Oxysterol and Diabetes Activate STAT3 and Control Endothelial Expression of Profilin-1 via OSBP1*\textsuperscript{t}\textsuperscript{s}

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Endothelial dysfunction plays a central role in diabetic vascular disease, but its molecular bases are not completely defined. We showed previously that the actin-binding protein profilin-1 was increased in the diabetic endothelium and that attenuated expression of profilin-1 protected against atherosclerosis. Also 7-ketocholesterol up-regulated profilin-1 in endothelial cells via transcriptional mechanisms. The present study addressed the pathways responsible for profilin-1 gene expression in 7-ketocholesterol-stimulated endothelial cells and in the diabetic aorta. In luciferase reporter assays, the response to 7-ketocholesterol within the 5′-flanking region of profilin-1 was dependent on a single STAT response element. In aortic endothelial cells, 7-ketocholesterol enhanced STAT3 activation, which required JAK2 and tyrosine 394 phosphorylation of oxysterol-binding protein-1. These changes were recapitulated in the aorta of diabetic rats. Also 7-ketocholesterol in cultured endothelial cells and diabetes in the aorta elicited the recruitment of STAT3 and relevant coregulatory factors to the oxysterol-responsive region of the profilin-1 promoter. These events were required for profilin-1 up-regulation. These studies identify a previously unrecognized oxysterol-binding protein-mediated mode of activation of STAT3 that controls the expression of the proatherogenic protein profilin-1 in response to 7-ketocholesterol and the diabetic milieu.

Dysfunction of the vascular endothelium precedes, and may contribute to, atheroma formation in response to a plethora of cardiovascular noxae, including diabetes (1, 2), hyperlipidemia (3, 4), and systemic as well as local inflammatory mediators (5). Although several markers of endothelial dysfunction have already garnered interest in the clinical arena (6, 7), the molecular bases of endothelial injury are still not fully understood. A growing body of evidence indicates that cytoskeletal dynamics regulate essential antiadhesive, anti-inflammatory, and antiatherogenic properties in endothelial cells (EC)\textsuperscript{2} (8, 9).

Our laboratory demonstrated that the actin-binding protein profilin-1 (Pfn) plays a role in endothelial dysfunction and atherosclerosis. Pfn is a well characterized regulator of cytoskeletal architecture because of its multifaceted function in actin filament assembly (10) and depolymerization (11). Of note, little is known regarding the transcriptional regulation of Pfn in mammalian cells either constitutively or in response to factors promoting vascular disease. We showed previously that Pfn protein levels were increased in the diabetic endothelium and within atherosclerotic plaques (12). Also attenuation of Pfn levels protected against atherosclerosis and endothelial dysfunction upon high fat feeding (13). Finally 7-ketocholesterol (oxysterol), but not high glucose concentrations, elevated Pfn expression in EC in part via transcriptional mechanisms (12). Collectively these studies suggest that Pfn levels are regulated by oxidized lipids and contribute to vascular injury associated to diabetes and a high fat diet.

Oxysterols are naturally occurring oxygenated products of cholesterol that play a major role in cholesterol homeostasis (14) and are enriched in foam cells and atherosclerotic plaques (15). In addition to interacting and regulating the activity of members of the liver X receptor family of transcription factors (for a review, see Ref. 16), oxysterols bind with varying affinity to the cytosolic protein oxysterol-binding protein-1 (OSBP1), which was first identified by Taylor et al. (17), and a growing list of OSBP1-related proteins (18). Oxysterol binding to the sterol-binding domain of OSBP1 elicits its translocation and tethering to the Golgi, which is mediated by the PH domain located in the N-terminal region of the protein (19). Recently OSBP1 was identified as an essential component of a multiprotein complex that regulates p44/42 mitogen-activated protein kinase activity in response to cholesterol and oxysterol (20), thus defining an unexpected role for OSBP1 as a scaffolding for the assembly of signaling modules.

Based on our previous findings, we reasoned that elucidating the mechanisms for oxysterol- and diabetes-dependent up-regulation of Pfn would shed new light on the pathophysiology of

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\textsuperscript{2} The abbreviations used are: EC, endothelial cells; RAEC, rat aortic endothelial cells; Pfn, profilin-1; JAK2, Janus-activated kinase-2; STAT, signal transducers and activators of transcription; OSBP, oxysterol-binding protein; PH, pleckstrin homology; ChIP, chromatin immunoprecipitation; sh, short hairpin; SH2, Src homology 2; STZ, streptozotocin; D.U., densitometric unit(s); GAP, GTPase-activating protein; IL-6, interleukin-6; IL-6R, interleukin-6 receptor; WT, wild-type; HDAC, histone deacetylase; DM, 5-month STZ diabetic; CBP, CAMP-responsive element-binding protein (CREB)-binding protein; SRC1, steroid receptor coactivator 1; H3, histone 3; ACh3, acetylated H3; BRG1, Brahma-related gene 1.
vascular injury associated with the metabolic syndrome. Here we show that OSBP1 is required for oxysterol-dependent nucleation and activation of the JAK2/STAT3 pathway, which in turn regulates Pfn gene expression in EC. Similarly diabetes increases the activation of STAT3 and its recruitment to the Pfn promoter in large vessels in vivo.

