

## Pathway-oriented profiling of lipid mediators in macrophages <sup>☆</sup>

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### Abstract

Macrophages produce various kinds of lipid mediators including eicosanoids and platelet-activating factor. Since they are produced from common precursors, arachidonic acid-containing phospholipids, regulations of metabolic pathways underlie the patterning of lipid mediator production. Here, we report a pathway-oriented profiling strategy of lipid mediators by a newly developed multiplex quantification system. We profiled mouse peritoneal macrophages in different activation states. The analysis of kinetics revealed the differences in the production time course of various lipid mediators, which also differed by the macrophage types. Scatterplot matrix analysis of the inhibitor study revealed correlations of lipid mediator species. The changes of these correlations provided estimates on the effects of lipopolysaccharide priming. We also found a highly linked production of 11-hydroxy-eicosatetraenoic acid and prostaglandin E<sub>2</sub>, implying the *in vivo* property of cyclooxygenase-mediated 11-hydroxyeicosatetraenoic acid production. The present approach will serve as a strategy for understanding the regulatory mechanism of lipid mediator production.

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Lipid mediator production is a consequence of lipid metabolism in tissues and cells, in which enzyme activities involved in the specific metabolic pathway determine the species and quantities of lipid mediators. Arachidonic acid-containing membrane phospholipids are the common precursors for various lipid mediators including eicosanoids such as prostaglandins (PGs), leukotrienes (LTs), and hydroxyeicosatetraenoic acids

(HETEs), and platelet-activating factor (PAF). Lipid mediators exhibit diverse biological actions, either synergistic or reverse direction *in vivo*, and play roles in self-defense as well as maintaining homeostasis [1,2]. Since these mediators are generated through a cascade pathway, regulatory enzymes such as phospholipase A<sub>2</sub> (PLA<sub>2</sub>), cyclooxygenases (COX), and lipoxygenases (LO) affect the profile of lipid mediator production (Fig. 1). It has also been suggested that one of the regulatory mechanisms involves a functional coupling of multiple enzymes [3,4], suggesting that the sum of enzyme activities does not necessarily determine profiles of the lipid mediators.

Macrophages are known to exist in various forms as tissue-associated resident types (e.g., peritoneal macrophages, pulmonary alveolar macrophages, Kupffer cells, and microglia) and elicited ones that accumulate to inflammatory sites [5]. It has been suggested that

<sup>☆</sup> Abbreviations: PG, prostaglandin; LT, leukotriene; HETE, hydroxyeicosatetraenoic acid; PAF, platelet-activating factor; PLA<sub>2</sub>, phospholipase A<sub>2</sub>; COX, cyclooxygenase; LO, lipoxygenase; MS, mass spectrometry; MeOH, methanol; DMSO, dimethylsulfoxide; EDTA, ethylenediaminetetraacetic acid; HBSS, Hanks' balanced salt solution; LPS, lipopolysaccharide; TG, thioglycolate; FBS, fetal bovine serum; LC, liquid chromatography; ESI, electrospray ionization; SRM, selected reaction monitoring.

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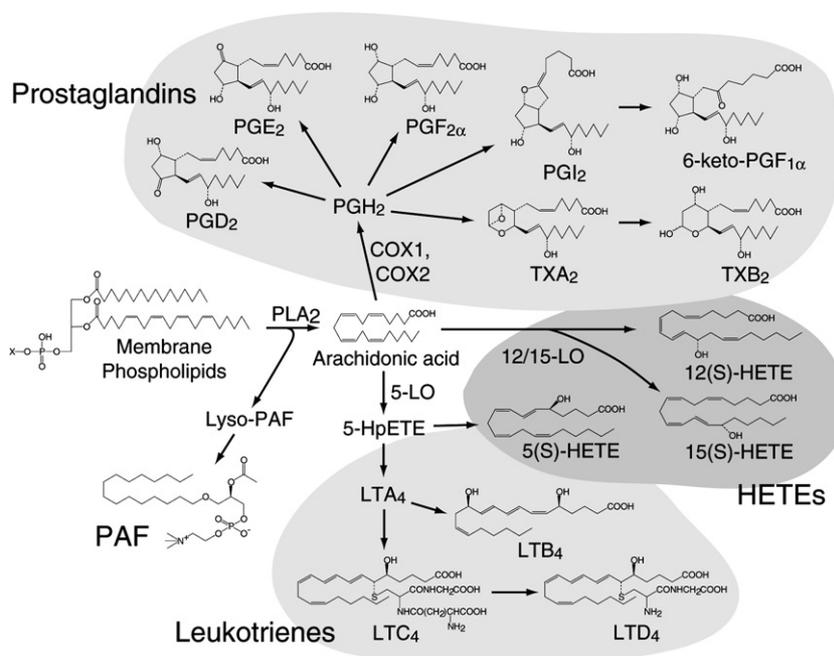


Fig. 1. Metabolic pathway of lipid mediator production. All the lipid mediators quantified in the present study are the metabolites of arachidonic acid-containing phospholipids. Arachidonic acid produced by PLA<sub>2</sub> enzymes is further metabolized to PGs, LTs, and HETEs by the downstream enzymes such as COX, LO, and various terminal synthases. PLA<sub>2</sub> also produces lyso-PAF, which can be metabolized into PAF. 5-HpETE, 5-hydroperoxyeicosatetraenoic acid.

macrophages exhibit different lipid mediator production according to their activation states [6–8]. This may be explained by changes in the regulatory state of lipid mediator production pathways as described above.

