Staurosporine induces endothelial cell apoptosis via focal adhesion kinase dephosphorylation and focal adhesion disassembly independent of focal adhesion kinase proteolysis

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The survival of endothelial cells is dependent on interactions between the matrix and integrins mediated through focal adhesions. Focal adhesion kinase (FAK) is thought to play a key role in maintaining focal adhesion function and cell survival, whereas caspase-mediated FAK proteolysis is implicated in focal adhesion disassembly during apoptosis. We examined the relationship between changes in FAK phosphorylation and proteolysis during apoptosis of primary porcine aortic endothelial cells (PAEC) induced by staurosporine, a widely used apoptogenic agent in diverse cell types. Staurosporine-induced PAEC apoptosis was detected after 1 h and was preceded by disruption and loss of FAK localization to focal adhesions within a few minutes, whereas staurosporine-induced cleavage of FAK occurred only after 8–24 h. Staurosporine induced a very rapid dephosphorylation of FAK at Tyr^{861} and Tyr^{397} and caused dissociation of phosphorylated FAK from focal adhesions as early as 30 s. The effect of staurosporine was very potent with striking inhibition of Tyr^{861} and Tyr^{397} phosphorylation and focal adhesion disruption occurring in the range 10–100 nM. Selective inhibition of a known target of staurosporine, protein kinase C, using GF109203X, and of phosphoinositide 3′-kinase using wortmannin, did not reduce FAK tyrosine phosphorylation at Tyr^{861} and Tyr^{397}, or cause disruption of focal adhesions. Cycloheximide, the protein synthesis inhibitor, induced PAEC apoptosis more slowly than staurosporine, but did not induce FAK dephosphorylation or rapid focal adhesion disruption, and instead caused a slower loss of focal adhesions and a marked increase in FAK proteolysis. These studies show that FAK dephosphorylation and focal adhesion disassembly are very early events mediating the onset of staurosporine-induced endothelial cell apoptosis and are dissociated from FAK proteolysis. Cycloheximide induces apoptosis through a pathway involving FAK proteolysis without dephosphorylation.

Key words: cycloheximide, src, survival.

INTRODUCTION

Focal adhesions are multicomponent juxtamembrane structures, which play a critical role in the adhesion, survival and migration of endothelial and other adherent cell types [1]. Focal adhesion kinase (FAK) is an important component of the focal adhesion complex [2–4], essential for embryonic development and implicated in cell migration, adhesion, survival, and cell-cycle control [5–9]. The FAK C-terminal non-catalytic domain contains a 159-residue region (the focal adhesion targeting domain or FAT) required for targeting to focal adhesions [10] and also mediates FAK association with paxillin, which co-localizes to focal contacts and is a potential FAK substrate [11,12].

FAK tyrosine phosphorylation is thought to play a central role in signal transduction through the focal adhesion complex triggered by diverse extracellular signals [13–17]. Phosphorylation at Tyr^{397}, the major FAK autophosphorylation site, creates a high-affinity binding site for the Src homology 2 (SH2) domains of $pp60^{src}$ and $pp59^{fyn}$ [18], and also mediates association with the p85 subunit of phosphoinositide 3′-kinase (PI 3-kinase) [19], whereas phosphorylation at Tyr^{576} and Tyr^{577} in the activation loop region of the catalytic domain enhances FAK kinase activity [20]. Phosphorylated Tyr^{925} is a binding site for the SH2 domain growth-factor-receptor-bound protein 2 [21]. FAK is also phosphorylated by Src at Tyr^{407} and Tyr^{861} residues [22], but the function of these sites is unclear.

The survival of endothelial and epithelial cells is critically dependent upon interactions between the matrix and integrins mediated through focal adhesions. Disruption of these interactions induces anoikis, a class of apoptosis characterized by membrane blebbing, loss of focal adhesions and retraction from the substrate followed by cell detachment [23]. FAK is thought to play a major role in survival signalling in several cell types [8,24–26]. Caspase-3-mediated FAK cleavage in human umbilical-vein endothelial cells (HUVEC) has been implicated in focal adhesion disassembly and apoptosis [27,28], but it is unclear whether FAK proteolysis is a cause of focal adhesion disassembly or a consequence of focal adhesion disaggregation and endothelial cell detachment. Furthermore, neither the relationship between FAK tyrosine phosphorylation and proteolysis nor the role of changes in phosphorylation during disassembly of focal adhesions are understood.

Given the importance of changes in FAK activity for the regulation of focal adhesion integrity and cell survival, we sought to determine the temporal relationships between changes in FAK tyrosine phosphorylation, FAK proteolytic cleavage and focal adhesion disassembly during endothelial cell apoptosis induced by the microbial alkaloid, staurosporine, one of the most widely used apoptogenic agents [29–31]. The mechanism of staurosporine-induced apoptosis is not fully understood and recent findings suggest that this agent may act through caspase-independent pathways distinct from those of other apoptotic

Abbreviations used: Bcl-2, B-cell lymphocytic leukaemia proto-oncogene 2; DAPI, 4,6-diamidino-2-phenylindole; DMEM, Dulbecco's modified Eagle's medium; FAK, focal adhesion kinase; HUVEC, human umbilical-vein endothelial cells; PAEC, porcine aortic endothelial cells; PI 3-kinase, phosphoinositide 3′-kinase; PKC, protein kinase C; SH2 domain, Src homology domain; TUNEL, terminal transferase deoxytidyl uridine end labelling.

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agents [32–34]. Here, we show that staurosporine induces rapid FAK dephosphorylation at Tyr^{861} and Tyr^{397} and focal adhesion disruption in aortic endothelial cells preceding apoptosis, and temporally dissociated from FAK proteolysis. In contrast, cycloheximide, the protein synthesis inhibitor induced apoptosis more slowly than staurosporine and caused a marked increase in FAK proteolysis without FAK dephosphorylation. These findings identify FAK as a novel target for staurosporine and indicate that FAK dephosphorylation is an important early step in initiating focal adhesion disassembly and apoptosis induced by this compound. These results have implications for the mechanisms underlying the caspase-independent induction of apoptosis by staurosporine in tumour cells.

EXPERIMENTAL

Cell culture

Porcine aortic endothelial cells (PAEC) were isolated from fresh porcine aorta (Fresh Tissue Supplies). Aortas were dissected free of fibro-fatty tissue and washed several times in sterile PBS. Branching blood vessels were ligated and the lumen of the aorta treated with 1 mg/ml of collagenase type I (Sigma) in PBS for 20 min at 37 °C. Collagenase treatment was terminated by the addition of Dulbecco's modified Eagle's medium (DMEM) with 10 % foetal bovine serum, and endothelial cells were collected by centrifugation. Primary cultures of PAEC were characterized on the basis of 'cobblestone' morphology and immunofluorescent staining of the endothelial cell-specific marker, VE cadherin. PAEC were maintained in DMEM (low glucose) supplemented with 10 % foetal bovine serum, penicillin–streptomycin (100 μg/ml), gentamicin (50 μg/ml) and L-glutamine (2 mM)m, and were used between passages 2 and 7 in experiments.

