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Role of E-type prostaglandin receptor EP3 in the vasoconstrictor activity evoked by prostacyclin in thromboxane-prostanoid receptor deficient mice

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Prostacyclin, also termed as prostaglandin I₂ (PGI₂), evokes contraction in vessels with limited expression of the prostacyclin receptor. Although the thromboxane-prostanoid receptor (TP) is proposed to mediate such a response of PGI₂, other unknown receptor(s) might also be involved. TP knockout (TP^{-/-}) mice were thus designed and used to test the hypothesis. Vessels, which normally show contraction to PGI₂, were isolated for functional and biochemical analyses. Here, we showed that the contractile response evoked by PGI₂ was indeed only partially abolished in the abdominal aorta of TP^{-/-} mice. Interestingly, further antagonizing the E-type prostaglandin receptor EP3 removed the remaining contractile activity, resulting in relaxation evoked by PGI₂ in such vessels of TP^{-/-} mice. These results suggest that EP3 along with TP contributes to vasoconstrictor responses evoked by PGI₂, and hence imply a novel mechanism for endothelial cyclooxygenase metabolites (which consist mainly of PGI₂) in regulating vascular functions.

Cyclooxygenase (COX), which exists mainly as COX-1 and -2 isoforms, mediates the metabolism of arachidonic acid (AA) to produce vasoactive prostanoids^{1–4}. Among them, thromboxane (Tx) A₂ and prostacyclin (prostaglandin I₂; PGI₂) have been considered to represent two opposing regulatory mechanisms in the cardiovascular system. TxA₂ is mainly produced in platelets and it acts on the thromboxane-prostanoid receptor (TP) to mediate vasoconstriction and platelet-aggregation. In contrast, PGI₂ is mainly synthesized in the vascular endothelium and is proposed to activate the PGI₂ receptor (IP) that mediates vasodilatation and opposes the effects of TP. An imbalance between the effects derived from endothelial PGI₂ and those of platelet-produced TxA₂ is thought to result in the development of cardiovascular disorders, such as hypertension^{1–6}.

On the other hand, in some vascular beds (including certain human vessels), PGI₂ or endothelial COX metabolites (which consist mainly of PGI₂) evoke contraction via the activation of TP^{7–24}. Studies have further revealed that vasomotor reactions to PGI₂ are modulated by both IP and TP; hence a vasoconstrictor response evoked by PGI₂ or endothelial COX metabolites reflects limited expression or function of IP, which leads to the uncovering of vasoconstrictor activity derived from concurrently activated TP^{8,20–22,25–32}. However, in some vessels, such as mouse abdominal aorta where IP is expressed (although to a lesser extent as compared to vessels showing dilation to the agonist), PGI₂ does not evoke relaxation even after TP blockade^{28,30}. Also, in some vascular beds, the contraction evoked by PGI₂ or endothelial COX metabolites is less sensitive to TP blockade^{11,22}. We propose that in addition to TP, other receptor(s) can also be involved in PGI₂-evoked vasoconstrictor activity. However, the existence of such a mechanism or the identity of the additional receptor(s) remains to be elucidated. In addition, the involvement of TP in the vasoconstrictor activity of PGI₂ has been primarily based on results with pharmacological blockade, which also inhibits contractions evoked by other PGs or AA-related metabolites^{8,33,34}. Thus, it would also be of interest to evaluate the precise role of TP in PGI₂-evoked vasoconstrictor responses with genetic manipulation.

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Figure 1. Mutation in TP^{-/-} mice and phenotype. (a) mutation in the TP locus. Top: WT DNA sequencing showing the surrounding sequences and the fragment to be deleted (underlined) in TP^{-/-} mice. Bottom left: sequencing of mutated DNA showing the deletion of 22 bp DNA fragment in TP^{-/-} mice. The bar separates the upper and down stream sequences of the deleted fragment. Bottom right: partial sequences of TP mRNA transcripts or those of proteins to be translated in WT (upper) and TP^{-/-} mice (lower). (b) RT-PCR showing the expressions of un-mutated mRNAs in WT and TP^{-/-} mouse aortas. Bands were visualized with a SYBR Safe DNA gel stain (Thermo Scientific) and the image was captured by an electrophoresis imaging cabinet (Universal Hood II; Bio-rad, Hercules, CA, USA). M: 100 bp ladder size marker (Thermo Scientific). (c) bleeding time in TP^{-/-} and WT mice. Values are expressed as mean ± SEM; n = 5; **P < 0.01.

