Role of the JAK/STAT Pathway in the Regulation of Interleukin-8 Transcription by Oxidized Phospholipids in Vivo and in Atherosclerosis in Vivo*

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Oxidized 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphorylcholine (Ox-PAPC) and its component phospholipid, 1-palmitoyl-2-epoxyisoprostane-sn-glycero-3-phosphorylcholine, induce endothelial cells (EC) to synthesize chemotactic factors, such as interleukin 8 (IL-8). Previously, we demonstrated a role for c-Src kinase activation in Ox-PAPC-induced IL-8 transcription. In this study, we have examined the mechanism regulating IL-8 transcription by Ox-PAPC downstream of c-Src. Our findings demonstrate an important role for JAK2 in the regulation of IL-8 transcription by Ox-PAPC. Treatment of human aortic EC with Ox-PAPC and 1-palmitoyl-2-epoxyisoprostane-sn-glycero-3-phosphorylcholine induced a rapid yet sustained activation of JAK2; activation of JAK2 by Ox-PAPC was dependent on c-Src kinase activity. Furthermore, pretreatment with selective JAK2 inhibitors significantly reduced Ox-PAPC-induced IL-8 transcription. In previous studies, we also demonstrated activation of STAT3 by Ox-PAPC. Here we provide evidence that STAT3 activation by Ox-PAPC is dependent on JAK2 activation and that STAT3 activation regulates IL-8 transcription by Ox-PAPC in human EC. Transfection with small interfering RNA against STAT3 significantly reduced Ox-PAPC-induced IL-8 transcription. Using chromatin immunoprecipitation assays, we demonstrated binding of activated STAT3 to the sequence flanking the consensus γ-interferon activation sequence (GAS) in the IL-8 promoter; site-directed mutagenesis of GAS inhibited IL-8 transcription by Ox-PAPC. Finally, these studies demonstrate a role for STAT3 activation in atherosclerosis in vivo.

We found increased staining for activated STAT3 in the inflammatory regions of human atherosclerotic lesions and reduced fatty streak formation in EC-specific STAT3 knock-out mice on the atherogenic diet. Taken together, these data demonstrate an important role for the JAK2/STAT3 pathway in Ox-PAPC-induced IL-8 transcription in vitro and in atherosclerosis in vivo.

Cardiovascular disease (CVD)2 is a major cause of morbidity and mortality in Western nations. It is estimated that 80 million Americans have one or more forms of CVD. Atherosclerosis, a common cause of CVD, is a chronic inflammatory condition, involving enhanced monocyte/endothelial cell interactions. Clinical studies suggest that the inflammatory index, as measured by levels of C-reactive protein or myeloperoxidase activity, is an important independent predictor of the risk of atherosclerosis. Our laboratory has demonstrated that oxidation products of palmitoyl-2-arachidonoyl-sn-glycero-3-phosphorylcholine (PAPC) accumulate in atherosclerotic lesions and other sites of chronic inflammation. Oxidized PAPC (Ox-PAPC) and its component phospholipid, 1-palmitoyl-2-epoxyisoprostane-sn-glycero-3-phosphorylcholine (PEIPC), activate human aortic endothelial cells (HAEC) in vitro to bind monocytes. Furthermore, these oxidized phospholipids increase the expression and secretion of chemokines known to activate monocytes; elevated levels of these proatherogenic chemokines have also been shown to accumulate within the vessel wall (1). Thus, we propose that Ox-PAPC plays an important role in regulating atherosclerosis.

Interleukin 8 (IL-8), an important mediator of monocyte transmigration and retention in vessel wall, is one such chemokine strongly induced in HAEC treated with Ox-PAPC. IL-8 plays an important role in the regulation of atherosclerosis. Boisvert et al. (2) demonstrated that knock-out mice of the homologue of IL-8 had reduced levels of atherosclerotic lesions. We recently demonstrated a role for c-Src kinase in the regulation of Ox-PAPC- and PEIPC-induced IL-8 synthesis in HAEC (3, 4). In these studies, we also presented evidence that activation of signal transducer and activator of transcription (STAT) 3 might be involved in Ox-PAPC-induced IL-8 transcription. Downstream of c-Src, however, the mechanism of IL-8 transcription by Ox-PAPC remained to be examined. In this study,

