Expression of Plasma Platelet-activating Factor Acetylhydrolase Is Transcriptionally Regulated by Mediators of Inflammation*

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Platelet-activating factor (PAF) is a potent phospholipid with diverse physiological and pathological actions, and it is inactivated by PAF acetylhydrolase. In this study, we analyzed the tissue distribution of the plasma PAF acetylhydrolase mRNA in humans. We isolated a 3.5-kilobase fragment containing the 5′ genomic sequence of the plasma PAF acetylhydrolase gene and further characterized the promoter activity. We determined the transcriptional initiation site by primer extension. We then prepared constructs containing various lengths of 5′ genomic fragments fused to a luciferase reporter gene and transfected these constructs into COS-7 cells. We found that there is more than one region in the 1.3-kilobase 5′ genomic sequence conferring promoter activity and that a very short 5′-flanking region (72 base pairs) is sufficient for more than 65% of the basal activity. In parallel, we examined the regulation of expression of the PAF acetylhydrolase gene. We found that interferon-γ (IFNγ) and lipopolysaccharide (LPS) significantly inhibited synthesis of PAF acetylhydrolase, whereas other cytokines, including IFNα, interleukin (IL) 1α, IL4, IL6, tumor necrosis factor-α, granulocyte/macrophage colony-stimulating factor, and macrophage colony-stimulating factor, had a smaller or no effect in human monocyte-derived macrophages. Furthermore, transfection of the promoter/reporter construct into macrophage RAW264.7 cells revealed that IFNγ and LPS decreased the promoter activity by 35% and 50%, respectively, whereas PAF stimulated it by 52% via its receptor. The promoter activity was much lower in monocytic U937 cells compared with the basal level in COS-7 cells, while the activities in P388D1 and RAW264.7 macrophagic cells were considerably higher than the basal level in COS-7 cells. There are multiple regions in the PAF acetylhydrolase promoter that contain responsive elements for signal transducer and activators of transcription-related proteins, and also for myeloid-specific transcription factors. Our data indicate that the opposite of mRNA expression in monocytes versus macrophages is due to inhibition of the promoter activity in the former and activation in the latter cells.

Platelet-activating factor (PAF, 1-alkyl-2-acetyl-sn-glycero-3-phosphocholine) is a potent phospholipid with diverse physiological and pathological effects in a variety of cells and tissues (for review, see Refs. 1–3). The biological effects of PAF include activation of platelets, polymorphonuclear leukocytes, monocytes, and macrophages. PAF also increases vascular permeability, decreases cardiac output, induces hypotension, and stimulates uterine contraction (2). The actions of PAF may contribute to the physiological processes associated with pregnancy, fetal development, parturition, and kidney function. PAF has been implicated in pathological processes, such as inflammation and allergy (4). Moreover, there are indications that PAF might be involved in human immunodeficiency virus pathogenesis and in carcinogenesis (5, 6).

The levels of PAF in plasma and tissues are determined by the balance of synthesis and degradation (7). A key mechanism for the removal of PAF is hydrolysis catalyzed by PAF acetylhydrolase, which converts PAF to the biologically inactive lysophosphatidic acid (8). This enzymatic activity has been detected in plasma and in the cytosolic fraction of some cells and tissues (9, 10). Molecular studies of the PAF acetylhydrolase from various sources indicate that the plasma and cytosolic forms of this enzyme are encoded by different genes (11–13). Several lines of evidence indicate that monocyte-derived macrophages are a major source of plasma PAF acetylhydrolase. Messenger RNA for the plasma PAF acetylhydrolase is induced and a large amount of this activity is secreted when monocytes differentiate to macrophages (11, 14). Furthermore, the properties of the macrophage-secreted PAF acetylhydrolase are identical to those of the plasma form, and the secreted enzyme is recognized by an antibody raised against the purified plasma PAF acetylhydrolase (15). Thus, monocyte-derived macrophages clearly secrete the plasma form of PAF acetylhydrolase. Tjoelker et al. (11, 16) cloned the plasma PAF acetylhydrolase cDNA by screening a human macrophage cDNA library. The encoded protein is composed of 441 amino acid residues with a catalytic triad GXSG, which is conserved in neutral lipases and serine esterases.

Plasma PAF acetylhydrolase dramatically reduces the inflammatory action of PAF both in vitro and in vivo, suggesting that this enzyme plays an important role in the regulation of PAF levels in blood and tissues (11). Moreover, PAF acetylhydrolase also catalyzes the hydrolysis of oxidatively fragmented...
phospholipids, which have been detected in oxidized low density lipoprotein and have been shown to be mitogenic and inflammatory under certain circumstances (17). Therefore, PAF acetylhydrolase may protect against oxidative damage.

The secretion of PAF acetylhydrolase activity has been shown to be regulated by many cytokines and hormones in cultured macrophages and hepatocytes. Previously, we showed that HepG2 cells and primary cultures of hepatocytes secrete PAF acetylhydrolase activity (although at relatively low levels compared with macrophages) (18). We found no substantial effect of dexamethasone on the secretion of this activity by hepatocytes, while Narahara et al. (19) showed that dexamethasone increases secretion from HL-60 cells that have differentiated to macrophages. In addition, lipopolysaccharide (LPS), interleukin (IL) 1α, IL1β, and tumor necrosis factor-α (TNFα) reduced the secretion from decidual macrophages (20). It was observed that estrogen administration to animals decreased PAF acetylhydrolase secretion (21). In in vitro studies, however, the effect depended on the cell type; estrogen reduced the secretion from HepG2 cells but had no effect on differentiated HL-60 cells (18, 19). Another regulator of PAF acetylhydrolase expression is its substrate. Satoh et al. (22) showed that PAF, but not the product of the reaction, lysoPAF, stimulated the secretion of PAF acetylhydrolase from HepG2 cells. In summary, previous studies have shown that macrophages secrete high levels of PAF acetylhydrolase activity, which can be influenced by inflammatory mediators. However, the regulatory mechanism was not examined in any of the published studies.