**EXPERIMENTAL PROCEDURES**

**Reagents**—7-Ketocholesterol (hereafter, oxysterol) and 25-hydroxycholesterol (Steraloids, Inc.) were the oxidized cholesterol derivatives used for cell stimulation. Other reagents were purchased from Sigma-Aldrich unless otherwise specified.

**Cells**—Rat aortic endothelial cells (RAEC) were isolated and cultured as described previously (12). Stimulation with oxysterols was performed at a final concentration of 10 μg/ml (~25 μM) in ethanol. Cell viability (trypan blue exclusion test) and apoptosis, assessed by photometric detection of mono- and oligonucleosomes (Cell Death Detection ELISA PLUS, Roche Applied Science), were comparable in untreated and oxysterol-treated cells over the short stimulation periods of our experiments. Stimulation with rat recombinant IL-6 (50 ng/ml; R&D Systems) and rat recombinant granulocyte-macrophage colony-stimulating factor was performed after overnight culture in medium with 0.2% bovine serum albumin and 0.5% fetal bovine serum.

**Animals and Organ Isolation**—Induction of diabetes by streptozotocin (55 mg/kg of body weight intravenously) in 8-week-old Sprague-Dawley male rats, insulin treatment, and procedures for perfusion and aorta isolation were described previously (12). Glycohemoglobin was measured (Glyc-Affin, Pierce) before sacrifice to assess the degree of hyperglycemia (14.1 ± 1.6% in diabetic versus 4.4 ± 0.3% in control rats). After isolation, the aorta was either used for extraction of protein lysates from the endothelium (12) or for chromatin immunoprecipitation experiments.

**Chromatin Immunoprecipitation (ChIP) in RAEC and Rat Aorta**—In RAEC (1.5 × 10⁷ cells per condition), ChIP assays were performed as described in the manufacturer’s manual (Upstate) with several modifications. After stimulation, medium was added with formaldehyde to a 1% final concentration for 5, 7.5, or 10 min. Fixation for 5 min greatly enhanced the efficiency of the subsequent STAT3 and steroid receptor coactivator 1 (SRC1) immunoprecipitation procedure. Following addition of glycine (125 mM) for 5 min at room temperature and three washes in ice-cold phosphate-buffered saline, cells were collected in ice-cold phosphate-buffered saline. Protease inhibitors leupeptin (10 μM), pepstatin (1 μM), aprotinin (5 μg/ml), and phenylmethylsulfonyl fluoride (1 mM) were added to buffers throughout the procedure. After centrifugation at 3,000 × g for 5 min, the pellet was resuspended in 1 ml of lysis buffer (50 mM Tris-HCl (pH 8.0), 2 mM EDTA, 10% glycerol, and 0.1% Nonidet P-40), rotated for 10 min at 4 °C, briefly vortexed, and subjected to 20 strokes with a Dounce homogenizer pestle B. After centrifugation (1,000 × g for 5 min), the supernatant was discarded, and the nuclear pellet was resuspended in 100 μl of nuclear lysis buffer (50 mM Tris-HCl (pH 8.0), 10 mM EDTA, and 1% SDS). Sonication was then performed on ice (four cycles of 10-s pulses interspersed by a 30-s pause, constant setting, 1/4 of maximum power in a Branson Sonifier 450) to yield DNA fragments of ~0.3–1.0-kb size. After centrifugation at maximum speed for 15 min in a microcentrifuge, the supernatant was diluted 20 times with ChIP dilution buffer (1.0% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl (pH 8.1), and 167 mM NaCl) and used for immunoprecipitation. A 2% aliquot was saved to quantify the starting material (“input”). Samples were precleared for 3 h at 4 °C using the respective non-immune IgG and either Protein A- or Protein G-agarose beads coupled with salmon sperm. The conditions and washing buffers for immunoprecipitation varied according to the antibody as indicated in Table 1. Following immunoprecipitation, the chromatin-protein-antibody complex was eluted from the beads by three 10-min incubations with 150 μl of freshly prepared elution buffer (1% SDS and 0.1 mM NaHCO₃). DNase-free RNase (Roche Applied Science) was added at a final concentration of 50 μg/ml for 1 h at 37 °C followed by incubation at 65 °C for 6–8 h to reverse cross-linking. Finally DNA-complexed proteins were digested for 2 h at 45 °C with nickase-, endonuclease-free proteinase K (Sigma; 100 μg/ml in 40 mM Tris-HCl (pH 6.5) and 10 mM EDTA). After DNA extraction by standard procedures, the pfn promoter regions were amplified by PCR using the primers Forward (~1025 to ~999) (5′-TGA GTCC CGG TCT GTG AAC CGG GTC GGC-3′) and Reverse (~410 to ~436) (5′-ACC ACT TTT CCG CAG AAG GAG GAA ACC-3′), yielding a 616-bp fragment. The ChIP assay in the aorta was optimized using several modifications of the procedure in cells. Anesthetized control and diabetic rats were perfused first with phosphate-buffered saline (3 min) and then with 1% formaldehyde (2 min). The whole descending aorta was dissected and cleaned from perivadienditial tissue. Samples were carefully minced, fixed again for 5 min, and subjected to 20 strokes with a Dounce homogenizer pestle B. Tissue fragments were lysed in nuclear lysis buffer followed by sonication (five cycles of 10-s pulses interspersed by a 60-s pause, constant setting, ½ of maximum power in a Branson Sonifier 450). Samples from 7–10 rats were pooled for each ChIP assay.

