Published in final edited form as:

Prostaglandins Other Lipid Mediat. 2009 November ; 90(1-2): 31–36. doi:10.1016/j.prostaglandins.2009.07.003.

Inhibition of prostaglandin E₂ signaling through the EP₁ receptor does not affect prostacyclin production in human endothelial cells

Tatsuya Kaneshiro^a, Masae Okumura^a, Samer Maalouf^a, Andre Uflacker^a, Takayuki Maruyama^b, and Toshihiko Kawamori^{a,*}

^a Department of Pathology & Laboratory Medicine, Medical University of South Carolina, Charleston, SC 29425 USA

^b Minase Research Institute, Ono Pharmaceutical Company, Osaka, Japan

Abstract

Accumulating evidence suggests that cyclooxygenase-2 (COX-2) and prostaglandin E₂ (PGE₂) may play an important role in colon carcinogenesis. Thus, blockage of this pathway may be a suitable strategy for colon cancer chemoprevention. Recent clinical studies suggest that COX-2 inhibitors cause adverse cardiovascular effects due to prostacyclin (PGI₂) inhibition. To test our hypothesis that inhibition of PGE₂ signaling through E-prostanoid (EP) receptors may offer a safer cardiovascular profile than COX-2 inhibition, we analyzed expression of 6-keto PGF_{1α}, a hydrated form of PGI₂ and PGI₂ synthase, which was stimulated with cytokines in human umbilical vein endothelial cells (HUVECs) treated with the EP₁ receptor antagonist ONO-8711 or the COX-2 inhibitor celecoxib. ONO-8711 did not inhibit both 6-keto PGF_{1α} production and PGIS expression, whereas celecoxib did in HUVECs. ONO-8711 also inhibited cytokine-induced tissue factor expression in HUVECs. These results suggest that ONO-8711 may be a safer chemopreventive agent with respect to cardiovascular events.

Keywords

EP₁ receptor antagonist; PGE₂; COX-2; colon cancer chemoprevention; PGIS; PGI₂; tissue factor; HUVECs

1. Introduction

Cancer continues to be a major health concern in developed nations despite aggressive screening guidelines for early detection and detailed knowledge of critical events underlying cancer pathogenesis. Colorectal cancer is the 2nd leading cause of all cancer-related deaths, annually causing ~56,000 deaths in the US alone [1]. Cancer chemoprevention—pharmacological intervention to arrest or reverse cancer development—is considered one of the most effective and economic strategies to reduce colorectal cancer prevalence and mortality [2]. The arachidonic acid cascade, especially cyclooxygenases (COXs), which are rate-limiting

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*Correspondence should be addressed to Toshihiko Kawamori, MD, PhD, Department of Pathology & Laboratory Medicine, 165 Ashley Avenue, Suite 309, Charleston, SC 29425 USA, Phone: 1-843-792-4171; Fax: 1-843-792-0368; Kawamori@musc.edu.

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enzymes of prostaglandin (PG) synthesis, and PGE₂, a major cascade metabolite, have been identified as potential targets for colorectal cancer chemoprevention [3]. Inhibition of the inducible form of COX, COX-2, reduces colon carcinogenesis [4] and exogenous PGE₂ enhances colon carcinogenesis [5]. However, recent studies suggest that selective COX-2 inhibitors may increase the risk of myocardial infarction and atherothrombotic events [6,7].

PGE₂ exerts physiological functions by binding to its E-prostanoid (EP) receptor subtypes 1-4 in target organs. Our recent results demonstrate the following: a) EP₁ receptor knockout (KO) mice have significantly fewer azoxymethane (AOM)-induced preneoplastic lesions, aberrant crypt foci (ACF) [8], and colon cancer development [9]; and b) ONO-8711, a selective EP₁ receptor antagonist, significantly reduces AOM-induced ACF formation and intestinal polyp formation in Apc^{Min} (Min) mice [8]. These results lead us to hypothesize that ONO-8711 may be a promising colon cancer chemopreventive agent.