To resolve the above issues, in this study we generated a strain of TP^{-/-} mice on a C57BL/6 background. Aortas, carotid and/or renal arteries, where PGI₂ evokes vasoconstrictor response under normal conditions^{26,28,30,35}, were isolated for biochemical and/or functional analyses.

Results

Mutation in TP^{-/-} mice and phenotype. As shown in Fig. 1a, sequencing of TP DNA PCR products revealed that as compared with that of wild-type (WT) mice, exon 3 of the TP locus in TP^{-/-} mice has a 22 bp fragment deletion (CTG GGG GCC TGC TTT CGC CCG G) in the coding area, which was 18 bp after the start codon (NCBI Reference Sequence: NM_009325.3). This resulted in a frame-shift in TP mRNA transcript and a premature termination of protein translation (only 7 amino acids were coded before the appearance of a stop codon (TGA) in TP^{-/-} mice; Fig. 1a, bottom right). Indeed, RT-PCR revealed that un-mutated TP mRNAs, which were abundant in WT aortas, were not detected in the TP^{-/-} counterparts (Fig. 1b). Also, compared to WT controls, TP^{-/-} mice had an elongated bleeding time (Fig. 1c). However, these mice appear normal, and show no overt abnormality in mean arterial blood pressure (MAP; 92.3 ± 3.3 vs. 95.0 ± 2.8 mmHg in WT mice, n = 5 for each; P > 0.05) or in reproduction.

Figure 2. Responses to U46619 and PGs in L-NAME-treated WT and $TP^{-/-}$ abdominal aortas. (a,b) comparison of contractions evoked by the TP agonist U46619 (a), and PGI_2 or $PGF_{2\alpha}$ (b) in WT and $TP^{-/-}$ vessels. (c,d) contraction to PGE_2 (c) or PGD_2 (d) in WT or $TP^{-/-}$ mice and that of $TP^{-/-}$ vessels treated with the TP antagonist SQ29548 (10 μ M; +SQ) or the EP3 antagonist L798106 (1 μ M; +L). Values are expressed as mean $\pm$ SEM; $n = 5$ for each. ** $P < 0.01$ vs. the value of WT mice; +++ $P < 0.01$ vs. $TP^{-/-}$ mice.

Effect of $TP^{-/-}$ on contractions evoked by PGI_2 and other prostanoids. Abdominal aortas were then isolated for functional analyses. Vessels were treated with the NO synthase (NOS) inhibitor N^w -nitro-L-arginine methyl ester (L-NAME; 1 mM). In WT vessels, the TP agonist U46619 evoked potent contraction as noted previously²⁸; however, in $TP^{-/-}$ mice, U46619 did not evoke any response (Fig. 2a). Interestingly, not only the contraction evoked by PGI_2 (Fig. 2b), but that to $PGF_{2\alpha}$ (Fig. 2b), PGE_2 (Fig. 2c), or PGD_2 (Fig. 2d) was also diminished or largely removed in $TP^{-/-}$ vessels. At the same time, the contraction to low concentrations (0.1–0.3 μ M) of PGE_2 remained intact in $TP^{-/-}$ vessels, and this contraction was abolished by the E-type prostaglandin receptor EP3 antagonist L798106 (1 μ M), but not by the TP antagonist SQ29548 (10 μ M; Fig. 2c). In addition, L798106 abolished the remaining contraction evoked by PGD_2 in $TP^{-/-}$ vessels (Fig. 2d).

PGI_2 -induced response in $TP^{-/-}$ abdominal aortas precontracted with PE. Next, we determined whether PGI_2 -evoked contractile response in $TP^{-/-}$ vessels was masked by a dilator effect of the agonist. To this end, L-NAME-treated or endothelium-denuded abdominal aorta were precontracted with phenylephrine (PE; 2 μ M), under which the vasoconstrictor response to an agonist is more readily detectable compared to baseline conditions²⁸. Under either condition, PGI_2 (1 μ M) evoked an increase of force on PE-induced contraction, which was however reversed by the EP3 antagonist L798106 (1 μ M) into relaxation (Fig. 3a,b). In contrast, the EP1 antagonist SC19220 (10 μ M) had no effect (Fig. 3a). Also, L798106, but not SC19220 inhibited a similar response evoked by PGE_2 (0.1 μ M), although no relaxation was observed (Fig. 3c,d). Meanwhile, forces of PE-evoked contractions were found to be comparable among vessel groups that had been treated with L-NAME (Fig. 3a,c bottom panels).

