Published in final edited form as:

Prostaglandins Leukot Essent Fatty Acids. 2012 ; 87(0): . doi:10.1016/j.plefa.2012.07.002.

Acute Effects of Prostaglandin E₁ and E₂ on Vascular Reactivity and Blood Flow *in situ* in the Chick Chorioallantoic Membrane

D.R. Harland^{#1}, L.D. Lorenz^{#1}, K. Fay¹, B.E. Dunn^{3,4}, S.K. Gruenloh¹, J. Narayanan², E.R. Jacobs^{1,4}, and M. Medhora²

¹Division of Pulmonary and Critical Care, Department of Medicine, Medical College of Wisconsin, Milwaukee, WI 53226

²Division of Pulmonary and Critical Care, Department of Radiation Oncology, Medical College of Wisconsin, Milwaukee, WI 53226

³Division of Pulmonary and Critical Care, Department of Pathology, Medical College of Wisconsin, Milwaukee, WI 53226

⁴Clement J. Zablocki VA Medical Center, Milwaukee WI 53295

These authors contributed equally to this work.

Abstract

1. The chick chorioallantoic membrane (CAM) subserves gas exchange in the developing embryo and shell-less culture affords a unique opportunity for direct observations over time of individual blood vessels to pharmacologic interventions. We tested a number of lipids including prostaglandins PGE_{1&2} for vascular effects and signaling in the CAM. Application of PGE_{1&2} induced a decrease in the diameter of large blood vessels and a concentration-dependent, localized, reversible loss of blood flow through small vessels. The loss of flow was also mimicked by misoprostol, an agonist for 3 of 4 known PGE receptors, EP₂₋₄, and by U46619, a thromboxane mimetic. Selective receptor antagonists for EP₃ and thromboxane each partially blocked the response. This is a first report of the effects of prostaglandins on vasoreactivity in the CAM. Our model allows the unique ability to examine simultaneous responses of large and small vessels in real time and *in vivo*.

Introduction

The chorioallantoic membrane (CAM) of the developing chick embryo is responsible for gas exchange. Hence, the vessels in the CAM function as the equivalent of mammalian pulmonary arteries and veins. Shell-less cultures of chick embryos permit ready access to vessels in the CAM such that effects of pharmacologic interventions can be directly observed and recorded in real time. This model affords the following unique advantages: (1) visualization of temporal changes of vessels *in vivo* permitting determination of reversibility (2) simultaneous assessment of reagents on large vessel diameters and small vessel flow (3)

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Corresponding Author: Meetha Medhora, MFRC 4076, Medical College of Wisconsin, 8701 W Watertown Plank Road, Milwaukee, WI 53226, mmedhora@mcw.edu, Phone (414) 456-5612, Fax (414) 456-6515.

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characterization of localized vascular effects *in situ* while the host remains stable or unchanged through the experiment (4) cost effective studies requiring minimal equipment.

Our interests are to study modulation of vascular tone and blood flow by eicosanoids and lipid mediators. We have successfully used the CAM to report a hyperemic response of the cytochrome P450 derivatives of arachidonic acid, epoxyeicosatrienoic acids (EETs) [1]. Prostaglandin (PG) derivatives of the essential fatty acid arachidonic acid via the cyclooxygenase (COX)/PG synthase pathway are important homeostatic mediators [2,3] involved in inflammation, pain and fever [4-6]. In the present study we tested PGs specifically of the “E- ring” (PGEs) which are reported to modulate the tone of human pulmonary vessels [7,8], constrict pig large cerebral arteries [9] and have a biphasic effect on renal afferent arterioles [10]. To our knowledge the acute effect of PGE₁ or PGE₂ on vasoreactivity and blood flow in the CAM has not been reported.

At the molecular level, the complex actions of PGE₁ and PGE₂ are attributable to their ability to engage at least five different receptors: EP₁, EP₂, EP₃, EP₄ [2,11-14] and thromboxane (TP) [15,16]. Thromboxane is also an arachidonic acid derivative. Our aim in this study is to use the CAM as a novel model to investigate acute vasoactive effects of biological lipids *in vivo*. The vasculature in the developing CAM starts maturing from day 8 [17]. The number of visible vessels continues to increase with time. For our study we used CAMs from embryos between 9-18 days of age.