In addition to the colon, ONO-8711 inhibits tumor development in several other sites. For example, ONO-8711 inhibits breast cancer induced by 2-amino-1-methyl-6-phenylimidazo [4,5-*b*]pyridine in female rats [10] and tongue cancer induced by 4-nitroquinoline 1-oxide in male rats [11]. Han *et al.* also reported that ONO-8711 effectively inhibits PGE₂-induced epidermal growth factor receptor (EGFR) phosphorylation followed by enhanced invasiveness of human hepatocellular carcinoma cells [12]. These findings suggest that an EP₁ receptor antagonist may be an excellent chemopreventive agent for multiple organs.

In terms of vascular function and thrombosis, the most relevant PGs are prostacyclin (PGI₂) and thromboxane A₂ (TxA₂). Unwanted vascular side effects caused by COX-2 inhibitors are due to a reduction in antithrombotic product, PGI₂, which is not accompanied by changes in the prothrombotic product, TxA₂. PGE₂ signaling through the EP₁ receptor is ideally independent to PGI₂ synthesis. Therefore, we hypothesize that inhibition of PGE₂ signaling through the EP₁ receptor may not affect PGI₂ production in endothelial cells. To test our hypothesis, we analyzed effects of ONO-8711 on production of 6-keto PGF_{1α}, a hydrated form of PGI₂, and PGI₂ synthase (PGIS) expression and compared these results to the effects of the selective COX-2 inhibitor, celecoxib, in human umbilical vein endothelial cells (HUVECs). We also analyzed whether PGE₂ signaling through the EP₁ receptor is involved in expression of tissue factor (TF), a primary regulator of blood coagulation. Our description of the antithrombotic function of an EP₁ antagonist may represent a safer chemopreventive agent regarding cardiovascular events.

2. Materials and methods

2.1. Cells culture and materials

HUVECs were purchased from the American Type Culture Collection (ATCC, Manassas, VA) and maintained in an endothelial media system (EGM-2, Cambrex, East Rutherford, NJ) at 37 ° in a 5% CO₂ incubator. Human recombinant tumor necrosis factor-α (TNF) and interleukin-1β (IL-1) were purchased from PeproTech (Rocky Hill, NJ). ONO-8711, 6-[(2S, 3S)-3-(4-chloro-2-methylphenylsulfonyl-ammomethyl)-bicyclo(2.2.2.)octan-2-yl]-5Z-hexenoic acid was chemically synthesized at Ono Pharmaceutical Co. (Osaka, Japan). Celecoxib is purchased from LKT Laboratories (St. Paul, MN). Peroxisome proliferator-activated receptor (PPAR) response element (PPRE)3-tk-luciferase reporter vector in which luciferase gene expression is driven by three tandem repeats of the PPRE from the acyl-CoA oxidase gene was kindly provided from Dr. Raymond N. DuBois, UT, MD Anderson Cancer Center [13].

2.2. EP receptors expression

Expression of EP receptors in HUVECs was analyzed by reverse transcription-polymerase chain reaction (RT-PCR). RNA was extracted from cell lysates using Trizol reagent (Invitrogen, Carlsbad, CA) per the manufacturer's instructions. cDNA was synthesized from these RNA using M-MLV reverse transcriptase (Promega, Madison, WI) and oligo d (T)₁₂₋₁₆. Primers for β -actin and EP receptor genes are listed in the previous study [14]. PCR was carried out with PCR Master mix (Promega), according to the manufacturer's instructions. PCR amplifications were performed in a thermocycler GeneAmp PCR System 9700; Perkin-Elmer Applied Biosystems, Foster City, CA) with 40 cycles of 95 °C for 30 seconds, 60 °C for 30 seconds, and 72 °C for one minute using specific primer sets. PCR products were then analyzed by electrophoresis on 1.5% agarose gel.

