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Inhibition by pentoxifylline of TNF-α-stimulated fractalkine production in vascular smooth muscle cells: evidence for mediation by NF-κB down-regulation

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1 Fractalkine is a CX_3C chemokine for mononuclear leukocytes that is expressed mainly by vascular cells, and regulated by pro-inflammatory cytokines. This study investigated signal transduction mechanisms by which tumor necrosis factor (TNF)-α stimulated fractalkine expression in cultured rat vascular smooth muscle cells (VSMCs), and the modulatory effect of a haemorrheologic agent, pentoxifylline, on its production.

2 TNF-α (1–50 ng ml^{-1}) stimulated fractalkine mRNA and protein expression in concentration- and time-dependent manners. Pretreatment with calphostin C (0.4 μM, a selective inhibitor of protein kinase C (PKC), and PD98059 (40 μM), a specific inhibitor of p42/44 mitogen-activated protein kinase (MAPK) kinase, attenuated TNF-α-stimulated fractalkine mRNA and protein expression. In contrast, H-89 (2 μM), a selective inhibitor of cAMP-dependent protein kinase, wortmannin (0.5 μM), a selective inhibitor of phosphatidylinositol 3-kinase, and SB203580 (40 μM), a specific inhibitor of p38 MAPK, had no discernible effect.

3 The ubiquitin/proteosome inhibitors, MG132 (10 μM) and pyrrolidine dithiocarbamate (200 μM), suppressed activation of NF-κB as well as stimulation of fractalkine mRNA and protein expression by TNF-α.

4 TNF-α-activated phosphorylation of PKC was blocked by calphostin C, whereas TNF-α-augmented phospho-p42/44 MAPK and phospho-c-Jun levels were reduced by PD98059. Neither calphostin C nor PD98059 affected TNF-α-induced degradation of I-κBα or p65 nuclear translocation.

5 Pretreatment with pentoxifylline (0.1–1 mg ml^{-1}) decreased TNF-α-stimulated fractalkine mRNA and protein expression, which was preceded by a reduction in TNF-α-activated phosphorylation of PKC, p42/44 MAPK and c-Jun as well as degradation of I-κBα and p65/NF-κB nuclear translocation.

6 These data indicate that activation of PKC, p42/44 MAPK kinase, and NF-κB are involved in TNF-α-stimulated fractalkine production in VSMCs. Down-regulation of the PKC, p42/44 MAPK, and p65/NF-κB signals by PTX may be therapeutically relevant and provide an explanation for the anti-fractalkine effect of this drug.

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Keywords: Atherosclerosis; fractalkine; mitogen-activated protein kinase; protein kinase C; transcription factor(s); vascular smooth muscle cells

Abbreviations: AP-1, activator protein-1; DMEM, Dulbecco's modified Eagle's media; FCS, foetal calf serum; H-89, N-[2-bromocinnamyl (amino)ethyl]-5-isoquinolinesulphonamide; I-κBα, inhibitory protein of NF-κB; MAPK, mitogen-activated protein kinases; NF-κB, nuclear factor-κB; PDTC, pyrrolidine dithiocarbamate; PI 3-K, phosphatidylinositol 3-kinase; PKA, protein kinase A; PKC, protein kinase C; PTX, pentoxifylline; RT–PCR, reverse transcription-polymerase chain reaction; TNF-α, tumour necrosis factor-α; VSMCs, vascular smooth muscle cells

Introduction

Inflammatory phenomena at sites of atherosclerotic plaques are increasingly thought to be major determinants of the progression and clinical outcome of atherosclerotic diseases (Ross, 1999). Inflammation involved in atherogenesis is mediated largely by monocyte-derived macrophages and specific subtypes of T cells, which emigrate from the blood and multiply within the atherosclerotic lesions (Gerszten *et al*., 2000). Attraction of mononuclear leukocytes to atherosclerotic lesions involves a series of complex interactions between cellular adhesion molecules and chemotactic cytokines expressed by leukocytes and vessel cells (Braun *et al*., 1999; Price & Loscalzo, 1999; Reape & Groot, 1999; Sasayama *et al*., 2000). Recently, a novel CX_3C chemokine known to capture and direct migration of mononuclear leukocytes has been identified. This chemokine is known as fractalkine (Bazan *et al*., 1997), and it acts primarily as an

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adhesive molecule in capturing circulating monocytes, T cells, and natural killer cells that express its cognate receptor, CX_3CR1 (Imai *et al*., 1997; Fong *et al*., 1998). When cleaved proximal to the membrane by metalloproteinases, cell-bound fractalkine is shed and functions as a soluble chemoattractant (Chapman *et al*., 2000a; Garton *et al*., 2001). In humans, high levels of fractalkine mRNA have been localized to advanced atherosclerotic lesions (Greaves *et al*., 2001). Moreover, CX_3CR1 V249I polymorphism is associated with a significant decrease in fractalkine-binding affinity as well as a reduced risk of coronary artery disease and improved endothelium-dependent vasodilation (Moatti *et al*., 2001; McDermott *et al*., 2001). Together, these data suggest that the fractalkine-CX_3CR1 system is involved in the pathogenesis of atherosclerotic disease (Umehara *et al*., 2001a).