One of the most dramatic examples of regulation of PAF acetylhydrolase synthesis is during the differentiation from monocytes to macrophages. Low levels of PAF acetylhydrolase mRNA were detected in isolated human monocytes by Northern analysis. In contrast, large amounts were detected once these cells differentiated to macrophages (11). This agreed with a previous report, in which the enzymatic activity was shown to increase during the maturation process (15). PAF acetylhydrolase mRNA also was expressed differentially among tissues; it was highly expressed in tonsil and placenta but not in a number of others (11). Taken together, the above results strongly suggest that plasma PAF acetylhydrolase is regulated in a tissue- and differentiation-dependent manner.

We report here the cloning of a 3.5-kb 5′ genomic fragment of the plasma PAF acetylhydrolase gene. We demonstrate that this fragment exhibits various levels of promoter activity in monocytic and macrophagic cell lines, and characterized the basic features of this promoter. Furthermore, we found that LPS and interferon γ (IFNγ) inhibit the promoter activity while PAF stimulates it.

**EXPERIMENTAL PROCEDURES**

**Materials and Cell Lines**—Hexadecyl-2-[3H-acetyl]-sn-glycero-3-phosphocholine was purchased from NEN Life Science Products. 1-Alkyl-2-acetyl-sn-glycero-3-phosphocholine (PAF) was obtained from Avanti Polar Lipids, Inc. (Alabaster, AL). All chemicals and reagents were from Sigma unless otherwise specified. All of the human and murine cytokines were from R&D Systems. PAF antagonists BN52021 and CV3988 were obtained from Bismol (Plymouth Meeting, PA). γ-32P[ATP (10 mCi/ml) was obtained from Amersham Life Science. Cell culture reagents and restriction enzymes were purchased from Life Technologies, Inc. except where specified. The monkey kidney cell line COS-7, human monocyte cell line U937, and mouse macrophage cell lines RAW264.7 and P388D1 were from American Type Culture Collection (Rockville, MD). The human embryonic kidney cell line 293 that was stably transfected with the PAF receptor, and the rabbit anti-PAF PAF acetylhydrolase antibody were provided by ICOS Corp. (Bothell, WA). The mouse monoclonal antibody against actin was from ICN Biomedicals, Inc. (Costa Mesa, CA).

**Isolation of a PAF Acetylhydrolase Genomic Clone**—A human λ genomic library (Stratagene, La Jolla, CA) was screened with a 32P-labeled Smal-Kpn1 cDNA fragment containing 277 bp of the 5′ end of the cDNA sequence. The probe was labeled with [α-32P]dCTP by random priming, and hybridization was carried out at 42 °C for 16 h. Positive plaques were detected by autoradiography. The λ DNA insert in one of the positive plaques was subcloned into pBluescript (SK), and Southern analysis was performed to confirm the identity of exon 1. A 3.5-kb NotI-BamHI genomic fragment hybridized with the exon 1 probe, and then was subcloned into pBluescript and termed pDS.

**mRNA Dot Hybridization**—A human RNA master blot was purchased from CLONTECH. The amount of poly(A)+ RNA from different tissues on this blot was normalized for eight housekeeping genes by the manufacturer. A human placenta PAF acetylhydrolase cDNA clone (11) was linearized by Smal digestion, and the 2.3-kb fragment was purified and used as template for RNA probe preparation. The antisense RNA was synthesized and labeled with the DIG RNA-labelling kit (Boehringer Mannheim) by an in vitro transcription reaction. The generated 1.2-kb antisense RNA probe then was hybridized to the blot following the manufacturer’s instructions. The levels of PAF acetylhydrolase mRNA were quantified by scanning using Photoshop software and analyzed using NIH Image.

**Primer Extension**—The 5′ end of an antisense oligonucleotide (RAH-40) was labeled using T4 polynucleotide kinase (Promega, Madison, WI) and purified through a NucTrap probe purification column (Stratagene). The primer extension reaction was performed using an AMV reverse transcriptase (Boehringer Mannheim) reverse transcription extension kit from Promega according to the manufacturer’s instructions.

The product was analyzed on a 6% denaturing polyacrylamide gel.

**Generation of Various Lengths of 5′ Genomic Sequence/Luciferase Reporter Constructs**—Various lengths of 5′ genomic fragments were prepared by restriction digestion of pDS, and then ligated into a luciferase reporter vector pGL3basic (Promega). Constructs A and B contain the longest insertion and were generated by digesting pDS with SmaI and NotI, filling protruding ends with Klenow, and inserting into the SmaI site of pGL3basic. The promoter direction is the same as that of the reporter gene in construct A and the opposite direction in construct B. Construct C was made by digestion of pDS with HindIII and insertion into the HindIII site of pGL3basic. The promoter direction is the same as that of the reporter construct. Construct D has the same insert as construct C but the opposite orientation. Construct E was prepared in two steps; pDS was digested with PstI, and the generated 1.15-kb fragment was inserted into pBluescript. The resulting plasmid was cut with BamHI and HindIII and then ligated into pGL3basic previously digested with BglII and HindIII. Construct F was made by digestion of pDS with EarI and EcoRV. The resulting 1-kb Earl-EcoRV fragment was then digested with SmaI and ligated into SmaI-digested pGL3basic. Construct G was prepared by EcoRI digestion of pDS followed by Klenow fill-in, and the 650-bp fragment was purified and inserted into SmaI-digested pGL3basic. Constructs H and I were derived from construct C: construct H was digested with XhoI and the 5.3-kb fragment was purified and self-ligated to generate construct H. Construct I was made in a similar way as construct H except KpnI was used instead of XhoI. Construct J was generated by EcoRI digestion of construct C, and the 5.9-kb fragment was purified and self-ligated. The identity and orientation of the constructs were verified by multiple restriction mapping and partial sequencing.

