Impaired Anaphylactic Responses with Intact Sensitivity to Endotoxin in Mice Lacking a Platelet-activating Factor Receptor

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Summary

Platelet-activating factor (PAF) is a potent phospholipid mediator with diverse biological activities in addition to its well-known ability to stimulate platelet aggregation. Pharmacologic studies had suggested a role for PAF in pregnancy, neuronal cell migration, anaphylaxis, and endotoxic shock. Here we show that disruption of the PAF receptor gene in mice caused a marked reduction in systemic anaphylactic symptoms. Unexpectedly, however, the PAF receptor-deficient mice developed normally, were fertile, and remained sensitive to bacterial endotoxin. These mutant mice clearly show that PAF plays a dominant role in eliciting anaphylaxis, but that it is not essential for reproduction, brain development, or endotoxic shock.

Key words: platelet-activating factor • platelet-activating factor receptor • anaphylaxis • endotoxic shock • gene targeting

Platelet-activating factor (PAF; 1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine) is a potent phospholipid mediator with various biological activities, including platelet activation, airway constriction, hypotension, and vascular permeation (1–4). PAF acts by binding to a G protein-coupled seven transmembrane receptor (5). Through G proteins, PAFR links to signal transduction mechanisms, including turnover of phosphatidylinositol, elevation in intracellular calcium concentration (6), and activation of many kinases, e.g., phosphatidylinositol-3 kinase (7), protein kinase C (8), and mitogen-activated protein kinase (9). We and others have cloned PAFR cDNAs of guinea pig (10), human (11–14), and rat (15) and a PAFR gene of mouse (16). A unique PAFR has been identified in each species by the molecular cloning technique, although possible existence of PAFR subtypes has been suggested by biochemical or pharmacologic methods (17, 18).

PAF is synthesized not only in various blood cells but also in many other tissues, e.g., early mammalian embryo (19), fetal lung (20), kidney (21), and brain (22) under physiological conditions. Thus, PAF, even at low concentrations, has been considered to constitutively regulate various physiological processes such as reproduction (19, 23), blood pressure (24), neural development (25), and hippocampal long-term potentiation (18). Despite extensive studies, the physiological importance of PAF in vivo remains largely unknown.

In addition to its role as a physiological mediator, PAF has been believed to be associated with the pathology of anaphylaxis and endotoxic shock. Systemic anaphylaxis is a rapid, often fatal type-I allergic reaction characterized by acute airway constriction, heart rate (HR) alteration, hypotension, and vascular leakage (26, 27); many anaphylactic symptoms can be mimicked by PAF injection in animals (4, 5). Indeed, PAF is released rapidly from antigen-stimulated mast cells (28) and basophils (29) in vitro. In studies in vivo, an increase in blood PAF level is also detected after antigen challenge to sensitized animals (30). PAFR antagonists, which inhibit the binding of PAF to the receptor, exert protective effects on anaphylaxis in animal models (30–32).
However, the precise role of PAF among a lot of allergic mediators in the development of anaphylactic responses is unclear.

Endotoxic or septic shock is the result of activation of the immune system by endotoxin/LPS, a component of Gram-negative bacterial cell wall. In this state, macrophages produce large amounts of cytokines such as TNF-α and IL-6, as well as other inflammatory mediators, including PAF, that are released into the circulation (33, 34). Thus, these events lead to hypotension, tissue injury, vascular leakage, and frequently, death. As is the case in anaphylaxis, PAF per se is capable of eliciting many symptoms associated with endotoxic shock (4, 5). Further, it has been reported that the blood PAF level increased during endotoxia (30, 35) and the PAF acetylhydrolase activity decreased in plasma of patients with sepsis (36). The administration of PAFR antagonists to animals and humans protects them from the deleterious effects of endotoxin (30, 32, 37-40). Our recent demonstration that transgenic mice overexpressing PAFR are hypersensitive to endotoxin has suggested that PAF plays an exaggerating role in the in vivo response to endotoxin (41).

To date, our ability to address the pathophysiological role of PAF has largely depended on the use of structurally diverse PAFR antagonists. However, pharmacologic studies often encounter problems related to the specificity of drugs. Indeed, several PAFR antagonists are demonstrated to have an inhibitory effect on the activity of intracellular PAF acetylhydrolase, an inactivating enzyme of PAF (42, 43). Some other antagonists inhibit histamine effects through the interaction with its G protein–coupled receptor (40, 44).

