

Tumor Necrosis Factor- α Develops Late Anaphylactic Reaction through Cytosolic Phospholipase A₂ Activation

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Key Words

Late anaphylaxis · Tumor necrosis factor- α · Cytosolic phospholipase A₂ · Mitogen-activated protein kinases

Abstract

Background: We have recently reported that tumor necrosis factor (TNF)- α plays an important role in the development of a late anaphylactic reaction, but the downstream pathway beyond TNF- α remains unclear. **Objective:** It was the aim of this study to examine whether TNF- α induces late-phase anaphylaxis via the activation of cytosolic phospholipase A₂ (cPLA₂). **Methods:** Using a murine model of active systemic anaphylaxis to penicillin V, the induction of the late phase of anaphylaxis was quantified by measuring the increase in hematocrit value as well as the plasma level of platelet-activating factor in TNF- α knockout mice. Phosphorylation of mitogen-activated protein kinases (MAPKs) and cPLA₂ was measured by immunoprecipitation. cPLA₂ activity was assessed by using 1-stearoyl-2-[1-¹⁴C] arachidonyl-sn-glycero-3-phosphocholine as the substrate. **Results:** Phosphorylation and enzymatic activity of cPLA₂, and phosphorylation of the 3 known MAPKs, i.e. p38, extracellular signal-regulat-

ed kinase 1/2 (ERK1/2) and c-Jun NH₂-terminal kinase, were markedly increased in a TNF- α -dependent way in the lungs of mice undergoing anaphylaxis. A specific cPLA₂ inhibitor significantly attenuated the late anaphylactic symptoms. Either p38 or an ERK inhibitor significantly attenuated not only cPLA₂ phosphorylation and activity, but also the late-phase anaphylaxis. **Conclusion:** TNF- α induces cPLA₂ activation through the pathway involving p38 MAPK and ERK activation and appears to be the key mechanism leading to the development of late-phase anaphylaxis.

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Introduction

Anaphylaxis has the potential for a recurrent or biphasic pattern with an incidence of 5–20% [1–4]. Although the molecular mechanism(s) leading to late anaphylactic reaction remains to be clarified, we have previously shown that tumor necrosis factor (TNF)- α , which is pro-

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duced as a result of platelet-activating factor (PAF)-mediated nuclear factor- κ B activation, induces the development of late anaphylactic reactions [5].

TNF- α is the major pleiotropic proinflammatory cytokine, playing an essential role in inflammation. The proinflammatory properties of TNF- α can be attributed to its ability to activate the enzyme cytosolic phospholipase A₂ (cPLA₂) [6]. cPLA₂ specifically releases arachidonic acid from the *sn*-2 position of glycerophospholipids to provide the precursor to eicosanoids such as prostaglandins and thromboxane, which are cyclooxygenase products, leukotrienes, which are 5-lipoxygenase products, and PAF [7, 8]. On the basis of findings that induction of anaphylaxis results in a biphasic increase in serum levels of PAF, and that TNF- α is associated with the secondary phase of PAF synthesis [5], it is possible to speculate a link between TNF- α and cPLA₂ in the development of late anaphylactic reaction.

In this study, we investigated this possibility and demonstrated that TNF- α induces late anaphylactic reaction through cPLA₂ activation, in which p38 mitogen-activated protein kinase (MAPK) and extracellular signal-regulated kinase 1/2 (ERK1/2) play important roles.

Materials and Methods

Animals

Specific pathogen-free female C57BL/6 mice were purchased from Orient Bio (Gapyeong, Republic of Korea) and were housed throughout the experiments in a laminar flow cabinet and maintained on standard laboratory chow ad libitum. TNF- α knockout mice (TNF- $\alpha^{-/-}$) were derived from breeder pairs of B6;129S-Tnfr1Gk1 stock and the original wild-type strain (B6129SF/J), which were obtained from The Jackson Laboratory (Bar Harbor, Me., USA). Mice were 7–8 weeks old at the start of each experiment. All animal experiments conformed to the protocol approved by the Institutional Animal Care and Use Committee of the Chonbuk National University Medical School.