**Cell Culture**—COS-7 and RAW264.7 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and HyClone Laboratories, Logan, UT. U937 and P388D1 cells were cultured in RPMI 1640 supplemented with 10% and 15% fetal bovine serum, respectively.

**Transient Transfection and Reporter Activity Assays**—Transfection in COS-7 and RAW264.7 cells was carried out using LipofectAMINE (Life Technologies, Inc.). One μg of promoter construct DNA, and 1 μg of pSV-β-galactosidase control plasmid (Promega) were cotransfected into ~ 4 × 105 cells. After 48 h, the cells were harvested and the luciferase and β-galactosidase activities were determined with assay kits from Promega and Tropix, Inc. (Bedford, MA), respectively. Transfection of U937 and P388D1 cells was conducted by electroporation with a Bio-Rad Gene Pulser at 300 V and 960 microfarads. Fifteen μg of promoter construct and 10 μg of pSV-β-galactosidase DNA were co-transfected into ~ 7 × 106 cells, which were harvested after 17 h. The luciferase and β-galactosidase activity assays were performed according to the manufacturer’s instructions.

**PAF Acetylhydrolase Enzymatic Assay**—Secreted PAF acetylhydrolase activity was assessed by collecting conditioned medium and then determined directly as described (23).

**Reverse Transcriptase PCR**—Total RNA was isolated from macrophages using TRI Reagent (Molecular Research Center, Inc., Cincinnati, OH).
were lysed with CHAPS lysis buffer (20 mM Tris-HCl, pH 7.5, 1 mM EDTA, 16 mM CHAPS, 0.5 mM dithiothreitol, 1 mM benzamidine HCl, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 10 μg/ml soybean trypsin inhibitor), and the protein concentration was determined by the BCA protein assay (Pierce). Fifty μg of protein were loaded on a 12% SDS-polyacrylamide gel. Western blots were carried out as described (24). A rabbit polyclonal plasma PAF acetylhydrolase antibody was used as the primary antibody. The secondary antibody was a horseradish peroxidase-conjugated anti-rabbit IgG (Amersham Life Science). The immunodetection was performed using an ECL Western blotting detection system (Amersham). The intensity of the bands was quantified as described above.

Gel Mobility Shift Assays—Nuclear extracts from monocytes and macrophages were prepared as described previously (25). The oligonucleotide probes were chemically synthesized and then were annealed in STE buffer at 100 °C for 10 min and slowly cooled down to room temperature. The annealed, double-stranded nucleotides were separated from the single-stranded oligonucleotides on a 5% low melt agarose gel. The double-stranded oligonucleotides were purified by phenol extraction of the recovered fragment. The probes were labeled with γ-32P]ATP using T4 polynucleotide kinase and then separated from free [γ-32P]ATP through a NucTrap probe purification column. The binding reaction was performed using a gel shift assay system from Promega. Nuclear extracts (5 μg) were incubated with ~50,000 cpm of radioactive probe. The bound products then were analyzed on a 6% native polyacrylamide gel. Gels were dried and exposed to Kodak BioMax MR films (Eastman Kodak Co.) overnight at ~70 °C.

RESULTS

Tissue Distribution of Plasma PAF Acetylhydrolase—Our original studies using Northern blot analysis (11) did not detect mRNA for the plasma form of PAF acetylhydrolase in liver, which conflicted with studies that observed secretion of the activity by cultured hepatocytes (18, 22, 26). Therefore, we examined the mRNA expression with a more sensitive method and a more extensive sample of tissue mRNAs. This experiment (Fig. 1) revealed that there is widespread expression of the mRNA for PAF acetylhydrolase but that the levels differed by as much as 4-fold. The mRNA was expressed in all parts of the brain, but at low levels except for hippocampus, which had the highest expression of the brain tissues. The tissues with the highest levels of expression included ovary, placenta, liver, lymph node, and thyroid gland in adult, and the spleen in the fetus. The lung had a lower, but easily detectable, amount. Thus, this experiment confirmed most of the previously reported pattern but, significantly, identified the liver as a potential source. This widespread distribution is consistent with two interpretations; either many cell types express the mRNA for PAF acetylhydrolase, or one cell type that is found in many tissues expresses it. The latter seemed more probable because we had found previously that macrophages, which are in all tissues, secrete high levels of enzyme activity. However, endothelial cells also are found in most organs, but we found no mRNA and no secretion of the PAF acetylhydrolase activity from endothelial cells (data not shown). Thus, we conclude that the expression of the PAF acetylhydrolase in many tissues is most likely, the result of expression in tissue macrophages. In the studies reported here, we focused on the regulation of PAF acetylhydrolase expression in macrophages.

