Agonist-induced down-regulation of platelet-activating factor receptor gene expression in U937 cells

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Prolonged exposure (8–24 h) of human promonocytic U937 cells to 100 nM 1-*O*-hexadecyl-2-*N*-methylcarbamyl-*sn*-glycero-3-phosphocholine (carbamyl-PAF), a non-metabolizable analogue of platelet-activating factor (PAF), reduced the numbers of PAF receptors by 50–75%, as determined by the radioligand-binding assay. To clarify whether the down-regulation of receptor numbers is due to decreased expression level of the PAF-receptor gene, the effect of carbamyl-PAF on the steady-state level of PAF-receptor mRNA was examined by a highly sensitive reverse-transcriptase PCR method. A 50% decline in the level of PAF-receptor mRNA was observed in U937 cells pretreated with 100 nM carbamyl-PAF for 24 h. The effect of carbamyl-PAF was dose-dependent, with an EC₅₀ value around 10 nM. A PAF-

receptor antagonist, SRI-63675, was able to attenuate the effect of carbamyl-PAF. Furthermore lysoPAF, at 1 μ M, was unable to induce a significant decrease in PAF-receptor mRNA after incubation for 24 h, indicating that the effect of carbamyl-PAF was specific. The half-life of the PAF-receptor mRNA measured in the presence of actinomycin D was unaffected by carbamyl-PAF treatment. In contrast, nuclear run-off experiments demonstrated that the transcription rate of the PAF-receptor gene in carbamyl-PAF-treated cells was about 65% of that in control cells. These results suggest that the PAF receptor in U937 cells is subject to down-regulation by agonist, at least partly, at the transcriptional level.

INTRODUCTION

Platelet-activating factor (PAF) is a potent inflammatory mediator which exerts diverse biological effects on a variety of cells, including platelets, neutrophils, macrophages, smooth-muscle cells, endothelial cells and nerve cells (Hanahan, 1986; Braquet et al., 1987; Snyder, 1990; Venable et al., 1993). The biological effects of PAF are mediated by specific receptor proteins on cell surfaces. Studies on a number of cell types have demonstrated that activation of the PAF receptor leads to membrane polyphosphoinositide breakdown, calcium influx, and tyrosine phosphorylation (Shukla, 1992; Shimizu et al., 1992). Recently, the cDNA for the PAF receptor has been cloned from guinea-pig lung (Honda et al., 1991) and human leucocytes (Nakamura et al., 1991; Ye et al., 1991; Kunz et al., 1992). The deduced amino acid sequence revealed that the receptor contains a seven- α -helix transmembrane structure, which is characteristic of the G-protein-coupled receptors (Dohlman et al., 1991). As commonly observed in many receptors, activation of the PAF receptor by agonist leads to loss of responsiveness or 'desensitization' of receptor (Morrison and Shukla, 1988; Homma and Hanahan, 1988; Schwertschlag and Whorton, 1988; Barzaghi et al., 1989; Grandison, 1990), but the underlying mechanism has not yet been clarified. Nevertheless, studies on other G-protein-coupled receptors, such as the β_2 -adrenergic receptor and the muscarinic acetylcholine receptor, have demonstrated that desensitization occurring in the early phase after activation results from phosphorylation of the receptor proteins, sequestration or decreased numbers of receptors on the cell surfaces and uncoupling of the receptors from effectors (Galper et al., 1982; Bouvier et al., 1988; Benovic et al., 1988; Lohse et al., 1990). During the later phase (up to several hours), down-regulation of the receptor transcripts has been observed (Hadcock and Malbon, 1988;

Hadcock et al., 1989; Habecker and Nathanson, 1992; Longone et al., 1993; Steel and Buckley, 1993). Apparently the receptors are subject to regulation by agonists at both protein and mRNA levels. Therefore we were interested to know whether the PAF receptor is also subject to regulation by agonist in a similar manner. In this study, we used human promonocyte-like U937 cells, which are known to respond to PAF (Barzaghi et al., 1989; Ong et al., 1991) as a model to study the effect of agonist stimulation on the expression of the PAF-receptor gene. A highly sensitive reverse-transcriptase PCR (RT-PCR) method was employed to quantify the steady-state level of PAF-receptor mRNA, which is normally not abundant in cells. It was shown that long-term incubation of the cells with 1-*O*-hexadecyl-2-*N*-methylcarbamyl-*sn*-glycero-3-phosphocholine (carbamyl-PAF), a non-metabolizable analogue of PAF (O'Flaherty et al., 1987; Tessner et al., 1989), led to a decline in the level of PAF-receptor mRNA by a maximum of 50%. The decrease in receptor mRNA was mainly due to a decrease in the transcription rate of the receptor gene in the agonist-treated cells.