2. Materials and Methods

1.1 Shell-less culture of chick embryos

CAMs were developed in embryos grown in shell-less culture as previously described [1,18]. Fertilized eggs of White Leghorn chickens were purchased from Sunnyside Inc., Beaver Dam, WI. Eggs were incubated for 3 days at 37.5°C with 62-72% humidity in a commercial incubator equipped with automatic turning trays that changed the angle of orientation every 3 hours. After 3 days the eggs were dipped briefly in 70% ethanol, dried and carefully opened with a razor blade [1]. The contents were poured into clear plastic wrap (Roundy's Quality distributed by Roundy's Inc., Milwaukee, WI) suspended in custom designed, disinfected tripods with holders made from 3 inch diameter plastic drainpipe and covered with the top half of a sterile Petri dish [18]. The developing embryos and yolk sac vascular system were visible at this time. Embryos were incubated in a water-jacketed tissue culture incubator at 37.5°C, 99 % humidity and 1.5 % CO₂ mixed with room air. Survival rates to 10 days were above 60% and to 18 days approximately 25%. No embryos were allowed to incubate beyond 18 days.

1.2 Application of Prostaglandins

Prostaglandin E₁ (PGE₁) and prostaglandin E₂ (PGE₂) (Cayman Chemical, Ann Arbor, MI) were dissolved in ethanol at a concentration of 1 mg/mL. Working concentrations were prepared by dilution in phosphate-buffered saline (PBS) (Sigma, St. Louis, MO) containing fatty-acid-free bovine serum albumin (BSA at 0.05 % w/v). Up to five silicone rings of 1 cm diameter (Marco Rubber and Plastics Products Inc., North Andover, MA) were placed onto each CAM and 10 µL of 2 µM, 10 µM, 50µM or 250 µM PGE₁, PGE₂, or vehicle control were carefully placed in the center of the ring, avoiding touching the membrane to prevent damage to the vessels. After 1 hour (unless otherwise mentioned) emulsified fat (heavy whipping cream, Roundy's Quality distributed by Roundy's Inc., Milwaukee, WI) warmed to 37°C was injected under the CAM below each ring for contrast and in part to obstruct the underlying vessels of the vitelline membrane [1,19,20]. Images were obtained at constant magnification with a Nikon SMZ-1000 stereo microscope using external fiber optic lighting,

and were captured with a fitted Insight Firewire Color Mosaic Camera (Fryer Company, Huntley, IL).

1.3 Reversibility and spatial localization of vascular changes

After baseline images were obtained, 10 μ L of 50 μ M PGE₁, PGE₂, or vehicle control was carefully placed in the center of the ring and the CAM was incubated at room temperature for 10 minutes, at which point images inside and in some cases outside the ring (for comparison) were obtained. The CAM was then incubated at 37°C in saturation humidity and images were taken every hour for up to five hours. Similar results were observed in CAMs that were not treated with emulsified fat, verifying the effects were not due to the heavy whipping cream which was being used only as a contrast agent.

1.4 Specificity of vascular responses

To assess for non-specific responses of the CAM, experiments analogous to those with PGEs were performed applying α -linolenic acid and α -estradiol (2 μ M – 3 mM).

1.5 Quantitation of visible vessels and measurement of vessel diameters

Acquired images of CAM were loaded into the image analysis software Metamorph (Version 6.1, Universal Imaging Corp, 2003). A fixed area grid with 0.4 mm divisions was superimposed on images of the treated area of the CAM. Vessels that intersected the grid were counted and the number of intersections in the drug-treated CAMs were compared to those of vehicle control. To measure vessel diameters, the Single Line Tool in Metamorph was used to measure the pixelated width of the largest vessel and two of its respective secondary vessels within the application area. Measurements for vessel diameters were taken in triplicate and data were transferred to Microsoft Excel for analysis.

1.6 Application of EP Receptor Agonist

Misoprostol methyl acetate (an EP₂₋₄ receptor agonist, Cayman Chemical, Ann Arbor, MI) was dissolved in ethanol at a concentration of 1 mg/mL and further diluted to working concentrations of 10 nM, 100 nM, 1 μ M, 10 μ M and 30 μ M respectively in PBS containing BSA (0.05 % w/v). A volume of 10 μ L was applied in an area bounded by a silicone ring on the CAM and the embryos were incubated and imaged as previously described.

1.7 Application of TP Agonist

U46619 (a thromboxane mimetic, Cayman Chemical, Ann Arbor, MI) was dissolved in ethanol at a stock concentration of 1.5 mM. Working concentrations of 150 nM were made in PBS+BSA (0.05 % w/v). A volume of 10 μ L of the agonist or vehicle control was placed on the CAM and incubated for 5 minutes before images were acquired.

1.8 Application of EP_{1,2 & 4} Receptor Antagonists

SC 19220 (EP₁ receptor antagonist, Cayman Chemical, Ann Arbor, MI) and AH 23848 (EP₄ and TP receptor antagonist, Sigma, St. Louis, MO) were dissolved in ethanol at a concentration of 1 mg/mL. AH 6809 (EP_{1&2} receptor antagonist, Cayman Chemical, Ann Arbor, MI) was dissolved in ethanol at a concentration of 0.5 mg/mL. Working concentrations of 250 μ M of each antagonist were dried to near completion under nitrogen and resuspended in PBS containing BSA (0.05 % w/v). A volume of 10 μ L of the antagonist or vehicle control was carefully placed in the center of a silicone ring on the CAM and incubated for 15 minutes at 37°C. Subsequently, 10 μ L of either 50 μ M PGE₁ or PGE₂ or 30 μ M misoprostol was added and incubated for an additional hour. Images were acquired as previously described.