2.3. PPAR transcriptional activity

HUVECs were transiently co-transfected with 0.3 μ g PPRE3-tk-luciferase reporter vector and pSV- β -galactosidase control vector as an internal control using Lipofectamine 2000 (Invitrogen) for 24 h. Then cells were incubated with PGE₂ and ONO-8711 at different concentrations for 4 h. The lysates were assayed for luciferase and β -galactosidase activities with the Bright-Glo luciferase assay system (Promega) and the All-in-One β -Galactosidase Assay Reagent (Pierce Biotechnology, Inc., Philadelphia, PA) using a luminometer and a spectrophotometer, respectively. Luciferase activity was normalized according to β -galactosidase activity, and the results are expressed as relative luciferase activity.

2.4. 6-keto PGF_{1 α} and PGE₂ measurements

Because PGI₂ is non-enzymatically hydrated to 6-keto PGF_{1 α} ($t_{1/2}$ =2–3 min), PGI₂ was measured with a 6-keto PGF_{1 α} enzyme immunoassay (EIA) kit (Cayman Chemical Co., Ann Arbor, MI). PGE₂ was also measured by PGE₂ EIA kit (Cayman Chemical Co.). The culture supernatants of HUVECs were collected after they were incubated with vehicle, TNF (5 nM), or IL-1 (2.5 ng/ml) and celecoxib or ONO-8711 at the indicated doses in growth media for the indicated time points and assayed for 6-keto PGF_{1 α} and PGE₂ production using a 6-keto PGF_{1 α} EIA kit and PGE₂ EIA kit, respectively, according to the manufacturer's instructions. The results are expressed as picograms per milliliter, calculated according to the 6-keto PGF_{1 α} and PGE₂ standard curve. All assays were performed in triplicate.

2.5. Real-time RT-PCR

RNA was extracted from cell lysates and cDNA was synthesized from RNA by the methods described above. Real-time PCR was performed on an iCycler iQ Multicolor real-time PCR Detection System (Bio-Rad Laboratories, Inc., Hercules, CA). The standard real-time PCR reaction volume was 25 μ l, including 12.5 μ l iQ SYBR Green Supermix (BioRad), 1 μ l forward primer and 1 μ l reverse primer, and 1 μ l cDNA. The primers for PGIS are following: forward primer, 5'-AGGAGAAGCACGGTGACATC-3'; reverse primer, 5'-GCAGCGCCTCAATTCCGTAA-3' and the primers for actin are listed in the previous study [14]. The primers for tissue factor (TF) are following: forward primer, 5'-GTGATTCCTCCCGAACAGTT-3'; reverse primer, 5'-CTGGCCCATACACTCTACCG-3' [15]. Cycles (n=40) consisted of a 10-sec melt at 95 °C, followed by 1 min annealing/extension at 60 °C. The final step was 1 min incubation at 72 °C. All reactions were performed in triplicate. Threshold cycle (CT) analysis for all samples was set at 0.15 relative fluorescence units. The data were analyzed using Q-Gene software [16] and expressed as mean normalized expression (MNE). MNE is directly proportional to the amount of RNA of the target gene (PGIS or TF) relative to the amount of RNA of the reference gene actin.

2.4. Data analysis

Data represent the mean $\pm$ SD. Results of 6-keto PGF_{1 α} and PGE₂ productions were analyzed by one-way ANOVA, and Dunnett's test was applied for multiple-comparison testing to compare to control. Results of PPAR transcriptional activity (relative luciferase activity), PGIS and TF MNE were analyzed by one-way ANOVA and Bonferroni's multiple comparison test among groups. Differences were considered statistically significant at $p < 0.05$.