2 The abbreviations used are: CVD, endothelial cell; siRNA, small interfering RNA; CHIP, chromatin immunoprecipitation; GAS, γ-interferon activation sequence; Ox-PAPC, oxidized 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphorylcholine; PEIPC, 1-palmitoyl-2-epoxyisoprostane-sn-glycero-3-phosphorylcholine; HAEC, human aortic endothelial cells; IFN, interferon; IL, interleukin; STAT, signal transducers and activators of transcription; ELISA, enzyme-linked immunosorbent assay; qRT, quantitative real time; HMEC, human microvascular endothelial cells.
we have defined the mechanism of c-Src-mediated IL-8 transcription by Ox-PAPC.

Previous studies by others had demonstrated interaction between Src kinases and JAK kinases, including their role in regulating several inflammatory processes (5–7). The JAK family consists of four members in mammals, JAK1–3, and TYK2 (8). Although JAK1, JAK2, and TYK2 are expressed in all cell types (9), including human endothelial cells, the expression of JAK3 is restricted to cells of the myeloid and lymphoid lineages (10). JAK activation is mediated by phosphorylation of specific tyrosine residues (9); phosphorylation of tyrosine residues 1007/1008 is a marker of JAK2 activation (11). JAKs are activated by autophosphorylation via direct association with cell surface receptors (9), such as the interferon (IFN) receptor (12), or through interaction with tyrosine kinases, such as the Src family of kinases (13). The major action of JAK is to promote gene transcription by activating STAT proteins (14). To date, seven mammalian STAT proteins have been identified, referred to as STAT1–4, -5A, -5B, and -6 (15). STAT3 activation can be detected as phosphorylation of tyrosine 705 and serine 727 (16). Once activated, STAT proteins homo- or heterodimerize and translocate into the nucleus, where they activate gene transcription through binding to specific promoter response elements (17). Most STAT dimers recognize and bind to members of the γ-IFN activation sequence (GAS) (18) or the IFN-stimulated response element (19) family of enhancers to promote gene transcription; to date, homodimerized STAT3 has only been shown to have affinity for and bind to the GAS (20, 21).

In this study, we have demonstrated that in response to Ox-PAPC treatment, c-Src kinase activates JAK2, which subsequently phosphorylates and activates STAT3. Activated STAT3 then translocates into the nucleus and binds to a GAS element in the IL-8 promoter, which regulates IL-8 transcription. We have also demonstrated a role for endothelial STAT3 in atherosclerosis in mice, as well as the presence of activated STAT3 in the inflammatory regions of human atherosclerotic lesions. These findings suggest that STAT3 activation by oxidized phospholipids may be an important therapeutic target for treatment of atherosclerosis.

**EXPERIMENTAL PROCEDURES**

**Material and Reagents**—M199 medium for HAEC was purchased from Irvine Scientific. MCDB131 medium for HMEC was purchased from Invitrogen. Fetal bovine serum was obtained from Hyclone. PAPC was purchased from Avanti Polar Lipids. Oxidized phospholipids were prepared as described previously (22). Rabbit polyclonal phosphospecific antibodies against JAK1 Tyr-1022/1023, JAK2 Tyr-1007/1008, and STAT3 Tyr-705, STAT3 Ser-727, Src Tyr-416, as well as the STAT3 blocking peptide against phospho-STAT3 Tyr-705 were purchased from Cell Signaling Laboratories, and rabbit polyclonal antibodies against JAK2, STAT3, and glyceraldehyde-3-phosphate dehydrogenase were purchased from Santa Cruz Biotechnology. For the ChIP studies, STAT3 antibody was purchased from Transduction Laboratories, and STAT3 Tyr-705 was from Upstate Biotechnology. PP2, PP3, AG490, AG9, and IFNγ were purchased from Calbiochem.

Cell Culture—HAEC were isolated from the aortic rings of explanted donor hearts and cultured as described previously (23). HMEC, obtained from the Center for Disease Control and Prevention (24), were cultured as described previously (4). Treatment with lipids and other activating agents was performed in media supplemented with 1–2% (v/v) fetal bovine serum.

Cell Lysate Preparation—Confluent endothelial cells (HAEC and HMEC) were harvested and lysed in radioimmunoprecipitation assay (RIPA) buffer as described previously (25). After centrifugation at 5,000 rpm for 10 min, the supernatants (cell lysates) were collected. Nuclear extracts were prepared as described previously (23).

