British Journal of Pharmacology (2009), 156, 846–856

0007-1188/09
www.brjpharmacol.org

RESEARCH PAPER

The integrin $\alpha_2\beta_1$ agonist, aggretin, promotes proliferation and migration of VSMC through NF-kB translocation and PDGF production

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Background and purpose: During the development of atherosclerotic plaques, vascular smooth muscle cells (VSMCs) migrate from the media to the intima through the basement membrane and interstitial collagenous matrix, and proliferate to form neointima. Here, we investigate the mechanism of VSMC migration and proliferation caused by aggretin, a snake venom integrin $\alpha2\beta1$ agonist.
Experimental approach: Cultures of rat and human VSMCs were treated with aggretin and the signal transduction pathways induced by this agonist were examined by Western blotting, immunoprecipitation and electrophoretic mobility shift assay techniques.
Key results: Aggretin-induced VSMC proliferation was blocked by a monoclonal antibody (mAb) against integrin $\alpha2$ (AII2E10) or against the platelet-derived growth factor receptor (PDGFR)-β. Proliferation was also blocked by inhibition of the tyrosine kinase Src with PP2, phospholipase C (PLC) with U73122, extracellular signal-regulated kinase (ERK) with PD98059 or nuclear factor-kappa B (NF-kB) activation with pyrrolidine dithiocarbamate (PDTC). VSMC migration towards immobilized aggretin was increased in a modified Boyden chamber and this effect was blocked by $\alpha2\beta1$-Src-PLC-MAPK axis inhibitors, but not by PDTC, PDGFR-β mAb, or a phosphoinositide-3 kinase inhibitor, LY294002. Aggretin stimulated the phosphorylation of PDGFR-β, Src and ERK in a time-dependent manner. NF-kB translocation and platelet-derived growth factor (PDGF)-BB production were also observed. The ERK activation, NF-kB translocation and PDGF-BB production were blocked by PP2, U73122 and PD98059.
Conclusions and implications: Aggretin induces VSMC proliferation and migration mainly through binding to integrin $\alpha2\beta1$, and subsequently activates Src, PLC and ERK pathways, inducing NF-kB activation and PDGF production.
British Journal of Pharmacology (2009) **156**, 846–856; doi:10.1111/j.1476-5381.2008.00095.x; published online 23 February 2009

Keywords: aggretin; integrin $\alpha2\beta1$; smooth muscle cell; NF-kB; PDGF

Abbreviations: ECM, extracellular matrix; EMSA, electrophoretic mobility shift assay; ERK, extracellular signal-regulated kinase; GPIb, glycoprotein Ib; HASMC, human aortic smooth muscle cell; HUVEC, human umbilical vein endothelial cell; MAPK, mitogen-activated protein kinase; NF-kB, nuclear factor-kappa B; PDGF, platelet-derived growth factor; PDTC, pyrrolidine dithiocarbamate; PI3K, phosphoinositide-3 kinase; PLC, phospholipase C; VSMC, vascular smooth muscle cell

Introduction

Vascular smooth muscle cells (VSMCs) contribute to the pathogenesis of atherosclerosis and restenosis by proliferation, migration from the media to the intima, and deposition of abundant extracellular matrix (ECM) in the neointima. In fact, several matrix proteins produced after vascular injury, including fibronectin, tenascin and type I collagen, stimulate VSMC proliferation and/or migration *in vitro* (Gotwals *et al.*, 1996). The nature of the local environment, particularly the components of the ECM, dramatically regulate cell behaviour (Skinner *et al.*, 1994). A majority of cell-matrix interactions is mediated by specific membrane receptors of the integrin family. Occupancy and clustering of integrins can activate intracellular signalling pathways and induce the formation of transcription factors and the subsequent gene expression (Hynes, 1992).

Collagen is known to stimulate the migration and growth of numerous cell types, including VSMCs (Xiang *et al.*, 2000). These actions of collagen are mediated through cell surface receptors, including integrins (Hynes, 1992). The ligation of

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Received 16 July 2008; revised 25 September 2008; accepted 21 October 2008

integrin is known to activate signalling pathways including the activation of the c-Src and Ras/extracellular signal-regulated kinase (ERK) pathways (Giancotti and Ruoslahti, 1999). Different ECM proteins interact with distinct integrins on cells (Stupack, 2005). For example, VSMCs adhere to collagen through the integrins α2β1 and α1β1, while they adhere to fibronectin through the integrin α2β1 (Glukhova *et al.*, 1994).

Snake venoms contain many unique components that affect cell-matrix interaction. Aggretin, a potent platelet-aggregating protein purified from *Calloselasma rhodostoma* venom, consists of α and β subunits which share sequences homologous to those of C-type lectins (Chung *et al.*, 1999). In previous studies, we showed that aggretin induced platelet aggregation, most likely via integrin α2β1 and glycoprotein Ib (GPIb) receptors (Chung *et al.*, 2001). In addition, aggretin exhibits pro-angiogenic activities, including promotion of human umbilical vein endothelial cell (HUVEC) proliferation, migration and Matrigel-induced capillary tube formation *in vitro*, and induction of neovascularization in a chick chorio-allantoic membrane angiogenesis model *in vivo*. The major target site of aggretin on HUVECs is integrin α2β1 (Chung *et al.*, 2004). Thus, we investigated if aggretin exerted any effect on VSMCs through α2β1 ligation.

Nuclear factor-kappa B (NF-kB) is a dimeric transcription factor involved in inflammatory and immune responses. Cellular stimulation by proinflammatory cytokines and other agents activates an IkB kinase complex to phosphorylate IkB proteins. Subsequent polyubiquitination and proteasomal degradation of IkB leads to the translocation of NF-kB into the nucleus (Karin and Ben-Neriah, 2000). Activated nuclear NF-kB has been detected in smooth muscle cells after balloon injury to rat carotid arteries and in the smooth muscle cells of human atherosclerotic lesions. It was further identified *in situ* in macrophages, endothelial cells, and VSMCs in the intima and media of atherosclerotic vessel sections (Landry *et al.*, 1997). These data strongly suggest a causative role for NF-kB in the development and maintenance of atherosclerosis.

In this study, we investigated the effect of aggretin on VSMC proliferation and migration, and the signal transduction pathways involved. To evaluate contribution of aggretin in modulating the integrin α2β1-mediated signalling pathway in VSMCs, we studied the Src, phospholipase C (PLC), and ERK cascades and their cross-regulation. Our analysis revealed that aggretin induced the activation of Src, platelet-derived growth factor receptor (PDGFR)-β, ERK phosphorylation and NF-kB translocation, leading to production of platelet-derived growth factor (PDGF)-BB.

Methods

Cell culture

Human aortic smooth muscle cells (HASMCs) purchased from Clonetics were cultured in smooth muscle cell growth medium-2 (SM-GM2) containing 2 ng·mL^{-1} human basic fibroblast growth factor, 0.5 ng·mL^{-1} human epidermal growth factor, 50 ng·mL^{-1} amphotericin-B, 5% fetal bovine serum (FBS); 50 μg·mL^{-1} gentamicin and 5 μg·mL^{-1} bovine insulin (all purchased from Clonetics). For all experiments, early-passage (passages 5 to 7) HASMCs were grown to 80% confluence, and made quiescent by serum starvation (0.4% FBS) for 24 h.

