Injury-induced Platelet-derived Growth Factor Receptor-α Expression Mediated by Interleukin-1β (IL-1β) Release and Cooperative Transactivation by NF-κB and ATF-4

IL-1β FACILITATES HDAC-1/2 DISSOCIATION FROM PROMOTER**

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Platelet-derived growth factors are a family of potent mitogens and chemottractants for fibroblasts and other cells of mesenchymal origin. Platelet-derived growth factor (PDGF) dimeric ligands (composed of A-, B-, C-, and D-chains) exert their biological activity through high affinity interactions with cell surface receptor subunits (α and β). PDGF-receptor-α is widely implicated in the pathogenesis of hyperplastic fibrotic disease, yet the molecular mechanisms controlling its expression in response to injury are poorly understood. Here we show that PDGF-Rα expression is induced in fibroblasts by mechanical injury and interleukin (IL)-1β, which was abolished by neutralizing IL-1β antibodies in the culture supernatant or inhibitors of NF-κB. Chromatin immunoprecipitation and electrophoretic mobility shift assay support the existence of a new NF-κB binding site at the PDGF-Rα promoter. We have recently demonstrated that IL-1β is also induced by injury (Malabanan, K. P., Kanellakis, P., and Khachigian, L. M., Circ. Res. 103, 378–387), and we demonstrate here that the IL-1β-stimulated NF-κB transcriptional induction, and ATF-4 and NF-κB interaction with the PDGF-Rα promoter, whereas the HDAC inhibitors suberoylanilide hydroxamic acid and trichostatin A potentiate IL-1β induction of PDGF-Rα transcription. These findings, taken together, demonstrate that injury stimulates IL-1β secretion by fibroblasts, which activates NF-κB and ATF-4 and stimulates interaction with the PDGF-Rα promoter, triggering PDGF-Rα transcription. Physical and functional interactions between NF-κB and ATF-4 have not been reported in any gene. This is also the first report of HDAC regulation of PDGF-Rα transcription.

Wound healing is pivotal for reduced scarring, chronic wounding, and the prevention of infection through barrier restoration (1) and involves the complex interplay of growth factors and cytokines with multiple cell types including fibroblasts (1). Platelet-derived growth factors (PDGF) are a family of potent mitogens and chemottractants for fibroblasts and other cells of mesenchymal origin (2, 3). PDGF is a family consisting of four different ligands (A, B, C, and D) and two different receptor tyrosine kinase (TK) receptor subunits (α and β) (4). PDGF-Rα and PDGF-Rβ have common domain structures consisting five extracellular Ig loops and a split intracellular TK domain. PDGF binding induces α/β-receptor dimerization, autophosphorylation, and signal transduction pathways (2). PDGF-A, whereas PDGF-AA is the most selective member of the PDGF family. PDGF-AA activating PDGFR-α and PDGF-CC activates PDGFR-β. It is not known whether PDGF-AC, PDGF-AD, PDGF-BD, or PDGF-CD occur naturally. Studies in mice indicate that PDGF-Rα and PDGF-Rβ drive physiologically distinct signaling pathways. For example, PDGF-Rα is important in gastrulation and the development of the cranial and cardiac neural crest, lung, skin, skeleton, and central nervous system, whereas PDGF-Rβ signaling mediates blood vessel formation and early hematopoiesis (6). PDGF-α mediates fibroblast proliferation (7) and migration (8), and PDGF-Rα-deficient mice knockouts display dermal defects. PDGF-Rα-null mice have severe dermal mesenchymal hypoplasia and skin blistering (9). PDGF-Rα is induced during wound healing (10,11). However, the mechanisms underlying the activation of PDGF-Rα expression after injury are poorly understood. The molecular mechanisms that govern PDGF-Rα gene transcription are also incompletely characterized.

The human PDGF-Rα gene lacks a TATA box but contains GATA, Sp1, AP-1, AP-2, and Oct motifs and a CCAAT box (12). CCAAT-enhancer-binding proteins (C/EBPs) β and δ have shown to bind to regions within the PDGF-Rα pro-