Western Blot Analysis—Western blot analyses were performed as described previously (23). Cells were lysed in RIPA buffer and separated on 4–20% acrylamide gels. Peroxidase-labeled secondary antibody was employed and developed with ECL plus. Films were scanned for quantitation of the levels of antibody staining.

Enzyme-linked Immunosorbent Assay (ELISA)—IL-8 levels in cell media were measured with an IL-8 ELISA kit (Quantikine, R & D Systems) according to the manufacturer’s protocol.

Plasmids—Luciferase-reporter plasmids containing the human IL-8 promoter (pIL8-Luc, −1481 to +44 bp) were obtained from Dr. K. Matsushima (University of Tokyo, Japan) (26). Dominant-negative JAK2 (DN-JAK2) construct, which contains a double mutation in C-terminal kinase domain, was provided by Dr. Sunil Srivastava (University of Cincinnati, Ohio) (27).

PCR Site-directed Mutagenesis—Mutations in the GAS element in the pIL-8-Luc were created with a commercially available site-directed mutagenesis kit (QuikChange, Stratagene), and protocols were provided by the manufacturer. Mutation primers were designed as described previously (3) and ordered from Invitrogen. The potential GAS element, found at −537, was mutated from 5′-ttcctgaa-3′ to 5′-tgcagca-3′.

Transient Transfection of Plasmids—HMEC were plated in 48-well culture dishes (2.0 × 10⁵ cells/plate). After 48 h, with cells at 90% confluence, transfections were performed in Opti-MEM (Invitrogen) with 0.125 μg of total DNA (0.05 μg of p-IL8-Luc, 0.025 μg of phRL-Renilla, and 0.05 μg of either the empty vector, pEFBos, or DN-JAK2) per well and Lipofectin (Promega), and Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. After 48 h, cell media were measured with an IL-8 ELISA kit (Quantikine, R & D Systems) according to the manufacturer’s protocol.

Transient Transfection of Small Interfering RNA (siRNA)—siRNA against STAT3, GC %–matched scrambled control (scRNA), and Lipofectamine 2000 were purchased from Invitrogen. Conditions were optimized from the manufacturer’s suggested 50 nM siRNA and 5 μl of Lipofectamine 2000 by testing 5, 20, and 100 nM siRNA. It was found that 5 nM, along with a slightly reduced amount of Lipofectamine 2000, yielded the greatest mRNA knockdown and eliminated transfection-induced IL-8 mRNA synthesis. HAEC were transfected in a 6-well dish at 50% confluence for 3 h with 5 nM siRNA or...
scRNA, and 4 μl of Lipofectamine 2000 in 2 ml of Opti-MEM minimal serum-free media per well. Afterward, cells were rinsed and plated in full-growth media, and allowed to grow an additional 48 h, until confluent.

Quantitative Real Time PCR (qRT-PCR) Using SYBR Green Chemistry—Primers for IL-8 and glyceraldehyde-3-phosphate dehydrogenase were designed using Primer Quest online design tool (Integrated DNA Technologies). Sequences were as follows: glyceraldehyde-3-phosphate dehydrogenase, 5′-tcatgccctcagaccacttttg-3′ and 5′-accaccctgtgctgttagaatc-3′; IL-8, 5′-accacctgccaggccagaaat-3′ and 5′-tccagacagagctctcttccatcaga-3′; STAT3, 5′-aggttagctagatcactaggtgga-3′ and 5′-ttctgttccaaaagggccagattg-3′. qRT-PCR was performed, and data were analyzed as described previously (23).

Chromatin Immunoprecipitation (ChIP)—ChIP assays were performed using the ChIP-IT™ chromatin immunoprecipitation kit (Active Motif, Carlsbad, CA) according to the manufacturer's instructions with the average size of sheared fragments ∼500 bp. The following forward and reverse primers that targeted sequence flanking GAS element (5′-ttctctgaa-3′, 537 bp upstream of START), in the IL-8 gene promoter were used: 5′-ggttttcacagtgctttcac-3′ and 5′-tttccctctttgagtcatgc-3′. To identify the GAS element in the IL-8 promoter, we used Gene2Promoter program (Genomatix) to identify potential transcription factor binding sites. Approximately 1-kb sequence upstream and 100-bp sequence downstream of the transcription start site were examined. This GAS element was also confirmed by the MOTIF program. For confirmation, the promoter sequence for the gene of interest was obtained from the UCSC genome browser and analyzed using the MOTIF program. Approximately 2 kb upstream of the start site in the IL-8 promoter were examined. Similar results with ChIP assays were obtained in two experiments.

