

# Thromboxane A synthase-independent production of 12-hydroxyheptadecatrienoic acid, a BLT2 ligand<sup>S</sup>

Takehiko Matsunobu,\* Toshiaki Okuno,<sup>1,\*†</sup> Chieko Yokoyama,<sup>§</sup> and Takehiko Yokomizo<sup>\*†</sup>

Department of Medical Biochemistry,\* Kyushu University, Fukuoka 812-8582, Japan; Department of Biochemistry,<sup>†</sup> Juntendo University School of Medicine, Tokyo 113-8421, Japan; and Department of Metabolism and Atherosclerosis,<sup>§</sup> Osaka University, Osaka 565-0871, Japan

**Abstract** 12(*S*)-hydroxyheptadeca-5*Z*,8*E*,10*E*-trienoic acid (12-HHT) has long been considered a by-product of thromboxane A<sub>2</sub> (TxA<sub>2</sub>) biosynthesis with no biological activity. Recently, we reported 12-HHT to be an endogenous ligand for BLT2, a low-affinity leukotriene B<sub>4</sub> receptor. To delineate the biosynthetic pathway of 12-HHT, we established a method that enables us to quantify various eicosanoids and 12-HHT using LC-MS/MS analysis. During blood coagulation, 12-HHT levels increased in a time-dependent manner and were relatively higher than those of TxB<sub>2</sub>, a stable metabolite of TxA<sub>2</sub>. TxB<sub>2</sub> production was almost completely inhibited by treatment with ozagrel, an inhibitor of TxA synthase (TxAS), while 12-HHT production was inhibited by 80–90%. Ozagrel-treated blood also exhibited accumulation of PGD<sub>2</sub> and PGE<sub>2</sub>, possibly resulting from the shunting of PGH<sub>2</sub> into synthetic pathways for these prostaglandins. In TxAS-deficient mice, TxB<sub>2</sub> production during blood coagulation was completely lost, but 12-HHT production was reduced by 80–85%. HEK293 cells transiently expressing TxAS together with cyclooxygenase (COX)-1 or COX-2 produced both TxB<sub>2</sub> and 12-HHT from arachidonic acid, while HEK293 cells expressing only COX-1 or COX-2 produced significant amounts of 12-HHT but no TxB<sub>2</sub>. These results clearly demonstrate that 12-HHT is produced by both TxAS-dependent and TxAS-independent pathways *in vitro* and *in vivo*.—Matsunobu, T., T. Okuno, C. Yokoyama, and T. Yokomizo. **Thromboxane A synthase-independent production of 12-hydroxyheptadecatrienoic acid, a BLT2 ligand.** *J. Lipid Res.* 2013. 54: 2979–2987.

**Supplementary key words** 12-HHT • cyclooxygenase • TxAS • mass spectrometry • eicosanoid

12(*S*)-hydroxyheptadeca-5*Z*,8*E*,10*E*-trienoic acid (12-HHT) was identified as a product of prostaglandin H<sub>2</sub>

(PGH<sub>2</sub>), which is biosynthesized from arachidonic acid (AA). Until recently, 12-HHT has been considered merely a by-product of thromboxane A<sub>2</sub> (TxA<sub>2</sub>) biosynthesis (1). The cleavage of PGH<sub>2</sub> into 12-HHT and malondialdehyde (MDA) is catalyzed by thromboxane A synthase (TxAS), the enzyme responsible for TxA<sub>2</sub> biosynthesis (2, 3). During aggregation of human and mouse platelets, 12-HHT and TxA<sub>2</sub> are produced in a 1:1 ratio in larger amounts than other prostaglandins (PG) (4–12). Nonenzymatic conversion of PGH<sub>2</sub> to 12-HHT *in vitro* has also been reported to occur via a process that involves glutathione and heme (13). Although PGs and TxA<sub>2</sub> mediate various physiological and pathophysiological roles in many tissues and cells through the activation of their specific G protein-coupled receptors (GPCR), the biological roles of 12-HHT have yet to be elucidated (1). In contrast, the biological roles of TxA<sub>2</sub> have been studied in detail. TxA<sub>2</sub> stimulates platelet aggregation and increases blood pressure by inducing the contraction of vascular smooth muscles through activation of the TxA<sub>2</sub>-specific GPCR, TP (14). For these reasons, ozagrel, an inhibitor of TxAS, is used clinically as a therapeutic drug to improve brain blood circulation after brain hemorrhage (15).

Recently, we discovered that 12-HHT functions as an endogenous ligand for BLT2, a low-affinity receptor for leukotriene B<sub>4</sub> (16, 17). Although the physiological functions of the 12-HHT/BLT2 axis *in vivo* remain unclear, we reported that BLT2 plays a protective role in murine inflammatory colitis (18). To understand the physiological functions of 12-HHT and BLT2, the molecular mechanism of 12-HHT biosynthesis needs to be clarified. In this report, we show that 12-HHT is produced by TxAS-dependent

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Abbreviations: AA, arachidonic acid; BLT2, leukotriene B<sub>4</sub> receptor 2; COX, cyclooxygenase; GPCR, G protein-coupled receptor; HEK, human embryonic kidney; 12-HHT, 12(*S*)-hydroxyheptadeca-5*Z*,8*E*,10*E*-trienoic acid; KO, knockout; MDA, malondialdehyde; PG, prostaglandin; TxA<sub>2</sub>, thromboxane A<sub>2</sub>; TxAS, thromboxane A synthase; TxB<sub>2</sub>, thromboxane B<sub>2</sub>; WT, wild-type.

<sup>1</sup>To whom correspondence should be addressed.

e-mail: tokuno@juntendo.ac.jp

<sup>S</sup>The online version of this article (available at <http://www.jlr.org>) contains supplementary data in the form of four figures.

and TxAS-independent pathways in vitro and in vivo using TxAS-deficient mice and various inhibitors of enzymes responsible for the biosynthesis 12-HHT.

## MATERIALS AND METHODS

### Materials

Arachidonic acid, 12-HHT, 12-HETE, LTB<sub>4</sub>, TxB<sub>2</sub>, PGD<sub>2</sub>, PGE<sub>2</sub>, PGH<sub>2</sub>, ozagrel, and rabbit anti-TxAS antibody (Ab) were purchased from Cayman Chemical (Ann Arbor, MI). Aspirin and thrombin were from Sigma. Goat anti-COX-1 (C-20) and goat anti-COX-2 (C-20) antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-goat IgG-HRP was from Jackson ImmunoResearch Laboratories (West Grove, PA).