3. Results

3.1. Effects of cytokines on PG production in HUVECs (Fig 1)

We first confirmed that cytokines stimulated HUVECs to release PGs, 6-keto PGF_{1 α} and PGE₂. HUVECs were incubated with TNF at 5 nM or IL-1 at 2.5 ng/ml for 4, 12, and 24 h and their media were analyzed for PG production with the EIA method. Figure 1 summarizes the results of 6-keto PGF_{1 α} and PGE₂ stimulated by cytokines in HUVECs. Data show that 6-keto PGF_{1 α} was significantly increased in HUVECs incubated with TNF for 12 and 24 h ($p < 0.05$ and $p < 0.01$, respectively) in Fig. 1A. Fig. 1B summarizes 6-keto PGF_{1 α} production in HUVECs incubated with IL-1. Levels of 6-keto PGF_{1 α} were significantly increased in HUVECs incubated with IL-1 for 4, 12, and 24 h ($p < 0.01$). Figs. 1C and D summarize PGE₂ production in HUVECs incubated with cytokines, which can activate HUVECs to release PGE₂. Incubation of HUVECs for more than 12 h with cytokines significantly induces PGE₂ production in media. With TNF stimulation, levels of PGE₂ were significantly increased in HUVECs for 12 and 24 h ($p < 0.05$ and $p < 0.01$, respectively) in Fig. 1C. With IL-1 stimulation, levels of PGE₂ were increased in HUVECs for 12 and 24 h ($p < 0.01$) in Fig. 1D. These results confirm that cytokines stimulate HUVECs to release PGE₂ and 6-keto PGF_{1 α} . Next, we confirmed expression of EP receptors in HUVECs using PCR. EP₁ and EP₄ receptors are clearly expressed in HUVECs (Fig. 1E).

3.2. ONO-8711 does not inhibit PGE₂ production induced by cytokines (Fig. 2)

We first confirmed that celecoxib reduced PGE₂ production induced by cytokines in HUVECs. We measured PGE₂ production in HUVECs incubated with TNF (Fig. 2A) at 5 nM or IL-1 (Fig. 2B) at 2.5 ng/ml and celecoxib at different concentrations (1 nM–10 μ M) for 24 h. As expected, celecoxib (10 nM or greater) significantly reduced PGE₂ production induced by TNF and IL-1 in HUVECs. We next investigated the effects of ONO-8711 on PGE₂ production induced by cytokines in HUVECs (Figs. 2C & D). Levels of PGE₂ production induced by TNF (Fig. 2C) or IL-1 (Fig. 2D) in HUVECs incubated with ONO-8711 (1 nM–10 μ M) for 24 h were analyzed. ONO-8711 did not reduce PGE₂ production induced by cytokines in HUVECs. Interestingly, incubation with ONO-8711 significantly increased PGE₂ production at 10 μ M with TNF treatment ($p < 0.05$). These results confirm that celecoxib significantly reduces cytokine-stimulated PGE₂ production in HUVECs. As expected, ONO-8711 does not reduce cytokine-stimulated PGE₂ production.

3.3. ONO-8711 does not inhibit 6-keto PGF_{1 α} production (Fig. 3)

We investigated the effect of ONO-8711 or celecoxib on 6-keto PGF_{1 α} production induced by cytokines in HUVECs. As shown in Fig. 3, incubation with celecoxib for 24 h at concentrations greater than 10 nM significantly reduced 6-keto PGF_{1 α} production induced by TNF (Fig. 3A, $p < 0.01$). IL-1-induced 6-keto PGF_{1 α} production was also significantly reduced by incubation with celecoxib for 24 h at concentrations greater than 1 nM (Fig. 3B, $p < 0.01$). Incubation with ONO-8711 did not reduce 6-keto PGF_{1 α} production except at concentrations of 10 μ M with IL-1 stimulation (Fig. 3C & D). Interestingly, incubation with ONO-8711 (10 or 100 nM) significantly upregulated 6-keto PGF_{1 α} production induced by TNF stimulation ($p < 0.05$ and $p < 0.01$, respectively) in Fig. 3C and incubation with ONO-8711 at 100 nM significantly

increased 6-keto PGF_{1α} production induced by IL-1 stimulation ($p<0.01$) in Fig. 3D. These results clearly suggest that ONO-8711 does not reduce 6-keto PGF_{1α} production in HUVECs, but that celecoxib does significantly reduce 6-keto PGF_{1α} production.

