A potential role for nuclear factor of activated T-cells in receptor tyrosine kinase and G-protein-coupled receptor agonist-induced cell proliferation

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We have studied the role of nuclear factor of activated T-cells (NFAT) transcription factors in the induction of vascular smooth muscle cell (VSMC) growth by platelet-derived growth factor-BB (PDGF-BB) and thrombin, the receptor tyrosine kinase (RTK) and G-protein-coupled receptor (GPCR) agonists, respectively. NFATc1 but not NFATc2 or NFATc3 was translocated from the cytoplasm to the nucleus upon treatment of VSMCs with PDGF-BB or thrombin. Translocation of NFATc1 was followed by an increase in NFAT–DNA binding activity and NFAT-dependent reporter gene expression. Cyclosporin A (CsA), a potent and specific inhibitor of calcineurin, a calcium/calmodulin-dependent serine phosphatase involved in the dephosphorylation and activation of NFATs, blocked NFAT–DNA binding activity and NFAT-dependent reporter gene expression induced by PDGF-BB and thrombin. CsA also completely inhibited PDGF-BB- and thrombin-induced VSMC growth, as measured by DNA synthesis and cell number. In addition, forced expression of the NFAT-competing peptide VIVIT for calcineurin binding significantly attenuated the DNA synthesis induced by PDGF-BB and thrombin in VSMCs. Together, these findings for the first time demonstrate a role for NFATs in RTK and GPCR agonist-induced growth in VSMCs.

Key words: DNA synthesis, platelet-derived growth factor BB, thrombin, vascular smooth muscle cells.

INTRODUCTION

Increased vascular smooth muscle cell (VSMC) growth is one of the major events in the thickening of arterial wall in the pathogenesis of atherosclerosis and restenosis [1]. A large number of molecules, including peptide growth factors, hormones, eicosanoids and oxidants that are generated at the site of arterial injury, can influence the growth of VSMCs [1–5]. Indeed, increased levels of growth factors such as platelet-derived growth factor (PDGF), eicosanoids such as hydroxyeicosatetraenoic acids ('HETEs') and oxidants such as oxidized low-density lipoprotein have been reported in the atheromatous arteries as compared with normal arteries [3–8]. As these molecules utilize divergent early mitogenic signalling events in the induction of VSMC growth [9–11], targeting inhibition of the activity of a single mitogen might not be able to suppress VSMC growth and lesion formation. However, identifying the mechanisms that are less redundant and are involved in the mitogenic activities of many of these molecules may advance the therapeutic developments against VSMC growth and vessel wall lesions.

The nuclear factors of activated T-cells (NFATs) are a multigene family of transcription factors that were initially characterized in T-lymphocytes [12]. They are NFATc1 (also known as NFATc or NFAT2), NFATc2 (also known as NFATp or NFAT1), NFATc3 (also known as NFAT4 or NFATx) and NFATc4 (also known as NFAT3) and each of these appeared to be expressed as several isoforms by alternative splicing [12–14]. A new member of the NFAT family of transcription factors is the recently cloned NFAT5 [15]. Although all five NFATs exhibit a similar DNA-binding specificity, NFAT5 differs from the other four members in two ways. First, NFAT5 is constitutively present in the nucleus and its phosphorylation state and distribution are insensitive to calcineurin. Second, it exhibits lack of co-operativity with Fos or Jun proteins [15]. With regard to the functional role of these transcription factors, earlier studies have reported the presence of NFATc1, NFATc2 and NFATc3, mainly in immune cells, regulating the expression of cytokine genes [16]. However, later studies have demonstrated the presence of all five NFAT proteins in non-immune cells as well [12,17,18]. Studies with knockout mice also established a role for NFATs in non-immune cells as observed when (i) knockout mice for NFATc1 failed to develop normal cardiac valves [19,20], (ii) knockout mice for NFATc2 and NFATc3 exhibited reduced skeletal muscle size, and (iii) mice with disruption of NFATc3/c4 genes died around embryonic day 11 with defects in vessel wall assembly [21–23]. Interestingly, one of the members of the NFAT transcription factors, namely NFATc4, was reported to play a role in cardiac hypertrophy [24]. NFATc1–NFATc4 exist as phosphoproteins in the resting state and their activation requires dephosphorylation. A calcium/calmodulin-dependent serine phosphatase known as calcineurin has been reported specifically to dephosphorylate and activate these NFATs [16]. Activated NFATs bind to a consensus DNA sequence GGAAAAT, present in the promoter regions of genes, as monomers or dimers via their Rel homology domain and activate transcription [12]. However, other than the phenotypic observations made from the knockout mice models [19–23], the functional significance of NFATs in non-immune cells is largely unclear. Here, we tested the role of NFATs in the regulation of VSMC growth by both the receptor tyrosine kinase (RTK) and

Abbreviations used: BrdU, 5-bromo-2′-deoxy-uridine; CsA, cyclosporin A; DMEM, Dulbecco's modified Eagles's medium; FBS, fetal bovine serum; GFP, green fluorescent protein; GPCR, G-protein-coupled receptor; IL, interleukin; NFAT, nuclear factor of activated T-cells; PDGF, platelet-derived growth factor; RTK, receptor tyrosine kinase; TCA, trichloroacetic acid; VSMC, vascular smooth muscle cell.