Immunohistochemistry—Human carotid endarterectomy samples from seven different donors were obtained. Specimens were placed directly on ice and fixed within 1 h in fixative containing 3% buffered neutral formalin and subsequently paraffin-embedded. Four-μm-thick sections were de-paraffinized and rehydrated. Immunostaining was performed as described previously (28). For incubation with blocking peptide 5 μg/ml of peptide were employed and incubated with antibody for 0.5 h before use in immunostaining.

Mice and Atherosclerotic Lesion Quantitation—Mice with a conditional STAT3 allele (STAT3f/f) were obtained from the laboratory of Dr. Xin-Yuan Fu (Yale University, New Haven, CT) (29). Cre-recombinase transgenic mice, in which Cre expression was regulated by the VE-cadherin promoter (VE-Cre), were obtained from the laboratory of Dr. Luisa Iruela-Arispe (UCLA) (30). VE-Cre mice were backcrossed a total of six generations onto the C57BL/6j background and then bred with STAT3f/f mice, which were already on the C57BL/6j background. The resulting heterozygotes (Cre STAT3f/+′) were backcrossed with STAT3f/f to generate 12 STAT3f/f (eSTAT3+/+) and 12 Cre STAT3f/f mice (eSTAT3−/−). At 8 weeks of age, mice were transferred onto the atherogenic diet containing 15% fat, 1.25% cholesterol, and 0.5% sodium cholate (TD 90221, Harlan Teklad) for 16 weeks. Mice were subsequently fasted for 12 h, weighed, and bled by retro-orbital puncture for lipid profile analysis. Cholesterol measurements were performed in triplicates as described previously (31). Sectioning of the heart and quantitative analysis of lesion size were performed as described previously (31). Macrophage content was also examined after staining with CD68 antibody of five sections from each mouse. The number of nuclei in the lesion area stained positively with CD68 was determined. We only included cells with an identifiable nucleus.

RESULTS

Ox-PAPC and PEIPC Treatment of HAEC Activates JAK2—To determine whether treatment of HAEC with Ox-PAPC and its most active component, PEIPC, activated JAK2, phosphoryl-
JAK2 activation regulates Ox-PAPC- and PEIPC-induced IL-8 synthesis. A, HAEC were pretreated for 30 min with the indicated concentrations of AG490 or vehicle and then stimulated for 4 h with Ox-PAPC (45 μg/ml) or control media. IL-8 production in the cell media was analyzed using ELISA. B, HAEC were pretreated for 30 min with 10 μM AG490 or vehicle and then stimulated for 4 h with PEIPC (1 μg/ml) or control media as indicated. IL-8 production in the cell media was analyzed using ELISA. C, HAEC were pretreated for 30 min with 10 μM AG490, 10 μM AG9, or vehicle and then stimulated for 4 h with Ox-PAPC (45 μg/ml) or control media as indicated. Relative IL-8 mRNA levels were analyzed using qRT-PCR. D, HMEC, co-transfected with pIL-8-Luc and phRL-Renilla, and either the DN-JAK2 construct or its empty vector were treated for 12 h with either Ox-PAPC or PEIPC (1 μg/ml) or control media as shown. Luciferase activation, normalized to Renilla, was analyzed. For A–D, values are mean ± S.D. (n = 4); *, p < 0.05; **, p < 0.01. Data shown are representative of four separate experiments with similar results. DN-JAK2, dominant-negative JAK2 containing double mutation in the kinase domain. DMSO, Me₂SO.