**TABLE 1 Primary antibodies and conditions used in ChIP assays**

| Protein | Source, reference no. | Species | Washing buffers (n times) |
|---------|-----------------------|---------|--------------------------|
| Acetyl-H3 (Ly-9/14) | Upstate, 06-599 | Rabbit | LS-I (2), HS-I (2), LiCl (1) |
| Acetyl-H3 | Cell Signaling, 9671 | Rabbit | LS-I (2), HS-I (2), LiCl (1) |
| STAT3 | Upstate, 06-596 | Rabbit | LS-II (2), HS-II (2), LiCl (1) |
| STAT3 | Santa Cruz, sc-482 | Rabbit | LS-II (2), HS-II (2), LiCl (1) |
| SRC1 | Santa Cruz, sc-6096 | Goat | LS-II (2), HS-II (2), LiCl (1) |
| CBP | Santa Cruz, sc-369 | Rabbit | LS-II (2), HS-II (2), LiCl (1) |
| CBP | Upstate, 06-294 | Rabbit | LS-II (2), HS-II (2), LiCl (1) |
| pCAF | Upstate, 07-141 | Rabbit | LS-II (2), HS-II (2), LiCl (1) |
| BRG1 | Upstate, 07-478 | Rabbit | LS-II (2), HS-II (2), LiCl (1) |
| HDAC3 | Upstate, 06-890 | Rabbit | LS-II (2), HS-II (2), LiCl (1) |
Nuclear Extracts—Cell fractionation was carried out using 1.5–2.0 × 10^6 RAEC. After washes with ice-cold STE (10 mM Tris (pH 7.4), 150 mM NaCl, and 1 mM EDTA) and ST (10 mM Tris (pH 7.4) and 150 mM NaCl), cells were harvested in ice-cold ST supplemented with 2 mM Na_4VO_4, 5 μg/ml aprotinin, 10 μM leupeptin, 1 μM pepstatin, and 1 mM phenylmethylsulfonyl fluoride. After centrifugation and a short wash in Buffer A (20 mM HEPES (pH 7.9), 25% glycerol, 1.5 mM MgCl_2, 0.2 mM EDTA, and 0.5 mM dithiothreitol), hypotonic lysis was performed in Buffer A for 15 min on ice followed by 30 strokes with a prechilled Dounce homogenizer pestle B. Nuclei were collected by centrifugation at 2,500 × g for 5 min; the supernatant was used as “cytosolic” extract. The nuclear pellet was resuspended in 200 μl of high salt Buffer C (20 mM HEPES (pH 7.9), 25% glycerol, 1.5 mM MgCl_2, 20 mM KCl, 0.2 mM EDTA, and 0.5 mM dithiothreitol), and then 200 μl of high salt Buffer C (20 mM HEPES (pH 7.9), 25% glycerol, 1.5 mM MgCl_2, 0.2 mM EDTA, 0.8 mM KCl, and 0.5 mM dithiothreitol) were added dropwise, ensuring thorough resuspension of nuclei. Following five strokes with a Dounce homogenizer, extracts were gently rotated for 1 h at 4 °C. After centrifugation at 13,000 × g for 15 min, the resulting supernatant was used as the “nuclear” extract. For immunoprecipitation, nuclear extracts were diluted 4 times in low salt buffer yielding a ~100 mM KCl final concentration.

Western Blot and Immunoprecipitation—For total cell lysate extracts, RAEC were harvested in radioimmune precipitation assay buffer and processed for immunoprecipitation or immunoblot analysis as described previously (12). Protein lysates from the aortic endothelium of control and diabetic rats were obtained as detailed previously (12). Immunoprecipitation for endogenous OSBP1 (goat polyclonal antibody, Novus Biologicals) in rat aorta was performed using 1 mg of lysates extracted from the endothelium of combined thoracic and abdominal aorta (12). All other immunoprecipitation experiments were performed using 0.5 mg of total RAEC or thoracic aorta lysates. Immunoprecipitation was performed after preclearing with the respective non-immune IgG and Protein G– or Protein A-agarose (Santa Cruz Biotechnology, Inc.). A list of antibodies used for immunoblot and the apparent molecular weight of the detected bands is reported in Table 2. Densitometric values were normalized for RasGAP, here used as a loading control (12, 13).