Fractalkine was originally discovered in human umbilical vein endothelial cells upon stimulation with tumour necrosis factor (TNF)-α or interleukin-1β (Bazan *et al*., 1997). In addition, fractalkine production has been found in non-endothelial cells which include neurons and astrocytes in the central nervous system (Harrison *et al*., 1998; Schwaeble *et al*., 2001), dendritic cells within the tonsil and skin (Kanazawa *et al*., 1999; Papadopoulos *et al*., 1999), and epithelial cells in the gut (Lucas *et al*., 2001). More recently, fractalkine expression has been induced in TNF-α-activated vascular smooth muscle cells (VSMCs) (Ludwig *et al*., 2002). The precise signal pathways leading to fractalkine induction have not been fully elucidated. In cultured endothelial cells, Garcia *et al*. (2000) suggest that fractalkine production by TNF-α is nuclear factor (NF)-κB-dependent. However, they did not provide additional information regarding other TNF-α-activated signalling pathways that may mediate fractalkine production. TNF-α is a pleiotropic cytokine that can initiate distinct cellular signals upon binding to its receptors. In addition to the well-known stimulatory effect on NF-κB activity (Malinin *et al*., 1997), TNF-α also activates an array of signalling pathways, notably the cAMP-dependent protein kinase (PKA), protein kinase C (PKC), phosphatidylinositol 3-kinase (PI 3-K), and mitogen-activated protein kinases (MAPKs) (Vilcek & Lee, 1991; Heller & Kronke, 1994). Stimulation of these protein kinases leads to activation of distinct transcription factors that includes c-Jun/activator protein (AP)-1 and NF-κB (Berghe *et al*., 1998; Lallena *et al*., 1999; Reddy *et al*., 2000). The present study investigated the signal transduction mechanisms that mediated TNF-α-stimulated fractalkine production in VSMCs, and the modulation of fractalkine production by pentoxifylline (PTX), a clinically available haemorrheologic agent which we have demonstrated exerts anti-proliferative and anti-fibrogenic effects on VSMCs (Chen *et al*., 1999).

In this study, we show that TNF-α activation of PKC, p42/44 MAPK, and NF-κB are involved in TNF-α-stimulated fractalkine expression in VSMCs. Down-regulation of the PKC, p42/44 MAPK, and NF-κB signals may contribute to PTX inhibition of fractalkine gene transcription by TNF-α.

Methods

Reagents

Dulbecco's modified Eagle's media (DMEM), foetal calf serum (FCS), and other tissue culture reagents were obtained from Gibco BRL (Rockville, MD, U.S.A.). Culture flasks and plates were purchased from Costa Corning (Cambridge, MA, U.S.A.). PTX was purchased from Sigma (St. Louis, MO, U.S.A.). N-[2-bromocinnamyl (amino)ethyl]-5-isoquinolinesulphonamide (H-89), calphostin C, PD98059, SB203580, wortmannin, MG132, pyrrolidine dithiocarbamate (PDTC), GM 6001, and actinomycin D were obtained from Calbiochem (La Jolla, CA, U.S.A.). Recombinant rat TNF-α and goat anti-rat fractalkine were obtained from R & D Systems (Minneapolis, MN, U.S.A.). Rabbit anti-p42/44 MAPK, and mouse anti-phospho-p42/44 MAPK, anti-phospho-PKC(pan) (including α, β_I, β_{II}, ε, η and δ isoforms) were obtained from New England BioLab (Beverly, MA, U.S.A.). Mouse anti-phospho-c-Jun, and rabbit anti-c-Jun, anti-PKC β_{II}, anti-PKCζ/ι, anti-phospho-PKCζ/ι, anti-p65/NF-κB and anti-inhibitory protein of NF-κB (I-κB)α were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA, U.S.A.). Mouse anti-β-actin was obtained from Sigma. All chemicals used for total RNA isolation, reverse transcription-polymerase chain reaction (RT–PCR), Northern blot analysis and Western blot analysis were of molecular grade and were obtained from Sigma or Roche Molecular Biochemicals (Mannheim, Germany) unless otherwise specified.

Cell culture

Primary culturing of rat aortic VSMCs was performed as described previously (Chen *et al*., 1999). Cells were characterized as VSMCs on the basis of the presence of α-smooth muscle actin staining with the avidin-biotin-peroxidase method, using diaminobenzidine as the chromogen. VSMCs between 10–20 passages were used and grown in DMEM containing 10% FCS.

TNF-α-stimulated fractalkine expression by VSMCs: Regulation through protein kinases and NF-κB, and modulation by PTX

These experiments were performed to determine the regulatory role of various protein kinases, on TNF-α-stimulated fractalkine expression by VSMCs. The effects of PKA, PKC, PI 3-K, p42/44 MAPK, and p38 MAPK on VSMC fractalkine gene expression were evaluated by incubating cells with various protein kinase inhibitors. Cells were first grown in DMEM containing 10% FCS until reaching 90% confluency. The medium was then replaced by DMEM containing 0.5% FCS for 24 h before treatment with a specific PKC inhibitor calphostin C (0.4 μM for 1 h), a selective PKA inhibitor H-89 (2 μM for 30 min), a p42/44 MAPK kinase inhibitor PD98059 (40 μM for 30 min), a p38 MAPK inhibitor SB203580 (40 μM for 30 min), or a PI-3K inhibitor wortmannin (0.5 μM for 30 min). After preincubation, cells were stimulated with TNF-α (5 ng ml^{-1}) for 4 or 24 h at 37°C. Further experiments were conducted to examine the role of NF-κB on TNF-α-stimulated fractalkine expression by VSMCs. The NF-κB inhibitors, MG132 (10 μM) and PDTC (200 μM) were incubated with VSMCs for 1.5 and 1 h, respectively. Then, cells were stimulated with TNF-α (5 ng ml^{-1}) for 4 or 24 h at 37°C. Additional studies were designed to examine the role of PTX in VSMC fractalkine expression. PTX (0.1–1 mg ml^{-1}) was first

incubated with VSMCs in the absence of TNF-α stimulation for 4 h at 37°C to determine the role of PTX on basal VSMC fractalkine expression. Further experiments were performed to examine the role of PTX on TNF-α-stimulated VSMC fractalkine expression. Cells were preincubated with PTX for 45 min, followed by TNF-α (5 ng ml^{-1}) for 4 or 24 h at 37°C. After incubation for the given periods, cell monolayers were washed and used for RNA (4-h stimulation) or protein (24-h stimulation) isolation as described below.