Regulation of Plasma PAF Acetylhydrolase Expression by Inflammatory Mediators—Several cytokines have been shown to alter the levels of PAF acetylhydrolase activity in vivo, and in some in vitro studies. To determine the basis for this response, we examined the PAF acetylhydrolase activity secreted by monocyte-derived macrophages with or without cytokine treatment. Compared with cells without treatment, cells that were treated with IFNα, IL1α, IL4, IL6, TNFα, GM-CSF (granulocyte/macrophage colony-stimulating factor), M-CSF (macrophage colony-stimulating factor), IFNγ, and LPS all secreted less PAF acetylhydrolase activity, although the effect was modest in most cases (data not shown). We also measured the intracellular activity in the same samples to ensure that the effect on the extracellular activity did not result just from an inhibition of the secretory process; the intracellular levels paralleled (but were more pronounced than) the changes observed in the supernatant, indicating that the effect of cytokines was to decrease the synthesis of PAF acetylhydrolase (data not shown). IFNγ and LPS caused the most marked inhibition on PAF acetylhydrolase secretion by 25% and 34%, respectively. These two cytokines have been shown to synergistically stimulate the expression of the nitric oxide synthase in a macrophage cell line (27). This prompted us to test the effect of IFNγ and LPS in combination. Macrophages were incubated with IFNγ, LPS, or IFNγ plus LPS for 24 h, and the secreted PAF acetylhydrolase activity was determined. The combined treatment resulted in a 25% reduction of the secreted activity, which is the same level as that observed using IFNγ alone (data not shown). Therefore, there was no synergistic or additive effect of IFNγ and LPS on PAF acetylhydrolase expression.

To further examine the level at which the negative regulation by LPS and IFNγ is exerted, we measured the amounts of PAF acetylhydrolase protein and mRNA in cytokine-treated, and control, macrophages. Twenty-four hours after the addition of IFNγ, the levels of mRNA decreased by about 40% (Fig. 2A) and levels of the protein by 50% (Fig. 2B). The levels of PAF acetylhydrolase mRNA decreased to similar levels after LPS treatment (data not shown). These results indicated that the most likely mechanism for the inhibition was an effect on transcription, which we tested in the experiments below.

In addition to cytokines, the substrate of the enzyme, PAF, has been shown to regulate the synthesis of PAF acetylhydrolase although in the opposite direction, stimulation, and only hepatocytes have been examined (18, 22). For example, Satoh...
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![Image](http://www.jbc.org/)

**Fig. 2. IFNγ inhibits PAF acetylhydrolase synthesis by human macrophages.** Human monocyte-derived macrophages were kept in M199 with 1% diisopropyl fluorophosphate-treated serum for 16 h, and then changed to serum-free M199 + IFNγ (50 ng/ml). After 1, 4, 8, and 24 h, a portion of the cells was collected, and total RNA and whole lysate protein were prepared. Sample C, negative control, was collected from the cells without IFNγ treatment at 1 and 24 h, and the level shown represents the means of these two time points. Reverse transcriptase PCR was performed using total RNA. The PCR reaction was carried out using two primers corresponding to the coding region of PAF acetylhydrolase: sense strand, +690 to +1215, TGACCTGGCATCTCATGGGT-TATAG; antisense strand, +1227 to +1252, TTCTGGTGACTGAAC-CCCTGATTG. This pair of primers amplifies a 562-bp cDNA fragment. A pair of gyceraldehyde-3-phosphate dehydrogenase primers was also used for normalization: 5′ primer, ACCACGTCATGCGCATCAC; 3′ primer, TCCACCACCTGTTGCTG7A (CLONTECH). The amplified 452-bp gyceraldehyde-3-phosphate dehydrogenase fragment provided an internal control for the quantitative PCR reaction (A). The protein level was determined by immunoblotting (B), and normalized to the β-actin expression by blotting the same membrane with an anti-β-actin antibody. The data in the plots are the means of two independent experiments.

et al. (22) found that PAF stimulated PAF acetylhydrolase secretion by HepG2 cells by 5-fold. To explore the mechanism for this, we first asked whether PAF had the same effect on monocyte-derived macrophages. We found that treatment of cultured macrophages with PAF (1 μM) increased PAF acetylhydrolase secretion by 47%. The activity in the medium under control conditions was 0.20 μmol/ml/h, and increased to 0.29 μmol/ml/h when PAF was supplemented to the medium for 24 h. Moreover, PAF antagonists BN52021 and CV3988 almost completely blocked the stimulation; the levels of the enzymatic activity returned to 0.22 and 0.20 μmol/ml/h, respectively. This indicates that the effect of PAF was mediated by the PAF receptor. Thus, PAF stimulates PAF acetylhydrolase secretion by macrophages, and the relative change is much lower than that observed in hepatocytes (18, 22). However, the absolute difference is greater since macrophages secrete a much higher basal level (11, 15). We hypothesized that this result, like the cytokine effect, resulted from an effect on transcription.

**Fig. 3. Determination of the transcriptional initiation site of the PAF acetylhydrolase gene.** An antisense oligonucleotide RAH + 40, GACTGCTTCTCAGCACGC, which is 40 bases downstream of the 5′ end of previously identified cDNA, was used to perform the primer extension reaction using avian myeloblastosis virus reverse transcriptase. The template used for the reaction was mRNA isolated from monocyte-derived macrophages, which had been cultured for 10 days. The extension product was separated on a 6% denaturing polyacrylamide gel. The same primer also was used to perform a sequencing reaction using pDS as the template and the sequencing ladder served to determine the size of the primer extension product. Lane 1, dX174/Hind single-strand DNA size marker. Lane 2, primer extension product generated with the RAH + 40 primer. Lanes 3–6, sequencing ladder prepared using the RAH + 40 as primer and pDS as the template. The numbers indicate the size in bases. Two other primers were also used to confirm the position of +1. One primer is located 49 bases downstream the 5′ end of the cDNA construct and produced a 116-base extension product, while the other is located 15 bases upstream and resulted in an extension product of 52 bases.

