Regulation of platelet-activating factor receptor gene expression *in vivo* by endotoxin, platelet-activating factor and endogenous tumour necrosis factor

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A competitive PCR assay was developed to quantify platelet-activating factor (PAF) receptor (PAF-R) transcripts in rat tissues using a synthetic RNA as a competitor. We found PAF-R mRNA constitutively expressed in the eight organs tested, with the ileum containing the highest concentration [$(3.49 \pm 0.15) \times 10^7$ molecules/μg of RNA]. Significant but lower levels were also detected in the jejunum, spleen, lungs, kidneys, heart, stomach and liver. Furthermore we defined the regulatory role of inflammatory mediators in ileal PAF-R gene expression using a rat model of intestinal injury induced by PAF or lipopolysaccharide (LPS). Injection of LPS or low-dose PAF resulted in a marked increase in ileal PAF-R mRNA within 30 min. The up-regulation on PAF-R elicited by PAF was biphasic, peaking first at 90 min, then again at 6 h. In contrast, LPS elicited a weak monophasic response. The second phase of PAF-R mRNA increase after PAF administration was completely abolished by WEB 2170, a PAF antagonist, and partially inhibited by anti-tumour necrosis factor (TNF) antibody. These observations indicate the involvement of endogenous PAF and TNF in this event. In conclusion, we found: (a) preferential PAF-R expression in the ileum, suggesting a role for PAF in intestinal inflammation; (b) induction of PAF-R expression *in vivo* by its own agonist; (c) a complex regulation of PAR-R gene expression *in vivo* involving a network of various pro-inflammatory mediators.

INTRODUCTION

Platelet-activating factor (PAF) is a potent inflammatory mediator synthesized by various cells, such as monocytes/macrophages, polymorphonuclear leucocytes (PMNs), mast cells, endothelial cells and platelets [1,2]. The biological effects of PAF include shock [3,4], vasodilation or vasoconstriction [5], leucocyte–endothelium adhesion [6], increase in endothelial and epithelial permeability [7], as well as the expression of other inflammatory lipid mediators [2,8] and cytokines [9,10]. PAF has been found to be associated with many pathological conditions of the intestine [11–13], and particularly the small intestine, since systemically administered PAF causes necrosis of the bowel almost exclusively [11,14].

The biological effects of PAF are mediated by its specific receptor, as almost all actions of PAF can be prevented by blocking its receptor with antagonists. PAF receptor (PAF-R) exists in platelets, neutrophils, monocytes, macrophages and endothelium [15]. PAF-R is a member of the G-protein-coupled receptor superfamily characterized by seven transmembrane domains. Activation of PAF-R leads to the activation of several signal-transduction pathways [15,16]. Studies on comparative quantification of PAF-R in organ/tissue are rare, probably because of the technical difficulty of differentiating membrane binding and membrane intercalation of a phospholipid [2,17]. The recent development of quantitative PCR and cloning of PAF-R cDNA from guinea-pig [18], human [19,20] and rat [21] tissues has greatly facilitated our understanding of PAF-R gene expression and regulation. Recent studies *in vitro* on the regulation of PAF-R gene transcription by inflammatory stimuli have yielded contradictory results. Shirasaki et al. [22] showed that PAF rapidly doubled its receptor mRNA in human monocytes, whereas others showed that PAF markedly reduced its receptor mRNA in U937 cells [23], in keeping with earlier studies in PMNs [24] and Kupffer cells [25], which demonstrated that PAF caused a down-regulation of cell surface PAF-binding sites. Quantitative study of tissue PAF-R mRNA, especially of its change over time during inflammation, has so far been limited.

Our previous observations indicate that the small intestine (usually the ileum) is often the target of the injury induced by exogenous PAF and lipopolysaccharide (LPS), even though the challenging agent is administered systemically [11]. The reason is unclear. One possibility is preferential expression of PAF-R in the small intestine, but limited information is available on comparative PAF-R expression in different tissues and its changes after challenge with systemic inflammatory stimuli. The aims of the present study were: (a) to quantify and compare PAF-R gene transcripts in various organs by quantitative PCR; (b) to study intestinal PAF-R gene expression during inflammation *in vivo*; (c) to investigate the mechanism of intestinal PAF-R gene regulation; (d) to study the basis for the differences in the regulatory effects of these pro-inflammatory agents.