Figure 3. Effect of EP3 antagonism on the response to PGI₂ or PGE₂ in PE-pre-contracted TP^{-/-} abdominal aortas. (a) summaries (n = 5 for each) of responses (top) to 1 μ M PGI₂ and forces of PE-evoked contractions (pre-force; bottom) in control L-NAME-treated TP^{-/-} vessels or those additionally with the EP3 antagonist L798106 (1 μ M; +L) or the EP1 antagonist SC19220 (10 μ M; +SC). (b) representative traces with summarized values showing the control response to PGI₂ in endothelium-denuded TP^{-/-} vessels [TP^{-/-}/EC(-)] or that with L798106 (+L). * P < 0.05. (c) summaries (n = 5 for each) of responses (top) to 0.1 μ M PGE₂ and forces of PE-evoked contractions (bottom) as in (a). ** P < 0.01 vs. control value of TP^{-/-} vessels. In (a–c), * P < 0.05 or ** P < 0.01 vs. control value of TP^{-/-} vessels. Data are expressed as mean $\pm$ SEM. (d) representative traces showing the response to PGE₂ in L-NAME-treated TP^{-/-} vessels (TP^{-/-}) or that obtained with L798106 (+L).

Effect of EP3 antagonism on ACh-evoked contraction in TP^{-/-} or TP-inhibited abdominal aortas.

In the mouse abdominal aorta, the muscarinic agonist acetylcholine (ACh) activates endothelial COX to mainly produce PGI₂ and evoke contraction under NOS-inhibited conditions^{8,28,35}. Therefore, responses evoked by the maximal concentration of ACh were examined in L-NAME-treated TP^{-/-} or TP-inhibited abdominal aortas^{8,13–15}.

As compared to that of WT controls, the contraction evoked by ACh (10 μ M) in TP^{-/-} vessels was indeed mostly abolished; however, a minor contraction could still be produced (Fig. 4a). Moreover, in such-treated TP^{-/-} vessels precontracted with PE (2 μ M), ACh evoked relaxation, which was blunted by a biphasic force sensitive to the non-selective COX inhibitor indomethacin. Interestingly, the EP3 antagonist L798106 also abolished the biphasic force, resulting in relaxation, which was to a greater extent than that obtained with indomethacin (Fig. 4b,c). In addition, such an enhancement of relaxation resulting from L798106 was removed by the IP antagonist CAY10441 (1 μ M; Fig. 4c).

Likewise, in similar PE-precontracted WT vessels where the agonist usually evokes an increase of force²⁸, treatment with the TP antagonist SQ29548 (10 μ M) caused relaxation that was also blunted by a biphasic force in response to ACh. Again, the EP3 antagonist L798106 (1 μ M) abolished the force, resulting in an enhanced relaxation that was reduced by indomethacin (Fig. 4d top). No significant difference was found among forces of PE-evoked contractions in TP^{-/-} or WT vessels (Fig. 4c,d bottom panels).

Figure 4. Responses to ACh in L-NAME-treated $TP^{-/-}$ abdominal aortas or those of WT mice with TP inhibited. (a) representative traces with summarized values showing responses to ACh ($10\ \mu\text{M}$) under baseline conditions in WT (top) and $TP^{-/-}$ (bottom) vessels. $P < 0.01$ vs. WT vessels (b,c) representative traces (b) and/or summaries of time-courses of responses to ACh (c top) along with forces of PE-evoked contractions (pre-force; c bottom) in precontracted $TP^{-/-}$ vessels or those obtained with the non-selective COX inhibitor indomethacin ($10\ \mu\text{M}$; +IND), with the EP3 antagonist L798106 ($1\ \mu\text{M}$; +L) or with both L798106 and the IP antagonist CAY10441 ($1\ \mu\text{M}$; +L/CAY). (d) time-courses of responses to ACh (top) and forces of PE-evoked contractions (pre-force; bottom) in precontracted WT vessels in the presence of the TP antagonist SQ29548 (WT/SQ) or in those additionally treated with L798106 ($1\ \mu\text{M}$; +L) or both L798106 and indomethacin (+L/IND). In (c and d) $**P < 0.01$ vs. $TP^{-/-}$ or WT/SQ; $++P < 0.01$ vs. $TP^{-/-}$ /L or +L. Data were expressed as mean $\pm$ SEM ($n = 5$ for each).