Plasmid Construction and Expression—For stable expression of full-length rabbit OSBP1 and a PH-deficient version, the respective coding sequence was excised with Pmel from pcDNA3.1–OSBP1-myc-his and pcDNA3.1–ΔPH-myc-his (kindly provided by P.-y. Wang, University of Texas Southwestern Medical Center) and cloned into Stul sites of the retroviral vector pLNCX2 (Clontech). The cDNA coding for pLNCX2–OSBP1-myc-his mutant Y394F was constructed using the QuikChange XL site-directed mutagenesis kit (Stratagene). The forward primer for this construct was 5’-GCC ATG ATG GAC AGT TTA AGC ATC AGC TGG AGG AGA CC-3’ corresponding to positions 1246–1283 of GenBank™ accession number J05056 (the site of Tyr-394 mutated to Phe is underlined). cDNAs were verified by sequencing. After transfection of pLNCX2–OSBP1 constructs and empty vector into 293GPG cells (21), the virus-containing supernatant was collected, concentrated (25,000 × g for 90 min at 4 °C), and used to infect RAEC as described previously (12). Following selection with G418 (1 mg/ml), expression of OSBP1 plasmids was detected using anti c-MYC antibody (9E10), whereas total levels were monitored with goat polyclonal anti-OSBP1 (Novus Biologicals). With regard to STAT3 expression, RAEC were transiently transfected with the rat STAT3 expression vector (ATCC number 63359) or the pSVSport-1 empty vector using a combination of FuGENE (Roche Applied Science) and PLUS reagent (Invitrogen).

Short hairpin (sh)RNA—Knockdown of OSBP1 was achieved using the two oligos described previously by Wang et al. (20) that were engineered and cloned into the BamHI-EcoRI sites of RNAi-Ready pSIREN-RetroQ (Clontech BD Biosciences) according to the manufacturer’s instructions. RAEC were infected with virus, obtained as above, coding for the shRNA sequence 9E10 (the site of Tyr-394 mutated to Phe is underlined) and G418 (1 mg/ml).
target sequences or pSIREN empty vector and selected with puromycin (2 μg/ml).

Luciferase Reporter Assay—A PCR-based method was used to serially clone fragments of the 5′-region of the rat pfn gene upstream of the transcription initiation site into the pGL3-Basic vector (Promega). Genomic DNA from RAEC was used as a template for cloning. pGL3 constructs were named according to the size of the inserted promoter region (Table 3). The 5′-end of PCR forward primers included a KpnI site, and the 5′-end of the common reverse primer included a HindIII site (bold italic). The STAT-binding site at 945 to 1500 of the 717 fragment to oxysterol. Preincubation with synthetic agonists for the oxysterol-regulated liver X receptor family of transcription factors, T090137 (16) and GW3965 (22) both used at 5 μM final concentration, did not affect luciferase activity of pfn constructs (data not shown) in keeping with their lack of liver X receptor response elements (24). Together these studies indicated that oxysterol positively regulated pfn promoter activity, within the proximal 1.5-kb sequence of the 5′-flanking region, via a single STAT-binding site located at nucleotide −501. Although other transcription factors may play a critical role in basal transcriptional activity, we investigated the mechanisms for STAT activation and its function in Pfn regulation in response to oxysterol and to diabetes in vivo.

Diabetes and Oxysterol Activate STAT3 in EC—STATs are transcription factor that reside in the cytoplasm in a latent form and become activated by phosphorylation in response to a plethora of extracellular ligands (25), thus controlling processes such as inflammation and innate immunity (26) that may play a role in endothelial dysfunction. Phosphorylation and nuclear translocation of members of the STAT family were assessed using one-way analysis of variance followed by Bonferroni test. A p value of less than 0.05 was considered significant.

RESULTS

Oxysterol Regulates pfn Promoter Activity via STAT-Binding Site—Because 7-ketocholesterol (oxysterol) increases Pfn levels at least in part by transcriptional mechanisms (12), its effects on promoter activity of serial fragments of the 5′-flanking region of the rat pfn gene were assessed using a luciferase reporter assay. pfn promoter constructs were transfected in RAEC along with pSV-β-galactosidase as an internal control for the procedure. Luciferase reporter activity of fragments downstream of nucleotide −401, relative to the transcription initiation site, was not influenced by oxysterol (10 μg/ml; Fig. 1A). Conversely promoter activity of the fragment spanning from +59 to −717 was significantly increased in oxysterol-treated RAEC when compared with control cells (13.6 ± 1.29; versus 6.6 ± 0.33-fold over pGL3-Basic after normalization for β-galactosidase activity; p < 0.0005). Software-assisted analysis (MatInspector, Genomatix) of transcription factor response elements within this fragment revealed a single potential STAT-binding site, TTN5AA (23), starting at nucleotide −501. Notably site-directed mutagenesis of this consensus sequence largely abrogated response of the +59/−717 fragment to oxysterol. Promoter activity of this fragment was further elevated by co-transfection with a rat STAT3 expression vector (Fig. 1B), which resulted in a ~3-fold increase in total STAT3 protein levels accompanied by a modest increase in unstimulated STAT3 activation (data not shown). The effects of STAT3 over-expression were observed only in oxysterol-stimulated RAEC, thus suggesting that elevating STAT3 levels alone was not sufficient to influence pfn promoter activity. Furthermore forced expression of STAT3 did not modify promoter activity of the mutated +59/−717 fragment. Although 7-ketocholesterol was the primary type of oxidized cholesterol used for these experiments, exposure to 25-hydroxycholesterol (1 μg/ml) resulted in a comparable profile of promoter activity with values ~20% higher than those resulting from treatment with 7-ketocholesterol (data not shown). Finally preincubation with synthetic agonists for the oxysterol-regulated liver X receptor family of transcription factors, T090137 (16) and GW3965 (22) both used at 5 μM final concentration, did not affect luciferase activity of pfn constructs (data not shown) in keeping with their lack of liver X receptor response elements (24). Together these studies indicated that oxysterol positively regulated pfn promoter activity, within the proximal 1.5-kb sequence of the 5′-flanking region, via a single STAT-binding site located at nucleotide −501. Although other transcription factors may play a critical role in basal transcriptional activity, we investigated the mechanisms for STAT activation and its function in Pfn regulation in response to oxysterol and to diabetes in vivo.
levels, strongly suggesting an essential role for JAK2 in oxysterol-mediated STAT3 activation. Treatment with 25-hydroxycholesterol resulted in a similar pattern of STAT3 activation (data not shown).

