Fractalkine (CX3CL1) stimulated by nuclear factor κB (NF-κB)-dependent inflammatory signals induces aortic smooth muscle cell proliferation through an autocrine pathway

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Fractalkine (also known as CX3CL1), a CX3C chemokine, activates and attracts monocytes/macrophages to the site of injury/inflammation. It binds to CX3C receptor 1 (CX3CR1), a pertussis toxin-sensitive G-protein-coupled receptor. In smooth muscle cells (SMCs), fractalkine is induced by proinflammatory cytokines [tumour necrosis factor-α (TNF-α) and interferon-γ (IFN-γ)], which may mediate monocyte adhesion to SMCs. However, the mechanisms underlying its induction are unknown. In addition, it is unlear whether SMCs express CX3CR1. TNF-α activated nuclear factor κB (NF-κB) and induced fractalkine and CX3CR1 expression in a time-dependent manner in rat aortic SMCs. Transient transfections with dominant-negative (dn) inhibitory κB (IκB)-α, dnIκB-β, dnIκB kinase (IKK)-γ, kinase-dead (kd) NF-κB-inducing kinase (NIK) and kdIKK-β, or pretreatment with wortmannin, Akt inhibitor, pyrrolidinecarbodithioc acid ammonium salt ('PDTC') or MG-132, significantly attenuated TNF-α-induced fractalkine and CX3CR1 expression. Furthermore, expression of dn TNF-α-receptor-associated factor 2 (TRAF2), but not dnTRAF6, inhibited TNF-α signal transduction. Pretreatment with pertussis toxin or neutralizing anti-CX3CR1 antibodies attenuated TNF-α-induced fractalkine expression, indicating that fractalkine autoregulation plays a role in TNF-α-induced sustained fractalkine expression. Fractalkine induced its own expression, via pertussis toxin-sensitive G-proteins, phosphoinositide 3-kinase (PI 3-kinase), phosphoinositide-dependent kinase 1 (PDK1), Akt, NIK, IKK and NF-κB activation, and induced SMC cell–cell adhesion and cellular proliferation. Taken together, our results demonstrate that TNF-α induces the expression of fractalkine and CX3CR1 in rat aortic SMCs and that this induction is mediated by NF-κB activation. We also show that fractalkine induces its own expression, which is mediated by the PI 3-kinase/PDK1/Akt/NIK/IKK/NF-κB signalling pathway. More importantly, fractalkine increased cell–cell adhesion and aortic SMC proliferation, indicating a role in initiation and progression of atherosclerotic vascular disease.

Key words: atherosclerosis, autoregulation, cell adhesion, CX3C receptor 1 (CX3CR1), fractalkine (CX3CL1), mitosis, nuclear factor κB (NF-κB).

INTRODUCTION

Recruitment, activation and subsequent expansion of monocytes/macrophages in the arterial vessel walls lead to foam cell formation and proliferation of intimal smooth muscle cells (SMCs), resulting in stenosis or complete occlusion of vessels. In the context of coronary vessels, atherogenesis and plaque formation results in myocardial infarction, unstable angina pectoris and sudden cardiac death [1].

Chemokines, low molecular-mass cytokines (7–14 kDa), attract and activate specific subsets of immune cells during normal physiological immunosurveillance as well as during injury and inflammation [2–4]. Based on the spacing of N-terminus cysteine residues, chemokines are classified into C, CC, CXC and CX3C families. In the CX3C family, the first two conserved cysteine residues are separated by three non-conserved amino acids. Fractalkine (CX3CL1) is the only member recognized so far that belongs to the CX3C family [5]. Unlike other chemokines, which contain 70–80 amino acids, fractalkine is composed of 350 residues. It exists as a membrane-bound glycoprotein with four different domains: a chemokine domain tethered to the cell surface via a heavily glycosylated mucin-like stack which, in turn, is attached to transmembrane and cytoplasmic domains. Fractalkine exists in two forms: the membrane-anchored form, which acts as an adhesion molecule [6], and the soluble form, which is released upon cleavage by tumour necrosis factor-α (TNF-α)-converting enzyme ('TACE') and acts as a potent chemoattractant for monocytes/macrophages, T-cells and natural killer cells [7]. Fractalkine exerts its biological activity by binding to a high-affinity pertussis toxin-sensitive seven transmembrane G-protein-coupled receptor (GPCR) known as CX3C receptor 1 (CX3CR1) [8].

Recently, Garcia et al. [9] have shown induction of fractalkine in aortic endothelial cells by various inflammatory mediators.

Abbreviations used: AM, acetoxymethyl ester; Bad, Bcl-2/Bcl-X_L-antagonist, causing cell death; CX3CR1, CX3C receptor 1; DMEM, Dulbecco's modified Eagle's medium; dn, dominant-negative; EGFP, enhanced green fluorescent protein; EMSA, electrophoretic mobility-shift assay; Flag, Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys; GPCR, G-protein-coupled receptor; GSK-3, glycogen synthase kinase-3; HA, haemagglutinin; IFN-γ, interferon-γ; IκB, inhibitory κB; IKK, IκB kinase; kd, kinase-dead; LPS, lipopolysaccharide; NF-κB, nuclear factor κB; NIK, NF-κB-inducing kinase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide; PI 3-kinase, phosphoinositide 3-kinase; PDK1, phosphoinositide-dependent kinase-1; PDTC, pyrrolidinecarbodithioc acid ammonium salt; PTx, pertussis toxin; RGS3, regulator of G-protein signalling 3; RT, reverse transcription; SDF-1α, stromal cell-derived factor-1α; SMC, smooth muscle cell; ASMC, aortic SMC; TdR, thymidine; TNF-α, tumour necrosis factor-α; TRAF, TNF-α-receptor-associated factor; TNFR1, TNF-α-receptor 1; TRADD, TNFR1-associated death domain protein.

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Similarly, Harrison et al. [10] have demonstrated induction of fractalkine in rat myocardium in response to various proinflammatory stimuli. By using *in situ* hybridization and immunohistochemical techniques, fractalkine was shown be localized predominantly to vascular endothelial cells [10]. On the other hand, SMCs failed to show any positive immunoreactivity. However, Ludwig et al. [11] have demonstrated fractalkine induction in aortic SMCs (ASMCs) by TNF-α and interferon-γ (IFN-γ), but not by interleukin-1β. In the present study, we have investigated the mechanism(s) by which proinflammatory stimuli lead to fractalkine induction in SMCs and whether fractalkine has any autocrine effects on SMCs. Our results demonstrate that TNF-α induces fractalkine and CX3CR1 expression in aortic SMCs via nuclear factor κB (NF-κB) activation. Furthermore, fractalkine induced its own expression via heterotrimeric G_i-proteins, phosphoinositide 3-kinase (PI 3-kinase), phosphoinositide-dependent kinase 1 (PDK1), Akt, NF-κB-inducing kinase (NIK), inhibitory κB (IκB) kinase (IKK) and NF-κB. Most importantly, fractalkine autoregulation enhanced cell–cell adhesion and cellular proliferation.

MATERIALS AND METHODS

Materials and reagents

Recombinant rat TNF-α, fractalkine (chemokine domain), platelet-derived growth factor ('PDGF')-BB, neutralizing anti-(rat TNF-α) antibodies and normal goat IgG (preimmune) were obtained from R&D Systems (Minneapolis, MN, U.S.A.) The recombinant proteins contained <1 ng of endotoxin/μg of cytokine, as determined by the *Limulus* amoebocyte lysate method (manufacturer's technical data sheet). Rabbit anti-(rat fractalkine) and anti-(rat CX3CR1) antibodies were from Torrey Pines Biolabs (Houston, TX, U.S.A.). Anti-(IκB-α), anti-p85 and anti-p110γ antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Antibodies against phospho-IκB-α(Ser^{32}), Akt, phospho-Akt (Thr^{308}), Bad (Bcl-2/Bcl-X_L-antagonist, causing cell death) and phospho-Bad (Ser^{112}) were from Cell Signaling Technology (Beverly, MA, U.S.A.). All tissue culture supplies were from Life Technologies (Rockville, MD, U.S.A.). Radiochemicals ([α-^{32}P]dCTP, [γ-^{32}P]ATP and [α-^{32}P]UTP) were purchased from Amersham Biosciences (Piscataway, NJ, U.S.A.), and [^{3}H]thymidine (TdR) (6.7 Ci/mmol) was from NEN Life Sciences (Boston, MA, U.S.A.). Unless otherwise indicated, all other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Cell culture

Non-transformed rat ASMCs (a gift from Dr Sergei N. Orlov, Centre de Recherche, Centre Hospitalier de l'Université de Montréal, Montréal, Canada) [12] were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % (v/v) heat-inactivated fetal calf serum. When cells reached 70–80 % confluency, the media was replaced with DMEM + 0.5 % BSA. After overnight culture, TNF-α, fractalkine or vehicle (PBS, pH 7.4) was added for the indicated time periods. Treatment with TNF-α (10 ng/ml) or fractalkine (10 ng/ml) for up to 48 h did not induce cell death in ASMCs, as evidenced by Trypan Blue exclusion. In order to determine the role of heterotrimeric G-proteins, PI 3-kinase, PDK1 and Akt in fractalkine autoregulation, ASMCs were pretreated with pertussis toxin (PTx; 100 ng/ml in PBS for 1 h), wortmannin (a specific PI 3-kinase inhibitor; 100 nM in DMSO), LY294002 (a specific PI 3-kinase inhibitor; 20 μM in DMSO), Akt inhibitor [1L-6-hydroxymethyl-*chiro*-inositol 2-(*R*)-2-*O*-methyl-3-*O*-octadecylcarbonate; 1 μM in DMSO] [13], PBS or DMSO prior to the addition of TNF-α or fractalkine. The above inhibitors were obtained from Calbiochem–Novabiochem (San Diego, CA, U.S.A.). In dose-determining pilot experiments, the Akt inhibitor at 1 μM significantly inhibited Akt kinase activity without cell toxicity. Because TNF-α is known to induce free radical generation [14], we also treated ASMCs with pyrrolidinecarbodithioc acid ammonium salt (PDTC; 100 μM in PBS for 1 h; Sigma), a free radical scavenger, or MG-132 (carbobenzoxyl-L-leucil-L-leucil-L-leucinol; 5 μM in DMSO; Calbiochem–Novabiochem), a proteasome inhibitor, prior to TNF-α addition.