Effect of $TP^{-/-}$ on endothelial COX products and expressions of PG receptors. The production of PGI_2 and some other prostanoids evoked by ACh in $TP^{-/-}$ and WT aortas was then examined. As shown in Fig. 5a, in WT and $TP^{-/-}$ aortas ACh evoked an increase in the production of the PGI_2 metabolite 6-keto- $\text{PGF}_{1\alpha}$, which was comparable between the two mouse strains. Also, an increase of PGE_2 was obtained with ACh, although levels were ~ 10 -fold lower than those of 6-keto- $\text{PGF}_{1\alpha}$ (Fig. 5b). No significant difference was found in amounts of PGE_2 between $TP^{-/-}$ and WT vessels (Fig. 5b). In contrast, the TxA_2 metabolite TxB_2 was not increased by ACh in vessels from either mouse strain (Fig. 5c), similar to results reported previously^{34,36}.

The expressions of IP, EP3 and the $\text{PGF}_{2\alpha}$ receptor (FP) mRNAs were also determined. As shown by real-time PCR, the level of IP, EP3 or FP mRNAs normalized by that of β -actin in $TP^{-/-}$ aortas was comparable with that of WT counterparts (Fig. 5d).

Effect of EP3 antagonism on varied vasoconstrictor responses in WT vessels. The effect of EP3 antagonism was further determined in L-NAME-treated WT vessels. As shown in Fig. 6a, in WT abdominal aortas the EP3 antagonist L798106 ($1\ \mu\text{M}$) diminished the contraction evoked by PGI_2 . Also, in such vessels precontracted with PE ($2\ \mu\text{M}$), PGI_2 ($1\ \mu\text{M}$) or the COX substrate AA ($3\ \mu\text{M}$), whose response is sensitive to TP antagonism under baseline conditions³⁵, evoked an increase of force in the presence of the TP antagonist

Figure 5. Effect of TP^{-/-} on COX products and IP, EP3 and FP mRNA levels. (a–c) summaries of the PGI₂ metabolite 6-keto-PGF_{1α} (a), PGE₂ (b), and the TxA₂ metabolite TxB₂ (c) in TP^{-/-} and WT aortas under the basal and ACh (10 μM)-stimulated conditions. (d) real-time PCR detection of IP, EP3 and FP mRNAs in TP^{-/-} and WT aortas. The level of mRNAs was normalized by that of β-actin with the average value of WT assumed as 1.0. **P* < 0.05 and ***P* < 0.01; NS: not significant. Data are expressed as mean ± SEM (n = 6 for each).

SQ29548 (10 μM) but a relaxation that was sensitive to the IP antagonist CAY10441 (1 μM) when both SQ29548 and L798106 were present (Fig. 6b). No significant difference was found among forces of PE-evoked contractions (Fig. 6b bottom).

Interestingly, in WT abdominal aortas, L798106, which drastically diminished the contraction evoked by ACh, only slightly reduced that evoked by a sub-maximal concentration of PGE₂ (10 μM; Fig. 6c). However, this contraction to PGE₂ was very sensitive to the TP antagonist SQ29548 (Fig. 6c). In addition, L798106 diminished the contraction evoked by 1 or 10 μM PGI₂ in carotid and renal arteries (Fig. 6d).

Discussion

In this study we show that in NOS-inhibited WT mouse abdominal aortas PGI₂- or ACh (which activates endothelial COX to mainly produce PGI₂) evokes contraction that is diminished in TP^{-/-} counterparts. More importantly, in TP^{-/-} vessels or TP-inhibited vessels of WT mice, antagonizing EP3 abolishes the remaining vasoconstrictor responses to these agonists, resulting in relaxations sensitive to IP and/or COX blockade. EP3 antagonism also diminishes the contraction evoked by PGI₂ and/or ACh in NOS-inhibited WT abdominal aorta, carotid and renal arteries. These results not only demonstrate that TP contributes only partially to the contraction evoked by PGI₂, but also suggest that EP3 has an important involvement in the response.