RT–PCR and Northern blot analysis

Total RNA was extracted by the acid guanidinium thiocyanate phenol chloroform method (Chomczynski & Sacchi, 1987). Ten micrograms of total RNA were electrophoresed on formaldehyde-denatured 1% agarose gels and subsequently transferred to nylon membranes. cDNA fragments of rat fractalkine and GAPDH (glyceraldehyde-3-phosphate dehydrogenase) were first amplified by RT–PCR from glomerular RNA of Wistar rats using the following specific primer pairs: rat fractalkine, upstream 5′-attttccaagacagaggacc-3′, downstream 5′-gaagagtagaccaagaaagg-3′ (Harrison *et al*., 1998), and rat GAPDH, upstream 5′-tcattgacctcaactacatg-3′, downstream 5′-caaagttgtcatggatgacc-3′ (Tso *et al*., 1985). RT–PCR was performed by adding 3 μl (150 ng) of the native first-strand cDNA in a total of 10 μl containing 50 mM Tris, 0.25 mg ml^{-1} bovine serum albumin, 1 mM $MgCl_2$, 200 μM dNTPs, 33 pmoles of each primer, and 1 U DNA polymerase. The amplified products were eluted from polyacrylamide gels, and subcloned into pGEM-T vectors (Promega). The accuracy of the inserts were confirmed by DNA sequence analysis. The cloned cDNAs were linearized and used as templates for *in vitro* transcription of antisense digoxigenin-conjugated riboprobes, following the manufacturer's instructions (Roche Molecular Biochemicals). After hybridization, the blots were developed using CSPD® (Roche Molecular Biochemicals) as the substrate for alkaline phosphatase. The intensity of the signal was then quantified with computerized densitometry, and normalized against the signal of GAPDH messages.

Western blot analysis

VSMCs were washed and lysed in RIPA buffer containing 1% IGEPAL CA-630 and 0.25% deoxycholate (Sigma). Forty micrograms of cell lysates were heated at 100°C for 10 min, applied to 7.5% (for cell-bound fractalkine) or 9% (for PKC, p42/44 MAPK, c-Jun, IκBα and β-actin) SDS-polyacrylamide gels, and electrophoresed. For detection of soluble fractalkine in the conditioned medium of TNF-α-activated VSMCs, media were concentrated with Centricon-10® (Millipore, Bedford, MA, U.S.A.), and 50 μg of protein were electrophoresed on 7.5% SDS-polyacrylamide gels. A prestained marker was also electrophoresed as a molecular weight marker. After electrophoresis, proteins were transferred onto a PVDF membrane (Millipore) using a transblot chamber with Tris buffer. Western blots were incubated at 4°C overnight with primary antibodies. The next morning, membranes were washed with 1 × phosphate-buffered saline/5% Tween-20 at room temperature for 40 min, and incubated with peroxidase-conjugated second antibodies at room temperature for 1 h. After washing, the membranes were incubated with Renaissance® (NEN® Life Science, MA, U.S.A.) according to the manufacturer's instructions. The intensity of the signal was then quantified with computerized densitometry, and normalized against the signal of β-actin wherever appropriate.

Immunocytochemistry

For demonstration of p65/NF-κB nuclear translocation, VSMCs were incubated with TNF-α (5 ng ml^{-1}), or vehicles for 7.5, 15, or 30 min before fixation with 4% paraformaldehyde for 1 h at 4°C. The cells were then washed by 1 × phosphate-buffered saline/0.2% TritonX-100 for 15 min and incubated with rabbit anti-p65/NF-κB at 4°C overnight. The next day, after washing for 15 min, the cells were incubated with biotin-conjugated anti-rabbit IgG at room temperature for 1 h. Then, the cells were washed and incubated with the avidin-biotin-peroxidase reagent (Dako, Glostrup, Denmark) at room temperature for 1 h. After washing, immunodetection for p65/NF-κB was performed by adding 3-amino-9-ethylcarbazole chromogen (Dako) as substrate according to the manufacturer's instructions.

Statistics

Data are expressed as mean ± s.e.mean. All comparisons were done by analysis of variance followed by Dunnett's *t*-test using the StatView® package for the Macintosh computer (Abacus Concepts, CA, U.S.A.). A probability value of less than 0.05 was considered statistically significant.

Results

Effects of TNF-α on fractalkine mRNA and protein expression

VSMCs were first incubated with different concentrations of TNF-α (1 to 50 ng ml^{-1}) for varying time periods (4 to 24 h). The Northern and Western blot results showed that at basal state VSMCs expressed a low level of a single 3.8-kb fractalkine mRNA species and a ~90-kDa cell-bound fractalkine protein. Exogenous TNF-α stimulated fractalkine mRNA and cell-bound protein expression in time- and dose-dependent manners (Figure 1). Further, our immunoblot results showed the presence of a ~70 kDa fractalkine polypeptide in the conditioned media of TNF-α-activated VSMCs (Figure 2). By using a broad-spectrum inhibitor of matrix metalloproteinases, GM 6001 (2 μM), we found that the amount of soluble fractalkine could be reduced by GM 6001, in association with an increase of the cell-bound form (Figure 2). These results indicate that TNF-α can stimulate VSMCs to produce cell-bound and soluble fractalkine, with the latter deriving from cleavage of the cell-bound form.

