CXCL16 Signals via $G_i$, Phosphatidylinositol 3-Kinase, Akt, IκB Kinase, and Nuclear Factor-κB and Induces Cell-Cell Adhesion and Aortic Smooth Muscle Cell Proliferation*

Bysani Chandrasekar‡, Sailaja Bysani, and Srinivas Mummidi§

From the Department of Medicine, The University of Texas Health Science Center, San Antonio, Texas 78229-3900

CXCL16, a recently discovered transmembrane chemokine, is expressed in human aortic smooth muscle cell (ASMC). It facilitates uptake of low density lipoproteins by macrophages, resulting in foam cell formation. However, it is not known whether ASMC express CXCR6, the receptor for CXCL16, or whether CXCL16 affects ASMC biology. To dissect the biological and signal transduction pathways elicited by CXCL16, human aortic smooth muscle cells (HASMC) were treated with pharmacological inhibitors or transiently transfected with pathway-specific dominant-negative or kinase-dead expression vectors prior to the addition of CXCL16. HASMC expressed CXCR6 at basal conditions. Exposure of HASMC to CXCL16 increased NF-κB DNA binding activity, induced κB-driven luciferase activity, and up-regulated tumor necrosis factor-α expression in an NF-κB-dependent manner. However, treatment with pertussis toxin (G$i$ inhibitor), wortmannin or LY294002 (phosphatidylinositol 3-kinase (PI3K inhibitors)), or Akt inhibitor or overexpression of dominant-negative (dn) PI3K, dnPDK-1, kinase-dead (kd) Akt, kdIKK-β, dnIKK-γ, dnIκB-α, or dnIκB-β significantly attenuated CXCL16-induced NF-κB activation. Furthermore, CXCL16 increased cell-cell adhesion and induced cellular proliferation in an NF-κB-dependent manner. In conclusion, CXCL16 is a potent and direct activator of NF-κB and induces κB-dependent proinflammatory gene transcription. CXCL16-mediated NF-κB activation occurred via heterotrimeric G proteins, PI3K, PDK-1, Akt, and IκB kinase (IKK). CXCL16 induced IκB phosphorylation and degradation. Most importantly, CXCL16 increased cell-cell adhesion and induced κB-dependent ASMC proliferation, indicating that CXCL16 may play an important role in the development and progression of atherosclerotic vascular disease.

CXCL16 is a member of the CXC chemokine subfamily. Unlike other members of this subgroup, CXCL16 is structurally different and has four distinct domains: a chemokine domain tethered to the cell surface via a mucin-like stalk, which in turn is attached to transmembrane and cytoplasmic domains (1, 2). Fractalkine (CX3CL1), the only member described so far in the CX3C family (3), has a similar structure to that of CXCL16, and both CXCL16 and fractalkine act as adhesion molecules when expressed on cell surface, and upon cleavage from cell surface, the soluble chemokines act as chemoattractants (1–3). CXCL16 attracts CXCR6 expressing T, NK, and NKT cells to the site of inflammation/injury (4–8). Recently, Nakayama et al. (9) have reported enrichment of CXCR6 expressing plasma cells and myeloma cells in bone marrow and other target tissues via CXCR6/CXCL16 interaction. In addition, CXCL16, via its chemokine domain, facilitates phagocytosis of Gram-positive and Gram-negative bacteria by the antigen presenting macrophages and dendritic cells (10).

CXCL16 is originally identified as a scavenger receptor for phosphatidylserine and oxidized lipoprotein (SR-PSOX) (11). In human atherosclerotic lesions, Minami et al. (12) have localized CXCL16 expression predominantly to lipid-laden macrophages accumulated in the intima. In contrast, normal arterial wall failed to show CXCL16 immunoreactivity. However, in cultured aortic smooth muscle cells, Hofnagel et al. (13) have demonstrated CXCL16 expression by RT-PCR. 1 These reports indicate that CXCL16 is expressed in atherosclerotic lesions and that both macrophages and ASMC express CXCL16, and suggest that CXCL16 may play a role in the pathobiology of atherosclerosis. However, it is not known whether ASMC express CXCR6 and whether CXCL16 affects ASMC biology. In the present study, we demonstrate for the first time that human ASMC express CXCR6 and that treatment with CXCL16 activates NF-κB via heterotrimeric G proteins/Pi3K/PDK-1/Akt/IKK/IκB. Most importantly, treatment of ASMC with CXCL16 increases cell-cell adhesion and cellular proliferation.