In order to determine whether fractalkine-induced NF-κB activation is mediated via TNF-α induction, as shown previously for stromal cell-derived factor-1α (SDF-1α [15]), we treated ASMCs with a TNF-α antisense oligonucleotide (5′-CTGACTGCCTGGGCCAGAGGGCTGATTAG-3′) [16], which was phosphorothioated on three nucleotides on each end of the molecule. Specificity of antisense oligonucleotides was verified in pilot experiments, and our results indicated that these antisense oligonucleotides inhibited lipopolysaccharide (LPS)-induced TNF-α expression in ASMCs (results not shown). ASMCs were transfected with antisense oligonucleotides in Oligofectamine™ reagent, as recommended by the manufacturer (Invitrogen, Carlsbad, CA, U.S.A.), in DMEM + 0.5 % BSA and without antibiotics. After 3 h, cells were treated with fractalkine (10 ng/ml) for either 30 min (NF-κB DNA-binding activity) or 2 h (mRNA expression). Phosphorothioated scrambled oligonucleotides were used as controls. In order to demonstrate whether release of preformed membrane-bound TNF-α mediates fractalkine effects on NF-κB activation, we incubated ASMCs with neutralizing anti-(rat TNF-α) antibodies (5 μg/ml) 15 min prior to fractalkine (10 ng/ml) addition. After 30 min, cells were harvested for isolation of nuclear protein. Normal goat IgG served as a control.

In order to determine the role of fractalkine in TNF-α-induced sustained fractalkine induction, ASMCs were treated with PTx prior to TNF-α addition. In addition, ASMCs were treated with anti-CX3CR1 blocking antibodies (10 μg/ml for 1 h) prior to TNF-α addition. Cells were harvested after 2 h of TNF-α treatment and analysed for fractalkine mRNA expression by Northern blotting.

Electrophoretic mobility-shift assay (EMSA)

NF-κB DNA-binding activity was measured by EMSA as described previously [17,18] using NF-κB consensus double-stranded oligonucleotides (5′-AGTTGAGGGGACTTTCCCAGGC-3′; core consensus sequence is underlined; Santa Cruz Biotechnology). NF-κB mutant oligonucleotide containing a G→C substitution in the consensus sequence (5′-AGTTGAGG**C**GACTTTCCCAGGC-3′; substitution is bold) and octamer-1 ('Oct-1') (5′-TGTCGAATGCAAATCACTAGAA-3′; core consensus sequence is underlined) oligonucleotide served as controls.

Transient cell transfections and reporter assays

ASMCs were transfected with dominant-negative (dn) or kinase-dead (kd) expression vectors using LIPOFECTAMINE™ 2000 essentially as described previously [18]. The phosphorylation-deficient mutant of IκB-α [pCMX-IκB-α (S32A/S36A)] was a gift from Dr Inder Verma (The Salk Institute, La Jolla, CA, U.S.A.). dnIκB-β [pCMV-Tag3B-IκB-β (S19A/S32A)–Myc] was as described previously [18]. kdNIK [pRK7-NIK(KK429-430AA)–Flag] (where Flag is Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys), dn TNF-α-receptor-associated factor 2 (TRAF2)

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[pRK5-TRAF2(87–501)–Flag] and dnTRAF6 [pRK5-TRAF6-(289–522)–Flag] were gifts from Dr David V. Goeddel (Tularik Inc., South San Francisco, CA, U.S.A.). kdIKK-β (pRK5-IKK-β–Flag) and dnIKK-γ (pcDNA3-IKK-γ−-HA) (where HA is haemagglutinin) were obtained from Dr Tom Maniatis (Harvard University, Cambridge, MA, U.S.A.) and Dr Gabriel Nunez (University of Michigan Medical School, Ann Arbor, MI, U.S.A.) respectively. kdPDK1 [pcDNA3.1-PDK1(K114G)–Myc-HisA+], wild-type regulator of G-protein signalling 3 (RGS3) (pCMV-Tag3B-RGS3–Myc) and mutant RGS3 [pCMV-Tag3B (S264A)–Myc] were described previously [19–21]. To compensate for variations in transfection, the cells were co-transfected with pRL-*Renilla* luciferase vector (pRL-TK vector; Promega, Madison, WI, U.S.A.). Transfection efficiency of ASMCs was determined using pEGFP-N1 [18] (where EGFP is enhanced green fluorescent protein), and found to be approx. 32 % (32.3 ± 2.29; mean ± S.E.M.).

Analysis of RNA expression

Total RNA was extracted using TRIzol reagent (Invitrogen). A sample (20 mg) of RNA was electrophoresed in a 0.8 % formaldehyde gel and transferred on to a nitrocellulose membrane. Northern blotting, autoradiography and densitometry were performed as described previously [18,22–24]. Fractalkine and CX3CR1 probes were generated by reverse transcription (RT)-PCR using total RNA isolated from ASMCs. RT was carried out in 20 μl of reaction mixture containing 2 μg of total RNA. PCR was conducted using the RT product and gene-specific primers for fractalkine (GenBank® accession number AF030358) and CX3CR1 (GenBank® accession number RNU04808). The cDNA products were gel-purified, subcloned into pCR 2.1-TOPO (Invitrogen) and the sequence confirmed by restriction enzyme analysis and DNA sequencing. Fractalkine mRNA stability after TNF-α, fractalkine or vehicle (control) was determined using actinomycin D treatment essentially as described previously [18]. Nuclear run-on assays were performed to determine whether TNF-α and fractalkine induce fractalkine and CX3CR1 expression by increasing transcription. After treating ASMCs with TNF-α or fractalkine in M199 medium containing 0.5 % BSA, nuclei were isolated and nuclear run-on assays were performed as described previously [18].

Analysis for protein expression

Extraction of protein homogenates, Western blotting, autoradiography and densitometry were performed as described previously [18,22–24]. Rabbit anti-(rat fractalkine) and anti-(rat CX3CR1) antibodies were used at a concentration of 2 and 2.5 μg/ml respectively. β-Actin was used as an internal control. Fractalkine levels in culture supernatants were determined using rat fractalkine/CX3CL1 DuoSet ELISA Development kit (R&D Systems).

Measurement of PI 3-kinase and Akt kinase activities

PI 3-kinase lipid kinase assays were performed using either p85 (TNF-α-treated) or p110γ (fractalkine-treated) immunoprecipitates essentially as described previously [18,25,26]. Akt kinase activity in ASMCs was performed using a commercially available kit (Cell Signaling Technology). The assay is based on Akt-induced phosphorylation of glycogen synthase kinase-3 (GSK-3).

Cell proliferation by [^{3}H]TdR incorporation and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) colorimetric assay

Cells were seeded in triplicate at a concentration of 1×10^5 cells/well in 200 μl of DMEM containing 10 % (v/v) fetal calf serum in 96-well flat-bottom plates (Costar, Corning, NY, U.S.A.). After 24 h incubation, the complete medium was replaced with DMEM containing 0.5 % BSA, and were incubated for an additional 48 h. The cells were treated with fractalkine (10 ng/ml) for the indicated periods and were pulsed with 0.5 μCi/well [^{3}H]TdR for the last 16 h of the incubation period. Cells were then harvested on to membranes and the incorporated [^{3}H]TdR was measured using a liquid-scintillation counter (Beckman LS6500; Fullerton, CA, U.S.A.). SMC proliferation was also determined using a colorimetric assay kit based on the uptake of MTT by viable cells (Cell Proliferation Kit; Roche Applied Science, Indianapolis, IN, U.S.A.).

Cell-death-detection ELISA

After 48 h incubation in DMEM containing 0.5 % BSA, ASMCs were treated with fractalkine (10 ng/ml) for 24 h. Cells were harvested and analysed for mono- and oligo-nucleosomes in the cytoplasmic fraction of cell lysates by ELISA (Cell Death Detection ELISAPLUS kit; Boehringer Mannheim, Indianapolis, IN, U.S.A.). *S*-nitroso-*N*-acetyl-DL-penicillamine ('SNAP'; 500 μM in ethanol; Calbiochem–Novabiochem), a nitric oxide donor, was used as a positive control [27].

