Prostacyclin Production In Vitro by Rabbit Aortic Endothelium: Correction for Unstirred Diffusional Layers

By Eric F. Grabowski, Gregory J. Naus, and Babette B. Weksler

The degree of mixing in fluid layers immediately adjacent to the endothelial surface is a major variable in assessment of prostacyclin (PGI₂) production by cultured endothelial cells or intact vessel endothelium in vitro. Lack of adequate mixing should lead to underestimation of true production because PGI₂ immediately adjacent to endothelium would be only poorly sampled upon buffer collection. Thoracic aortas from 38 New Zealand white rabbits were therefore excised, opened longitudinally, and mounted endothelial side uppermost in a buffer-filled chamber which excluded cut tissue edges from study. Production of PGI₂ under unstirred and magnetically stirred conditions was measured by radioimmunoassay (RIA) for 6-keto-PGF₁α. For animals pretreated with the combination of papaverine and heparin (see below), unstimulated and arachidonate-stimulated 6-keto-PGF₁α increased with stirring rate toward limits of 2.9 and 28.5 ng/cm²/min, respectively. Unstimulated and stimulated 6-keto-PGF₁α measured at 650 rpm, for example, were greater than their values at 0 rpm by factors of 3.5 (2P < .01) and 3.7 (2P < .001), respectively. The process of vessel excision, however, produces another variable: degree of injury to endothelium caused by such factors as secondary vessel contraction and thrombin generation. Vessel contraction and thrombin generation can be minimized, respectively, by the use of a smooth muscle relaxant and heparin administered prior to killing of the animals. The rabbits were, therefore, grouped according to intravenous (IV) treatment, prior to killing, with saline, papaverine (4 mg/kg), heparin (200 U/kg) or the combination of papaverine and heparin (same doses). As compared with pretreatment with saline, papaverine alone, or heparin alone, pretreatment with the combination of papaverine and saline led to increases in stimulated 6-keto-PGF₁α of 1.6- to 2.8-fold. By transmission electron microscopy, endothelium from animals pretreated with saline showed ultrastructural changes, including disruption of cytoplasm, separation without detachment of most endothelial cells from subendothelium, and focal areas of denudation. In contrast, ultrastructural integrity of endothelium was preserved in aortas of animals pretreated with combined papaverine and heparin. These results support the hypothesis that unstirred diffusional layers lead, in vitro, to underestimation of PGI₂ production, especially when vessels are protected from excisional injury. The effect of stirring on in vitro PGI₂ measurement is therefore considerable, and assays for true PGI₂ production rates must take into account degree of fluid mixing.

MATERIALS AND METHODS

Stirring chamber. Figure 1 depicts the in vitro chamber in which aortic segments were mounted for study. This template-like device utilizes rubber gaskets to exclude cut tissue edges from study, and permits access to four prescribed areas (each 0.5 cm²) of endothelium to be simultaneously exposed to buffer solutions. In contrast to earlier templates, the present template fixes the aortic segments in the vertical plane, so that the adjoining wells (each 0.5 mL) can each accommodate 1 mm × 5 mm siliconized stirring bars, driven two at a time by a single magnetic stirrer (Model PC-351, Dow Corning, Midland, Mich), which turn in 0.5-mm circular depressions so that no possible direct contact of bar with endothelium can occur. The chamber itself is heated in a small water bath seated upon the stirrer. Water at 37°C is driven by a pump (Model 2E-N, Little Giant Pump Co, Oklahoma City) from a larger water bath to the smaller, where a spill-over drain then returns the water to the larger bath. Stirring rate was calibrated by a stroboscope (Strobotec, Type 1531-A, General Radio, Concord, Mass). Following a five-minute warm-up period, there was approximate linearity between rpm and stirring setting over the range of rpm from 160 to 700. Reproducibility of a

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given rpm was approximately 3% at 350 rpm, the variability decreasing at higher rpm.

**Experimental groups.** Thirty-eight white New Zealand rabbits of either sex, weighing 2.85 to 3.85 kg, were fed commercial rabbit ration (Purina Rabbit Chow, Ralston Purina, St Louis) and water ad lib. They were divided into four groups according to the use of IV papaverine (Eli Lilly, Indianapolis) and heparin (Elkins Sinn, Cherry Hill, NJ). Injection of these drugs was made into the lateral ear vein 2.5 and 2.0 minutes, respectively, before the animals were killed. Group I animals received saline only (0.2 mL/kg); group II received papaverine only (4 mg/kg); group III received heparin only (200 U/kg); and group IV received papaverine and heparin at the same doses. Under the assumption of uniform distribution in extracellular water by the time of killing, the above papaverine dose, similar to a therapeutic human dose on a per weight basis, led to a decreasing at higher rpm. 