1.9 Application of EP₃ Receptor Antagonist

ONO-AE3-240 (EP₃ receptor antagonist, ONO Pharmaceutical Co., Ltd., Osaka, Japan) was dissolved in DMSO (dimethyl sulfoxide, Sigma, St. Louis, MO) at a stock concentration of 9.4 mM and diluted in PBS (Sigma, St. Louis, MO) containing BSA (0.05 % w/v). A volume of 20 μ L of ONO-AE3-240 or vehicle control was carefully placed in the center of a silicone ring and the embryo was incubated for 30 minutes. Subsequently, 10 μ L of 50 μ M PGE₁ or vehicle was added and the CAM was incubated for an additional hour. Images were acquired as previously described. Working concentrations of ONO-AE3-240 (0.9-1.4 mM) were similar to those described previously [21].

1.10 Application of TP Receptor Antagonist

SQ 29,584 (a thromboxane receptor antagonist, Cayman Chemical, Ann Arbor, MI) was dissolved in ethanol at a stock concentration of 30 mM. Working concentrations of 1 μ M were made in PBS containing BSA (0.05 % w/v). A volume of 10 μ L of the antagonist or vehicle control was carefully placed on the CAM and incubated for 15 minutes at 37°C. Subsequently, 10 μ L of either 50 μ M PGE₁, PGE₂ or U46619 were added and incubated for 5 minutes to 1 hour.

1.11 Detection of EP₃ protein in CAMs

CAMs were dissected from the cultures, washed in PBS and homogenized in 10 mM potassium phosphate buffer (pH 7.7) containing 250 mM sucrose, 1 mM EDTA, and 10 mM MgCl₂. Kidneys from adult rats were also homogenized in the same buffer. The homogenates were centrifuged for 10 minutes at 9000 $\times$ g, and the supernatant centrifuged for 90 minutes at 100,000 $\times$ g to obtain a microsomal pellet [22]. Microsomal proteins from CAMs or rat kidneys (positive control) were solubilized in and extracted with RIPA buffer (50 mM Tris pH 8.0, 150 mM NaCl, 0.5 % SDS, 1 % Nonidet P40, 0.5 % sodium deoxycholate, 1 mM EDTA, 1 $\times$ protease inhibitor cocktail (Pharmingen, San Diego, CA)). Protein content of the microsomes was assayed with the Biorad DC Protein Assay Reagent (Biorad, Hercules, CA). Equal amounts of protein (50 μ g) from each sample were electrophoresed on a 10 % SDS-polyacrylamide gel with running buffer [23]. The membrane was treated with primary antibody (1:200 dilution of polyclonal rabbit anti-goat EP₃, Santa Cruz Biotechnology, Inc. Santa Cruz, CA) for 18 hours and then a secondary antibody (1:5000 dilution of anti-rabbit HRP). Chemiluminescence reagents were used to identify bands which corresponded to the positive control (MW between 55-78 kD).

1.12 Statistical Analyses

All values represent the mean $\pm$ standard error of the mean (SEM). Results were compared by unpaired t-tests for visible vessel numbers after treatment with vehicle or agent, or ANOVA with appropriate post-hoc tests for comparison of groups. For comparison of vessel diameters before and after vehicle or PGEs, we employed Mann Whitney rank sum tests. Sample sizes (n) are provided in each graph or figure legend. P values indicating differences between two groups are included in the figures or figure legends.

3.0 Results

3.1 PGE₁ and PGE₂ induce time dependent and reversible loss in visible blood vessels

Typical vasculature on a CAM (day 14) is depicted in Fig. 1a. Panel A depicts four images of the same field treated with vehicle and imaged at increasing intervals of time up to 5 hours. A single application of 50 μ M PGE₁ (Fig. 1b) or PGE₂ (Fig. 1c) but not vehicle (Fig. 1a) induced a reversible visible avascular zone directly under the area of application on a live CAM. This response appeared within 5-10 minutes of treatment with PGEs, continued

beyond this time and was marked by disappearance of smaller branches of the vasculature that ranged from 47-95 μm in diameter (tertiary and quaternary vessels). The effects of 50 μM of PGE_1 (Fig. 1b) and PGE_2 (Fig. 1c) were reversible and vessels generally reappeared by 1-5 hours after application of the eicosanoid, as demonstrated by colored arrows in Fig. 1. The red arrow (Fig. 1b1) follows a vessel that “disappears” by 10 minutes (Fig. 1b2) and begins to “reappear” at 1 hour (Fig. 1b3) after treatment. The blue arrow (Fig. 1c1) points to another vessel that disappears in 10 minutes (Fig. 1c2) and reappears by 5 hours but not 60 minutes (Fig. 1c4). A time lapse video of a representative response to PGE_1 appears in the online supplement to this paper (Fig. S1).