Signalling pathways mediating fractalkine expression by TNF-α

Because TNF-α activates multiple signalling pathways that include PKA, PKC, PI 3-K, p42/44 MAPK, and p38 MAPK (Vilcek & Lee, 1991; Heller & Kronke, 1994; Berghe *et al*., 1998; Lallena *et al*., 1999; Reddy *et al*., 2000) experiments

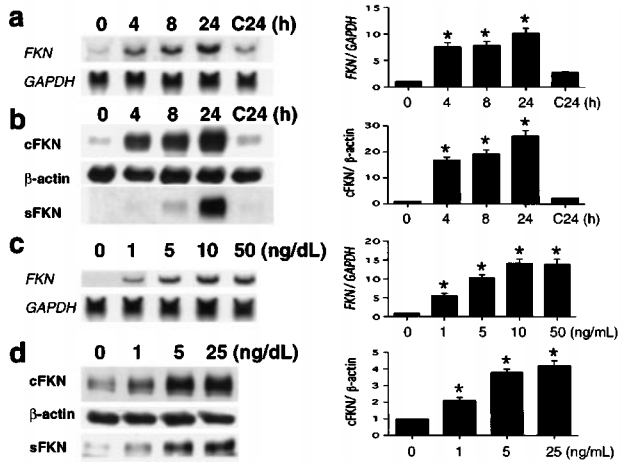

Figure 1 Time-course and dose-response of TNF-α stimulation on fractalkine mRNA and protein expression. VSMCs were incubated with TNF for the given periods. Ten micrograms of total RNA, forty micrograms of cell lysate, and fifty micrograms of concentrated condition media were analysed for fractalkine mRNA and protein expression as described in Methods. (a and b) Representative Northern and Western blots of fractalkine expression in response to TNF-α (5 ng ml^{-1}) at variable timepoints. *FKN*: fractalkine mRNA, *GAPDH*: glyceraldehyde-3-phosphate dehydrogenase mRNA, cFKN: cell-bound fractalkine in cell lysate, sFKN: soluble fractalkine in concentrated conditioned media. (c and d) Representative Northern and Western blots of fractalkine expression in response to TNF-α (1–50 ng ml^{-1}) at 4 and 24 h, respectively. Right panels show corresponding quantitative results of *FKN*/*GAPDH* mRNA and cFKN/β-actin ratios relative to that of control. Values are mean±s.e.mean of three experiments. *$P<0.05$ vs control at zero time.

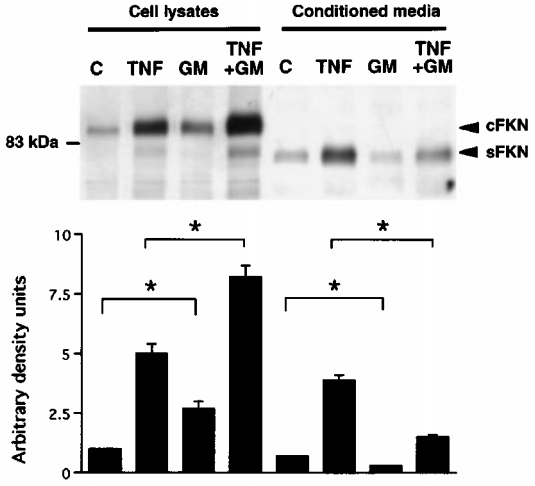

Figure 2 Effects of GM 6001 on TNF-α-stimulated cell-bound and soluble fractalkine expression. VSMCs were incubated with TNF-α (5 ng ml^{-1}) for 24 h, with or without pretreatment with GM (GM 6001, 2 μM) for 30 min. Upper panel shows representative Western blots. cFKN: cell-bound fractalkine, sFKN: soluble fractalkine. Lower panel shows quantitative results of cFKN (corrected for β-actin) and sFKN relative to that of control. Values are mean± s.e.mean of three experiments. *$P<0.05$ between the given experimental conditions.

were performed to identify the role of these pathways on TNF-α-stimulated fractalkine expression. Our results indicated that the incubation of VSMCs with calphostin C (0.4 μM), a selective inhibitor of PKC, or PD98059 (40 μM), a specific inhibitor of p42/44 MAPK kinase, significantly attenuated TNF-α-stimulated VSMC fractalkine mRNA and protein expression (Figure 3). In contrast, H-89 (2 μM), a selective inhibitor of PKA, wortmannin (0.5 μM), a selective inhibitor of PI 3-K, and SB203580 (40 μM), a specific inhibitor of p38 MAPK did not have discernible effects on fractalkine expression (Figure 3).

Effects of NF-κB inhibition on TNF-α-stimulated fractalkine expression

The preincubation of cells with the ubiquitin/proteosome inhibitors, MG132 (Yamakawa *et al*., 1999) and PDTC (Liu *et al*., 1999) resulted in complete inhibition of TNF-α-stimulated fractalkine mRNA and protein expression (Figure 4). At the concentrations used in the present study, both MG132 and PDTC reversed TNF-α-induced degradation of I-κBα (Figure 5a,b) and nuclear translocation of p65/NF-κB subunit (Figure 6). Furthermore, MG132 and PDTC have been reported as activators for c-Jun/AP-1 pathway (Yakoo & Kitamura, 1996; Nakayama *et al*., 2001) and our results showed that they did increase TNF-α-activated phospho-c-Jun levels (Figure 5a,b). In contrast, neither agent had discernible effects on the levels of TNF-α-stimulated phospho-PKC or phospho-44/42 MAPK (Figure 5a,b).

