC inhibitors, chelerythrine chloride [32] and Gö6850 [33], were used. When either of these inhibitors was added together with EGF during treatment of cells with wortmannin, the percentage of apoptosis resulting was statistically indistinguishable from that obtained with wortmannin alone. Consistently, the cleavage of PARP was not prevented by EGF in the presence of chelerythrine chloride.

4. Discussion

Previous studies have demonstrated that EGF protected cells against apoptosis under certain circumstances [19–21]. The details of the underlying mechanism are not known but the pro-survival protein Bcl-x_L is suggested to be involved [20,21]. We made use of CNE-2 cells which could be induced to undergo apoptosis by either staurosporine or wortmannin to address the possibility that the survival action of EGF requires the inhibition of caspase activity. The survival action of EGF on the CNE-2 cells was demonstrated by its ability to substantially reduce the percentage of cells with apoptotic features upon treatment with either of the two drugs (Fig. 2). The hydrolytic activity towards the caspase-specific substrate Ac-DEVD-AMC was also substantially reduced in cell lysates prepared from cells treated with either staurosporine or wortmannin, and EGF. This provides the first experimental evidence that caspase(s) could serve as a target for the survival action of EGF. This conclusion was supported by the result that when cells are treated with both EGF and staurosporine or wortmannin, the cleavage of PARP to form the 85 kDa fragment was severely inhibited (Fig. 4A).

Since PARP is a specific substrate for caspase-3 [7,34], the possibility that this caspase is a target for EGF is investigated. We therefore performed experiments to monitor the activation of pro-caspase-3 by following its cleavage during incubation with staurosporine or wortmannin alone or in the presence of EGF. These experiments gave rise to two novel observations. First, in cells treated with staurosporine and EGF together, the proteolytic processing of pro-caspase-3 was only transiently inhibited, resulting in a delay of that event for approximately 6 h so that complete proteolytic processing of pro-caspase-3 could only be seen after 12 h of incubation. However, even when almost all the pro-caspase-3 had undergone proteolytic processing and should become active, most of the PARP still remained in the uncleaved state (Fig. 4A,C) and the measured in vitro Ac-DEVD-AMC activity was only half of that seen in staurosporine-treated cells (Fig. 3). This result therefore suggests that the lack of PARP cleavage and the reduction of in vitro Ac-DEVD-AMC hydrolytic activity could not be accounted for simply by a lack of activation of pro-caspase-3 in these cells. Further experiments will be required to identify the mechanism that is responsible for the inhibition of the caspase activity, for example, by measuring the expression and activity of IAP, which could inhibit caspases activity [35]. Second, the proteolytic processing of pro-caspase-3 is undetectable for apoptosis stimulated by wortmannin, and the cleavage of PARP to form the 85 kDa form occurs to a much lesser extent than for the staurosporine-treated cells. This is the first indication that staurosporine and wortmannin induced apoptosis through different mechanisms in the same cells.

The stimulation of the EGF receptor is known to activate a number of signalling pathways, including the activation of breakdown of phospholipids, the activation of the MAP kinase cascade, and the mobilisation of calcium [36]. Two of these signalling pathways, the activation of PI 3-kinase and PKC, were shown in many other systems to have survival-promoting functions [12–14,17,18]. To see if these pathways were involved in the survival function of EGF, we used specific inhibitors to inhibit the activity of these kinases. Wortmannin at a concentration which is known to achieve full suppression of PI 3-kinase in most cell types [13,30] was able to abolish the ability of EGF to protect cells against staurosporine-induced apoptosis. Another inhibitor of PI 3-kinase, LY294002, was also able to inhibit the same action of EGF, providing convincing evidence that PI 3-kinase, which is known to be activated by the EGF receptor, serves in the anti-apoptosis signalling pathway of EGF. Since EGF is also capable of protecting cells against wortmannin-induced apoptosis, another pathway not involving PI 3-kinase must exist to mediate the protective action of EGF. The presence of staurosporine and two other PKC inhibitors, chelerythrine chloride and Gö6850, could totally abolish the anti-apoptotic action of EGF, suggesting that a PKC-mediated pathway is triggered by the activated EGF receptor to inhibit apoptosis. This is mirrored by the finding that TPA, which can activate PKC, was able to mimic the ability of EGF for inhibiting wortmannin-induced apoptosis.

In summary, our experiments demonstrate that the survival action of EGF is associated with a reduction of caspase activity but this cannot be explained entirely on the basis of inhibition of caspase-3 activation, which after all occurred only transiently. The inhibition of caspase activity seen in the presence of activation of pro-caspase-3 suggests that other mechanism(s), apart from delaying of pro-caspase-3 activation, is employed by EGF to inhibit apoptosis. Although staurosporine and wortmannin could induce apoptosis in the same cells, they may trigger two apoptotic pathways that may differ in the requirement for pro-caspase-3 activation. These two pathways could be respectively inhibited by a PI 3-kinase and a PKC-dependent mechanism, both of which are activated in the CNE-2 cells by EGF stimulation.

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