Reagents

Ovalbumin (OVA) and bovine serum albumin (BSA) were purchased from Sigma-Aldrich (St. Louis, Mo., USA). The cPLA₂ inhibitor, arachidonyl trifluoromethyl ketone (AACOCF₃), purchased from Biomol (Plymouth Meeting, Pa., USA), was administered intraperitoneally (20 mg/kg) 30 min before challenge. The p38 MAPK inhibitor (SB202190; 5 mg/kg), the ERK inhibitor (PD98059; 12.5 mg/kg) and the c-Jun NH₂-terminal kinase (JNK) inhibitor (SP600125; 12.5 mg/kg) from Calbiochem (San Diego, Calif., USA) were administered intraperitoneally 2 days before the challenge. Recombinant mTNF- α was purchased from R&D Systems (Minneapolis, Minn., USA). Control groups received the appropriate vehicles. *Bordetella pertussis* was obtained from the National Institute of Health (Seoul, Republic of Korea).

Induction of Active Systemic Anaphylaxis to Penicillin V

Penicillin V (Pen V)-OVA and Pen V-BSA conjugates were prepared as described previously [5, 9]. Mice were sensitized by intraperitoneal injection of 500 μ g of Pen V-OVA conjugate plus 2×10^9 *B. pertussis* and 1.0 mg of alum. Challenge was given as an intravenous injection of 100 μ g of Pen V-BSA conjugate 5 weeks later.

Determination of Hematocrit Values

Late anaphylaxis was quantified by measuring the increase in hematocrit (Ht) value as described previously [5]. Briefly, the heart was cut open, and small blood samples were collected into a heparinized microhematocrit tube (Proper Manufacturing Co., Long Island City, N.Y., USA). The tubes were centrifuged for 5 min using a microhematocrit centrifuge (International Equipment Co., Needham Height, Mass., USA). Percent Ht change was expressed as [(value at postchallenge – value at prechallenge)/value at prechallenge] $\times$ 100.

Immunoprecipitation and Western Blotting

Immunoprecipitation and Western blotting were performed as described previously [10]. The antibodies against cPLA₂, p38, ERK, JNK, activating transcription factor (ATF)2, c-Jun, phospho-cPLA₂, phospho-p38, phospho-ERK, phospho-JNK, phospho-ATF2 and phospho-c-Jun were employed. All antibodies were purchased from Cell Signaling Technology Inc. (Beverly, Mass., USA) except for anti-cPLA₂, which was from Santa Cruz Biotechnology (Santa Cruz, Calif., USA). The signal intensities of specific bands were analyzed quantitatively using a Fluor-STM Imager (Bio-Rad, Hercules, Calif., USA) and were plotted as relative intensity.

cPLA₂ Activity

The assay of cPLA₂ activity was performed using lung tissues as described previously [11]. The lungs were minced and sonicated in a buffer (50 mg tissue/ml) containing 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 150 mM NaCl and protease inhibitors with a tip-type sonicator (Branson Ultrasonics, Danbury, Conn., USA) at 20% amplitude for 3 min, followed by centrifugation at 1,000 g for 20 min. The supernatants were fractionated by passaging 20 times using a 26-gauge needle and ultracentrifugation at 85,000 rpm for 1 h. cPLA₂ activity in the supernatants was assayed with sonicated liposomes prepared as described previously [12].

Determination of Plasma PAF

The postchallenge blood was taken from the heart, which had been cut open, and was mixed with 0.1 volumes of 3.8% ice-chilled citrate solution, and then, was centrifuged immediately using an Eppendorf microfuge. The plasma was stored at -20°C until use. PAF was measured as previously described [5].

Statistical Analysis

Data are expressed as mean $\pm$ SD. Statistical comparison was performed using one-way ANOVA followed by the Fisher test. Significant differences between the groups were determined using the unpaired Student's *t* test. A *p* value <0.05 was accepted as an indication of statistical significance.