1 The abbreviations used are: RT, reverse transcription; ADAM, a disintegrin and a metalloprotease; ASMC, aortic smooth muscle cell; HASMC, human ASMC; Bad, Bcl-2/Bcl-XI-antagonist; dn, dominant-negative; kd, kinase-dead; EGFP, enhanced green fluorescent protein; ELISA, enzyme-linked immunosorbent assay; EMSA, electrophoretic mobility-shift assay; FLAG, Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GSK-3, glycogen synthase kinase-3; HA, hemagglutinin; IκB, inhibitory κB; IKK, IκB kinase; NF-κB, nuclear factor κB; MTT, 3(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide; PI3K, phosphoinositide 3-kinase; PI, phosphoinositide; PDK-1, phosphoinositide-dependent kinase-1; PDTC, pyrrolidinecarboxibolic acid ammonium salt; siRNA, small interfering RNA; TACE, TNF-α-converting enzyme; TNF-α, tumor necrosis factor-α; PBS, phosphate-buffered saline; SNAP, soluble NSF attachment protein; Luc, luciferase; h, human.
FIG. 1. CXCL16 activate NF-κB and induces κB-dependent luciferase activity in human aortic smooth muscle cells. ASMC express CXCR6 as seen by RT-PCR (A) and FACS analysis (B). – ve, negative control. Treatment with CXCL16 increased NF-κB DNA binding activity in a time-dependent manner with peak levels detected at 1 h following CXCL16 treatment (C). The dose-response studies at 1 h indicate peak levels of NF-κB activity at 50 ng/ml (D). Arrows in panels C and D indicate specific DNA-protein complexes, and solid circles at the bottom denote unincorporated labeled probe. Lanes 1–3 in panels C and D are defined as follows. Lane 1, competition with mutant NF-κB oligonucleotide. Protein extract from ASMC treated with CXCL16 for 1 h was preincubated with a 100-fold molar excess of unlabeled double-stranded mutant NF-κB oligonucleotide followed by the addition of 32P-labeled consensus B probe. Lane 2, competition with consensus NF-κB oligonucleotide. Protein extract from ASMC treated with CXCL16 for 1 h was preincubated with a 100-fold molar excess of unlabeled double-stranded consensus NF-κB oligonucleotide followed by the addition of 32P-labeled consensus B probe. Lane 3, no protein extract but contains 32P-labeled consensus κB probe. Results from EMSA were further confirmed in transient transfection assays (E). Similar to the EMSA results, CXCL16 (50 ng/ml) also induced κB-driven luciferase activity in ASMC, *, p < 0.001 (versus pEGFP-Luc transfected CXCL16-treated cells and pNF-κB-Luc-transfected control cells).
FIG. 2. **CXCL16 induces NF-κB activation in ASMC via heterotrimeric G proteins, PI3K, PDK-1, Akt, IKK and IκB phosphorylation.**

**A**, PI 3-kinase lipid kinase assays. Human ASMCs were treated with pertussis toxin (PTx) and wortmannin prior to CXCL16 addition. p110γ immunoprecipitates were prepared and analyzed for PI 3-kinase activity using phosphatidylinositol as a substrate. The reaction products were separated by TLC. The location of one such product, PtdIns(1,4,5)P3 (PI3P), is shown. As shown in **B**, phosphorylation at Thr308 activates Akt. ASMC were treated with CXCL16 (50 ng/ml) for the indicated periods. Whole cell lysates were prepared and analyzed for total Akt and phospho-Akt (Thr308) levels by Western blotting. β-Actin was used as an internal control and shows similar levels of protein loading/lane.