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WITHDRAWN

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The abbreviations used are: PDGF, platelet-derived growth factor; PDGF-R, PDGF receptor; IL, interleukin; TK, tyrosine kinase; siRNA, small interfering RNA; ATF, activation transcription factor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HDAC, histone deacetylase; SAHA, suberoylanilide hydroxamic acid; TSA, trichostatin A; PBS, phosphate-buffered saline; DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride; DMSO, dimethyl sulfoxide; RT-PCR, real-time PCR; qRT-PCR, quantitative real-time PCR; ELISA, enzyme-linked immunosorbent assay; ChIP, chromatin immunoprecipitation; EMSA, electrophoretic mobility shift assay.
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Promoter and can both positively and negatively regulate PDGF-Rα activity (13, 14). The proto-oncogene product Cbl negatively regulates PDGF-Rα by enhancing ubiquitination and degradation (15). Sp1 and Ets-1 under stimulation by peroxide and FGF-2, respectively, can increase PDGF-Rα expression. NF-κB and ATF-4 are induced by IL-1β and interact with the PDGF-Rα promoter, triggering PDGF-Rα transcription. Physical and functional interactions between NF-κB and ATF-4 have not been reported in any gene. This study also provides the first report of histone deacetylase (HDAC) regulation of PDGF-Rα transcription.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Reagents**—Transformed murine embryonic fibroblasts NIH-3T3 cells were purchased from ATCC. They were cultured in Dulbecco’s modified Eagles’ medium, pH 7.4, supplemented with 10% fetal bovine serum, 10 units/ml penicillin, and 10 μg/ml streptomycin at 37 °C and 5% CO2. The cells were passaged every 3–4 days in 75-cm² flasks. Cells were incubated for 24 h in serum-free medium to achieve quiescence before stimulation with agonists or inhibitors. Human recombinant IL-1β was purchased from R&D systems, according to manufacturer’s instructions. cDNA synthesis from total RNA was performed using SuperScript II reverse transcriptase (Invitrogen) in accordance with the manufacturer’s instructions. Thermal cycling conditions for qPCR were: PDGF-Rα, 94 °C for 10 s, 62 °C for 30 s, and 72 °C for 10 s for 22–27 cycles; and GAPDH, 94 °C for 10 s, 60 °C for 30 s, and 72 °C for 30 s for 25–30 cycles. Primer sequences for forward and reverse, 5'-AGA TAG CTT ACC CTA AGG AG-3', and reverse, 5'-TCC ACC GGA ACG GGG TCA GAG-3'. Densitometric analyses were performed using Quantity One Version 4.1.0 (Bio-Rad).

**Quantitative PCR**—Quantitative PCR was carried out using Rotor-Gene 3000 (Corbett Life Science). The reaction was set in a final volume of 10 μl containing 1 μl of cDNA, 5 μl of 2 × SYBR Green Master Mix (Applied Biosystems), 0.2 μl of 10 μM of forward and reverse primer (Sigma), and 3.6 μl of DNase-free water. Murine PDGF-Rα primers were: forward, 5’-CAA ACC CTG AGA CCA CAA TG-3’, and reverse, 5’-TCC CCC AAC AGT AAC CCA AG-3’. β-actin primers were: forward, 5’-AGC CAT GTA CGT AGC CAT CC-3’, and reverse, 5’-CTC TCA GCT GTG GTG TTG AA-3’. PCR conditions were: 95 °C for 10 min followed by 40 cycles at 95 °C for 20 s and 60 °C for 45 s, 72 °C for 20 s.

**Inhibitor Studies**—Inhibitors to the various components were preincubated with the cells 1 h before the addition of agonist or plasmid. MG132 (AG Scientific) was used at 10 μM, BAY 11-7082 (Sigma-Aldrich) was used at 10 μM, and Oridonin (Merck) was used at 5 μg/ml. Cells were extracted for RT-PCR or luciferase analysis.

**siRNA Studies**—Growth-quiescent cells in 6-well plates were transfected with 100 nM (3 μg) small interfering RNA (siRNA) targeting murine p65. After 16 h, the cells were stimulated with 10 ng/ml IL-1β for another 4 h, and the cells were then harvested for luciferase assay. siRNA targeting murine p65 and scrambled siRNA were purchased from Santa Cruz Biotechnology.

**Preparation of Nuclear Extracts**—Nuclear extracts were prepared essentially as described (20, 21). Briefly, fibroblasts were washed twice with ice-cold PBS, pH 7.4, and then scraped into
10 ml of cold PBS. The cells were pelleted by centrifugation at 250 × g for 15 min at 4 °C. The cells were lysed with the addition of ice-cold hypotonic Buffer A (consisting of 10 mM HEPES, pH 8.0, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM DTT, 200 mM sucrose, 0.5% Nonidet P-40, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 1 μg/ml leupeptin, and 1 μg/ml aprotinin). The suspension was recentrifuged, and nuclei were lysed in an ice-cold Buffer C (consisting of 20 mM HEPES, pH 8.0, 100 mM KCl, 0.2 mM EDTA, 20% glycerol, 1 mM DTT, 0.5 mM PMSF, 1 μg/ml leupeptin, and 1 μg/ml aprotinin). The nuclear fraction was combined with an equal volume of ice-cold Buffer D (containing 20 mM HEPES, pH 8.0, 100 mM KCl, 0.2 mM EDTA, 20% glycerol, 1 mM DTT, 0.5 mM PMSF, 1 μg/ml leupeptin, and 1 μg/ml aprotinin). Nuclear extracts were stored at −80 °C until use.