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G-protein-coupled receptor (GPCR) agonists, PDGF-BB and thrombin, respectively. PDGF-BB and thrombin activated NFATc1, as measured by its translocation from the cytoplasm to the nucleus in VSMCs. Cyclosporin A (CsA), a potent and specific inhibitor of calcineurin, completely blocked PDGF-BB- and thrombin-induced NFAT–DNA-binding activity, NFAT-dependent reporter gene expression and growth in VSMCs. In addition, forced expression of an NFAT-competing peptide VIVIT for calcineurin binding [25] also significantly attenuated the DNA synthesis induced by both PDGF-BB and thrombin. Together, these findings for the first time demonstrate a role for NFATs in the hyperplastic growth induction by both the RTK and GPCR agonists in VSMCs.

MATERIALS AND METHODS

Reagents

Aprotinin, PMSF, sodium orthovanadate, sodium deoxycholate, leupeptin, Hepes, dithiothreitol and thrombin were purchased from Sigma (St. Louis, MO, U.S.A.). CsA was obtained from Biomol (Plymouth Meeting, PA, U.S.A.). [γ-^{32}P]ATP (3000 Ci/mmol) and [^{3}H]thymidine (20 Ci/mmol) were obtained from NEN Life Science Products (Boston, MA, U.S.A.). Recombinant human PDGF-BB was bought from R&D Systems (Minneapolis, MN, U.S.A.). Anti-NFATc1 (catalogue no. SC-1149-R), anti-NFATc2 (SC-7296), anti-NFATc3 (SC-8321) and anti-NFATc4 (SC-1153) antibodies and NFATc consensus (5′-CGCCCAAAGAGGAAAATTTGTTTCATA-3′; SC-2577) and mutant (5′-CGCCCAAAGCTTAAAATTTGTTTCATA-3′, where the mutated sequence is underlined; SC-2578) oligonucleotides were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Monoclonal NFATc1 antibodies (catalogue no. MA3-024) were bought from Affinity Bioreagents (Golden, CO, U.S.A.). T4 polynucleotide kinase was procured from Gibco-BRL (Grand Island, NY, U.S.A.). FuGENE 6 transfection reagent was obtained from Roche Molecular Biochemicals (Indianapolis, IN, U.S.A.). Lipofectamine plus reagent was purchased from Invitrogen (Carlsbad, CA, U.S.A.). Luciferase assay kit was bought from Promega (Madison, WI, U.S.A.).

Cell culture

VSMCs were isolated from the thoracic aortae of 200–300 g male Sprague–Dawley rats by enzymic dissociation as described earlier [26]. Cells were grown in Dulbecco's modified Eagles's medium (DMEM) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS), 100 units/ml penicillin and 100 μg/ml streptomycin. Cultures were maintained at 37 °C in a humidified 95% air/5% CO_2 atmosphere. Cells were growth-arrested by incubating in DMEM containing 0.1% calf serum for 72 h and used to perform the experiments, unless otherwise stated.

Western blot analysis

Growth-arrested VSMCs were treated with and without PDGF-BB (20 ng/ml) or thrombin (0.1 units/ml) for the indicated times at 37 °C. After treatment, medium was aspirated, cells rinsed with cold PBS and frozen immediately in liquid nitrogen. Cells were lysed by thawing in 250 μl of lysis buffer (PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 100 μg/ml PMSF, 100 μg/ml aprotinin, 1 μg/ml leupeptin and 1 mM sodium orthovanadate) and scraped into 1.5 ml Eppendorf tubes. After standing on ice for 20 min, the cell lysates were cleared by centrifugation at 13400 ***g*** for 20 min at 4 °C. The protein content of the supernatants was determined using Micro BCA™ Protein Assay Reagent kit (Pierce, Rockford, IL, U.S.A.). Cell lysates containing equal amounts of protein were resolved by electrophoresis on 0.1% SDS/10% polyacrylamide gels. The proteins were transferred electrophoretically to a nitrocellulose membrane (Hybond; Amersham Biosciences, Piscataway, NJ, U.S.A.). After blocking in 10 mM Tris/HCl buffer, pH 8.0, containing 150 mM NaCl, 0.1% Tween 20 and 5% (w/v) non-fat dry milk, the membrane was treated with appropriate primary antibodies followed by incubation with horseradish peroxidase-conjugated secondary antibodies. The antigen–antibody complexes were detected using chemiluminescence reageant kit (Amersham Biosciences).

Electrophoretic mobility-shift assay

Growth-arrested VSMCs were treated with and without PGDF-BB (20 ng/ml) or thrombin (0.1 units/ml) in the presence and absence of CsA (10 μM) for the indicated times, and nuclear extracts were prepared according to the procedure described by Dignam et al. [27]. Protein–DNA complexes were formed by incubating 5 μg of nuclear protein in a total volume of 20 μl consisting of 15 mM Hepes (pH 7.9), 3 mM Tris/HCl (pH 7.9), 60 mM KCl, 1 mM EDTA, 1 mM PMSF, 1 mM dithiothreitol, 4.5 μg of BSA, 2 μg of poly(dI-dC), 15% glycerol and 100000 c.p.m. of ^{32}P-labelled NFAT consensus or mutant oligonucleotide probe for 30 min on ice. Protein–DNA complexes were resolved on a 4% polyacrylamide gel using 1× Tris/glycine/EDTA buffer (25 mM Tris/HCl, pH 8.5, 200 mM glycine and 0.1 mM EDTA). Double-stranded NFATc consensus and mutant oligonucleotides (see above for sequences) were labelled with [γ-^{32}P]ATP using a T4 polynucleotide kinase kit as per the supplier's protocol (Gibco-BRL).