### Mice

TxAS-knockout (KO) mice were generated using a strategy similar to that described previously for the generation of PGI<sub>2</sub> synthase-deficient mice (19). A clone of the murine TxAS gene was isolated from the 129SVJ murine genomic library in the Lambda FIX II vector (Stratagene, La Jolla, CA) with murine TxAS cDNA used as a probe. The targeting vector (supplementary Fig. IV) was designed to replace the *Xba*I-*Xho*I fragment, including exons 12 and 13, with a gene conferring neomycin resistance. *Kpn*I-*Xba*I fragment containing exon 11 was inserted into *Kpn*I/*Xba*I site between hsv-thymidine kinase and the neomycin-resistant genes of pPTN plasmid. A 7 kb fragment downstream of exon 13 was inserted at the *Xho*I-*Not*I site, which is upstream of the neomycin resistance gene. The targeting vector was transfected into the R1 embryonic stem cells by electroporation, and successful homologous recombination was confirmed by Southern blot and PCR analyses (data not shown). Positive clones were injected into eight cell-stage embryos of C57BL/6J mice and implanted into pseudopregnant ICR mice. Chimeric mice were generated and backcrossed eight times with C57BL/6J (Clea Japan Inc., Japan). All animal studies and procedures were approved by the Ethics Committees for Animal Experiments of Kyushu University.

### Preparation of human serum and washed platelets

Blood was collected from healthy volunteers who had been free of drugs for at least two weeks. Ethical approval was obtained from the Juntendo University Research Ethics Committee to use human blood. After incubation at 37°C for the indicated times, the blood was centrifuged at 5,000 *g* for 10 min to separate the serum fraction. A 50  $\mu$ l volume of serum was immediately mixed with 100  $\mu$ l of methanol containing 0.1% formic acid and a mixture of deuterium-labeled eicosanoids, which served as internal standards. To prepare washed platelets, 36 ml of human blood was collected into 4 ml of 3.2% sodium citrate. Platelet-rich plasma (PRP) was obtained by centrifugation at 200 *g* for 10 min. Platelets were isolated from PRP by centrifugation at 1,000 *g* for 10 min, washed twice with calcium-free HEPES-Tyrode's buffer (134 mM NaCl, 0.34 mM Na<sub>2</sub>HPO<sub>4</sub>, 2.9 mM KCl, 12 mM NaHCO<sub>3</sub>, 20 mM HEPES, 5 mM glucose, 1 mM MgCl<sub>2</sub>, pH 7.4), and resuspended in calcium-free HEPES-Tyrode's buffer.

### Production of 12-HHT and eicosanoids in thrombin-treated washed platelets

Washed human platelets ( $2 \times 10^5$  cells/100  $\mu$ l) in calcium-free HEPES-Tyrode's buffer were prewarmed for 5 min at 37°C. Reactions were initiated by the addition of 50  $\mu$ l of thrombin (0.5 NIH

unit/ml) in HEPES-Tyrode's buffer (134 mM NaCl, 0.34 mM Na<sub>2</sub>HPO<sub>4</sub>, 2.9 mM KCl, 12 mM NaHCO<sub>3</sub>, 20 mM HEPES, 5 mM glucose, 1 mM MgCl<sub>2</sub>, 1.8 mM CaCl<sub>2</sub>, pH 7.4), incubated at 37°C, and terminated by the addition of 150  $\mu$ l of ice-cold methanol containing 0.1% formic acid and a mixture of deuterium-labeled eicosanoids.

### RT-PCR

Isogen-LS (Nippon Gene) was used to extract total RNA from mouse whole blood as described by the manufacturer's instruction. Total RNA (1  $\mu$ g) was reverse-transcribed using Superscript II reverse transcriptase (Invitrogen). An equal quantity of cDNA was used to amplify TxAS and  $\beta$ -actin transcripts using the following conditions: 94°C for 15 s, 58°C for 30 s, and 68°C for 1 min for a total of 35 cycles (for TxAS), or 94°C for 15 s, 58°C for 30 s, and 68°C for 45 s for a total of 30 cycles (for  $\beta$ -actin). The sequences of the primers used are as follows: TxAS, 5'-GACCAGCAAAGCAGCAGAAGAGAG-3' (forward) and 5'-AGGTATGTGAACGGCCTCCG-3' (reverse);  $\beta$ -actin 5'-GTGGA-CCTCATGGCCTACAT-3' (forward) and 5'-GGGTGCAGCGAA-CTTATTG-3' (reverse). The polymerase chain reaction (PCR) products were analyzed by electrophoresis on a 2% agarose gel.

### Preparation of mouse serum

Mouse blood was collected from the caudal vena cava under urethane anesthesia and incubated at 37°C for 0, 1, 2, 3, and 5 min. After centrifugation at 5,000 *g* for 10 min at 4°C, the supernatant was recovered as serum. From this, 10  $\mu$ l was immediately mixed with 20  $\mu$ l of methanol containing 0.1% formic acid and a mixture of deuterium-labeled eicosanoids as internal standards.

### Plasmids

The cDNA of human COX-1 and COX-2 were a gift from Dr. Murakami (Tokyo Metropolitan Institute of Medical Science). The open-reading frame (ORF) of COX-1 was amplified by PCR using a sense primer (5'-AAGATATCATGAGCCGGAGTCTCTTG-3') and an antisense primer (5'-TTTCTCGAGTCAGAGCTCTGTGG-ATGG-3'), digested with *Eco*RV and *Xho*I, and subcloned into the *Eco*RV-*Xho*I site of pCXN2.1(+) (20). The ORF of COX-2 was amplified by PCR using a sense primer (5'-AAGGTACCA-TGCTCGCCCGCGC-3') and an antisense primer (5'-TTGAATTCTACAGTTCAGTCGAACGTTTC-3'), digested with *Kpn*I and *Eco*RI, and subcloned into the *Kpn*I-*Eco*RI site of pCXN2.1(+). The ORF of TxAS (21) was subcloned into the *Bam*HI-*Xba*I site in pcDNA3.1 Zeo(+). Entire sequences of the ORFs of these enzymes were confirmed by DNA sequencing.

### Cell culture and transfection

Human embryonic kidney (HEK)293 cells were cultured in Dulbecco's modified Eagle's medium (Wako, Osaka, Japan) containing 10% (v/v) fetal calf serum (Invitrogen), 100 IU/ml penicillin, and 100  $\mu$ g/ml streptomycin (Nacalai Tesque, Kyoto, Japan) at 37°C in 5% CO<sub>2</sub>. The cells were transfected with expression vectors using Lipofectamine LTX reagent (Invitrogen) according to the manufacturer's protocol.

### Western blotting analysis

HEK293 cells transfected with each expression vector were lysed with lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.25% sodium deoxycholate, 1% NP-40, 1 mM AEBSF, 0.8  $\mu$ M aprotinin, 15  $\mu$ M E-64, 20  $\mu$ M leupeptin, 50  $\mu$ M bestatin, and 10  $\mu$ M pepstatin A), and then centrifuged at 10,000 *g* for 10 min at 4°C. The supernatants were recovered, and protein concentrations were determined using a BCA assay kit (Nacalai Tesque). Proteins were diluted in SDS sample buffer (37.5 mM

Tris-HCl, 7.5% glycerol, 1.5% SDS, 1.5% 2-mercaptoethanol, and 0.075% bromophenol blue). Samples were then denatured for 5 min at 95°C, electrophoresed in 10% SDS-polyacrylamide gels, and transferred to PVDF membranes. After blocking with 1% skim milk in Tris-buffered saline (TBS), the membranes were incubated with primary antibodies against COX-1, COX-2, or TxAS (1:250–1:1000 dilution in TBS), and then with horseradish peroxidase-conjugated secondary antibodies (1:5000 dilution). After washing with TBS-T, the membranes were visualized with a chemiluminescence reagent kit (PerkinElmer) and an LAS-4000 mini-imaging system (Fujifilm).