Western-blotting system

Confluent cultures of PAEC were washed twice with serum-free medium, and incubated in serum-free DMEM with factors as indicated. Cellular protein was extracted from 2 × sample buffer [250 mM Tris (pH 6.8)/2 mM EDTA/25 % glycerol/4 % SDS/10 % 2-mercaptoethanol/0.1 % Bromophenol Blue] and heated to 95–100 °C for 5 min. Equal quantities of protein were separated by SDS/PAGE and transferred to Immobilon (Millipore, Watford, Herts., U.K.). Membranes were blocked with PBS/5 % non-fat milk for 30 min and incubated for 1 h with primary antibody (2 μg/ml) diluted in blocking buffer followed by horseradish peroxidase-conjugated secondary antibody for 1 h. Immunoreactive bands were detected by chemiluminescence using ECL® reagents (Amersham).

Terminal transferase deoxytidyl uridine end labelling (TUNEL) staining

Detection of apoptosis using the TUNEL method was performed using an apoptosis detection kit (Promega, Chilworth, Southampton, U.K.) according to the manufacturer's instructions. After treatment as indicated, cells on glass coverslips were fixed in 4 % formaldehyde for 25 min at 4 °C, washed in PBS and permeabilized with 0.2 % (v/v) Triton X-100 in PBS for 5 min on ice. The cells were preincubated in equilibration buffer (as supplied) for 10 min, followed by incubation in equilibration buffer plus nucleotide mix containing fluorescein-12–dUTP and terminal deoxnucleotidyl transferase for 1 h. The reaction was stopped by immersion in 2 × standard saline citrate (Life Technologies, Paisley, Renfrewshire, Scotland, U.K.) for 15 min at room temperature. Next the cells were stained with propidium iodide (1 μg/ml) for 15 min. After washing with PBS, coverslips were mounted using Pro-Long Anti-FADE (Molecular Probes) and TUNEL staining was visualized on a Zeiss immunofluorescence microscope and photographed using Kodak slide film.

Annexin V staining

Treated cells were trypsinized, collected by centrifugation, and stained with fluorescein-conjugated annexin V and propidium iodide (Boehringer Mannheim, Lewes, East Sussex, U.K.). After staining, the cells were sorted by flow cytometry using a FACScan (Becton Dickinson, Bedford, U.K.). Annexin V-positive staining cells are apoptotic cells. Cells that were propidium iodide-positive, annexin V-negative were not counted as apoptotic cells.

Immunofluorescent staining and microscopy

Cells on glass coverslips were fixed and permeabilized by immersion in precooled methanol for 10 min at −20 °C, washed thrice in PBS and then incubated in primary antibody (1–10 μg/ml) diluted in PBS/0.1 % BSA. The cells were washed thrice and incubated with fluorochrome-conjugated (FITC or tetramethylrhodamine β-isothiocyanate) secondary antibodies or 4,6-diamidino-2-phenylindole (DAPI) diluted in PBS/0.1 % BSA for 30 min. The coverslips were inverted on to microscope slides using Pro-Long Anti-FADE. Cells were viewed on a Zeiss Axiophot 100 M microscope using a × 63 oil immersion objective lens (numerical aperture 1.4) and image capture was performed using a Hamamatsu DCC camera and Improvision software, Openlab 2.2.4. In all experiments, control stains were performed with each antibody using secondary antibodies separately (results not shown).

Immunopreciptation and kinase assay

Confluent cells in 90 mm dishes were treated as indicated and lysed in RIPA buffer [10 mM Tris (pH 7.4)/150 mM NaCl/1 % Nonidet P40/0.5 % sodium deoxycholate/0.1 % SDS/10 μg/ml leupeptin/1 mM PMSF/25 μg/ml aprotinin] for 10 min on ice. Lysates were clarified by centrifugation at 15 000 ***g*** for 10 min and incubated overnight at 4 °C with constant mixing with 4 μg/ml antibody to the FAK C-terminus (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) followed by incubation with protein agarose AG+ (Santa Cruz) for 2 h at 4 °C. The protein agarose AG+ complex was washed three times in lysis buffer, followed by three washes in kinase assay buffer [50 mM Hepes (pH 7.4)/75 mM NaCl/0.1 mM EDTA/10 μg/ml/1 mM PMSF/25 μg/ml aprotinin]. The protein agarose AG+ complex was resuspended in kinase assay buffer containing 100 μM ATP, 10 mM $MgCl_2$ and 10 μCi per sample [γ-^{32}P]ATP (Amersham Biosciences) and incubated at 30 °C for 20 min after which the reaction was stopped by the addition of excess ice-cold RIPA buffer. Immunoprecipitates were washed thrice in RIPA buffer at 4 °C, resuspended in 2 × sample buffer and heated to 95–100 °C for 5 min. The samples were separated by SDS/PAGE (8 % gel). The gel was dried and exposed to X-ray film (Kodak) at −70 °C for 3 days following which the film was developed.

Materials

Staurospaurine, cycloheximide, anisomycin and actinomycin D were from the Sigma. GF109203X, wortmannin and ML-7 were from Calbiochem. Monoclonal antibodies to FAK and paxillin were obtained from Transduction Laboratories. Antibodies to

FAK N-terminal residues 2–18 (FAK-N), Src, B-cell lymphocytic leukaemia proto-oncogene 2 (Bcl-2) and VE-cadherin came from Santa Cruz Inc. Monoclonal N-terminal-specific FAK antibody (4.47) was from Upstate Biotechnology Inc. Phospho-specific antibodies to FAK phosphorylated at Tyr^{397} and Tyr^{861} and Src phosphorylated at Tyr^{418} were from Biosource. Horseradish peroxidase-conjugated secondary antibodies to mouse and rabbit immunoglobulins were from Amersham International (Little Chalfont, Bucks., U.K.). Fluorochrome-conjugated secondary antibodies were from Dako (Cambridge, U.K.). All other reagents were of the highest grade available.

RESULTS

Staurosporine was an effective inducer of PAEC apoptosis at 1 μM as determined by detection of cell surface binding of annexin V and TUNEL and propidium iodide staining of nuclei (Figure 1). Staurosporine treatment for 1 h increased annexin V staining to 33 % of the total cell population as compared with 7 % in control untreated cells incubated in serum-free medium. Annexin V staining increased to 90–95 % after addition of staurosporine for 24 h, whereas withdrawal of serum alone for 24 h caused no significant increase in annexin V staining (Figure 1A). TUNEL staining was readily detectable after treatment with staurosporine for 2 h and was significant after 4 h (Figure 1B).