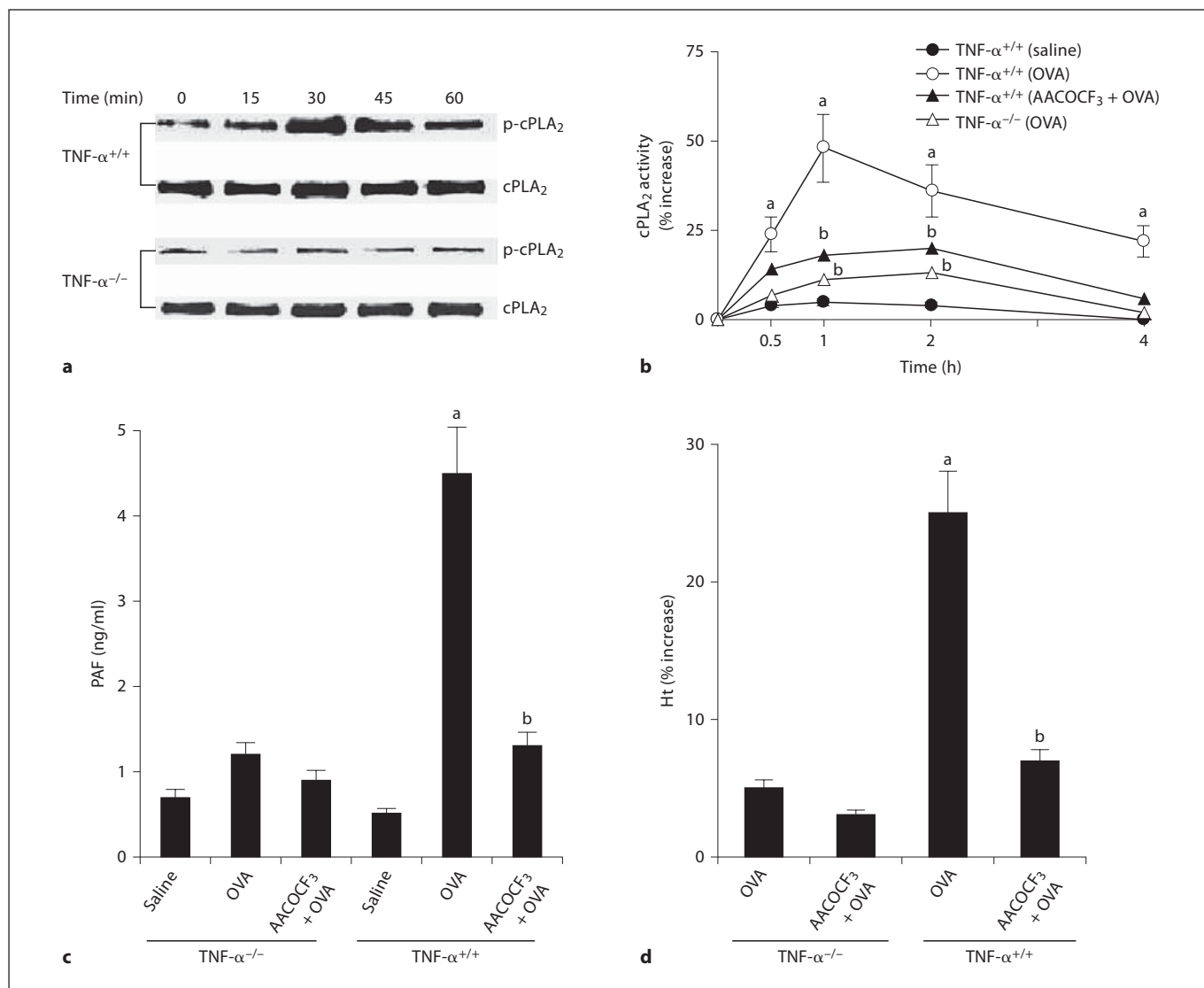


Fig. 1. TNF- α -induced cPLA₂ activation dependency of late anaphylactic symptoms. Lungs were removed at the indicated time points after the challenge, and time courses of cPLA₂ phosphorylation (**a**) and activity (**b**) were assessed in TNF- $\alpha^{+/+}$ and TNF- $\alpha^{-/-}$ mice. Lungs were removed at 1 h to assess cPLA₂ activity. Blood was collected 7.5 h after the challenge to assess plasma PAF levels

(**c**) and Ht values (**d**). AACOCF₃ (20 mg/kg) was administered intraperitoneally 30 min prior to the challenge. **a** Data are representative of 3 independent experiments with 2 or 3 mice per time point. **c, d** Data represent the mean $\pm$ SD of 3 independent experiments with 3–4 mice per group. ^a $p < 0.01$ versus the saline group; ^b $p < 0.01$ versus the TNF- $\alpha^{+/+}$ (OVA) group.