Cell adhesion assays

Cell adhesion assays were performed using a commercially available kit (Vybrant Cell Adhesion Assay kit; Molecular Probes, Eugene, OR, U.S.A.). The assay is based on labelling cells with an acetoxymethyl ester (AM) form of the cytoplasmic fluorescent dye calcein (calcein AM) [28]. Upon loading, calcein AM is cleaved by endogenous esterases to produce a highly fluorescent calcein that does not leak out the cells or interfere with cell adhesion [29]. ASMCs were plated on to a 96-well flat-bottomed microtitre plate (1000 cells/well) and, after overnight culture, the complete medium was replaced with DMEM containing 0.5 % BSA. After incubation for 2 h with fractalkine (10 ng/ml), the medium was changed to fresh DMEM containing 0.5 % BSA and immediately overlayed with calcein AM-loaded ASMCs that were treated with fractalkine (10 ng/ml) for 2 h. After incubation for 1 h at 37 °C, the medium was gently aspirated and washed with PBS at 37 °C to remove non-adherent cells. The remaining fluorescence was measured in a microplate reader at an excitation wavelength of 485 nm and an emission wavelength of 530 nm. A standard curve was generated using calcein AM-loaded cells in incremental numbers, and the results were expressed as relative fluorescence intensity. CX3CR1 blocking was attained by incubating ASMCs with anti-CX3CR1 antibodies (10 μg/ml for 1 h at 37 °C) prior to fractalkine addition.

Statistical analysis

Comparisons between controls and various treatments were performed for measures of NF-κB DNA-binding activity, NF κB-driven luciferase activity, mRNA and protein levels, cell proliferation, cell death and cell adhesion by ANOVA with post-hoc Dunnett's *t* tests. All assays were performed at least three times, and the error bars in Figures indicate the S.E.M.

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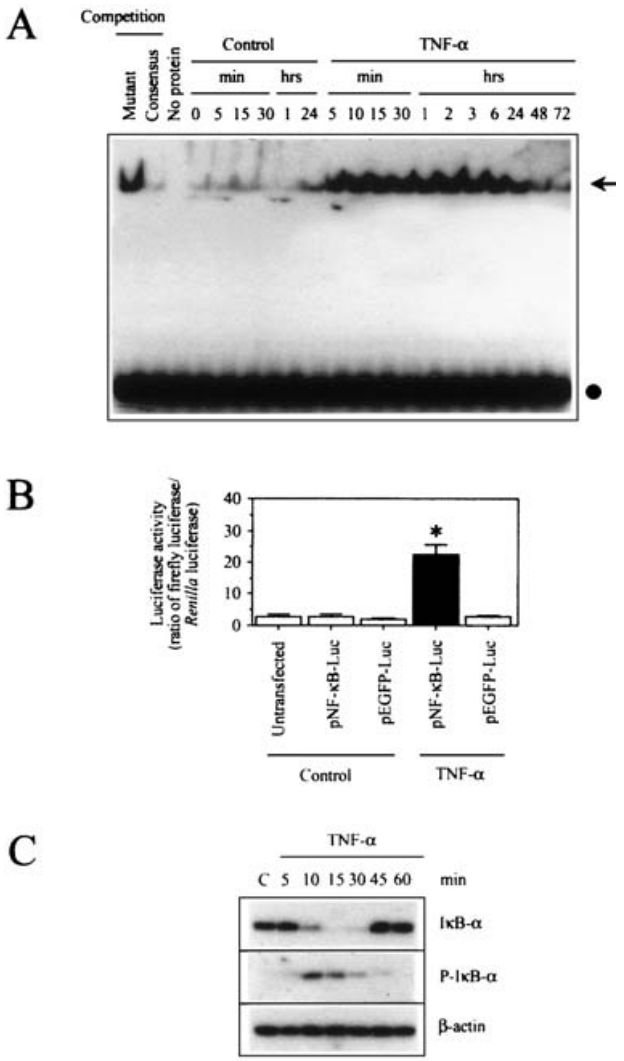

Figure 1 The proinflammatory cytokine TNF-α activates NF-κB and induces NF-κB-driven luciferase activity in rat ASMCs

(**A**) EMSA was performed using nuclear protein extracts isolated from TNF-α-treated ASMC and ^{32}P-labelled NF-κB consensus oligonucleotide. In competition experiments with mutant or consensus NF-κB oligonucleotides, protein extracts from ASMCs treated with TNF-α for 15 min were preincubated with 100-fold molar excess of either unlabelled double-stranded mutant or consensus NF-κB oligonucleotide respectively, followed by the addition of ^{32}P-labelled consensus NF-κB probe. The No protein lane contains all the components of the reaction mixture, except the nuclear protein extract. Arrow indicates NF-κB-specific DNA–protein complexes. Solid circle shows unincorporated labelled probe. (**B**) NF-κB-driven luciferase activity was measured in TNF-α-treated ASMCs that were transiently transfected with either pNF-κB-Luc or pEGFP-Luc. To compensate for transfection efficiency, cells were co-transfected with pRK-*Renilla*, and the results are presented as a ratio of firefly luciferase to *Renilla* luciferase. $^{*}P < 0.001$ compared with control. (**C**) Western blotting was performed to determine TNF-α effects on total IκB-α and phospho-IκB-α(Ser^{32}) levels in ASMCs.

RESULTS

TNF-α activates NF-κB DNA-binding activity and induces NF-κB-driven luciferase activity in ASMCs

TNF-α is a potent activator of NF-κB and induces the expression of various NK-κB-responsive genes [30,31]. We therefore determined its effects on NF-κB activation in ASMCs. EMSAs revealed low levels of NF-κB DNA-binding activity in ASMCs under basal conditions. However, a rapid and sustained activation of NF-κB was detected after TNF-α treatment (Figure 1A). Treatment with TNF-α also increased NF-κB-(6.81-fold; $P < 0.001$), but not EGFP-driven luciferase activity (Figure 1B). TNF-α mediated NF-κB activation occurred via phosphorylation and degradation of IκB-α (Figure 1C).

TNF-α up-regulates fractalkine and CX3CR1 expression in ASMCs

TNF-α has been shown previously [9] to induce fractalkine expression in endothelial cells via NF-κB activation. We found that TNF-α induced coordinated expression of both fractalkine and CX3CR1 in ASMCs, suggesting an autocrine pathway. Figure 2(A) shows that TNF-α induces fractalkine expression in ASMCs in a sustained manner. Although CX3CR1 expression was not detected under basal conditions, TNF-α induced its expression at 30 min, which remained elevated up to 48 h. Furthermore, incubation with neutralized TNF-α abrogated its effects on fractalkine and CX3CR1 expression (Figure 2B). Although, CX3CR1 mRNA expression was not detected by Northern blotting under basal conditions, RT-PCR revealed low levels of CX3CR1 expression in ASMCs (Figure 2C). Western blotting revealed peak levels of fractalkine and CX3CR1 protein levels around 2 h post-TNF-α treatment. However, their protein levels declined by 24 h (Figure 2D), suggesting decreased translation at later time periods, even in the presence of high levels of mRNA (Figure 2A). Although CX3CR1 protein levels were not detected under basal conditions by Western blotting, FACS analysis revealed CX3CR1 expression on ASMC surface (Figure 2E). In addition to inducing fractalkine mRNA and protein levels, TNF-α, but not neutralized TNF-α, significantly increased fractalkine secretion into culture supernatants (9-fold; $P < 0.001$; Figure 2F). Nuclear run-on assays and mRNA stability studies indicated that, although TNF-α increased both fractalkine and CX3CR1 mRNA transcripts (fractalkine 3.48-fold; $P < 0.01$ compared with control; CX3CR1, 5.34-fold; $P < 0.0001$ compared with control; Figure 2G), it had no significant effect on fractalkine mRNA stability (results not shown), indicating that increased transcription accounted for TNF-α-induced fractalkine mRNA expression.

In order to determine whether fractalkine autoregulation plays a role in TNF-α-induced sustained fractalkine mRNA expression, we treated ASMCs with the G_i-protein-specific inhibitor PTx prior to TNF-α addition. The results in Figure 2(H) show that PTx pretreatment significantly attenuated TNF-α-induced fractalkine mRNA expression. Similarly, neutralizing anti-CX3CR1 antibodies also attenuated TNF-α-induced fractalkine expression (Figure 2I), indicating that fractalkine autoregulation contributes to TNF-α-induced sustained fractalkine expression.

TNF-α induces fractalkine and CX3CR1 expression via TRAF2, NIK, IKK and NF-κB

Using various pathway-specific dn and kd expression vectors, we delineated the possible signal transduction pathway leading to TNF-α-mediated NF-κB activation and fractalkine and CX3CR1 expression in ASMCs. Expression of recombinant proteins was confirmed by Western blotting (Figure 3A). Although transfection with empty vectors had minimal to no effect on basal levels of NF-κB activation (results not shown) and on TNF-α-induced NF-κB activation (Figure 3B), transfection with dn and kd expression vectors significantly attenuated TNF-α-induced NF-κB activation and fractalkine and CX3CR1 expression. Furthermore, dnTRAF2, but not dnTRAF6, MG-132 and PDTC had similar inhibitory effects (Figures 3B and 3C), indicating that TNF-α signals