3.2 Vasoreactivity in the CAM is localized

A single application of 50 μM PGE_1 or PGE_2 induced a decrease in visible blood vessels directly under the area of application (Figs. 1 and 2). Vasculature outside the area of application remained unchanged (Fig. 2).

3.3 Vascular effects are not induced by α -linolenic acid or β -estradiol

To test for the specificity of PGE-evoked effects, we examined vascular changes secondary to two other lipids. Neither α -linolenic acid nor β -estradiol in concentrations from 2 μM -3 mM elicited acute vascular effects (Fig. 3). Vessels were quantitated by counting intersections of visible vasculature with lines of a grid that was superimposed on the image. The number of grid intersections in images of CAMs treated with 3 mM linolenic acid (70 ± 3 , mean SEM) matched the vehicle controls (70 ± 2) as did β -estradiol (66 ± 4) and its matched vehicle controls (63 ± 3), $n=3$.

3.4 PGEs cause vasoactive effects on both arteries and veins

Arteries and veins can be readily distinguished one from the other by the direction of blood flow and color. Arteries flow toward the CAM and, when whipping cream is injected below the CAM to improve contrast, appear blue-red in color since they carry de-oxygenated blood. In contrast, veins flow away from CAM toward the embryo and are bright red in color since they carry blood which has been oxygenated in the CAM chorionic sinus. The direction of blood flow is easily distinguished in real time as the transit of nucleated RBCs in vessels is visible under the higher power of the dissecting microscope. Fig. 4 shows CAM images with arteries and veins identified before and one hour after application of 50 μM PGE_1 . PGE_1 decreases the diameter of both arteries and veins.

3.5 Vascular effects of PGE are concentration-dependent

A decrease in the number of visible vessels was observed after applying PGE_1 or PGE_2 at all concentrations greater than 2 μM (Fig. 5a-d). Maximal effect was observed at the highest concentration applied (e.g. PGE_2 at 250 μM was more effective than 50 μM ($p<0.001$)). Reversibility of PGE effects was typically delayed with higher concentrations of lipids (less than 1 hour with 10 μM to more than 2 hours when 250 μM was applied).

3.6 The vascular effect of PGE_1 and PGE_2 is not dependent on the age of the embryo

The vascular changes evoked by PGE_1 and PGE_2 were observed in the CAM between 9 and 18 days of incubation. The CAM developed only after day 6 and continued to expand and mature through this time. For the purpose of analyses, we divided the CAMs into 3 different age groups: 9-11 days (young), 12-14 days (middle) and 15-18 days (old). Significant decreases in visible vessels were observed with PGE_1 and PGE_2 in all ages tested (Fig. 6).

3.7 Decrease in diameter of large vessels (35-300 μm internal diameter) by PGE₁ and PGE₂

We measured the effect of PGE₁ and PGE₂ on the diameter of large vessels of the CAM (Fig. 7a). Images were captured before and 5 minutes after application of PGE₁, PGE₂, or vehicle control by which time constriction was maximum. No change in vascular diameter was observed at low concentrations of the eicosanoids (50 nM-2 μM , results not shown). However, we observed a decrease in diameter of the larger vessels by application of PGE₁ or PGE₂ (50 μM each). There was an approximately 40% decrease in diameter in both primary and secondary vessels treated with eicosanoids (Fig. 7b & c) in contrast to the response of corresponding vessels of the same size in areas of the CAM treated with vehicle. For time lapse images over 5 minutes showing dynamic changes please see supplemental data online, Fig. S1.

3.8 Loss of visible vessels is partially mediated by an EP receptor

Misoprostol, an agonist for EP₂, EP₃ or EP₄ receptors [24-26] was applied to the CAM at three concentrations (100 nM, 10 μM or 30 μM). This drug induced a decrease in visible vessels at 10 μM and 30 μM in a magnitude which was comparable to that observed with 50 μM of PGE₂ (Fig. 8). These results support the presence of functional receptors for EP₂₋₄ in CAMs.