Following tyrosine phosphorylation, STAT dimerization takes place through the reciprocal interaction of Src homology 2 (SH2) domain with phosphotyrosine-binding domain to create either homo- or heterodimers, thus leading to STAT translocation to the nucleus and regulation of target genes (27). As expected, nuclear extracts from RAEC exposed to oxysterol showed a marked increase in phosphorylated STAT3, further demonstrating STAT3 activation upon oxysterol treatment (Fig. 2B).

Conversely oxysterol treatment did not induce either STAT5 or STAT1 phosphorylation in apparent contrast with previous evidence obtained in mouse embryo fibroblasts (28) (Fig. 2C). The activation of STAT3 by oxysterol in RAEC prompted us to test whether this event could be recapitulated in diabetes, which is associated with enhanced lipid oxidation (29, 30).

Phosphorylated STAT3 was significantly increased in the aortic endothelium of 5-month streptozotocin (STZ) diabetic rats when compared with age-matched nondiabetic rats (0.59 ± 0.04 versus nondiabetic 0.23 ± 0.03 arbitrary densitometric unit (D.U.); p < 0.0001; Fig. 2D). The elevation in STAT3 activation was also observed after a shorter duration of diabetes (10 weeks) but did not achieve statistical significance (p = 0.07; data not shown). Collectively these studies demonstrated that oxidized cholesterol species can acutely and transiently activate the JAK2/STAT3 pathway in aortic EC and that this event could also be observed in the diabetic macrovasculature.

**Diabetes and Oxysterol Induce Tyrosine Phosphorylation of OSBP1 and Its Association with JAK2**—Through the interaction of their SH2 domain with phosphorylated tyrosines of receptor complexes, STATs can be recruited and can operate as second messengers in numerous signaling pathways (31). We tested whether oxysterol triggered prototypical activators of the JAK2/STAT3 pathway, namely interleukin-6 receptor (32) (IL-6R) and granulocyte-macrophage colony-stimulating factor receptor (33). Oxysterol loading did not induce tyrosine phosphorylation of the gp130 chain, which is the intracellular transducer of IL-6R, whereas it did induce STAT3 phosphorylation (supplemental Fig. 1). Similarly granulocyte-macrophage colony-stimulating factor receptor was not Tyr phosphorylated by oxysterol (data not shown). In contrast, tyrosine phosphorylation of OSBP1 was detected in response to oxysterol with a time course comparable to that of STAT3 activation (Fig. 3A). Moreover we tested the interaction of OSBP1 and JAK2, the putative kinase responsible for STAT3 phosphorylation. Co-
immunoprecipitation studies showed that oxysterol markedly augmented OSBP1 association with JAK2 (Fig. 3B). Similar changes were observed in the macrovasculature of rats with STZ-induced diabetes, an established model of endothelial dysfunction. When compared with age-matched nondiabetic controls, the aorta of 5-month diabetic rats showed a significant increase in Tyr phosphorylation of the slower migrating form of OSBP1 (tyrosine phosphorylated/total OSBP1 ratio, 1.14 ± 0.23 versus nondiabetic 0.43 ± 0.16 arbitrary D.U.; p = 0.0005; Fig. 3C) accompanied by enhanced association of OSBP1 with JAK2 (OSBP1/JAK2 ratio, 2.08 ± 0.09 versus nondiabetic 0.93 ± 0.08 arbitrary D.U.; p < 0.0001; Fig. 3D). Of note, total OSBP1 levels were comparable in the two groups (data not shown). Based on these findings, we concluded that both oxysterol and diabetes induced STAT3 activation through a “non-classical” pathway and hypothesized that OSBP1 could play a role in this process.

Several lines of evidence defined OSBP1 as a rational candidate for mediating STAT3 activation. 1) OSBP1 contains a single YXXQ motif, which is the critical STAT3 SH2-binding consensus sequence (34). 2) As recently shown (20), OSBP1 can regulate the assembly of signaling modules in response to oxysterol. 3) As shown in Fig. 3, both diabetes and oxysterol loading result in OSBP1 tyrosine phosphorylation and its association with JAK2.