**Electrophoretic Mobility Shift Assays (EMSA)**—Binding reactions were performed essentially as described (22, 23). Briefly, these were in 20 μl of 10 mM Tris-HCl, 50 mM NaCl, 1 mM EDTA, 2 mM DTT, 5% glycerol, 0.5% Nonidet P-40, 1 mg/ml bovine serum albumin, 32P-labeled oligonucleotide probe (100,000 cpm), and added protein. Recombinant proteins used were 100 ng of p65 (Australian Biosearch), 100 ng of p50 (Promega), and 1 μg of ATF-4 (Abnova). Reactions were allowed to proceed for 30 min at 22 °C. Bound complexes were separated

**TABLE 1**

Sequences of 5′/3′ primers for ChIP amplicons

| Primer | Sequence (5′-3′) |
|--------|-----------------|
| Ampicon 1 5′-3′ F | CCC CCT CTT GGT TTG TTT |
| Ampicon 1 5′-3′ R | ACA GGA ATG GGC TCA AAA CCC |
| Ampicon 2 5′-3′ F | CGG TCC TCC AAA AAC TTA CCA |
| Ampicon 2 5′-3′ R |CAA AAC AAT AGC ACC CCC AC |
| Ampicon 3 5′-3′ F | TCC GAA GGG ATA AAG GTG GA |
| Ampicon 3 5′-3′ R | TGG GTG TGC TAT TGG TTT TG |
| Ampicon 4 5′-3′ F | GTC TCT CTC TGG GTG TCT TG |
| Ampicon 4 5′-3′ R | GCC TCC GGG GCC ACT CTC CT |
| VCAM-1 5′-3′ F | CAG CAT TGT CCT TTA TCT TT |
| VCAM-1 5′-3′ R | GAA ATA GAA AGT CTG TGC TT |

**FIGURE 1.** Mechanical injury induces PDGF-Rα expression in an IL-1β-dependent manner. A, PDGF-Rα is induced in NIH-3T3 by scraping injury as determined by conventional PCR (upper) and quantitative real-time PCR (lower). NIH-3T3 cells in 100-mm plates were repeatedly scraped, and after 24 h, total RNA was prepared for determination of levels of PDGF-Rα mRNA. B, IL-1β was released into the culture supernatant as determined by ELISA. Data were compared with a standard curve using recombinant IL-1β. C, injury-inducible PDGF-Rα expression is abrogated by the presence of 10 μg/ml neutralizing IL-1β antibodies (IL-1beta IgG), but not an equivalent concentration of IgG as indicated by conventional PCR (upper) and quantitative real-time PCR (lower). No antibody added (No Ab) represents a negative control. * denotes p < 0.05 as compared with no treatment control using Student’s t test. Error bars denote S.E.
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from free probe by loading samples onto a 6% non-denaturing polyacrylamide gel and electrophoresing at 100 V for 4 h. Gels were vacuum-dried at 80 °C and subjected to autoradiography overnight at −20 °C. PD-κB (containing NF-κB consensus element) sequence is 5′-CAA CGG CAG GGG AAT TCC CCT CTC TT-3′.

**FIGURE 2.** Recombinant IL-1β stimulates PDGF-Rα promoter, mRNA, and protein expression. **A**, NIH-3T3 cells were transfected with 1 μg of pLuc-α2 overnight and treated with various concentrations of IL-1β for 24 h. Levels of PDGF-Rα mRNA were determined by RT-PCR (upper) or qRT-PCR (lower). **B**, NIH-3T3 cells were incubated with IL-1β (10 ng/ml) or phorbol 12-myristate 13-acetate (PMA) (100 ng/ml) for 4 h, and levels of PDGF-Rα mRNA were determined by RT-PCR (upper) or qRT-PCR (lower). GAPDH is used as an unbiased loading control. qPCR was used to quantify results. * denotes p < 0.05 as compared with no treatment control using Student’s t test. Error bars denote S.E.