The deletion of TP in TP^{-/-} mice was confirmed by DNA sequencing, mRNA analyses and an elongation of bleeding time³⁷. Indeed, abdominal aortas from these mice (which possess a normal MAP, as reported previously³⁸) lost contraction in response to the TxA₂ analogue and TP agonist U46619 even under NOS-inhibited conditions. Notably, in such vessels, not only the contraction to PGI₂, but also that to PGF_{2α}, PGE₂, or PGD₂ was diminished. In contrast, levels of IP, FP and EP3 mRNAs were similar between TP^{-/-} and WT vessels, arguing against that the above reduced PG responses resulted from altered expressions of receptors. Thus, TP, which appears able to be activated by all vasoactive prostanoids that are structurally similar⁴, mediates PGI₂'s contractile activity. Due to practical reasons, we were unable to clearly detect IP, EP3, and FP at the protein level; however, our above idea concurs with results in WT mice and some other species obtained here or previously with pharmacological blockade^{8,28,34}. At the same time, responses evoked by low concentrations (0.1–0.3 μM) of PGE₂ in TP^{-/-} vessels reveal a functional role of EP3 unaffected by the TP antagonist used.

Interestingly, we further noted that antagonizing EP3 abolished the remaining contraction, resulting in relaxation in response to PGI₂ in either NOS-inhibited or endothelium-denuded TP^{-/-} abdominal aortas. This suggests that EP3, which exists in medial smooth muscles in a manner similar to that of IP and TP, mediates

Figure 6. Effects of EP3 antagonism on vasoconstrictor responses in L-NAME-treated WT vessels.

(a) control (CTL) responses evoked by PGI₂ under baseline conditions in the abdominal aorta (AAo), and that obtained with the EP3 antagonist L798106 (1 μM; +L) (b) responses (top) to PGI₂ (1 μM) or AA (3 μM) and forces of PE-evoked contractions (pre-force; bottom) in pre-contracted AAO treated with the TP antagonist SQ29548 (10 μM; WT/SQ) or those additionally with L798106 (1 μM; +SQ/L) or both L798106 and the IP antagonist CAY10441 (1 μM; +SQ/L/CAY). **or ***P < 0.01 vs. WT/SQ or +SQ/L, respectively. (c) effect of L798106 (1 μM; +L) or SQ29548 (10 μM; +SQ) on the contraction to PGE₂ (10 μM; PGE₂) or ACh (10 μM) in AAO. (d) effect of L798106 (1 μM; +L) on contraction to 1 or 10 μM PGI₂ in carotid (CA) and renal arteries (CA), respectively. In (a,c and d) *P < 0.05, and **P < 0.01 vs. WT control (WT/CTL). Data are expressed as mean ± SEM (n = 5 for each).

PGI₂'s contractile response, although its effect is largely offset by IP when TP is absent. In support of the idea, in NOS-inhibited, TP-antagonized WT vessels a relaxation sensitive to IP antagonism was also evoked by PGI₂ following EP3 blockade. Moreover, after EP3 antagonism the contraction to PGI₂ was minimal. This suggests that the part of EP3-mediated activity could be only slightly smaller than that of TP, which alone could also be largely masked by IP-mediated effect. Therefore, the robust contractile response to PGI₂ in WT vessels reflects activities from both TP and EP3 overcoming the effect of concomitantly activated IP. In contrast, EP1 (another vasoconstrictor PGE₂ receptor), EP2 and EP4 (dilator PGE₂ receptors) do not appear to have a role in the vessels studied, as suggested by the lack of effect caused by antagonism or the absence of relaxation to PGE₂ in TP^{-/-} vessels even after EP3 is antagonized^{39,40}.