To clearly identify both the nonredundant physiological role of PAF and the pathophysiological role of PAF without the use of possibly nonspecific PAFR antagonists, we generated animals in which the PAFR signaling was congenitally blocked by targeted disruption of the PAFR gene in murine embryonic stem (ES) cells. We report here that the resulting PAFR-deficient mice were fertile and developed normally. Furthermore, although these mice showed the markedly attenuated anaphylactic symptoms, the responses to endotoxin remained intact. Thus, our studies demonstrate that PAF plays a dominant role in eliciting anaphylaxis, and is not essential for reproduction, brain development, or endotoxic shock.

**Materials and Methods**

Reagents. Materials and chemicals were obtained from the following sources. α-[32P]dCTP (111 TBq/mmol) and transfer membrane (Hybond-N+) were from Amersham (Tokyo, Japan); restriction enzymes and DNA-modifying enzymes were from Takara (Kyoito, Japan) or Toyobo (Kyoito, Japan); agorases (Sea Kem and Nu Sieve) were from FMC (Rockland, ME); PAF (1-O-hexadecyl-2-acetyl-sn-glycero-3-phosphocholine) and leukotriene B4 (LTB4) were from Cayman Chemical (Ann Arbor, MI); [3H]JW2086 (499.5 GBq/mmol) and [1H]jacyt-PAF (370 GBq/mmol) were from Du Pont/New England Nuclear (Tokyo, Japan); fatty acid–free BSA, grade VI (OVA), and endotoxin (LPS; Escherichia coli, serotype O127:B8, code No. L-3129) were from Sigma Chemical Co. (St. Louis, MO); lyo-PAF was from Cascade Biochem (Berkshire, UK); pyrogen-free saline was from Tsuka Pharmaceuticals (Tokyo, Japan); RPMI 1640 medium was from Nissui (Tokyo, Japan). Other materials and reagents were of analytical grade. PAF and LTB4 were dissolved in pyrogen-free saline containing 0.25% BSA just before use. Endotoxin was suspended in saline. Unlabeled WEB 2086 was a gift from Boehringer Ingleheim (Ingelheim, Germany) and was dissolved in saline by sonication at a concentration of 0.5 mg/ml.

Targeted Disruption of Mouse PAFR Gene. The murine PAFR genomic DNA was isolated by screening a AFixII 129/Sv genomic library (Stratagene, La Jolla, CA) (16) with a guinea pig PAFR cDNA (10). The targeting vector was constructed in pBluescript vector (Stratagene) using a 10.7-kb EcoRI fragment of the genomic DNA, a PGK-neo cassette, and an MC1-1k cassette (Fig. 1a). The PGK-neo cassette was inserted into the Psil site in the exon. E14-1 cells were electroporated with Not1-linearized targeting vector and selected with G418 (225 μg active weight/ml) and fluoroiodoadenosyluracine (FIAU; 0.2 μM). The ES clones carrying the PAFR-targeted mutation on one allele through the homologous recombination event were screened by Southern blot analysis after ScI digestion with a 5′ flanking 0.6-kbSal–EcRI fragment (9′ probe shown in Fig. 1a). This fragment is located at the margin of the murine PAFR genomic DNA clone used; the Sal site derives from the polycloning sites of a FixII vector. The 9′ probe identified a 9.1-kb mutant band in addition to a 11.1-kb wild-type band (data not shown). Results were verified by hybridization of BamHI restriction digests with a 3′ flanking 1.4-kb EcoRI–EcoRI fragment (3′ probe shown in Fig. 1a). Hybridization of Scal restriction digests either with a 1.2-kb EcoRI–BglII fragment containing the entire exon (PAFR probe shown in Fig. 1a) or with a 1.1-kb Aat–BglII fragment of a neomycin resistance gene (Neo probe shown in Fig. 1a) was also performed to ensure a single integration site. The former probe identified 9.1- and 3.5-kb mutant bands in addition to a 11.1-kb wild-type band (data not shown). Results were verified by hybridization of BamHI restriction digests with a 3′ flanking 1.4-kb EcoRI–EcoRI fragment (3′ probe shown in Fig. 1a). Hybridization of Scal restriction digests either with a 1.2-kb EcoRI–BglII fragment containing the entire exon (PAFR probe shown in Fig. 1a) or with a 1.1-kb Aat–BglII fragment of a neomycin resistance gene (Neo probe shown in Fig. 1a) was also performed to ensure a single integration site. The former probe identified 9.1- and 3.5-kb mutant bands in addition to a 11.1-kb wild-type band (data not shown). Results were verified by hybridization of BamHI restriction digests with a 3′ flanking 1.4-kb EcoRI–EcoRI fragment (3′ probe shown in Fig. 1a). Hybridization of Scal restriction digests either with a 1.2-kb EcoRI–BglII fragment containing the entire exon (PAFR probe shown in Fig. 1a) or with a 1.1-kb Aat–BglII fragment of a neomycin resistance gene (Neo probe shown in Fig. 1a) was also performed to ensure a single integration site. The former probe identified 9.1- and 3.5-kb mutant bands in addition to a 11.1-kb wild-type band (data not shown).