Results

TNF- α Induces Late Anaphylactic Reaction through cPLA₂ Phosphorylation and Activation

Increases in cPLA₂ phosphorylation (fig. 1a) and enzymatic activity (fig. 1b) were observed in the lungs of TNF- $\alpha^{+/+}$ mice undergoing anaphylaxis with the peak levels at 30 and 60 min, respectively. In addition, late phase of PAF

synthesis (fig. 1c) and anaphylaxis (fig. 1d) occurred in TNF- $\alpha^{+/+}$ mice. However, the increases were not observed in TNF- $\alpha^{-/-}$ mice (fig. 1). Late anaphylaxis was monitored by measuring increases in Ht values, because hemoconcentration, which occurs due to PAF-induced extravasation of plasma, is the most prominent symptom in murine anaphylaxis [5]. To assess the possible involvement of cPLA₂ in the development of late-phase anaphylaxis, the

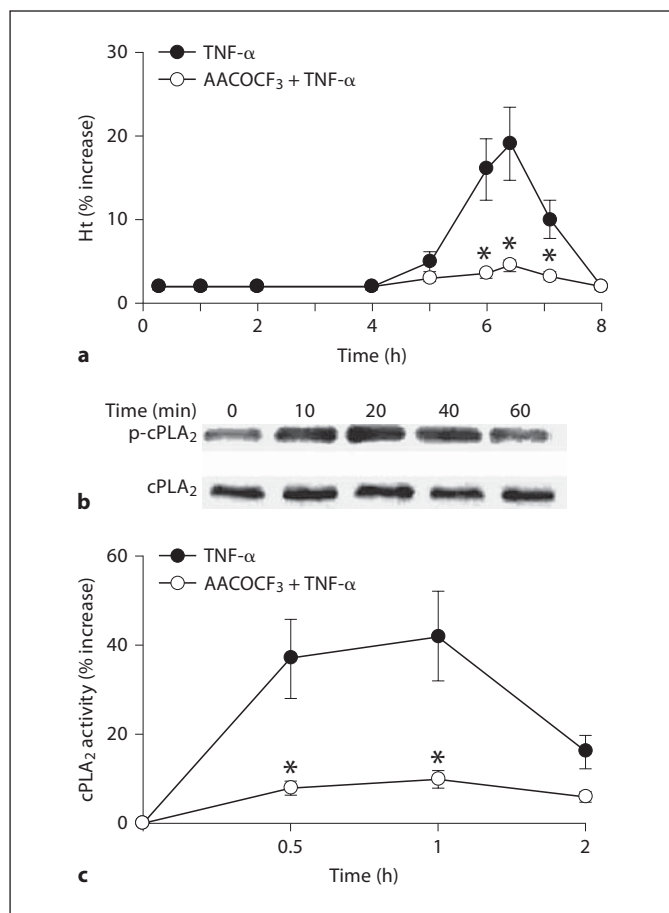


Fig. 2. TNF- α induces not only late-phase hemoconcentration but also cPLA₂ phosphorylation and activation in C57BL/6 mice. After the challenge, time courses of hemoconcentration (**a**), cPLA₂ phosphorylation (**b**) and activation (**c**) were assessed. **a, c** Data represent the mean $\pm$ SD of 2 independent experiments with 3 mice per time point. **b** Data are representative of 2 independent experiments with 3 mice per time point. * $p < 0.01$ versus the TNF- α group.