3.4. ONO-8711 does not affect PGI synthase (PGIS) mRNA levels (Fig. 4)

To elucidate the underlying mechanism by which celecoxib inhibits 6-keto PGF_{1α} production induced by cytokines in HUVECs, we analyzed PGIS expression levels in cell lysates using real-time RT-PCR. Cells were incubated with celecoxib or ONO-8711 at 0 or 100 nM in the presence or absence of cytokines (TNF at 5 nM and IL-1 at 2.5 ng/ml) for 24 h. The results are summarized in Fig. 4. Cytokines including TNF or IL-1 significantly induced PGIS expression in HUVECs ($p<0.001$). As expected, celecoxib treatment significantly reduced PGIS expression induced by cytokines in HUVECs ($P<0.001$). Interestingly, ONO-8711 treatment did not inhibit PGIS expression induced by cytokines in HUVECs. Levels of PGIS in cells treated with ONO-8711 induced by cytokines are significantly higher than those treated with celecoxib ($p<0.01$ for TNF and $p<0.001$ for IL-1 treatment). These results suggest that ONO-8711 does not affect PGIS expression in HUVECs whereas celecoxib inhibits cytokine-induced PGIS expression.

3.5. ONO-8711 reduces PGE₂-activated PPARE (Fig. 5)

It has been reported that PPAR plays a role in PGIS activation, resulting to PGI production in endothelial cells [15]. We first analyzed that PGE₂ activates PPARE (Fig. 5A). PGE₂ activated PPARE in a dose-dependent manner in HUVECs. Interestingly, ONO-8711 at 100 nM inhibited PGE₂-induced PPARE activity in a dose-dependent manner (Fig. 5B). These results suggest that PGE₂ activates PPAR expression through the EP₁ receptor in HUVECs, and that ONO-8711 has inhibitory effects on PGE₂-activated PPAR expression in HUVECs.

3.6. ONO-8711 reduces cytokine-stimulated TF (Fig. 6)

Cytokines (TNF and IL-1) induced TF expression in HUVECs about 5- and 11-fold compared to basal levels, respectively (Fig. 6). TF expression levels with celecoxib treatment are higher than cytokine treatment alone, but this difference was not significant. ONO-8711 treatment (100 nM) reduced TNF-induced TF expression by 45% compared with stimulation with TNF alone. It is noteworthy that ONO-8711 significantly decreased IL-1-induced TF expression by 92% ($P<0.001$). These results suggest that the EP₁ receptor pathway may be involved in thrombotic function by TF expression in HUVECs.

4. Discussion

The present study shows that ONO-8711 has no effects on PGI₂ production and PGIS expression, whereas celecoxib reduces PGI₂ production through a decrease in PGIS expression induced by cytokines in HUVECs. We also show that ONO-8711 decreases cytokine-induced TF expression in HUVECs.

We show that ONO-8711, an EP₁ receptor antagonist, reduced neither PGI₂ production nor PGIS expression in HUVECs and we also confirm that celecoxib, a selective COX-2 inhibitor, significantly reduced PGI₂ production as well as PGE₂ production induced by cytokines in HUVECs. The main providers of PGs in the cardiovascular system are platelets, which express only COX-1 and endothelial cells, which express both COX-1 and COX-2. TXA₂ and PGI₂ are functionally antagonistic prostanoids that are generated by the sequential metabolism of arachidonic acid by COX and specific synthases, TXA₂ synthase and PGIS. The data in this study are consistent with previous results indicating that celecoxib significantly reduced PGI₂ production through decreased PGIS expression in HUVECs. TXA₂ is generated predominantly by platelets and stimulates platelet aggregation and vasoconstriction, whereas