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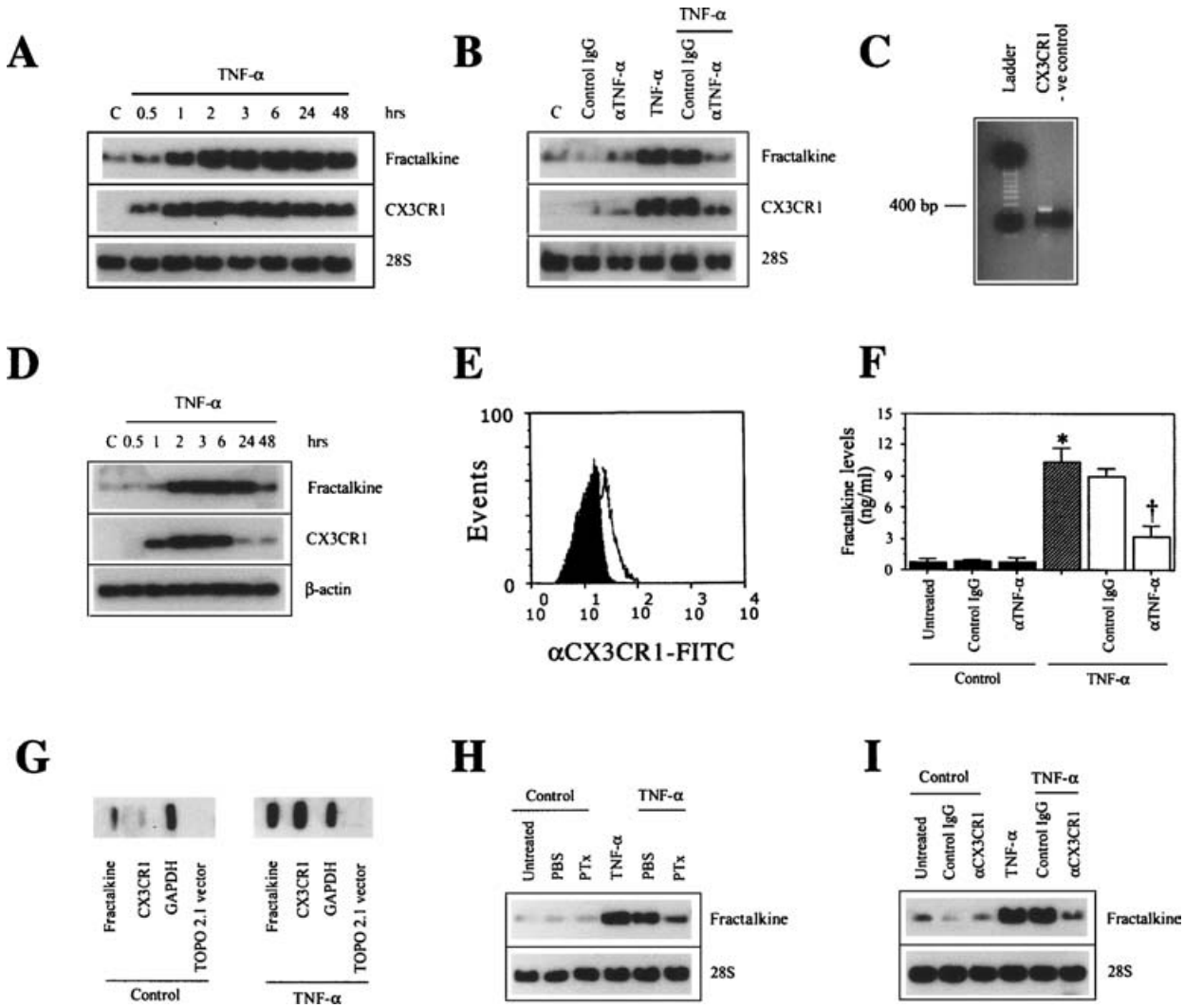

Figure 2 TNF-α induces fractalkine and CX3CR1 expression in rat ASMCs

(**A**) ASMCs were treated with TNF-α (10 ng/ml) for the indicated time periods, and total RNA was isolated, blotted on to nitrocellulose membranes and probed for fractalkine and CX3CR1 mRNA expression. 28 S rRNA was used as an internal control. (**B**) Northern blotting was performed to demonstrate TNF-α specificity on fractalkine mRNA expression. ASMCs were treated with TNF-α after neutralization with anti-TNF-α antibodies. Incubation with normal IgG served as a control. (**C**) Demonstration of CX3CR1 mRNA expression by RT-PCR in ASMCs under basal conditions. Negative control contained all components of the reaction mixture except the cDNA. Ladder, 100 bp ladder. (**D**) Western blotting showing temporal expression of fractalkine and CX3CR1 protein levels in TNF-α-treated ASMCs. β-Actin was used as an internal control. (**E**) CX3CR1 surface expression under basal conditions was analysed by FACS. ASMCs were incubated with FITC-conjugated rabbit anti-CX3CR1 antibodies. Normal rabbit IgG served as a control (black). (**F**) TNF-α-induced secreted fractalkine levels were quantified by ELISA. αTNF-α, ASMCs were treated with TNF-α after neutralization with anti-TNF-α antibodies. $^{*}P < 0.001$ compared with untreated cells; $\dagger P < 005$ compared with TNF-α alone. (**G**) Nuclear run-on assays were performed to investigate whether TNF-α-induced expression of fractalkine and CX3CR1 is regulated at transcriptional level. glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. TOPO 2.1 empty vector served as a negative control. (**H**) Northern blotting was performed to analyse whether fractalkine autoregulation plays a role in TNF-α-induced sustained fractalkine expression. As fractalkine signals via CX3CR1, a GPCR, we treated ASMCs with PTx (100 ng/ml in PBS for 1 h) prior to TNF-α addition. 28 S rRNA served as a control. (**I**) Role of fractalkine autoregulation was confirmed further by treating ASMCs with neutralizing anti-CX3CR1 antibodies prior to TNF-α addition, and total RNA was analysed by Northern blotting for fractalkine mRNA expression.

via TRAF2, NIK, IKK and NF-κB in inducing fractalkine and CX3CR1 expression.

TNF-α induces NF-κB activation via PI 3-kinase and Akt

TNF-α also signals via PI 3-kinase to activate NF-κB [32]. Therefore we explored the role of PI 3-kinase in TNF-α-induced NF-κB activation and fractalkine and CX3CR1 expression. Figure 4(A) shows the results of PI 3-kinase lipid kinase assays and demonstrates that TNF-α enhanced PI 3-kinase p85-mediated PtdIns(3,4,5)P_3 formation in ASMCs. Wortmannin, a specific PI 3-kinase inhibitor, inhibited TNF-α-induced PI 3-kinase activation. Similarly, treatment with LY 294002 also had similar inhibitory effects (results not shown). The phospholipids generated by PI 3-kinase act as potent second messengers in the activation of PDK1, which, in turn, regulate various downstream signalling pathways, including phosphorylation and activation of the serine/threonine kinase Akt/protein kinase B [33,34]. Phosphorylation at Thr^{308} and Ser^{473} activates Akt kinase activity. Treatment with TNF-α increased Akt kinase activity as seen by increased levels of phosphorylated GSK-3 (Figure 4B). Activated Akt also associates with IKK complex [34] and activates NF-κB following phosphorylation and degradation of IκB-α. Both

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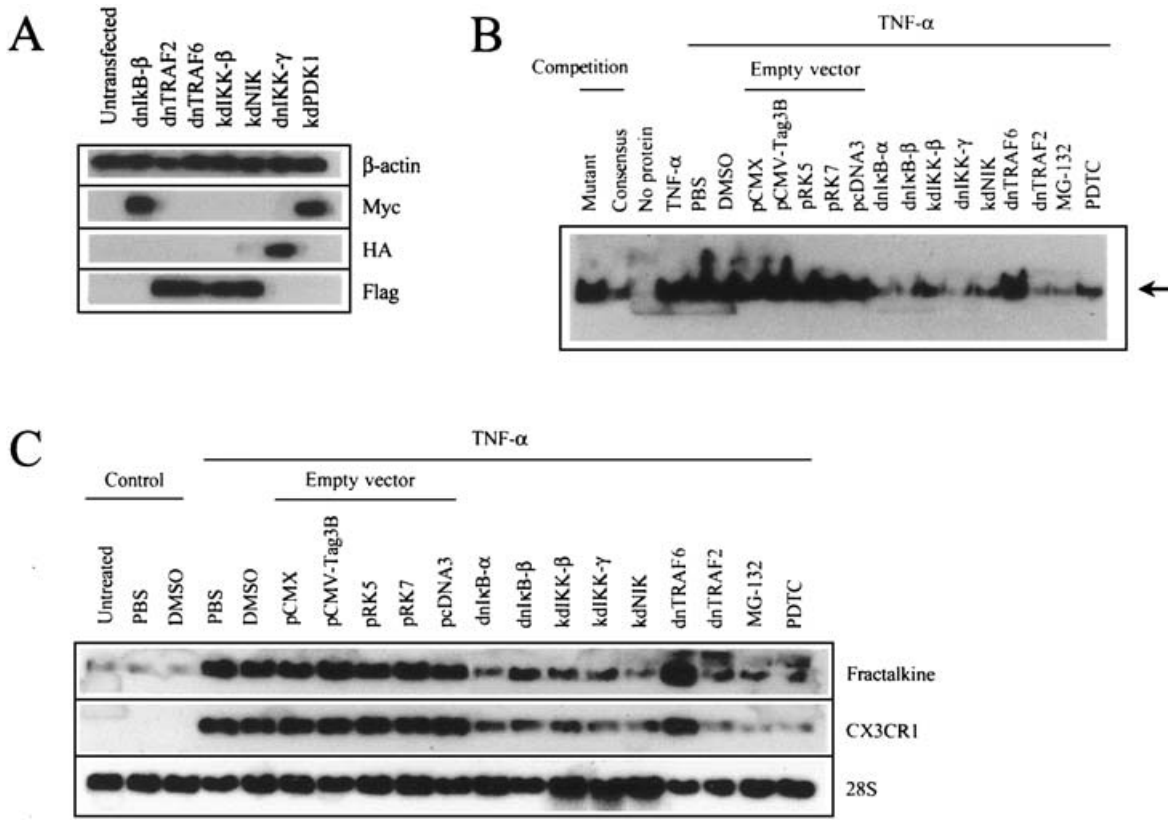

Figure 3 TRAF2, NIK, IKK and IκB mediate TNF-α-mediated NF-κB activation and fractalkine and CX3CR1 expression in ASMCs

(**A**) Western blotting was performed to confirm the expression of recombinant proteins in ASMCs transiently transfected with various NF-κB pathway-specific expression vectors by using antibodies against the Myc, HA and Flag tags. β-Actin served as a control. (**B**) EMSAs were performed to quantify NF-κB DNA-binding activity in TNF-α-treated ASMCs transiently transfected with the indicated expression vectors. Transfection with empty vectors served as controls. Arrow indicates NF-κB-specific DNA–protein complexes. (**C**) Northern blotting was carried out to analyse fractalkine and CX3CR1 mRNA expression in TNF-α-treated ASMCs. 28 S rRNA served as a control.

wortmannin and Akt inhibitor attenuated TNF-α-induced NF-κB activation (Figure 4C), NF-κB-driven luciferase activity (Figure 4D) and fractalkine and CX3CR1 expression (Figure 4E), indicating that TNF-α activates NF-κB in ASMCs via two independent signalling pathways; one involving the activation of PI 3-kinase, Akt and IKK, and the other involving TRAF2, NIK and IKK.