animals were pretreated with a selective cPLA₂ inhibitor (AACOCF₃). AACOCF₃ inhibited cPLA₂ enzymatic activity in response to the antigen challenge (fig. 1b) as well as the late phase of PAF synthesis (fig. 1c) and alleviated anaphylactic symptoms (fig. 1d) in TNF- $\alpha^{+/+}$ mice. We next examined whether the late-phase hemoconcentration could occur by injection of TNF- α (1 μ g/mouse, i.v.). The time kinetics of hemoconcentration after TNF- α injection revealed that TNF- α induced a single peak of increase in Ht value at 5.5–7.5 h (fig. 2a). A similar degree of hemoconcentration was observed by TNF- α ranging from 0.5 to 2 μ g/mouse (data not shown). TNF- α also increased cPLA₂ phosphorylation (fig. 2b) and cPLA₂ enzy-

matic activity (fig. 2c). Pretreatment with AACOCF₃ significantly inhibited TNF- α -induced hemoconcentration (fig. 2a) and cPLA₂ enzymatic activity (fig. 2c). These data indicate that TNF- α induced late anaphylactic reaction through cPLA₂ activation.

Either p38 MAPK or ERK Is Involved in Phosphorylation and Activation of cPLA₂ during Anaphylaxis

cPLA₂ has a consensus phosphorylation motif (containing Ser-505) for a group of serine/threonine-specific, proline-directed protein kinases, the MAPK family, including p38 kinases, ERK1/2 (p42/p44) and JNK [13]. Phosphorylation of cPLA₂ is a key step in the activation of this enzyme and subsequent arachidonic acid release and the generation of biologically important eicosanoids [14, 15]. Thus, we examined the effect of TNF- α on activation of MAPKs during anaphylaxis and assessed which MAPK was importantly involved in cPLA₂ activation. All 3 MAPKs were markedly activated during anaphylaxis in TNF- $\alpha^{+/+}$ mice, and the magnitude of activation of all the MAPKs was significantly reduced in TNF- $\alpha^{-/-}$ mice (fig. 3). These data suggest that activation of these MAPKs was a TNF- α -dependent process in anaphylaxis.

We next assessed the effects of specific inhibitors of MAPKs on cPLA₂ phosphorylation and activation as well as the development of late anaphylactic symptoms. We first verified whether the concentration of each MAPK inhibitor used in this in vivo study was effective in blocking each MAPK activation. In the case of ERK, we examined the effect of an ERK inhibitor (PD98059) on ERK phosphorylation because PD98059 blocks MEK1, the upstream kinase phosphorylating ERKs, and therefore, correctly inhibits ERK phosphorylation [16]. However, p38 MAPK and JNK inhibitors are both direct inhibitors of the activity of p38 and JNK themselves, and thus, cannot block p38 and JNK phosphorylation [17, 18]. In case of the p38 MAPK inhibitor SB202190 and the JNK inhibitor SP600125, the phosphorylation of ATF2 and c-Jun was examined, given that ATF2 and c-Jun are substrates that are phosphorylated by p38 [19] and JNK [20], respectively. Induction of anaphylaxis resulted in the phosphorylation of ATF2, ERK and c-Jun in the lungs, and each MAPK inhibitor inhibited the phosphorylation of its target molecule (fig. 4a). Either the p38 MAPK or ERK inhibitor significantly attenuated cPLA₂ activity (fig. 4b) as well as late-phase anaphylactic symptoms (fig. 4c). However, the JNK inhibitor did not display such inhibitions. These data suggest that p38 MAPK and ERK played a role in the activation of cPLA₂ during anaphylaxis.