**DMSO**, Me2SO. As shown in **C**, ASMC were pretreated with wortmannin or Akt inhibitor prior to CXCL16 addition, and Akt kinase activity was analyzed using a commercially available kit, which is based on the detection of Akt-mediated phosphorylation of GSK-3 (phospho-GSK-3α/β(Ser21/9)) by Western blotting. As shown in **D**, NF-κB DNA binding activity was analyzed by EMSA using nuclear protein extracts isolated from ASMCs treated...
Recombinant human CXCL16 (chemokine domain), human platelet-derived growth factor-BB, neutralizing anti-human TNF-α antibodies, and normal goat IgG were obtained from B&BD Systems (Minneapolis, MN). Anti-IκB-α, anti-p110γ, and anti-β-actin antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against phospho-IκB-α (Ser32), Akt, phospho-Akt (Thr308), Bad, phospho-Bad (Ser387), and NF-κB p65 were purchased from Cell Signaling Technology (Beverly, MA). Antibodies against FLAG, Myc, and HA were from Sigma, Roche Applied Science, and Covance Inc. (Princeton, NJ). Normal rabbit IgG (control IgG) was from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). Ham’s-F12 medium was from American Type Culture Collection (Manassas, VA). [3H]Thymidine (6.7 Ci/mmol) was from PerkinElmer Life Sciences. Endothelial cell growth supplement was from The Upstate Company (Charottesville, VA). Unless otherwise indicated, all other chemicals were purchased from Sigma.

**Cell Culture**—Human aortic smooth muscle cells (ASMC) were purchased from ATCC and were grown in Ham’s-F12 medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum and endothelial cell growth supplement (0.03 mg/ml). When cells reached 70–80% confluency, the medium was replaced with Ham’s-F12 containing 0.5% bovine serum albumin. 24 h later, rhCXCL16 or vehicle (PBS, pH 7.4) was added for the indicated time periods. Treatment with CXCL16 (up to 500 ng/ml) for up to 48 h did not induce cell death, as evidenced by trypan blue dye exclusion. To determine the role of heteroclinic G proteins, PI3K, PDK-1, and Akt, in CXCL16 signal transduction, ASMCs were transiently transfected with dnPI3K, dkPDK-1, or dnAkt or pretreated for 1 h with pertussis toxin (100 ng/ml in PBS), wortmannin (a specific PI3K inhibitor; 50 μM in Me2SO), Li294002 (a specific PI3K inhibitor; 20 μM in Me2SO), Akt inhibitor (1 μM in Me2SO), PBS, or Me2SO prior to the addition of CXCL16. To determine whether CXCL16 mediates NF-κB activation via free radical generation, ASMCs were pretreated for 1 h with pyrrolidinecarbodithioic acid amidonium salt (PDTC; 100 μM in PBS; Sigma), a free radical scavenger, or MG-132 (5 μM in Me2SO; Calbiochem-Novabiochem), a proteasome inhibitor, prior to CXCL16 addition.

**Electrophoretic Mobility Shift Assay (EMSA)**—NF-κB DNA binding activity was measured by EMSA as described previously (14, 15). **Transcript Transfections and Reporter Assays**—ASMCs were transfected with dominant-negative (dn) or kinase-dead (kd) expression vectors using LipofectAMINE Plus (Invitrogen) essentially as described previously (14, 15). The phosphorylation-deficient mutant of IkBα (pCMX-IκBα (S32A/S36A)), dnlIkBβ (pCMV-Tag3B-l-IκBβ (S19A/S32A-Myc)), dnlIKK-γ (pDNA3-IKK-γ-HA), dkPDK-1 (pDNA3-1-PDK-1-K114Q-Myc-HA+), dkIkBβ (pDNA3-1-IκBβ-FLAG), and dnlIKK-γ (pDNA3-IKK-γ-HA) have been described previously (14, 15). dmPDK3y (pDNA3-p110y-R833R) was a gift from Dr. M. P. Wymann (University of Fribourg, Fribourg, Switzerland). dkAkt (pDNA-A+Akt-K179A, T308A, 547SA) was obtained from Dr. Lester Liou (University of Texas Health Science Center, San Antonio, TX). Corresponding empty vectors were used as controls. Total RNA was isolated from transfected cells by the TRIzol method. Cells were co-transfected with pRL- Renilla luciferase vector (pRL-TK vector; Promega, Madison, WI). Transfection efficiency of ASMCs was determined using pEGFP-N1 and found to be 34.8% ± 2.13%. siRNA for TACE (sense, 5'-AGUUUGCUUGGACCACTTdTdT-3', 5'-AGUAAGGCCAACCGAGUUttdTdT-3', 5'-CAUGAAGCACCUGUUGUtttdTdT-3', 5'-CAUGAAGCACCUGUUGUtttdTdT-3', and 5'-CCUGCUUGCAGAAGAUGUtttdTdT-3') was described previously (16).