Mutant clones were injected into blastocysts of C57BL/6 mice and the resulting male chimeras were mated to C57BL/6 females to test for germine transmission of the mutation. Offspring derived from ES cells were identified by coat color and screened for the presence of the targeted allele by Southern blot analysis of Scal-digested tail DNA using 5′ probe (Fig. 1b). For the following experiments, homozygous mutants and wild-type littermates were obtained by intercrossing heterozygous mutant mice under specific pathogen-free conditions. The genotypes of mice were determined by Southern blot analysis of Scal-digested tail DNA using the PAFR probe (Fig. 1b).

Northern Blot Hybridization. Total RNA was prepared from 2% casein-induced neutrophils as previously described (16). The RNA (1 μg) was electrophoresed, transferred to a nylon membrane, and hybridized (16) with either the PAFR probe shown in Fig. 1a or a human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probe (Clontech, Palo Alto, CA).

Radioligand Binding Assays. Lung membranes were prepared and used for ligand binding assay as described (41). Peritoneal exudate macrophages were obtained using thioglycollate as described in reference 16. The cells were cultured in 6-well dishes at 106 cells/well. After incubation for 2 h, nonadherent cells were washed away with RPMI 1640 medium containing 10% heat-inactivated FCS (GIBCO BRL, Rockville, MD). Adherent mac-
rophages were then cultured for 1 or 2 d. Binding of \(^{3}H\)W E B 2086 (20 nM) or \(^{3}H\)jaceyl-PAF (10 nM) was competed with unlabeled W E B 2086 (20 \(\mu\)M) in H e p e s-Tyr o l e's buffer (pH 7.4) containing 0.1% BSA (H e p e s-B S A buffer) at 20°C for 90 min or 25°C for 60 min, respectively. The cells were washed three times with cold H e p e s-B S A buffer and lysed with 5% Triton X-100. The radioactivity associated with the cells was measured by liquid scintillation counting.

Measurement of Intracellular Calcium Concentration. Neutrophils were loaded with 3 \(\mu\)M Fura-2 AM (D o j i n o, K u m a m o t o, J a p a n) at 37°C for 1 h and resuspended in calcium-free H e p e s-Tyr o l e's buffer (pH 7.4) at 2 \(\times\) 10\(^5\) cells/ml. Intracellular calcium concentrations were measured using a fluorometer (C A F-110; J A S C O, T o k y o, J a p a n) as described previously (45).

S e nsitization of M i c e. \(p a f^{+/-}\) and \(p a f^{-/-}\) (18-22-wk-old) male mice were given an intraperitoneal injection of O V A (100 \(\mu\)g), aluminum hydroxide gel (1 mg), and pertussis toxin (300 ng; K a k e n Pharmaceutical, T o k y o, J a p a n) (26). Elicitation of anaphylaxis or bleeding were performed 18-21 d after the immunization. Serum levels of O V A-specific IgE and IgG1 were determined by E L I S A as described in the Pharmac. Ingan (S e n g a, D i a o n o, C A) protocol. In brief, 96-well flat-bottomed plates (C o r n i n g Gl a s s W o r k s, C o r n i n g, N Y) were coated with either rat anti-mouse IgE antibody (clone R 3 5-72) or O V A. Bound IgE or IgG1 was detected with biotinylated O V A in combination with horseradish peroxidase-conjugated streptavidin or horseradish peroxidase-conjugated anti-mouse IgG1 (clone G 1-6,5), respectively. 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (Sigma Chemical Co.) was used as a substrate to generate the color reaction, which was read at 405 nm. The Ig levels of the samples were estimated using serum of an O V A-immunized mouse as a standard, and were compared using the M a n n-W h i t n e y U test.