Also, the above effects of TP^{-/-}, TP and/or EP3 antagonism under NOS-inhibited conditions were similarly obtained in responses evoked by ACh or AA, which stimulates endothelial COX to mainly produce PGI₂^{28,35}. As seen from EIA measurements, the profile of COX-derived products in aortas was unaltered by TP^{-/-}. Thus, the mechanism for the contraction evoked by endothelial COX metabolites produced *in situ* is similar to that of PGI₂. Due to an endothelium-derived hyperpolarizing factor (EDHF)-mediated relaxation concomitantly activated^{28,41}, the effect of EP3 antagonism on the response evoked by ACh in NOS-inhibited, TP^{-/-} or TP-inhibited vessels was reflected by abolition of the contractile activity blunting EDHF-mediated relaxation and a relaxation that is sensitive to IP or COX blockade, but adds to that of EDHF. One must note that the EDHF activity in the vessel does not originate from non-COX AA metabolites, as we put forward previously³⁵. Indeed, this point is also supported here by the lack of IP-independent relaxation to AA after TP and EP3 were both antagonized.

Previously, the contractile role of EP3 in PGE₂-evoked vasoresponse was established in vessels of mice as well as those of humans^{32,42}. In the present study, our results further suggest an intimate link between EP3 and the contractile activity evoked by PGI₂. Notably, PGD₂ might also act on EP3 to mediate a minor contraction, as revealed by functional studies of its response in TP^{-/-} vessels. These results could again be possibly due to a structural similarity among PGs. In support of this, iloprost, a PGI₂ analog, also activates EP3⁴³. Moreover, EP3 antagonism exerts a greater inhibitory effect on PGI₂-evoked contraction than on that of PGE₂ (whose response via EP3 peaks at 0.3 μM, as seen from its response in TP^{-/-} vessels). This implies not only that the EP3 antagonist used has limited if any, unintended effects on TP, but also that PGI₂, although it might have a lower potency, is more effective on EP3 than PGE₂, underscoring the importance of PGI₂ in EP3-mediated vasoconstrictor activities. Besides, the effects of its antagonism among WT vessels studied further indicate that the involvement of EP3 in PGI₂'s vasoconstrictor activity is not limited to any specific vascular bed.

Therefore, our above results make important amendments to previous proposals on the mechanisms of PGI₂ or endothelial COX metabolites (which consist mainly of PGI₂) in mediating vasoconstrictor responses^{20,44}. It should be noted that the contraction to PGI₂ only exists in vessels with limited expression of IP⁴⁴. This is also true in the abdominal aorta examined here where we previously showed that IP expression is lower than in mesenteric arteries (where 0.3 μM PGI₂ almost completely relaxes 2 μM PE-evoked contraction)²⁸. A reason for this could be that PGI₂ (the prototype IP agonist) is more potent on IP than TP and/or EP3, leading to PGI₂ being more likely to evoke relaxation than to cause contraction. Indeed, this idea explains why PGI₂ has been recognized as a potent vasodilator in many vascular beds and used clinically as an effective therapy for pulmonary hypertension or peripheral arterial diseases^{2,6,45,46}.

On the other hand, it must also be emphasized that the minimal concentration of PGI₂ required to initiate vasoconstrictor activity could be 0.003–0.03 μM (under precontracted conditions), far below the amount (1 ng/mg 6-keto-PGF_{1α} can be translated into 2.7 μmol PGI₂ per kg of vessel) released by agonists, such as ACh, or similar to that of it (PGI₂) to evoke relaxation in vessels, such as mouse mesenteric arteries even after TP is antagonized^{28,30,44}. Also, PGI₂-mediated contraction or endothelial COX-derived vasoconstrictor activity has been found in many vessels across species (including those of humans), of which some are small or resistance arteries^{23,24,29,34,47}. Moreover, PGI₂'s contractile activity exists in vessels that show a dilator response to the agonist^{27,32}. As a result, although PGI₂ may cause a hypotensive effect in general, concurrent activities via TP and/or EP3 can negate some of its beneficial effects via IP, especially on local vascular pathology under disease conditions^{22,26,36}. For this reason, antagonizing TP and/or EP3 might be needed for an optimal therapeutic effect obtained with PGI₂ or its analogues under clinical conditions.