Rat A10 VSMCs were obtained from American Type Culture Collection (A10 CRL 14776). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM), containing 10% fetal calf serum (Gibco) supplemented with glutamax I (Gibco), 100 IU·mL^{-1} penicillin G (sodium salt), 100 mg·mL^{-1} streptomycin and 0.25 mg·mL^{-1} amphotericin B (antibiotic-antimitotic solution, Gibco).

Cell proliferation

Human aortic smooth muscle cells and VSMCs (5×10^3 cells per well) were seeded in 96-well plates (Costar) for attachment, and cells were grown in SM-GM2 or DMEM, or in the presence of aggretin or various inhibitors or antibodies for 48 h before assay. For the assay using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), cells were incubated with MTT at a final concentration of 0.5 mg·mL^{-1} for 4 h. After incubation, the medium was aspirated, and the cells were dissolved in dimethyl sulphoxide and then measured for developed colour absorbance at 550 nm. For control, HASMCs and VSMCs were grown in SM-GM2 or DMEM with or without 10% FBS for 48 h and then assayed for cell proliferation

Migration assay (haptotaxis)

HASMC and VSMC migration assays were performed using the modified Boyden chamber model (Transwell apparatus, 8.0 μmol·L^{-1} pore size, Costar), as previously described, with modification (Leavesley *et al.*, 1993). Polycarbonate filters (Transwell inserts) were coated with aggretin (0.6 μg), collagen (0.2 μg) or bovine serum albumin (BSA) (20 μg) overnight, respectively, and the lower chamber was filled with 0.6 mL DMEM. VSMCs (1×10^4 cells·mL^{-1}, 200 μL) were placed in the upper chamber of the Transwell in the absence or presence of A2IIE10 for 30 min. After 16 h incubation, all non-migrant cells were removed from the upper face of the Transwell membrane with a cotton swab, and the migrant cells were fixed and stained with 0.5% toluidine blue in 4% paraformaldehyde. Migration was quantified by counting the number of stained cells per 100× field with an inverted contrast phase microscope (Nikon, Japan), and then photographed.

Binding assays of aggretin towards VSMCs

Flow cytometric studies were performed to assay the binding and target receptor for aggretin in VSMCs. VSMCs were suspended in phosphate-buffered saline (PBS)/1% BSA and fixed with 1% paraglutaldehyde for at 4°C 30 min. Following washing with PBS/1% BSA, the cells were pretreated with A2IIE10, Agkistin or 7E3 at 4°C for 1 h. Treated cells were washed twice and then incubated with fluorescein-5-isothiocyanate (FITC)-conjugated aggretin for 30 min at 4°C with a continuous shaking. After incubation, cells were washed twice, resuspended in PBS and analysed immediately using a FACalibur (Becton Dickinson, CA, USA) at excitation

and emission wavelengths of 488 and 525 nm respectively. Fluorescence signals from 10 000 cells were collected to calculate the mean fluorescence intensity of a single cell and the percentage of positively stained cells.

Immunoprecipitation and Western blotting

Vascular smooth muscle cells were pretreated with various concentrations of aggretin for different time intervals. Reactions were terminated with lysis buffer, and after sonication of the cells, the supernatant was isolated by centrifugation. PDGFR was immunoprecipitated from the supernatant using anti-PDGFR antibody for 1 h, and then protein A/G plus Sepharose was added for 1 h. The pelleted protein A/G plus Sepharose was washed three times with 1 mL of Tris-saline buffer, and eluted with Laemmli sodium dodecyl sulphate (SDS) reducing buffer. The immunoprecipitates or VSMC lysates were separated by SDS-PAGE using 8% gels, and transferred to polyvinylidene difluoride membranes. The membranes were blocked in 5% (w/v) non-fat milk dissolved in Tris/phosphate/saline/Tween (TBS-T) and incubated with primary and secondary antibodies, which were diluted in TBS-T containing 1% non-fat milk. Blots were washed for at least 1 h in TBS-T after each incubation with antibodies, and developed using an enhanced chemiluminescence (ECL) detection system. Primary antibodies and horseradish peroxidase-conjugated secondary antibodies were used at a concentration of 1 $\mu g \cdot mL^{-1}$.

NF-kB translocation

For study of NF-kB p65 translocation, cells were rinsed with PBS and suspended in hypotonic buffer A (10 $mmol \cdot L^{-1}$ HEPES, pH 7.6, 10 mmol L^{-1} KCl, 1 mmol L^{-1} DTT, 0.1 $mmol \cdot L^{-1}$ EDTA and 0.5 $mmol \cdot L^{-1}$ phenylmethylsulphonyl fluoride) for 10 min on ice and vortexed for 10 s. The lysates were separated into cytosolic and nuclear fractions by centrifugation at 12 000 g for 2 min. The supernatants containing cytosolic proteins were collected. A pellet containing nuclei was suspended in buffer C (20 $mmol \cdot L^{-1}$ HEPES, pH 7.6, 1 $mmol \cdot L^{-1}$ EDTA, 1 $mmol \cdot L^{-1}$ DTT, 0.5 $mmol \cdot L^{-1}$ phenylmethylsulphonyl fluoride, 25% glycerol and 0.4 $mol \cdot L^{-1}$ NaCl) for 30 min on ice. The supernatants containing nuclei proteins were collected by centrifugation at 12 000 g for 20 min and stored at –70°C. All protein concentrations were determined by colorimetric assay using a Bio-Rad assay kit (Bio-Rad, Hercules, CA, USA). Equal amounts (40 μg) of each protein from cytosolic or nuclei fractions were separated by 10% polyacrylamide-SDS gel and then electrotransferred to polyvinylidene difluoride membranes.

Electrophoretic mobility shift assay (EMSA)

Electrophoretic mobility shift assay was performed by using LightShift® Chemiluminescent EMSA kit (Pierce Inc.) according to the manufacturer's protocol. Binding reaction was initiated by adding 5 μg nuclear extract to binding buffer, 1 μg poly(deoxyinosinic-deoxycytidylic acid) and 100 $nmol \cdot L^{-1}$ biotin-labelled target double-stranded oligonucleotide incubated for 30 minutes at 37°C. The reaction was terminated by adding 5 μL of 5X DNA loading dye and then placing samples on ice before electrophoresis on a 5% native polyacrylamide gel. The samples on gel were then transferred onto Hybone™-N Nylon membrane (Amersham, Buckinghamshire, UK). The membrane was cross-linked at 120 $mJ \cdot cm^{-1}$ for 1 min, and then developed by adding the blocking buffer and streptavidin-HRP conjugate. NF-kB consensus site probe sequence: 5′-AGTTGAGGGGACTTTCCCAGGC-3′ (Liu *et al.*, 2006).