To follow up these findings we pretreated areas of the CAM for 30 minutes with vehicle or antagonists for EP₁ (SC 19220), EP₁ and EP₂ (AH 6809), EP₃ (ONO-AE3-240) or EP₄/thromboxane receptors (TP) (AH 23848) [7,9,21]. We then treated the same locations with 50 μM PGE₁ or PGE₂, or 30 μM misoprostol (Fig. 9a & b, images for PGE₂ only shown). The antagonists for EP₁, EP₂, and EP₄ did not alter vessels by themselves nor did they change the decreases evoked by PGE₁, PGE₂ or misoprostol (Fig. 9c). These experiments were powered to detect 40% change from vehicle, such as we observed with PGEs and misoprostol. Similarly, no effects were observed with CAMs pretreated with receptor blockers then PGEs at 10 μM ; these data are not reported as the effects of PGEs at the lower concentrations were more transient. The antagonist for EP₃ partially attenuated PGE-induced loss of visible vessels (Fig. 10 shows results with PGE₁).

3.9 Loss of visible vessels is partially mediated by the thromboxane receptor (TP)

Thromboxane agonist U46619 was applied to the CAM at a concentration of 150 nM or 1.5 μM and induced a decrease in visible vessels (Fig. 11a). Next we pretreated the CAM with a selective TP antagonist (SQ 29,548) then treated the same area with U46619 (Fig. 11b) or PGE_{1&2} (Fig. 11c shows results with PGE₂). We observed that at higher concentrations of PGE (50 μM and greater) the decrease in visible vessels was blunted by pretreatment with the TP antagonist, while at lower concentrations of PGE (10 μM and less), the TP antagonist had little effect (Fig. 11c). These findings confirm the expression of functional TP receptors in CAM and suggest that at high concentrations PGE engages them [27].

3.10 Expression of EP₃ protein in the CAM

1.1. Western blots of CAM microsomes probed with EP₃ primary antibodies confirm immunospecific bands of ~50 and 78 kD (Fig. 12) consistent with EP₃ non-glycosylated and glycosylated proteins. Similar sized bands were observed in rat kidney microsomes.

4.0 Discussion

Models offering direct observation of vascular events in real time, particularly of both large and small pulmonary vessels, are limited. The CAM in developing chick embryos grown outside of the egg shell permits easy access to and direct visibility of the growing vascular

bed. Because of the robust vascular development in this organ, the CAM has been extensively used to study angiogenesis. We used this system previously to identify acute effects of lipid metabolites in a vascular bed which facilitates gas exchange, subserving the function of the mammalian lung [1]. In this study we report for the first time the acute effects of at least two vasoactive agents prostaglandins PGE₁ and PGE₂ on the CAM vasculature *in vivo*.

PGE₁ and PGE₂ evoked time- and concentration-dependent decreases in the diameters of large arteries and veins in the CAM (Figs. 1 & 5). Vessel diameters decreased within 5 minutes after application of PGEs (Fig. 7) and were restored within 5 hours, depending on the concentrations applied and the size of the vessels. Based on Poiseuille's law, the blood flow is proportional to the fourth power of the radius of a vessel. By decreasing the radius of a large blood vessel by 35% (similar to values observed with 50 μ M PGE₁ or 2) and keeping all other variables constant, the blood flow to downstream smaller vessels would be decreased by over 80%. Indeed we observed that PGEs, but not α -linolenic acid or α -estradiol (Fig. 3), induced concentration-dependent loss in visible blood flow in small vessels when applied to the CAM. Most smaller vessels were not visible for 60 minutes after application of 50 μ M PGE (Figs. 1, 5-8, 9 & 11). The vascular effect of PGE₁ & 2 was seen in CAMs ranging from 9-18 days of age, and confined to the area treated. Together, these data suggest that vessels in the CAM constrict to PGE₁ and PGE₂.

Because vasoactivity induced by PGEs is known to be modulated by EP receptors, we sought evidence for the role of these receptors in mediating the vascular responses we observed. Misoprostol, an agonist for EP₂, EP₃ and EP₄, induced a loss of visible vessels in a manner similar to that of PGE₁ and PGE₂. Antagonists to EP₁, EP₂, or EP₄/TP did not alter PGE-evoked changes. In contrast, the antagonist to EP₃ receptors, ONO-AE3-240, partially blocked the PGE-evoked decrease in small visible vessels. We also identified 50 and 78 kD immunospecific bands for EP₃ protein in CAMs. The size of the EP₃ receptor has been reported as low as 33-38 kD in splice variants [28,29] while the full length protein determined by amino acid comparison is estimated at ~55-63 kD. There are two sites of possible N-linked glycosylation, which can yield products of 65-75 kD that have been previously detected on Western blots [28,29]. Together these results suggest that constrictive effects of PGE₁ and PGE₂ are mediated at least in part by EP₃ receptors.