Additional experiments were performed to examine the effects of PD98059 and calphostin C on TNF-α-activated signalling cascades. Our results indicated that PD 98059, but not calphostin C, inhibited TNF-α-activated phospho-p42/44 MAPK and phospho-c-Jun levels whereas only calphostin C suppressed the levels of TNF-α-activated phospho-PKC (Figure 5c,d). Neither PD98059 nor calphostin C, however, affected TNF-α-induced degradation of I-κBα (Figure 5c,d) or nuclear translocation of p65/NF-κB (Figure 6).

Effects of PTX on TNF-α-stimulated fractalkine expression

The preincubation of VSMCs with PTX (0.1–1 mg ml^{-1}) alone had no effect on basal fractalkine mRNA or protein expression (data not shown). However, the pretreatment of VSMCs with PTX for 45 min dose-dependently attenuated TNF-α-stimulated fractalkine mRNA and protein expression (Figure 7a,b). Fractalkine transcript stability over time in cells incubated with or without PTX (1 mg ml^{-1}) is shown in Figure 8. Significant breakdown of fractalkine mRNA becomes detectable between 3 to 7 h after cessation of transcription by actinomycin D (20 μg ml^{-1}) in the control cells. In cells treated with PTX, no difference in the rate of fractalkine mRNA breakdown was apparent from control; a clear reduction in the number of fractalkine mRNA transcripts in the presence of PTX also occurs between 3 to 7 h. These results suggest that PTX reduced TNF-α-stimulated fractalkine mRNA levels *via* inhibition of transcription, rather than *via* enhancement of mRNA transcript breakdown.

To further explore the underlying mechanisms, PTX-pretreated VSMCs were stimulated with TNF-α (5 ng ml^{-1}) for 7.5 to 30 min. The results showed that PTX attenuated TNF-α-activated phosphorylation of PKC and p42/44 MAPK, and prevented TNF-α-induced degradation of I-

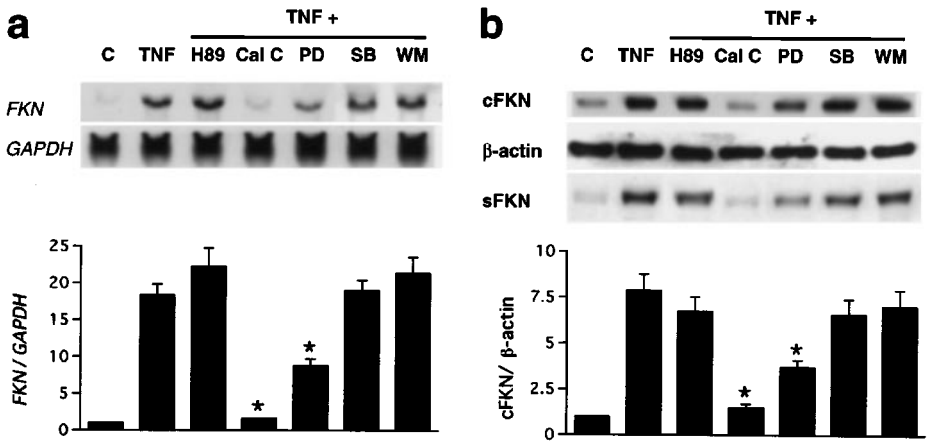

Figure 3 Effects of protein kinase inhibitors on TNF-α stimulated fractalkine mRNA and protein expression. VSMCs were incubated with TNF-α (5 ng ml^{-1}) for 4 or 24 h, with or without pretreatment with the given pharmacologic inhibitors (H-89, 2 μM; Cal C: calphostin C, 0.4 μM; PD: PD98059, 40 μM; SB: SB203580, 40 μM; WM: wortmannin, 0.5 μM). (a) Representative Northern blots. *FKN*: fractalkine mRNA. (b) Representative Western blots. cFKN: cell-bound fractalkine, sFKN: soluble fractalkine. Lower panels show corresponding quantitative results of *FKN*/*GAPDH* mRNA and cFKN/β-actin ratios relative to that of control. Values are mean±s.e.mean of three experiments. $^*P<0.05$ vs TNF-α-treated cells.

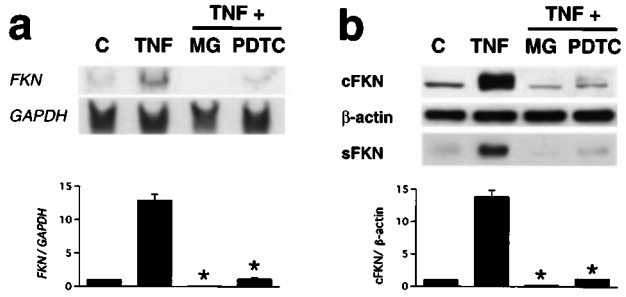

Figure 4 Effect of NF-κB inhibition on TNF-α stimulated fractalkine mRNA and protein expression. VSMCs were pretreated with MG (MG132, 10 μM) or PDTC (pyrrolidine dithiocarbamate, 200 μM) for 1.5 and 1 h, respectively, followed by TNF-α (5 ng ml^{-1}) for 4 or 24 h. (a) Representative Northern blots. *FKN*: fractalkine mRNA. (b) Representative Western blots. cFKN: cell-bound fractalkine, sFKN: soluble fractalkine. Lower panels show corresponding quantitative results of *FKN*/*GAPDH* and cFKN/β-actin ratios relative to that of control. Values are mean±s.e.mean of three experiments. $^*P<0.05$ vs TNF-α-treated cells.