To obtain the PAF acetylhydrolase promoter, we screened a human genomic library with a 32P-labeled fragment that contains the 5′ flanking region. The DNA insert from a positive plaque was subcloned into pBluescript (SK) vector, generating pDS which contains a −3.5-kb fragment 5′ to exon 1 of the PAF acetylhydrolase gene (Fig. 4). The sequence directly upstream of the transcriptional initiation site contains a −736 to −741. Other cis-acting elements for common transcription factors were found; there are two regions containing the CAAT consensus sequence at nucleotides −426 and −457, one octamer motif at nucleotide −977, which is far upstream; and multiple Sp1 sites around the +1 position. In addition, there are seven regions resembling the MS2 binding consensus sequence, and one Pu.1 box was found at nucleotide +228. Strikingly, there are many regions that contain STAT consensus sequences. From −1113 to −332, 11 regions match the sequence TT(N1)4eAA, which has been shown to bind STAT (signal transducer and activator of transcription) proteins (28, 29). This suggests that the gene is probably regulated by the JAK-STAT pathway.

Various lengths of the 5′ genomic fragments were prepared by digestion of pDS with appropriate restriction enzymes, followed by ligation into a luciferase reporter vector (Fig. 5). The...
identity and orientation of the truncated constructs were verified by restriction mapping and partial sequencing. Each of the constructs was transiently transfected into COS-7 cells, and the luciferase activity was determined. All of them, except B and D, in which the orientation of the promoter was opposite to that of the reporter, showed promoter activity (Fig. 5). Construct I, which contained only 72 bp of upstream sequence, had about 65% of the activity level observed with construct A. This indicates that a very short 5′-flanking region can drive expression. Construct J, which contains the potential TATA box but no Inr site, also showed promoter activity, although weaker than that from either constructs A or I.

**FIG. 4.** Sequence of the 5′ regulatory region of the human plasma PAF acetylhydrolase gene. The pDS clone that contains the 5′ genomic sequence and part of exon 1 was sequenced from both ends using an ABI sequencing system. +1 is the transcriptional initiation site determined by primer extension, and the numbering is relative to this site. The vertical arrow indicates the position of the 5′ end of the original cDNA clone. Potential cis-regulatory elements were determined by computer analysis of the sequence. These elements are underlined and labeled below the sequence to indicate the type of element. The boxes correspond to the sequence of oligonucleotides 1 and 2, which were used as probes in the gel shift experiments (Fig. 8).

**FIG. 5.** Definition of the basal promoter of the PAF acetylhydrolase gene. By restriction digestion and ligation, various lengths of 5′ genomic fragments were subcloned into the luciferase reporter vector, pGL3basic (see “Experimental Procedures”). The left panel depicts the lengths and locations of the 5′ genomic fragments that were inserted into the luciferase reporter vector in constructs A–J. The numbering is relative to the transcriptional initiation site, and the arrows represent the insert orientation. The constructs then were transiently transfected into COS-7 cells using LipofectAMINE™. One μg each of the promoter construct and pSV-β-galactosidase plasmid (Promega) were cotransfected into ~4 × 10⁵ cells. After 48 h, the cells were harvested and both luciferase and β-galactosidase activities were determined. The means of luciferase activity from triplicate assays, normalized by the cotransfected β-galactosidase activity, are shown in the right-hand panel. The fold induction in the vector, pGL3basic, is also shown as a negative control.
Inflammatory Mediators Alter PAF Acetylhydrolase Expression by Regulating Transcription—For studies regarding the effects of cytokines on PAF acetylhydrolase transcription, we used the mouse macrophagic RAW264.7 cell line RAW264.7 since it was much more readily transfected than human monocye-derived macrophages. This cell line is an appropriate model since several previous investigators have reported that this line expresses receptors for a variety of cytokines (30, 31), and we found that treatment of these cells with murine cytokines inhibited the synthesis of PAF acetylhydrolase as assessed by secreted activity (data not shown). Construct A, which contains the entire 3.5 kb of 5' genomic sequence, was transiently transfected into cells supplemented with various cytokines, and luciferase activity was determined 16 h later. We found that IFNγ and LPS had a marked inhibitory effect on PAF acetylhydrolase transcription (Fig. 6), which is in agreement with our activity determinations. IFNγ decreased the promoter activity by about 35%, and LPS reduced it by 50%. Thus, the inhibitory effects of IFNγ and LPS on PAF acetylhydrolase levels can be explained at a transcriptional regulation level.

The effect of PAF on the promoter activity was investigated using a human embryonic kidney cell line, 293, which had been stably transfected with a cDNA encoding the human PAF receptor. The cells were transiently cotransfected with construct A (see Fig. 5) and the β-galactosidase control plasmid. The cells were starved for 5 h and stimulated with 2-O-methyl-PAF at the concentrations indicated, and 17 h later the cells were assayed for luciferase and β-galactosidase activities. The results represent the mean ± standard deviation from three separate experiments and are statistically significant ($p < 0.05$). PAF receptor inhibitors BN52021 and CV3988 were transiently cotransfected with construct A (see Fig. 5) and the β-galactosidase control plasmid. The cells were starved for 5 h and stimulated with 2-O-methyl-PAF at the concentrations indicated, and 17 h later the cells were assayed for luciferase and β-galactosidase activities. The results represent the mean ± standard deviation from three separate experiments and are statistically significant ($p < 0.05$). PAF receptor inhibitors BN52021 (10 μM) and CV3988 (10 μM) were added 1 h before the PAF treatment, where indicated.