To investigate the role of FAK in staurosporine-induced PAEC apoptosis, we examined the effects of staurosporine on FAK localization and proteolysis. Staurosporine induced a significant decrease in FAK localization to focal adhesions after 30 min (Figure 2A) and caused a similar loss of paxillin staining at focal adhesions (results not shown). In marked contrast, staurosporine treatment of PAEC for up to 2 h caused no detectable increase in either FAK proteolytic fragments or any significant decrease in $p125^{FAK}$ immunoreactivity in adherent or detached apoptotic cells (Figure 2B). Staurosporine increased FAK proteolysis to 90 and 80 kDa fragments in adherent cells after 8 h, and markedly increased proteolytic fragments in both adherent and detached apoptotic cells after 24 h. Staurosporine-induced FAK proteolysis was accompanied after 24 h, but not at earlier times, by a marked reduction in full-length 125 kDa FAK expression (Figure 2B). In parallel cultures of PAEC to those shown in Figure 2(B), it was examined whether focal adhesion

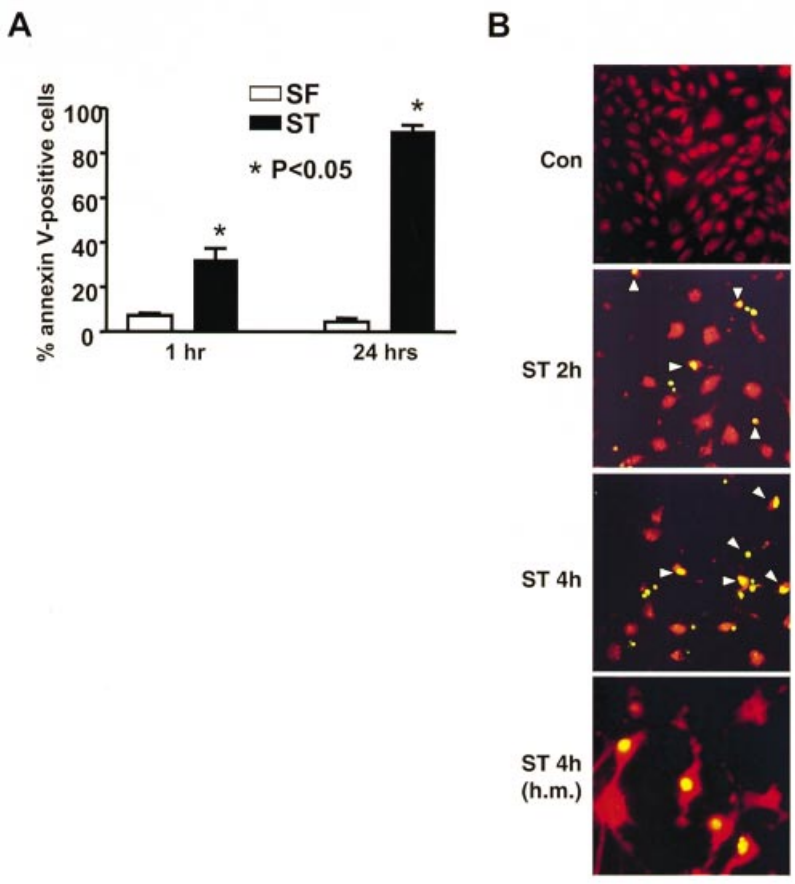

Figure 1 Staurosporine induces rapid apoptosis in PAEC

(**A**) Confluent cultures of PAEC were incubated for the times indicated (in h) in serum-free medium, in the absence (SF) or presence of 1 μM staurosporine (ST), and annexin V staining was determined by flow cytometry as described in the Materials and methods section. The results show the means ± S.E.M. for annexin V-positive cells. $^*P < 0.05$ for ST versus serum-free at 1 and 24 h. (**B**) Cells were treated with no addition (Con) or with 1 μM ST for the times indicated and TUNEL staining was performed as described in the Materials and methods section. Cells were counterstained with propidium iodide (red) and apoptotic nuclei (indicated by arrowheads) appear yellow due to merging of PI and green TUNEL stain. Photomicrographs were taken using a 40 × objective. The lower panel shows a high magnification (h.m.) photomicrograph of the 4 h treatment taken using a 63 × objective. Note that no TUNEL-positive cells are present in control, untreated cells. The results shown in (**A**) and (**B**) are representative of three independent experiments.

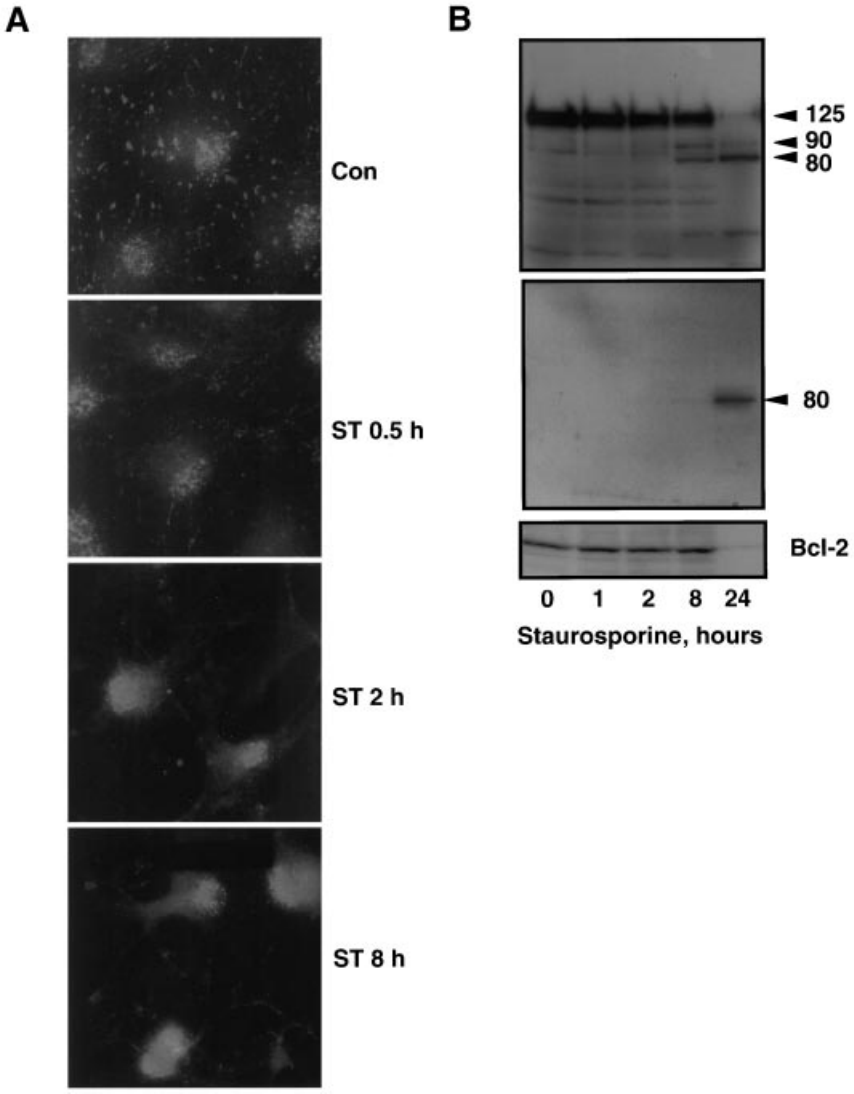

Figure 2 Staurosporine-induced apoptosis and disruption of FAK localization to focal adhesions dissociated from FAK proteolysis