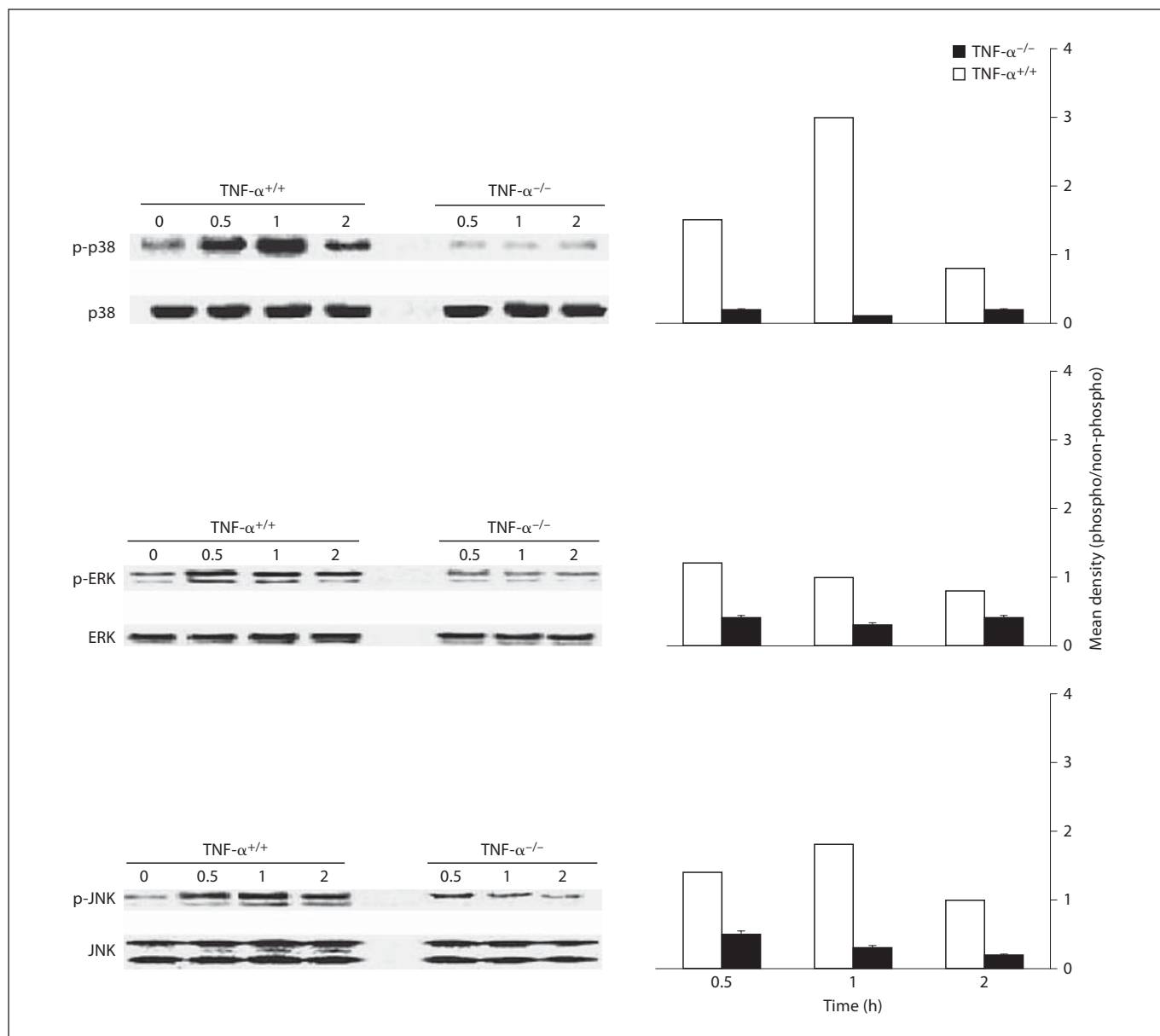


Fig. 3. TNF- α -dependent activation of MAPKs during anaphylaxis. After the challenge, lungs were removed at the indicated time points, and time courses of phosphorylation of MAPKs were assessed. Data are representative of 3 independent experiments with 2 or 3 mice per time point. The results of the activation of MAPKs were used for quantitation by densitometry (right panels).

Discussion

We have previously shown that TNF- α , which is produced as a result of PAF-mediated nuclear factor- κ B activation, induces the development of late anaphylactic reaction [5]. In this study, using the Pen-V-induced murine model of anaphylaxis, we demonstrate that TNF- α in-

duces late anaphylactic reaction through cPLA₂ phosphorylation and its activation.

cPLA₂ has been implicated in the inflammatory responses induced by TNF- α [21–23]. TNF- α -induced activation of cPLA₂ mediates the production of agonist-induced arachidonic acid release and plays an essential role in the release of PAF as well as of important inflam-