Recent approaches in addressing the complex regulatory mechanisms of metabolic pathways involve metabolomics, in which all metabolites in the relevant pathways are thoroughly detected and quantified [9]. For example, metabolome analysis of the changes in the levels of cationic- and anionic metabolites and nucleic acids during *Bacillus subtilis* sporulation [10], and metabolome analysis of sulfur-containing metabolites of the *Arabidopsis* in relation to sulfur deficiency [11], have been reported. Metabolic pathways and their regulation can be predicted from relations and changes in the metabolite levels. A similar strategy can be applied to lipid mediator production, if several mediators are simultaneously quantified.

Recently, we have developed a mass spectrometry (MS)-based multiplex quantification method for eicosanoids and PAF.<sup>1</sup> The multiplex quantification system allows high-throughput comprehensive analysis of PGs, LTs, HETEs, and PAF. In this paper, we report a pathway-oriented profiling of lipid mediator production in mouse peritoneal macrophages. We demonstrate that macrophages in different activation states exhibit different profiles as well as different kinetics of lipid mediator production. Comparative analysis within lipid mediator

species revealed the similarities and differences in the kinetics. We also show that one of the simple multivariate analyses, a scatterplot matrix analysis, provides means for the correlation analysis for multiple lipid mediators on the pathway.

## Materials and methods

**Reagents.** HPLC grade methanol (MeOH) and formic acid, ethanol, petroleum ether, dimethyl sulfoxide (DMSO), and ethylenediaminetetraacetic acid (EDTA) were obtained from Wako (Osaka, Japan). MS grade acetonitrile was obtained from Kanto (Tokyo, Japan). All eicosanoids and PAF (PAF C-16, 1-*O*-hexadecyl-2-*O*-acetyl-*sn*-glycero-3-phosphocholine) reference compounds including deuterium-labeled internal standards were obtained from Cayman (Ann Arbor, MI). RPMI-1640 medium, Hanks' balanced salt solution (HBSS), AA861, and lipopolysaccharide (LPS, from *Escherichia coli*, Serotype 0111:B4) were obtained from Sigma (St. Louis, MO). Thio-glycolate (TG) medium and fetal bovine serum (FBS) were obtained from Difco (Detroit, MI) and Equitech Bio (Kerrville, TX), respectively. A23187, SC560, and NS398 were obtained from Calbiochem (San Diego, CA). Pyrrolidine-1 was prepared as described [12,13].

**Animals.** Adult female C57BL/6J mice (CLEA Japan, Tokyo, Japan) were used in the study. All of the animals used were maintained in a specific pathogen free facility, and fed with a standard laboratory diet and water ad libitum. All animal studies were conducted in accordance with the Guidelines for Animal Research at the University of Tokyo and were approved by the Animal Ethics Committee of the University of Tokyo.

**Preparations of mouse peritoneal macrophages.** Mice were either injected intraperitoneally with 2 ml of 4% TG medium 3 days before sacrifice for the collection of TG-induced macrophages or not treated for the collection of resident macrophages. Mice were euthanized and peritoneal lavage fluids were collected by washing the peritoneal cavity

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twice with 5 ml each of cold PBS containing 2 mM EDTA. The cells in the lavage fluids were washed with PBS containing 2 mM EDTA, suspended in RPMI-1640 medium supplemented with 10% heat-inactivated FBS, and seeded onto 96-well culture plates at a density of  $1 \times 10^5$  cells per well. Two hours later, cells were washed with HBSS to remove non-adherent cells, and the medium was changed again to RPMI-1640 with 10% FBS. The adherent cells were maintained at 37 °C, 5% CO<sub>2</sub> in air and used in the experiments.

**Sample collection and pretreatment.** Macrophages were treated with or without 100 ng/ml LPS (LPS priming) in the presence of 10% FBS in RPMI-1640. After 12 h, the cells were washed twice with HBSS and stimulated with 100  $\mu$ l of 2  $\mu$ M A23187 in HBSS or vehicle (0.1% DMSO in HBSS) for indicated periods. For inhibitor studies, cells were pretreated for 30 min with various inhibitors at indicated concentrations in HBSS.