**FIGURE 3.** IL-1β and injury induction of PDGF-Rα is NF-κB-dependent. **A**, NIH-3T3 cells pretreated with 5 μg/ml MG132, BAY 11-7082, Oridonin, or DMSO for 1 h were incubated with IL-1β for 4 h. Levels of PDGF-Rα mRNA were determined by RT-PCR (upper) or qRT-PCR (lower). **B**, BAY 11-7082 and Oridonin pretreatment inhibits injury-inducible PDGF-Rα expression after 4 h, as determined by RT-PCR (upper) or qRT-PCR (lower). GAPDH is used as an unbiased loading control. qPCR was used to quantify results. * denotes p < 0.05 as compared with no treatment control using Student’s t test. Error bars denote S.E.
Chromatin Immunoprecipitation Analysis (ChIP) — The fibroblasts were incubated with 1% formaldehyde for 10 min and quenched with glycine (final concentration, 0.1M). The cells were washed twice with PBS, pH 7.4. ChIP buffer consisting of 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 0.5% Nonidet P-40, 50 mM Tris-HCl, pH 7.5, and 0.5 mM DTT was added to the cells, which were then scraped and collected. The cells were sonicated at four rounds of 15, 1 s each time. After spinning at 14,000 g for 10 min at 4 °C, the supernatant was collected and evenly divided, and 5 g of rabbit polyclonal antibodies was added to the indicated targets (Santa Cruz Biotechnology) or no antibody was added. After incubation at 22 °C for 1 h, sonication was performed at 4 °C in a water bath for 15 min. The suspension was spun at 14,000 g for 10 min at 4 °C, the supernatant was collected and evenly divided, and 5 g of rabbit polyclonal antibodies was added to the indicated targets (Santa Cruz Biotechnology) or no antibody was added. After incubation at 22 °C for 1 h, sonication was performed at 4 °C in a water bath for 1 min. The suspension was spun at 14,000 g for 10 min at 4 °C, and the supernatant was collected. Washed protein A- and G-Sepharose beads were added to the supernatant and spun at 4 °C for 1 h. The suspension was spun down, and supernatant was removed with a 30-gauge syringe. The beads were washed with ChIP buffer 5×. Chelex was added, and the suspension was boiled for 10 min. The suspension was treated with proteinase K at 55 °C for 30 min while spinning. The suspension was boiled again for 10 min. The suspension was spun down, and the supernatant was collected. Phenol-chloroform extraction and ethanol precipitation were conducted overnight at −20 °C. The fragment of PDGF-Rα promoter was amplified by PCR. For PDGF-Rα and VCAM-1 primers used, refer to Table 1. Thermal cycling conditions were as follows: 94 °C for 30 s, 58 °C for 30 s, 72 °C for 45 s for 45 cycles, generating products of expected length.

Co-immunoprecipitation — Cells were treated with IL-1β for 2 h and then extracted using the preparation for nuclear extraction method. The nuclear extracts were then precleared (Buffer C D) protein A- and G-Sepharose beads overnight at 4 °C while spinning. Polyclonal rabbit p65 antibodies were added to the supernatant, which was then incubated at 4 °C overnight with spinning. Prewashed protein A- and G-Sepharose beads were added again and incubated with the suspension overnight with spinning at 4 °C. The suspension was washed with Buffer C D three times. The supernatant was resuspended in 4× SDS sample buffer and analyzed by Western blotting and read using the Odyssey system.

Western Blotting — Cells were treated with 10 ng/ml IL-1β for 2 h and washed twice with ice-cold PBS before being lysed on ice in buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1% deoxycholate, 0.1% SDS) containing protease inhibitors (2 mM PMSF, 5 mM EDTA, 10 μg/ml leupeptin, and 1% aprotinin) or nuclear extracts. Cell lysates were collected after centrifugation at 14,000 rpm for 20 min at 4 °C, and protein...
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Mechanical Injury Induces PDGF-Rα Expression in an IL-1β-dependent Manner—Although PDGF-Rα expression is increased during wound healing (10, 11), the mechanisms underlying its induction by injury are completely unknown. We subjected murine fibroblasts to in vitro mechanical injury and demonstrated by conventional and real-time PCR an increase in PDGF-Rα mRNA (Fig. 1A). Levels of the proinflammatory cytokine IL-1β, which has also been implicated in the process of wound healing (24), increased in the cell supernatant after injury (Fig. 1B). There is no clear relationship between the two factors in the context of wound healing. Inclusion of neutralizing IL-1β antibodies in the culture medium prior to injury abolished injury-inducible PDGF-Rα expression (Fig. 1C). In contrast, isotype-matched antibodies (IgG) had no effect on injury-inducible PDGF-Rα expression (Fig. 1C). These data demonstrate that injury-inducible PDGF-Rα expression involves the release and paracrine activation by endogenous IL-1β.

Recombinant IL-1β Activates the PDGF-Rα Promoter, mRNA, and Protein Expression—Previous studies from other laboratories have shown that IL-1β can modulate the expression of different members of the PDGF family (25–27). We explored the direct influence of IL-1β on PDGF-Rα expression in the fibroblasts. Recombinant IL-1β increased, in a dose-dependent manner, PDGF-Rα promoter-dependent expression in fibroblasts transiently transfected with pLuc-α2, a Firefly luciferase construct driven by 1300 bp of the PDGF-Rα promoter.