**OSBP1 Is an Essential Mediator of Oxysterol-dependent STAT3 Activation—**To begin addressing this hypothesis, the requirement of Tyr-394, in the context of the only YXXQ motif within OSBP1, was assessed in RAEC stably expressing MYC-tagged rabbit wild-type (WT) OSBP1 or its Y394F-mutated version. Upon oxysterol stimulation, STAT3 activation was enhanced in WT-OSBP1 cells and significantly abrogated in Y394F cells when compared with empty vector cells (Fig. 4A). Conversely the profile of p44/42 phosphorylation was comparable in WT- and Y394F-OSBP1 cells, suggesting that Tyr-394 mutation did not affect OSBP-mediated regulation of p44/42 (20). Also Y394F cells displayed oxysterol-dependent translocation of OSBP1 to the Golgi and IL-6-dependent activation of STAT3 comparable to that in WT-OSBP1 cells, thus ruling out indirect effects of Tyr-394 mutation (supplemental Fig. 2). In addition, Y394F mutation largely prevented OSBP1 Tyr phosphorylation in response to oxysterol (tyrosine phosphorylated/total MYC ratio, 0.26 ± 0.09 versus Y394F 0.08 ± 0.29 arbitrary D.U.; p < 0.001; Fig. 4B). Collectively these experiments indicated that OSBP1 phosphorylation at Tyr-394 was required for oxysterol-mediated STAT3 activation and that Y394F mutation of OSBP1 could operate as a dominant negative of the endogenous protein.

**OSBP Is Required for Pfn Up-regulation in Response to Oxysterol—**The effects of OSBP1 on STAT3 activation were further investigated in RAEC following shRNA-mediated knockdown of OSBP1 at ~90% of the endogenous levels. Upon oxysterol treatment, shRNA-OSBP1 cells showed reduced nuclear accumulation of phospho-STAT3 when compared with empty vector control cells (Fig. 5A). These results, together with the luciferase reporter studies presented in Fig. 1, suggested that OSBP1 knockdown would abrogate Pfn up-regulation in response to oxysterol. As previously shown (12), oxysterol increased Pfn levels starting at 6 h in empty vector control cells. Pfn elevation was largely prevented in shRNA-
OSBP1 cells (Fig. 5B). In agreement with the role of OSBP1 in p44/42 activation (20), shRNA-OSBP1 cells showed a marked decrease in p44/42 phosphorylation both under basal and oxysterol-stimulated conditions. Pretreatment with PD98059, an inhibitor of the mitogen-activated protein kinase MEK1 upstream of p44/42, did not affect STAT3 activation or Pfn up-regulation in response to oxysterol (data not shown). These studies indicate that oxysterol triggers an OSBP-dependent, but p44/42-independent, pathway that mediates up-regulation of the proatherogenic protein Pfn.

**Activated STAT3 Is Recruited to the pfn Promoter in a Multisubunit Protein Complex**—As shown in Fig. 1, the STAT consensus sequence at −501 was required for oxysterol-dependent pfn gene expression in luciferase reporter experiments. Transcriptional activation is dynamically controlled by the interplay of coactivators with intrinsic histone acetyltransferase activity, histone deacetylases (HDACs), and a growing family of chromatin-remodeling factors (35). Thus, we investigated whether

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**Figure 3.** OSBP1 Tyr phosphorylation and association with JAK2 are enhanced by oxysterol and diabetes. A, after exposure to 7-ketocholesterol (oxysterol, 10 μg/ml) for the indicated time periods, RAEC overexpressing MYC-tagged OSBP1 were processed for immunoprecipitation (IP) with anti-MYC or non-immune mouse IgG (neg ctrl) followed by immunoblot (IB) analysis. The 0' lane represents cells exposed to buffer (ethanol; final concentration, 0.25%). Oxysterol induced OSBP1 Tyr phosphorylation (PY) beginning at 5 min (n = 5). B, immunoprecipitation with anti-JAK2 or non-immune rabbit IgG (neg ctrl) of lysates of RAEC overexpressing MYC-tagged OSBP1 followed by immunoblot analysis. Oxysterol resulted in enhanced association of JAK2 to OSBP1 (n = 5). C, immunoprecipitation with anti-OSBP1 or non-immune goat IgG (neg ctrl) followed by immunoblot analysis in whole aorta lysates from DM and age-matched control (C) rats. Diabetes enhanced Tyr phosphorylation of the slower migrating form of OSBP1. The bar graph represents the ratio (expressed in arbitrary D.U.) of Tyr phosphorylation (PY) over OSBP1 in aortic lysates of C, DM, and age-matched control (C) rats. Diabetes augmented JAK2 association to endogenous OSBP1. The bar graph represents the ratio (expressed in arbitrary D.U.) of JAK2 to OSBP1 in aortic samples (*, p < 0.0001; n = 6 per group). D, minutes.