Due to the similar chemical structure of PGE and thromboxane, PGE at high concentrations can cross-react with TP receptors [27]. Response to thromboxane has been reported in chickens [30]. Indeed in our hands, the thromboxane agonist U46619 also evoked concentration dependent decrease in visible vessels which was blocked by the TP antagonist SQ 29548 (Fig. 11). In addition SQ 29548 blocked vasoconstriction at higher concentrations of PGE (50 μ M), but did not prevent the vasoconstriction at a lower concentration of PGE (10 μ M). These data suggest that at lower concentrations PGE-evoked vasoconstriction seen in the CAM is mediated by the EP₃ receptor, while at higher concentrations both EP₃ and TP receptors may contribute as has been observed previously [27]. In some species receptors for prostaglandin D₂ (DP₂), a chemoattractant receptor-homologous molecule expressed on T-helper type 2 cells (CRTH2), and prostaglandin F (FP) have a greater affinity for PGE₂ than for TP [26]. We have not yet addressed the possible contribution of these receptors in our CAM model. There are a number of potential explanations for the lack of inhibitory effect of the EP₄/TP receptor antagonist AH23848 [31,32]) but not SQ 29,548, in PGE-evoked contraction in our experiments (Figs 9 & 11). SQ 29,548 (formula weight 387.5) is a highly selective TP receptor antagonist which binds to the human recombinant TP receptor with a K_i of 4.1 nM and blocks U-46619-induced contraction of rat and guinea pig tracheal, arterial, and venous smooth muscles with EC₅₀ in the range of 1-50 nM [33]. On the other hand, AH23848 (much larger with a formula weight 1011.3) is reported to block U46619

evoked contraction in human bronchial smooth muscles at *micromolar* concentrations [34]. Thus it is possible that concentrations of AH23848 sufficient to block receptors in CAM vessels were not achieved.

Molecular cloning has confirmed at least four G protein-coupled EP receptor subtypes, EP₁, EP₂, EP₃ and EP₄ [11], each coded by different genes. EP₂ & 4 receptor subtypes have been shown to exist in chicken by molecular cloning [35], and our data support the existence of EP₃ receptors in the developing CAM (Fig 12). EP₃ receptors mediate contraction of the uterus, inhibition of gastric acid secretion, modulation of neurotransmitters, lipolysis, sodium and water reabsorption in kidney tubules and secretion of catecholamines [4,25,29,36-38]. The vasoactive effects of PGEs on pulmonary arteries appear to depend upon a variety of factors including the state of activation of the vascular smooth muscle prior to exposure to the lipid, whether the vessels are arteries or veins and/or the subtype of EP receptor expressed. Norel *et al.*, [7] reported that PGE₁ induces relaxation in human pulmonary arteries and veins, though engagement of EP₃ receptors alone in both vessel types evokes constriction. Walch *et al.*, [8] observed no effect of PGE₂ on precontracted human pulmonary arteries and PGE-evoked either constriction or relaxation of human pulmonary veins. PGE vasodilation is observed almost exclusively after precontraction, presumably because pulmonary vessels are dilated at rest. We attempted to precontract CAM vessels with norepinephrine (NE) or phenylephrine (PE) with the intention of testing the effects of PGEs under these conditions. In data not shown, neither NE nor PE in concentrations as high as 1 mM resulted in vasoconstriction. However, PGE₁ added after constriction with 150 nM U46619 still resulted in disappearance of small vessels and decrease in the diameter of large vessels (data not shown). Thus, while we cannot exclude the possibility that PGEs may relax CAM arteries or veins in certain situations, we observed only vasoconstriction under the conditions employed.

An important limitation of our model is uncertainty regarding the actual concentrations of pharmacological agents and blockers at the level of the vasculature in the CAM. All reagents had to be applied at high concentrations to be effective, raising the possibility for off target effects. For example, PGE₁ at a concentration of 40 nM inhibits human platelet aggregation [39] and PGE₂ has an affinity constant (K_d) of <25 nM for all four EP receptor subtypes [11]. We observed vascular effects on the CAM at concentrations of 10 μM. The need for higher concentrations of reagents in the CAM could be due to the presence of the ectoderm above the mesenchymal layer of the CAM in which the blood vessels are embedded [1,40,41]. The lipophilic reagents we tested are likely soluble in the lipid membranes of the ectoderm, and may not diffuse efficiently beyond the ectoderm to the adjacent cells below [42]. In addition, the total volume of PGEs applied to the CAMs was relatively small. We limited the volume of buffer applied to prevent covering a large surface area of the CAM which would block gas exchange necessary for survival of the embryo. Since PGEs are lipophilic they would be more soluble in the cell membranes than the buffer used in the application. Many models of PG vasoactivity assess diameters of blood vessels in organ baths with ratios of buffer to cellular “sinks” much larger than we used in the CAM. Since the lipids preferentially partition to cells and tissues from the surrounding buffer, higher total amounts of lipids may be taken up by vessels in organ baths as compared to those embedded within the CAM in our study. As a result, the effective lipid concentrations at the level of the mesodermal vessels in our studies are likely to be much lower than in the applied solutions.