Inhibition of JAK2 Activation Decreases Ox-PAPC- and PEIPC-induced IL-8 Synthesis—To test the hypothesis that JAK2 plays a role in Ox-PAPC-induced IL-8 synthesis, we employed the selective JAK2 inhibitor, AG490. This inhibitor represses JAK2 activity with an IC₅₀ in the low (<10 μM) micromolar range, and concentrations as high as 50 μM have been shown to have no effect on the activity of Src kinases (33). In our studies, using 10 μM AG490, we also observed no effect on phosphorylation of Tyr-418 of c-Src in response to OxPAPC (data not shown). Pretreatment of HAEC with AG490 caused a significant dose-dependent decrease in Ox-PAPC-induced IL-8 protein synthesis, as measured by ELISA (Fig. 2A). Pretreatment of HAEC with 10 μM AG490 also significantly reduced the induction of IL-8 protein synthesis by PEIPC (Fig. 2B). Because the induction of IL-8 protein synthesis by Ox-PAPC is mediated at the transcription level, we examined the role of JAK2 in mediating Ox-PAPC-induced IL-8 phosphorylation within 2 min (and for up to 4 h) following treatment with Ox-PAPC (45 μg/ml; see Fig. 1A, top panel) and PEIPC (1 μg/ml; see Fig. 1B, top panel). Total levels of JAK2 remained unchanged at all time points following treatment with Ox-PAPC (Fig. 1A, middle panel) and PEIPC (Fig. 1B, middle panel). Using densitometry, we calculated the fold activation of JAK2 by measuring the ratio of Ox-PAPC- or PEIPC-induced phosphor-JAK2 band intensity to that of PAPC-induced phosphor-JAK2 band intensity. Over several experiments, at their respective 1-h peaks, Ox-PAPC treatment of HAEC induced a 4-fold increase in JAK2 phosphorylation (Fig. 1A, bottom panel), whereas PEIPC treatment induced a 3-fold increase (Fig. 1B, bottom panel). We also examined the activation state of JAK1 following Ox-PAPC treatment. Our findings demonstrated that Ox-PAPC treatment of HAEC did not alter the levels of JAK1 Tyr-1022/1023 phosphorylation, a marker of JAK1 activation (data not shown). These findings demonstrated that treatment of HAEC with Ox-PAPC and PEIPC activated JAK2, but not JAK1, in a rapid, yet sustained manner.

In a previous study (4), we demonstrated activation of c-Src kinase in HAEC following Ox-PAPC treatment. The interaction between c-Src and JAK2 has been reported in other systems (13, 32). Because our findings demonstrated that Ox-PAPC-induced JAK2 activation was sustained for up to 4 h, whereas c-Src activation was rapid and transient, we hypothesized that activation of JAK2 was downstream of c-Src activation. To address whether Ox-PAPC-induced JAK2 activation was mediated through c-Src kinase, we utilized the chemical inhibitor of c-Src kinase activity, PP2, and its inactive analogue, PP3. Pretreatment of HAEC with PP2, but not PP3, decreased Ox-PAPC-induced JAK2 Tyr-1007/1008 phosphorylation (Fig. 1C, top panel), whereas total JAK2 levels remained unchanged (Fig. 1C, middle panel). Densitometry from four experiments revealed a 50% reduction in JAK2 activation with PP2 (Fig. 1C, bottom panel). Furthermore, we observed that the inhibitory effect of PP2 on JAK2 activation was specific to Ox-PAPC, because PP2 did not affect the activation of JAK2 by IFNγ, a known JAK2 activator (Fig. 1C).

In the previous study (4), we demonstrated activation of c-Src kinase in HAEC following Ox-PAPC treatment. The interac-
JAK/STAT in Inflammation and Atherosclerosis

**FIGURE 3. JAK2 regulates STAT3 activation by Ox-PAPC and STAT3 activation regulates IL-8 transcription.** A and B, HAEC were transfected with JAK2 siRNA or scRNA and treated with Ox-PAPC (50 μg/ml) or control media for 1 h. A, JAK2 mRNA levels were analyzed using qRT-PCR. B, STAT3 activation was analyzed by Western analysis using phosphospecific antibody to STAT3 (top panel) and phosphorylation state-independent antibody to STAT3 (middle panel) for normalization. Fold induction of Ox-PAPC-induced STAT3 phosphorylation relative to control-induced STAT3 phosphorylation was analyzed from three pooled experiments (bottom panel). C and D, HAEC were transfected with STAT3 siRNA or scRNA and treated with Ox-PAPC (50 μg/ml) or control media (C) for 4 h. C, STAT3 mRNA levels were analyzed using qRT-PCR; D, IL-8 mRNA levels were analyzed using qRT-PCR. For A–D (bottom panel of B), values are mean ± S.D. (pooled from the three repeated experiments). **, p < 0.01.

with AG490 10 μM for 30 min followed by a 1-min treatment with Ox-PAPC (the optimal time for Src phosphorylation).