In contrast to our findings, EP1 antagonism has also been suggested to diminish PGI₂-evoked contraction⁴⁸. However, the EP1 antagonist used was also a partial antagonist of TP, which was deleted in the vessels we studied⁴⁹, not to mention the variation that might exist among species or vascular beds. Also, the COX inhibitor indomethacin may cause off-target effects^{50,51}; however, this agent has been shown not to alter ACh responses in similar vessels of COX-1^{-/-} mice²⁸. Indeed, IP blockade inhibited the relaxation in a manner similar to that of indomethacin. Thus, the effect of indomethacin noted here can be considered to result mainly from COX inhibition. However, the precise structural properties responsible for different PGs to activate the same receptor or for one PG to act on different receptors still require further investigation. Also, reasons for one PG to evoke contraction mainly through receptors other than its own, e.g. FP of PGF_{2α} need to be resolved, given that contractions evoked by endothelial COX metabolites can result from non-PGI₂ products, including PGF_{2α}^{19,20,34,52,53}.

In summary, our results demonstrate that TP, which appears able to be activated by all vasoactive prostanoids, only partially mediates PGI₂'s vasoconstrictor activity. Interestingly, our data further suggest that PGI₂ also effectively activates EP3, whose activity along with that of TP can overcome the dilator effect of concomitantly activated IP to produce a robust vasoconstrictor response, and hence imply a novel mechanism for endothelial COX metabolites (which consist mainly of PGI₂) in regulating vascular functions.

Material and Methods

Chemicals and solution. L-NAME, ACh, PE, AA, and the non-selective COX inhibitor indomethacin were purchased from Sigma (St Louis, MO, USA). The TP agonist U46619, PGI₂, PGF_{2α}, PGE₂, and PGD₂, the TP antagonist SQ29548, the IP antagonist CAY10441, the EP3 antagonist L798106, and the EP1 antagonist, SC19220 were bought from Cayman Chemical (Ann Arbor, MI, USA). The composition of physiological salt solution (PSS; pH 7.4 with 95% O₂–5% CO₂) was as follows (in mM): NaCl 123, KCl 4.7, NaHCO₃ 15.5, KH₂PO₄ 1.2, MgCl₂ 1.2, CaCl₂ 1.25, and D-glucose 11.5. The 60 mM K⁺-PSS (K⁺) was prepared by replacing an equal molar of NaCl with KCl.

L-NAME, PE, AA, and ACh were dissolved in distilled water (purged with N₂ for dissolving AA), while PGI₂ was dissolved in carbonate buffer (50 mM, pH 10.0). PGF_{2α}, PGE₂, PGD₂, CAY10441, SQ29548, L798106, and indomethacin were dissolved in dimethyl sulfoxide (DMSO). The final ratio of a solvent (distilled water, carbonate buffer, or DMSO) to working PSS was 0.5/1,000, which doesn't alter the final pH value of the working buffer (pH 7.4). The concentration of an inhibitor or antagonist used was based on previous reports, which would selectively inhibit the effect of its intended target^{27,54,55}.

Animals and tissue preparation. All procedures were in conformance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996), and approved by The Institutional Animal Research and Use Committee of Shantou University.

The breeder male and female TP^{-/-} mice (C57BL/6 background) were custom produced by Viewsolid Biotech (Beijing, China), using transcription activator-like effector nuclease technology that targets the TP gene to result in deletion of a 22 bp DNA fragment, 18 bp after the start codon in exon 3 of the TP locus⁵⁶. WT mice (C57BL/6) were purchased from Vital River (Beijing, China). Male WT mice or TP^{-/-} progenies of 8–12 wk of age were used

for experiments. Mice were killed by CO₂ inhalation. For *in vitro* functional and biochemical analyses, aortas, carotid and/or renal arteries were isolated and dissected free of adherent tissues with the help of a binocular microscope.

DNA sequencing. The gene mutation in TP^{-/-} mice was verified by sequencing PCR products (the sense and anti-sense PCR primers were 5'-GAA AGG GTA TTT TGT TCC TGA GGC-3' and 5'-GCT ACC CCC ATG AAG TAG CAC AGG-3', respectively) of DNA isolated from tail biopsies and performed by Sangon Biotech (Shanghai, China).

RT-PCR and real-time PCR. The preparation of total RNA from whole sections of mouse aortas and RT reactions were performed as described elsewhere previously²⁸. First-strand cDNA was synthesized using total RNA (250 ng) and oligo(dT)15 primers (TaKaRa; Dalian, China).