For collection of samples, 100  $\mu$ l of MeOH–formic acid (100:0.2; v/v) was added to each well to stop lipid mediator production, and the entire sample (~200  $\mu$ l) was transferred to glass vials after the addition of 10  $\mu$ l of a mixture of internal standards. Then, another 100  $\mu$ l of MeOH–formic acid (100:0.2) was added to the well and mixed for 1 h at room temperature. After the addition of 100  $\mu$ l of water–formic acid (100:0.03) to the well, the solution (~200  $\mu$ l) was transferred to the collection vial (final volume, ~400  $\mu$ l). The samples were submitted to solid-phase extraction using an Oasis HLB 96-well cartridge cluster (5 mg, Waters, Milford, MA). Briefly, samples were diluted with water–formic acid (100:0.03) to give a final MeOH concentration of ~20% by volume, applied to preconditioned cartridges, and washed serially with water–formic acid (100:0.03), water–ethanol–formic acid (90:10:0.03), and petroleum ether. Samples were eluted into glass vials with 200  $\mu$ l MeOH–formic acid (100:0.2) by centrifugation (100g, 1 min) and stored at 4 °C in the autosampler while awaiting injection.

**Quantification of lipid mediators.** A recently developed column-switching liquid chromatography (LC)–electrospray ionization (ESI)–MS/MS method<sup>1</sup> was used for the simultaneous quantification of PGs, LTs, HETEs, and PAF. Separations of these target compounds in a reversed-phase HPLC were achieved within 10 min in the

system that provide a throughput of 96 samples per 24 h. A TSQ-7000 triple stage quadrupole MS instrument equipped with an ESI interface (Thermo Electron, Waltham, MA) was operated in selected reaction monitoring (SRM) mode for the detection of target compounds. Quantification was performed by internal standard method using deuterium-labeled compounds as references. Compounds for which corresponding deuterium-labeled internal standards were not available were calibrated using deuterium-labeled PGB<sub>2</sub> (for LTC<sub>4</sub> and LTD<sub>4</sub>) or deuterium-labeled 15-HETE (for 8-HETE and 11-HETE).

**Data analysis.** For peak detection, integration, and quantitative calculation of MS data, the Xcalibur version 1.2 software (Thermo Electron, Waltham, MA) was used. Statistics including scatterplot analysis were conducted by the JMP version 5.1.1 software package (SAS Institute, NC).

## Results

### Lipid mediator production profiles of mouse peritoneal macrophages

We used resident- and TG-induced mouse peritoneal macrophages as two different types of macrophages. These macrophages have been suggested to exhibit different lipid mediator production [6,7], as well as different cytokine production and bacteriocidal activity [14,15]. We started with describing the basic properties of these macrophages with regard to their lipid mediator production. For this purpose, we stimulated the macrophages with a calcium ionophore, A23187, and the net production of lipid mediators within 30 min was quantified.

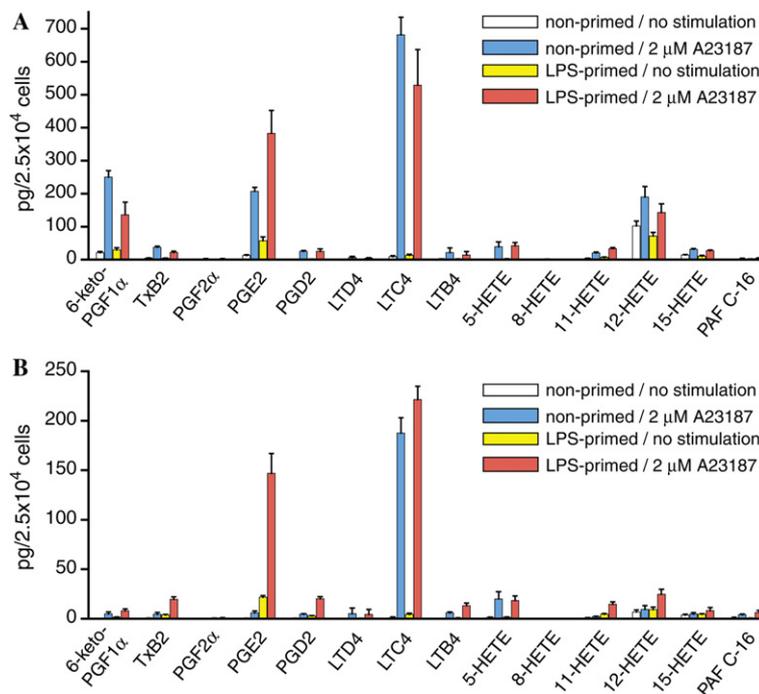


Fig. 2. Lipid mediator production profiles of mouse peritoneal macrophages. Resident (A) and TG-induced (B) macrophages with or without 12-h LPS priming were stimulated with vehicle (0.1% DMSO) or 2  $\mu$ M A23187, and lipid mediator production within 30 min was quantified. Values are expressed as means  $\pm$  SD.