Transfection with small interfering RNA (siRNA)

Vascular smooth muscle cells (1×10^6 cells) were transfected with integrin α2 siRNA or non-targeting siRNA (negative siRNA) in 100 μL serum-free media with RNAi-Mate. A mixture of three siRNA sequences targeting the integrin α2 was used at total concentration of 1.5 μg per sequence. After 48 h, flow cytometry analysis for the integrin α2 was performed to confirm RNA suppression.

The integrin α2 sequences were as follows: sequence 1, sense UGAAUUGUCUGGCGUAUAATT, antisense UUAUACG CCAGACAAUUCATT; sequence 2, sense CAACUGGGAUCU GUUCUGATT, antisense UCAGAACAGAUCCCAGUUGTT; sequence 3, sense GCCAAUGAGCCGAGAAUUATT, antisense UAAUUCUCGGCUCAUUGGCTT (Jafri *et al.*, 2008).

PDGF-BB production

Platelet-derived growth factor-BB concentrations of cell lysates were determined, following the manufacturer's instructions, using a quantitative enzyme-linked immunosorbent assay (R&D Systems, Minneapolis, MN, USA). All analyses and calibrations were carried out in triplicate. PDGF-BB immunoreactivity was determined as compared with recombinant PDGF-BB standards. Optical densities were determined at 450 nm using a microtiter plate spectrophotometer.

Statistical analysis

All values are presented as mean ± standard error. Differences between groups were assessed by one-way ANOVA and Newman-Keuls multiple comparison test where appropriate. P values less than 0.05 ($P < 0.05$) were considered significant difference.

Materials

Aggretin and agkistin were purified from *Calloselasma rhodostoma* and Formosan *Agkistrodon acutus* venom, as described previously (Huang *et al.*, 1995; Yeh *et al.*, 2000). Protein A/G plus-Sepharose, peroxidase-conjugated anti-mouse antibody, anti-PDGFR, anti-NF-kB p65, peroxidase-conjugated anti-rabbit antibody, anti-phospho-c-Src α polyclonal antibody (Tyr 216), anti-α-tubulin antibody (TU-02), anti-nucleolin antibody (sc-8031), anti-phospho-ERK1/2 (E-4) and an ECL detection system were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA); anti-α2 monoclonal antibody (mAb) (A2IIE10), anti-α1 mAb (5E8D9) and anti-phosphotyrosine mAb (4G10) were obtained from Upstate Biotechnology Inc. (Lake Placid, NY, USA); FITC was from

Molecular Probes (USA); Collagen type I, MTT, pyrrolidine dithiocarbamate (PDTC), LY294002, PD98059 and BSA were from Sigma Co., (USA); PP2 was obtained from Calbiochem (San Diego, CA, USA); U73122 was obtained from Calbiochem. (La Jolla, CA, USA); DMEM, FBS and all culture reagents were purchased from Gibco BRL (USA); RNAi-Mate transfection reagent, integrin α2 siRNA and non-targeting siRNA were obtained from MDBio Inc. (Taipei, Taiwan). Our drug/molecular target nomenclature conforms to the BJP Guide to Receptors and Channels (Alexander *et al.*, 2008).

Results

Effect of aggretin on VSMC and HASMC proliferation and migration

Our working hypothesis is that integrin α2β1 plays an important role in SMC proliferation. Thus, we used this natural, specific integrin α2β1 agonist, aggretin to examine its effect in stimulating VSMCs and HASMCs. The SMC proliferation assay was performed by determining cell metabolic activity with MTT. As shown in Figure 1A,B, aggretin significantly increased cell proliferation in a concentration-dependent manner, compared with serum-free controls (10~80% increase). VSMC proliferation were partially inhibited by an integrin α2 mAb, A2IIE10 (50 μg·mL^{-1}, about 60% inhibition) and almost completely blocked by the Src inhibitor, PP2 (10 μmol·L^{-1}) (Salazar and Rozengurt, 1999), a PLC inhibitor, U73122 (10 μmol·L^{-1}) (Tatrai *et al.*, 1994), an ERK inhibitor, PD98059 (30 μmol·L^{-1}) (Dudley *et al.*, 1995) and the NF-kB activation inhibitor, PDTC (20 μmol·L^{-1}) (Sherman *et al.*, 1993) as compared with those of the control (Figure 1A). HASMC proliferation was also partially inhibited by an integrin α2 mAb, A2IIE10 (50 μg·mL^{-1}, about 62% inhibition) and almost completely blocked by the ERK inhibitor, PD98059 (30 μmol·L^{-1}, about 94% inhibition), compared with that in