**Figure 4.** OSBP1 is required for oxysterol-mediated STAT3 activation. A, immunoblot analysis in RAEC expressing empty vector (EV; pLNCX2), MYC-tagged WT-OSBP1, or its Y394F-mutated version. STAT3 phosphorylation in response to oxysterol was increased in WT-OSBP1 and significantly blunted in Y394F cells when compared with empty vector cells (10 min: empty vector versus Y394F, p < 0.0001). In contrast, WT-OSBP1 and Y394F showed comparable levels of phosphorylated p44/42 that were moderately higher than in cells expressing empty vector. Immunoblot with an antibody that recognizes both endogenous (rat) and cDNA-expressed (rabbit) OSBP1 demonstrated a similar increase of total OSBP1 (~3-fold) in WT and Y394F when compared with empty vector. RasGAP was used as a control for loading (n = 4). B, RAEC expressing MYC-tagged WT-OSBP1 or its Y394F-mutated version were processed for immunoprecipitation (IP) with anti-MYC or non-immune mouse IgG (IgB) analysis. Tyrosine phosphorylation (PY) in response to oxysterol was largely, although not completely, prevented in Y394F cells. The bar graph represents the ratio (expressed in arbitrary D.U.) of tyrosine phosphorylation over MYC (OSBP1) at 5 min of oxysterol treatment (*, p < 0.0001; n = 4), minutes; P, phospho-

**Figure 5.** OSBP1 knockdown prevents oxysterol-induced Pfn up-regulation. A, nuclear and cytosolic extracts were obtained from RAEC stably expressing sh empty vector (EV; pSIREN-RetroQ) or sh tRNA-OSBP1 that were stimulated with 7-ketocholesterol (10 μg/ml) or buffer (ethanol; final concentration, 0.25%; 0 min). Immunoblot analysis of shRNA-OSBP cell fractions showed almost complete OSBP1 knockdown in cytosolic extracts paralleled by decreased nuclear levels for phospho-STAT3 upon oxysterol treatment when compared with empty vector cells. Lamin and RasGAP were used as loading control for nuclear and cytosolic fractions, respectively (n = 4). B, immunoblot analysis showed that Pfn up-regulation upon oxysterol treatment was largely abrogated in shRNA-OSBP cells, which displayed almost complete OSBP1 knockdown when compared with empty vector cells. RasGAP was used as loading control (n = 4). P, phospho-.
activated STAT3 could associate with relevant coactivators and be recruited on the endogenous pfn promoter upon oxysterol treatment and in 5-month STZ diabetic (DM) rats. First, immunoprecipitation studies addressed the association of STAT3 with CREB-binding protein (CBP) and SRC1, two histone acetyltransferase proteins that have been shown to bind STAT3 (36, 37). Upon oxysterol treatment, the increased nuclear pool of STAT3 interacted with both SRC1 and CBP (Fig. 6A). Notably STAT3 association with SRC1 peaked earlier than that with CBP in keeping with SRC1 function as an “adapter” for CBP docking to the transcriptional machinery (38). Oxysterol did not change SRC1 and CBP total levels (Fig. 6B). Second, we investigated the effects of oxysterol on pfn gene activation and recruitment of coregulatory proteins on the pfn promoter using ChIP. Oxysterol loading resulted in histone 3 (H3) hyperacetylation, an event typically associated with gene activation, of the −410/-1025 5′-flanking region of pfn that spans the STAT3 response element (Fig. 7A). ChIP analysis with antibodies to acetylated H3 (AcH3) at Lys-9/14 or Lys-9 alone showed similar results. This finding is in agreement with the oxysterol-dependent expression of the −401/-717 region in luciferase reporter studies (Fig. 1). Moreover oxysterol resulted in STAT3 recruitment to the target pfn chromatin, an event that was prevented by pretreatment with AG-490 (Fig. 7B). The time course of STAT3 binding to pfn showed strong correlation with the profile of STAT3 phosphorylation and nuclear translocation (Fig. 2). In addition to STAT3, oxysterol led to recruitment of relevant histone acetyltransferase proteins, HDAC, and chromatin-remodeling factors on the target pfn region (Fig. 7C). The profile of CBP association closely mirrored STAT3, whereas SRC1 interaction with the target region was transient and preceded that of STAT3 and CBP. Conversely an antibody to p300/CBP-associated factor failed to immunoprecipitate pfn target chromatin (data not shown). Also ChIP experiments for prototypical class I (HDAC1 and -3) and class II (HDAC4 and -7) deacetylases showed that HDAC3 was exclusively detected on the pfn target chromatin at 60 min, thus preceding the decline in STAT3 and CBP recruitment at 120 min. Finally oxysterol stimulation resulted in a progressive binding of the Brahma-
related gene 1 (BRG1), which is a critical subunit of the ATP-dependent chromatin-remodeling complex SWI/SNF (39). In summary, oxysterol-induced H3 hyperacetylation at the target gene chromatin in association with the ordered recruitment of STAT3 and other chromatin-remodeling factors.