Fractalkine activates NF-κB via heterotrimeric G_i-proteins

In the next series of experiments, we investigated whether fractalkine activated NF-κB in ASMCs and determined the role of heterotrimeric G-proteins in fractalkine-induced NF-κB activation. We employed RGS3, which accelerates GTPase activity of $G_{\alpha q}$ and $G_{\alpha i}$ and negatively modulates G_q/G_i-mediated signalling [20,35], as well as its phosphorylation-deficient mutant, RGS3 (S264A), which elicits higher potency due to inability to bind and to be scavenged by 14-3-3 [21]. To ascertain the role of G_i, we employed PTx, which ADP-ribosylates $G_{\alpha i}$ subunits and inhibits G_i-mediated signalling. As shown in Figure 5(A), fractalkine increased NF-κB activation at 15 min. Its levels increased further at 30 min and remained high until 24 h. Pretreatment with PTx or transient overexpression of wild-type RGS3 or mutant RGS3 (S264A) (expression was confirmed by Western blotting; Figure 5B) significantly inhibited fractalkine-mediated NF-κB activation (Figure 5C). As predicted, mutant RGS3 was more potent than wild-type RGS3 in inhibiting fractalkine-mediated NF-κB activation. These results indicate that fractalkine signals via heterotrimeric G_i-proteins to activate NF-κB.

Fractalkine activates PI 3-kinase and Akt kinase

GPCRs signal via a wide variety of signalling pathways, including activation of PI 3-kinase. Figure 5(D) demonstrates that fractalkine increased PI 3-kinase activity in p110γ immunoprecipitates (4.82-fold; $P < 0.001$ compared with untreated controls), and both PTx ($P < 0.01$) and wortmannin ($P < 0.005$) inhibited its activation. PDK1 acts as a downstream signalling molecule for PI 3-kinase-mediated 3′-phosphorylated phospholipids [34] and, in turn, acts on several downstream kinases, including Akt, resulting in its phosphorylation at Thr^{308}. Figure 5(E) shows that, although fractalkine had no effect on total Akt levels, it increased phospho-Akt (Thr^{308}) levels. Furthermore, pretreatment with wortmannin, but not DMSO, inhibited fractalkine-induced phospho-Akt levels (Figure 5F). Phosphorylation of Akt results in its activation and activated Akt, in turn, phosphorylates several downstream target proteins, including GSK-3. Akt negatively regulates GSK-3α/β kinase activity via phosphorylation at $Ser^{21/9}$. Figure 5(G) shows that fractalkine increased Akt kinase activity, as seen by increased phospho-GSK-3α/β

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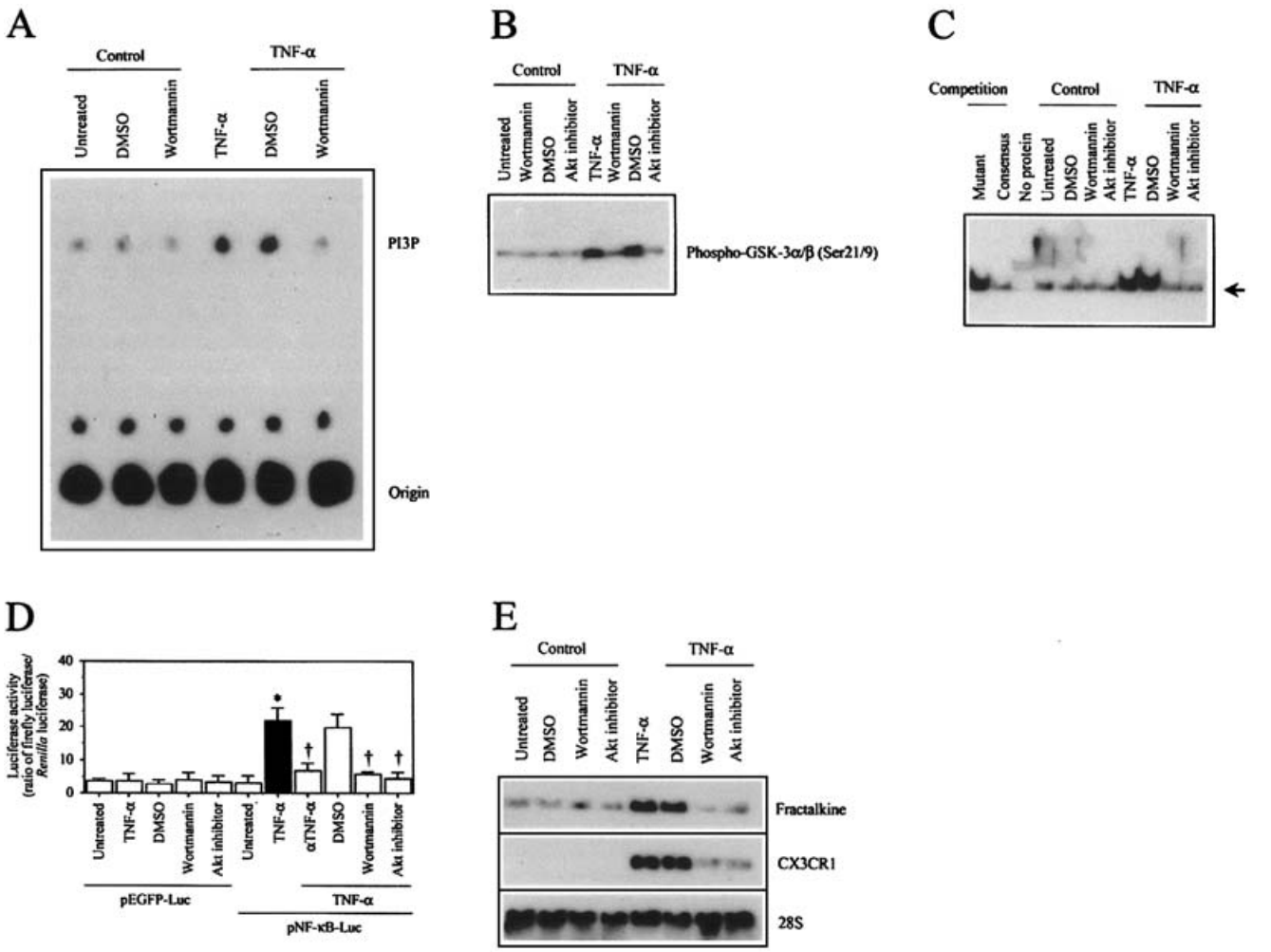

Figure 4 PI 3-kinase and Akt kinase mediate TNF-α-induced NF-κB activation in ASMCs

(**A**) PI 3-kinase lipid kinase assays. Rat ASMCs were treated with wortmannin or Akt inhibitor prior to TNF-α addition. p85 immunoprecipitates were prepared and analysed for PI 3-kinase activity using phosphatidylinositol as a substrate. The reaction products were separated by TLC. The location of one such product PtsIns(1,4,5)P_3 (PI3P) is shown. (**B**). Akt kinase activity was performed using a commercially available kit, and is based on the detection of Akt-mediated phosphorylation of GSK-3 (phospho-GSK-3α/β(Ser$^{21/9}$) by Western blotting. (**C**) NF-κB DNA-binding activity was determined by EMSA using nuclear protein extracts isolated from ASMCs pretreated with wortmannin or Akt inhibitor prior to TNF-α addition. Competition studies were performed as described in the legend to Figure 1(A). Arrow indicates NF-κB-specific DNA–protein complexes. (**D**) ASMCs transiently transfected with pNF-κB-Luc were pretreated with wortmannin or Akt inhibitor prior to TNF-α addition, and luciferase activity was determined as described in the Materials and methods section. pEGFP-Luc served as a control. $^*P < 0.001$ compared with untreated cells; $\dagger P < 0.05$ compared with TNF-α treated pNF-κB-Luc-transfected cells. (**E**) Northern blotting was performed to determine the role of PI 3-kinase and Akt in TNF-α-mediated fractalkine and CX3CR1 mRNA expression.

(Ser$^{21/9}$) levels, and both wortmannin and the Akt inhibitor attenuated its activity (Figure 5G), indicating that fractalkine signals via PI 3-kinase and Akt.