### Production of 12-HHT and eicosanoids in HEK293 cells

HEK293 cells were seeded at  $1 \times 10^6$  cells/well onto a 6-well plate 24 h before transfection with 2.5  $\mu$ g of plasmid DNA. After 48 h, the cells were washed with Hank's balanced salt solution (HBSS)-HEPES (20 mM, pH 7.4) and treated with 10  $\mu$ M arachidonic acid in 150  $\mu$ l of HBSS-HEPES at 37°C. The reactions were terminated by the addition of 300  $\mu$ l of methanol containing 0.1% formic acid and a mixture of deuterium-labeled eicosanoids as internal standards.

### Production of 12-HHT and eicosanoids in homogenate of HEK293 cells

HEK293 cells ( $2 \times 10^6$ ) transfected with/without TxAS were suspended in 200  $\mu$ l of 0.1 M Tris-HCl, pH 7.4, and then sonicated for 15 min. Heat inactivation was carried out at 95°C for 5 min. After a 5 min preincubation at 25°C, 1  $\mu$ M PGH<sub>2</sub> (200 pmol dissolved in 1  $\mu$ l of acetone) was added to the cell homogenates to initiate the reaction. The reaction was carried out at 25°C and terminated by the addition of 800  $\mu$ l of methanol containing 0.1% formic acid. A 200  $\mu$ l aliquot was analyzed by LC-MS/MS.

### Quantification of 12-HHT and eicosanoid levels

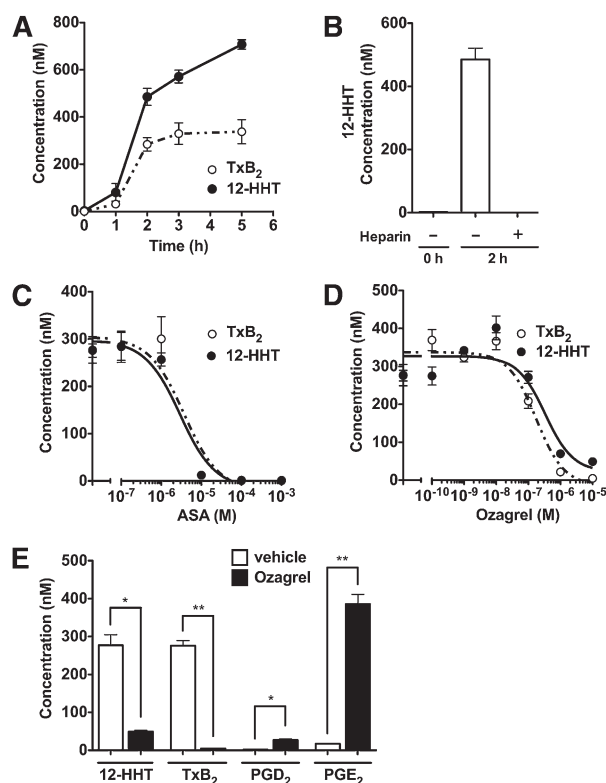
Samples were diluted with water to yield a final methanol concentration of 20% and then loaded on Oasis HLB cartridges (Waters). The column was sequentially washed with water containing 0.1% formic acid, 15% methanol containing 0.1% formic acid, and petroleum ether containing 0.1% formic acid. The samples were eluted with 200  $\mu$ l of methanol containing 0.1% formic acid. Each sample was analyzed by LC-MS/MS as described previously (22). For LC-MS/MS analysis, a Shimadzu liquid chromatography system consisting of four LC-20AD pumps, a SIL-20AC autosampler, a CTO-20AC column oven, a FCV-12AH six-port switching valve, and a TSQ Quantum Ultra triple quadrupole mass spectrometer equipped with an electrospray ionization (ESI) ion source (Thermo Fisher Scientific) were used. An aliquot of each sample (50  $\mu$ l) was injected into the trap column, an Opti-Guard Mini C18, at a total flow rate of 500  $\mu$ l/min. Three minutes after sample injection, the valve was switched to introduce the trapped sample to the analytical column, a Capcell Pak C18 MGS3 (Shiseido, Tokyo, Japan). Separation of lipids was achieved by a step gradient using mobile phase A and mobile phase B at ratios of 64:36 (0–6 min), 55:45 (6–7 min), and 35:65 (7–13 min). The compositions of mobile phases A and B were water:formic acid (100:0.1) and MeCN:formic acid (100:0.1), respectively. The total flow rate was 120  $\mu$ l/min, the column temperature was set at 46°C, and the LC column eluent was introduced directly into a TSQ Quantum Ultra. All compounds were analyzed in a negative ion polarity mode. 12-HHT and eicosanoids were quantified by selective reaction monitoring (SRM). The SRM transitions monitored were  $m/z$  279  $\rightarrow$  179 for 12-HHT,  $m/z$  369  $\rightarrow$  195 for TxB<sub>2</sub>, and  $m/z$  351  $\rightarrow$  271 for PGE<sub>2</sub> and PGD<sub>2</sub>. For accurate quantification, a mixture of deuterium-labeled eicosanoids was used as the internal standard. As deuterium-labeled 12-HHT was not available, 12-HHT

was calibrated using deuterium-labeled LTB<sub>4</sub>. Automated peak detection, calibration, and calculation were carried out by the Xcalibur 1.2 software package.

## RESULTS

### Production of 12-HHT and TxB<sub>2</sub> during blood coagulation

Previous studies have shown that 12-HHT is abundantly produced during blood coagulation (23, 24). To confirm this, we collected human blood from three healthy volunteers and measured 12-HHT and eicosanoid levels during blood coagulation (Fig. 1, supplementary Figs. I and II). Representative data is shown in Fig. 1. The concentration of 12-HHT in the serum increased in a time-dependent



**Fig. 1.** 12-HHT production during human blood coagulation is resistant to TxAS inhibition. **A:** Production of 12-HHT and TxB<sub>2</sub> during human blood coagulation. Human blood from a healthy volunteer-1 was incubated at 37°C for the indicated periods, and then the amounts of 12-HHT and TxB<sub>2</sub> in the serum were quantified by LC-MS/MS. Data represent the means  $\pm$  SE (n = 4). **B:** Effects of heparin on the production of 12-HHT. Human blood was incubated with or without heparin at 37°C for the indicated periods and 12-HHT levels were quantified. Data represent the means  $\pm$  SE (n = 3). **C and D:** Effects of aspirin (C) or the TxAS inhibitor ozagrel (D) on the production of 12-HHT (filled circles) and TxB<sub>2</sub> (open circles) during human blood coagulation. Human blood was incubated with or without inhibitors at 37°C for 2 h, and then 12-HHT and TxB<sub>2</sub> levels were quantified. Data represent the means  $\pm$  SE (n = 4). **E:** Effect of the TxAS inhibitor ozagrel (10  $\mu$ M) on the production of 12-HHT, TxB<sub>2</sub>, PGD<sub>2</sub>, and PGE<sub>2</sub> during human blood coagulation. Data represent the means  $\pm$  SE (n = 4). \**P* < 0.05; \*\**P* < 0.01; \*\*\**P* < 0.001 (unpaired *t*-test).