MATERIALS AND METHODS

Materials

PAF (1-*O*-hexadecyl-2-acetyl-*sn*-glycero-3-phosphocholine) and molecular-biology reagents were purchased from Sigma (St. Louis, MO, U.S.A.). PAF was prepared fresh in saline containing

Abbreviations used: PAF, platelet-activating factor; PAF-R, PAF receptor; LPS, lipopolysaccharide; TNF, tumour necrosis factor α; RT-PCR, reverse transcriptase-PCR; Q-PCR, quantitative RT-PCR; cRNA, competitor RNA; WEB, PAF antagonist; WBC, white blood cell count; PMN, polymorphonuclear leucocyte; i.p., intraperitoneal; i.v., intravenous.

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5 mg/ml BSA. LPS from *Salmonella typhosa* was purchased from Difco (Detroit, MI, U.S.A.). WEB 2170, a PAF antagonist, was a gift from Dr. H. Heuer, Boerhinger-Ingelheim, Mainz, Germany. Rabbit anti-[mouse tumour necrosis factor (TNF)] antibody, which cross-reacts with rat TNF, was generated in the laboratory of D.G.R. The antibody was diluted 1:1 with saline before injection. GeneAmp DNA amplification kit and GeneAmp Thermostable r*Tth* RNA PCR kit were supplied by Perkin–Elmer (Norwalk, CT, U.S.A.). SYBR Green I was purchased from Molecular Probes (Eugene, OR, U.S.A.).

Animal experiments

Young male Sprague–Dawley rats (70–120 g) were anaesthetized with Nembutal [65 mg/kg, intraperitoneal (i.p.)] and placed under warm light. The carotid artery and jugular vein were catheterized for continuous blood pressure recording, drug injection and blood sampling. Blood samples were collected at 0, 30, 90, 180 and 360 min for white blood cell count (WBC) and assessment of haematocrit. The rats were divided into five groups: (a) sham-operated, vehicle (saline) only, 1 ml/kg, intravenous (i.v.); (b) PAF (1.5 μg/kg, i.v.); (c) LPS (5 mg/kg, i.v.); (d) 1:1 diluted rabbit anti-(mouse TNF) antibody (10 ml/kg, i.p.) 30 min before and 150 min after PAF administration; and (e) WEB 2170, 60 min (0.5 mg/kg, i.v.) and 3 h (1 mg/kg, i.v.) after PAF injection. The rats were killed at different time points after PAF or LPS stimulation, and organs were removed, minced and immediately frozen in liquid nitrogen. The ileum and jejunum were rinsed with ice-cold saline to remove the intestinal content before freezing.

To examine the specificity of the effect of PAF and LPS, intestinal injury/inflammation was induced by two consecutive daily subcutaneous injections of indomethacin (7.5 mg/kg per day) on day 0 and 1 [26]. The rats were killed on day 0, 4 and 8, and ileal PAF-R mRNA was quantified (see below) and routine histological examination carried out to confirm intestinal injury.

RNA preparation

Total RNA was isolated from frozen tissues as previously described [9]. The concentration of RNA was determined by measuring A_{260}, and the purity was checked by the A_{260}/A_{280} ratio (greater than 1.8) and by electrophoresis. The remaining preparation was stored at −80 °C.

Reconstruction of pGRPR plasmid containing a site-specific mutated rat PAF-R cDNA fragment