RESULTS

Mechanical Injury Induces PDGF-Rα Expression in an IL-1β-dependent Manner—Although PDGF-Rα expression is increased during wound healing (10, 11), the mechanisms underlying its induction by injury are completely unknown. We subjected murine fibroblasts to in vitro mechanical injury and demonstrated by conventional and real-time PCR an increase in PDGF-Rα mRNA (Fig. 1A). Levels of the proinflammatory cytokine IL-1β, which has also been implicated in the process of wound healing (24), increased in the cell supernatant after injury (Fig. 1B). There is no clear relationship between the two factors in the context of wound healing. Inclusion of neutralizing IL-1β antibodies in the culture medium prior to injury abolished injury-inducible PDGF-Rα expression (Fig. 1C). In contrast, isotype-matched antibodies (IgG) had no effect on injury-inducible PDGF-Rα expression (Fig. 1C). These data demonstrate that injury-inducible PDGF-Rα expression involves the release and paracrine activation by endogenous IL-1β.
(Fig. 2A) (14). The cytokine also increased levels of PDGF-Rα mRNA (Fig. 2B) and protein (Fig. 2C) within 4 h.

The NF-κB Pathway Mediates IL-1β and Injury Induction of PDGF-Rα Expression—A canonical downstream target of IL-1β is the transcription factor NF-κB. NF-κB normally resides in the cytoplasm bound to its inhibitor, IκB, which upon ubiquitination is degraded by proteasomes, allowing NF-κB translocation to the nucleus (28). The peptide-aldehyde MG132 (carboxenzoxy-leucinyl-leucinyl-leucinal-H) is a proteasome inhibitor that prevents this action (29). Preincubation of the fibroblasts with MG132 blocked IL-1β induction of PDGF-Rα mRNA (Fig. 3A). This suggests that proteasome degradation is an important step in IL-1β up-regulation of PDGF-Rα transcription. Because proteasome degradation affects NF-κB-dependent and-independent gene expression, we used two additional inhibitors to identify a role for NF-κB in PDGF-Rα expression. The cells were treated with BAY 11-7082, a synthetic IκB kinase (IKK) inhibitor (30), and the diterpenoid Oridonin, an inhibitor of NF-κB (p65) DNA binding (31) prior to exposure to IL-1β; each abolished IL-1β stimulation of PDGF-Rα expression (Fig. 3A). BAY 11-7082 and Oridonin also inhibited injury-inducible PDGF-Rα expression (Fig. 3B). These findings demonstrate a role for the NF-κB pathway in IL-1β and injury induction of PDGF-Rα expression.

IL-1β Stimulates PDGF-Rα Promoter-dependent Activity via NF-κB p65—To demonstrate that NF-κB can directly activate PDGF-Rα at the transcriptional level, the fibroblasts were transfected with pLuc-α2 and an NF-κB p65 expression vector (pcDNA3-p65), and luciferase activity measured 24 h. p65 activated the PDGF-Rα promoter in these experiments, p65 also stimulated PD-κB, a palindromic DNA sequence, whose formation was abolished by mutation of the NF-κB binding site (32) (Fig. 4B). The cytokine also increased levels of PDGF-Rα mRNA (Fig. 2A) (14). The cytokine also increased levels of PDGF-Rα mRNA (Fig. 2B) and protein (Fig. 2C) within 4 h.

NF-κB p65 Binds and Activates the PDGF-Rα Promoter—To delineate whether IL-1β-dependent p65 activation of PDGF-Rα involved the interaction of endogenous NF-κB with the authentic PDGF-Rα promoter, we performed ChIP analysis using antibodies to p50 and p65 and PCR amplification of the promoter. No detectable p50 or p65 binding activity was detected in untreated cells (Fig. 5A and data not shown). After a 1-h incubation with IL-1β, p65 bound the PDGF-Rα promoter, whereas p50 did not associate with the promoter (Fig. 5A and data not shown). The integrity of the p50 antibody as a protein/DNA precipitation reagent was validated by its ability to pull down a region of the human VCAM-1 promoter (−267/−6) containing two well-established NF-κB binding elements (33) (Fig. 5A, lower). Because PDGF-Rα promoter does not contain a consensus NF-κB recognition element (Table 2), we used TESS (Transcription Element Search System, the TRANSFAC data base) to identify a candidate motif. A 32P-labeled double-stranded oligonucleotide (32P-oligonucleotide PDGF-Rα) spanning this element (−531/−521) was used in EMSA with nuclear extracts of cells untreated or treated with IL-1β for 1 h. This produced an IL-1β-inducible nucleoprotein complex, which was eliminated by inclusion of p65 antibodies in the binding reaction (Fig. 5B). In contrast, this complex was not eliminated with antibodies to p50 or USF-1 (Fig. 5B).