manner during blood coagulation and was higher than that of  $\text{TxB}_2$ , a stable metabolite of  $\text{TxA}_2$  in two volunteers (Fig. 1A, supplementary Fig. II-A). One volunteer showed similar levels of production of 12-HHT and  $\text{TxB}_2$  (supplementary Fig. I-A). We next examined the effect of the anticoagulant heparin on 12-HHT production. Pretreatment of human blood with heparin almost completely inhibited 12-HHT production in all three volunteers (Fig. 1B, supplementary Figs. I-B and II-B). We also examined the involvement of COX and TxAS in 12-HHT production during blood coagulation. Pretreatment of human whole blood with aspirin, a COX inhibitor, inhibited 12-HHT and  $\text{TxB}_2$  production in a dose-dependent manner, with a concentration of 10  $\mu\text{M}$  aspirin inducing almost complete inhibition of 12-HHT and  $\text{TxB}_2$  production (Fig. 1C). Pretreatment of blood with ozagrel, an inhibitor of TxAS, also inhibited 12-HHT and  $\text{TxB}_2$  production in a dose-dependent manner. Although 10  $\mu\text{M}$  ozagrel almost completely inhibited  $\text{TxB}_2$  production, 12-HHT production was only partially inhibited (Fig. 1D, E). Ozagrel treatment at 10  $\mu\text{M}$  also increased the production of  $\text{PGD}_2$  and  $\text{PGE}_2$  (Fig. 1E). The effects of heparin, aspirin, and ozagrel were similarly observed among three volunteers (Fig. 1, supplementary Figs. I and II).

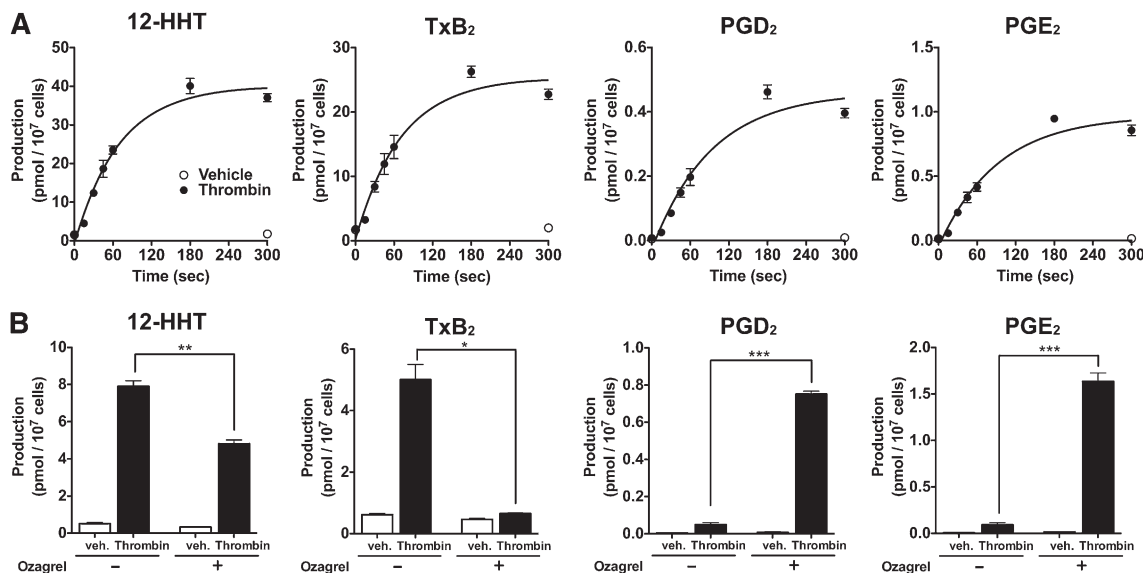
### Production of 12-HHT and $\text{TxB}_2$ in human platelets

Next, we examined thrombin-stimulated production of 12-HHT and eicosanoids in washed human platelets from two volunteers with similar results (Fig. 2, supplementary Fig. III). Expression of COX-1 and TxAS in human platelets was confirmed by Western blotting (data not shown). Thrombin treatment stimulated 12-HHT production in a time-dependent manner, and the level of 12-HHT (40 pmol/ $10^7$  platelets) was higher than that of  $\text{TxB}_2$

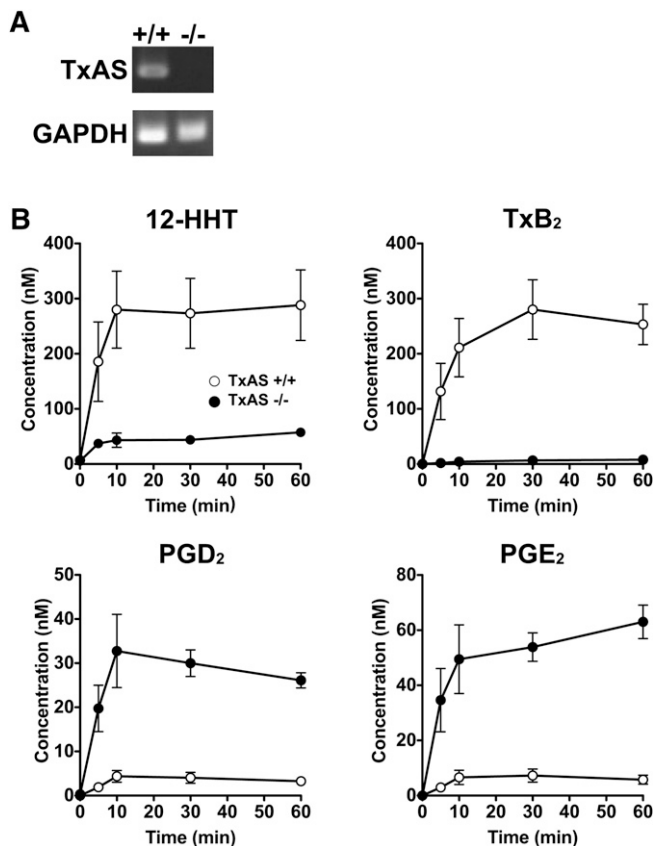
(25 pmol/ $10^7$  platelets) at 300 s after stimulation (Fig. 2A). Thrombin stimulation also resulted in  $\text{PGD}_2$  and  $\text{PGE}_2$  production, although the amounts of  $\text{PGD}_2$  and  $\text{PGE}_2$  were much lower than those of 12-HHT and  $\text{TxB}_2$  (Fig. 2A). Treatment of human platelets with 10  $\mu\text{M}$  ozagrel almost completely inhibited thrombin-induced  $\text{TxB}_2$  production, and 12-HHT production was inhibited by 40–60% (Fig. 2B, supplementary Fig. III-B). Conversely, production of  $\text{PGD}_2$  and  $\text{PGE}_2$  was markedly increased by ozagrel treatment. These results suggest that two biosynthetic pathways of 12-HHT production, TxAS-dependent and TxAS-independent, are present in human platelets and that ozagrel treatment increases  $\text{PGD}_2$  and  $\text{PGE}_2$  production by shunting  $\text{PGH}_2$  to the  $\text{PGD}_2$  and  $\text{PGE}_2$  synthetic pathways.