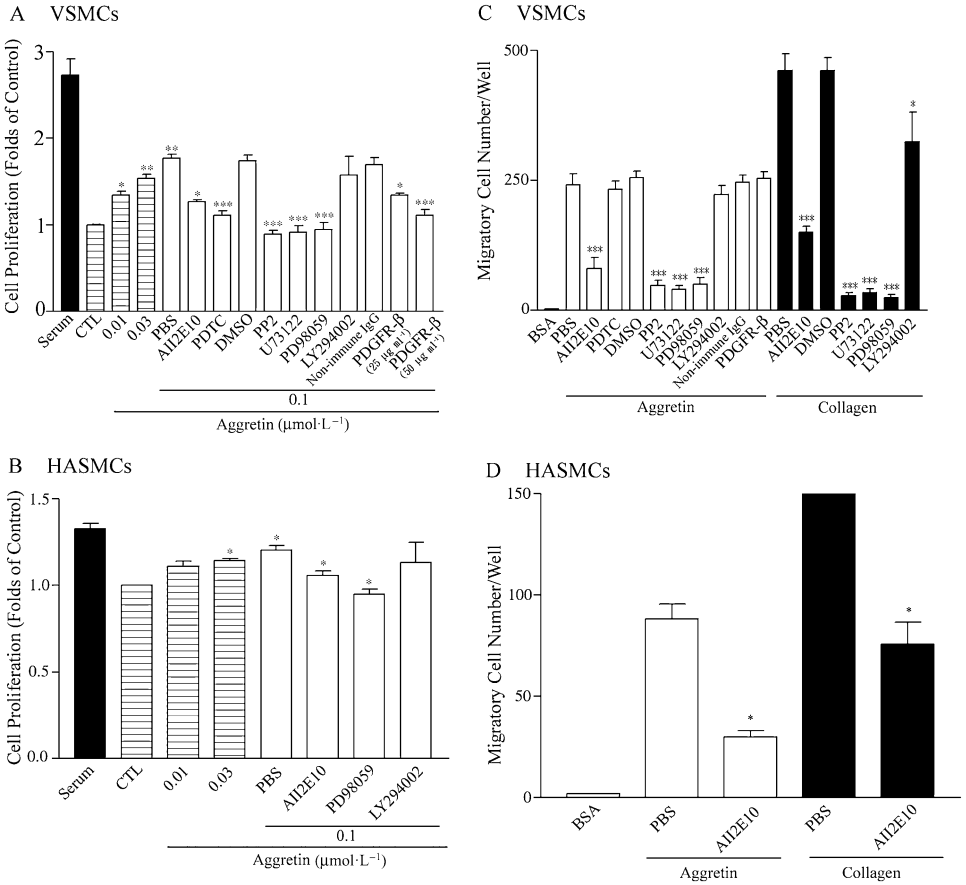

Figure 1 Effects of aggretin on proliferation (A,B) and migration (C,D) of rat VSMCs and HASMCs. (A, B) VSMCs or HASMCs (1×10^4 cell·mL^{-1}) were seeded on 24-well plates. After attachment, cells were treated with an indicated concentration of aggretin for 48 h in the absence or presence of various inhibitors, and followed by MTT assay. Results are expressed as cell viability relative to the control cells (CTL, as 1.0) in the absence of serum (DMEM). The inhibitor studies with 0.1 μmol·L^{-1} aggretin were compared with the PBS (0.1 μmol·L^{-1} aggretin alone) group. (C, D) Migration of VSMCs and HASMCs (5×10^4 cells·mL^{-1}) incubated in the absence or presence of various inhibitors, was followed by placing them in the upper chamber of a Transwell containing a gelatin-coated filter membrane, using collagen (0.2 μg), aggretin (0.6 μg) or BSA (20 μg) as the coating of the lower filter. Haptotaxis was allowed to proceed for 16 h. After fixation and removing non-migrated cells, cells that migrated to the underside of the filter membrane were quantified by a phase-contrast light microscope under a high power field (HPF, magnification × 100). All experiments were conducted in triplicate and similar results were repeated at least four times. Data are presented as mean ± SEM ($n = 4$). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ as compared with control. BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagle's medium; HASMCs, human aortic smooth muscle cells; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; PBS, phosphate-buffered saline; VSMCs, vascular smooth muscle cells.

the control cultures (Figure 1B). On the other hand, the proliferation effect induced by aggretin was not affected by LY294002, a phosphoinositide-3 kinase (PI3K) inhibitor (10 μmol·L^{-1}) (Baumann and West, 1998). PP2, U73122, PD98059 and LY294002 use dimethyl sulfoxide (DMSO) as their solvent. Vehicle controls showed no significant effects in this assay. These results suggest that aggretin-SMC interaction promotes cell proliferation and this effect is mainly mediated by integrin α2β1, and that the activation of Src, PLC, ERK and NF-kB are involved in the proliferation cascades. However, the activation of the PI3K/Akt cascade was not involved in VSMC and HASMC proliferation. To evaluate if growth factor was involved in VSMC proliferation, PDGFR-β mAb was used. PDGFR-β mAb concentration-dependently inhibited VSMC proliferation (25 and 50 μg·mL^{-1}, 45% and 75% inhibition).

As shown in Figure 1C,D, VSMCs and HASMCs migrated through aggretin or collagen-coating inserts, and both of these cell migrations were similarly blocked by A2IIE10 (50 μg·mL^{-1}), indicating that α2 integrin is essential for cell migration. VSMC migration was also abolished by PP2, U73122, PD98059, but not by LY294002, indicating that Src, PLC, ERK and PI3K/Akt may exert their effects in diverse signalling pathways. As the activation of integrin α2β1 in VSMCs may increase PDGF production (Figure 6), the involvement of PDGF-BB in VSMC migration was investigated. In the presence of PDGFR-β mAb, however, VSMC migration was not affected (Figure 1C). Thus, VSMC migration caused by aggretin is primarily modulated through integrin α2β1 activation, but is not correlated with activation of the PDGFR-β.

Binding assay of aggretin towards VSMC

To study the target site of aggretin on VSMCs, the cells were incubated with FITC-conjugated aggretin to examine the binding reaction. The increment of relative fluorescence intensity of the bound FITC-aggretin was concentration-dependent, reaching saturation at a concentration of 10 μmol·L^{-1} (Figure 2A). 5E8D9, a mAb against α1 integrin, agkistin, a snake venom GPIb antagonist and antibody 7E3 raised against integrin αvβ3 showed little effect on FITC-aggretin binding towards VSMCs (Figure 2B). In contrast, A2IIE10 (25 and 50 μg·mL^{-1}) inhibited aggretin binding (39 and 69%) to VSMCs in a concentration-dependent manner (Figure 2B).

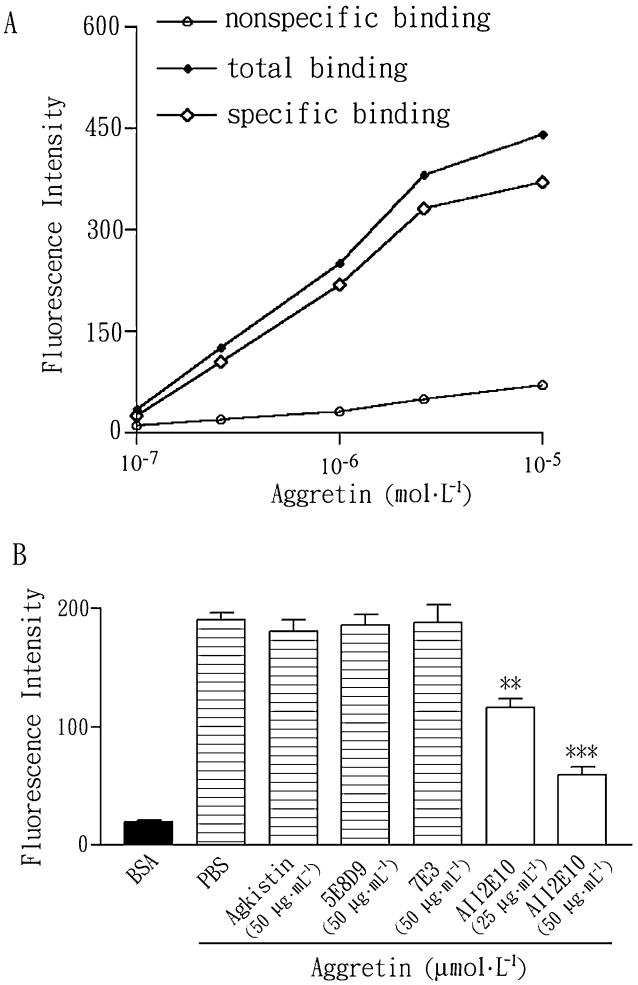

Figure 2 Effects of anti-α2 integrin mAb, anti-α1 integrin mAb and GPIb antagonist on the binding of FITC-conjugated aggretin to VSMCs. (A) VSMCs (1×10^6 cells) were incubated with various concentrations of FITC-conjugated aggretin or FITC-conjugated BSA for 30 min and then analysed by flow cytometry. This is representative of three similar results. (B) Quantitative analyses of FITC-aggretin and FITC-BSA with the various mAb and inhibitors, are presented as mean fluorescence intensity. Data are presented as mean ± SEM ($n = 3$). $^{**}P < 0.01$, $^{***}P < 0.001$ as compared with that of control. BSA, bovine serum albumin; FITC, fluorescein-5-isothiocyanate; GPIb, glycoprotein Ib; mAb, monoclonal antibody; VSMCs, vascular smooth muscle cells.