κBα (Figure 7c) and nuclear translocation of p65/NF-κB (Figure 6). In addition, PTX attenuated phosphorylation of c-Jun activated by TNF-α (Figure 7c).

Discussion

The present study shows that cultured VSMCs at steady-state express a low level of fractalkine mRNA and protein, which could be up-regulated by TNF-α in both time- and dose-dependent manners. In addition to cell-bound fractalkine, this study shows that the conditioned media of TNF-α-activated VSMC contain a ~75-kD soluble fractalkine, which is likely shed from the cell-bound fractalkine. This finding is consistent with recent observations that cleavage of the cell-bound form by metalloproteinases is the major source for soluble fractalkine in various cultured cell systems, including VSMCs (Chapman *et al*., 2000a; Garton *et al*., 2001; Ludwig *et al*., 2002). The biological significance of the cleaved soluble form of fractalkine remains controversial. While the soluble fractalkine was originally described as a novel chemoattractant (Bazan *et al*., 1997), recent *in vitro* studies performed in cultured endothelial cells and VSMCs fail to support such a notion (Ludwig *et al*., 2002; Chapman *et al*., 2000b; Umehara *et al*., 2001b). On the other hand, in the central nervous system soluble fractalkine shed from neurons has been reported to act as a chemoattractant for monocytes, or as a neurotransmitter for microglial cells (Chapman *et al*., 2000a; Harrison *et al*., 1998; Tong *et al*., 2000).

The signal pathways initiated by TNF-α include those that activate protein kinases such as PKA, PKC, PI 3-K, p42/44 MAPK, and p38 MAPK (Vilcek & Lee, 1991; Heller & Kronke, 1994; Berghe *et al*., 1998; Lallena *et al*., 1999; Reddy *et al*., 2000), and transcription factors such as NF-κB and AP-1 (Heller & Kronke, 1994; Malinin *et al*., 1997). This study shows that TNF-α-stimulated VSMC fractalkine mRNA and protein expression is attenuated by pharmacologic inhibitors of PKC (calphostin C) and p42/44 MAPK kinase (PD98059), but not PKA (H-89), PI-3K (wortmannin), or p38 MAPK (SB203580), indicating that the intracellular signals mediating TNF-α-stimulated fractalkine expression involve activation of PKC and p42/44 MAPK, rather than PKA, PI 3-K, or p38 MAPK pathways.

NF-κB is a key nuclear factor regulating the transactivation of genes involved in a variety of chronic inflammatory disorders, including atherosclerosis (Barnes & Karin, 1997; Bourcier *et al*., 1997; Brand *et al*., 1997). This study shows that inhibition of the NF-κB signal by the ubiquitin/proteosome inhibitors, MG132 and PDTC, abolished TNF-α-stimulated VSMC fractalkine mRNA and protein expression, indicating that NF-κB plays a crucial role in fractalkine gene transcription in VSMCs. Parallel to this finding, a recent study shows that fractalkine induction in rat aortic endothelial cells by inflammatory cytokines is NF-κB-dependent (Garcia *et al*., 2000).

In this study, we show that suppression of the classical/novel and atypical PKC pathways by calphostin C causes an inhibition in TNF-α-stimulated fractalkine gene expression at a concentration that did not affect nuclear translocation of p65/NF-κB. This suggests that mechanisms for the anti-fractalkine effect of calphostin C is either beyond p65/NF-κB nuclear translocation or through another transcription factor. Consistent with this notion, PKCζ has been shown to positively regulate κB-dependent transcription activity *via* direct phosphorylation of the transactivation domain of p65/NF-κB (Anrather *et al*., 1999). Thus, it is possible that inhibition of TNF-α-activated PKCζ by calphostin C may directly down-regulate the transactivation potential of NF-κB in the nucleus without involvement of IκB modulation. Similarly, PD98059 was found to inhibit TNF-α-stimulated fractalkine gene expression at a concentration that did not affect nuclear translocation of p65/NF-κB. Inhibition of p42/44 MAPK kinase by PD98058 has been reported to directly suppress nuclear p65/NF-κB transactivation potential without IκB regulation (Berghe *et al*., 1998), and overexpression of a dominant negative p42/44 MAPK mutant has been shown to block κB-dependent promotor activation by TNF-α, in association with a reduction in AP-1 but not NF-κB nuclear levels (Berra *et al*., 1995). Because c-Jun/AP-1 protein has been shown to mediate TNF-α-activated gene expression *via* interaction with p65/NF-κB (Stein *et al*., 1993; Ahmad *et al*., 1998; Kyriakis, 1999), we speculate that down-regulation of the c-Jun/AP-1 pathway by PD98059 may attenuate the transactivation potential of nuclear NF-κB. Our result that PD98059 decreases phospho-c-Jun levels induced by TNF-α was consistent with such a possibility. While AP-1 may synergize with NF-κB for fractalkine gene transcription, activation of c-Jun/AP-1 alone, without NF-κB, may not be sufficient for fractalkine gene expression. This notion is supported at least partially by the present data that MG132 and PDTC completely blocks TNF-α-stimulated fractalkine mRNA expression despite the presence of an augmented phospho-c-Jun level.