Johnston and colleagues (19) have shown that treatment of HL-60 cells with phorbol esters caused the cells to differentiate to macrophage-like cells and to markedly increase the secretion of PAF acetylhydrolase. To assess whether this response is the consequence of differentiation or whether it is because PAF acetylhydrolase is an early inducible gene, we treated monoocyte-like U937 cells with 12-O-tetradecanoylphorbol-13-acetate (TPA) or vehicle control for 17 h after transfection with construct A, and then determined the luciferase activity in the cells. This protocol was chosen because the time period was too short to reflect changes resulting from the differentiation program but was one at which early transcriptional changes should be well established. The promoter activity, as indicated by the luciferase activity normalized to pGL3basic, was not altered by TPA treatment (Table II). This result suggests that PAF acetylhydrolase is unlikely to be an early inducible gene but that its transcription is stimulated during the later stages of macrophage differentiation.

The marked change in transcriptional activity during differentiation suggests that there are alterations in specific trans-acting factors that regulate such events. As noted above, one of

**Fig. 6. The effect of cytokines on PAF acetylhydrolase promoter activity.** Mouse macrophagic RAW264.7 cells were cotransfected with construct A (see Fig. 5; 2 μg) and the β-galactosidase control plasmid (1 μg). Cells were starved in Dulbecco’s modified Eagle medium plus 1% fetal bovine serum overnight, and murine cytokines were added to the culture for 16 h. The cytokines and their concentrations were: 1, no cytokine, negative control; 2, IFNγ (100 units/ml); 3, IFNα (100 units/ml); 4, IL-1α (10 ng/ml); 5, IL-6 (25 ng/ml); 6, IL-6 (10 ng/ml); 7, TNFα (10 ng/ml); 8, LPS (100 ng/ml). The relative promoter activity was calculated as the ratio of normalized luciferase activity between cytokine treatment and no treatment. The results shown represent the means of three independent experiments in which triplicate determinations were performed.

**Fig. 7. PAF stimulates transcription of PAF acetylhydrolase.** This experiment was performed in a line of 293 cells that had been stably transfected with a cDNA encoding the PAF receptor. The cells were transiently cotransfected with construct A (see Fig. 5) and the β-galactosidase control plasmid. The cells were starved for 5 h and stimulated with 2-O-methyl-PAF at the concentrations indicated, and 17 h later the cells were assayed for luciferase and β-galactosidase activities. The results represent the mean ± standard deviation from three separate experiments and are statistically significant ($p < 0.05$). LysoPC, lysoPAF, and PC were tested at concentrations up to 10-fold higher and resulted in no significant stimulation. PAF receptor inhibitors BN52021 (10 μM) and CV3988 (10 μM) were added 1 h before the PAF treatment, where indicated.

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the most striking structural features of the 5′ genomic region is the presence of multiple STAT consensus sequences. We asked whether these regions are targets for trans-acting factors that change during the differentiation of monocytes to macrophages. To assess this, we synthesized two oligonucleotides that correspond to the regions containing STAT consensus sequences; oligonucleotide 1 was from nucleotide −692 to −712, and oligonucleotide 2 was from nucleotide −631 to −656 (see Fig. 4). These constructs were incubated with nuclear extracts from human peripheral blood monocytes or from macrophages cultured for either 6 or 10 days. Oligonucleotide 1 formed a single, intense band with the nuclear extract from monocytes cultured for 10 days (lane 2). In contrast, the same amount of nuclear extract from macrophages (grown for either 6 or 10 days) yielded more bands, which were of lesser intensity (Fig. 8A, lanes 2 and 3). This differentiation-dependent binding pattern was also observed with oligonucleotide 2 (Fig. 8B). Interestingly, a similar binding pattern was observed in both regions using nuclear extracts from U937 cells treated with TPA and cultured for 3, 6, and 8 days (Fig. 8, C and D). We carried out two controls to ensure that the binding was specific. First, we supplemented our reactions with unlabeled oligonucleotides 1 or 2 and found that they completely abolished binding to the labeled probes; second, oligonucleotides that were mutated to disrupt specific STAT binding also abolished the binding (data not shown). In conclusion, the regions containing STAT consensus sequences bind to different nuclear factors in monocytes as compared with macrophages, and it is likely that these regions are involved in the differentiation-dependent regulation of the promoter.

DISCUSSION

In this study, we found that LPS and IFNγ, which activate many of the functions of macrophages during inflammation, transcriptionally repress PAF acetylhydrolase expression. In contrast, PAF acting via its receptor is a positive transcription regulator of this gene. In addition, different levels of promoter activity were observed in monocytic and macrophagic cells, strongly suggesting that the PAF acetylhydrolase promoter is regulated in both positive and negative ways during differentiation.

To perform these studies, we cloned and characterized the PAF acetylhydrolase promoter and mapped the transcriptional initiation site to an adenosine residue located 67 bases upstream of the 5′ end of the cDNA construct. The promoter does not contain a TATA box, which is typically found upstream of the transcriptional initiation site of many eukaryotic genes. This type of promoter is commonly found in housekeeping genes and in hematopoietic lineage-specific genes (32, 33). Many of the TATA-less promoters share a weak consensus sequence (initiator element, INr) around the transcriptional initiation site, CA1NT/AYY (34). The sequence around the +1 site in the PAF acetylhydrolase promoter, CA1GGC, is quite similar to the consensus INr sequence. The presence of three Sp1 sites (GC boxes) at −203 and +18 in a short distance from the initiation site implies that Sp1 tran-

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**Table I**

| Fold inductiona (luciferase/β-gal) | Cell lines |
|-----------------------------------|-----------|
| -fold to pGL3basic               |           |
| 3.29 ± 0.14                      | U937      |
| 8.25 ± 1.28                      | RAW264.7  |
| 15.96 ± 1.45                     | P388D.1   |

a Fold induction, an indication of the promoter activity, was calculated as the enzymatic activity of the luciferase reporter normalized by the cotransfected β-galactosidase activities. The means of two to four independent determinations are shown.