PGI₂ is produced predominantly by the endothelium and inhibits platelet aggregation and vasoconstriction [17]. Thus, COX-2 inhibitors selectively affect endothelial cells to suppress PGI₂ production resulting in the predominance of TXA₂ in the vascular system [18], which may give rise to thrombogenic events. We show that incubation with ONO-8711 inhibited neither PGI₂ production nor PGIS expression in HUVECs. Also, we used RT-PCR and PGE₂-activated-PPRE luciferase activity to show that EP₁ receptor expression in HUVECs was blocked by ONO-8711, confirming that ONO-8711 blocks PGE₂ signaling through the EP₁ receptor in HUVECs. These results suggest that PGE₂ signaling through the EP₁ receptor is not involved in PGIS expression. Interestingly, with TNF stimulation, ONO-8711 (10 μM) enhanced PGE₂ production (Fig. 4A). This may be caused by a feedback activation of COX enzymes for reduction of functional PGE₂ in the cells. Also, ONO-8711 enhanced PGI₂ production by increasing PGIS expression induced by cytokines in HUVECs. These results suggest that EP₁ receptor antagonists including ONO-8711 are safer than COX-2 inhibitors with respect to cardiovascular risks.

We show that ONO-8711 reduced cytokine-induced TF expression in HUVECs whereas celecoxib did not alter TF expression. TF, the key enzyme for initiation of the coagulation cascade, plays a pivotal role in thrombus formation and atherosclerotic vascular diseases [19]. Two reports have been published regarding the effects of TF expression in celecoxib treatment. One study indicated that celecoxib inhibited TNF-induced TF expression through inhibition of c-Jun terminal NH₂ kinase (JNK) in human aortic endothelial cells [20]. In contrast, neither rofecoxib nor NS-398 altered TNF-induced TF expression. The other study indicated that celecoxib enhanced lipopolysaccharide (LPS)-induced TF expression in HUVECs through the PPARδ pathway [15]. These two reports employed different cell systems with differing stimulation, such as human aortic endothelial cells vs. HUVECs and TNF and LPS. In the current study, we employed TNF stimulation in HUVECs. TNF is a potent inducer of TF expression and both are expressed in atherosclerotic plaques [19,21-23]. In our experiments, celecoxib did not alter TF expression induced by TNF in HUVECs. Interestingly, celecoxib enhanced IL-1-induced TF expression in HUVECs, but this did not reach significance. Therefore, our results are consistent with the previous study using HUVECs. We also confirmed that ONO-8711 inhibited PGE₂-activated PPRE in HUVECs, suggesting that PGE₂ signaling through the EP₁ receptor is involved in TF expression in HUVECs. In addition, ONO-8711 can block not only the EP₁ receptor at 1.7 nM (*K_i*) but also the TP receptor (receptor of TxA₂) at 7.6 nM (*K_i*), indicating that ONO-8711 may block the TP receptor to prevent platelet aggregation, explaining the safer cardiovascular profile. Certainly, *in vivo* animal models to investigate the role of EP₁ receptor antagonists on cardiovascular function are still warranted as arterial thrombosis is dependent on a ratio between the COX-1-dependent generation of TXA₂ in platelets and the COX-2-dependent generation of PGI₂ in the endothelium [24].

In conclusion, the present study shows that ONO-8711 did not inhibit PGE₂ and 6-keto PGF_{1α} production but that celecoxib did. In addition, ONO-8711 inhibited cytokine-induced TF expression in HUVECs. These results suggest that ONO-8711 may be a promising candidate for cancer chemoprevention because of its safer cardiovascular profile.

Acknowledgments

This study was supported by grants from the NIH (R01CA124687) and a seed grant from the Hollings Cancer Center (HCC), MUSC. We thank the Animal Pathobiology Core of the SC COBRE in Lipidomics and Pathobiology (P20RR17677) and Animal Carcinogenesis Core in the HCC, MUSC.