Transient transfection and luciferase assay

VSMCs were plated evenly on to 35 mm dishes and grown in DMEM supplemented with 10% (v/v) heat-inactivated FBS, 100 units/ml penicillin and 100 μg/ml streptomycin. At 50–80% confluence, medium was replaced with DMEM containing 0.1% calf serum and cells were transfected with pT8NFAT-Luc plasmid using FuGENE 6 transfection reagent according to the manufacturer's instructions (Roche Molecular Biochemicals). The luciferase is under the control of interleukin (IL)-2 promoter containing three tandem NFAT-activator protein-1 enhancer sequences [28]. After transfection (30 h), VSMCs were treated with and without PGDF-BB (20 ng/ml) or thrombin (0.1 units/ml) in the presence and absence of CsA (10 μM) for 6 h and cell lysates were prepared. VSMC lysates were normalized for protein and assayed for luciferase activity using the Luciferase Assay System (Promega) and a Turner Designs TD-20/20 luminometer.

In parallel experiments, VSMCs were plated evenly on to 60 mm dishes or chamber slides (Lab-Tek II) the day before transfection and grown in DMEM supplemented with 10% (v/v) heat-inactivated FBS, 100 units/ml penicillin and 100 μg/ml streptomycin. Cells were transfected with pGFP or pGFP-VIVIT plasmid DNA (20 μg/60 mm dish or 1 μg/chamber) using the calcium phosphate precipitation method [29] or Lipofectamine plus reagent. Cells were washed with PBS 16 h after transfection and incubated in DMEM containing 0.1% calf serum for 36 h at 37 °C. Cells were then treated with and without PDGF-BB (20 ng/ml) or thrombin (0.1 units/ml) for 36 h and DNA synthesis was measured by pulse-labelling cells with either 1 μCi/ml [^{3}H]thymidine or 5-bromo-2′-deoxyuridine (BrdU; 10 μM) labelling reagent for the last 24 h of the 36 h treatment period as described below.

DNA synthesis and cell counts

VSMCs with and without appropriate treatments were pulse-labelled with 1 μCi/ml [^{3}H]thymidine for the indicated times. After labelling, cells were washed with cold PBS, trypsinized and collected by centrifugation. The cell pellet was suspended in cold 10 % (w/v) trichloroacetic acid (TCA) and vortexed vigorously to lyse cells. After standing on ice for 20 min, the cell lysis mixture was passed through a glass fibre filter (GF/C; Whatman). The filter was washed once with cold 5 % TCA and once with cold 70 % (v/v) ethanol. The filter was dried, placed in a liquid-scintillation vial containing the scintillant fluid, and the radioactivity was measured in a liquid-scintillation counter (LS 5000TA; Beckman). In the case of BrdU incorporation, after labelling with BrdU, cells were fixed in ethanol containing 15 mM glycine (pH 2.0) and incubated with monoclonal anti-BrdU antibodies (Roche Molecular Biochemicals). Cells were washed three times with cold PBS and incubated with Cy3-conjugated sheep anti-mouse IgG. After washing and mounting, cells were observed for fluorescence under confocal microscope (MRC 1024; Bio-Rad) and pictures were taken using Comos software. Images were processed with NIH Image and Adobe Photoshop. In parallel experiments, growth-arrested VSMCs were treated with and without PGDF-BB (20 ng/ml) or thrombin (0.1 units/ml) in the presence and absence of CsA (10 μM) for 48 h and cell number was determined by Trypan Blue dye exclusion assay using a haemocytometer.

Statistics

All the experiments were repeated at least three times with similar patterns of results. Results for [^{3}H]thymidine incorporation, cell number and luciferase activities are presented as means $\pm$ S.D., and the treatment effects were analysed by Student's t test. P values of less than 0.05 were considered to be statistically significant.

RESULTS AND DISCUSSION

To determine whether NFATs play a role in VSMC hyperplasia, growth-arrested cells were treated with and without PDGF-BB (20 ng/ml) or thrombin (0.1 units/ml) for various times, and cell lysates were prepared. Cell lysates containing equal amounts of protein from the control and each treatment were analysed by Western blotting for NFAT levels using their respective antibodies. NFATc1, NFATc2 and NFATc3 were detected in VSMCs. Treatment of VSMCs with PDGF-BB or thrombin caused a slight increase (1.5–2-fold) in the steady-state levels of NFATc1 and NFATc2 (Figure 1). NFATc3 and NFATc4 levels were barely detectable in growth-arrested cells and treatment with PDGF-BB or thrombin resulted in an increase of 3-fold in NFATc3 levels but not NFATc4. Earlier studies have also reported a lack of the presence of NFATc4 in VSMCs [18].

NFATs exist as phosphoproteins in the resting state, and are activated via dephosphorylation [12]. Calcineurin, a calcium/calmodulin-dependent serine phosphatase, specifically dephosphorylates NFATs, particularly NFATc1–NFATc4, leading to their activation and translocation from the cytoplasm to the nucleus [12]. In the nucleus they bind as monomers or dimers via their Rel homology domain to a consensus DNA sequence, GGAAAAT, which is present in the promoter region of genes, and induce transcription [24]. To find which of these transcription factors are activated in response to RTK and GPCR agonists, growth-arrested VSMCs were treated with and

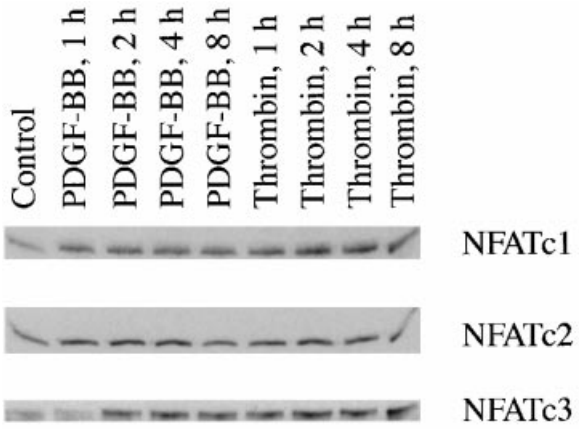

Figure 1 Western blot analysis of NFAT levels in VSMCs

Growth-arrested VSMCs were treated with and without PDGF-BB (20 ng/ml) or thrombin (0.1 units/ml) for the indicated times and cell lysates were prepared. Cell lysates containing equal amounts of protein from control and each treatment were analysed by Western blotting for NFAT levels using their respective antibodies.