### 12-HHT production is partially inhibited by TxAS deficiency

We next measured the production of 12-HHT and eicosanoids during blood coagulation in TxAS-deficient mice. The lack of TxAS expression in  $\text{TxAS}^{-/-}$  mice was first confirmed by RT-PCR analysis of whole blood cells (Fig. 3A). Next, whole blood was collected from wild-type (WT) and TxAS-deficient mice, and 12-HHT and eicosanoid levels were measured in the serum. In WT mouse serum, the concentrations of 12-HHT and  $\text{TxB}_2$  increased in a time-dependent manner and reached maximum concentrations of 286 and 268 nM, respectively, after 10 min of incubation (Fig. 3B). In the serum from  $\text{TxAS}^{-/-}$  mice, 50 nM 12-HHT was detected after 60 min of incubation, while no  $\text{TxB}_2$  was detected (Fig. 3B). In addition,  $\text{PGE}_2$  and  $\text{PGD}_2$  levels in the serum of  $\text{TxAS}^{-/-}$  mice were significantly higher than in the serum of WT mice. These results indicate that 12-HHT is produced during blood coagulation in mice in manner similar to that in humans (Fig. 1A,



**Fig. 2.** 12-HHT production in human platelets is resistant to TxAS inhibition. A: Production of 12-HHT,  $\text{TxB}_2$ ,  $\text{PGD}_2$ , and  $\text{PGE}_2$  in human platelets (volunteer-4) stimulated with thrombin. Washed human platelets were stimulated with thrombin (0.5 NIH unit/ml) for the indicated periods at 37°C, and then 12-HHT and PG levels were quantified. Data represent the means  $\pm$  SE (n = 3). B: Effect of the TxAS inhibitor ozagrel on the production of 12-HHT,  $\text{TxB}_2$ ,  $\text{PGD}_2$ , and  $\text{PGE}_2$ . Washed human platelets pretreated with or without ozagrel were stimulated with 1 NIH unit of thrombin for 45 s at 37°C, and then 12-HHT and PG levels were quantified. Data represent the means  $\pm$  SE (n = 3). \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$  (unpaired *t* test).



**Fig. 3.** Effect of TxAS deficiency on 12-HHT production during mouse blood coagulation. **A:** Lack of TxAS transcripts in whole blood cells of TxAS<sup>-/-</sup> mice was confirmed by RT-PCR. Beta-actin served as a positive control. **B:** The effect of TxAS deficiency on the production of 12-HHT, TxB<sub>2</sub>, and PGE<sub>2</sub>. Blood from WT (open circle) and TxAS<sup>-/-</sup> (filled circle) mice was incubated for the indicated periods at 37°C, and then 12-HHT and PG levels were quantified. Data represent the means ± SE (n = 5).

supplementary Figs. I-A and II-A). These results also show that significant amounts of 12-HHT are biosynthesized in the blood even in the absence of TxAS, and they suggest that accumulated PGH<sub>2</sub> caused by TxAS deficiency is used for the production of PGD<sub>2</sub> and PGE<sub>2</sub>.

### Involvement of COX-1 and TxAS

To examine the contribution of TxAS and COXs to the production of 12-HHT, we measured 12-HHT and eicosanoid levels after arachidonic acid treatment of HEK293 cells transfected with the expression vectors for COX-1 or COX-2 with or without TxAS. HEK293 cells do not intrinsically express COX-1, COX-2, or TxAS; proper expression of each enzyme in transfected HEK293 cells was confirmed by Western blotting (Fig. 4A). 12-HHT production was observed in COX-1-expressing cells with or without TxAS after the addition of 10 μM arachidonic acid (Fig. 4B, 12-HHT), while TxB<sub>2</sub> production was observed only in cells expressing both COX-1 and TxAS (Fig. 4B, TxB<sub>2</sub>). PGE<sub>2</sub> production was obvious in cells expressing COX-1 alone, and coexpression of COX-1 and TxAS abolished PGE<sub>2</sub> production (Fig. 4B, PGE<sub>2</sub>). A significant amount of 12-HETE production was observed, which