**Analysis of mRNA Expression**—RT-PCR was performed using the following gene-specific primers: CXCR6 (GenBank accession number AF007545, 763 bp; sense, 5'-CTGCTTGTTTGTGTCGTGSG-3' (bases 196–215), antisense, 5'-GGCTGTGACAGGAGCATAGAC-3' (bases 939–958); ADAM12 (GenBank accession number NM_021411, 479 bp), sense, 5'-GGCAAAATCTCAAGAGTTTC-3' (bases 1950–2012), antisense, 5'-AGCAAGAAAGCAGAGTGGT-3' (bases 2472–2452); and GAPDH (GenBank accession number BT008893, 1,442 bp), sense, 5'-ATACGCTGCAACCGAAAG-3' (bases 541–560), antisense, 5'-AATGAGTCACACCCCTGTT-3' (bases 983–984). No amplification was observed when reverse transcriptase was replaced with water during cDNA synthesis or when cDNA was replaced with water during PCR. NF-κB mRNA expression was analyzed by RNase protection assay using RiboQuant RNase protection assay kit (PharMingen). The template antisense 32P-labeled cRNA probes were specific for hTNF-α (unprotected 436 nucleotides, protected 407 nucleotides; PharMingen). 2 μg of hGAPDH (124 unprotected and 96 protected nucleotides) served as an internal control, with results expressed as a ratio of the specific gene to the corresponding hGAPDH mRNA.

**Western Blotting, ELISA, and FACS Analysis**—Extraction of protein homogenates, Western blotting, autoradiography, and densitometry were performed as described previously (14, 15). β-Actin was used as an internal control. TNF-α levels in culture supernatants were determined using human Quantikine colorimetric sandwich ELISA (R&D Systems). Surface expression of CXCR6 was analyzed by FACS analysis.

**Measurement of PI 3-Kinase and Akt Kinase Activities**—PI 3-kinase lipid kinase assays were performed in p110y immunoprecipitates essentially as described previously (14). Akt kinase activity in ASMCs was performed using a commercially available kit (Cell Signaling Technology); this assay is based on Akt-induced phosphorylation of glycogen synthase-3 (GSK-3) (14).

**Cell Proliferation, Cell Adhesion, and Cell Death Detection**—Cell proliferation was assessed by [3H]thymidine incorporation. Cell death was analyzed by Cell Death Detection ELISA PLUS kit (Roche Applied Science). S-nitroso-N-acetyl-penicillamine (SNAP; 500 μM in ethanol), a nitric oxide donor, was used as a positive control (14). To determine the role of NF-κB in CXCL16-mediated cellular proliferation, ASMCs were transfected with expression vectors for human p65 or IKK-α siRNA (Ingemex, San Diego, CA). Vector containing scrambled control (SC) or IKK-α siRNA (IKK-α-G500-6) was used as a control. Cells were transfected with 3 μg of plasmid using LipofectAMINE PLUS; 3 days later, cells were treated with CXCL16 for 5 days. Knockdown of p65 and IKK-α was confirmed by Western blotting using primary antibodies supplied by the manufacturer. Cell adhesion assays were performed using a commercially available kit (Vibrant cell adhesion assay kit; Molecular Probes, Eugene, OR) (14).

**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 analysis of variance with post hoc Dunnett’s t tests. All assays were performed at least three times, and the error bars in the figures indicate the S.E.

**RESULTS**

**Human ASMC Express CXCR6**—It is not known whether HASMC express CXCR6. Using gene-specific primers, we have analyzed total RNA isolated from ASMC by RT-PCR. Our results indicate that human ASMC express CXCR6 at basal conditions (Fig. 1A). These results were further confirmed by FACS analysis that revealed surface expression of CXCR6 on ASMC (Fig. 1B).