EXPERIMENTAL

Materials

RPMI 1640 medium, Opti-MEM reduced serum medium, Maloney leukaemia reverse transcriptase, yeast tRNA, dATP, dGTP, dCTP and dTTP were purchased from Gibco BRL. RNasin and random hexamers were from Promega. AmpliTaq DNA polymerase was from Perkin-Elmer Cetus. Carbamyl-PAF was obtained from Biomol. Uridine 5'-[α -³²P]triphosphate ([α -³²P]UTP; specific radioactivity 3000 Ci/mmol) and 1-*O*-[³H]alkyl-2-acetyl-*sn*-glycerophosphocholine ([³H]PAF; specific radioactivity 120 Ci/mmol) were from Amersham.

Abbreviations used: PAF, platelet-activating factor; carbamyl-PAF, 1-*O*-hexadecyl-2-*N*-methylcarbamyl-*sn*-glycero-3-phosphocholine; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RT-PCR, reverse-transcriptase PCR; NP-40, Nonidet P-40.

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Cell culture and treatment

Human U937 cells were subcultured in RPMI 1640 medium with 10% (v/v) fetal-calf serum at a density of 2.5×10^5 cells/ml. After incubation in a humidified atmosphere of 95% air/5% CO₂ at 37 °C for 2 days, cells were harvested, washed twice with Opti-MEM and resuspended in Opti-MEM at a density of 2.5×10^5 /ml. Cells (1×10^7) were then incubated with medium vehicle or carbamyl-PAF at indicated concentrations for various times at 37 °C.

Determination of PAF-receptor-binding activity

U937 cells (1×10^8) were incubated with or without 100 nM carbamyl-PAF at 37 °C for indicated times. BSA was then added to the medium to achieve a final concentration of 2.5% and the incubation was continued for 10 min. Cells were harvested by centrifugation at 500 *g* for 5 min and washed once with cold PBS. Membrane fractions were prepared and the PAF-receptor-binding activity was measured by a radioligand-binding assay as described previously (Chau et al., 1992).

Total RNA extraction

Total RNA was isolated from cells by the acid guanidium thiocyanate-phenol-chloroform extraction method (Chomczynski and Sacchi, 1987) with some modification. Briefly, following the first extraction by acid guanidinium thiocyanate-phenol-chloroform, the RNA in the aqueous phase was re-extracted once by adding an equal volume of phenol and a one-tenth volume of a chloroform-isoamyl alcohol mixture (49:1, v/v). After mixing, the sample was centrifuged again at 10000 *g* for 20 min at 4 °C. The aqueous phase was removed and the RNA was precipitated by adding a half volume of ethanol and placing at -70 °C for at least 1 h. The quality of the RNA was checked by the ratio of the A_{260}/A_{280} (2–1.8) and agarose gel electrophoresis.

Quantitative RT-PCR

A sample (1 µg) of total RNA was reverse-transcribed into cDNA by incubating it with 200 units of Maloney leukemia transcriptase in 20 µl of reaction buffer containing 10 units of RNasin, 0.2 µg of random hexamers and 0.8 mM dNTPs at 37 °C for 1 h. The reaction was terminated by heating at 95 °C for 10 min and the mixture diluted to 500 µl with deionized water. Aliquots were taken for PCR amplification with either PAF-receptor-specific primers or glyceraldehyde-3-phosphate dehydrogenase (GAPDH)-specific primers. The sequences of the sense and antisense primers for the PAF receptor were: sense-5'-TATAACCGCTTCCAGGCAGT-3', antisense-5'-GAAACAGTAGATAACAGGGTC-3'. The sense and antisense primers for GAPDH were: 5'-CCACCCATGGCAAATTCCATGGCA-3' and 5'-TCTAGACGGCAGGTCCAGGTCCACC-3'. The PCR was performed in 50 µl of 10 mM Tris/HCl, pH 8.3, containing 50 mM KCl, 1.5 mM MgCl₂, 0.001% (w/v) gelatin, 0.2 mM dATP, 0.2 mM dCTP, 0.2 mM dTTP, 0.2 mM dGTP, 1 µM of each primer, and 1.25 units of AmpliTaq DNA polymerase. The reaction proceeded for 25 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min, and extension at 72 °C for 1 min. Amplified cDNAs were electrophoresed on 1% (w/v) agarose gels containing ethidium bromide (1 µg/ml). The expected sizes of the cDNA fragments for both the PAF receptor and GAPDH were around 0.55 kb. The cDNA was quantified by an in-house assembled laser-excited confocal fluorescence gel scanner (Glazer et al., 1990; Rye et al., 1991). In brief, a