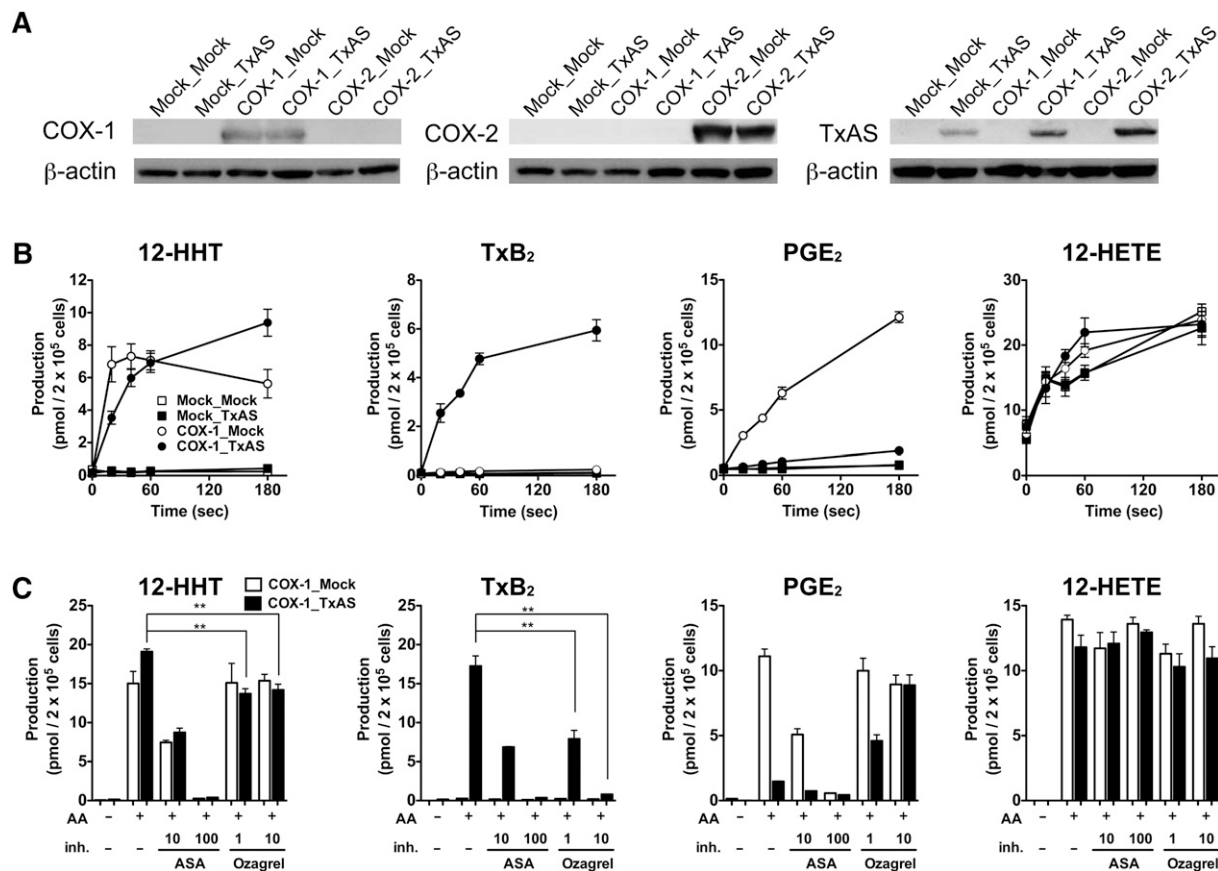
was not affected by expression of COX-1, COX-1, or TxAS (Fig. 4B, 12-HETE). Recently, Isobe et al. reported that HEK293 cells produced a significant amount of 12-HETE after incubation with 10 μM arachidonic acid (25), suggesting that excess arachidonic acid is converted to 12-HETE in HEK293 cells, possibly by endogenous 12-lipoxygenase. Figure 4 suggests that TxAS is dispensable for 12-HHT production but indispensable for TxB<sub>2</sub> production in COX-1-expressing cells. To confirm TxAS-independent production of 12-HHT, HEK293 cells were pretreated with ozagrel before the addition of arachidonic acid. In cells expressing both COX-1 and TxAS, pretreatment with 10 μM ozagrel only partially inhibited 12-HHT production, while TxB<sub>2</sub> production was completely inhibited (Fig. 4C). In addition, 12-HHT production was not affected by pretreatment with ozagrel in cells expressing only COX-1 (Fig. 4C, 12-HHT). These results show that TxAS catalyzes the production of 12-HHT from PGH<sub>2</sub> produced by COX-1 and that 12-HHT is produced by TxAS-dependent and TxAS-independent pathways.

### Involvement of COX-2 and TxAS

We performed similar experiments using COX-2 and TxAS. 12-HHT production was observed in COX-2-expressing cells with or without TxAS after the addition of 10 μM arachidonic acid (Fig. 5A, 12-HHT). Interestingly, 12-HHT production in cells expressing COX-2 alone was higher than in cells expressing both COX-2 and TxAS (Fig. 5A, 12-HHT). TxB<sub>2</sub> production was observed only in cells cotransfected with COX-2 and TxAS (Fig. 5A, TxB<sub>2</sub>), and PGE<sub>2</sub> production was detected only in cells expressing COX-2 alone (Fig. 5A, PGE<sub>2</sub>). These results also suggest that TxAS is not required for 12-HHT production but is vital for TxB<sub>2</sub> production in COX-2-expressing cells, as is the case for COX-1-expressing cells. To confirm TxAS-independent production of 12-HHT, HEK293 cells were pretreated with ozagrel. In cells expressing both COX-2 and TxAS, pretreatment with 10 μM ozagrel did not inhibit 12-HHT production but completely inhibited TxB<sub>2</sub> production (Fig. 5B). Pretreatment with ozagrel also increased PGE<sub>2</sub> production, suggesting that accumulated PGH<sub>2</sub> was used for the production of PGE<sub>2</sub> (Figs. 4B and 5B). 12-HETE production was not affected by the pretreatment with ozagrel, suggesting that the 12-lipoxygenase pathway is independent of the COX pathway. These results indicate that TxAS is indispensable for TxA<sub>2</sub> production but not for 12-HHT production.

### TxAS-independent production of 12-HHT from PGH<sub>2</sub>

To confirm TxAS-independent 12-HHT production, we next examined the conversion of PGH<sub>2</sub> into TxB<sub>2</sub> and 12-HHT using a heat-denatured homogenate of HEK293 cells (Fig. 6). Incubation of PGH<sub>2</sub> with a homogenate of HEK293 cells that had been transfected with TxAS resulted in the accumulation of TxB<sub>2</sub>; this effect was completely inhibited by heat-denaturing the homogenate (Fig. 6, TxB<sub>2</sub>). In addition, homogenate of mock-transfected cells did not convert PGH<sub>2</sub> into TxA<sub>2</sub>. These results show that intact TxAS is required for the conversion of PGH<sub>2</sub> to TxA<sub>2</sub>. By contrast, apparent conversion of PGH<sub>2</sub> into 12-HHT was observed in the homogenate of mock-transfected cells; this



**Fig. 4.** Coupling of COX-1 and TxAS induces 12-HHT production. **A:** Western blotting of COX-1, COX-2, and TxAS. HEK293 cells transfected with the expression vectors for these enzymes were lysed and analyzed by Western blotting. **B:** Production of 12-HHT, TxB<sub>2</sub>, PGE<sub>2</sub>, and 12-HETE from arachidonic acid in HEK293 cells. The cells were incubated with 10 μM arachidonic acid at 37°C for the indicated periods, and then 12-HHT and eicosanoid levels were quantified. Data represent the means ± SE (n = 3). **C:** Effects of aspirin and ozagrel on the production of 12-HHT, TxB<sub>2</sub>, PGE<sub>2</sub>, and 12-HETE. HEK293 cells pretreated with aspirin (ASA) or ozagrel were incubated with 10 μM arachidonic acid for 20 s at 37°C, and then 12-HHT and eicosanoid levels were quantified. Data represent the means ± SE (n = 3). \*\**P* < 0.01 (unpaired *t*-test).