**Table 2**

| Murine PDGF-Rα promoter region |
|---------------------------------|
| Predicted genomic organization of the murine PDGF-Rα promoter following UC Santa Cruz (UCSC) Genome Bioinformatics analysis of accession number NM_011058 is shown. Sequence letters in lower case denote the untranslated region was predicted by the software, and numbering is relative to this TSS. Nucleotides indicated in bold underlining indicate transcription factor elements in the rat or human promoter, and promoter usage. The two transcription factors which are the subject of this study are indicated in white text, highlighted in black. Previously identified motifs for Ets at −46/−42 (16) and Sp1 at −64/−52 (17) in the rat PDGF-Rα promoter are located at −47/−44 and −64/−55 in the murine promoter. |

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**IL-1β, NF-κB, and ATF-4 Activate PDGF-Rα**

To delineate whether IL-1β-dependent p65 activation of PDGF-Rα involved the interaction of endogenous NF-κB with the authentic PDGF-Rα promoter, we performed ChIP analysis using antibodies to p50 and p65 and PCR amplification of the promoter. No detectable p50 or p65 binding activity was detected in untreated cells (Fig. 5A and data not shown). After a 1-h incubation with IL-1β, p65 bound the PDGF-Rα promoter, whereas p50 did not associate with the promoter (Fig. 5A and data not shown). The integrity of the p50 antibody as a protein/DNA precipitation reagent was validated by its ability to pull down a region of the human VCAM-1 promoter (−267/−6) containing two well-established NF-κB binding elements (33) (Fig. 5A, lower). Because PDGF-Rα promoter does not contain a consensus NF-κB recognition element (Table 2), we used TESS (Transcription Element Search System, the TRANSFAC data base) to identify a candidate motif. A 32P-labeled double-stranded oligonucleotide (32P-oligonucleotide PDGF-Rα) spanning this element (−531/−521) was used in EMSA with nuclear extracts of cells untreated or treated with IL-1β for 1 h. This produced an IL-1β-inducible nucleoprotein complex, which was eliminated by inclusion of p65 antibodies in the binding reaction (Fig. 5B). In contrast, this complex was not eliminated with antibodies to p50 or USF-1 (Fig. 5B).

Incubation of 32P-oligonucleotide PDGF-Rα with recombinant p65 homodimers also resulted in a nucleoprotein complex, which corresponded to that produced using 32P-PPD-kB, whose formation was abolished by mutation within the −531/−521 region of the PDGF-Rα promoter (Fig. 5C). These results demonstrate that this element in the PDGF-Rα promoter supports binding of IL-1β-inducible endogenous and recombinant p65. **ATF-4 Binds and Activates the PDGF-Rα Promoter**—Recent studies in our laboratory have determined that mechanical stimulation of the fibroblasts with MG132 blocked IL-1β induction of PDGF-Rα mRNA (Fig. 3A). This suggests that proteasome degradation affects NF-κB-dependent and-independent gene expression, we used two additional inhibitors to identify a role for NF-κB in PDGF-Rα expression. The cells were treated with BAY 11-7082, a synthetic IκB kinase (IKK) inhibitor (30), and the diterpenoid Oridonin, an inhibitor of NF-κB (p65) DNA binding (31) prior to exposure to IL-1β; each abolished IL-1β stimulation of PDGF-Rα expression (Fig. 3A). BAY 11-7082 and Oridonin also inhibited injury-inducible PDGF-Rα expression (Fig. 3B). These findings demonstrate a role for the NF-κB pathway in IL-1β and injury induction of PDGF-Rα expression.

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Injury, albeit of vascular smooth muscle cells, induces the expression of activation transcription factor ATF-4, a member of the ATF/CREB (activation transcription factor/cAMP-response element-binding protein) family of basic leucine zipper transcription factors (34). Co-transfection of fibroblasts with an ATF-4 expression vector and pLuc-α2 revealed that ATF-4 activates the PDGF-Rα promoter (Fig. 6A). We noted a putative recognition element for ATF-4 in the PDGF-Rα promoter at position −259/−254 (Table 2). 32P-oligonucleotide PDGF-Rα−271/−244 spanning this element bound recombinant ATF-4 homodimers in an EMSA (Fig. 6B). Mutation within the −259/−254 element abolished complex formation (Fig. 6B). This is the first demonstration of ATF-4 regulation of PDGF-Rα or any other member of the PDGF ligand/receptor family.