(**A**) Confluent PAEC were treated in the absence (Con) or presence (ST) of 1 μM staurosporine (ST) for the times indicated, fixed, permeabilized and then immunostained with an antibody directed against FAK. The FAK localization to focal adhesions is almost completely eliminated after ST treatment for 0.5 h. In this and in subsequent Figures, cells were viewed and photographed using a Zeiss Axiophot 100M microscope using a 63 $\times$ oil immersion objective lens (numerical aperture 1.4). (**B**) PAEC were treated for the times indicated with 1 μM ST in serum-free medium and cell extracts were prepared from both adherent (upper and lower) and detached floating (middle) cells. Extracts of adherent and detached cells were immunoblotted with an antibody directed against the FAK N-terminus (upper and middle) and extracts of adherent cells were immunoblotted with antibody to Bcl-2 (lower). The positions of full-length 125 kDa FAK and of 90 and 80 kDa proteolytic fragments are indicated by arrowheads. The results shown in (**A**) and (**B**) are representative of five independent experiments.

disruption or FAK proteolysis occurred as subsequent to decreased expression of the anti-apoptotic component Bcl-2. The results showed that staurosporine had no effect on the level of Bcl-2 immunoreactivity after treatment for up to 8 h (Figure 2B). Serum deprivation of PAEC for 24 h caused no significant decrease in immunoreactive $p125^{FAK}$ in adherent cells, and little detectable increase in the generation of lower molecular mass FAK fragments in either adherent or detached cells (results not shown).

The results in Figure 2 indicated that a significant increase in FAK proteolysis occurred hours after staurosporine induced a striking loss of FAK localization to focal adhesions. The relationship between loss of FAK localization to focal adhesions and changes in FAK tyrosine phosphorylation during the apoptotic response to staurosporine was examined next. Two major constitutively tyrosine-phosphorylated species of 120–130 kDa, and 68 kDa were present in PAEC. Staurosporine caused a marked decrease in the phosphotyrosine content of both bands as shown by immunoblots with anti-phosphotyrosine antibody. The 120–130 kDa component is similar in apparent molecular mass to full-length $p125^{FAK}$, whereas the 68 kDa band is likely to correspond to paxillin, a candidate substrate for FAK known to be prominently phosphorylated at tyrosine in other cell types [11,16,17]. Western-blotting of PAEC with antibodies to specific phosphorylated tyrosines indicated that FAK was constitutively phosphorylated predominantly at two sites, the major autophosphorylation site Tyr^{397} and Tyr^{861}. Staurosporine produced a rapid decrease in phospho-Tyr^{861} and phospho-Tyr^{397}

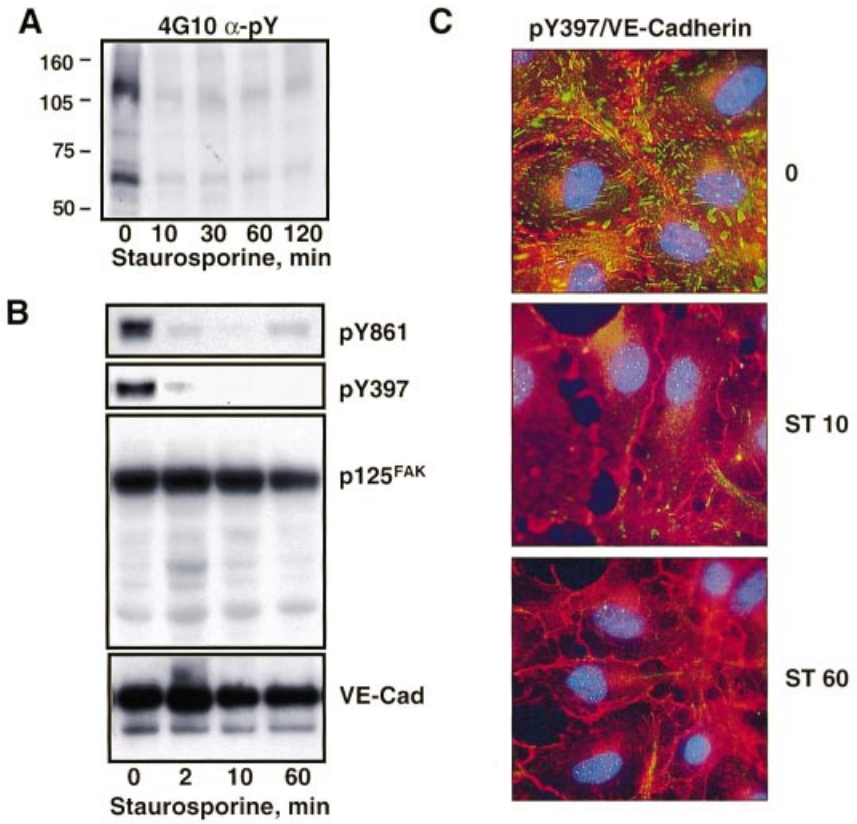

Figure 3 Staurosporine induces rapid FAK dephosphorylation

PAEC were treated for the times indicated with 1 μM staurosporine (ST) and cell extracts were then prepared and immunoblotted with (**A**) 4G10 phosphotyrosine antibody, or (**B**) with antibodies directed against FAK phosphorylated at Tyr^{861} or Tyr^{397}, antibody 4.47 to the FAK N-terminus ($p125^{FAK}$) or VE-cadherin (VE-Cad). (**C**) PAEC were treated for the times indicated (in min) with 1 μM ST and immunostained with antibodies directed against FAK phosphorylated at Tyr^{397} (green) and VE-cadherin (red). Cells were counterstained with DAPI (blue).

immunoreactivity, which was marked after 2 min and reached a maximum after 10 min (Figure 3B). Loss of phospho-Tyr^{861} and phospho-Tyr^{397} immunoreactivity was not due to decreased $p125^{FAK}$ expression or increased FAK proteolysis as shown by blotting of parallel samples with N-terminal FAK antibody.

Since adherens junctions play a key role in maintaining the integrity of endothelial monolayers, it was examined whether staurosporine-induced changes in focal adhesion integrity and associated FAK dephosphorylation could result from the disruption of adherens junctions and/or rapid proteolytic cleavage of adherens junction components. As shown in Figure 3, staurosporine-induced FAK dephosphorylation was not accompanied by any detectable decrease in the expression of VE-cadherin (Figure 3B). Co-immunostaining of VE-cadherin and FAK phosphorylated at Tyr^{397} showed that whereas staurosporine caused a complete loss of phospho-Tyr^{397} FAK staining in focal adhesions after 10 min, VE-cadherin staining of adherens junctions, though disrupted, was still readily detectable after 1 h (Figure 3C).