Measurement of M a n n A r t i a l Pressure and H R. Under urethane anesthesia (1.5 g/kg, intraperitoneal), mice were cannulated with PE-10 polyethylene tubing (C l a y A d a m s, P a r s i p p a n y, N J) into the right femoral artery. The tubing was filled with heparinized saline and connected to a pressure transducer (T P-200T; N i h o n K o h d e n, T o k y o, J a p a n). The blood pressure signals were amplified (A P-600G; N i h o n K o h d e n) and stored into a tape recorder. H R was computed using a tachometer (AT-601G; N i h o n K o h d e n, T o k y o, J a p a n) and were compared using a computer (P C9801RX; N E C, T o k y o, J a p a n) to calculate the data were fed into a computer (P C9801RX; N E C, T o k y o, J a p a n) to calculate the mean values. Body temperature was maintained at 37°C with a heating pad by monitoring rectal temperature. The cannulated mice were injected with 200 \(\mu\)g/kg of P A F (10 ml/kg), 200 \(\mu\)g/kg of lyso-PAF (10 ml/kg), or 150 \(\mu\)g of O V A (100 \(\mu\)l/head) via the right femoral vein.

Bronchopulmonary Responses. Total lung resistance (R\(_L\)) was measured as previously described (41, 46, 47). Mechanically ventilated mice were challenged with 500 \(\mu\)g O V A via the jugular vein in a 100-\(\mu\)l bolus. The lungs were removed intact 5 min after the challenge of O V A. For histological analysis, the lungs were frozen in liquid nitrogen, fixed in Carnoy's solution, and stained with hematoxylin and eosin (46). In other experiments, the dissected lungs were weighed and placed in a gravity convection oven at 90°C for 72 h to determine dry weights for calculation of wet weight/dry weight ratios (46).

Determination of Cytokine Levels. A dherent peritoneal macrophages were prepared as described in reference 16 and were cultured on 48-well dishes at 2.5 \(\times\) 10\(^5\) cells/well in 250 \(\mu\)l of R P M I 1640 medium supplemented with 10% heat-inactivated F C S (M o r e g a t e, M o u l b o r n e, A u s t r a l i a). The cells were exposed to endotoxin (100 ng/ml) plus mouse rIFN-\(\gamma\) (100 U/ml; G e n z y m e, Cambridge, M A) for 24 h. The supernatants were harvested and stored at \(-80°C\). Concentrations of T N F-\(\alpha\) and I L-6 were determined in duplicate using murine ELISA kits obtained from G e n z y m e and A m e r s h a m, respectively. Limits of detection for T N F-\(\alpha\) and I L-6 were 35 and 40 pg/ml, respectively. Data shown are averages from two separate experiments. Unstimulated macrophages released undetectable levels of the cytokines.

Results and Discussion

G eneration of P A F R-deficient M i c e. A gene-targeting construct was generated in which an open reading frame of the G protein–coupled P A F R gene was inserted by a PGK-neo cassette (Fig. 1 a). The construct was electroporated into E 14-1 E 5 cells. Two targeted ES clones were identified after screening 670 ES clones resistant to both G 418 and f u r a i o d o d e n o s u l a r u c e by Southern blot analysis of Scal-digested genomic DNA using the 5' probe shown in Fig. 1 a (data not shown). Further Southern blot analyses with the 3' probe after BamH I digestion, and Nco probe and P A F R probe after Scal digestion, demonstrated the absence of rearrangements or extra insertional events in these two clones (data not shown). The targeted clones were injected into C 5 7 B L /6 blastocysts, and the resulting male chimeras bred to C 5 7 B L /6 females to produce mice heterozygous for the disrupted P A F R allele. Crossings between F 1 or F 2 heterozygous mice under specific, pathogen-free conditions yielded 82 wild-type (p a f\(^{+/-}\)), 140 heterozygous (p a f\(^{-/-}\)) mutant, and 72 homozygous (p a f\(^{-/-}\)) mutant mice, consistent with a normal 1:2:1 Mendelian distribution of offspring (\(\chi^2\) test). p a f\(^{-/-}\) mouse lines were established from both clones.