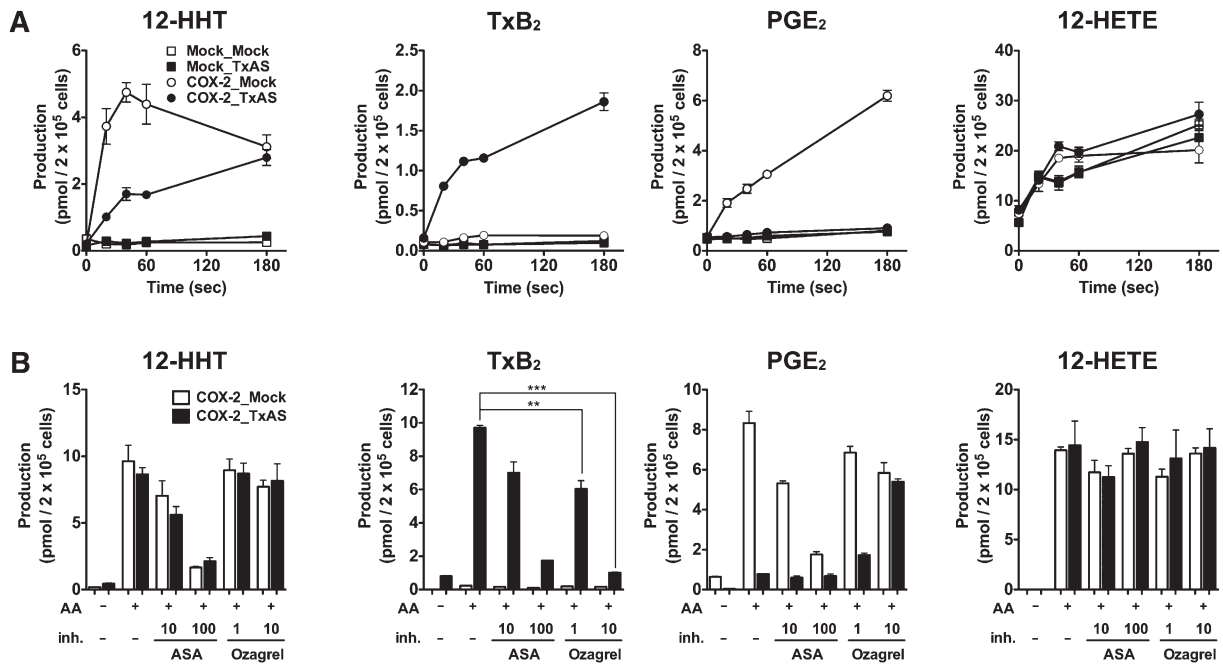
effect was not decreased by heat treatment (Fig. 6, 12-HHT). Conversion of PGH<sub>2</sub> into 12-HHT was greater in the homogenate of TxAS-expressing cells than mock cells, and this increment was sensitive to heat denaturation. Conversion of PGH<sub>2</sub> into PGE<sub>2</sub> and PGD<sub>2</sub> was also observed in mock cells, and this reaction was not sensitive to heat treatment (Fig. 6, PGD<sub>2</sub> and PGE<sub>2</sub>). The conversion of PGH<sub>2</sub> into PGE<sub>2</sub> and PGD<sub>2</sub> was decreased in TxAS-expressing cells and was increased by heat treatment to a level similar to that observed in mock-transfected cells. These results clearly show that intact TxAS is required for the conversion of PGH<sub>2</sub> into TxA<sub>2</sub>, whereas the conversion of PGH<sub>2</sub> into 12-HHT, PGD<sub>2</sub>, and PGE<sub>2</sub> is possibly nonenzymatically catalyzed. The reduced conversion of PGH<sub>2</sub> into PGD<sub>2</sub> and PGE<sub>2</sub> in TxAS-transfected cells can be explained by the rapid enzymatic conversion of PGH<sub>2</sub> into TxA<sub>2</sub> by intact TxAS.

## DISCUSSION

12-HHT was first identified by Hamberg and Samuelsson as an enzymatic product of arachidonic acid metabolism downstream of COXs (26). TxAS simultaneously catalyzes

the conversion of PGH<sub>2</sub> to TxA<sub>2</sub>, 12-HHT, and MDA in an equimolar ratio. TxA<sub>2</sub> potentially stimulates platelet aggregation and vascular constriction through activating the TP receptor, a member of the GPCR family (27). TxA<sub>2</sub> has also been shown to play a major role in thrombosis, vasoconstriction, proliferation of vascular smooth muscle cells, and immune regulation (28). However, the physiological roles and biosynthetic pathway of 12-HHT remain elusive.

In this report, we show that 12-HHT is produced by both TxAS-dependent and TxAS-independent pathways *in vitro* and *in vivo* using LC-MS/MS. Until now, the nonenzymatic conversion of 12-HHT from PGH<sub>2</sub> by heme or reduced glutathione had been reported only in an *in vitro* cell-free system (13, 29). Yu et al. reported the successful establishment of TxAS-deficient mice with normal thrombopoiesis and lymphocyte differentiation (30), and they examined 12-HHT production by washed platelets stimulated with [<sup>14</sup>C] arachidonic acid using thin-layer chromatography (TLC). Although they reported that the amount of 12-HHT in TxAS<sup>-/-</sup> platelets was reduced but not completely lost, they did not address this issue in detail. In this report, we used LC-MS/MS

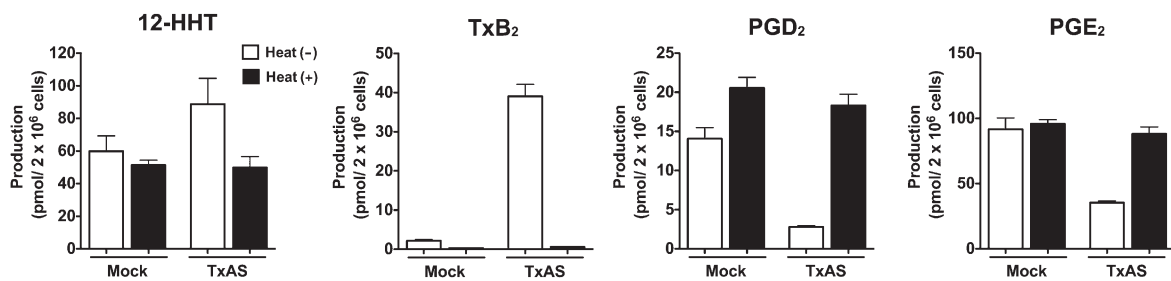


**Fig. 5.** Coupling of COX-2 and TxAS induces 12-HHT production. A: Production of 12-HHT, TxB<sub>2</sub>, PGE<sub>2</sub>, and 12-HETE in HEK293 cells incubated with arachidonic acid. The cells were incubated with 10  $\mu$ M arachidonic acid for the indicated periods at 37°C, and then 12-HHT and eicosanoid levels were quantified. Data represent the means  $\pm$  SE (n = 3). B: Effects of aspirin and ozagrel on the production of 12-HHT, TxB<sub>2</sub>, PGE<sub>2</sub>, and 12-HETE. HEK293 cells pretreated with aspirin (ASA) or ozagrel were incubated with 10  $\mu$ M arachidonic acid for 20 s at 37°C, and then 12-HHT and eicosanoid levels were quantified. Data represent the means  $\pm$  SE (n = 3). \*\**P* < 0.01; \*\*\**P* < 0.001 (unpaired *t*-test).

to quantify simultaneously the levels of 12-HHT and various eicosanoids using TxAS-deficient mice. During blood coagulation, 12-HHT levels reached concentrations of 500–900 nM (human blood) and 300 nM (mouse blood), which are high enough to activate endogenous BLT2, a GPCR for 12-HHT (16). In the blood of volunteer-1 and volunteer-3, the levels of 12-HHT were higher than those of TxB<sub>2</sub> (Fig. 1A, supplementary Fig. II-A), and the level of 12-HHT was comparable to TxB<sub>2</sub> in volunteer-2 (supplementary Fig. I-A). These individual differences might be due to the different expression levels of TxAS in platelets. In fact, volunteer-2's blood produced greater amounts of TxB<sub>2</sub> and 12-HHT (supplementary Fig. I-A) than did the blood of volunteer-1 and volunteer-3 (Fig. 1A, supplementary Fig. II-A), and the individual differences of TxB<sub>2</sub> and 12-HHT generation require future analysis. Although we did not confirm