Effect of aggretin on PDGFR-β and Src phosphorylation in VSMCs

There is Src-dependent crosstalk between α2β1 integrin and PDGFR-β (Hollenbeck *et al*., 2004). PDGF is a well-known SMC agonist and the phosphorylated receptor transduces its signal by binding to intracellular signalling proteins, including the Src family of non-receptor tyrosine kinases, which in turn activates the downstream pathway implicated in cellular proliferation, including ERK (Bornfeldt *et al*., 1995). We evaluated the effect of aggretin on PDGFR-β and Src phosphorylation. After incubation of VSMCs with aggretin for various time intervals, the PDGFR-β of cell lysates was immunoprecipitated and immunoblotted. Figure 3A shows the tyrosine phosphorylation of immunoprecipitated aggretin-treated VSMC lysates. Phosphorylation of PDGFR-β induced by aggretin (0.1 μmol·L^{-1}) was significantly elevated, as compared with that of the control after 1/12 h of aggretin pretreatment, and this effect persisted for 3 h; the sequential phosphorylation of PDGFR-β was observed after 24 h of aggretin treatment. This long-term effect may be related to PDGF-BB production. As Src is also important in integrin α2β1 signalling (Inoue *et al*., 2003), the activation of Src was evaluated in aggretin-stimulated VSMCs. After aggretin pretreatment, aggretin induced Src activation of VSMCs in a time-dependent manner. This effect was evident at time points ranging from 5 min to 1 h ($P < 0.05$) after aggretin stimulation (Figure 3B).

ERK activation and its regulation

It is documented that matrix proteins and growth factors are able to stimulate ERK phosphorylation in migratory cells

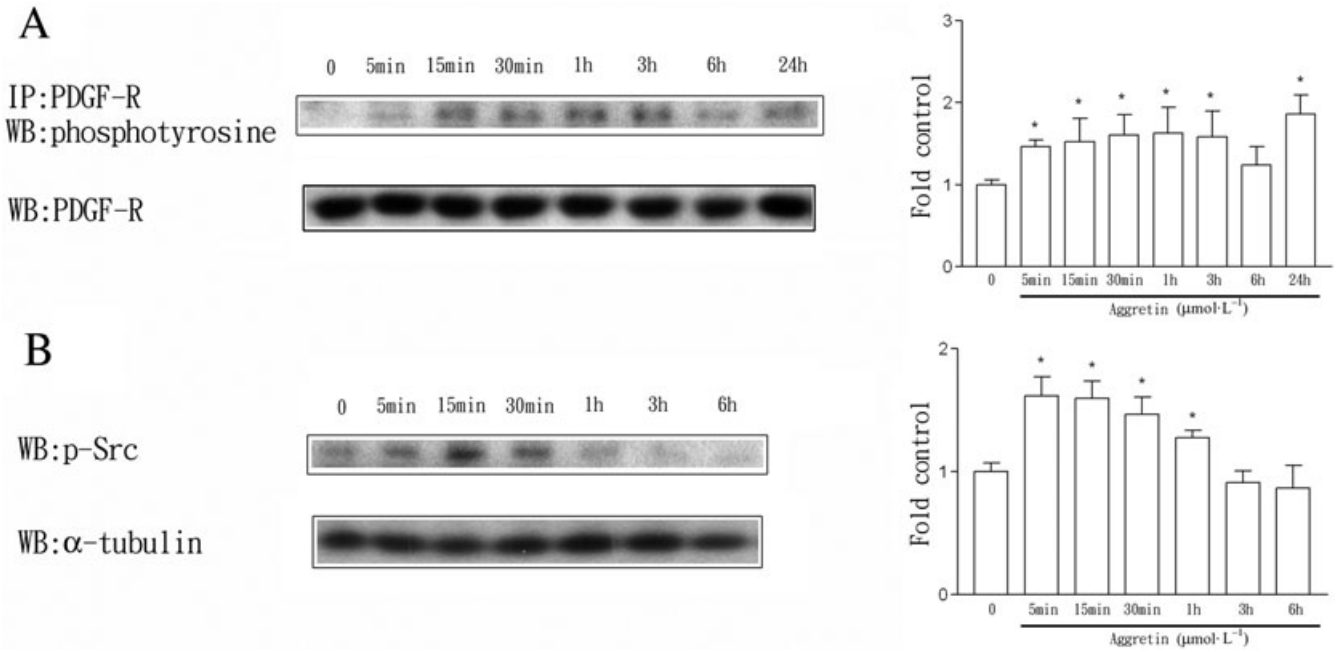

Figure 3 Effect of aggretin on VSMC PDGFR-β and Src phosphorylation. (A) VSMCs (1×10^6 cells) were cultured in the presence of aggretin (0.1 μmol·L^{-1}) for various times and the reactions were stopped with lysis buffer. Immunoprecipitates (IP) of PDGFR-β were formed as described in the methods. VSMC lysates were applied to SDS-PAGE. Tyrosine-phosphorylated proteins were detected with Western blot (WB) using anti-phosphotyrosine mAb (4G10) coupled with ECL, and then reprobed with PDGFR-β mAb. (B) VSMCs (1×10^6 cells) were cultured in the presence of aggretin (0.1 μmol·L^{-1}) for different times, and the reactions were stopped with lysis buffer. Src activation was detected with WB using anti-phospho-Src pAb (Tyr 216) coupled with ECL, whereas α-tubulin was taken as an internal control. Summary data of PDGFR-β and Src phosphorylation were presented (on the right) as mean density, as determined by a densitometer. Densitometric band intensities were normalized to static controls, and fold increases were calculated. The data are representative of at least three experiments. Data are presented as mean ± SEM ($n = 4$). *$P < 0.05$ as compared with control. ECL, enhanced chemiluminescence; PDGFR-β, platelet-derived growth factor receptor-β; SDS, sodium dodecyl sulphate; VSMC, vascular smooth muscle cell.

(Robinson and Cobb, 1997), and the ERK inhibitor, PD98059, can also affect VSMC proliferation and migration, so we were interested in identifying ERK involvement in integrin-ligand interaction. Utilization of the phosphorylation-specific antibody against ERK1/2 for immunoblotting revealed activation of the ERK1/2 pathway in aggretin-stimulated VSMCs. After VSMC exposure to aggretin (0.1 μmol·L^{-1}) for various time intervals, ERK1/2 phosphorylation was markedly increased in a time-dependent manner (Figure 4A), and the ERK of VSMCs lysates were phosphorylated within 5 min and partially inactivated at 1 h. Moreover, the activation of ERK1/2 was abolished by the Src inhibitor, PP2 (10 μmol·L^{-1}) and the PLC inhibitor, U73122 (10 μmol·L^{-1}) (Figure 4B).