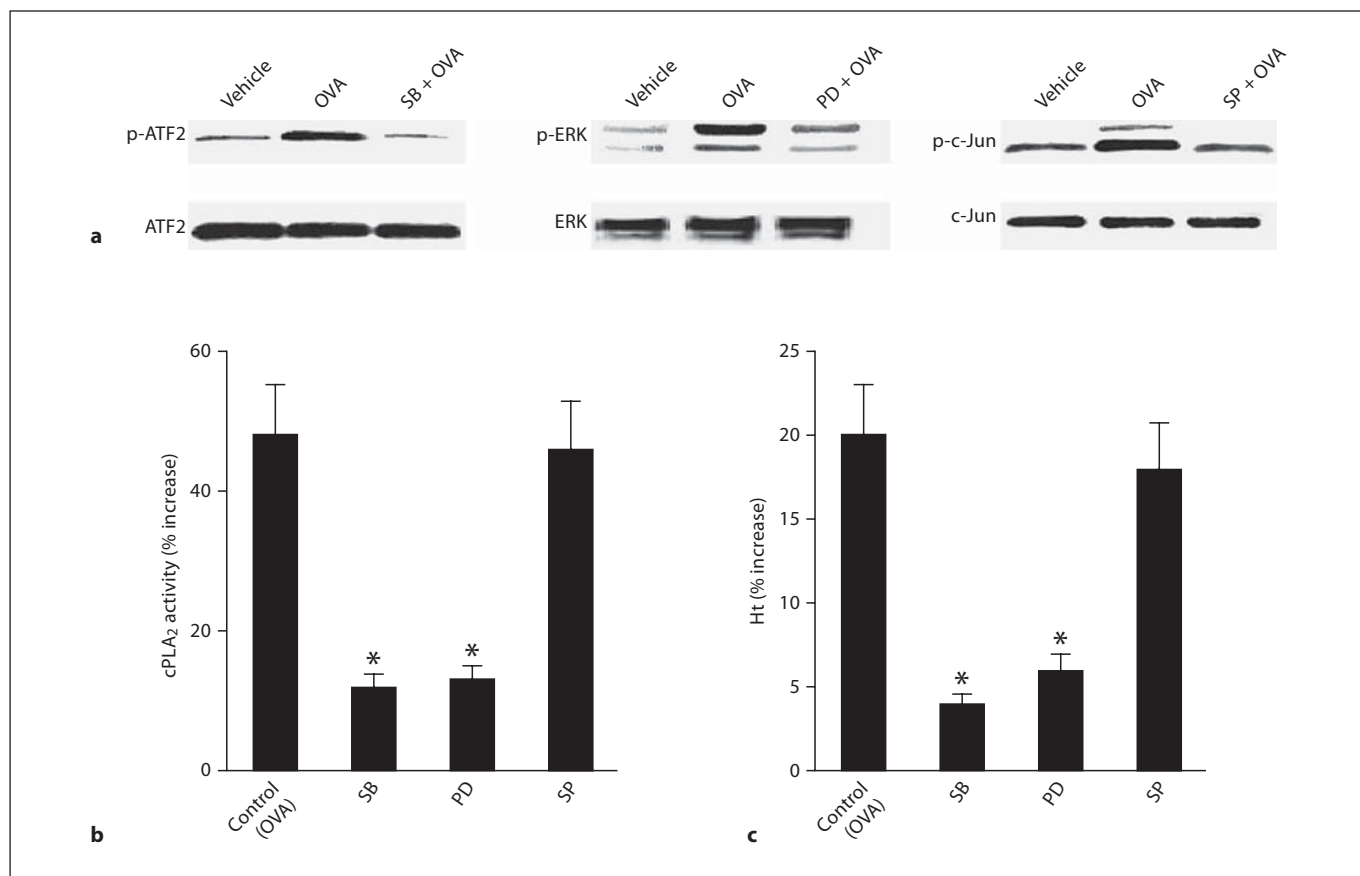


Fig. 4. Effects of MAPK inhibitors on cPLA₂ activation and late-phase anaphylactic symptoms. Lungs were removed 30 min and 1 h after the challenge to assess the effects of MAPK inhibitors on phosphorylation of ATF2, ERK and c-Jun (**a**) and cPLA₂ enzymatic activity (**b**), respectively. **c** Blood was collected 7.5 h after the challenge. The p38 MAPK inhibitor (SB202190, 5 mg/kg), ERK inhibitor (PD98059, 12.5 mg/kg) and JNK inhibitor