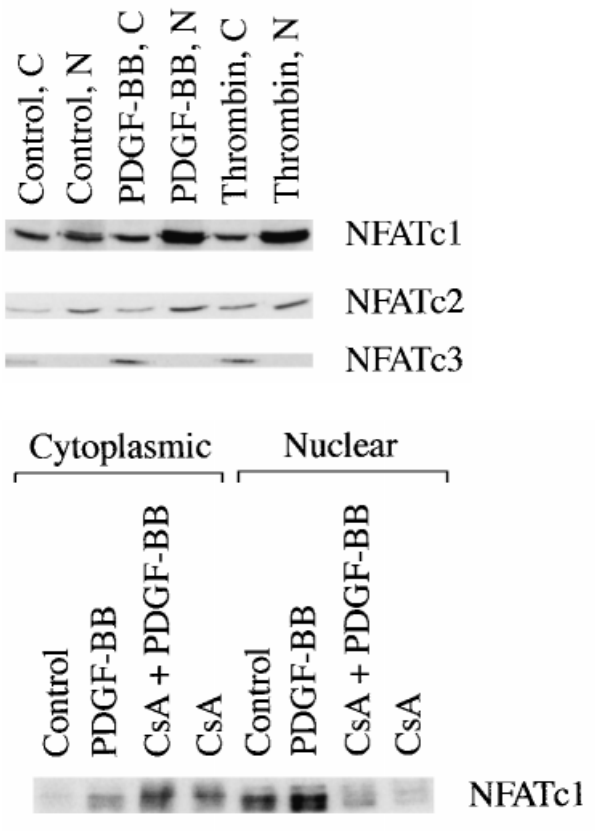

Figure 2 Effect of PDGF-BB and thrombin on translocation of NFATs from the cytoplasm to the nucleus

Upper panel: growth-arrested VSMCs were treated with and without PDGF-BB (20 ng/ml) or thrombin (0.1 units/ml) for 2 h and the cytoplasmic and the nuclear extracts were prepared. Equal amounts of protein from the cytoplasmic (C) and the nuclear (N) extracts of control and agonist-treated VSMCs were analysed by Western blotting for NFAT levels using their respective antibodies. Lower panel: conditions were the same as for the upper panel except that cells were treated with and without PDGF-BB (20 ng/ml) in the presence and absence of CsA (10 μM) for 2 h and cytoplasmic and the nuclear extracts were prepared.