NF-kB activation is involved in integrin α2β1 ligation

An important role for NF-kB in controlling the proliferation of various cell types has been demonstrated (Bargou *et al*., 1997). It is possible that integrin ligation may also involve NF-kB activation. To monitor NF-kB activation in VSMCs after aggretin stimulation, we studied the translocation of NF-kB from cytosol to nucleus. Figure 5A displays a decrease of cytosolic NF-kB and an increase in the nucleus in a time-dependent manner. This translocation effect was more pronounced at 15 and 30 min (increases of 1.98 ± 0.56- and 2.30 ± 0.81-fold respectively). We also investigated the influence of pharmacological inhibitors on NF-kB activation in VSMCs. In parallel studies (Figure 5B), preincubation of VSMCs with Src, PLC and ERK inhibitors eliminated the NF-kB translocation effect induced by aggretin. NF-kB DNA binding and transcriptional activity were also confirmed by EMSA analysis (Figure 5C). Results were similar to NF-kB translocation studies (Figure 5A,B).

To precisely evaluate the role of integrin α2β1, the relevant siRNA was used to suppress the expression of α2-subunit and was confirmed by flow cytometry. The expression of integrin α2β1 was dramatically decreased, compared with non-transfected cells (Figure 5D). Aggretin-induced activation of NF-kB was significantly inhibited in A10 cells transfected with integrin α2 siRNA (Figure 5E). Cells transfected with non-targeting siRNA showed no difference in integrin α2β1 expression (data not show) and NF-kB activation (Figure 5E), compared with non-transfected cell.

Regulation of PDGF-BB production by aggretin

We next explored the contribution of PDGF-BB production to aggretin-stimulated VSMCs. As shown in Figure 6, no increment in PDGF-BB production was observed within the first 6 h, but a dramatic increase detected after 24 h exposure to aggretin. As compared with the PDGFR-β phosphorylation pattern, PDGF-BB production 24 h after aggretin treatment may result in sequential phosphorylation of PDGFR-β (Figure 3A, 24 h). To investigate the signalling proteins required for PDGF-BB

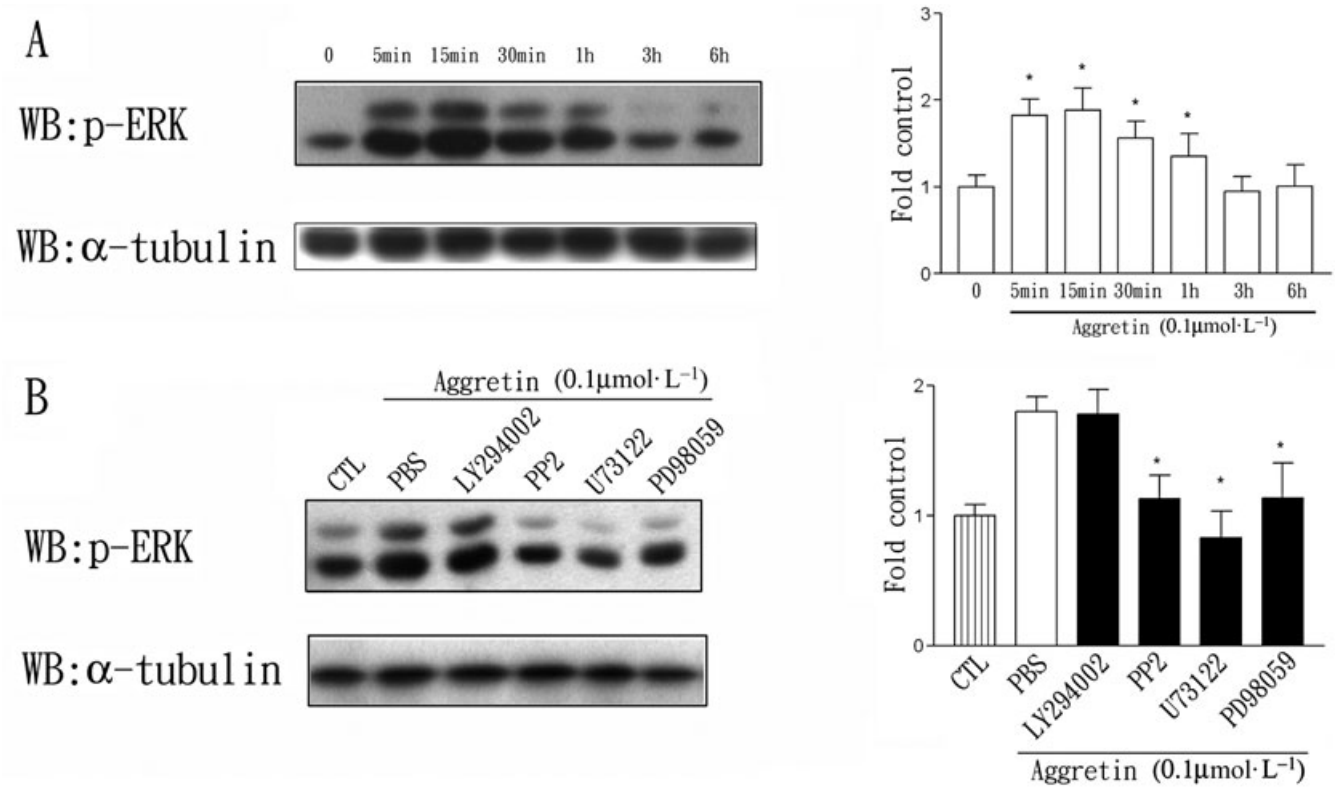

Figure 4 Aggretin causes ERK activation and its regulation. VSMCs (1×10^6 cells) were cultured in the presence of aggretin (0.1 μmol·L^{-1}) for different times (A) or in the presence of various inhibitors for 30 min (B). The reactions were stopped with lysis buffer. ERK activation was detected with Westen blots (WB) using anti-phospho-ERK1/2 mAb coupled with ECL, with α-tubulin was taken as an internal control. Summary data of ERK phosphorylation are presented (on the right) as mean density, determined by densitometer. Densitometric band intensities were normalized to static controls, and fold increases were calculated. The data are representative of at least three experiments. Data are presented as mean ± SEM ($n = 4$). *$P < 0.05$ as compared with control. ECL, enhanced chemiluminescence; ERK, signal-regulated kinase; VSMCs, vascular smooth muscle cells.

production in aggretin-stimulated VSMCs, we measured the level of PDGF-BB in cells pretreated with various kinase inhibitors. PP2, U73122 and PD98059 almost completely eliminated the enhancing effect of aggretin on PDGF-BB production, but LY294002 did not affect PDGF production (Figure 6). PDTC (20 μmol·L^{-1}) also effectively suppressed PDGF-BB production. These data suggest that in VSMCs, the Src/PLC/ERK cascade and NF-kB play crucial roles in promoting PDGF-BB production triggered by aggretin.