Coherent Innova 200 argon-ion laser was used as the excitation light source to deliver 488 nm wavelength excitation with 40 mW average power at the sample. An objective lens (Zeiss, LD-EpiPlan 20X) was used to focus the laser beam and to collect fluorescence emitted from the sample. A spatial filter placed in the optical path of the induced fluorescence emission served to implement confocal arrangement and to reject out-of-focus lights. A bandpass filter which transmits ethidium bromide fluorescence emissions of around 610 nm was used to provide spectral rejection of background interference. Fluorescence emissions were gathered by a Hamamatsu R928 photomultiplier tube and signals processed by a photon counter (Hananatsu C3866) and a counter/timer board (Advantec CTM-5) in an IBM PC-compatible computer. The gel containing cDNA was placed on an X-Y motor-driven stage (Newport PM500) which translated ethidium bromide-stained cDNA bands to pass the laser beam. The same IBM-compatible computer was used to control the X-Y translation stage, to acquire data, and to display an image on the screen simultaneously. The fluorescence imaging was grey-scale encoded to represent different intensity levels. Image analysis and quantification were done by the Image program developed by Wayne Rasband of National Institutes of Health (Bethesda, MD, U.S.A.).

Determination of mRNA decay

U937 cells were pretreated with or without 100 nM carbamyl-PAF at 37 °C for 24 h in Opti-MEM. Actinomycin D was then added to the medium to achieve a final concentration of 10 µg/ml, at which concentration cell viability was not significantly affected during a 6-h incubation. Cells were harvested at the indicated time points and the total RNA was isolated. Ethidium bromide-staining of the RNA isolated at time zero and after 1 h and 3 h showed no sign of degradation; whereas some degree of RNA breakdown of the 28S RNA was noticed at 6 h. The PAF-receptor transcript was quantified by RT-PCR as described above.

Nuclear run-off

Cells [$(1-2) \times 10^7$] pretreated with or without 100 nM carbamyl-PAF for 24 h were harvested and washed twice with cold PBS. The cell pellet was resuspended in 1 ml of Nonidet P-40 (NP-40) lysis buffer containing 10 mM Tris/HCl (pH 7.4), 10 mM NaCl, 3 mM MgCl₂ and 0.5% (v/v) NP-40. After incubation at 4 °C for 5 min, nuclei were collected by centrifugation at 500 *g* for 5 min at 4 °C and washed once with lysis buffer. Nuclei were then resuspended in 100 µl of 50 mM Tris/HCl (pH 8.3), 0.1 mM EDTA, 5 mM MgCl₂ and 40% (v/v) glycerol. Reaction buffer (100 µl) containing 10 mM Tris/HCl (pH 8.0), 5 mM MgCl₂, 300 mM KCl, 1 mM ATP, 1 mM CTP, 1 mM GTP and 100 µCi [α -³²P]UTP was added and incubation was conducted at 30 °C for 30 min. The radiolabelled nascent RNA was isolated by the guanidinium isothiocyanate extraction method and precipitated using ethanol. Samples of radiolabelled RNA (1×10^6 c.p.m./ml) were hybridized to nylon membranes slot-blotted with the probes (10 µg/slot). A human PAF-receptor cDNA (1.8 kb), which contains the entire coding region, was isolated from the human endothelial cDNA library constructed in bacteriophage λ gt11 (Clontech), subcloned into PTZ19R, and used as probe. A plasmid pIBI30GAPDH was used as the internal control. The hybridization was conducted at 42 °C for 24 h in the hybridization solution containing 50% formamide, 5×SSC (SSC, 0.15 M NaCl/0.015 M sodium citrate), 0.1% SDS, 5% dextran sulphate, 1×Denhardt's soln. and 0.1 mg/ml of yeast tRNA. Membranes were washed at 50 °C with 0.1×SSC containing 1% (w/v) SDS,

then dried and quantified using a phosphor imager (Molecular Dynamics).