The effect of staurosporine on focal adhesion localization of FAK phosphorylated at Tyr^{861} and Tyr^{397} was potent and rapid. Staurosporine strikingly decreased localization of phospho-Tyr^{861}FAK to focal adhesions over the range 10–100 nM and this was accompanied by loss of total FAK staining of focal adhesions (Figure 4A). Treatment with 1 μM staurosporine for 30 s caused a marked reduction in phospho-Tyr^{861}FAK localization to focal adhesions (Figure 4B), and focal adhesion staining was abolished after 1–2 min. Concomitant with dissociation of tyrosine phosphorylated FAK from focal adhesions, association of total FAK with focal adhesions was also markedly reduced by staurosporine as indicated by tetramethylrhodamine β-isothiocyanate (red) fluorescence 30 s to 2 min after addition of staurosporine (Figure 4B). A similar concentration and time dependence was observed for the effect of staurosporine on focal adhesion localization of FAK phosphorylated at Tyr^{397} (results not shown).

Since Src is known to phosphorylate FAK at Tyr^{861} and Tyr^{397}, it was examined whether staurosporine-induced FAK dephosphorylation was a consequence of decreased Src expression or inhibition of Src activity. Western-blot analysis of cell extracts prepared from staurosporine-treated cells with antibodies to either total Src protein or active Src phosphorylated at Tyr^{418}, showed that staurosporine caused little decrease in either total Src expression or Src phosphorylation at Tyr^{418} at times up to 2 h (Figure 5A). After 4 h treatment, some decrease in the level of total Src was observed, but no decrease in Tyr^{418} phosphorylation was detectable. The effect of staurosporine on intrinsic FAK kinase activity was also examined. Staurosporine treatment of PAEC caused no detectable decrease in FAK kinase activity measured in FAK immunoprecipitates prepared from treated cells (Figure 5B). It was further investigated whether staurosporine might be inhibiting FAK by blocking the activity

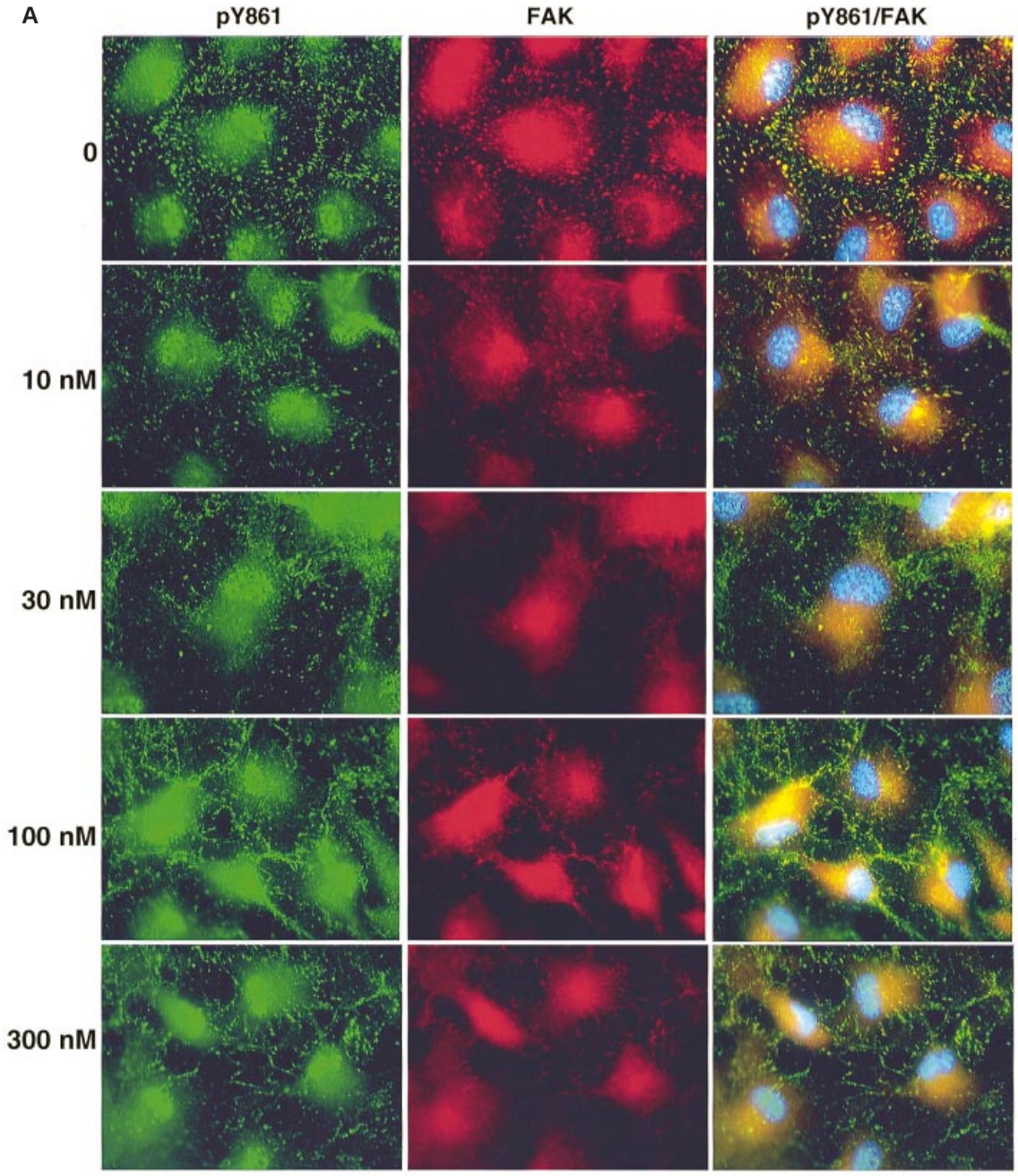

Figure 4 For legend see facing page

of protein kinase C (PKC), a known target of this agent [35]. In addition, because PI 3-kinase has been implicated in mediating FAK tyrosine phosphorylation [36], we examined the effect of wortmannin, an inhibitor of PI 3-kinase and PI 3-kinase-dependent activation of the anti-apoptotic kinase Akt/PKB. Neither GF109203X, a widely used PKC inhibitor, nor wortmannin had any effect on phosphorylated FAK immunostaining of focal adhesions (Figure 5C), and these agents caused no detectable increase in TUNEL staining after both 4 and 24 h treatment (results not shown). ML-7, a selective inhibitor of myosin light-chain kinase, another staurosporine-inhibitable kinase [37,38], also caused no decrease in FAK phosphorylation, or immunostaining at focal adhesions (results not shown).