Abbreviations

EP, prostanoid E receptor

cyclooxygenase, COX
 prostaglandin, PG
 prostacyclin, PGI₂
 HUVECs, human umbilical vein endothelial cells
 PGIS, PGI₂ synthase
 ACF, aberrant crypt foci
 AOM, azoxymethane
 TxA₂, thromboxane A₂
 TNF, tumor necrosis factor- α
 IL-1, interleukin-1 β
 EIA, enzyme immunoassay
 RT-PCR, reverse transcription-polymerase chain reaction
 MNE, mean normalized expression.

References

1. Jemal A, Siegel R, Ward E, et al. Cancer statistics, 2006. CA: a cancer journal for clinicians 2006;56(2):106–30. [PubMed: 16514137]
2. Prevention of cancer in the next millennium: Report of the Chemoprevention Working Group to the American Association for Cancer Research. Cancer research 1999;59(19):4743–58. [PubMed: 10519377]
3. Gupta RA, Dubois RN. Colorectal cancer prevention and treatment by inhibition of cyclooxygenase-2. Nature reviews 2001;1(1):11–21.
4. Kawamori T, Rao CV, Seibert K, Reddy BS. Chemopreventive activity of celecoxib, a specific cyclooxygenase-2 inhibitor, against colon carcinogenesis. Cancer research 1998;58(3):409–12. [PubMed: 9458081]
5. Kawamori T, Uchiya N, Sugimura T, Wakabayashi K. Enhancement of colon carcinogenesis by prostaglandin E2 administration. Carcinogenesis 2003;24(5):985–90. [PubMed: 12771044]
6. Solomon SD, McMurray JJ, Pfeffer MA, et al. Cardiovascular risk associated with celecoxib in a clinical trial for colorectal adenoma prevention. The New England journal of medicine 2005;352(11):1071–80. [PubMed: 15713944]
7. Bresalier RS, Sandler RS, Quan H, et al. Cardiovascular events associated with rofecoxib in a colorectal adenoma chemoprevention trial. The New England journal of medicine 2005;352(11):1092–102. [PubMed: 15713943]
8. Watanabe K, Kawamori T, Nakatsugi S, et al. Role of the prostaglandin E receptor subtype EP1 in colon carcinogenesis. Cancer research 1999;59(20):5093–6. [PubMed: 10537280]
9. Kawamori T, Kitamura T, Watanabe K, et al. Prostaglandin E receptor subtype EP(1) deficiency inhibits colon cancer development. Carcinogenesis 2005;26(2):353–7. [PubMed: 15564292]
10. Kawamori T, Uchiya N, Nakatsugi S, et al. Chemopreventive effects of ONO-8711, a selective prostaglandin E receptor EP(1) antagonist, on breast cancer development. Carcinogenesis 2001;22(12):2001–4. [PubMed: 11751431]
11. Makita H, Mutoh M, Maruyama T, et al. A prostaglandin E2 receptor subtype EP1-selective antagonist, ONO-8711, suppresses 4-nitroquinoline 1-oxide-induced rat tongue carcinogenesis. Carcinogenesis 2007;28(3):677–84. [PubMed: 17052996]
12. Han C, Michalopoulos GK, Wu T. Prostaglandin E2 receptor EP1 transactivates EGFR/MET receptor tyrosine kinases and enhances invasiveness in human hepatocellular carcinoma cells. Journal of cellular physiology 2006;207(1):261–70. [PubMed: 16331686]
13. Wang D, Wang H, Shi Q, et al. Prostaglandin E(2) promotes colorectal adenoma growth via transactivation of the nuclear peroxisome proliferator-activated receptor delta. Cancer Cell 2004;6(3):285–95. [PubMed: 15380519]
14. Shoji Y, Takahashi M, Kitamura T, et al. Downregulation of prostaglandin E receptor subtype EP3 during colon cancer development. Gut 2004;53(8):1151–8. [PubMed: 15247185]
15. Ghosh M, Wang H, Ai Y, et al. COX-2 suppresses tissue factor expression via endocannabinoid-directed PPARdelta activation. J Exp Med 2007;204(9):2053–61. [PubMed: 17724132]