TP mRNA transcripts were detected with RT-PCR. Primers for TP were 5'-CTG GGG GCC TGC TTT CGC CCG G-3' (sense; using the deleted fragment) and 5'-GTC AGG AAG CAC CAA GAG CC-3' (antisense), while those for β -actin (internal control) were as described previously²⁸. The expected sizes of the RT-PCR products were 530 bp for TP and 300 bp for β -actin.

Expressions of IP, EP3 or FP mRNAs were analyzed by real-time PCR. Primers for EP3 or FP were as follows: 5'-CAG AAT CAC CAC GGA GAC G-3' (EP3 sense) and 5'-TGC ATT GCT CAA CCG ACA T-3' (EP3 antisense), and 5'-TCC TTG GAC ACC GAG ATT AT-3' (FP sense) and 5'-GCA ACG ACT GGC AAG TTT AT-3' (FP antisense). Those for IP and β -actin (internal control) were described previously²⁸. Real-time PCR was performed using a SYBR PrimScript RT-PCR kit (Thermo Scientific, Carlsbad, CA, USA).

Blood pressure measurement. In some experiments, blood pressure in mice (body weight of 26–30 g) was measured using a computerized noninvasive blood pressure system (Kent Scientific Corporation, Torrington, CT, USA). Mice were accustomed to tail-cuff blood pressure measurements for 3 consecutive days, and then blood pressure was measured on the 4th day. MAP taken from the averaged value of three measurements was used for analysis.

Tail bleeding time assay. To evaluate *in vivo* bleeding time, WT and TP^{-/-} tails (age, tail size and length matched) were cut 2 mm from the tips, and wounds were then gently wiped with sterilized filter paper every 30 s, until no more blood was visible. The bleeding time was calculated from the ending of cutting to the time when no more blood would be seen on paper.

Analysis of vascular function. Abdominal sections of aortas and main stems of carotid or renal arteries were cut into 1 mm rings. Analysis of vascular function was performed with isometric force measurement as described elsewhere previously^{28,30}. For some experiments, the endothelium was denuded by rotating vessel rings around two wires with passive tension kept at 100 mg (endothelial removal was confirmed by absence of relaxation to 10 μ M ACh at the end of experiment).

To remove the influence of endothelial NO, vessels were treated with the NOS inhibitor L-NAME (1 mM), under which the response of arteries appears similar to that of eNOS^{-/-} mice¹⁴. Inhibitors or solvents were added 30 min before the vessel was contracted with an agonist, and was kept in the solution throughout the experiment. The response elicited by an agonist under baseline conditions was expressed relative to the contraction evoked by 60 mM K⁺, while that during the contraction evoked by PE (2 μ M) was expressed as a change of force relative to the value before the application of the agent.

Assay of COX-derived metabolites. Measurement of the PGI₂ metabolite 6-keto-PGF_{1 α} , the TxA₂ metabolite TxB₂, or PGE₂ was performed by EIA^{28,36}. Briefly, after being rinsed of blood components, whole sections of aortas were incubated with PSS at 37 °C for 30 min, followed by exposure to PSS (300 μ l) and ACh (10 μ M) in 300 μ l PSS (37 °C) for 15 min each. Thereafter, vessels were taken out, and 1, 10, or 100 μ l of reaction solutions was used for 6-keto-PGF_{1 α} , PGE₂, or TxB₂ measurements, respectively (2 replicates for each single measurement), using protocols according to instructions of the manufacturer. The amount of 6-keto-PGF_{1 α} , TxB₂, or PGE₂ was expressed in ng per mg of wet tissue.

Data analysis. Values were expressed as means $\pm$ SEM from n numbers or pools of vessels from different animals. The normality of data sets with n of 5 or more was confirmed using the Kolmogorov-Smirnov test. Thereafter, statistical analyses were performed with a Student's t-test (unpaired) or ANOVA (1-way or 2-way), followed by Bonferroni's or Dunnett's post-hoc test. For some data sets with undeterminable normality (n = 3), the Mann-Whitney U test was used. *P* < 0.05 was considered to be statistically significant.

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Author Contributions

Y. Zhou and B.L. conceived and designed the study; Z.L., Y. Zhang, B.L., W.L. and H.L. performed the experiments. Y. Zhou and B.L. analyzed and interpreted data. Y. Zhou wrote the manuscript. Y. Zhou and B.L. provided financial support for the project. All authors reviewed the manuscript.

Additional Information

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