Fractalkine induces fractalkine expression via activation of NF-κB

Because fractalkine induced NF-κB activation, we next sought to determine whether fractalkine induces its own expression via NF-κB activation. Figure 6(A) shows that ASMCs express low levels of fractalkine mRNA at basal conditions and fractalkine up-regulated its own expression with peak levels detected at around 2 h. Nuclear run-on assays revealed increased fractalkine-induced fractalkine and CX3CR1 mRNA transcripts (fractalkine, 4.01-fold; $P < 0.001$ compared with control; CX3CR1, 6.81-fold; $P < 0.0001$ compared with control; Figure 6B). Actinomycin D pulse studies showed increased fractalkine-induced fractalkine mRNA stability (between 2.5–3 h compared with 1 h for control; Figure 6C), indicating both increased transcription and mRNA stability contributed to fractalkine-induced fractalkine mRNA expression. Furthermore, TNF-α antisense oligonucleotides (Figure 6E) or neutralizing TNF-α antibodies (results not shown) had no effect on fractalkine-induced NF-κB activation (Figure 6D), fractalkine mRNA expression (Figure 6E) and on secreted fractalkine protein levels (Figure 6F), indicating that fractalkine effects were direct and were not mediated by TNF-α, as shown previously for stromal cell-derived factor-1 α (SDF-1α) [15].

Because fractalkine activated NF-κB and increased its own expression, we next determined the signalling pathway involved in fractalkine-mediated NF-κB activation. Although treatment with wortmannin, Akt inhibitor or transient transfections with empty vectors had minimal or no effect on basal levels of NF-κB (results not shown), they significantly inhibited fractalkine-induced NF-κB activation (Figure 7A) and fractalkine mRNA

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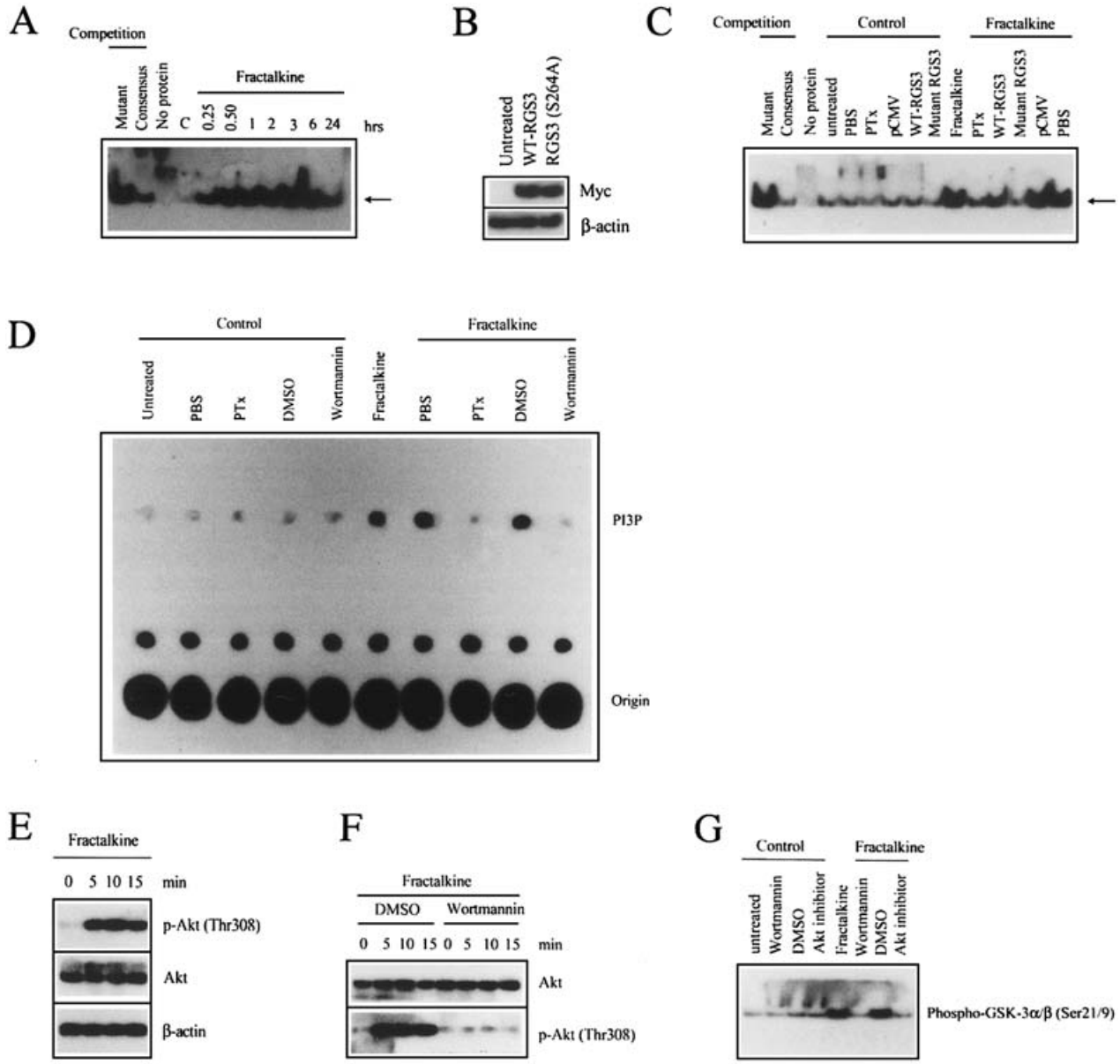

Figure 5 Fractalkine activates NF-κB via PTx-sensitive GPCR, PI 3-kinase and Akt

(**A**) EMSA was Rat ASMCs were treated with fractalkine (10 ng/ml) for the indicated periods, and nuclear protein extracts were isolated and analysed for NF-κB DNA-binding activity by EMSA. Competition studies were performed as described in the legend to Figure 1(A). Arrow indicates NF-κB-specific DNA–protein complexes. (**B**) Western blotting was performed to confirm the expression of recombinant proteins in ASMCs transiently transfected with the indicated expression vectors. Untransfected cells served as controls. (**C**) Effects of PTx, wild-type RGS3 (WT-RGS3) and mutant RGS3 on NF-κB DNA-binding activity were determined by EMSA. Treatment with PBS and transfection with empty vectors served as controls. Arrow indicates NF-κB-specific DNA–protein complexes. (**D**) PI 3-kinase lipid kinase assays were performed to determine whether fractalkine signals via PI 3-kinase using p110γ immunoprecipitates. (**E**) Levels of total Akt and fractalkine-induced phospho-Akt (Thr^{308}) were quantified by Western blotting. (**F**) Western blotting was performed to determine the effects of wortmannin on total Akt and fractalkine-induced phospho-Akt (Thr^{308}) protein levels. (**G**) Fractalkine treatment increased Akt kinase activity in ASMCs as seen by increased phospho-GSK3 levels in a Western blotting.

expression (Figure 7B). Transient transfection with dnIκB-α, kdIKK-β, kdPDK1 and kdNIK also had similar inhibitory effects, indicating that fractalkine autoregulation involves PI 3-kinase, PDK1, Akt, NIK, IKK and NF-κB.

Fractalkine promotes cell–cell adhesion and cellular proliferation

Activation of PI 3-kinase/Akt pathway has been shown to mediate survival signals [34]. Because fractalkine activated this pathway, we investigated its effects on cell adhesion, cell survival and cellular proliferation. Figure 8(A) shows that fractalkine significantly increased ASMC cell–cell adhesion as seen by increased calcein fluorescence ($P < 0.001$ compared with untreated cells), and antibody blockade of CX3CR1 attenuated cell adhesion ($P < 0.01$). Both [^{3}H]TdR incorporation (Figure 8B) and MTT cell proliferation assays (Figure 8C) revealed that fractalkine promoted ASMC proliferation. Although the effects were less pronounced during early periods, a significant increase in cell numbers was detected at day 5 post-treatment ([^{3}H]TdR incorporation, 36% more compared with controls at similar