A C<u>A</u>AATTCC sequence exists in the cDNA of rat PAF-R from bp 258 to 265 [21]. The adenine at bp 259 (underlined) could be changed to guanine using a site-specific mutagenesis PCR [27], resulting in a cDNA fragment containing a mutated C<u>G</u>AATTCC sequence, which can be digested with *Eco*RI restriction enzyme. Single-stranded cDNA was synthesized from 0.5 μg of ileal total RNA by reverse transcription (RT) reaction and amplified by PCR as previously described [28]. During the primary PCR, the upstream mismatched fragment (from bp −119 to bp 270) was synthesized with primers 5′-1 (5′-GCCACAACACAGAGGCTTGA-3′, sense, −119 to −100) and 3′-1 (5′-GCACAGGAATTCGTGTACAAT-3′, antisense, 270–250), and the downstream one (from bp 250 to bp 848) was synthesized with primers 5′-2 (5′-ATTGTACACGAATTCCTGTGC-3′, sense, 250–270) and 3′-2 (5′-GTGCTCAGGAGGCAGAGGGT-3′, antisense, 848–829). The PCR products were purified by GeneCAPSULE (Geno Technology, St. Louis, MO, U.S.A.). An overlapping region containing the same mutation introduced via primer mismatch was present in both upstream and downstream fragments. After the two purified fragments had been mixed in a tube, a recombinant DNA fragment was amplified with 30 cycles of PCR by using primers 5′-1 and 3′-2. The 967 bp fragment was purified and subcloned into pGEM-T vector (Promega). This new plasmid was named pGRPR. Both strands of the inserted fragment were confirmed by DNA sequencing with Sequenase version 2.0 kit (USA Amersham), which indicated that the 5′ end of the sense strand is located downstream of the SP6 RNA polymerase-binding site, and the 3′ end is followed by a *Nco*I site of the pGEM vector.

Synthesis of competitor RNA (cRNA)

The RiboMax Large Scale RNA Production System (Promega) was used for synthesis of cRNA. Digestion of pGRPR with *Nco*I restriction enzyme resulted in a linearized DNA fragment. Using this fragment as template, we synthesized sense-strand cRNA by SP6 RNA polymerase. The template DNA was subsequently removed by adding RNase-free DNase (1 unit/μg of DNA) and incubating at 37 °C for 60 min. After extraction with phenol/chloroform, the cRNA was precipitated with ethanol, washed, resuspended in water, quantified and stored at −80 °C until use.

Quantitative RT-PCR (Q-PCR)

The first step, an initial titration assay, was performed to estimate the number of molecules of PAF-R gene transcript in the total RNA sample isolated from different organs. Briefly, a serially diluted cRNA mixed with 0.25–1.5 μg of total RNA was added to a reaction mixture (10 μl) containing 1 × r*Tth* RT buffer, 1 mM $MnCl_2$, 200 μM each dNTP, 2.5 units of r*Tth* DNA polymerase and 1.5 μM primer 3′-3 (5′-CTGTGGCCAGGAAGTAGGAT-3′, antisense, 466–447). The reaction mixture was incubated at 70 °C for 15 min and then quickly chilled on ice. PCR was carried out in a total volume of 50 μl containing 10 μl of RT reaction mixture, 1 × chelating buffer, 2.5 mM $MgCl_2$ and 0.3 μM 5′-1 primer. The reaction mixtures were heated at 95 °C for 2 min and immediately carried through 30 cycles of PCR with a denaturing step for 1 min at 95 °C, and an annealing and extension combined step for 2 min at 68 °C. In the final cycle, a 5 min extension step at 68 °C was included. After PCR, the DNA products were digested with *Eco*RI at 37 °C for 60 min and separated by electrophoresis on 1.2% agarose gel containing 1:10000 diluted SYBR Green I. The second step is a quantitative assay. Based on the results of the initial titration assay, a dilution series of cRNA for each organ was used for each RT-PCR cycle. The RT-PCR and post-PCR procedure were the same as described above in the initial titration assay. After separation of *Eco*RI-digested PCR products by electrophoresis, the density of each DNA band was evaluated with a storm Phosphorimager (model 860; Sony) and analysed by a computer software ImageQuant™ (Molecular Dynamics, Sunnyvale, CA, U.S.A.). To correct the error due to the difference in molecular masses of the DNA bands generated from cRNA (380 or 205 bp) and target RNA (585 bp), the density of the DNA band correlated to cRNA was multiplied by 0.65 (380/585 bp) or 0.35 (205/585 bp). A standard curve was established on the basis of the ratio of the two DNA bands (generated from cRNA and target RNA). The concentration of PAF-R mRNA in each sample was calculated by reference to its own standard curve established by Q-PCR.