Discussion

Many matrix molecules that promote cell migration, such as collagen, act as attachment factors because it is necessary for a cell to adhere to a substrate to gain traction for migration (Elliott *et al.*, 2005). It is interesting to connect these behaviours of VSMCs with integrin signalling and so activation of kinase cascades coupled with integrin ligation has been investigated. In this study, ligand-binding demonstrated that the major binding site of aggretin is integrin α2β1, but not α1β1, GPIb or integrin αvβ3 expressed on VSMCs (Figure 2B). Skinner *et al.* (1994) showed that cultured SMCs expressed α2, α3, α5 and α v subunits with little α1 or β3 (Skinner *et al.*, 1994). Given that the rate of matrix turnover in normal blood vessels is slow, it is most likely that expression of the α2β1 collagen receptor, which can mediate migration, collagen turnover and gel contraction, and α1β1 may be expressed only during periods of active remodeling, such as in development and wound repair (Franco *et al.*, 2002). In other study, Katsue *et al.* identified a novel aggretin (i.e. rhodocytin)-binding 32-kDa surface megakaryocyte/platelet-specific protein, the C-type lectin receptor, CLEC-2 (Suzuki-Inoue *et al.*, 2006). However, α2β1 integrin is expressed primarily on cultured VSMCs and there is no literature suggesting that CLEC-2 is expressed in VSMCs, thus excluding the involvement of CLEC-2 as a target for aggretin in VSMCs.

The VSMC response to aggretin is mainly mediated by the α2 integrin receptor, because both the enhancement of migration and proliferation effects induced by aggretin were blocked by A2IIE10, a mAb against α2 integrin. Ligation of integrins induces a cascade of intracellular signals, regulates gene expression and contributes to proliferation, migration and differentiation (Stromblad *et al.*, 1996). Therefore, we investigated the integrin α2-mediated signalling pathways of VSMCs induced by aggretin. Some studies have demonstrated that integrin α2β1 enhances PDGF-BB-dependent proliferation and the synergistic effect of collagen on PDGF-BB-induced VSMC proliferation appears to reflect transactivated PDGFR-β receptor (Hollenbeck *et al.*, 2004). In this report,

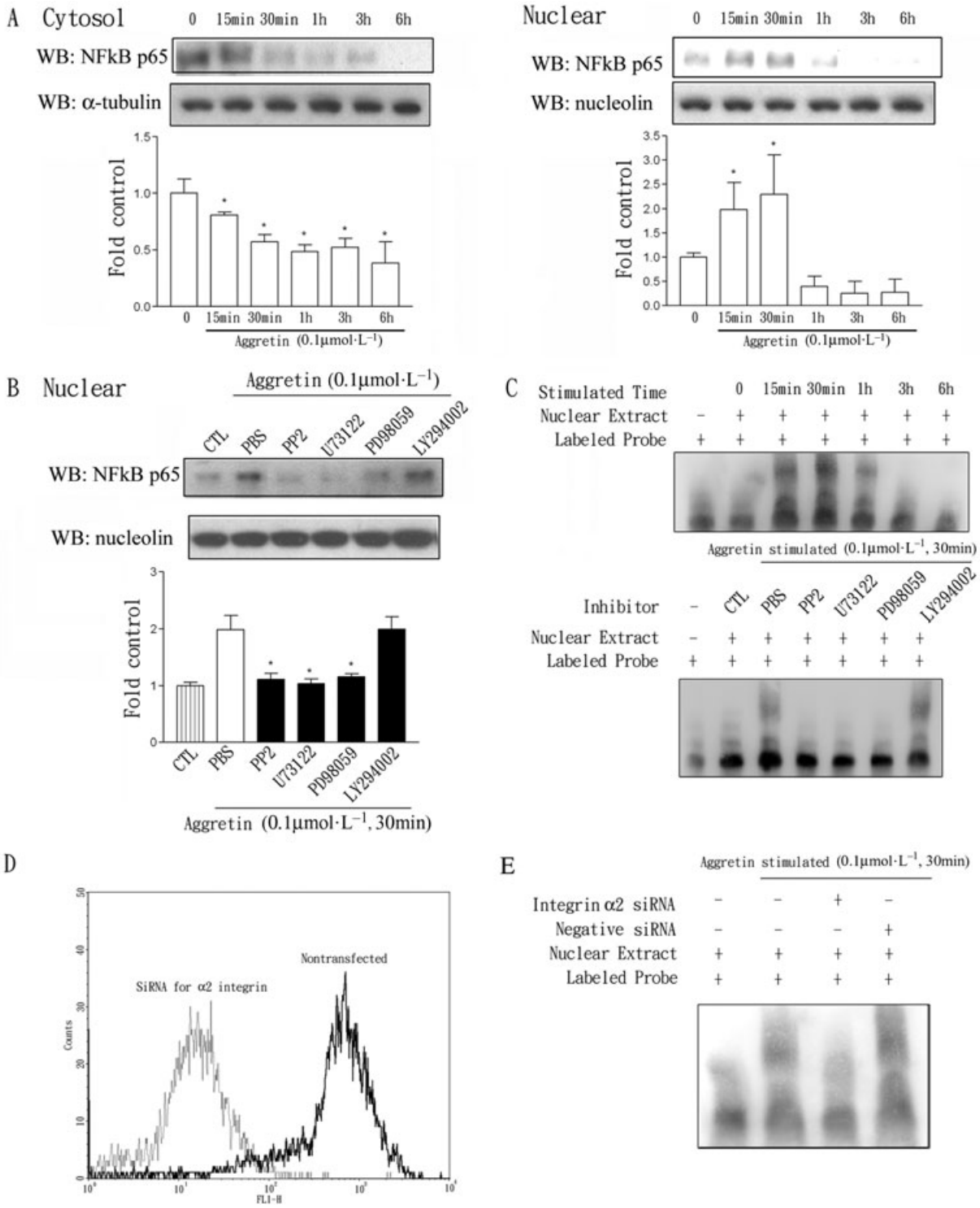

Figure 5 Integrin α2β1 ligation triggers NF-kB activation. VSMCs (1×10^7 cells) were cultured in the presence of aggretin (0.1 μmol·L^{-1}) for different times (A) or in the presence of various inhibitors for 30 min (B). The cytosolic and nuclear fractions were obtained as described in the methods. The reactions were stopped with lysis buffer. NF-kB activation was detected with Western blots using anti-NF-kB p65 pAb coupled with ECL. Summary data of NF-kB translocation are presented below the blots, as mean density, determined by densitometer. Densitometric band intensities were normalized to static controls, and fold increases were calculated. (C) EMSA was performed in the absence or presence of NF-kB binding sequence to confirm the NF-kB activation condition in A and B. VSMCs were also pretreated with aggretin (0.1 μmol·L^{-1}) for different time intervals or in the presence of various inhibitors for 30 min. Nuclear extracts were preincubated with biotin-labelled double-stranded oligonucleotide NF-kB, and reaction products were analysed on 5% nondenaturing polyacrylamide gels. (D) FACS analysis for integrin α2β1 expression after siRNA interference. (E) EMSA was performed in the absence or presence of integrin α2 siRNA in aggretin-induced NF-kB activation. The data are representative of at least three experiments. Data are presented as mean ± SEM ($n = 4$). *$P < 0.05$ as compared with control. ECL, enhanced chemiluminescence; EMSA, electrophoretic mobility shift assay; NF-kB, nuclear factor-kappa B; siRNA, small interfering RNA; VSMCs, vascular smooth muscle cells.