**CXCL16 Activates NF-κB DNA Binding Activity and Induces κB-driven Luciferase Activity**—CXCL16 and fractalkine are the

with pertussis toxin, wortmannin, Akt inhibitor, MG-132, PDTC, or corresponding vehicle prior to CXCL16 addition. Competition studies were performed as described in the legend for Fig. 1, panels C and D. The arrow indicates NF-κB-specific DNA-protein complexes. As shown in E, ASMCs transiently transfected with pNF-κB-Luc were pretreated with pertussis toxin, wortmannin, Akt inhibitor, dnPI3K7x, hdPDK-1, or PDTC or corresponding vehicle prior to CXCL16 addition, and luciferase activity was determined as described under “Materials and Methods.” pEGFP-Luc served as a control. *p < 0.0001 when compared with control cells treated with the inhibitors alone; †p < 0.01 when compared with CXCL16 treated pNF-κB-Luc transfected cells. As shown in F, ASMCs were transiently transfected with dnIkBα, dnIkBβ, dnIkBγ, or dkPDK-1 expression vectors. Cells transfected with empty vectors (pCMX, pCMV-Tag3B, pR5K, pDNA3, and pDNA3.1) served as controls. 24 h after transfection, the medium was changed, and the cells were treated with CXCL16 (50 ng/ml) for 1 h (expression of recombinant proteins was confirmed by Western blotting; panel F). The results indicate that overexpression of dnIkBα, or dnIkBβ inhibited CXCL16-induced NF-κB activation and κB-driven luciferase activity (B). Similar results were obtained by the overexpression of dkIkB-β, dkPDK-1, and dkIkB-γ. To investigate the role of NF-κB in DNA binding and degradation, ASMCs were treated with CXCL16 (50 ng/ml) for up to 60 min. At the indicated time periods, nucleus-free protein homogenates were prepared and analyzed for total IκB-α and phosphorylated-IκB-α (Ser32) levels by Western blots. β-Actin was used as an internal control. Our results indicate that CXCL16 induces IκB-α phosphorylation followed by degradation (I).
FIG. 3. CXCL16 treatment up-regulates the eB-responsive proinflammatory cytokine TNF-α, increased transcription rate, but not mRNA half-life in human ASMC. After ASMC reached 70–80% confluency, the complete medium was replaced with medium containing 0.5% bovine serum albumin. After overnight culture, CXCL16 (50 ng/ml) was added, and the incubation continued for up to 4 h. At the end of experimental period, medium was separated, and the cells were rinsed with ice-cold PBS. Cells were then processed for mRNA expression by RNase
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two known transmembrane chemokines described so far. We have demonstrated previously that fractalkine is a potent inducer of NF-κB activation in ASMC (14). However, it is not known whether CXCL16 exerts similar biological effects in HASMC. Fig. 1C shows an EMSA and indicates that HASMC express low levels of NF-κB at basal conditions, and treatment with rhCXCL16 (50 ng/ml) increases its activation. An increase in NF-κB DNA binding activity was detected at 30 min. Its levels peaked at 1 h, persisted at high levels up to 4 h, and decreased gradually by 48 h (Fig. 1C). Fig. 1D is a dose-response study and shows peak levels of NF-κB activation at 50 ng/ml. No further increase in NF-κB activation was detected when CXCL16 concentrations were increased to 100 ng/ml. Therefore, in all subsequent EMSAs, CXCL16 was used at 50 ng/ml for 1 h. We further confirmed our EMSA results in transient cell transfection assays using pNF-κB-Luc that contained five tandem repeats of NF-κB-driven luciferase activity (6.8-fold, p < 0.001; Fig. 1E).

CXCL16 Signals via Heterotrimeric G Proteins, PI3K, Akt, and IKK—In the next series of experiments, we investigated possible signal transduction pathways involved in CXCL16-mediated NF-κB activation. At first, we verified whether CXCL16 transduces signals via heterotrimeric G proteins. In fact, treatment with pertussis toxin significantly attenuated CXCL16-mediated NF-κB activation (Fig. 2D) and NF-κB-driven luciferase activity (Fig. 2E). Because G-protein-coupled receptors transduce signals via diverse signaling pathways including activation of PI3K and Akt (see Ref. 23), we explored the role of PI3K and Akt in CXCL16-mediated NF-κB activation. Although treatment with CXCL16 increased PI3K activity in p110γ immunoprecipitates (5.1-fold; p < 0.001 versus untreated controls), both pertussis toxin (p < 0.01) and wortmannin (p < 0.005) inhibited its activation (Fig. 2A). Similar results were obtained when LY294002 was used (data not shown), indicating that CXCL16 signals via heterotrimeric G proteins/PI3K. We next studied the effects of CXCL16 on activation of Akt, a downstream signaling molecule to PI3K. Fig. 2B shows that CXCL16 indeed induced Akt activation as evidenced by increased p-Akt (Thr189) levels. In addition, CXCL16 increased Akt kinase activity, as seen by increased phospho-GSK-3α/β (Ser21/Ser27) levels (Fig. 2C), and both wortmannin and Akt inhibitor attenuated Akt activation (Fig. 2B) and Akt kinase activity (Fig. 2C). Furthermore, treatment with wortmannin, Akt inhibitor, or overexpression of dnPI3K, kdPDK-1, kdAkt attenuated CXCL16-induced NF-κB activation (Fig. 2D) and NF-κB-driven luciferase activity (Fig. 2E), indicating that CXCL16 signals via heterotrimeric G proteins/PI3K/PDK-1/Akt.