Inactivation of the P A F R gene was confirmed by genomic Southern analysis (Fig. 1 b) and by Northern analysis of RNA isolated from neutrophils (Fig. 1 c). Neither peritoneal macrophages nor lung tissues from p a f\(^{-/-}\) mice displayed any detectable binding sites for the P A F R antagonist W E B 2086 (16, 48; data not shown), confirming the loss of surface receptors. In p a f\(^{-/-}\) mice, challenge with P A F no longer induced an increase in intracellular calcium concentration in neutrophils (49) (Fig. 1 d) or a decrease in mean arterial pressure (M A P; reference 50; Fig. 1 e). These results provide compelling evidence that the P A F R plays an essential role in P A F-induced calcium mobilization in neutrophils and in systemic hypotension.

Development, Reproduction, and Cardiac Function. P a f\(^{-/-}\) mice were healthy in appearance and grew normally up to 1 yr of age. There were no changes in blood cell populations, differential leukocyte counts, or serum biochemical parameters (data not shown). Light microscopic examination of major organs (brain, heart, lung, liver, kidney, pituitary, thyroid, adrenal, thymus, spleen, gastrointestinal tract, bone, bone marrow, and skin) revealed no differences in morphology between p a f\(^{+/-}\) and p a f\(^{-/-}\) mice at 13-16 wk old (data not shown). A requirement for P A F in neural cell migration had previously been proposed based on the finding that mutation in the L I S 1 gene encoding a subunit of brain P A F acetylhydrolase causes M i l l e r-D i e k e r lissencph-
aly (25). The apparent lack of brain malformation in paf<sup>−/−</sup> mice suggests, however, that the LIS1 subunit may have other function(s) relevant to brain development that are unrelated to PAF. This is consistent with a recent report that LIS1 reduces microtubule catastrophe events by interacting with tubulin (51).

PAF has also been proposed to play significant roles in reproductive functions such as implantation and parturition.
neutrophils. Changes in intracellular calcium in response to 10 nM PAF or 10 nM LTβ2 were determined using Fura-2AM. The agonists were added at the times indicated by the arrows. (e) MAP measurements of pafr<sup>+/+;</sup> and pafr<sup>−/−</sup> mice. PAF (200 μg/kg) was administered intravenously at the times indicated by the arrows. Lyso-PAF (200 μg/kg) had no effects on MAP values in either group. (data not shown). N = early identical data were obtained with two animals in each group.

in various species (52–54). In fact, human preembryos produced by in vitro fertilization show an increased rate of implantation after exposure to PAF (53) and transgenic mice that overproduce PAfR display reproductive disorders (41). Hence, there existed a possibility that pafr<sup>−/−</sup> mice might be infertile. Contrary to this expectation, however, the homozygous pafr<sup>−/−</sup> mice were fertile and produced healthy litters of normal size (6.2 ± 1.2 [pafr<sup>−/−</sup> × pafr<sup>−/−</sup>; n = 5] versus 8.0 ± 0.7 [pafr<sup>+/+</sup> × pafr<sup>−/−</sup>; n = 7]; mean litter size ± SEM, P = 0.2, the unpaired t test [two-tailed]). These results suggest that PAF is not essential for normal development and reproduction of mice, although we cannot rule out the possible existence of additional PAfR subtypes as previously suggested by biochemical and pharmacologic experiments (17, 18). Furthermore, it is also possible that there may be receptor-independent actions of PAF.

MAP was measured through a catheter placed in the femoral artery. There were no significant differences in the baseline of both parameters between pafr<sup>+/+</sup> and pafr<sup>−/−</sup> mice (101 ± 5 versus 107 ± 4 mmHg; mean ± SEM, n = 5 each, P = 0.4, the unpaired t test [two-tailed]). Although intravenously administered PAF has a potent hypotensive effect, as shown in Fig. 1 e, the lack of difference in MAP between pafr<sup>+/+</sup> and pafr<sup>−/−</sup> mice suggests that PAF may not be a mediator involved in setting the basal tone of vascular smooth muscle. H R of pafr<sup>+/+</sup> and pafr<sup>−/−</sup> mice were also indistinguishable (586 ± 22 versus 560 ± 28 beats/min; mean ± SEM, n = 5 each, P = 0.5, the unpaired t test [two-tailed]). However, on the contrary, we observed in OVA-sensitized mice (see below) that H R of pafr<sup>−/−</sup> mice (648 ± 7 beats/min; mean ± SEM, n = 10) was significantly higher than that of pafr<sup>+/+</sup> mice (570 ± 18 beats/min; n = 12, P = 0.003, the Mann-Whitney U test), whereas MAPs were comparable between pafr<sup>+/+</sup> and pafr<sup>−/−</sup> mice. Sensitization for active anaphylaxis may be associated with many changes, e.g., in levels of antigen-specific Igs of different isotypes, numbers of various effector cells, and local concentrations of immunomodulatory cytokines. It is possible that some of these changes cause the observed increase in the basal H R.