To avoid errors caused by contamination, each PCR result was considered valid only when the negative control (RT-PCR mixture with no competitor or sample RNA) showed no DNA

band on the gel. Genomic DNA, a possible impurity in the RNA extract, was not considered an important source of interference. This is because (i) the 5′-1/3′-3 primer pair walks through a large intron [21], and (ii) this large intron could not be amplified by PCR under the conditions of our experiment, as reported by Bito et al. [21].

Statistical analysis

A non-paired Student's t test program was used for comparison of differences between means.

RESULTS

Quantitative RT-PCR method for analysis of rat PAF-R mRNA

A Q-PCR for PAF-R mRNA was developed using the synthesized cRNA as a competitor. The following special points were addressed to ensure the accuracy of the assay system.

(i) To avoid the error due to amplification efficiency, which may vary with the length of sequence to be amplified, the designed competitor has a length equal to its wild-type, with only one base difference in the sequence.

(ii) Target–competitor heteroduplex might be formed during the annealing period of PCR, because the wild-type DNA and mutated DNA have 99.8% of sequence in common. This heteroduplex, which cannot be digested by *Eco*RI, will migrate with the wild-type DNA, resulting in a falsely high concentration of wild-type DNA product. To avoid self-annealing of the products and subsequent heteroduplex formation during the plateau phase, we kept the PCR in linear phase by limiting the cycle number to 30. Thus only homoduplex is formed since almost all the double-stranded DNA is a template–replicate pair at the end of each cycle.

(iii) An optimal original competitor/target ratio is required to achieve close amplification efficiency for both of them. As the content of the target transcripts varies with each organ examined, the amount of competitor is chosen individually on the basis of the result of titration assay of PAF-R mRNA from each tissue.

In the initial Q-PCR experiment using the synthetic cRNA as a competitor, we found that PAF-R mRNA in rat tissues could be quantified, and the method was sufficiently sensitive to detect changes in competitor concentration as low as 1.5-fold.

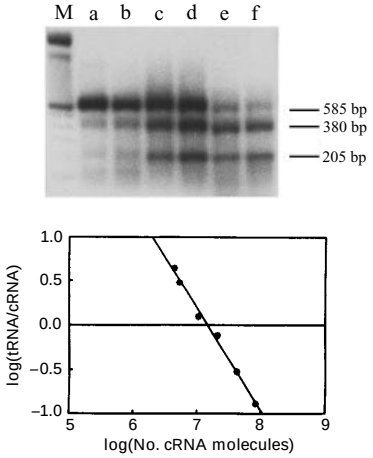

Figure 1 Analysis of PAF-R mRNA in rat ileum by quantitative PCR

Upper panel shows a typical electrophoresis gel of RT-PCR amplifying wild-type DNA (585 bp) and mutated DNA (380 and 205 bp). Ileal total RNA (0.4 μg) was co-amplified by RT-PCR in the presence of 2.6×10^6 (lane a), 5.2×10^6 (lane b), 1.04×10^7 (lane c), 2.08×10^7 (lane d), 4.16×10^7 (lane e) and 8.32×10^7 (lane f) molecules of cRNA. After *Eco*RI digestion, both competitor and target DNA were analysed by 1.2% agarose-gel electrophoresis followed by staining with SYBR Green I. Lane M, marker of 100 bp ladder. A standard curve was constructed by determining the ratio of wild-type DNA and mutated DNA in each sample (lower panel).

Table 1 Quantitative analysis of PAF-R mRNA in various organs

Results are means ± S.E.M. (n = 3–5).

Organ	PAF-R mRNA (molecules/μg of total RNA)	PAF-R mRNA (% of that in ileum)
Ileum	$(3.49 \pm 0.15) \times 10^7$	100.00
Jejunum	$(1.96 \pm 0.10) \times 10^7$	56.16
Spleen	$(1.03 \pm 0.16) \times 10^7$	29.51
Lung	$(9.10 \pm 1.50) \times 10^5$	0.03
Kidney	$(3.02 \pm 0.28) \times 10^5$	0.01
Heart	$(2.24 \pm 0.17) \times 10^5$	0.006
Stomach	$(1.71 \pm 0.21) \times 10^5$	0.005
Liver	$(1.12 \pm 0.28) \times 10^5$	0.003