One of the downstream signaling molecules of Akt is IKK (18). In addition to direct interaction, Akt has been shown to activate IKK and IKK-mediated IκB phosphorylation and degradation (19). Therefore, we carried out transient transfections with kdIKK-β or dnIKK-γ (expression of recombinant proteins was confirmed by Western blotting (Fig. 2F)). Our results indicate that overexpression of kdIKK-β or dnIKK-γ significantly attenuated CXCL16-mediated NF-κB activation (Fig. 2G) and κB-driven luciferase activity (Fig. 2H). Activation of NF-κB occurs following IκB phosphorylation and degradation. Fig. 2I shows that CXCL16 induced IκB-α phosphorylation in a time-dependent manner with increased phosphorylated-IκB-α (Ser32) levels detected at 20 min. Furthermore, overexpression of phosphorylation-deficient mutants of IκB-α (S32A/S36A) or IκB-β (S19A/S23A) or pretreatment with the proteasomal inhibitor MG-132 attenuated CXCL16-mediated NF-κB activation (Fig. 2J) and κB-driven luciferase activity (Fig. 2, E and H). However, PDTC, a free radical scavenger, failed to modulate CXCL16-mediated NF-κB activation (Fig. 2, D and E), suggesting that free radicals may not play a role in CXCL16-mediated NF-κB activation in ASMC.

CXCL16 Induces TNF-α Expression Via NF-κB Activation—TNF-α is a κB-responsive proinflammatory cytokine and plays a role in atherosclerotic vascular disease by inducing the expression of adhesion molecules and chemokines involved in monocyte-ASMC/endothelial cell interactions. As CXCL16 is a strong inducer of NF-κB in ASMC, we determined whether CXCL16-induced NF-κB activation leads to TNF-α induction in ASMC. ASMC expressed low basal levels of TNF-α mRNA (Fig. 3A) and protein (Fig. 3B), and treatment with CXCL16 significantly increased its mRNA (9-fold, p < 0.01 versus untreated control; Fig. 3A), protein (11-fold, p < 0.001, Fig. 3B), and secretion (8-fold, p < 0.001, Fig. 3C). Furthermore, increased transcription (Fig. 3E), but not mRNA stability (Fig. 3F), contributed to CXCL16-induced TNF-α mRNA expression. We next determined whether TNF-α induction by CXCL16 is in fact mediated via NF-κB activation. ASMC were either transfected with p65 siRNA expression vector or treated with MG-132 prior to CXCL16 addition. Transfection with siRNA for NF-κBp65 (knockdown of p65 protein was confirmed by Western blotting; Fig. 3D) or pretreatment with MG-132 significantly attenuated CXCL16-induced TNF-α mRNA expression (mRNA, Fig. 3A; protein, Fig. 3B; and secretion, Fig. 3C), indicating that CXCL16 is an inflammatory chemokine, and induces TNF-α expression via activation of NF-κB.

Effects of CXCL16 on NF-κB Activation Are Direct and Are Not Mediated by TNF-α—in addition to being a κB-responsive cytokine, TNF-α is a potent inducer of NF-κB activation. Therefore, we investigated whether CXCL16-induced NF-κB activation is mediated by TNF-α. TNF-α-converting enzyme (TACE/ADAM17) cleaves the transmembrane form of TNF-α at its physiological processing site (20). Therefore, we pretreated ASMC with siRNA for TACE to prevent TNF-α cleavage or incubated ASMC with TNF-α neutralizing antibodies to prevent release of preformed membrane-bound TNF-α. Fig. 4 shows TACE mRNA expression in ASMC by RT-PCR (Fig. 3G)
CXCL16 promotes cell-cell adhesion and aortic smooth muscle cell proliferation. Cell death was analyzed by an ELISA. SNAP, a nitric oxide donor, served as a positive control. Unlike SNAP, treatment with CXCL16 failed to induce cell death (A). *p < 0.01 versus untreated controls and ethanol. We then determined CXCL16 effects on cell proliferation. ASMC proliferation was determined by[^3H]thymidine incorporation (B, *, p < 0.05; **, p < 0.01 versus control) and MTT assay (C). Platelet-derived growth factor-BB (PDGF-BB) was used as a positive control. (*, p < 0.05, and **, p < 0.001 versus control at 5 days). We then performed Western blotting to study the effects of CXCL16 on Bad and phospho-Bad (Ser112) levels in ASMCs, and our results indicate that CXCL16 induces phosphorylation of Bad (D). Inhibition of NF-κB activation by p65 and IKK-α siRNA expression vectors significantly attenuated xB-driven luciferase activity (E, *, p < 0.001 versus untreated; †, p < 0.01.
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and its protein levels by immunoblotting (Fig. 3H). Transfection with TACE siRNA, but not ADAM12 siRNA, inhibited TACE mRNA (Fig. 3G) and protein levels (Fig. 3H) and attenuated CXCL16-mediated TNF-α secretion (Fig. 3I). However, TACE inhibition or TNF-α neutralization failed to modulate CXCL16-induced NF-κB activation (Fig. 3J), indicating that the effects of CXCL16 on NF-κB activation are direct and are not mediated by TNF-α as has been reported previously for SDF-1 (21).