RESULTS AND DISCUSSION

Regulation of receptor expression and function in response to agonist stimulation is commonly observed in many receptors. Desensitization of PAF-receptor function following short-term stimulation by PAF has been observed in a number of cell types

Table 1 Effect of carbamyl-PAF treatment on the specific [3 H]PAF-binding sites in U937 cells

Cells were incubated with 100 nM carbamyl-PAF for the indicated times. Cell membranes were then prepared and the PAF-binding activity was assessed as described in the Experimental section. The results are expressed as a percentage of the amount of specific [3 H]PAF-binding activity in control cells (without carbamyl-PAF treatment) at each time point. Each value represents the mean $\pm$ S.D. of three independent experiments performed in triplicate. Significant difference from control cells: * $P < 0.01$; ** $P < 0.002$.

Time (h)	Specific [3 H]PAF-binding (% of control)
1	86.5 $\pm$ 1.5
8	52.0 $\pm$ 13.0*
24	23.0 $\pm$ 11.2**

Figure 1 RT-PCR analysis of the expression of PAF-receptor mRNA and GAPDH mRNA in U937 cells

Total RNA was isolated from U937 cells and 1 μ g was used for RT reaction. Various aliquots taken from the RT reaction were used in PCR for analysis of PAF-receptor and GAPDH transcripts. After 25 cycles of PCR amplification, 10 μ l aliquots were taken and separated on a 1% (w/v) agarose gel containing ethidium bromide (1 μ g/ml). The PCR products were then quantified by a laser-excited fluorescence gel scanner. Insets: ethidium bromide-staining of the PCR products.

(Morrison and Shukla, 1988; Homma and Hanahan, 1988; Schwertschlag and Whorton, 1988; Barzaghi et al., 1989; Grandison, 1990). Very little is known about the prolonged effect of PAF on its own receptor. It has been shown that some cytokine receptors, such as the interleukin-2 receptor and the interleukin-4 receptor, are up-regulated upon prolonged exposure to their agonists (Lee et al., 1990; Renz et al., 1991; Espinoza-Delgado et al., 1992). In contrast, studies on the G-protein-coupled receptors, including the β_2 -adrenergic receptor and the muscarinic receptor, have demonstrated down-regulation of these receptors by their agonists (Haddock and Malbon, 1988; Haddock et al., 1989; Habecker and Nathanson, 1992; Longone et al., 1993; Steel and Buckley, 1993). In an attempt to elucidate the possible regulation of the PAF receptor by agonist, we treated U937 cells with carbamyl-PAF, a non-metabolizable bioactive PAF analogue (O'Flaherty et al., 1987; Tessner et al., 1989), for various times and examined the changes in receptor levels. As shown in Table 1, treatment of U937 cells with 100 nM carbamyl-PAF in culture for long periods of time (> 1 h) caused a marked decrease in the number of receptors, as assessed by the radioligand-binding assay. The number of receptors was reduced to almost half of the control value following an 8-h incubation and continued to decrease up to 24 h, which was the longest time point tested. The reduction in the receptor numbers might have been due to an increase in protein turnover rate or a decrease in receptor expression level. To address this issue, we examined further the steady-state level of the PAF receptor mRNA in cells following prolonged incubation with carbamyl-PAF. As the PAF receptor is not abundant in the cells, quantification of its mRNA by standard Northern-blot analysis appeared to be a difficult task. Therefore, we developed a highly sensitive RT-PCR method in conjunction with a fluorescence-scanning and imaging system to quantify the receptor mRNA. A 1 μ g sample of cellular RNA was reverse-transcribed into cDNA followed by PCR with primers for the PAF receptor and GAPDH (internal standard) respectively. As depicted in Figure 1, the amounts of cDNA produced were proportional to the inputs of the RT-PCR products for both the PAF receptor and GAPDH. DNA sequencing of the PCR fragments confirmed they were the right products (results not shown). Therefore, in subsequent experiments, the amounts of reverse-transcriptase products taken for PCR amplification of the PAF receptor and GAPDH were within the linear ranges, in order to ensure that the amounts of cDNA produced truly reflect the levels of mRNA in the original samples. When U937 cells were incubated with 100 nM carbamyl-PAF for long periods of time (> 8 h), the PAF-receptor mRNA level declined significantly i.e. by 50% after 24 h (Figure 2). However, the time course for the decline in PAF-receptor mRNA appeared to be much slower than that for the decrease in receptor numbers (Table 1). Furthermore, the percentage of the receptors lost after carbamyl-PAF treatment was much greater than could be accounted for solely by the decrease in receptor mRNA. We speculated that prolonged exposure of cells to carbamyl-PAF might also lead to increased degradation of the PAF receptor, as observed for the muscarinic receptor (Klein et al., 1979; Galper and Smith, 1980). The effect of carbamyl-PAF was dose-dependent, with an EC_{50} value of around 10 nM (Figure 3), which is comparable with the EC_{50} value for eliciting a degranulation response in neutrophils (O'Flaherty et al., 1987). Although the reduction in PAF-receptor mRNA caused by the prolonged exposure to carbamyl-PAF was only 50% maximally, this nevertheless represented a considerable contribution to the profound drop in receptor numbers. To confirm that the carbamyl-PAF-induced down-regulation of PAF-receptor gene expression was mediated through the PAF receptor, an ex-