As shown in Figs. 2A and B, one of the major differences of these two macrophage types was the total lipid mediator production; it was higher in resident macrophages as compared with TG-induced macrophages ( $\sim 3.5$  pmol/ $2.5 \times 10^4$  cells and  $\sim 0.5$  pmol/ $2.5 \times 10^4$  cells for resident- and TG-induced macrophages, respectively). The profiles of lipid mediator species also differed. Resident macrophages produced LTC<sub>4</sub>, PGE<sub>2</sub>, 6-keto-PGF<sub>1 $\alpha$</sub> , and 12-HETE as major products after calcium ionophore stimulation (Fig. 2A, blue bars). In contrast, TG-induced macrophages predominantly produced LTC<sub>4</sub>, followed by much smaller amounts of other lipid mediators (Fig. 2B, blue bars).

LPS has been shown to activate macrophages through its cognate receptor, TLR4, causing changes in gene expressions [16]. Therefore, we evaluated the effect of LPS priming in these macrophage types. In TG-induced macrophages, the changes were mainly characterized by dramatic increases in the basal and calcium ionophore-stimulated production of PGE<sub>2</sub> (Fig. 2B, yellow and red bars). In contrast, LPS priming ef-

fects were relatively small in the resident macrophages, although PGE<sub>2</sub> upregulation was significant (Fig. 2A, yellow and red bars). Interestingly, LPS did not much affect the total lipid mediator production. In contrast to 60-fold increase in PGE<sub>2</sub> production, the sum of lipid mediator amounts showed only a twofold increase in TG-induced macrophages ( $\sim 0.5$  and  $\sim 1.2$  pmol/ $2.5 \times 10^4$  cells in non-primed and LPS-primed cells, respectively). In addition, no significant changes of total lipid mediators were observed in resident macrophages ( $\sim 3.5$  and  $\sim 3.3$  pmol/ $2.5 \times 10^4$  cells in non-primed and LPS-primed cells, respectively). These observations suggest that LPS priming changed the profile of lipid mediator species, rather than affecting the entire pathway.

#### Time courses of lipid mediator production

To further characterize lipid mediator production of these macrophages, we compared the kinetics of lipid mediator production. As shown in Fig. 3, we found several distinct patterns of kinetics. PG production oc-

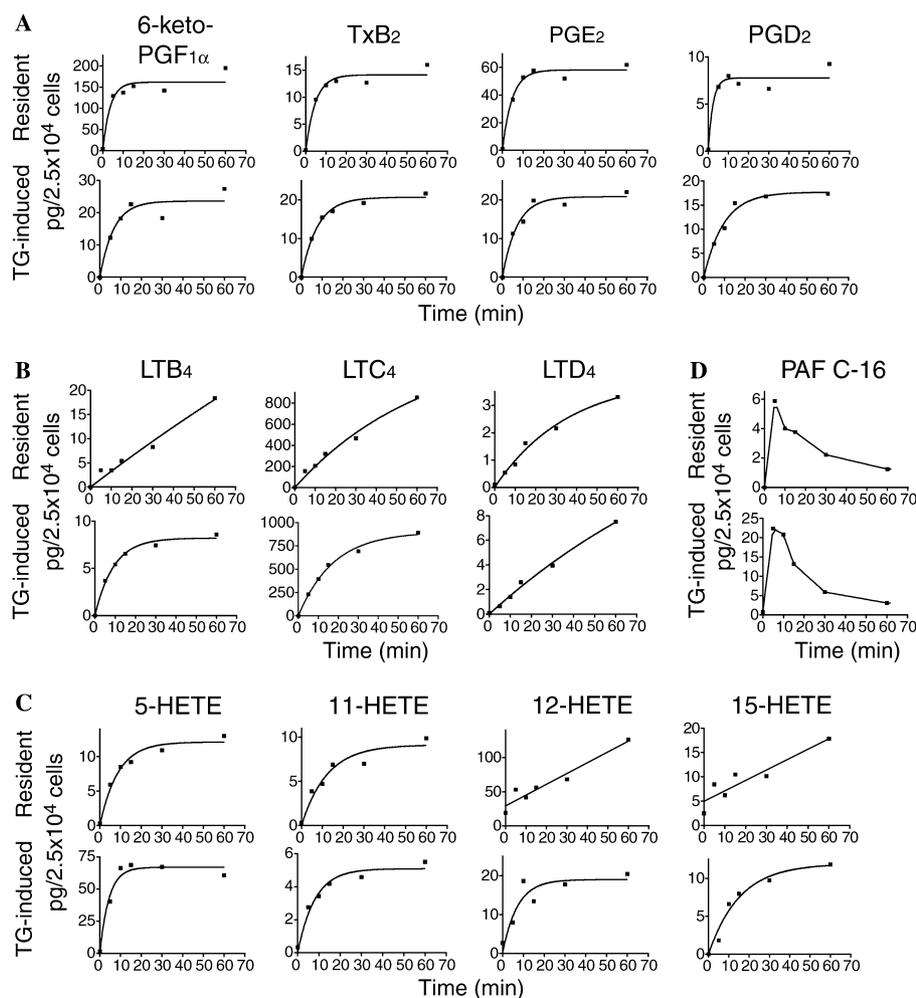


Fig. 3. Kinetics of lipid mediator production. Non-primed resident- and TG-induced macrophages were stimulated with  $2 \mu\text{M}$  A23187 for indicated periods, and lipid mediator production was quantified. Results for PGs (A), LTs (B), HETEs (C), and PAF (D) are shown.