CXCL16 Induces Cell-Cell Adhesion and Cellular Proliferation—From the aforementioned studies, it is clear that CXCL16 is a strong inducer of NF-κB in HASMC, and this induction of NF-κB is mediated through Akt. Activation of Akt regulates survival signals induced by cytokines, chemokines, and growth factors (18). Upon activation, Akt phosphorylates downstream pro-apoptotic signaling molecules such as Bad, rendering them inactive (18). Furthermore, Akt activates NF-κB (22), and activation of NF-κB plays a role in growth factor-induced cell proliferation (23). Because CXCL16 activated Akt and induced Akt-dependent NF-κB activation, we hypothesized that CXCL16 may promote ASMC survival and proliferation. Fig. 4A shows low levels of oligonucleosomal fragmented DNA in untreated controls, and unlike SNAP, a nitric oxide donor, CXCL16, failed to induce cell death. In addition, MTT reduction and [3H]thymidine incorporation assays indicated that CXCL16 is in fact mitogenic to ASMC (Fig. 4, B and C). However, CXCL16 increased ASMC proliferation was more pronounced at days 5 and 7 after treatment. Platelet-derived growth factor-BB was used as a positive control and induced HASMC proliferation. Our results also demonstrated that CXCL16 induced phosphorylation of Bad at Ser112 (Fig. 4D), indicating that CXCL16 is pro-mitogenic and not a pro-apoptotic chemokine. To verify whether CXCL16 induces HASMC proliferation via NF-κB activation, we performed transient transfection assays using NF-κBp65 or IKK-α siRNA expression vectors. Fig. 4E shows that transfection with p65siRNA or IKK-α siRNA significantly inhibited CXCL16-mediated NF-κB-driven luciferase activity and significantly increased oligonucleosomal fragmented DNA (Fig. 4F). Inhibition of NF-κB also significantly reduced HASMC numbers following CXCL16 addition, indicating that CXCL16 induces cell proliferation via NF-κB activation (Fig. 4G). Because cell-cell signaling plays a role in cellular proliferation, we investigated whether CXCL16 increases cell-cell adhesion. Fig. 4H shows that treatment with CXCL16 significantly increased cell-cell adhesion as seen by increased calcein acetoxymethyl ester fluorescence. Together, these results indicate that CXCL16 increases cell-cell adhesion and ASMC proliferation.

DISCUSSION

Results from the present study indicate for the first time that human aortic smooth muscle cells express CXCR6. Treatment with CXCL16 activates NF-κB via heterotrimeric G proteins/PI3K/PDK-1/Akt/IKK/IκB and induces TNF-α expression in an NF-κB-dependent manner. Furthermore, CXCL16 increases cell-cell adhesion and ASMC proliferation, indicating that CXCL16 may play a role in the development and progression of human atherosclerotic vascular disease.