Figure 2 Time course of down-regulation of PAF-receptor mRNA induced by carbamyl-PAF

Cells were incubated without (control) or with 100 nM carbamyl-PAF for indicated times. Total RNA was isolated and the mRNAs for PAF receptor and GAPDH were analysed by RT-PCR. Data are normalized by the ratio of PAF-receptor mRNA/GAPDH mRNA and expressed as percentages of the control. Results shown are the mean $\pm$ S.D. of five independent experiments. Significant difference from control cells: * $P < 0.01$.

Table 2 Effects of PAF antagonist and lysoPAF on PAF-receptor mRNA expression in U937 cells

Cells were treated with 1 μ M lysoPAF or 100 nM carbamyl-PAF in the absence or presence of 500 nM SRI-63675 for 24 h. The mRNA levels for PAF-receptor (PAFR) and GAPDH were analysed as described in the Experimental section. Results shown are the mean $\pm$ S.D. of three independent experiments. Values given in parentheses are the percentage of the control value. (* $P < 0.01$).

Treatment	Ratio of PAFR mRNA/ GAPDH mRNA
None (control)	0.078 $\pm$ 0.013 (100)
LysoPAF (1 μ M)	0.063 $\pm$ 0.008 (80 $\pm$ 10)
Carbamyl-PAF (100 nM)	0.035 $\pm$ 0.006 (47 $\pm$ 18)*
Carbamyl-PAF (100 nM) and SRI-63675 (500 nM)	0.068 $\pm$ 0.010 (86 $\pm$ 10)

periment was conducted to examine the effect of a PAF antagonist. As shown in Table 2, when the PAF antagonist SRI-63675 (Handley et al., 1987) was present during the incubation with carbamyl-PAF, the decrease in PAF-receptor mRNA was significantly attenuated. Furthermore, lysoPAF, the inactive metabolite of PAF, at a concentration of 1 μ M did not exert a significant effect on PAF-receptor mRNA after incubation for 24 h. Taken together, these results indicate that the PAF receptor, in analogy to the β_2 -adrenergic receptor and the muscarinic

Figure 3 Dose-dependence of carbamyl-PAF-induced down-regulation of PAF-receptor mRNA

Cells were incubated with various concentrations of carbamyl-PAF for 24 h. The mRNAs for PAF receptor and GAPDH were analysed by RT-PCR. Data shown are mean $\pm$ S.D. of three independent experiments.