curred immediately after calcium ionophore stimulation in 5–10 min, and virtually no further production was observed thereafter (Fig. 3A). In contrast, kinetics of LT production exhibited a linearly increasing profile in resident macrophages, showing a continuous production during the observation up to 60 min (Fig. 3B, upper panels). Interestingly, TG-induced macrophages exhibited different LT production kinetics as compared with those in resident macrophages (Fig. 3B, lower panels). In these cells, the production of LTB<sub>4</sub> and LTC<sub>4</sub> plateaued at 15–20 min after calcium ionophore stimulation. The difference of kinetics between PGs and LTs may be explained in part by the self-inactivation of COX enzymes [17]. The continuous LT production observed in resident macrophages suggests the continuous supply of substrate arachidonic acid even after PG production was terminated. In contrast, the kinetics observed in TG-induced macrophages, where both PG- and LT production exhibited deceleration, suggests the arachidonic acid depletion or inactivation of enzymes involved in the synthesis of PGs and LTs.

As shown in Fig. 3C, HETE production kinetics showed several patterns. 5-HETE and 11-HETE production exhibited kinetics similar to those of PGs, reaching a plateau after 10–15 min. In contrast, 12-HETE and 15-HETE production kinetics differed between resident- and TG-induced macrophages. It showed a linear profile in resident macrophages, whereas it reached a plateau in TG-induced macrophages. These observations, taken together with the result for PG and LT kinetics, suggest an inactivation of entire arachidonic acid pathway within 10–15 min in TG-induced macrophages.

The kinetics of PAF production was completely different from those of eicosanoids. As shown in Fig. 3D, PAF levels reached a peak around 3–5 min after calcium ionophore stimulation, and then decreased with a half-life of 15–20 min. Since PAF was not released into assay buffer (data not shown), PAF may be associated with cells. It may exist intracellularly or may be associated with plasma membrane. Although the mechanism of PAF degradation cannot be specified from our experiment, a role of intracellular-type of PAF acetylhydrolases is suggested [18].

### *Inhibitor study*

Next, we performed an inhibitor study to analyze lipid mediators by their dependency on enzymes of the biosynthetic pathway. We collected lipid mediator production profiles representing the effects of various doses of inhibitors for cPLA<sub>2</sub>α, COX-1, COX-2, and 5-LO. All the data obtained were submitted to the correlation analysis (see Correlation analysis of lipid mediators).

Here, to provide a primary characterization of resident- and TG-induced macrophages, the results for

PGE<sub>2</sub> and LTC<sub>4</sub> are shown in Fig. 4. A selective cPLA<sub>2</sub>α inhibitor, pyrrolidine-1, effectively inhibited PGE<sub>2</sub> and LTC<sub>4</sub> in both types of macrophages, suggesting that productions of these mediators are equally dependent on cPLA<sub>2</sub>α (Figs. 4A and B). In resident macrophages, a selective COX-1 inhibitor, SC560, effectively inhibited PGE<sub>2</sub> production, independently of LPS priming state (Fig. 4A, left panels). In TG-induced macrophages, SC560 less effectively inhibited PGE<sub>2</sub> production of LPS-primed macrophages as compared with non-primed macrophages (Fig. 4B, left panels). In contrast, the effect of a selective COX-2 inhibitor, NS398, was most prominent for the inhibition of PGE<sub>2</sub> produced by TG-induced macrophages primed with LPS. The results suggest that COX-1 plays a dominant role in resident macrophages independently of LPS priming, while a shift from COX-1 to COX-2 occurs after LPS priming in TG-induced macrophages. AA861, a selective 5-LO inhibitor, equally inhibited LTC<sub>4</sub> production in both cell types, showing similar contributions of 5-LO to the LTC<sub>4</sub> production (Figs. 4A and B, right panels). It is also noteworthy that cells treated with COX inhibitors, especially SC560-treated cells, produced more LTC<sub>4</sub> as compared with control cells. This can be explained by the increased arachidonic acid availability for LTC<sub>4</sub> production by the blockade of the COX pathway.