Quantification of PAF-R gene transcripts in various organs

The numbers of PAF-R gene transcripts in the ileum, jejunum, spleen, lungs, kidneys, heart, stomach and liver were quantified by Q-PCR. Figure 1 illustrates the quantification of PAF-R mRNA in the normal rat ileum after construction of the standard curve by the ratio of wild-type DNA and mutated DNA. Table 1 demonstrates the amounts of PAF-R gene transcripts in the eight organs tested. Representative photographs used to determine these values are shown in Figure 2. Although some variation exists between individual animals, a relatively consistent pattern of PAF-R gene expression was found in normal unstimulated rats. As shown in Table 1, of the organs examined, the ileum contained the highest number of PAF-R gene transcripts [$(3.49 \pm 0.15) \times 10^7$ molecules/μg of total RNA].

Regulation of ileal PAF-R gene expression by PAF and LPS

I.v. injection of PAF (1.5 μg/kg) or LPS (5 mg/kg) up-regulated ileal PAF-R gene expression within 30 min (Figure 3). These doses did not cause gross intestinal injury, although histological examination revealed minimal epithelial damage at the villus tips in some animals (not shown). Figure 3 shows the time course of changes in ileal PAF-R mRNA in sham-operated, PAF- and LPS-treated rats. PAF induced a biphasic increase in PAF-R gene transcripts. There was an initial rapid increase within 30 min, which peaked at 90 min (1.6-fold of baseline, $P < 0.05$), followed by a fall to baseline at 3 h. A second increase was observed at 6 h (1.6-fold of baseline, $P < 0.05$). The response to LPS was weaker than to PAF. A rapid increase was observed within 30 min (1.5-fold of baseline, marginally significant), which returned to baseline at 3 h. In contrast with PAF, only one peak was observed after LPS addition within 6 h.

A PAF antagonist or anti-TNF antibody was given to the PAF-challenged animals to investigate the mechanism responsible for the second peak of PAF-R mRNA. Figure 4 shows the time-dependent changes in ileal PAF-R mRNA content in animals injected with PAF, with or without treatment with anti-TNF or WEB 2170. PAF antagonist completely abolished the second peak of PAF-R mRNA in the ileum. Interestingly,

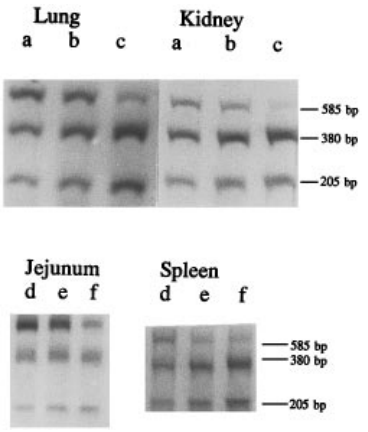

Figure 2 Analysis of PAF-R mRNA in different organs by quantitative PCR

Total RNA (0.4 μg) extracted from lungs, kidneys, jejunum and spleen was co-amplified with RT-PCR in the presence of 6.5×10^5 (lane a), 1.3×10^6 (lane b), 2.6×10^6 (lane c) 5.2×10^6 (lane d), 1.04×10^7 (lane e), 2.08×10^7 (lane f) molecules of cRNA and analysed as in Figure 1.

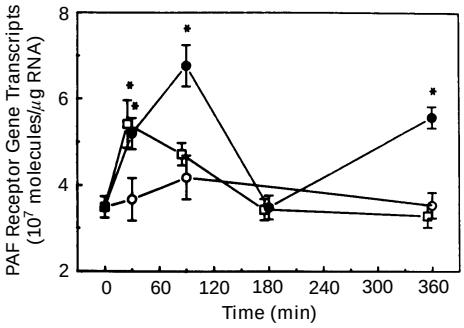

Figure 3 Time course of PAF- or LPS-induced up-regulation of PAF-R transcripts in the rat ileum

○, Sham-operated; ●, PAF (1.5 μg/kg, i.v.); □, LPS (5 mg/kg, i.v.). PAF and LPS were injected at time 0. Animals were killed at different time points for PAF-R mRNA quantification. $^*P < 0.05$ compared with sham-operated animals. Results are means ± S.E.M. ($n = 3$–4).