**Table II**

| Luciferase activity | TPA concentrationa (nM) |
|---------------------|-------------------------|
|                     | 0          | 30         | 60         | 200        |
| -fold to pGL3basic  | 2.02 ± 0.02 | 1.49 ± 0.12 | 1.62 ± 0.10 | 1.48 ± 0.05 |

a TPA was added to the cells immediately after the transfection. After 17 h, cells were harvested and assayed for luciferase activity. The data here indicate the means of two independent determinations.

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**Fig. 8.** Differentiation of monocytes to macrophages results in a different pattern of protein binding to STAT consensus sequences in the promoter. The 5′ ends of the oligonucleotide probes were labeled and added to 5 μg of nuclear extracts, initiating the binding reaction. Competition experiments were carried out in the presence of 100-molar excess of unlabeled double-stranded oligonucleotide, which has the same sequence as the radioactive probe in the binding reaction. A negative control for competition was always performed using 100-molar excess of unlabeled non-related oligonucleotide, which failed to compete for binding. Oligonucleotide 1, sense strand: TTTCAAACTACTTCTCTCTAAG; complementary antisense strand: CTTAGGGAAAAGTGTGGTTTGAAG. They correspond to −692 to −712 of the 5′ genomic sequence of the PAF acetylhydrolase gene. Oligonucleotide 2, sense strand: TTTATTAGAAAGTGGATTGAA; antisense strand: TTCTTTCAATCCACTTTTCTAAATAAA. They correspond to −631 to −656 (see Fig. 4). Mutated oligonucleotide 1 (only sense strand shown): TTTCACAGCATTigtagTAAG; mutated oligonucleotide 2 (sense strand): TggctCAcctGAggctGAGAA. Lower-case letters in the sequences indicate mutated bases. The binding to the monocyte and macrophage nuclear factors was completely abolished using these two mutated oligonucleotides as probes, resulting in no detected bands on the gels (data not shown). A and C, with oligonucleotide 1, B and D, with oligonucleotide 2. A and B, the probes were incubated with nuclear extracts from isolated human monocytes (lane 1), monocytes plated for 6 days (lane 2), and monocytes plated for 10 days (lane 3), respectively. The result with fresh monocytes in B is also shown as a light exposure. C and D, the probes were incubated with nuclear extracts from U937 cells. Lane 1 indicates no TPA treatment; cells were harvested 3 days (lane 2), 6 days (lane 3), and 8 days (lane 4) after 50 nM TPA treatment. The results shown here are representative of at least two independent experiments.
scription factors contribute to the precise transcription initiation.

Sequence comparison among the promoter regions of many genes expressed by myeloid cells revealed three consensus motifs: CCCCACCC, CCCCTCCC, and CCTTCTCC (35). The first two motifs have been demonstrated to bind to transcription factor MS2, which is expressed only by differentiated myeloid cells but not by undifferentiated counterparts (36, 37). The MS2 consensus sequence is almost always found in the promoters of myeloid-specific and developmentally controlled genes. The third motif probably binds to another myeloid-cell specific general transcription activator. In the PAF acetylhydrolase promoter, there are seven regions resembling the MS2 consensus sequence. In addition, the sequence from +207 to +217, GGGAGGAAGGG, contains overlapping binding sites for MS2 (GGGAGG) (36), Pu.1/MS1 transcription activator (GAGGAA), and MS1/MS2 (GAGGAG). Pu.1 is a member of the Ets family of transcription activators that is only expressed in macrophages and B cells, and MS1 is a general transcription factor expressed exclusively in myelomonocytic cells (35, 36). The presence of binding sites for MS1 and Pu.1 in the PAF acetylhydrolase promoter strongly suggests that the plasma PAF acetylhydrolase gene is a myeloid-specific gene. Moreover, multiple potential binding sites for MS2 indicate that the expression of this gene is under tight differentiation control, which is likely to be primarily mediated by the MS2 transcription activator. The role of MS2 in the regulation of expression of the PAF acetylhydrolase gene is currently under investigation. Finally, tissues known to contain large numbers of macrophages such as ovary, placenta, liver, lymph node, thyroid gland, and fetal spleen also express higher levels of PAF acetylhydrolase mRNA, which is in agreement with the idea that expression of the PAF acetylhydrolase gene is specific for mature myeloid cells.