Diabetes Increases AcH3 and STAT3 Recruitment on the pfn Gene in Vivo—Both oxysterol in vitro and diabetes in vivo increase Pfn levels in aortic EC (12) in addition to enhancing STAT3 activation (Fig. 2). Oxysterol-induced changes detected at the pfn target chromatin led us to hypothesize that diabetes could recapitulate these events in the aorta in vivo. To this end, we optimized a ChIP procedure in the rat aorta using a pool of 7–10 specimens per assay. Indeed AcH3 at the pfn target chromatin was significantly increased in the aorta of STZ diabetic rats when compared with nondiabetic age-matched controls (AcH3/input ratio, 3.6 ± 0.98 versus nondiabetic 0.82 ± 0.49 arbitrary D.U.; p < 0.005; Fig. 8A). Moreover diabetes magnified the recruitment of STAT3 on the pfn promoter (STAT3/input ratio, 2.53 ± 0.32 versus nondiabetic 0.91 ± 0.21 arbitrary D.U.; p = 0.005; Fig. 8B). These studies demonstrate that pfn, a hitherto unappreciated target gene for STAT3, is activated in diabetic vessels and underscore a striking similarity between the effects exerted by oxysterol and the diabetic milieu in EC.

DISCUSSION

The present work addressed the mechanisms that control Pfn expression in EC upon stimulation with oxysterol or in diabetes in vivo. The rationale for these investigations stems from our recent report showing that Pfn levels play a critical role in early atheroma formation and vascular inflammation (13). Here we present several novel observations that indicate striking similarities between pathways triggered by oxysterol and the diabetic milieu in EC. Indeed oxysterol-mediated activation of STAT3 and its recruitment to the pfn promoter were recapitulated in the diabetic aorta (Fig. 9). Although STAT3-dependent Pfn expression may be a novel contributing factor to the accelerated atherosclerosis observed in diabetic patients, it should be acknowledged that mechanisms and consequences of STAT3 activation in the diabetic macrovasculature may extend beyond the findings presented here. As recently shown by Banes-Berceli et al. (40), STAT3 activation in the diabetic aorta can occur through an angiotensin II-, endothelin-dependent pathway involving JAK2. Our studies implicate OSBP as a critical mediator of JAK2/STAT3 activation in response to oxysterol. Thus, STAT3 may be at the crossroad of, and possibly integrate, several pathways of diabetic vascular injury. Unregulated activation of STAT3 may play a role in inducing or maintaining the chronic, low grade inflammation that accompanies both diabetes (41) and atherosclerosis (42) by regulating a maladaptive program of gene expression.
STAT3 Activation upon Oxysterol Treatment and in Diabetes

Independently of the activating ligand(s), ChIP experiments in the aorta demonstrated the relevance of the STAT3-dependent regulation of pfn in the setting of diabetic vascular disease and to the best of our knowledge represent the first application of ChIP to the vasculature in vivo. In light of the emerging role of STAT3 in diabetic complications, characterizing the specificity versus redundancy of its target genes upon different stimuli should be a primary goal. For instance, we found that IL-6 could not induce STAT3 recruitment to pfn promoter while triggering a robust STAT3 activation.3 Chromatin target specificity may result, among other factors, from the discrete profile of coregulatory proteins that are engaged by oxysterol and IL-6. In this view, oxysterol regulated a fine tuned scheme of recruitment of coregulatory proteins on the pfn promoter (Fig. 7) that could not be mimicked by IL-6 stimulation.3

Another aspect of novelty of these studies originates from the observation that OSBP1 is an essential mediator of oxysterol-dependent STAT3 activation. Although initially characterized as a cholesterol sensor, OSBP1 (and perhaps other members of the OSBP1-related protein family) can influence cholesterol signaling through its recently recognized scaffolding function as shown for p44/42 activation (20). Based on this emerging evidence, we postulated that OSBP1 could similarly “nucleate” the assembly of a JAK2/STAT3 module upon oxysterol treatment. We found that phosphorylation of Tyr-394, in the context of the unique STAT3 SH2-binding motif YXXQ, was required for STAT3 activation. Tyr-394 is located just within the sterol-binding domain of OSBP1 (amino acids 382–798) and is predicted to be highly accessible under basal conditions (Scansite). Thus, oxysterol binding to OSBP1 could induce a conformational change necessary to recruit JAK2 to OSBP1 as supported by their enhanced association in the diabetic aortic endothelium and in cultured EC exposed to oxysterol (Fig. 3). Also it appears that OSBP1 controls activation of STAT3 and p44/42 via largely independent mechanisms as abrogation of STAT3 activation was not associated with changes in p44/42 phosphorylation (Fig. 4A), and vice versa MEK1 inhibition did not affect STAT3 activation (data not shown). Such an oxysterol-initiated, OSBP1-mediated mode of activation of STAT3 plays a critical role in Pfn up-regulation in EC and, one may speculate, in the diabetic endothelium, which shows increased Pfn levels. These experiments corroborate the emerging role of OSBP1 as a signaling platform for pathways regulating cholesterol metabolism, cell growth/migration, and inflammation.

Collectively these studies set the foundation to assess the impact of the OSBP/STAT3 pathway in models of diabetic vascular disease. Measures aimed at preventing Tyr-394 phosphorylation would allow assessment of the relative contribution of oxysterol/STAT3 to the pathogenesis of diabetic endothelial dysfunction without affecting STAT3 prosurvival properties in response to other stimuli (43). Although our experiments focused on Pfn as a proof-of-principle STAT3 target under these conditions, it is likely that excess STAT3 activation in the diabetic aorta will regulate a diverse program of gene expression possibly relevant to diabetic vascular injury.

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