This study shows that PTX decreases the levels of TNF-α-stimulated fractalkine mRNA transcripts, which are thus unavailable for translation into protein. Based on the present study on fractalkine-RNA transcript stability, PTX appears to act by inhibiting TNF-α-augmented fractalkine transcription rather than enhancing fractalkine-mRNA breakdown. Furthermore, our results show that PTX blocks TNF-α-induced I-κBα degradation and p65/NF-κB nuclear translocation, indicating that PTX may suppress TNF-α-stimulated

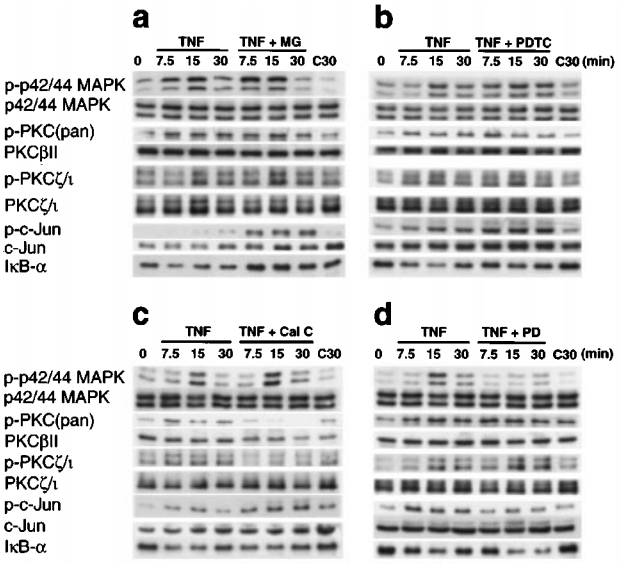

Figure 5 Effects of MG132, PDTC, calphostin C, and PD98059 on TNF-α-activated phospho-p42/44 MAPK, phospho-PKC, phospho-c-Jun and I-κBα levels. VSMCs were incubated with TNF-α (5 ng ml^{-1}) for varying timepoints, with or without pretreatment with the given pharmacologic inhibitors. Graphs showing representative Western blots from three independent experiments with similar results. The concentrations of inhibitors used in the signalling studies were MG: MG132 (10 μM); PDTC: pyrrolidine dithiocarbamate (200 μM); Cal C: calphostin C (0.4 μM); PD: PD98059 (40 μM). PKC(pan): PKC of classical/novel isoforms; PKCζ/ι: PKC of atypical isoformζ/ι.

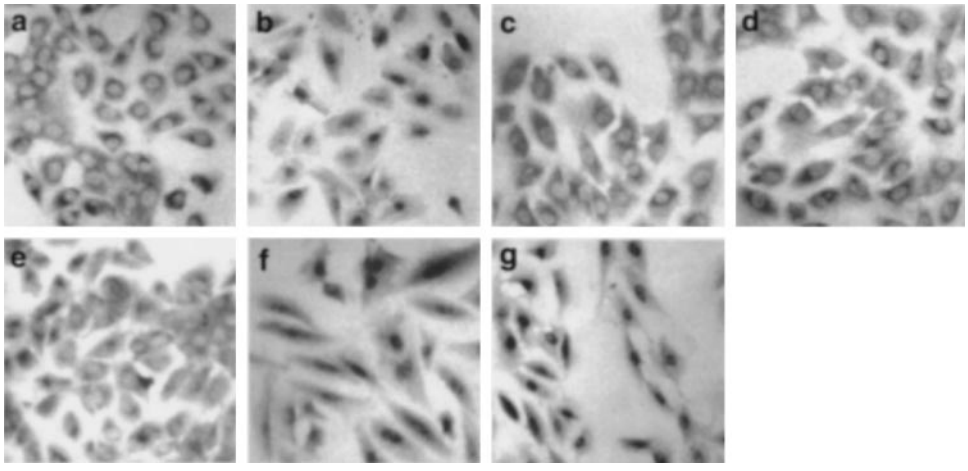

Figure 6 Immunostaining of nuclear translocated NF-κB/p65 induced by TNF-α: effects of MG132, PDTC, and pentoxifylline. (a) control. (b) incubation of VSMCs with TNF-α (5 ng ml^{-1}) for 15 min induced nuclear translocation of p65/NF-κB. (c, d, e) MG132 (10 μM), pyrrolidine dithiocarbamate (200 μM), and pentoxifylline (1 mg ml^{-1}) blocked translocation of p65/NF-κB induced by TNF-α. (f, g) Calphostin C (0.4 μM) and PD98059 (40 μM) did not affect NF-κB/p65 nuclear translocation (original magnification × 300).