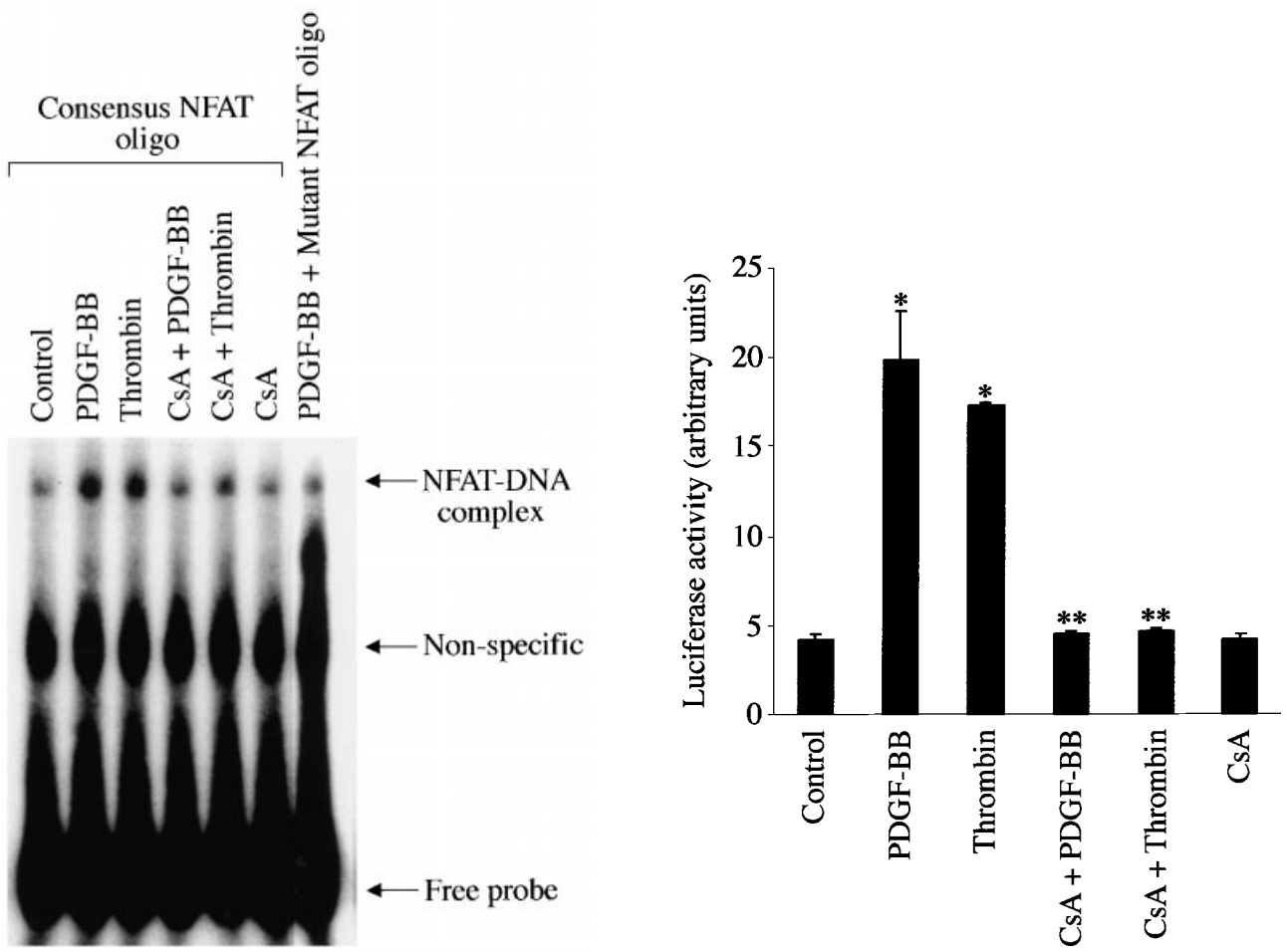

Figure 3 Effect of PDGF-BB and thrombin on transactivating activity of NFATs

Left-hand panel: growth-arrested VSMCs were treated with and without PDGF-BB (20 ng/ml) or thrombin (0.1 units/ml) in the presence and absence of CsA (10 μM) for the indicated times and nuclear extracts were prepared. Protein (5 μg) from nuclear extracts of control and agonist-treated VSMCs were incubated with 100 000 c.p.m. of ^{32}P-labelled NFAT consensus or mutant oligonucleotide probe for 30 min on ice and the protein–DNA complexes were resolved by electrophoresis on 4% polyacrylamide gels. Right-hand panel: growth-arrested VSMCs were transfected with pT8NFAT-Luc using FuGENE 6 transfection reagent, treated with and without PDGF-BB (20 ng/ml) or thrombin (0.1 units/ml) in the presence and absence of CsA (10 μM) for 6 h and cell lysates were prepared. Cell lysates containing equal amounts of protein from control and each treatment were assayed for luciferase activity. *$P < 0.001$ versus control; **$P < 0.001$ versus PDGF-BB or thrombin treatment alone.

without PDGF-BB (20 ng/ml) or thrombin (0.1 units/ml) for 2 h, and the cytoplasmic and the nuclear extracts were prepared. The cytoplasmic and the nuclear extracts of control and agonist-treated VSMCs that were normalized for protein were analysed by Western blotting for NFAT levels. As shown in Figure 2 (upper panel), NFATc1, although detected in both the cytoplasmic and nuclear fractions, was found to be 2-fold higher in the nuclear fraction of PDGF-BB and thrombin-treated VSMCs compared with the control. No significant changes were observed in the cytoplasmic NFATc1 levels between control versus PDGF-BB or thrombin-treated cells. With regard to NFATc2, it was present in higher abundance in the nuclear fractions compared with the cytoplasmic factions of VSMCs. Furthermore, the cytoplasmic/nuclear ratio of NFATc2 was not affected between control and agonist-treated VSMCs. This result suggests that upon treatment of VSMCs with PDGF-BB or thrombin, there is no net translocation of NFATc2 from the cytoplasm to the nucleus. In the case of NFATc3, its levels were barely detectable in control VSMCs. However, treatment of VSMCs with PDGF-BB or thrombin increased its levels by 3-fold as compared with the control. Interestingly, despite an increase in the expression of NFATc3 in response to PDGF-BB and thrombin, its distribution was restricted only to the cytoplasm. Together, these results indicate that treatment of VSMCs with PDGF-BB or thrombin caused dephosphorylation and translocation of only NFATc1, but not NFATc2 or NFATc3, from the cytoplasm to the nucleus.

To test whether the shuttling of NFATs between the cytoplasm and nucleus is calcineurin-sensitive, growth-arrested VSMCs were treated with and without PDGF-BB (20 ng/ml) in the presence and absence of CsA (10 μM) for 2 h, and the cytoplasmic and nuclear extracts prepared. Equal amounts of protein from the cytoplasmic and the nuclear fractions of control and PDGF-BB-treated VSMCs were analysed by Western blotting for NFATc1 levels. As shown in Figure 2 (lower panel), CsA significantly inhibited the PDGF-BB-induced translocation of NFATc1 from the cytoplasm to the nucleus, thereby resulting in an increase in NFATc1 levels in the corresponding cytoplasmic fractions. Consistent with this effect, NFATc1 levels were also found to be higher in the cytoplasmic fractions of VSMCs treated with PDGF-BB in the presence of CsA than PDGF-BB