In addition to MS2, other differentiation-induced transcription factors may be involved in the regulation of expression of the PAF acetylhydrolase gene. A well characterized macrophage-specific transcription factor is the zinc finger protein Egr-1, which has been shown to be essential for differentiation of myeloblast cells along the macrophage lineage (38). No region in the 1.3-kb 5’ genomic sequence perfectly matches the consensus sequence CCGGGGGCCG for Egr-1 binding. However, there is a sequence from +208 to +216, CCGGGGGCCG, which differs in only one nucleotide from the consensus motif. Whether Egr-1 binds to this region and is involved in the regulation of expression of the PAF acetylhydrolase gene awaits further investigation. Recently, there have been reports that differentiation of myeloid cells is accompanied by the activation of another transcription factor, DIF (differentiation-induced factor) (39, 40). In U937 cells, DIF was shown to be activated by phorbol esters, IFNγ, GM-CSF, and CSF-1. This myeloid differentiation-related transcription factor was further shown to be a STAT5-related transcription factor that binds to a STAT consensus sequence (41). Our data indicate that nuclear factors from monocytes and macrophages bind to regions containing STAT consensus sequences in a differentiation-dependent manner (Fig. 8). Therefore, it is possible that the STAT5-related DIF is involved in the differentiation-dependent regulation of the PAF acetylhydrolase promoter by interacting with regions containing the STAT consensus sequences, i.e., −692 to −712 and −631 to −656. Since nuclear factors from both monocytes and macrophages bind to these two regions, we anticipate that the regulatory mechanism in these two regions involves negative regulation in monocytes and positive regulation in macrophages. Our observation on the different levels of promoter activity in monocytes and macrophages (Table I) supports this possibility.

We found that PAF stimulates the expression of its own inactivating enzyme. PAF has been observed to increase the expression of other genes related to inflammation and injury. Bazan and colleagues reported that PAF rapidly activated transcription of the proto-oncogene c-fos in neuroblastoma and corneal epithelial cells, and the responsive cis region was mapped to an 80-bp promoter region (42). They also found that human immunodeficiency virus long terminal repeat promoter activity was elevated by PAF (5). In addition, PAF induced collagen type I transcription in corneal tissues, and PAF and retinoic acid synergistically induced the expression of prostaglandin synthase II (43, 44). Our data suggest that PAF functions as a physiological feedback regulator of its catalytic enzyme: an elevated level of PAF in plasma or tissues leads to enhanced PAF acetylhydrolase expression and secretion. A recent in vivo study using a rat model observed a rapid increase in PAF receptor expression in the ileum after PAF injection (45). Taken together with our data, it is likely that PAF can precisely modulate cellular responses, and therefore probably the entire body can adjust the capacity and magnitude of inflammatory and immune responses partly by this autoregulatory mechanism.

Previous evidence indicates that most of the physiological actions of PAF can be achieved at very low concentrations (10^-9 to 10^-12 M) (2). In the promoter-reporter system used, we did not see a stimulatory effect of PAF until the concentration reached 10 nM (10^-8 M) (Fig. 7). Several possibilities can explain the requirement of higher PAF concentrations in our experiments. Specific plasma or membrane factors may facilitate access of PAF to its receptor in vivo, and these factors may be absent in our experimental approach (i.e., cultured cells utilized, culture medium used, etc.). Alternatively, our experimental conditions may have hindered ready access of PAF to the promoter (for example sequestration by albumin or other proteins present in the system could account for the higher EC50). In addition, the sensitivity of the assay may have limited our ability to detect small alterations in promoter activity at lower PAF levels. In related studies, other research groups have also shown that higher PAF concentrations in vitro are necessary to elicit biological effects that are characteristic of the actions of PAF in vivo (6, 42).

In our studies, the effect of PAF was mediated specifically through its receptor since the stimulation was completely blocked by pretreatment of cells with PAF antagonists (Fig. 7). The PAF receptor is a G-protein-coupled molecule that can activate several signal transduction pathways, including phospholipases C, D, and A2, protein kinase C, and tyrosine kinases (46–48). Although PAF regulates the expression of a number of genes, the nature of the activated transcription factors and the cis-responsive elements have not been characterized yet. One study showed that PAF induced NFκB activation through a G-protein-coupled pathway (49), but the PAF acetylhydrolase promoter does not have a perfect match for the NFκB consensus sequence.

LPS and IFNγ were found to inhibit PAF acetylhydrolase promoter activity in our studies. IFNγ exerts important biological functions in the immune response including activation of macrophages, promotion of differentiation, and antiviral action (50). It sometimes functions synergistically with other stimuli such as LPS (endotoxin), which is a cell wall constituent of Gram-negative organisms that can trigger inflammatory reactions during infections. The effect of IFNγ is known to be mediated through the JAK-STAT pathway, which targets the STAT consensus sequence (also termed GAS elements) (28).
The effect of LPS was demonstrated to be mediated in part by NFκB and TNFα receptors in decidual macrophages (20). Moreover, the effect of LPS has been shown to involve several transcription factors including NFκB and NFIIL6 (51). Recently, one study reported that LPS induced the binding of NFκB components NFκB1 and RelA to the STAT consensus sequence (31). A significant synergism has been observed between IFNγ and LPS on transcription of nitric oxide synthase (27) in the macrophage cell line RAW264.7, which was also used in our study. The regulation of the PAF acetylhydrolase gene had a different pattern, in that IFNγ and LPS did not show a synergistic or even additive effect. This probably ruled out the possibility that IFNγ and LPS bind to two cis-regulatory elements, and that the two regions either interact with each other (synergistic) or function independently (additive) during the regulation. Therefore, IFNγ and LPS probably share the same responsive cis-region in the PAF acetylhydrolase promoter. Regardless of the precise mechanism, both stimuli lowered PAF acetylhydrolase expression and secretion in our study, suggesting that they may potentiate the inflammatory response by this mechanism.

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Note added in proof—While this manuscript was under review, the effect of LPS on PAF acetylhydrolase was reported in Kupffer cells in vivo (11). Tjoelker, L. W., Wilder, C., Eberhardt, C., Stafforini, D. M., Dietsch, G., Stafforini, D. M., Prescott, S. M., and Gray, P. W. (1995) J. Biol. Chem. 270, 1289–1296

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