(SP600125, 12.5 mg/kg) were administered intraperitoneally 2 days before the challenge. **a** Data are representative of 2 independent experiments with 3 mice per group. **b, c** Data represent the mean $\pm$ SD of 3 independent experiments with 3–5 mice per group. SB = SB202190; PD = PD98059; SP = SP600125. * $p < 0.01$ versus the control group.

matory lipid mediators [7, 8]. Therefore, the fact that the late-phase synthesis of PAF occurs at 7.5 h after antigen challenge and that it can be abrogated by either TNF- α blockade [5] or cPLA₂ inhibitor (fig. 1d) suggests that TNF- α -induced activation of cPLA₂ is involved in the late-phase synthesis of PAF, which eventually induces the late anaphylactic symptom of hemoconcentration. Although we only measured PAF in the circulation during anaphylaxis and observed PAF-induced hemoconcentration in this study, other lipid mediators whose synthesis are regulated by cPLA₂, such as cyclooxygenase and 5-lipoxygenase products, must be involved in the development of other late anaphylactic symptoms. Presently, we also demonstrated that injection of TNF- α resulted in the

development of late-phase hemoconcentration at 5.5 h and cPLA₂ activation, which were blocked by the pretreatment with AACOCF₃. Taken together, these data indicate that TNF- α induces late anaphylaxis through the activation of cPLA₂.

cPLA₂ has a consensus primary sequence (Pro-Leu-Ser-Pro) for phosphorylation by MAPKs [12]. Presently, we demonstrate that all 3 MAPKs, ERK, p38 and JNK, are activated in a TNF- α -dependent way during anaphylaxis. Although little evidence exists regarding activation of MAPKs by TNF- α in vivo, in vitro studies have reported that TNF- α rapidly mediates the phosphorylation of different MAPKs depending on the cell types used. For example, activation of JNK and p38 MAPK is a crucial event

in TNF- α signaling in Hela cells [24] and human neutrophils [25], respectively. On the other hand, ERK is activated in mouse macrophages stimulated by TNF- α [26].

Importantly, in the present study, we found that either the p38 MAPK or ERK inhibitor significantly inhibits cPLA₂ activity as well as the late anaphylactic symptom. Although ERK-2 has been proposed to cause phosphorylation and activation of cPLA₂ [13, 27], recent studies with hematopoietic cells and other cell lines have indicated more prominent roles of p38 [28, 29] in the activation of cPLA₂ induced by collagen [30] and thrombin [31]. In human neutrophils treated with TNF- α , the activation of cPLA₂ appears to be regulated by p38 MAPK rather than by ERK [25]. Moreover, other investigators have also reported that an inhibitor of p38 MAPK abolishes TNF- α -induced cPLA₂ phosphorylation [32, 33]. These findings suggest that TNF- α -induced cPLA₂ phosphorylation and activation appears to be regulated by p38 MAPK rather than by ERK. However, our in vivo study clearly demonstrates that an ERK inhibitor is comparable with a p38 MAPK inhibitor in blocking cPLA₂ phosphorylation and activity as well as the late anaphylactic symptom. Thus, this study suggests that p38 MARK and ERK are involved in the phosphorylation and activation of cPLA₂ in the

pathogenesis of the diseases in which TNF- α plays an important role.

In conclusion, we demonstrated the molecular mechanisms underlying the development of a late anaphylactic reaction. The pathway comprises TNF- α synthesis, phosphorylation of p38 MAPK and ERK, cPLA₂ activation and late-phase synthesis of PAF and cPLA₂ metabolites. Therefore, if a similar mechanism operates in human anaphylaxis, selective inhibitors that target the various components of the pathway (for example, anti-TNF- α antibody, MAPK inhibitor or cPLA₂ inhibitor) might be of great therapeutic value for controlling the late phase of human anaphylaxis.

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