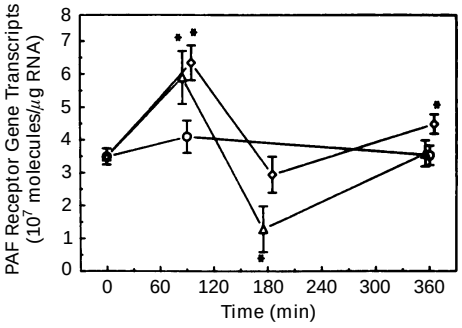

Figure 4 Inhibition of PAF-induced ileal PAF-R gene expression by WEB 2170 (a PAF antagonist) and anti-TNF antibody

PAF (1.5 μg/kg, i.v.) was injected at time 0. ◇, Anti-TNF antibody (10 ml/kg, i.p.) was given 30 min before and 150 min after PAF administration. △, WEB 2170 was injected i.v. 60 min (0.5 mg/kg) and 3 h (1 mg/kg) after PAF, and the intestine was removed at different time points for PAF-R mRNA determination. ○, Sham-operated. $^*P < 0.05$ compared with sham-operated animals. Results are means ± S.E.M. ($n = 3$–4).

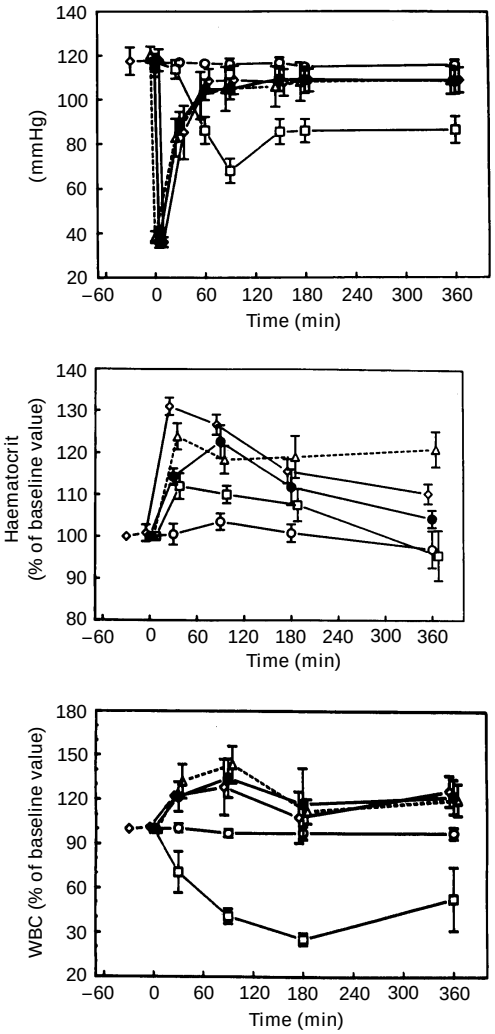

Figure 5 Changes in mean arterial pressure (top), haematocrit (middle) and WBC (bottom) after administration of PAF or LPS

See the legends of Figure 3 and 4 for explanation of symbols. Results are means ± S.E.M. ($n = 3$–15).

treatment with PAF antagonist 60 min after PAF administration led to suppressed PAF-R gene expression: the PAF-R mRNA content was significantly lower than that of sham-treated animals at 3 h ($P < 0.01$). Pretreatment with anti-TNF antibody partially blocked the second increase in PAF-R mRNA in the ileum (1.24-fold less compared with PAF only; $P < 0.05$).

Systemic pathophysiological changes after administration of PAF or LPS are presented in Figure 5. PAF at a dose of 1.5 μg/kg, i.v., induced only a transient hypotension at 5 min with recovery at 60 min (Figure 5, top). PAF also caused a mild increase in peripheral WBC and haematocrit (Figure 5, middle and bottom panels). There was no statistical difference in WBC and systemic blood pressure among the groups receiving PAF, PAF with anti-TNF and PAF with WEB 2170. However, a signficant increase in haematocrit was observed at 6 h in all three groups (Figure 5). LPS (5 mg/kg, i.v.) caused mild hypotension beginning at 30 min after injection (Figure 5, top), which persisted to the end of the experiment (6 h). Persistent leucopenia (Figure

5, bottom) and a transient increase in haematocrit (Figure 5, middle) in response to LPS were also noted.