### *Correlation analysis of lipid mediators*

To analyze correlations of each lipid mediator, all data obtained in a single set of inhibitor study were visualized in a scatterplot matrix as shown in Fig. 5A. The matrix consists of a series of scatterplots that provide pairwise correlations of each lipid mediator. Analyses of the matrix are based on the concept that those lipid mediator productions that are regulated by similar mechanisms will exhibit high correlation. As shown in Figs. 5B and C, we found that 6-keto-PGF<sub>1α</sub> and TxB<sub>2</sub> showed high correlation in both types of macrophages. These results suggest that these lipid mediators are under similar regulatory mechanism with regard to cPLA<sub>2</sub>α, COX-1, and COX-2. LPS priming did not change the ratio of these two lipid mediators (Figs. 5B and C, panels for 6-keto-PGF<sub>1α</sub> vs. TxB<sub>2</sub>; compare blue and red groups). In contrast, PGE<sub>2</sub> exhibited a different mode of correlation with 6-keto-PGF<sub>1α</sub> and TxB<sub>2</sub>. LPS priming caused changes in the ratio of PGE<sub>2</sub> against 6-keto-PGF<sub>1α</sub> and TxB<sub>2</sub> (Figs. 5B and C, panels for PGE<sub>2</sub> vs. 6-keto-PGF<sub>1α</sub> or TxB<sub>2</sub>; compare blue and red groups). Interestingly, we still observe linear correlation within each priming state, suggesting that the inhibitors did not exhibit selective inhibition of specific PG species. For example, COX inhibitors did not exhibit selective inhibition of PGE<sub>2</sub> against 6-keto-PGF<sub>1α</sub> or TxB<sub>2</sub>.

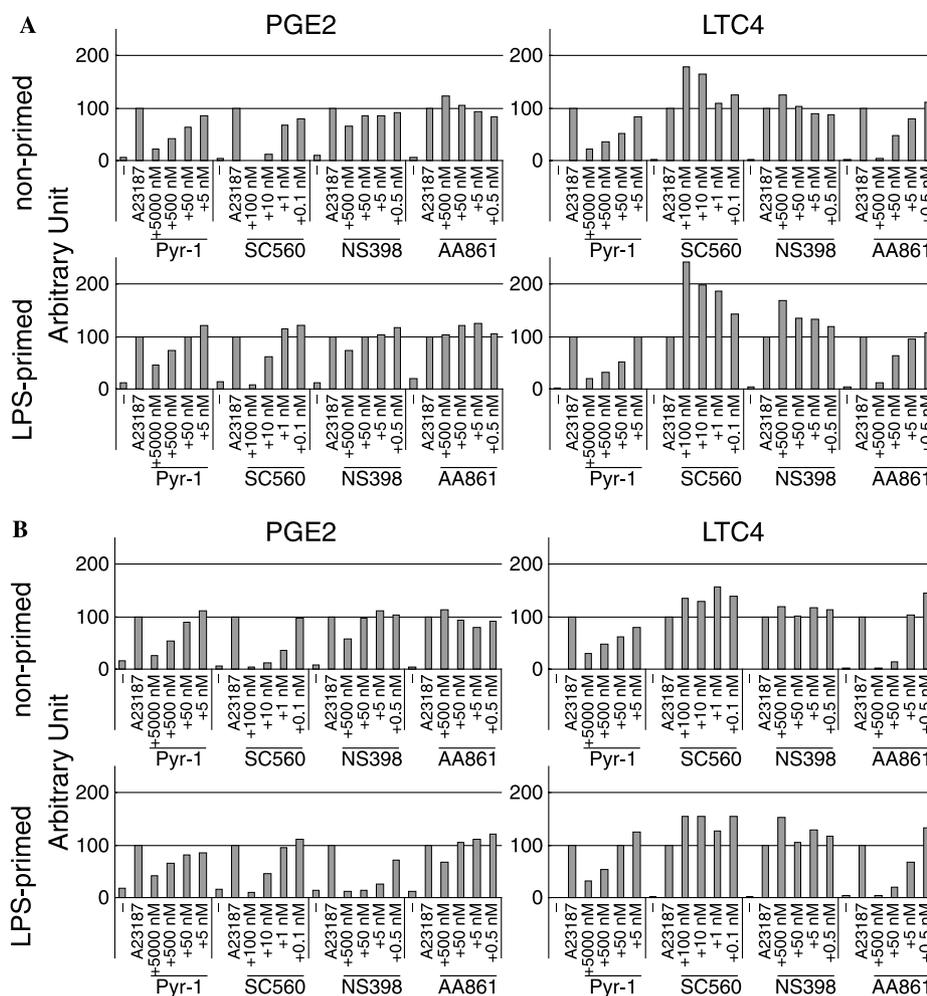


Fig. 4. Effects of various inhibitors on lipid mediator production. Resident- (A) and TG-induced (B) macrophages with or without 12-h LPS priming were pretreated with indicated concentrations of pyrrolidine-1 (Pyr-1), SC560, NS398 or AA861 for 30 min. Lipid mediator production after 30 min stimulation with 2  $\mu$ M A23187 in the presence of inhibitors was measured. Results for PGE<sub>2</sub> (left panels) and LTC<sub>4</sub> (right panels) are shown. The data are normalized with A23187-stimulated controls. Minus (–), non-stimulated control.