A second limitation to our model is the scant information regarding the pharmacological profiles of EP or TP receptors in the developing CAM. Because there are widely varying species-specific affinities to agonists and antagonists, negative results cannot exclude participation of EP₁, EP₂ or EP₄ receptors in the CAM which are not blocked by antagonists

that are effective in mammals. In this regard, cloned chicken receptors in expression systems that would permit identification of Kds to a range of agonists/ligands or antagonists would greatly enhance the utility of this model.

PGs or antagonists are used clinically for treatment of a number of diseases making it very important to understand their effects on vascular beds *in vivo*. For example, PGE₁ (alprostadil) is prescribed in some countries as a vasodilator for the treatment of peripheral vascular diseases and to inhibit platelet aggregation [43]. PGE₁ may promote angiogenesis [44,45] and it is well established that PGE₂ is pro-angiogenic [46-49]. PGE₂ at low concentrations is a potent stimulator of uterine contractions and promotes labor [38]. It can induce bronchoconstriction as well as hypertension [7,36,50]. Non-steroidal anti-inflammatory drugs (NSAIDs), well known inhibitors of PGE synthase, are commonly used against inflammation, pain, cardiovascular diseases and colon cancer [51,52]. Such wide applications of modulators of PGs make it essential to understand the consequences of their vascular effects in different organs.

In conclusion, we demonstrate that PGE₁ and PGE₂ induce rapid and dramatic vasoconstriction in the CAM. Further, our observations using a living embryonic model *in situ* demonstrate, for the first time, a unique loss of visible blood flow in small vascular branches that is reversible within hours. The apparent loss of blood flow in small vessels may be due to vasoconstriction of the small vessels *per se* and/or to decreased blood flow due to vasoconstriction of the upstream vessels. This is a first step in visually examining the effect of PGs *in situ* in living models.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors would like to thank ONO Pharmaceutical Co., Ltd. for providing the ONO-AE3-240 compound and Dr. Sandra Pfister for her valuable suggestions and expertise in the use of PG and TP receptor agonists and antagonists. This work was supported by the National Institutes of Health, National Heart, Lung, and Blood Institute grants HL 069996 (to MM), 68627 (to ERJ), 49294 (to ERJ). The work was supported by facilities at the Clement J. Zablocki VA Medical Center.

Sources of Support: This work was supported by the National Institutes of Health, National Heart, Lung, and Blood Institute grants HL 069996 (to MM), 68627 (to ERJ), 49294 (to ERJ).

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Fig.1.

PGE₁ and PGE₂ induce loss of visible blood vessels in the CAM in a time dependent and reversible manner. CAMs were imaged at 0 minutes, 10 minutes, 1 hour and 5 hours (columns 1-4) after a single application of (a) vehicle (control) (b) 50 μ M PGE₁ (c) 50 μ M PGE₂. The red and blue arrows follow the loss (dotted arrows) of blood vessels after 10 minutes. Maximal effects were generally observed in minutes and largely persist for 60 minutes with 50 μ M PGEs. A few vessels reappear prior to one hour (solid red arrow) and virtually all return by 5 hours (solid blue arrow).

Fig. 2. Vascular effects of PGEs are limited to the site of application. Representative images show loss of visible vessels 60 minutes after application of 50 μ M PGE₁ within the silicone rings (Fig. 2a), but not outside (Fig. 2b).

Fig. 3. Exclusion of non-specific lipid effects. Representative images (from 3 experiments) at 30 minutes after the CAMs were treated with 3 mM (the highest concentration tested) linolenic acid or β -estradiol. These agents had no visible effect on the CAM vasculature.

Fig. 4.

PGEs affect both arteries and veins in the CAM. Arteries can be distinguished from veins in the CAM based upon color and direction of blood flow. This figure shows images of a CAM marked with “A” for arteries and “V” for veins before (a) and 60 minutes after (b) application of 50 μM PGE₁. Both directional flow (visible under the dissecting microscope in real time) and color of the vessels are different as described in the text. Lymphatics are not visible in this model.

Fig. 5.

Loss of visible blood vessels is dependent on the concentration of PGE_{1&2} (only PGE₂ imaged). CAMs were treated with increasing concentrations of (a) PGE₂ (2 μ M) (b) PGE₂ (50 μ M) (c) PGE₂ (250 μ M). Images were captured at constant magnification 60 minutes after applications. (d) Graphical representation of visible vessels after quantitation using a grid. The Y-axis represents the number of vessels as a % of vehicle (control). The numbers within the bars represent number of CAMs analyzed.

Fig. 6.