Alteration of ileal PAF-R mRNA in indomethacin-induced gut injury

Two consecutive daily injections of indomethacin produced extensive inflammation of the intestine, which lasted at least 2 weeks, as previously reported [26]. Histological examination showed villous necrosis and marked infiltration of PMNs and macrophages into the mucosa (not shown). At day 4 after the first injection, ileal PAF-R mRNA fell below baseline level, to 52% of that in sham-operated rats ($P < 0.05$). The amount of PAF-R gene transcripts returned to normal at day 8.

DISCUSSION

The biological effects of PAF are initiated by binding its receptor in various target organs. Few studies investigating tissue PAF-Rs *in vivo* have been reported, and only relative amounts of PAF-R mRNA have been assessed [21]. Previous studies *in vitro* have demonstrated various effects of PAF on PAF-R binding and PAF-R gene transcription, but the results have been contradictory [22–25], and may be irrelevant to inflammation *in vivo*. Using a highly sensitive Q-PCR method to quantify accurately PAF-R transcripts, we report here high expression of PAF-R in the small intestine, especially the ileum, during both normal and inflamed states. This observation suggests a unique susceptibility of this organ to PAF-related intestinal inflammation. Furthermore PAF may be a physiological regulator of intestinal PAF-R expression, since WEB 2170 suppressed PAF-R expression to below the basal level of normal rats. Further, PAF at low dosage (1.5 μg/kg, i.v.) stimulates PAF-R gene transcription in the ileum. We previously showed that PAF is detectable in the normal small intestine [29], and rises during endotoxaemia, especially in the ileum [30]. These observations indicate a correlation between endogenous PAF and PAF-R expression in the intestinal tract. PAF-induced PAF-R expression is probably not a non-specific response to injury and inflammation, as, in indomethacin-induced injury, suppression of PAF-R mRNA, rather than an elevation, is observed. Thus PAF rapidly stimulates the expression of its own receptors *in vivo*, thereby potentiating the inflammatory response.

Previous studies *in vitro* have shown that LPS up-regulates PAF-R expression and potentiates the PAF effect [31–33]. Our study showed that LPS only slightly and transiently increased the level of ileal PAF-R mRNA *in vivo*. However, a delayed response to LPS cannot be ruled out because of the experimental design, which limits the observation period to only 6 h.

The biphasic increase in ileal PAF-R mRNA after PAF injection is of special interest. PAF has been shown, by Northern-blot analysis, to increase its own receptor expression *in vitro* [22]. Thus it is probable that the first up-regulation of PAF-R gene expression is a direct response to exogenous PAF, since it starts immediately after agonist challenge, too soon for the generation of a secondary mediator. The second peak of ileal PAF-R mRNA is especially intriguing. Our data suggest that this peak is caused by PAF-induced production of various endogenous pro-inflammatory mediators, including PAF itself. We previously reported that exogenous PAF elicits production of endogenous PAF [8] and TNF [9] in the small intestine. We demonstrated that WEB 2170, a PAF antagonist, completely abolished the second peak of PAF-R gene transcription, indicating a crucial role for endogenous PAF in PAF-R induction. Anti-TNF antibody also partially blocked the second peak of PAF-R gene transcription. Since our earlier studies showed that PAF induces gene transcription as well as protein synthesis of TNF in the small intestine [9] and TNF causes PAF production [29], we hypothesize that exogenous PAF induces production of endogenous TNF, which in turn causes up-regulation of PAF-R gene transcription via endogenous PAF. The physiological function of the second late phase of up-regulation of PAF-R expression may be more complex. It is possible that this increase is also related to a late event of inflammation, such as proliferation or healing [34,35].

The detailed mechanism of how PAF up-regulates the expression of its own receptor is unclear. We recently showed that a low dose (1.0 μg/kg) of PAF induces intestinal NF-κB [28]. Other investigators also demonstrated that PAF activates NF-κB [36] and up-regulates the expression of human PAF-R transcripts via NF-κB *in vitro* [37]. Thus, in inflammation, PAF may up-regulate PAF-R expression via NF-κB.

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