receptor, is subject to down-regulation by agonist at the mRNA level. Since the decrease in the steady-state level of the PAF-receptor mRNA might have resulted either from a decrease in the rate of transcription of the gene encoding the PAF receptor, or from an increase in the rate of degradation of the PAF-receptor transcript, we investigated the underlying mechanism by which the PAF-receptor mRNA was down-regulated by agonist further. Cells were preincubated with or without 100 nM carbamyl-PAF for 24 h and then harvested at 0, 1, 3, or 6 h after the addition of the transcription blocker actinomycin D at a concentration of 10 μ g/ml. As illustrated in Figure 4, the half-life of the PAF-receptor mRNA in U937 cells was about 2 h, which is similar to that reported for peripheral blood monocytes (Thivierge et al., 1993). Pretreatment of U937 cells with carbamyl-PAF did not significantly decrease the half-life of PAF-receptor mRNA, suggesting that the changes in the PAF-receptor mRNA in agonist-treated cells were not due to altered stability of the receptor transcript. Therefore, nuclear run-off experiments were conducted to monitor the rate of transcription of the PAF receptor in U937 cells after agonist stimulation. Figure 5 clearly shows that the rate of PAF-receptor-gene transcription in nuclei isolated from carbamyl-PAF-treated cells was about 65% of that in nuclei from control cells, which correlates with the percentage decline in PAF-receptor mRNA in agonist-treated cells. This observation differs from that reported for the β_2 -adrenergic receptor in which agonist-induced down-regulation of receptor transcripts resulted from destabilization of the receptor mRNA (Hadcock et al., 1989). Nevertheless, an agonist-induced decrease in receptor-gene transcription has also been suggested for the muscarinic receptor (Longone et al., 1993). Although the down-regulation of PAF-receptor mRNA occurred during a later phase, signals produced in the early phase following stimulation by agonist might conceivably participate in modulating transcriptional factor activities which in turn regulate PAF-receptor gene expression. For instance, cyclic AMP has been shown to be responsible for the down-regulation of the β_2 -

Figure 4 PAF-receptor mRNA stability in control and carbamyl-PAF-treated U937 cells

Cells were treated without (Control) or with 100 nM carbamyl-PAF for 24 h and actinomycin D (10 μ g/ml) was then added for various times. Total RNA was isolated and the PAF-receptor mRNA was quantified by RT-PCR. (a) Fluorescence imaging of the PCR products. (b) Ethidium bromide staining of the RNA. (c) Relative mRNA levels were plotted versus time of actinomycin D treatment. Data shown here are representative of two independent experiments with similar results.

adrenergic receptor (Haddock and Malbon, 1988), and the second messengers derived from both the adenylate cyclase and phospholipase C pathways have also been shown to be involved in regulation of muscarinic receptor gene expression (Habecker et al., 1993). Stimulation of U937 cells by PAF leads to activation of phospholipase C (Barzaghi et al., 1989) as well as phospholipase D (Balsinde and Mollinedo, 1991) and the subsequent generation of the second messengers diacylglycerol, inositol trisphosphate, and phosphatidate, which can then trigger activation of protein kinase C and release of Ca^{2+} from intracellular stores (Moolenaar et al., 1986; Berridge, 1987). Whether these signals are involved in mediating agonist-induced down-regulation of PAF-receptor gene transcription remains to be clarified.

In summary, the present study demonstrates that the PAF receptor is down-regulated following prolonged agonist treatment of U937 cells. The marked reduction in receptor numbers was due, at least in part, to a decline in the steady-state level of receptor mRNA, which was correlated with a decrease in the transcriptional rate of the receptor gene. Although the molecular mechanism underlying transcriptional regulation of the PAF receptor is not yet clear, agonist-induced down-regulation of

Figure 5 Relative transcriptional rate of the PAF-receptor gene in U937 cells treated with or without (Control) carbamyl-PAF

Nuclei were isolated from cells treated with or without 100 nM carbamyl-PAF for 24 h and allowed to transcribe in the presence of radiolabelled nucleotide. Nascent RNA transcripts were isolated and hybridized to filters on which plasmid DNA containing human PAF receptor or GAPDH had been blotted. PTZ19R plasmid was also included as a negative control. Following hybridization, the signal on each slot was determined by a phosphorimager (Molecular Dynamics). Data presented are the mean $\pm$ S.D. of three independent experiments performed in duplicate. (* $P < 0.025$.)

receptor-gene expression represents an important feed-back route for controlling the expression and function of the receptor following long-term exposure to PAF.

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