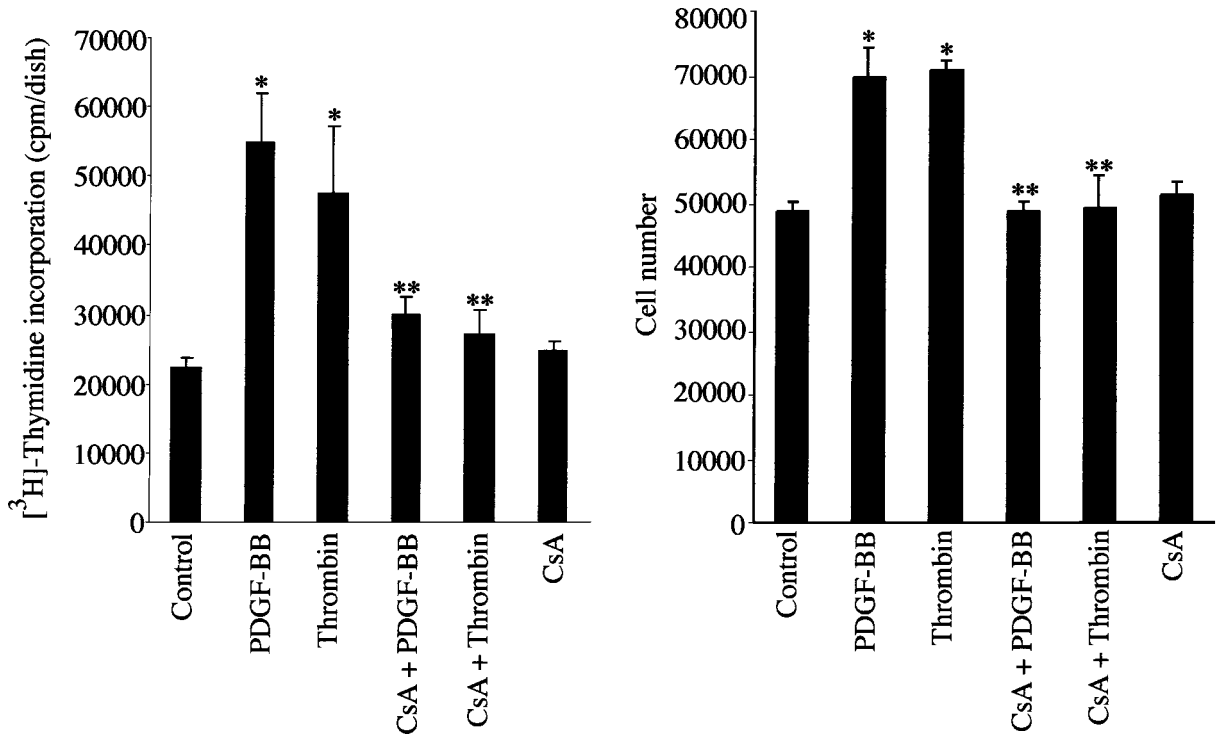

Figure 4 CsA inhibits PDGF-BB- and thrombin-induced growth in VSMCs

Left-hand panel: growth-arrested VSMCs were treated with and without PDGF-BB (20 ng/ml) or thrombin (0.1 units/ml) in the presence and absence of CsA (10 μM) for 24 h and DNA synthesis was measured by pulse-labelling cells with 1 μCi/ml [^{3}H]thymidine for the last 20 h of the 24 h treatment period. Right-hand panel: growth-arrested VSMCs were treated with and without PDGF-BB (20 ng/ml) or thrombin (0.1 units/ml) in the presence and absence of CsA (10 μM) for 48 h and cell number was measured. *$P < 0.01$ versus control; **$P < 0.05$ versus PDGF-BB or thrombin treatment alone.

alone. Interestingly, the basal nuclear NFATc1 levels were decreased in CsA-treated VSMCs compared with untreated VSMCs, with a concomitant increase in NFATc1 levels in the corresponding cytoplasmic fractions. This finding suggests the presence of calcinuerin in the nucleus and that the inhibition of its activity by CsA facilitates the export of NFATc1 from the nucleus to the cytoplasm.

To test whether translocation of NFATc1 from the cytoplasm to the nucleus is followed by an increase in NFAT–DNA-binding activity, growth-arrested VSMCs were treated with and without PDGF-BB (20 ng/ml) or thrombin (0.1 units/ml) in the presence and absence of CsA (10 μM) for 3 h, and nuclear extracts were prepared. Nuclear extracts containing 5 μg of protein from control and PDGF-BB or thrombin-treated VSMCs were incubated with 100000 c.p.m. of ^{32}P-labelled NFAT consensus or mutant oligonucleotide probe, and the protein–DNA complexes were separated by electrophoresis on polyacrylamide gels. PDGF-BB and thrombin increased NFAT–DNA-binding activity 2–3-fold as compared with the control (Figure 3, left-hand panel). CsA significantly attenuated the NFAT–DNA-binding activity induced by both PDGF-BB and thrombin. Use of a mutant NFAT oligonucleotide as a probe, however, did not reveal any increase in protein–DNA-binding activity in VSMCs in response to PDGF-BB or thrombin, a finding that suggests that the observed increases in protein–DNA-binding activities in PDGF-BB- and thrombin-treated VSMCs with NFAT consensus oligonucleotide as a probe were sequence-specific. To find whether the increased NFAT–DNA-binding activities lead to corresponding increases in NFAT-dependent transcription, VSMCs were transfected with a plasmid (pT8NFAT-Luc) in which the expression of a luciferase gene is controlled by three tandem NFAT-activator protein-1 enhancer sequences present in the IL-2 promoter [28]. Cells were then treated with and without PDGF-BB (20 ng/ml) or thrombin (0.1 units/ml) for 6 h in the presence and absence of CsA (10 μM), and cell lysates were prepared. Cell lysates from control and agonist-treated VSMCs that were normalized for protein were assayed for luciferase activity. Consistent with the NFAT–DNA-binding activities, luciferase activities were increased 3-fold in PDGF-BB- and thrombin-treated VSMCs compared with control (Figure 3, right-hand panel). CsA completely blocked both the PDGF-BB- and thrombin-induced increases in luciferase activities (Figure 3, right-hand panel). CsA alone had no effect on basal luciferase activity.