A dive Systemic Anaphylaxis. To directly evaluate the pathophysiological role of PAF in systemic anaphylaxis, pafr<sup>+/+</sup> and pafr<sup>−/−</sup> mice were sensitized with OVA in the presence of adjuvants (26). This treatment caused mice of both genotypes to develop comparable and significant levels of OVA-specific IgE and IgG1 (data not shown), which mediate systemic anaphylaxis (27). The sensitized animals were injected intravenously with OVA to elicit systemic anaphylaxis. Cardiovascular or bronchopulmonary parameters were measured to assess the severity of the shock. Soon after challenge with OVA, all pafr<sup>+/+</sup> mice developed severe and irreversible hypotension (Fig. 2 a). In this shock state, H R response to OVA was biphasic. OVA caused a rapid onset of tachycardia that reached a maximal increase of ~20% after 10 min, followed by bradycardia (Fig. 2 b). The tachycardia may be a compensatory response due to baroreceptor reflex. Five of six pafr<sup>−/−</sup> mice died within 25 min (Fig. 2), and the remaining mouse died after 48 min. In contrast, the fall in MAP was much milder in pafr<sup>−/−</sup> mice compared with pafr<sup>+/+</sup> mice (Fig. 2 a). Furthermore, H R remained stable upon the administration of OVA (Fig. 2 b). Only one of seven pafr<sup>−/−</sup> mice died after 36 min. Lethality among pafr<sup>−/−</sup> mice monitored 50 min after the an-
tigen challenge was significantly lower than that of pafr+/+ mice (P < 0.005, as determined by Fisher’s exact test).

Next, ṘL was measured in tracheostomized, mechanically ventilated mice. All pafr+/+ mice showed a rapid increase in ṘL after the intravenous injection of OVA (Fig. 3a). In comparison to the response in pafr+/+ mice, antigen challenge of pafr−/− mice produced a significantly smaller increase in ṘL (Fig. 3a). Lung tissues in pafr+/+ mice given OVA were characterized by airway constriction associated with folding of airway epithelium, alveolar wall thickening, alveolar distortion, and edema (Fig. 3b). Pulmonary edema due to increased vascular permeability was further assessed by measuring wet weight/dry weight ratios (Fig. 3c). In marked contrast to pafr+/+ mice, pafr−/− mice displayed no response to the antigen challenge, either in the wet weight/dry weight ratios (Fig. 3c) or in lung histology (Fig. 3b).

These results clearly demonstrate a dominant role for PAF in the anaphylactic responses. Because pafr−/− mice still show milder symptoms of anaphylaxis, chemical mediators other than PAF, such as eicosanoids (55), histamine, or serotonin, probably also contribute to the anaphylactic responses, albeit to a much lesser extent.

The results of this study elucidated a large contribution of PAF to allergen-induced airway obstruction. As for the effects of PAF on the bronchopulmonary system, we reported that PAFR overexpressing mice show a remarkable airway hypersensitivity to methacholine (41). Thus, PAF is likely to play an important role in the pathophysiology of the bronchopulmonary system. However, the murine airways are somehow unresponsive to the intravenously administered PAF (41, 50, 56), whereas PAF given in the same manner elicited severe hypotension in mice (Fig. 1a; reference 50). This is in contrast to our present data from immunologically stimulated mice which shows that PAF is deeply involved in both hypotension and bronchoconstriction. The murine airways, therefore, do not appear to respond to the blood-released PAF, which exerts deleterious effects on the cardiovascular system. Only PAF produced in the lung may act on the airways in a paracrine fashion.