Loss of visible blood vessels is induced by PGs 60 minutes after application in CAM vessels from embryos of all ages. Embryos were divided by age into 3 groups, young (9-11 days), middle (12-14 days) and old (15-18 days) and each were treated with high concentration of PGE_{1&2} (250 μ M) or vehicle control. Typical loss of visible blood vessels was observed in all embryos treated with PGEs but not vehicle control, demonstrating that this PGE effect is independent of age of the embryo. The Y-axis represents vessel numbers depicted by grid intersections. Visible vasculature increased with age of the embryo. The numbers within the bars represent number of CAMs analyzed.

Fig. 7.

PGE₁ and PGE₂ induce acute constriction of vessels in the CAM. (a) Images of CAMs treated with vehicle, PGE₁ and PGE₂ at 0 minutes and 5 minutes after application of PGEs. The white arrows point to examples of blood vessels in the CAM (termed primary) that were scored along with secondary vessels (black arrows). Vessels decreased in diameter by up to 40% within 5 minutes after treatment with 50 μ M PGE₁ or PGE₂ but only ~10% with time and vehicle controls. (b) The graphs show internal diameters of primary vessels immediately before and 5 minutes after application of vehicle or 50 μ M PGE₁ or PGE₂. The broken black lines represent average values for the group. P values for Mann-Whitney Rank Sum tests to detect changes from baseline appear below the data for each group. The p value for changes

in diameter of large vessels treated with PGE₁ was 0.05 and for PGE₂ was 0.01. (c) analogous studies on the diameters of secondary vessels exposed to vehicle, PGE₁, or PGE₂.

Fig. 8. Misoprostol, an agonist of EP₂₋₄ receptors, induces concentration dependent visible loss in blood vessels. CAMs were treated with (a) vehicle control, (b) 100 nM, (c) 10 μM and (d) 30 μM of misoprostol and images obtained 60 minutes later. Note loss in visible blood vessels similar to that induced by PGE_{1&2} (bar 1 Fig. 8e). (e) Graphical representation of visible vessel intersections in CAMS treated with increasing concentrations of misoprostol, confirm that 10 μM and 30 μM but not <1 μM are effective. The Y-axis represents the number of visible vessels as a % of vehicle. The numbers within the bars represent number of CAMs/group analyzed for quantitation.

Fig. 9.

Inhibitors for EP₁, EP₂ and EP₄/TP receptors do not prevent loss in visible blood vessels. CAMs were pretreated for 30 minutes locally with vehicle control or receptor inhibitors as labeled in the figure prior to application of PGEs/vehicle. Images were obtained 60 minutes after PGEs were applied. (a) Images of CAM treated with 50 μ M PGE₂ show loss in visible blood vessels. (b) Application of PGE₂ on CAMs pretreated with receptor blockers (as marked) demonstrate loss in visible blood vessels (PGE₁ and misoprostol not imaged) (c) Graph showing pretreatment with EP₁, EP₂ and EP₄/TP antagonists fails to block loss in blood vessels in CAMs treated with PGE₁, PGE₂, or misoprostol. The numbers within the bars represent number of CAMs analyzed. The Y-axis represents the number of vessels intersecting the grid.

Fig. 10. EP₃ receptor antagonist (ONO-AE3-240) prevents loss in visible blood vessels with PGE₁. (a) images of CAM treated with vehicle for the EP₃ antagonist, PGE₁ alone and pretreated with EP₃ antagonist followed by PGE₁ in the same protocol for antagonists in Fig. 9. (b) Graph representing grid intersections (% vehicle) after treatments with EP₃ receptor antagonist and PGE₁. Note the increased numbers of visible vessels in CAMs pretreated with ONO-AE3-240 when compared to CAM treated with 50 μ M PGE₁ alone.

Fig. 11.

Thromboxane receptor (TP) antagonist (SQ 29,548) prevents loss of visible blood vessels when CAMs are treated with thromboxane agonist (U46619) or PGE. (a) CAMs with vehicle control, 150 nM or 1.5 μ M U46619 were imaged 60 minutes after application of treatments. (b) Loss of visible blood vessels by U46619 is reduced by pretreatment with TP antagonist SQ 29,548. (c) No differences in visible vessels were observed when comparing 10 μ M PGE₂ in CAMs pretreated with vehicle or TP antagonist. However at 50 μ M PGE₂ there was a reduction in the loss of visible blood vessels compared to pretreatment with SQ 29,548. Vessel numbers are quantified as % of vehicle i.e. those treated with vehicle for U46619 only. *p<0.01 when compared to pretreatment with corresponding vehicle.

Fig. 12.

EP₃ protein in CAMs. Western blots using polyclonal primary antibodies against EP₃ revealed immunospecific bands with molecular weights of ~78 kD which corresponds to the glycosylated form of the receptor. A fainter band is observed around 50 kD. Microsomes from rat kidney revealed bands of similar size. The representative image of a western using the polyclonal antibody is similar to 4 additional samples that were tested but not shown.