In the matrices, we also found that 11-HETE correlates with PGE<sub>2</sub>, in both resident- and TG-induced macrophages (Figs. 5D and E). In TG-induced macrophages, LPS-priming enhanced both 11-HETE and PGE<sub>2</sub> production without changing their ratio, suggesting a highly linked production of 11-HETE and PGE<sub>2</sub> (Fig. 5E). 11-HETE showed a correlation also with other PGs such as 6-keto-PGF<sub>1 $\alpha$</sub>  or TxB<sub>2</sub> (Fig. 5A). These observations suggest that the 11-HETE production is related to COX branch of arachidonic acid cascade. The mechanism of 11-HETE production may be explained by COX itself, since COX has been shown to produce small amount of 11-HETE *in vitro* [19]. It is noteworthy that LPS priming significantly changed the ratio of 11-HETE against 6-keto-PGF<sub>1 $\alpha$</sub>  and TxB<sub>2</sub>. This contrasts with the observation that LPS priming did not affect the ratio of 11-HETE and PGE<sub>2</sub>, suggesting that the profile of 11-HETE production is much similar to that of PGE<sub>2</sub> rather than those of other types of PGs.

## Discussion

In the present study, we profiled lipid mediator production of macrophages in different activation states using a multiplex quantification system. Lipid mediators differ from peptide hormones or cytokines in that they are metabolites rather than gene products, and their levels are regulated by concerted actions of enzymes that biosynthesize and metabolize lipid mediators [1,2,20]. Therefore, a number of researches have been carried out to characterize individual enzymes.

Recent approaches of metabolomics analysis are based on the concept that changes in the quantity and proportion of metabolites may reflect the regulatory mechanism of the relevant metabolic pathway [9]. In these strategies, regulation of metabolic pathways is inductively explained by profiling the metabolites. A similar approach can be applied to lipid mediators and responsible enzymes. For this purpose, we used a re-

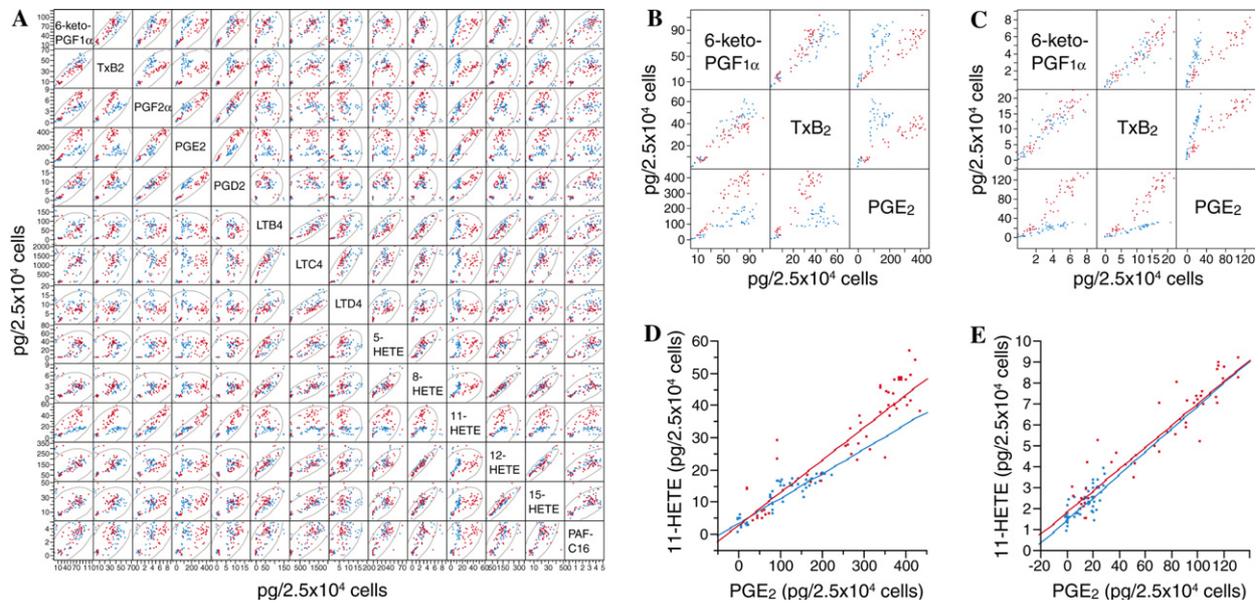


Fig. 5. Correlation analysis of lipid mediators. (A) Pairwise correlations of lipid mediators are visualized in a scatterplot matrix. Each panel in the matrix contains a scatterplot for a pair of lipid mediators, and each dot in the scatterplot corresponds to a single measurement. Results for a single set of inhibitor experiment in the resident macrophages are shown. Blue and red dots indicate results from non-primed and LPS-primed cells, respectively. Density ellipses,  $\alpha = 0.95$ . (B,C) A close-up view of scatterplot matrix for 6-keto-PGF $_{1\alpha}$ , TxB $_2$ , and PGE $_2$  for resident- (B) and TG-induced (C) macrophages. (D,E) Correlation of PGE $_2$  and 11-HETE in the resident- (D) and TG-induced (E) macrophages. Lines are linear fittings for non-primed (blue) and LPS-primed (red) cells.

cently developed simultaneous quantification system that covers PGs, LTs, HETEs, and PAF $^1$ .