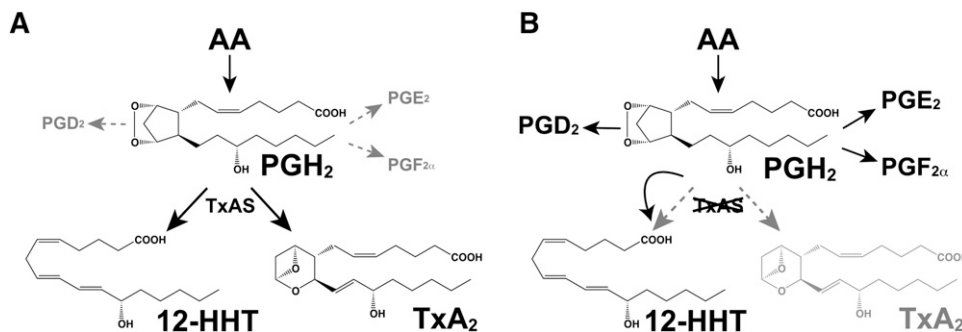
the chirality of 12-HHT in our study, it is reasonable to assume that nonenzymatically generated 12-HHT is 12(S)-HHT, based on the chirality of PGH<sub>2</sub>. Our results are important, because this is the first report to quantitatively show TxAS-independent 12-HHT production using TxAS-deficient mice. Recently, Bui et al. reported that cytochrome P450 protein CYP2S1 is able to metabolize PGG<sub>2</sub> and PGH<sub>2</sub> to 12-HHT (31). Further study is required to identify the other 12-HHT-producing enzymes.

The other important finding of this study is the possible functional coupling of COX and TxAS enzymes. TxAS deficiency or ozagrel treatment caused a compensatory increase in PGD<sub>2</sub> and PGE<sub>2</sub> levels (Figs. 3B, 4B and C, and 5). These results strongly suggest that in the absence or inhibition of TxAS, PGH<sub>2</sub> produced from AA by COX is shunted into PGE<sub>2</sub> and PGD<sub>2</sub>, possibly through nonenzymatic pathways. This is supported by the findings that



**Fig. 6.** TxAS-independent production of 12-HHT. PGH<sub>2</sub> (1  $\mu$ M) was incubated at 25°C for 2 min with homogenates of HEK293 cells that had been heat-denatured at 95°C for 5 min or untreated, and then 12-HHT and eicosanoid levels were quantified. Data represent the means  $\pm$  SE (n = 4).





**Fig. 7.** TxAS-dependent and TxAS-independent 12-HHT production. A: Enzymatic production of TxAS<sub>2</sub> and 12-HHT by TxAS. Arachidonic acid (AA) is converted into PGH<sub>2</sub> by COX-1 and COX-2. TxAS simultaneously catalyzes the conversion of PGH<sub>2</sub> to TxAS<sub>2</sub> and 12-HHT. B: TxAS-independent production of 12-HHT from PGH<sub>2</sub>. In the presence of inhibition or deficiency of TxAS, 12-HHT is produced from PGH<sub>2</sub> while TxAS<sub>2</sub> production is completely lost. Inhibition or deficiency of TxAS results in the production of PGD<sub>2</sub>, PGE<sub>2</sub>, and PGF<sub>2α</sub> by shunting PGH<sub>2</sub> from the TxAS<sub>2</sub> synthetic pathway.

PGH<sub>2</sub> is rapidly converted to 12-HHT, PGD<sub>2</sub>, and PGE<sub>2</sub> through synthetic pathways in aqueous solutions (data not shown) and that the expression levels of other PG-producing enzymes are low in platelets and HEK cells.

The contribution of TxAS for 12-HHT generation seems to be greater in whole blood (Figs. 1 and 2) and platelets (Fig. 3) than in COXs and TxAS-transfected HEK cells (Figs. 4 and 5), possibly because TxAS expression is much higher in platelets than in transfected HEK cells. The other possibility is different coupling of COXs and TxAS in platelets and HEK cells. In platelets, most PGH<sub>2</sub> generated by COX-1 is used for TxAS<sub>2</sub> and 12-HHT generation in a TxAS-dependent manner. If COXs and TxAS in transiently transfected HEK cells are weakly coupled, PGH<sub>2</sub> generated by COXs will be easily and nonenzymatically converted to 12-HHT independently of TxAS. The 12-HHT generation in HEK cells expressing only COXs is faster than in cells expressing both COXs and TxAS (Figs. 4B and 5A), possibly because nonenzymatic conversion of 12-HHT is faster than TxAS-dependent generation of 12-HHT. In addition to the homogenates of TxAS-transfected heat(-) cells, the homogenates of mock cells and TxAS-transfected heat(+) cells generate a lot of 12-HHT (Fig. 6), possibly by nonenzymatic generation of 12-HHT. TxAS-transfected heat(+) cells generate more PGD<sub>2</sub> and PGE<sub>2</sub> than do TxAS-transfected heat(-) cells (Fig. 6). In TxAS-transfected heat(+) cells, PGH<sub>2</sub> could not be converted to TxAS<sub>2</sub>, and the accumulated PGH<sub>2</sub> would nonenzymatically be converted to PGD<sub>2</sub> and PGE<sub>2</sub>.

The use of TxAS inhibitors as antithrombotic agents offers an advantage over aspirin in that they redirect arachidonic metabolism toward PGI<sub>2</sub> and other protective eicosanoids. However, the therapeutic effects of these agents have been disappointing (32), partly because inhibition of TxAS also results in accumulation of PGH<sub>2</sub>, which can activate TP receptors at higher concentrations (30). Although dual inhibition of TxAS and TP may be a potential treatment option (33), the unexpected production of PGs observed in this study might evoke some side effects associated with TxAS inhibitors, including headache and fever. Lipidomics analysis using LC-MS/MS will be a

useful tool to monitor the changes in metabolic pathways induced by inhibitors of enzymes of the arachidonic cascade.

In summary (Fig. 7), we demonstrated that 12-HHT is produced by TxAS-independent pathway in addition to a TxAS-dependent pathway both in vitro and in vivo. Serum from TxAS<sup>-/-</sup> mice contained significant amounts of 12-HHT. PGE<sub>2</sub> and PGD<sub>2</sub> production in TxAS<sup>-/-</sup> mouse serum was significantly higher than that in WT mouse, suggesting that PGH<sub>2</sub> is shunted into the synthetic pathways for PGE<sub>2</sub> and PGD<sub>2</sub>. We used LC-MS/MS to quantify 12-HHT and eicosanoid levels and revealed the novel shunting of PGH<sub>2</sub> from the TxAS<sub>2</sub> synthetic pathway to PG synthetic pathways following the inhibition of TxAS. **FIG 7**

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