Next, we compared the effects of staurosporine on FAK phosphorylation and focal adhesion localization in PAEC with those of protein synthesis inhibitors, a distinct class of apoptogenic compounds. Treatment of PAEC with cycloheximide for 1 h caused no increase in apoptosis as judged by annexin V surface binding, but after 24 h induced a striking apoptotic effect (Figure 6A). Similar to the effect of staurosporine, cycloheximide caused a striking increase in FAK proteolysis to 90 and 80 kDa fragments after 8 h, which was readily detectable in adherent and

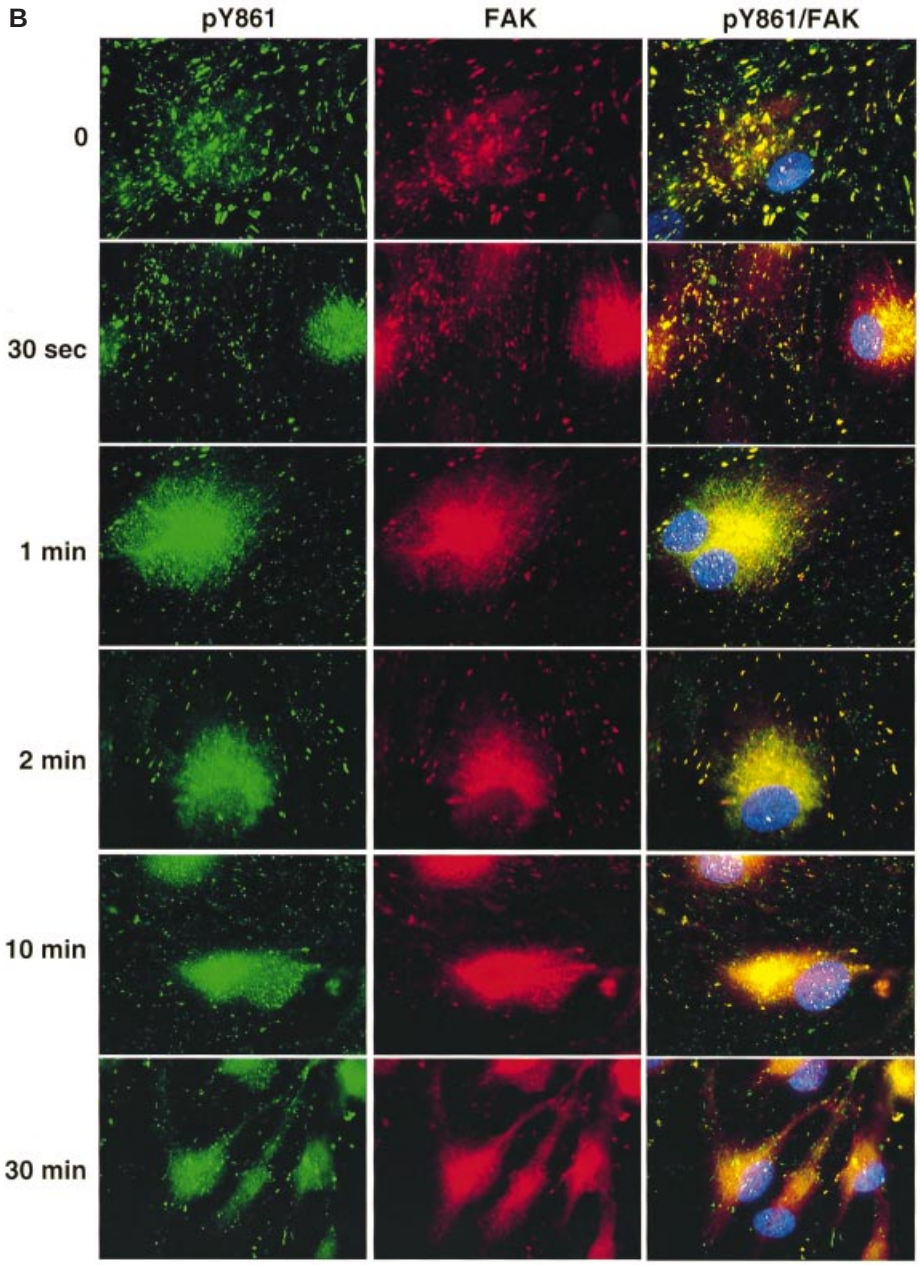

Figure 4 Concentration dependence and time course for staurosporine-induced dephosphorylation of FAK at focal adhesions

Confluent PAEC were either treated with the indicated concentrations staurosporine (ST) for 1 h (**A**), or for the times indicated with 1 μM ST (**B**), fixed and permeabilized, and then immunostained with antibodies raised against FAK phosphorylated at Tyr^{861} [phospho-Tyr^{861} (pY861), green], or antibody 4.47 to the FAK N-terminus (FAK, red) followed by incubation with secondary antibodies conjugated to either FITC (pY861) or rhodamine (FAK 4.47). The merge in (**A**) and (**B**) shows co-localization of pY861 FAK with total FAK (pY861/FAK). Cells were counterstained with DAPI.

detached cells (Figure 6B). It was observed that the accumulation of FAK proteolytic fragments in detached cells was more significant in cycloheximide-treated PAEC after 24 h compared with the effect of staurosporine in parallel cell cultures (cf. Figure 6B with Figure 2B). After 24 h, $p125^{FAK}$ expression was almost undetectable in cycloheximide-treated adherent cells (Figure 6B). Similar to cycloheximide, treatment with anisomycin, another protein synthesis inhibitor, caused complete proteolytic degradation of $p125^{FAK}$ after 24 h, and the mRNA synthesis inhibitor actinomycin D over a similar time course caused similar changes in FAK expression and proteolytic cleavage to those produced by cycloheximide in parallel cultures (results not shown).

Unlike staurosporine, cycloheximide did not cause a rapid change in either PAEC morphology, or loss of normal FAK staining of focal adhesions. Instead, the protein synthesis inhibitor caused a more gradual reduction in FAK focal adhesion staining which was evident after 4 h and significant after 8 h, when many cells were depleted in FAK-stained focal adhesions (Figure 7A). In marked contrast to the effect of staurosporine, cycloheximide had no effect on FAK tyrosine phosphorylation

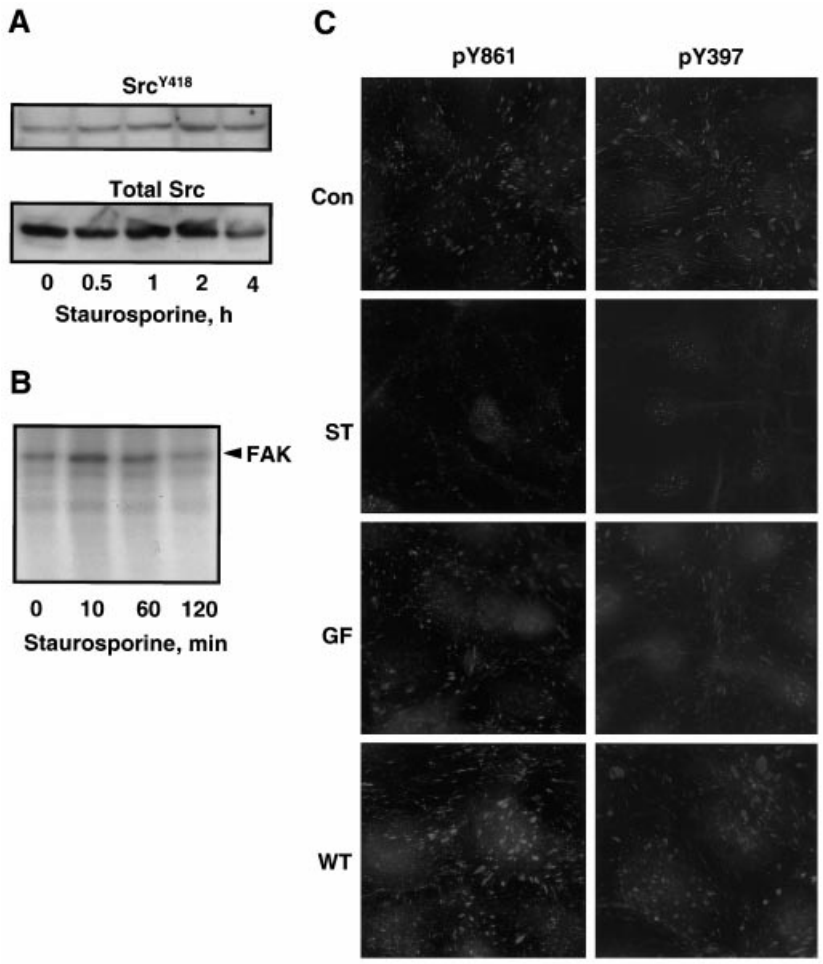

Figure 5 Staurosporine-induced FAK dephosphorylation is not mediated via inhibition of Src, intrinsic FAK kinase activity, PKC or PI 3-kinase