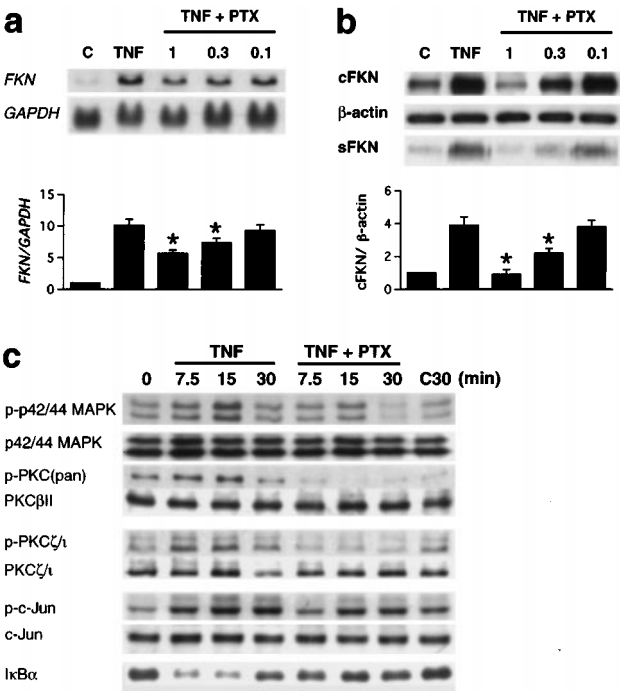

Figure 7 Effects of pentoxifylline on TNF-α stimulated fractalkine mRNA and protein expression as well as TNF-α-activated phospho-p42/44 MAPK, phospho-PKC, phospho-c-Jun and I-κBα levels. VSMCs were incubated with TNF-α (5 ng ml^{-1}) for 4 or 24 h, with or without pretreatment with PTX (pentoxifylline, 1–0.1 mg ml^{-1}) for 45 min. (a) Representative Northern blots. *FKN*: fractalkine mRNA. (b) Representative Western blots. cFKN: cell-bound fractalkine, sFKN: soluble fractalkine. Lower panels show corresponding quantitative results of *FKN*/*GAPDH* mRNA and cFKN/β-actin ratios relative to that of control. Values are mean ± s.e.mean of three experiments. $^*P<0.05$ vs TNF-α-treated cells. (c) Representative Western blots showing the effects of PTX (1 mg ml^{-1}) on TNF-α-activated signalling pathways. These experiments were performed three times, and similar results were obtained. PKC(pan): PKC of classical/novel isoforms; PKCζ/ι: PKC of atypical isoformζ/ι.

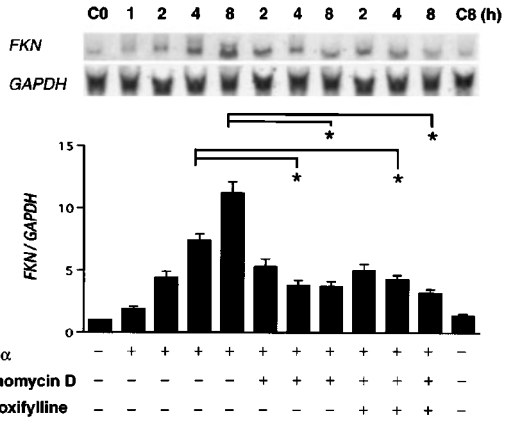

Figure 8 Northern blot of TNF-α-stimulated fractalkine mRNA stability. Cells were exposed to TNF-α (5 ng ml^{-1}) for 1 h, followed by actinomycin D (20 μg ml^{-1}) with or without pentoxifylline (1 mg ml^{-1}) to stop further mRNA transcription. Cells were harvested at serial timepoints as indicated to assess the rate of fractalkine mRNA breakdown. Upper panel shows representative Northern blots. Significant fractalkine mRNA transcript breakdown appears to occur between 3 to 7 h after addition of actinomycin D. Lower panel shows quantitative results of *FKN*/*GAPDH* mRNA ratios relative to that of control. Values are mean ± s.e.mean of three experiments. $^*P<0.05$ vs corresponding TNF-α-treated cells.

fractalkine gene transcription *via* down-regulation of NF-κB activation. The ability of PTX to antagonize NF-κB activity in VSMCs has been reported elsewhere, but the exact mechanism remains poorly elucidated (Bellas *et al*., 1995; Bretschneider *et al*., 1997). As demonstrated by this study, the anti-NF-κB activity of PTX is mediated, at least in part, by cytoplasmic retention of I-κBα-p65 complexes. Because PTX is an inhibitor of cyclic 3′,5′-nucleotide phosphodiesterase (Chen *et al*., 1999), one would speculate that this effect is initiated by activation of the cAMP-PKA cascade, which in turn suppresses the *raf*-1/ p42/44 MAPK pathway (Pinzani *et al*., 1996; D'Angelo *et al*., 1997) and the resultant activation of NF-κB (Neumann *et al*., 1995). Consistent with this notion, our results show that PTX decreases TNF-α-activated phospho-p42/44 MAPK levels. On the other hand, Lee *et al*. (1997) have reported that the anti-NF-κB activity of PTX is not mediated by phosphodiesterase inhibition, and Biswas *et al*. (1994) have shown that PTX inhibits NF-κB activation *via* a PKC-, but not PKA-dependent mechanism. Thus, the anti-NF-κB activity of PTX may not be cAMP-PKA-dependent. In this study, we found that PTX reduced TNF-α-induced phospho-PKC and phospho-c-Jun levels, suggesting that PTX may also modulate NF-κB activity in a way similar to calphostin C and PD98059.

In conclusion, we have demonstrated that TNF-α stimulates fractalkine gene and protein expression in rat VSMCs, and blockade of PKC, p42/44 MAPK kinase, and NF-κB nuclear translocation correlates with inhibition of fractalkine gene and protein expression. In view of the emerging importance of fractalkine in the recruitment of mononuclear leukocytes, these data may open possibilities for designing novel interventions or for using currently available agents to modulate the inflammatory response within the vessel wall. One example presented in this study is suppression of TNF-α-stimulated fractalkine gene and protein expression by the haemorrheologic agent PTX through down-regulation of the PKC, p42/44 MAPK, and p65/NF-κB pathways.

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