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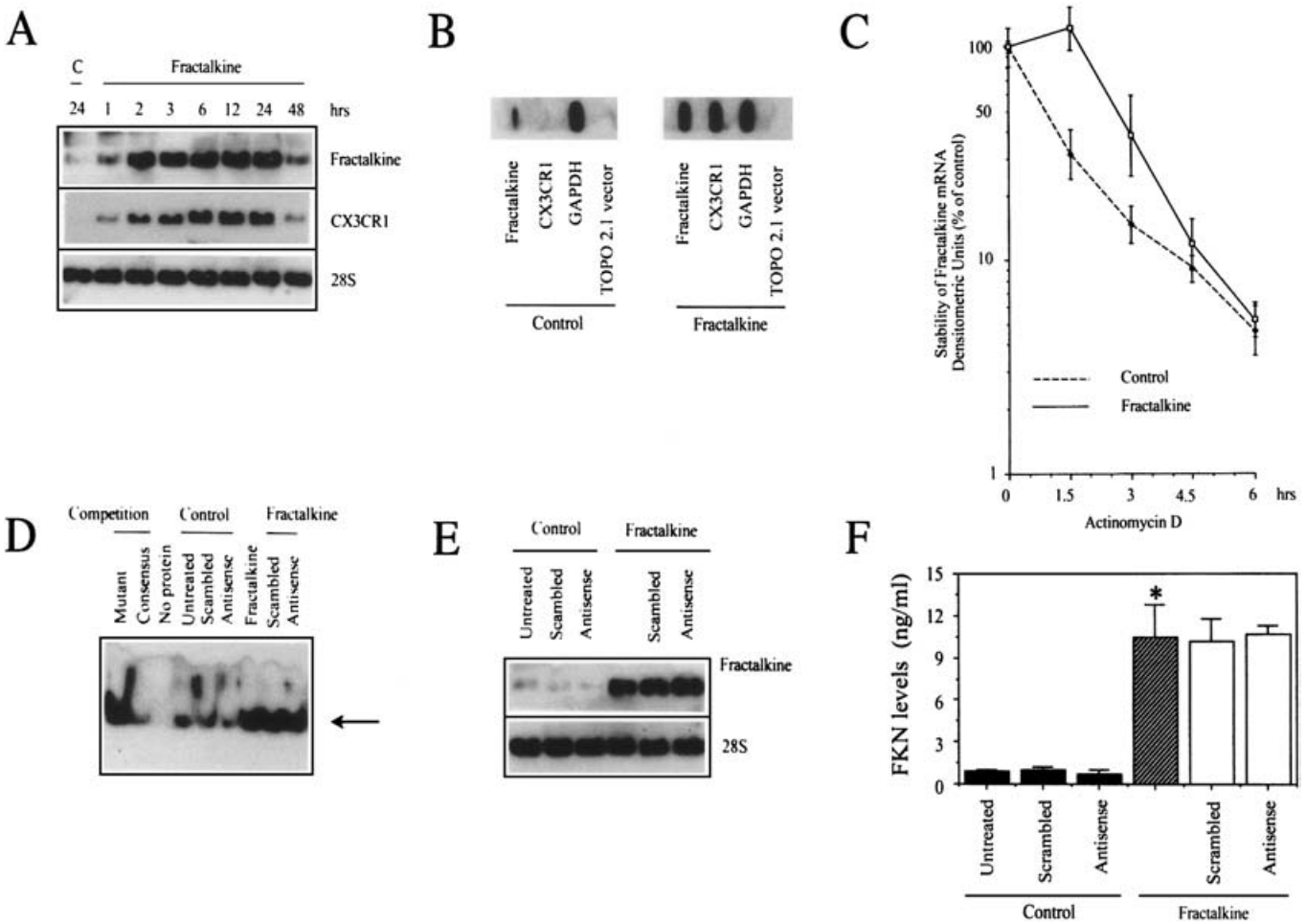

Figure 6 Fractalkine induces fractalkine mRNA expression via increased transcription and mRNA stability

(**A**) Fractalkine-induced fractalkine and CX3CR1 mRNA expression were analysed by Northern blotting. (**B**) Nuclear run-on assays revealed increased fractalkine-induced fractalkine mRNA transcription. GAPDH was used as an internal control. TOPO 2.1 empty vector served as a negative control. (**C**) Fractalkine mRNA stability was determined by actinomycin D pulse experiments. The autoradiographic signals obtained in Northern blotting were semi-quantified and equalized to respective 28 S rRNA. Fractalkine expression at 0 h of actinomycin D was considered as 100 %, and the results are shown as the percentage change from control. (**D**, **E**) To determine whether TNF-α mediates fractalkine-induced fractalkine expression, as shown previously for SDF-1α, ASMCs were treated with TNF-α antisense or scrambled oligonucleotides prior to fractalkine addition. NF-κB DNA-binding activity was measured by EMSA (**D**) and fractalkine mRNA expression by Northern blotting (**E**). (**F**) Secreted fractalkine (FKN) levels in culture supernatants were quantified at 24 h post-fractalkine treatment by ELISA. $^*P < 0.001$ compared with untreated cells.

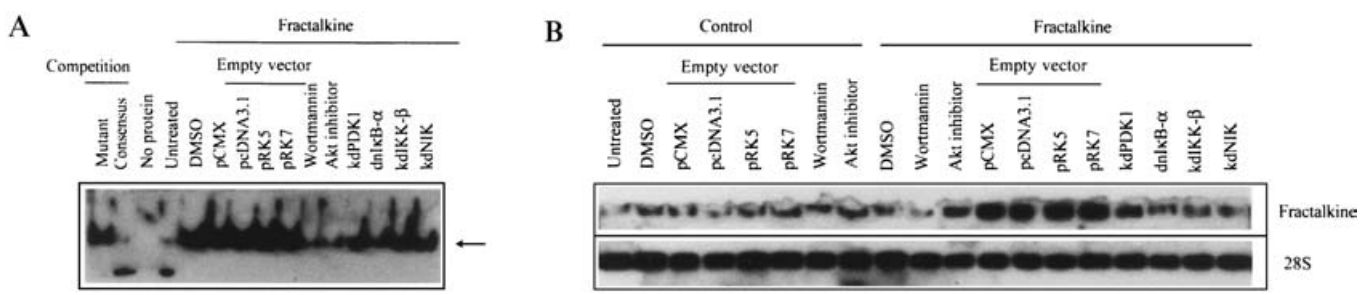

Figure 7 Fractalkine autoregulation involves PI 3-kinase, PDK1, Akt, NIK, IKK and IκB

NF-κB DNA-binding activity was measured by EMSA in fractalkine-treated ASMCs that were pretreated with PI 3-kinase or Akt kinase inhibitors, or transiently transfected with the indicated expression vectors. Pretreatment with DMSO and transfection with empty vectors served as controls. Competition studies were performed as described in the legend to Figure 1(A). Arrow indicates NF-κB-specific DNA–protein complexes. (**B**) Northern blotting was performed to determine the role of PI 3-kinase, Akt and NF-κB in fractalkine-induced fractalkine mRNA expression in ASMCs.

period of time; $P < 0.01$). Furthermore, treatment with fractalkine failed to induce cell death (Figure 8D).

Since one of the downstream targets of Akt is Bad and phosphorylation of Bad at Ser[112] renders it inactive and thereby preventing it from inactivating either Bcl-X_L or other anti-apoptotic members of Bcl-2 family [36,37], we considered this as a possible mechanism for the increased cell survival and proliferation. Immunoblotting using Bad- and phospho-Bad-specific

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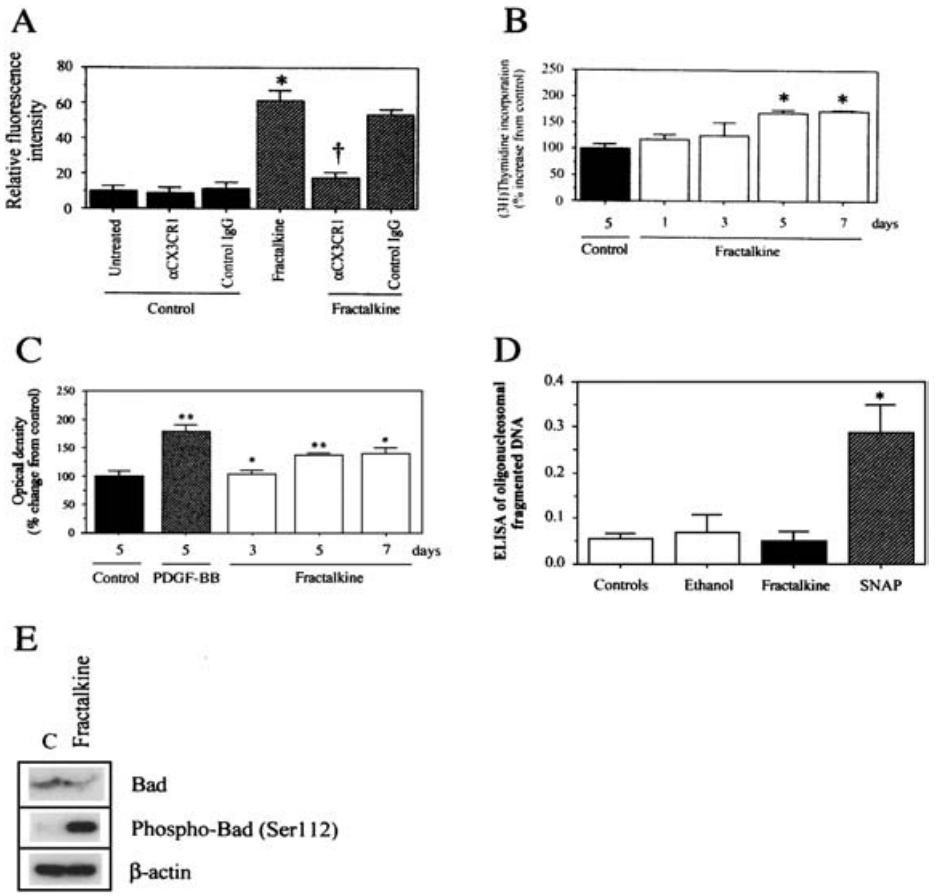

Figure 8 Fractalkine promotes ASMC cell–cell adhesion and cellular proliferation

(**A**) Cell adhesion assays were performed using Vybrant Cell Adhesion Assay kit as described in the Materials and methods section. Fractalkine-treated ASMCs loaded with calcein AM were overlayed on fractalkine-treated ASMCs, washed to remove non-adherent cells and the fluorescence remaining was measured in a spectrophotometer. Blocking experiments were performed by incubating cells with anti-CX3CR1 antibodies for 1 h prior to fractalkine addition. Normal IgG served as a control. $P < 0.001$ compared with untreated cells; †$P < 0.01$ compared with fractalkine. (**B**) Fractalkine-induced ASMC proliferation was determined by [^{3}H]TdR incorporation ($P < 0.01$ compared with control). (**C**) Fractalkine-induced ASMC proliferation was also determined by MTT assay. Platelet-derived growth factor-BB (PDGF-BB) was used as a positive control. *$P < 0.05$ and **$P < 0.001$ compared with control at 5 days. (**D**) Cell death was analysed by ELISA. *S*-nitroso-*N*-acetyl-DL-penicillamine (SNAP), a nitric oxide donor, served as a positive control. *$P < 0.001$ compared with controls and ethanol. (**E**) Western blotting was performed to study the effects of fractalkine on Bad and phospho-Bad (Ser^{112}) levels in ASMCs treated with fractalkine.

antibodies revealed increased phospho-Bad (Ser^{112}) levels following fractalkine treatment of ASMCs (Figure 8E). Collectively, these results indicate that fractalkine enhances cell–cell adhesion and cell proliferation.