Endotoxic Shock. To assess the importance of PAF and PAFR in the development of endotoxic shock, PAFR-deficient mice were examined for their sensitivity to endotoxin using three kinds of assays. First, pafr+/+ and pafr−/− mice were treated with endotoxin given intravenously at a dose range of 7.5–30 mg/kg. As shown in Table 1, there was no significant difference in lethality in this model between pafr+/+ and pafr−/− mice. At each dose, mice of both genotypes exhibited apparently equivalent symptoms of endotoxic shock: lethargy, piloerection, and diarrhea. We next measured MAP of anesthetized mice in endotoxemia for 100 min as shown in Fig. 2a. pafr−/− mice treated with endotoxin given intravenously at a dose range of 7.5–30 mg/kg. As shown in Table 1, there was no significant difference in lethality in this model between pafr+/+ and pafr−/− mice. At each dose, mice of both genotypes exhibited apparently equivalent symptoms of endotoxic shock: lethargy, piloerection, and diarrhea. We next measured MAP of anesthetized mice in endotoxemia for 100 min as shown in Fig. 2a. pafr−/− mice treated with endotoxin given intravenously at a dose range of 7.5–30 mg/kg. As shown in Table 1, there was no significant difference in lethality in this model between pafr+/+ and pafr−/− mice. At each dose, mice of both genotypes exhibited apparently equivalent symptoms of endotoxic shock: lethargy, piloerection, and diarrhea.
equivalent levels of TNF-α (10,250 and 10,544 pg/ml, respectively) and IL-6 (4,352 and 4,314 pg/ml, respectively). During endotoxic shock, a strong hypotensive factor, nitric oxide (NO), is synthesized. A PAFR antagonist is reported to inhibit the endotoxin-stimulated NO production in cultured murine macrophages (57), suggesting that endotoxin-induced hypotension is mediated by PAF and NO in this order. However, our results demonstrate that endotoxic shock can be elicited without the action of PAF.

Contrary to our expectation based on the previous pharmacologic reports, PAFR-deficient mice failed to acquire resistance to endotoxin. The intact susceptibility to endotoxin is consistent with our present data that macrophages from PAFR-deficient mice produced TNF-α and IL-6, the inflammatory cytokines induced by endotoxin, to the same extent as those from wild-type mice. Since we have previously reported an increased susceptibility to endotoxin in PAFR overexpressing mice (41), it is very intriguing that
PAFR-deficient mice show normal sensitivity to endotoxin. It may be that PAF functions in vivo not as an essential, but as an exaggerating factor for endotoxic shock. There are noteworthy pharmacologic reports exhibiting that ID$_{50}$ values of various PAF antagonists for death due to anaphylaxis are nearly equal to those for PAF-induced death (32), whereas the doses of PAFR antagonists for inhibiting endotoxin-induced death are usually 10 times higher than those for inhibiting PAF-induced death (39). These data may suggest that PAFR antagonists attenuate the symptoms of anaphylaxis and endotoxic shock in different ways. The latter might be PAFR independent.

Concluding Remarks. Although PAF has pleiotropic biological activities in vitro, the effects of deleting the PAFR gene were restricted to anaphylactic responses, i.e., PAFR-deficient mice were fertile and grew normally and were as sensitive to endotoxin as the wild-type mice. Thus, we conclude that although PAF is a dominant anaphylactic mediator, it may not be required for reproduction, neural development, or endotoxic shock. PAFR antagonists should, therefore, be useful in preventing anaphylactic responses in humans without causing serious mechanism-based side effects. Recent mouse models demonstrated that neither IgE (26) nor its high-affinity receptor, Fc$\varepsilon$RI (27), is required for active systemic anaphylaxis, further supporting the validity of PAF as a target mediator for preventing and treating the disorder.

Table 1. Lethal Toxicity of Bacterial Endotoxin

| Dose of endotoxin (mg/kg) | paf$^{-/-}$ | paf$^{+/+}$ |
|---------------------------|------------|------------|
| 7.5                       | 0/3        | 0/3        |
| 15.0                      | 3/8        | 3/8        |
| 22.5                      | 5/8        | 4/8        |
| 30.0                      | 7/8        | 7/8        |

Sex- and age-matched paf$^{-/-}$ and paf$^{+/+}$ mice (unanesthetized, 14–27 wk old) were injected with endotoxin in a volume of 10 ml/kg via tail vein. Survival was monitored for 3 d. There were no deaths after 3 d. No statistical significance was observed at any doses in paf$^{-/-}$ mice compared with paf$^{+/+}$ mice (Fisher's exact test).

Figure 4. Change in MAP during endotoxemia. paf$^{+/+}$ and paf$^{-/-}$ mice are represented by the open and closed squares, respectively. Data from female mice are shown as in Fig. 2 a. The baselines of paf$^{+/+}$ and paf$^{-/-}$ mice were 101 ± 5 and 107 ± 4 mmHg (mean ± SEM; n = 5 each). No mice died during the recordings.

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