To further test the role of JAK2 in regulating Ox-PAPC-induced IL-8 transcription, we examined the effect of a dominant-negative kinase-deficient JAK2 construct (DN-JAK2) on IL-8 promoter (pIL-8-Luc) activation. In HMEC, transient transfection of DN-JAK2 caused a significant reduction in Ox-PAPC-induced IL-8 promoter activation by ~40%, as compared with empty vector (Fig. 2D). These findings demonstrated that JAK2 activation regulates the induction of IL-8 transcription by Ox-PAPC.

**JAK2 Activation Regulates IL-8 Transcription through STAT3—**We next examined the mechanism by which JAK2 activation regulated Ox-PAPC-induced IL-8 transcription. Previously, we had proposed a role for STAT3 in IL-8 transcription by Ox-PAPC. Therefore, we examined the role of JAK2 on the activation of STAT3 by Ox-PAPC. For these studies, siRNA to JAK2 was employed. Treatment of HAEC with siRNA to JAK2 down-regulated JAK2 mRNA levels by greater than 90%, as compared with transfection with scRNA oligonucleotides (Fig. 3A). Furthermore, JAK2 siRNA completely inhibited Ox-PAPC-induced STAT3 activation, as measured by Tyr-705 phosphorylation (Fig. 3B, top panel), but has no significant effect on total STAT3 levels (Fig. 3B, middle panel). This result was observed in several experiments (Fig. 3B, bottom panel). Next, we examined the role of STAT3 in Ox-PAPC-induced IL-8 transcription using siRNA against STAT3. STAT3 is composed of two isoforms, STAT3α (86 kDa) and STAT3β (79 kDa); the siRNA used in our studies targeted the STAT3α isoform. Our findings demonstrated a 90% knockdown in relative STAT3α mRNA levels following transfection with the STAT3 siRNA, as compared with transfection with scRNA oligonucleotides (Fig. 3C). Furthermore, STAT3 siRNA significantly reduced the levels of IL-8 mRNA induced by Ox-PAPC in HAEC, as compared with transfection with scRNA (Fig. 3D). These findings demonstrated that JAK2 activation regulated IL-8 transcription in HAEC through STAT3 activation.

**Identification of the GAS Element in the IL-8 Promoter and Its Role in IL-8 Transcription by Ox-PAPC—**To determine the mechanism by which STAT3 regulated IL-8 transcription by Ox-PAPC, we first examined the human IL-8 promoter for a sequence homologous to the known STAT3 response element, GAS. A sequence was identified in the IL-8 promoter (5′-TTTCCTAGAA-3′), 537 bp upstream of START, which was identical to the consensus GAS (5′-TTTCN₂⁻⁴GAA-3′) in all relevant positions. To determine the relevance of the consensus GAS element in Ox-PAPC signaling, ChIP assays were performed to investigate whether STAT3 would directly bind to the IL-8 promoter. Our findings demonstrated that Ox-PAPC treatment induced the binding of STAT3 (Fig. 4A, top panel) to the sequence flanking the consensus GAS in the IL-8 promoter. Furthermore, the bound form of STAT3 was demonstrated to be the activated, Tyr-705 phosphorylated form of STAT3 (Fig. 4A, 2nd panel). Specificity for the binding of STAT3 to the IL-8 promoter was also demonstrated using nonspecific IgG, which did not bind to this promoter sequence (Fig. 4A, 3rd panel). Treatment with INFγ was used as positive control, and the input levels across all conditions were equal (Fig. 4A, bottom panel).