CXCL16 belongs to the α chemokine subfamily due to the presence of an N-terminal CXC motif (1). However, phylogenetically, it is more closely related to β-chemokines (1). It has structural similarities with fractalkine, a transmembrane protein that belongs to the CX3C subfamily, suggesting that, unlike other chemokines described to date, CXCL16 has similarities with a broad range of chemokines from the CC, CXC, and CX3C subfamilies (1). It exclusively binds CXCR6, a seven-transmembrane G-protein-coupled receptor, known previously as STRIL3/BONZO/TYMSTR. However, to date, no reports are available describing the signal transduction pathways elicited by CXCL16 and its effects on ASMC biology. Using pharmacological inhibitors and pathway-specific dominant-negative, kinase-dead, and siRNA expression vectors, we demonstrate that in human aortic smooth muscle cells, CXCL16 activates NF-κB via heterotrimeric G proteins/PI3K/PDK-1/Akt/IKK/IκB.

Our results clearly demonstrate that CXCL16 is a potent activator of NF-κB, a property also shared by the other transmembrane chemokine, fractalkine (14). Although fractalkine activates NF-κB and induces its own expression, CXCL16 failed to regulate its own expression, indicating that CXCL16 is not a κB-responsive chemokine. In fact, it has been recently demonstrated that both IL-1α (interleukin-1α) and TNF-α, two potent activators of NF-κB, failed to modulate CXCL16 expression in human aortic smooth muscle cells and umbilical vein endothelial cells (13), indicating that CXCL16 expression is not NF-κB dependent and may be regulated by other transcription factors. On the other hand, CXCL16 induced TNF-α expression in an NF-κB-dependent manner. However, TNF-α, a potent inducer of NF-κB activation, did not mediate CXCL16-induced NF-κB activation. Both neutralizing antibodies and inhibition of TACE failed to modulate CXCL16-induced NF-κB activation, indicating that CXCL16 is a direct and potent activator of NF-κB. In addition to TNF-α, TACE has also been shown to shed fractalkine (24, 25). Because inducible fractalkine release is TACE-dependent (24) and fractalkine induces NF-κB activation and ASMC proliferation (14), our TACE knockdown studies further suggest that neither TNF-α nor fractalkine mediated CXCL16-induced NF-κB activation.

CXCL16 mediates NF-κB activation via Akt/PKB. Activation of Akt generally transmits survival signals. Akt directly phosphorylates and inactivates proteins involved in cell death such as Bad, procaspase 9, and members of the Forkhead transcription factor family (18). In the present study, we have demonstrated that treatment with CXCL16 failed to induce cell death, and in fact, promotes cell survival and proliferation. In addition, it induced phosphorylation of Bad (Ser112), providing further evidence that CXCL16 is an anti-apoptotic chemokine. Upon phosphorylation, Bad is sequestered in the cytoplasm due to its interaction with 14-3-3 protein (26) and becomes unavailable to the pro-apoptotic machinery. Akt has also been shown to induce phosphorylation of procaspase 9 at Ser183 (18). Furthermore, phosphorylation and inactivation of FKHRL1, a member of the Forkhead transcription factor family, promotes cell survival (26). Although we have shown phosphorylation of Bad by CXCL16, its effects on procaspase 9 and the members of the Forkhead transcription factor family are not known and are under investigation.

In addition to inactivating pro-apoptotic mediators, Akt has...
CXCL16 is a potent and direct inducer of NF-κB activation (18). Activation of NF-κB promotes cell survival in a cell- and stimulus-specific manner. For example, platelet-derived growth factor-induced cell survival is mediated by Akt-dependent NF-κB activation (23). In ASMC, fractalkine activated NF-κB via Akt and induced ASMC proliferation (14). In the present study, we demonstrated that CXCL16 induces HASMC proliferation via NF-κB activation as inhibition of NF-κB led to a significant increase in cell death as evidenced by increased oligonucleosomal fragmented DNA and reduced cell numbers, indicating that in addition to chemoattraction, chemokines also play a role in cell survival, proliferation, angiogenesis, and malignancy (27–29).

Taken together, our results clearly demonstrate that CXCL16 is a potent and direct inducer of NF-κB activation, signals via heterotrimeric G proteins, PI3K, PDK-1, Akt, IKK, and IκB phosphorylation, and induces the expression of TNF-α, a proinflammatory and pro-atherogenic cytokine. Most importantly, CXCL16 induces cell-cell adhesion and HASMC proliferation, indicating that CXCL16 may play an important role in atherosclerosis.

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CXCL16 Signals via G<sub>i</sub>, Phosphatidylinositol 3-Kinase, Akt, IκB Kinase, and Nuclear Factor-κB and Induces Cell-Cell Adhesion and Aortic Smooth Muscle Cell Proliferation

Bysani Chandrasekar, Sailaja Bysani and Srinivas Mummidi

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