To investigate the functional significance of activation of NFATs by vascular mitogens, growth-arrested VSMCs were treated with and without PDGF-BB (20 ng/ml) or thrombin (0.1 units/ml) in the presence and absence of CsA (10 μM) for 24 h, and DNA synthesis was measured by pulse-labelling cells with [^{3}H]thymidine for the last 20 h of the 24 h treatment period. PDGF-BB and thrombin stimulated VSMC DNA synthesis 2–3-fold as compared with untreated cells (Figure 4, left-hand panel). CsA inhibited the PDGF-BB- and thrombin-induced DNA synthesis by 70%. To confirm these findings further, growth-arrested VSMCs were treated with and without PDGF-BB (20 ng/ml) or thrombin (0.1 units/ml) in the presence and absence of CsA (10 μM) for 48 h, and cell number was measured. PDGF-BB and thrombin increased VSMC number by 40% as

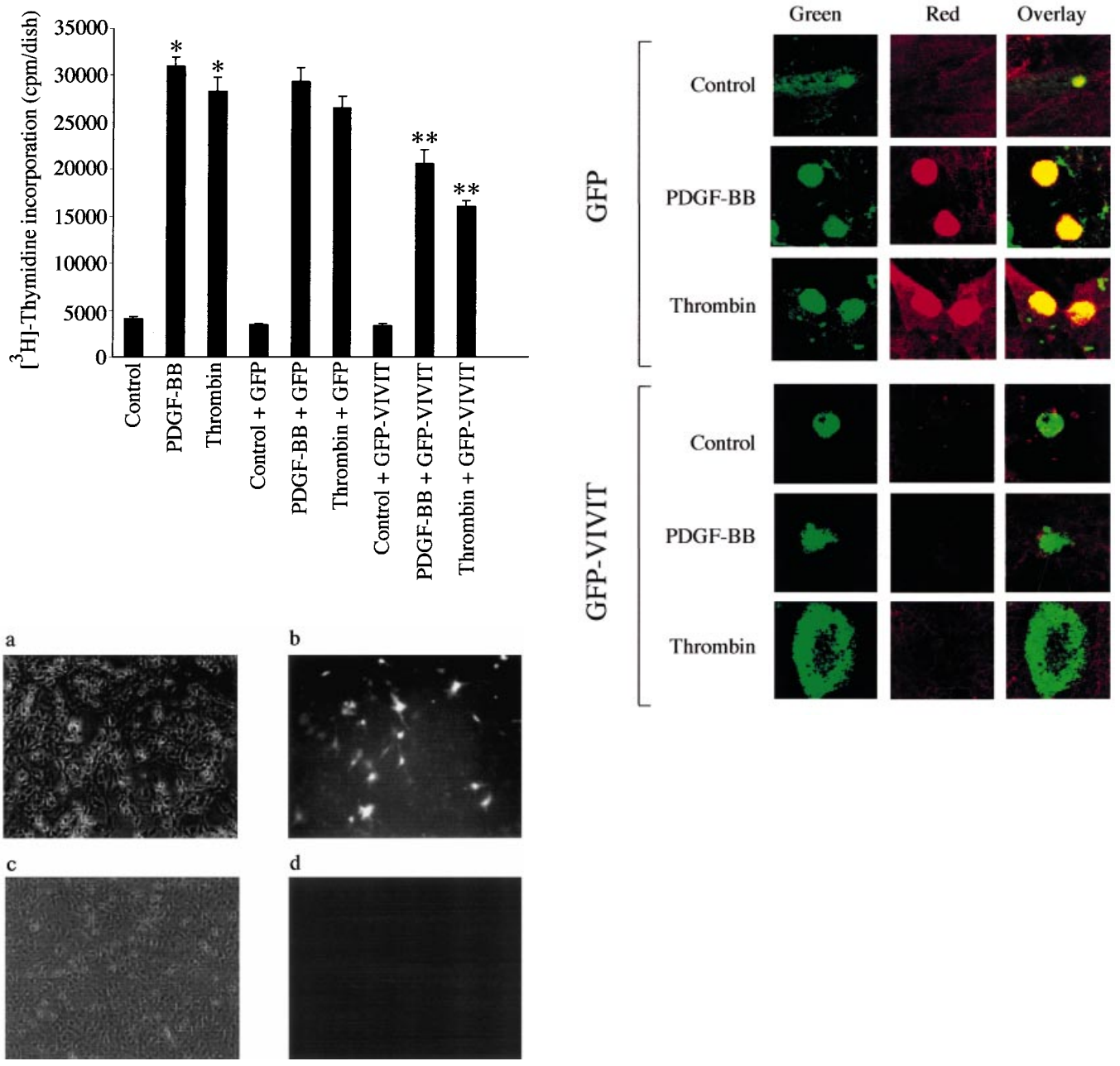

Figure 5 Expression of VIVIT, a NFAT-competent peptide, inhibits PDGF-BB- and thrombin-induced DNA synthesis in VSMCs