We next examined the importance of this GAS element in IL-8 promoter activation by Ox-PAPC by creating a mutant construct of the pIL-8-Luc, containing 4-bp mutations in this sequence (as described under "Experimental Procedures"). HMEC were transfected with either the wild type pIL-8-Luc (1481) or the mutant pIL-8-Luc (1481ΔGAS), and IL-8 promoter activation following Ox-PAPC treatment was analyzed. Our results demonstrated that the GAS mutation significantly
reduced activation of the IL-8 promoter by Ox-PAPC (Fig. 4B). Phorbol 12-myristate 13-acetate, which induces IL-8 transcription through activation of the AP-1 and NF-κB response elements (34), was used as a negative control and demonstrated equivalent activation of both constructs (Fig. 4B). These data demonstrated a direct role for STAT3 in the regulation of IL-8 transcription and identified the GAS element as an important response element mediating IL-8 promoter activation by Ox-PAPC.

In Vivo Evidence for the Role of STAT3 in Atherosclerosis in Humans—To determine whether STAT3 activation plays a role in atherosclerosis in vivo, we first examined sections of human atherosclerotic lesions from carotid endarterectomy specimens for activated STAT3 staining. We examined both noninflammatory and inflammatory areas of the lesion for binding of the activated phospho-STAT3 (Tyr-705) antibody. In inflammatory areas of atherosclerotic lesions, there was strong staining for activated phospho-STAT3 (Tyr-705) in the nuclei of endothelial cells and also in the nuclei of some inflammatory cells. Blocking antibody was mixed with the blocking peptide before staining. Magnifications ×200.

endothelial STAT3 activation might play an important role in the recruitment of inflammatory cells into the human atherosclerotic lesion and that oxidized phospholipids might contribute to this STAT3 activation.

In Vivo Evidence for the Role of STAT3 in Atherosclerosis in Mice—We next examined the role of endothelial STAT3 in atherosclerosis in vivo, using Cre-Lox technology. Endothelial STAT3 knockout mice were generated by breeding STAT3f/f mice (on a C57/BL6 background) with VE-cadherin Cre (VE-Cre) transgenic mice (on this same background), in which the VE-cadherin promoter regulated Cre-recombinase expression. VE-cadherin is an endothelial cell adhesion molecule that is expressed and active in both embryonic and adult endothelial cells (35). Using the Rosa26R reporter mouse, Alva et al. (30) demonstrated that the VE-Cre mice expressed Cre throughout the endothelium (and to a lesser extent, in a small compartment of hematopoietic cells). Endothelial STAT3 knockout (eSTAT3−/−) and wild type (eSTAT3+/+) mice were generated and placed on the atherogenic diet for 16 weeks. Despite no significant differences in weight (eSTAT3+/+, 24.0 ± 2.3 g; eSTAT3−/−, 22.9 ± 3.8 g) or total cholesterol levels following the diet (eSTAT3+/+, 240.8 ± 109.8 mg/dl; eSTAT3−/−, 225.7 ±
JAK/STAT in Inflammation and Atherosclerosis

FIGURE 6. Endothelial STAT3 knock-out mice have smaller atherosclerotic lesions. A, mean lesion areas per section through the entire aortic root of the indicated eSTAT3+/+ and eSTAT3−/− mice were analyzed. Average lesion areas with S.E. (error bars) for each group are shown: eSTAT3+/+ = 7,750 ± 1658; eSTAT3−/− = 3575 ± 1002; p < 0.05. S.E. = standard error. 12 mice were analyzed for each group. B, representative sections of aortic root from eSTAT3+/+ (WT) and eSTAT3−/− (KO) mice stained with Oil Red O. C, average number of macrophage/5 sections for all 12 mice in each group.

128.5 mg/dl), eSTAT3−/− mice had significantly smaller lesions than the eSTAT3+/+ mice (Fig. 6A). The lesions in these mice were all fatty streak lesions as shown in representative sections (Fig. 6B). Total macrophage content per five sections was determined after staining with CD68 antibody and was also reduced in the eSTAT3 null mice (Fig. 6C). These findings further supported the role for STAT3 as an important gene regulating atherosclerosis in vivo.

DISCUSSION

This study suggests an important role for the c-Src/JAK2/STAT3 pathway in atherosclerosis. Our in vitro studies identify an important pro-atherogenic response controlled by this pathway, the induction of IL-8 transcription by oxidized phospho-

FIGURE 7. Schematic model for Ox-PAPC- and PEIPC-induced IL-8 transcription. Ox-PAPC and its component phospholipid, PEIPC, through a yet unidentified receptor (1), induce a rapid and transient increase in c-Src kinase activity (2). Activated c-Src kinase induces the phosphorylation and activation of JAK2 (3), which subsequently phosphorylates and activates STAT3. Activated STAT3 monomers dimerize and translocate into the nucleus (4), where they selectively bind to the GAS element in the IL-8 promoter and induce IL-8 transcription.