Using resident- and TG-induced peritoneal macrophages, we studied two different aspects of lipid mediator production, kinetics and correlation. The kinetics that differed by lipid mediator species suggested differences in the enzyme property (Fig. 3). In the previous report by Gonchar et al. [21], the contribution of COX self-inactivation in the kinetics of PG production has been discussed. Their observation that PG production plateaus by 5–10 min agrees with our results. Thus, COX self-inactivation may characterize the PG production kinetics. We also demonstrated the different kinetics between the macrophage types. In TG-induced macrophages, the inactivation of entire arachidonic acid pathway within 10–15 min after calcium ionophore stimulation was suggested. Resident macrophages did not exhibit such an inactivation, as continuous LT production was observed. A possible explanation for this is the changes in arachidonic acid supply by PLA $_2$ . There is a report on the reduced PLA $_2$  activity in TG-induced macrophages as compared with resident macrophages [6]. It is also possible that phospholipid compositions and arachidonic acid contents differ between these cell types [22]. Thus, the reduction in arachidonic acid-containing phospholipids and/or reduced PLA $_2$  activity may explain the different kinetics between the macrophage types.

In the correlation analysis, we demonstrated that the data visualization by the scatterplot matrix serves as a

tool to find correlation of lipid mediators (Fig. 5A). When applied to an inhibitor study, the correlation analysis profiled the relationship of lipid mediators with regard to dependency on enzymes of the pathway. It has been discussed that various terminal PG synthases are differentially coupled with COX-1 and COX-2 [23–25]. However, we did not observe selective inhibition of specific PG species by COX inhibitors (Figs. 5B and C). The possible explanation for the result is that the macrophages examined in our experiments were mostly dependent on a single COX isotype, COX-1 or COX-2. It has been reported that administration of COX-2 selective inhibitors preferentially decreases systemic prostacyclin levels as compared with those of thromboxane [26]. Thus, systemic inhibition of COX-2 may tilt the prostacyclin/thromboxane balance in favor of thromboxane [27]. Here, our results for macrophages suggest that systemic changes in the balance, if any, are explained by the difference in cell types that produce prostacyclin and thromboxane, rather than changes in prostacyclin/thromboxane ratio in a single cell type. It is of interest that a COX-1 selective inhibitor increased LTC $_4$  production upon calcium ionophore stimulation of the cells, while the effect was rather obscure in the cells treated with a COX-2 selective inhibitor (Fig. 4). Our result suggests that arachidonate shunt occurs preferentially for COX-1. This agrees with the safety of COX-2 selective inhibitors in aspirin-induced asthma patients [28], where an increased LTC $_4$  production by aspirin has been suggested to play a role in its pathology.

Independently of these mechanisms, we observed that LPS priming affected the ratio of PG production, which increased the PGE<sub>2</sub> ratio (Figs. 5B and C). It has been shown that LPS treatment of macrophages causes increase in microsomal PGE synthase (mPGES) expression levels, as well as upregulation of COX-2 levels [29,30]. In addition, a concerted action of mPGES and COX-2 has been reported [31].

Although it has been shown that the purified COX produces 11-HETE from arachidonic acid [19], in vivo property of 11-HETE production has not been described. In this paper, we described in vivo correlation of 11-HETE and PGE<sub>2</sub> (Figs. 5D and E). It has been reported that purified COX enzyme also produces 15-HETE in addition to 11-HETE [32]. However, we did not observe such a COX-linked production of 15-HETE, suggesting that COX may preferentially produce 11-HETE rather than 15-HETE in the peritoneal macrophages. These observations were not limited to a specific COX isotype, as they were inhibited by selective inhibitors for both COX-1 and COX-2 (Fig. 5A). The observation for 11-HETE also provides an insight into the mechanism of PGE<sub>2</sub> upregulation by LPS priming. The constant 11-HETE/PGE<sub>2</sub> ratios imply that the COX activity is the primary determinant of PGE<sub>2</sub> production in the macrophages. Although the contribution of mPGES upregulation has been suggested as described above, the impact seems less than that of COX. By contrast, changes observed in the ratio of 6-keto-PGF<sub>1 $\alpha$</sub>  and TxB<sub>2</sub> against 11-HETE after LPS priming suggest that production of these mediators is determined by specific terminal PG synthases, prostaglandin I synthase and thromboxane synthase, as well as COX. It is reasonable that productions of 6-keto-PGF<sub>1 $\alpha$</sub>  and TxB<sub>2</sub> are regulated independently by specific terminal PG synthases, since prostacyclin and thromboxane exert opposing biological actions with regard to vascular constriction and platelet aggregation [2].

In conclusion, we established a pathway-oriented profiling strategy for lipid mediators. We showed that multiplex quantitative data can be used for the correlation analysis of multiple lipid mediators, as well as kinetic analyses of individual lipid mediator. The present top-down strategy will serve as an important tool for understanding events that involve lipid mediators, especially when integrated with the existing genomics/proteomics strategy.

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