(**A**) Confluent PAEC were treated for the times indicated with 1 μM staurosporine (ST), cell extracts were prepared and immunoblotted with antibodies recognizing either active Src phosphorylated at Tyr^{418} or total Src. (**B**) PAEC were treated for the times indicated with 1 μM ST, lysed and total FAK immunoprecipitates were prepared and used for *in vitro* kinase assay as described in the Experimental section. (**C**) Confluent PAEC were treated for 60 min with serum-free medium either with no addition (Con) or containing 1 μM ST, 3 μM GF109203X (GF), or 100 nM wortmannin (WT). Cells were then immunostained with antibodies against FAK phosphorylated at Tyr^{861} (pY861) or Tyr^{397} (pY397) and secondary antibody conjugated to FITC. The results in (**A**–**C**) are representative of two independent experiments.

at Tyr^{861} or Tyr^{397} after 8 h. Tyrosine phosphorylation at both sites decreased after 24 h in parallel with decreased expression of p125^{FAK} (Figure 7B).

DISCUSSION

Disassembly of focal adhesions is a key event causing the detachment of many adherent cell types, resulting in apoptotic cell death or anoikis. FAK is the only kinase, which is known to localize predominantly and specifically to these structures, and is therefore a strong candidate as a key regulator of focal adhesion integrity, and phosphorylation of other focal adhesion proteins. Although increased FAK tyrosine phosphorylation occurs in response to diverse extracellular stimuli, FAK maintains a relatively high basal level of phosphorylation and activity [16,17, 39]. The role of this constitutive phosphorylation in normal cellular function is not fully understood, but may be important for maintaining cell survival signalling and focal adhesion integrity in the resting state. A major finding of the present paper is that the potent apoptogenic agent staurosporine induces a potent and rapid dephosphorylation of FAK at Tyr^{861} and Tyr^{397} in PAEC, which occurs concomitantly with disengagement of FAK from focal adhesions and precedes apoptosis. Staurosporine-induced dephosphorylation of FAK was maximal within seconds and loss of FAK immunostaining of focal adhesions was very significant within 10 min, both effects therefore occurring before the earliest detectable increase in TUNEL or annexin V staining (1–2 h) and 8 h before the onset of detectable FAK proteolysis. These findings suggest strongly that whereas decreased expression and proteolysis of FAK may be late consequences of the terminal execution phase of staurosporine-induced apoptosis, loss of FAK phosphorylation is an early trigger for disruption of the focal adhesion complex and loss of survival signals relayed through focal adhesions. Moreover, staurosporine did not affect expression of Bcl-2, or VE-cadherin at times up to 1–4 h, indicating that staurosporine-induced FAK dephosphorylation, focal adhesion disruption and apoptosis are independent of the inhibition of a well-known anti-apoptotic pathway and loss of a major constituent of intercellular adherens junctions. FAK tyrosine dephosphorylation was previously shown to

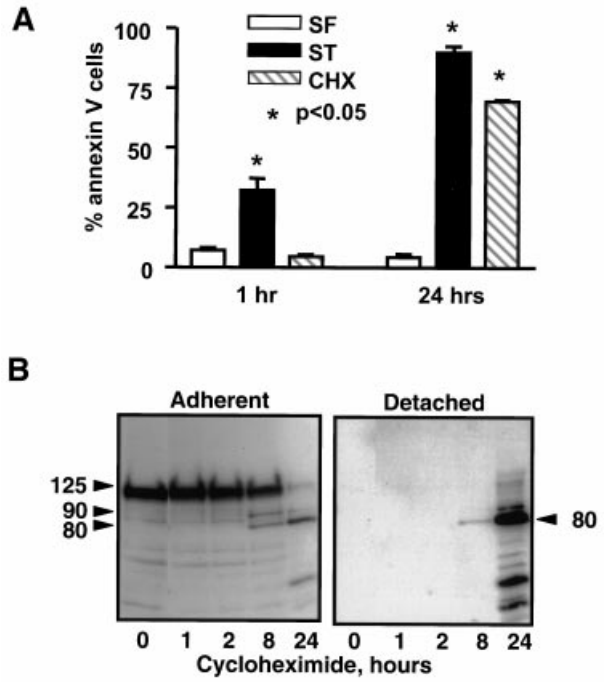

Figure 6 Apoptotic effects of inhibitors of protein and mRNA synthesis involve FAK proteolysis: comparison with staurosporine

(**A**) Cells were incubated for the times indicated in serum-free medium containing either no addition (unfilled bars), addition of 1 μM staurosporine (ST, black bars) or 25 μM cycloheximide (CHX, striped bars). Results show the percentage of annexin V-positive cells in the total cell population determined by flow cytometry. $^*P < 0.05$ for CHX versus serum-free at 24 h. (**B**) Parallel cultures of PAEC were treated with 25 μM CHX for the times indicated and cell extracts were prepared from adherent and detached cells and immunoblotted with antibody 4.47 to the FAK N-terminus. The positions of full-length 125 kDa FAK and 90 and 80 kDa proteolytic fragments are indicated by arrowheads.