This study has also identified an enhancer element in the IL-8 promoter, 537 bp upstream of START, which is identical to the consensus GAS element. Using ChIP analysis, we demonstrated the binding of activated STAT3 to a sequence flanking the GAS element in the IL-8 promoter (Fig. 4A). Inhibitors of JAK2 were able to inhibit Ox-PAPC-induced IL-8 mRNA and protein synthesis (Fig. 2, A–D). We also demonstrated that JAK2 mediates STAT3 activation by Ox-PAPC (Fig. 3B), and that STAT3 activation plays a direct role in regulating IL-8 transcription by Ox-PAPC (Fig. 3D). We focused on STAT3 in these studies because we previously found that Ox-PAPC treatment of HAEC activated STAT3 but not STAT1, -5, or -7 (3). This was examined by measuring the phosphorylation and nuclear translocation of STAT1, -3, -5, and -7.

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also been demonstrated in the signaling pathways induced by other inflammatory mediators, including IL-4 (37) and IL-6 (38). Importantly, a previous study demonstrated that activated STAT3 and nuclear factor-κB (NFκB) were required to increase IL-8 expression in adenocarcinoma cells (39). Interestingly, we demonstrated previously that Ox-PAPC treatment does not activate NFκB yet still induces IL-8 expression in HAEC. This suggests that either STAT3 alone is sufficient to stimulate IL-8 expression in response to Ox-PAPC or that a different co-activator is required.

Previously, we reported that Ox-PAPC induction of IL-8 transcription was elevated for at least 24 h (3). We therefore propose that the c-Src/JAK2/STAT3 pathway regulates the early phase of IL-8 induction, because activation of this pathway returns to control levels within 6 h following Ox-PAPC treatment. In contrast, we have identified a second pathway, involving the activation of endothelial nitric-oxide synthase and sterol regulatory element-binding protein, which is activated after 2 h of Ox-PAPC treatment and sustained for at least 16 h; we propose that the endothelial nitric-oxide synthase/sterol regulatory element-binding protein pathway regulates the sustained induction of IL-8 transcription by Ox-PAPC (23).

This study has also examined the role of STAT3 activation in atherosclerosis in vivo. We have demonstrated significantly greater STAT3 activation in the endothelium of the inflammatory areas of human atherosclerotic lesions (Fig. 5). Activated STAT3 staining was also present in monocytes in the human lesions, suggesting a possible role in this cell type as well as in endothelial cells. Furthermore, using the Cre-Lox system, we have generated STAT3 knock-out mice, in which STAT3 was knocked out in the endothelium, and demonstrated reduced fatty streak formation in these mice, as compared with their wild type littermates (Fig. 6A). Although we attribute the reduction in atherosclerosis in these mice to the deletion of STAT3 in the endothelium, we cannot rule out the effect of STAT3 deletion in hematopoietic cells on atherosclerosis, because Cre was expressed in a subset of hematopoietic cells (30).

Our studies are the first to report a role for STAT3 in atherosclerosis. However, activation of the JAK/STAT pathway has been previously implicated in other cardiovascular pathways. In a recent study, Maziere et al. (40) demonstrated activation of JAK2 in fibroblasts in response to oxidized low density lipoprotein. Shibata et al. (41) demonstrated that STAT3 activation in smooth muscle cells regulated neointima formation in mice, and IL-13 regulated 15-lipoxygenase transcription in macrophages (16). Taken together, these studies suggest that STAT3 activation plays an important role in vessel wall homeostasis.

Ox-PAPC and its component phospholipids accumulate not only in atherosclerotic tissue but also in other sites of chronic inflammation. STAT3 activation has been demonstrated to play a role in other chronic inflammatory diseases, including rheumatoid arthritis (42), psoriasis (43), and systemic lupus erythematosus (44). Therefore, this study suggests that activation of STAT3 by oxidized phospholipids may be an important interventional target for atherosclerosis as well as other chronic inflammatory diseases.

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