Upper left panel: VSMCs that were transfected with and without expression plasmids for pGFP-VIVIT or pGFP were growth-arrested for 36 h, treated with and without PDGF-BB (20 ng/ml) or thrombin (0.1 units/ml) for 36 h and DNA synthesis was measured by pulse-labelling cells with 1 μCi/ml [^{3}H]thymidine for the last 24 h of the 36 h treatment period. *$P < 0.001$ versus control; **$P < 0.05$ versus PDGF-BB or thrombin treatment alone. Lower left panels: in a parallel experiment 72 h after transfection with pGFP-VIVIT or pGFP plasmid, growth-arrested VSMCs were observed for the expression of GFP under deconvolution confocal microscope. Transfection efficiency was calculated by counting cells for green fluorescence expression in a population of 1000 cells in four randomly selected areas per dish. (**a**) Phase-contrast photomicrograph of GFP-VIVIT-transfected cells; (**b**) fluorescence photomicrograph of GFP-VIVIT-transfected cells; (**c**) phase-contrast photomicrograph of untransfected cells; (**d**) fluorescence photomicrograph of untransfected cells. Right-hand panel: conditions were the same as in the upper left panel except that DNA synthesis was measured by immunofluorescence labelling of BrdU and confocal microscopy as described in the Materials and methods section. Green, cells expressing GFP or GFP-VIVIT; Red, cells stained for BrdU; Overlay, cells were examined for the presence of both green and red fluorescence.

compared with control, and this response was significantly blunted by CsA (Figure 4, right-hand panel). CsA alone had no significant effect on either basal DNA synthesis or cell number. These results are consistent with its effect on PDGF-BB- and thrombin-induced NFAT–DNA-binding activity.

To gain additional evidence for the role of NFATs in PDGF-BB- and thrombin-induced DNA synthesis, we have used an approach in which NFAT activation is specifically inhibited by expression of a peptide that competes with NFATs for binding to calcineurin. Calcineurin binds to and dephosphorylates

NFATs, leading to their activation [16]. NFATc1–NFATc4 possess a highly conserved calcineurin-binding site, PXIXIT, in their regulatory domain at the N-terminus. Based on this information, a peptide, namely VIVIT, which specifically competes with NFATs for binding to calcineurin, was developed [25]. Expression of green fluorescent protein (GFP)-VIVIT in T-cells inhibited only calcineurin-sensitive NFAT-dependent but not calcineurin-sensitive NFAT-independent inducible expression of IL-2, IL-3, IL-13, tumour necrosis factor-α, granulocyte-macrophage colony-stimulating factor and macrophage inflammatory protein-1α expression by PMA and ionomycin [25]. VSMCs were transfected with pGFP or pGFP-VIVIT, growth-arrested for 36 h, treated with and without PDGF-BB (20 ng/ml) or thrombin (0.1 units/ml) for 36 h and DNA synthesis was measured by either [^{3}H]thymidine incorporation or BrdU labelling for the last 24 h of the 36 h treatment period. Expression of pGFP-VIVIT but not pGFP attenuated PDGF-BB- and thrombin-induced [^{3}H]thymidine incorporation into TCA-precipitable material by 25–30 % (Figure 5, upper left panel). To relate the growth-inhibitory effect of pGFP-VIVIT to that with CsA, transfection efficiency was measured. A 20–25 % of VSMCs exposed to pGFP-VIVIT were found to be positive for expression of GFP (Figure 5, lower left panels). Therefore, the inhibition of PDGF-BB- and thrombin-induced DNA synthesis by pGFP-VIVIT correlated with its transfection efficiency. In addition, cells expressing pGFP-VIVIT but not pGFP failed to incorporate BrdU, as detected by immunofluorescence staining using confocal microscopy (Figure 5, right-hand panel).

The important finding of the present study is that NFAT activation is required for the hyperplastic growth of VSMCs induced by both RTK and GPCR agonists. This conclusion is based on the following observations. (i) CsA, a potent and specific inhibitor of calcineurin, significantly blocked PDGF-BB- and thrombin-induced NFAT–DNA-binding activity, NFAT-dependent reporter gene expression and growth in VSMCs. (ii) Forced expression of VIVIT, a peptide that specifically competes with NFATs for binding to calcineurin, significantly attenuated both PDGF-BB- and thrombin-induced DNA synthesis. The present results also reveal that, among all five NFAT members, NFATc1 is the likely candidate mediating the mitogenic effects of PDGF-BB and thrombin in VSMCs. This notion is supported by the following findings: (i) NFATc1 is translocated from the cytoplasm to the nucleus in response to PDGF-BB and thrombin; (ii) NFATc2 is present both in the cytoplasmic and the nuclear fractions of growth-arrested VSMCs and its distribution was not affected by treatment with PDGF-BB or thrombin; and (iii) despite its induction of expression by PDGF-BB and thrombin NFATc3 did not translocate from the cytoplasm to the nucleus. Earlier reports have demonstrated that NFATc4 plays a role in cardiac hypertrophy [24]. Because the present investigation and previous studies from other laboratories [18] failed to detect the presence of NFATc4 in VSMCs, it is unlikely that this transcription factor mediates PDGF-BB- and thrombin-induced hyperplastic growth in this cell type. Similarly, a role for NFAT5 in PDGF-BB- and thrombin-induced VSMC growth is unlikely because this event is sensitive to the calcineurin inhibitor CsA, whereas NFAT5 is insensitive to this drug. In summary, the present study demonstrates that NFATs, most likely NFATc1, mediates the mitogenic effects of PDGF-BB and thrombin in VSMCs.

We thank Dr Anjana Rao and Dr Michael P. Bell for providing us with pGFP-VIVIT and pT8NFAT-Luc plasmids, respectively. We also thank Dr Anjana Rao for her critical reading of the manuscript and suggestions. This work was supported in part by National Institutes of Health grant HL 69908 (to G. N. R.).

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Received 28 February 2002/18 July 2002; accepted 21 August 2002
Published as BJ Immediate Publication 21 August 2002, DOI 10.1042/BJ20020347