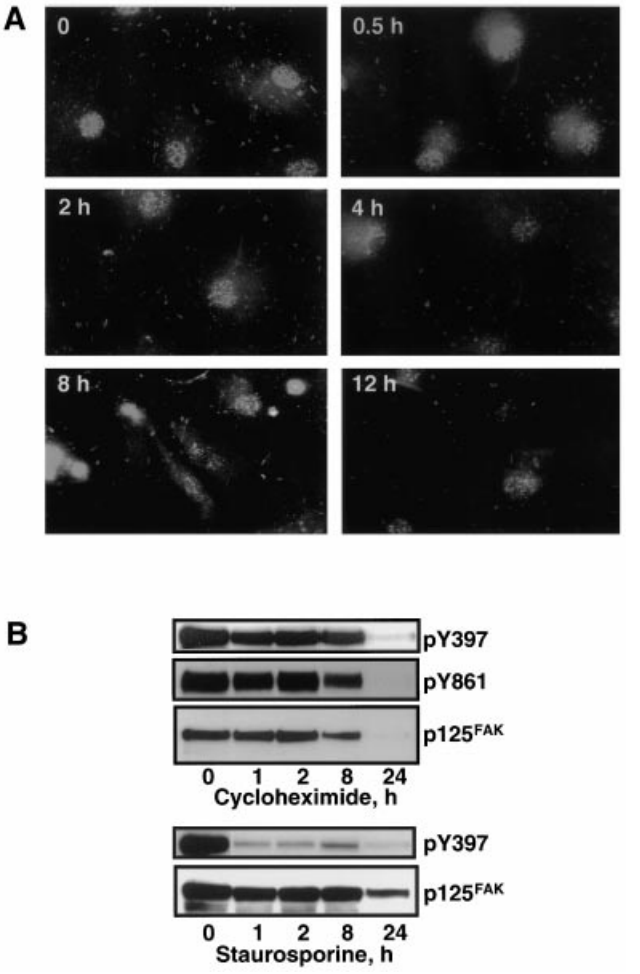

Figure 7 Cycloheximide-induced FAK dissociation from focal adhesions and FAK proteolysis are not associated with FAK dephosphorylation

(**A**) Confluent PAEC were treated for the times indicated with 25 μM cycloheximide (CHX), fixed and permeabilized, and then immunostained with antibody 4.47 to the FAK N-terminus followed by incubation with secondary antibody conjugated to FITC. (**B**) PAEC were treated for the times indicated with 25 μM CHX or 1 μM staurosporine and cell extracts were prepared from adherent cells and immunoblotted with antibodies directed against FAK phosphorylated at Tyr^{397} or Tyr^{861}, or antibody 4.47 to the FAK N-terminus (p125^{FAK}). The results shown in (**A**) and (**B**) are representative of three independent experiments.

precede its cleavage during *S*-(1,2-dichlorovinyl)-L-cysteine-induced apoptosis in renal epithelial cells, but this study examined changes in FAK phosphorylation only after 4 h, did not identify specific phosphorylation sites and did not show that dephosphorylation preceded apoptosis [40]. Previous studies have not reported staurosporine to be an inhibitor of FAK phosphorylation or focal adhesion localization.

The possibility that dephosphorylation may be a crucial trigger for focal adhesion disassembly is supported by the very rapid kinetics of staurosporine-induced dephosphorylation in PAEC (marked within 30 s) and by the finding that loss of localization of FAK phosphorylated at Tyr^{861} and Tyr^{397} either preceded or paralleled dissociation of total FAK from focal adhesions at early times of treatment (up to 2 min).

Previous studies in HUVEC and other cell types suggested that FAK proteolysis and/or disruption of focal adhesions are integral to the apoptotic response. One explanation for differences in results obtained with HUVEC and PAEC is that HUVEC may be particularly susceptible to apoptosis and not typical of adult arterial endothelial cells. Indeed, the resistance of PAEC to the apoptotic effects of serum deprivation is in striking contrast to the sensitivity of HUVEC to serum withdrawal [27]. Consistent with the results presented here, our findings show that whereas staurosporine induced FAK proteolysis more rapidly in HUVEC than in PAEC, Tyr^{861} and Tyr^{397} dephosphorylation also preceded and was temporally dissociated from FAK cleavage in HUVEC (M. Lobo and I. Zachary, unpublished work). This indicates that rapid staurosporine-induced FAK dephosphorylation is a mechanism common to other endothelial cell types.

Since staurosporine is a non-specific kinase inhibitor, it is not precluded that staurosporine exerts its effects on FAK phosphorylation through inhibition of other kinases. However, specific inhibitors of kinase pathways, including GF109203X, wortmannin and ML-7 had no comparable effects on FAK localization to focal adhesions or FAK phosphorylation. FAK is phosphorylated by pp60$^{c\text{-}src}$ at Tyr^{861} *in vitro* and *in vivo* [20,22,39], implicating inhibition of Src in staurosporine-induced FAK dephosphorylation. However, staurosporine did not decrease either the total level of pp60$^{c\text{-}src}$ expression or pp60$^{c\text{-}src}$ phosphorylation at Tyr^{418}, a site required for pp60$^{c\text{-}src}$ activation, indicating that pp60$^{c\text{-}src}$ inhibition is unlikely to mediate the rapid effects of staurosporine on FAK phosphorylation status. Furthermore,

specific inhibition of Src in HUVEC selectively blocked phosphorylation at Tyr^{861} without affecting Tyr^{397} phosphorylation and did not cause a rapid loss of FAK from focal adhesions [39]. Identification of the staurosporine-inhibitable kinases responsible for maintaining FAK phosphorylation at Tyr^{397} and Tyr^{861} will be an important future goal.

The mechanism by which changes in phosphorylation at Tyr^{861} and Tyr^{397} could disrupt focal adhesion targeting of FAK is unclear. Tyr^{397} is the major autophosphorylation site in FAK, and its phosphorylation forms a high-affinity binding site for the SH2 domains of pp60$^{c\text{-}src}$ and pp59$^{c\text{-}fyn}$. FAK with a mutation of Tyr^{397} cannot form FAK–Src complexes, and in contrast to wild-type FAK, is unable to stimulate migration when overexpressed in Chinese-hamster ovary cells [7]. Dephosphorylation at Tyr^{397} is therefore likely to have a marked effect on normal FAK function, which is likely to be increased in combination with dephosphorylation at Tyr^{861}.

The effects of staurosporine on FAK phosphorylation and its localization to focal adhesions diverged sharply from those of cycloheximide, the protein synthesis inhibitor. Whereas cycloheximide was an effective apoptotic agent, it induced apoptosis more slowly than staurosporine and did not promote either the rapid morphological changes or loss of FAK focal adhesion localization and tyrosine phosphorylation caused by staurosporine. Instead, the protein synthesis inhibitor induced a slower loss of FAK focal adhesion staining which occurred concomitantly with increased FAK proteolysis. FAK dephosphorylation is therefore not obligatory for apoptosis, but appears to be an important part of the apoptotic pathway, specifically induced by staurosporine. The fact that staurosporine induced a more rapid onset of apoptosis than cycloheximide, suggests that the rapid loss of FAK phosphorylation and focal adhesion localization are mechanisms underlying temporal differences in the induction of apoptosis by these agents.

Staurosporine has been widely used as an apoptogenic agent, but the molecular basis of its action has not been fully elucidated. A growing body of evidence indicates, however, that staurosporine differs from other apoptogenic agents such as DNA-damaging anti-cancer drugs and radiation, by inducing apoptosis via caspase-independent as well as caspase-dependent pathways, and it has been proposed that this may account for its ability to induce cell death in tumour cells normally resistant to chemotherapeutic drugs [32–34]. The identification of FAK as a target for staurosporine will help to elucidate the caspase-independent mechanisms underlying the apoptotic effects of staurosporine and raises the possibility that inhibition of FAK tyrosine phosphorylation may contribute to the induction of apoptosis by this agent in tumour cells. Our findings also suggest that inhibition of FAK phosphorylation may be a rational target for strategies aimed at suppression of endothelial cell survival in order to block neovascularization.

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