ATF-4 Interacts with NF-κB p65 and Cooperatively Activates the PDGF-Rα Promoter—We next performed co-immunoprecipitation analysis to determine whether ATF-4 and p65 interact. Nuclear extracts of IL-1β-treated fibroblasts were precipitated with p65 antibodies and then blotted with antibodies to p65 or ATF-4. IL-1β activated p65 and ATF-4 and stimulated the physical interaction of these factors within 1 h (Fig. 6C). BAY 11-7082, which blocks p65 activation and PDGF-Rα expression (Figs. 3, A and B, and 6C), also abrogated IL-1β-inducible ATF-4/p65 complex formation (Fig. 6C). ChIP experiments revealed that ATF-4 and p65 both co-occupy the PDGF-Rα promoter in fibroblasts exposed to IL-1β (Fig. 6D). p65 was more prominently associated with amplicon −617/−314, and ATF-4 was more prominently associated with amplicon −339/+21 (Fig. 6D). In contrast, p50 was not associated with either amplicon (Fig. 6D). Transfection analysis revealed that ATF-4 and p65 transactivated the PDGF-Rα promoter in a cooperative manner.

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Histone Modification during IL-1β Induction of PDGF-Rα Expression—Inducible gene expression not only involves an orchestrated network of transcription factor protein–protein and protein–DNA interaction but histone modification in chromatin by enzymes such as HDACs, which generally serve to silence gene expression. Inhibitors of HDAC are an emerging class of anticancer agents (35). The role of histone modification in PDGF-Rα gene expression is completely unexplored. We used the HDAC inhibitor suberoylanilide hydroxamic acid (SAHA, also known as Vorinostat/Zolinza) to determine

(Fig. 6E, upper). Consistent with these findings, ATF-4 induction of the PDGF-Rα promoter–dependent expression was completely blocked in the presence of BAY 11-7082 (Fig. 6E, middle). Mutation within the −531/−521 element abolished p65 transactivation and attenuated ATF-4 transactivation (Fig. 6E, lower). Mutation within the −259/−254 element abolished both p65 and ATF-4 transactivation (Fig. 6E, lower). These findings demonstrate that ATF-4 forms a complex with p65 and cooperatively activates the PDGF-Rα promoter.

FIGURE 7. Treatment with IL-1β, NF-κB, and ATF-4 activates the PDGF-Rα promoter. A, NIH-3T3 cells were pretreated with the HDAC inhibitor SAHA for 24 h before transfection with p65, ATF-4, or pcDNA3 (10 ng/ml). Levels of PDGF-Rα mRNA were determined by RT-PCR (left) or qRT-PCR (right). * denotes p < 0.05 as compared with no treatment control using Student’s t test. Error bars denote S.E. B, NIH-3T3 cells were pretreated with TSA for 1 h prior to exposure to IL-1β. EMSA was performed with 32P-oligonucleotide PDGF-Rα motif (Fig. 6A) (271/−244) and recombinant ATF-4 (1 μg). The sequence of oligonucleotide PDGF-Rα motif underlined and mutants bold. C, nuclear extracts of IL-1β-treated (1 h) or untreated cells were pulled down (IP) with p65 antibodies, and then Western blot (IB) analysis was performed either with p65 antibodies or with ATF-4 antibodies (upper). Western blots (lower) for p65 or ATF-4 antibodies serve as loading controls. D, ChIP analysis was performed with cells treated with or without IL-1β (10 ng/ml). Total input, No antibody (No Ab), total input. No antibody (No Ab) was used as negative control. E, upper, NIH-3T3 cells were transfected with 1 μg of pcDNA3-p65 and 10 μg of pcDNA3-ATF-4. After 24 h, Firefly luciferase readings were normalized to Renilla. Lower, cells were transfected with 1 μg of pcDNA3-p65 and 10 μg of pcDNA3-ATF-4, after 24 h, Firefly luciferase readings were normalized to Renilla. * denotes p < 0.05 as compared with no treatment control using Student’s t test. Error bars denote S.E.
**IL-1β, NF-κB, and ATF-4 Activate PDGF-Rα**

**FIGURE 8. Possible mechanism underlying injury induction of PDGF-Rα expression.** IL-1β is released by injury, which activates PDGF-Rα expression via NF-κB and ATF-4 and release of HDAC-1/2 from the PDGF-Rα promoter. Ub denotes ubiquitination, and P denotes phosphorylation.

whether HDACs are involved in IL-1β control of PDGF-Rα expression. SAHA increased levels of IL-1β-inducible PDGF-Rα mRNA in a dose-dependent manner up to 1.5 μM SAHA (Fig. 7A). A second HDAC inhibitor, trichostatin A (TSA), also potentiated IL-1β-inducible PDGF-Rα expression (Fig. 7B). ChiP analysis revealed that both HDACs dissociate from the PDGF-Rα promoter (Fig. 7C). Following injury HDAC-1/2 release from the PDGF-Rα promoter, relieving the promoter from repression and facilitating inducible, cooperative transcription by p65 and p50.