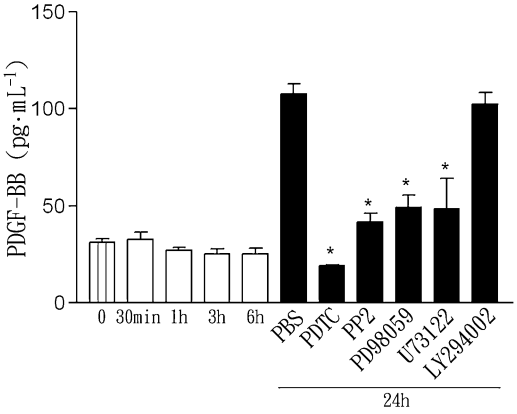

Figure 6 Aggretin induced PDGF-BB production and its regulation. VSMCs (1×10^7 cells) were cultured in the presence of aggretin (0.1 μmol·L^{-1}) for different times or in the presence of various inhibitors for 24 h. The cell lysates were collected, and PDGF-BB concentrations of cell lysates were determined by quantitative enzyme-linked immunosorbent assay. Aggretin did not induce PDGF-BB production until 24 h of incubation. This late phase output was inhibited by all the compounds tested except LY294002, a PI3K inhibitor. The data are representative of at least three experiments. Data are presented as mean ± SEM ($n = 4$). *$P < 0.05$ as compared with control. PDGF-BB, platelet-derived growth factor-BB; VSMCs, vascular smooth muscle cells.

when smooth muscles were treated with aggretin, the tyrosine phosphorylation of PDGFR-β and Src were markedly increased within 5 min in parallel, compared with the serum-free control (Figure 3). Src is necessary and sufficient for smooth muscle cell proliferation and migration (Cho *et al*., 2005). The Src kinase activity is also required for integrin-mediated NF-kB activation (Courter *et al*., 2005). We showed that Src activation was involved in integrin ligation and may be related to PDGFR-β phosphorylation. Accordingly, some authors have described the role of the Src family of kinases in αvβ3/EGF and α2β1/PDGFR crosstalk (Moro *et al*., 2002; Hollenbeck *et al*., 2004); however, the pathways linking integrin ligation to activation of Src, and the downstream signalling proteins involved, are not yet fully defined.

The proliferation of several phenotypes of cells is mediated by growth factor or cytokine-induced mitogen-activated protein kinases (MAPKs), a family of serine-threonine proteins. MAPKs consist of ERK, p38 MAPK (p38) and c-Jun NH2-terminal kinase (JNK) (Cowan and Storey, 2003). The activation of ERK induced by various substances, such as PDGF, increased proliferation of human airway smooth muscle cells (Karpova *et al*., 1997). Studies also have revealed that loss of the α7β1 integrin results in VSMC hyperplasia through ERK activation (Welser *et al*., 2007). The mechanism of ERK activation in aggretin-stimulated VSMCs was elucidated in this study (Figure 4A). The phosphorylation of other MAPK pathways, p38 and JNK, were not observed with aggretin-stimulated VSMCs (data not shown). These findings suggest that aggretin-induced VSMC proliferation is related to the activation of the ERK pathway, and Src and PLC may be the upstream signalling molecules.

The PI3K/Akt/PKB cascade is another integrin-mediated cell signalling pathway. PI3K is known to be activated by integrin engagement and is important in cell spreading in normal fibroblastd (Berrier *et al*., 2000). Although PI3K/Akt was also activated after aggretin treatment of VSMCs (data not shown), pretreatment of VSMCs with a PI3K inhibitor did not affect the proliferation and migration induced by aggretin (Figure 1A,C). These results indicate that ERK and PI3K/Akt may contribute to the diverse signalling pathway modulating the different function of VSMCs. As the phosphorylation of p38 and JNK were not observed in aggretin-stimulated VSMCs (data not shown), they are not involved in aggretin-mediated cell proliferation and migration.

Various reports suggest a role for the ubiquitous transcription factor NF-kB in the mitogenic growth control of a variety of cell types. The activated NF-kB binds to specific promoter regions of target genes in the nucleus. Previous studies demonstrated that NF-kB activation is enhanced in intimal lesions (Wilson *et al*., 2000). Furthermore, evidence indicates that NF-kB activation was increased in endothelial cells of regions pre-disposed to lesion formation and, additionally, that NF-kB can bind to the promoter regions of PDGF-BB (Hajra *et al*., 2000). Li *et al*. also reported that angiotensin II and oxidized-LDL promoted NF-kB activation, which mediated, at least in part, PDGF-BB expression in cultured endothelial cells (Zhou *et al*., 2003). According to our experiments, incubation with aggretin stimulates VSMCs, resulting in NF-kB activation in 15–30 min (Figure 5A). These findings also indicate that the aggretin-induced NF-kB translocation in VSMCs was dependent on Src, PLC and ERK, thus implicating integrin α2β1 ligation for triggering NF-kB activation (Figure 5B,C). *In vitro*, siRNA transfection also supported an important role for integrin α2β1 in aggretin-induced signal transduction in VSMCs. Aggretin-induced NF-kB activation was almost completely inhibited in A 10 cells silenced with siRNA for integrin α2 (Figure 5D,E). These results indicate that the integrin α2β1 is an important receptor in aggretin-mediated VSMC signalling.

As PDGF-BB production was blocked by the NF-kB inhibitor, PDTC, the consequence of NF-kB activation is the promotion of PDGF-BB expression (Figure 6), suggesting a close relationship between the induction of NF-kB activation and the expression of PDGF-BB *in vivo*. Although aggretin induced PDGFR-β trans-activation in the early phase, after 24 h the activation of PDGFR-β following aggretin stimulation may be through PDGF-BB release, as no significant PDGF-BB release was detected during the first 6 h of incubation.

In conclusion, we have shown that aggretin exhibited a significant promoting effect on VSMC proliferation and migration. The binding of FITC-conjugated aggretin to VSMCs was specifically inhibited by a mAb A2IIE10 raised against α2 integrin. Aggretin stimulated Src and ERK phosphorylation and, in turn, induced NF-kB activation. Aggretin-induced NF-kB activation was abolished by transfection with siRNA for integrin α2, indicating that the NF-kB activation may primarily through α2β1 ligation. Additionally, an increase in NF-kB translocation enhances PDGF-BB expression in aggretin-stimulated VSMCs. Taken together, we demonstrate that an increased proliferation and migration of VSMCs is initially triggered by α2β1 ligation of aggretin, and eventually a delayed elevation of PDGF-BB production, linking α2β1 engagement, the activation of PDGFR-β, Src-PLC-ERK, NF-kB translocation, PDGF production and feedback activation of PDGFR in mediating cell proliferation.

Acknowledgement

We appreciate the financial support of National Science Council, Taiwan.

Conflict of interest

None.

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