16. Muller PY, Janovjak H, Miserez AR, Dobbie Z. Processing of gene expression data generated by quantitative real-time RT-PCR. *BioTechniques* 2002;32(6):1372–4, 6, 8-9. [PubMed: 12074169]
17. Mitchell JA, Warner TD. COX isoforms in the cardiovascular system: understanding the activities of non-steroidal anti-inflammatory drugs. *Nat Rev Drug Discov* 2006;5(1):75–86. [PubMed: 16485347]
18. Grosser T, Fries S, FitzGerald GA. Biological basis for the cardiovascular consequences of COX-2 inhibition: therapeutic challenges and opportunities. *J Clin Invest* 2006;116(1):4–15. [PubMed: 16395396]
19. Moons AH, Levi M, Peters RJ. Tissue factor and coronary artery disease. *Cardiovasc Res* 2002;53(2):313–25. [PubMed: 11827681]
20. Steffel J, Hermann M, Greutert H, et al. Celecoxib decreases endothelial tissue factor expression through inhibition of c-Jun terminal NH2 kinase phosphorylation. *Circulation* 2005;111(13):1685–9. [PubMed: 15795326]
21. Liu Y, Pelekanakis K, Woolkalis MJ. Thrombin and tumor necrosis factor alpha synergistically stimulate tissue factor expression in human endothelial cells: regulation through c-Fos and c-Jun. *J Biol Chem* 2004;279(34):36142–7. [PubMed: 15201277]
22. Mechtcheriakova D, Schabbauer G, Lucerna M, et al. Specificity, diversity, and convergence in VEGF and TNF-alpha signaling events leading to tissue factor up-regulation via EGR-1 in endothelial cells. *FASEB J* 2001;15(1):230–42. [PubMed: 11149911]
23. Libby P. Inflammation in atherosclerosis. *Nature* 2002;420(6917):868–74. [PubMed: 12490960]
24. Fitzgerald GA. Coxibs and cardiovascular disease. *The New England journal of medicine* 2004;351(17):1709–11. [PubMed: 15470192]

Fig. 1. Levels of 6-keto PGF_{1α} and PGE₂ induced by TNF (A & C) and IL-1 (B & D) in HUVECs, respectively (* $p < 0.05$ and ** $p < 0.01$ vs. 0 h). PCR results (E) show EP₁ and EP₄ expression in HUVECs.

Fig. 2. Effects of celecoxib (A & B) and ONO-8711 (C & D) on PGE₂ production induced by TNF (A & C) and IL-1 (B & D) in HUVECs (* p <0.05, ** p <0.01, and *** P <0.001 vs. 0 h)

Fig. 3.
Effects of celecoxib (A & B) and ONO-8711 (C & D) on 6-keto PGF_{1 α} production induced by TNF (A & C) and IL-1 (B & D) in HUVECs (* p <0.05 and *** p <0.001 vs. control)

Fig. 4. Levels of PGIS expression in HUVEC incubation with cytokines (TNF at 5 nM and IL-1 at 2.5 ng/ml) and celecoxib or ONO-8711 at 100 nM for 24 h using real-time RT-PCR (* $p < 0.001$ and ** $p < 0.01$).

Fig. 5. PGE₂ activates PPRE in HUVECs (A). ONO-8711 blocks PGE₂-activated PPRE in HUVECs (B).

Fig. 6. Effects of celecoxib and ONO-8711 at 100 nM for 24 h incubation on TF expression induced by cytokines (TNF at 5 nM and IL-1 at 2.5 ng/ml) in HUVECs (** $P < 0.01$ and *** $P < 0.001$ vs. control). ONO-8711 treatment significantly reduced IL-1-induced TF expression (*** $P < 0.001$).