**DISCUSSION**

In this study, we report that PDGF-Rα expression induced by injury is mediated by the release and paracrine activation by IL-1β. IL-1β stimulates PDGF-Rα expression via NF-κB p65 and ATF-4, which, in turn, interact with previously unrecognized sites in the PDGF-Rα promoter. ATF-4 and NF-κB physically interact, occupy the authentic PDGF-Rα promoter, and induce PDGF-Rα transcription in a cooperative manner. Further, we have demonstrated that IL-1β causes the dissociation of HDAC-1 and HDAC-2 from the PDGF-Rα promoter. HDAC inhibition with SAHA or TSA potentiates IL-1β induction of PDGF-Rα transcription. These findings, taken together, suggest that fibroblast injury stimulates IL-1β secretion, which activates NF-κB and ATF-4, and stimulates interaction with the PDGF-Rα promoter, triggering PDGF-Rα transcription (Fig. 8).

The ability of tissues to heal after injury is pivotal for reduced scarring, chronic wounding, and the prevention of infection through barrier restoration (1). This involves a complex interplay of growth factors and cytokines and multiple cell types including fibroblasts (1). The PDGF family has long been recognized as a key player in the process of wound healing, and PDGF-Rα mediates the proliferation (7) and migration (8) of fibroblasts. PDGF-Rα is induced during wound healing (10, 36), and PDGF-Rα-null mice display severe skin abnormalities (9). All PDGF dimeric ligands except PDGF-DD induce PDGF-Rα activation and dimerization (37). The mechanisms underlying the activation of PDGF-Rα expression after injury are poorly understood. IL-1β levels have previously been found to increase following pulmonary vascular endothelial injury (38) and spinal cord injury (39). However, a mechanistic link between PDGF-Rα and IL-1β has not been established as a central event in context of injury. Cytokine-induced PDGF-Rα expression has been linked with the local mitogenic and chemotactic response to PDGF in response to injury. These findings are reminiscent of previous studies demonstrating that ATF-4 and NF-κB in the wound are potentiated by NF-κB inhibitor, in the context of injury, as well as by activation of NF-κB by IL-1β (40).

In this study, we have demonstrated that ATF-4 and NF-κB act as a convergence point for many cellular stress pathways (41). ATF-4 is also known to be involved in hematopoietic development (42). ATF-4 up-regulates eIF2a, which is a convergence point for many cellular stress pathways (43, 44). We recently demonstrated that ATF-4 is activated by injury and mediates neointima formation in rat arteries after balloon injury (34). ATF-4 and NF-κB interact, bind the PDGF-Rα promoter, and induce PDGF-Rα transcription in a cooperative manner. Physical and functional interactions between NF-κB and ATF-4 have not previously been reported. The present study also provides the first demonstration of ATF-4 regulation of any member of the PDGF family.

The use of inhibitors in our studies was pivotal in the identification of NF-κB in the transcriptional control of a PDGF receptor. MG132 has been widely used in cancer research (45, 46) and acute pancreatitis protection (47). BAY 11-7082, like MG132, has been used to define a causal role for NF-κB in diseases such as cancer (48), renal failure (49), and the regulation of stress signal transduction pathways (50). Oridonin has only been recently described as an NF-κB inhibitor, in the context of immunosuppression (51) and apoptosis in cancer (52). BAY 11-7082, Oridonin, and siRNA thus define a key role for p65 in PDGF-Rα transcription. p50 did not associate with...
the PDGF-Rα promoter, nor did it transactivate the PDGF-Rα promoter.

Gene expression is influenced by the dynamic interaction of histone acetylation and deacetylation. Acetylation of histone lysine residues is preferentially associated with transcriptionally active chromatin, whereas histone deacetylases are generally involved in transcriptional repression (53). This study provides the first report of HDAC regulation of PDGF-Rα transcription. TSA and SAHA potentiated IL-1β induction of PDGF-Rα. Moreover, exposure to IL-1β prompted the dissociation of HDAC-1 and HDAC-2 from the PDGF-Rα promoter and the association of NF-κB and ATF-4. Histone remodeling thus regulates transcription factor access and occupancy of the PDGF-Rα promoter and altered transcription of this gene.

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Supplemental Material can be found at:
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