DISCUSSION

Our results in the present study indicate that rat ASMCs express fractalkine and CX3CR1 and their co-expression leads to an autocrine effect of fractalkine-induced fractalkine expression. We also determined that these autocrine effects are mediated via heterotrimeric G-proteins, PI 3-kinase, PDK1, Akt, IKK and NF-κB (Figure 9). Furthermore, fractalkine autocrine regulation increased ASMC cell–cell adhesion and cellular proliferation. We demonstrated further that TNF-α, a proinflammatory cytokine, induced expression of fractalkine and CX3CR1 in ASMCs by PI 3-kinase and NF-κB activation.

Fractalkine is the only ligand so far discovered for CX3CR1 [5]. It acts both as an adhesion molecule and a chemoattractant. It functions as a chemoattractant and proinflammatory cytokine by attracting activated leucocytes to the site of inflammation or injury, and acts as an adhesion molecule by binding to CX3CR1 expressed on leucocytes. Our present study demonstrates that TNF-α and fractalkine not only up-regulated fractalkine mRNA and protein expression, but they also increased fractalkine secretion, indicating that ASMC-expressed fractalkine may exert both adhesion and chemoattractant effects.

A wide array of inflammatory stimuli, including interleukin-1β, TNF-α and LPS, induce fractalkine expression in ventricular endocardium, coronary endothelial cells and ASMCs [9–11]. Thus fractalkine, once induced, may contribute to vascular injury and atherogenesis [4,38]. In the present study, we demonstrated that TNF-α induces the expression of fractalkine and CX3CR1 via two signalling pathways both converging at IKK activation. Binding of inherently trimeric TNF-α to TNF-α-receptor 1 (TNFR1) induces receptor trimerization and recruitment of several signalling proteins to the cytoplasmic domains of the receptors [39,40]. Binding to TNFR1 recruits TRADD (TNFR1-associated death domain protein) followed by receptor-interacting protein ('RIP') and TRAF2. The TRADD–TRAF2 complex then leads to activation of NIK, IKK and NF-κB. In ASMCs, transient transfection with dnTRAF2 (but not dnTRAF6), dnIKK-γ, dnIκB-α, dnIκB-β, kdNIK and kdIKK-β inhibited the

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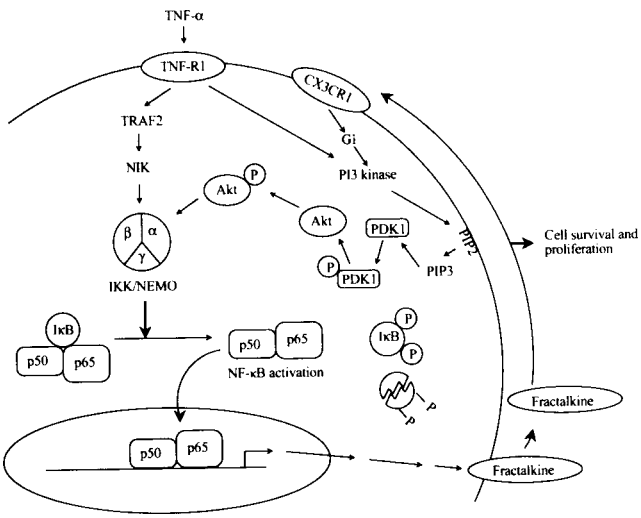

Figure 9 Possible signalling pathways involved in TNF-α- and fractalkine-induced NF-κB activation, fractalkine induction and ASMC proliferation

The proinflammatory cytokine TNF-α activates NF-κB in ASMCs via two different signalling pathways, both converging at IKKs. Activation of NF-κB occurred via TNFR1, TRAF2, NIK and IKK. TNF-α also induced NF-κB activation via PI 3-kinase, Akt, NIK and IKK. Activation of NF-κB resulted in the induction of fractalkine and CX3CR1 expression. Fractalkine induced its own expression via PI 3-kinase, PDK1, Akt, NIK and IKK, and fractalkine autoregulation is mitogenic to ASMCs.

TNF-α-induced expression of fractalkine and CX3CR1. Predictably, treatment with MG-132, a proteasomal inhibitor, inhibited the TNF-α-induced expression of fractalkine and CX3CR1. Furthermore, pretreatment with PDTC, a free radical scavenger [41], decreased TNF-α-induced NF-κB activation and fractalkine expression, indicating that fractalkine and CX3CR1 are oxidative-stress-responsive genes.

TNF-α is also known to activate survival signals via activation of PI 3-kinase/Akt signalling pathway. In the present study, we also investigated whether TNF-α signals via PI 3-kinase and Akt in inducing the expression of fractalkine and CX3CR1 in ASMCs. Indeed, pretreatment with wortmannin and Akt inhibitor attenuated TNF-α induced PI 3-kinase and Akt kinase activities as well as NF-κB activation. Although the present study demonstrates that NF-κB activation is downstream of PI 3-kinase and Akt, Meng et al. [42] have demonstrated the opposite, that is Akt is a downstream target of TNF-α and LPS-induced NF-κB activation in NIH3T3 cells and primary cerebellar granule neurons. In their study, stimulation of Akt was detected only after IκB-α degradation following TNF-α or LPS treatment. Activation of NF-κB and translocation of NF-κB p65 occurred before Akt kinase activation, and pretreatment with NF-κB inhibitors blocked TNF-α-induced Akt activation. In contrast, pretreatment with wortmannin inhibited activation of PI 3-kinase and Akt, but failed to modulate NF-κB activation, placing NF-κB upstream of PI 3-kinase and Akt. These results are in agreement with several other reports [32], which demonstrated that PI 3-kinase and Akt lie upstream of NF-κB. Akt has also been shown to directly associate with IKKs. Akt activates IKK-α by phosphorylating it at Thr^{23}, and mutation of this amino acid has also been shown to block Akt-induced IKK-α phosphorylation and NF-κB activation [32]. Taken together, these studies indicate that TNF-α signals via diverse and complex signalling pathways that culminate in NF-κB activation and fractalkine and CX3CR1 expression in ASMCs.

Fractalkine exerts its biological effects by binding to CX3CR1. However, Inngjerdingen et al. [43] have recently proposed that fractalkine may signal via receptors other than CX3CR1. These workers demonstrated that non-activated and non-adherent NK cells that did not express surface CX3CR1 responded to fractalkine in promoting chemotaxis, and neutralizing anti-CX3CR1 antibodies failed to modulate fractalkine-induced NK cell chemotaxis. Although we do not rule out this possibility, our results clearly indicate that ASMCs express both fractalkine and CX3CR1 at the mRNA and protein level.

Our present studies also indicate that fractalkine signals via heterotrimeric G-proteins, and the PI 3-kinase/Akt pathway plays a role in CX3CR1 signal transduction. This is in agreement with the observations of Meucci et al. [44], who demonstrated that fractalkine mediates its protective effects in hippocampal neurons through the Akt pathway. Although treatment with fractalkine induced NF-κB activation, overexpression of the regulator of G_q/G_i-mediated signalling RGS3 and its more potent mutant RGS3 (S264A) [21], as well as pretreatment with G_i-specific PTx, attenuated fractalkine-induced NF-κB activation, indicating that G_i proteins play a role in fractalkine signalling. Interestingly, blocking G-protein function by PTx was shown previously [45] to have no effect on fractalkine-induced cell adhesion, suggesting that the signalling pathways leading to adhesion and NF-κB activation may be different.

Another significant outcome of the present study is that fractalkine autoregulation increases cell–cell adhesion and cellular proliferation. Both cell adhesion and cell proliferation play a pathological role in atherosclerosis and restenosis following transluminal coronary angioplasty. Wong et al. [46] have demonstrated fractalkine immunoreactivity in intima, media and adventia in atherosclerotic coronary arteries. Within the atherosclerotic lesions, macrophages, foam cells and SMCs showed positive immunoreactivity. Furthermore, Faure et al. [47] and McDermott et al. [48] have recently identified gene polymorphisms in human CX3CR1 at amino acids 249 ($Val^{249} \rightarrow Ile$) and 280 ($Thr^{280} \rightarrow Met$), and Moatti et al. [49] have shown that Ile^{249} heterozygosity was associated with a significantly decreased CX3CR1 expression and fractalkine binding activity and was associated with a markedly decreased risk of acute coronary events. Although low basal expression of fractalkine and CX3CR1 may not be sufficient enough to induce more fractalkine and fractalkine-mediated SMC proliferation under physiological conditions, when expressed at high levels they may play a pathological role. Taken together, results from the present study and those reported previously indicate that the fractalkine/CX3CR1 axis could play an important role in the pathogenesis of atherosclerosis.

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