**Thoracic aorta.** Thirty-eight white New Zealand rabbits of either sex, weighing 2.85 to 3.85 kg, were fed commercial rabbit ration (Purina Rabbit Chow, Ralston Purina, St Louis) and water ad lib. They were divided into four groups according to the use of IV papaverine (Eli Lilly, Indianapolis) and heparin (Elkins Sinn, Cherry Hill, NJ). Injection of these drugs was made into the lateral ear vein 2.5 and 2.0 minutes, respectively, before the animals were killed. Group I animals received saline only (0.2 mL/kg); group II received papaverine only (4 mg/kg); group III received heparin only (200 U/kg); and group IV received papaverine and heparin at the same doses. Under the assumption of uniform distribution in extracellular water by the time of killing, the above papaverine dose, similar to a therapeutic human dose on a per weight basis, led to a calculated in vivo concentration (5 x 10^4 mol/L) which approximated that used in the initial (collection) buffer.

**Experimental procedure.** Thoracic aortas were carefully dissected free of extraneous connective tissue, opened along the posterior aspect between the rows of intercostal ostia, and then mounted in the chamber so that their endothelial surfaces were immediately bathed in 0.5 mL of buffer at 37 °C, pH 7.4, and at an oxygen tension of 134 ± 4.0 mm Hg (mean ± SD). Magnetic stirring rate was adjusted to a preselected value: 0, 160, 350, or 650 rpm for group IV aortas; or 0 or 350 rpm for aortas from the other three groups. Papaverine was absent from all buffer used subsequent to mounting of aortas in the chamber. Buffer in all wells was replaced by fresh buffer for a period of two minutes. The fresh buffer was collected for determination of unstimulated (baseline) PGI₂ production using RIA for 6-keto-PGF₁α. Buffer containing 25 µmol/L Na arachidonate was next added to each well for a period of two minutes and then also collected for RIA. The latter measurement yielded stimulated PGI₂ production. As many as four 0.5-cm² areas could be studied from the same thoracic aorta. Paired values for production of 6-keto-PGF₁α at 350 rpm from neighboring areas of seven group II thoracic aortas yielded a coefficient of variation of 0.17 for segments of the same aorta.

For three group IV aortas, the response to 25 µmol/L Na arachidonate was studied as a function of time using stirring rates of 0 and 350 rpm for segments of the same aorta (tested in parallel). After the addition of Na arachidonate at time zero, buffer was removed and completely replaced at time 2, 5, 7, 10, 12, 15, and 17 minutes. Buffer collected at 2, 7, 12, and 17 minutes was stored frozen for RIA.

For four other group IV aortas, the response to 0.5 U/mL (final concentration) human thrombin was determined at 350 rpm in order to aid in the interpretation of results involving heparin pretreatment. After the addition of thrombin solution (freshly thawed at 4 °C) at time zero, buffer was removed, completely replaced, and collected as above for Na arachidonate. **RIA.** Samples were thawed and allowed to incubate at 37 °C for one hour. Dilutions were made as necessary with standard buffer at the time of the RIA for 6-keto-PGF₁α. Details of the RIA itself are presented elsewhere. Results were expressed as nanograms of 6-keto-PGF₁α per square centimeter of endothelial surface, and were numerically equivalent to nanograms per 0.5 mL times two minutes, or milliliter times minutes. Range of sensitivity was 0.025 to 5.0 ng/cm²/min, depending on degree of sample dilution.

**Statistical methods.** Initial values for production of PGI₂ were compared, for the various stirring rates and animal pretreatments, using the two-tailed Student's t test, and the two-tailed sign test. **Endothelial removal by abrasion.** Endothelial integrity following mounting of vessels in the chamber was assessed in two ways. First, for each of two group IV aortas, a wire loop was used to abrade the endothelium in one of two adjacent wells for 40 seconds. Baseline and stimulated PGI₂ production were determined.

Second, for four other group IV aortas, the initial chamber buffer from each of two adjacent wells was collected after a period of ten minutes and examined for detached cells. The endothelium in one of the wells had always been abraded, as above, in order to provide positive controls. All buffer samples, diluted with an equal volume of 50% ethanol, were placed in a cytocentrifuge (Shandon Scientific Co. LTD. London) at 138 g for four minutes. Resulting stained (Papanicolau method) slides were examined in 20 fields at 450 x in order to determine the mean number of cells per field.

**Morphological studies.** Two aortas from each of groups I and IV were also examined by light microscopy, and by scanning and transmission electron microscopy utilizing methods previously described. In particular, specimens for transmission electron microscopy measured up to 2 mm in length. These were taken from the distal thoracic and abdominal aortas of each animal and were viewed with a Phillips 301 transmission electron microscope at an accelerating voltage of 80 kV.

**RESULTS**

**PGI₂ production and stirring.** For group IV aortas, values for unstimulated (baseline) and stimulated production of 6-keto-PGF₁α increased markedly with increased stirring rate (Fig 2). Values for baseline and stimulated production at 650 rpm, in fact, were greater than values at 0 rpm by...
PROSTACYCLIN AND BUFFER STIRRING

Fig 2. Rate of production of 6-keto PGF$_{1\alpha}$ vs rpm, where $N$ is the number of different aortas studied at a given rpm. Open and closed circles denote unstimulated and stimulated values, respectively. Increases in unstimulated production at 160, 350, and 650 rpm over production at 0 RPM had 2P values of >10, <.01, and <.01 by Student's t test. Corresponding 2P values for stimulated production were <.001, <.001, and <.001.

Because production of baseline and stimulated PG$_I_2$ appeared to approach a limit as rpm were increased, an alternative way to interpret the data is by means of a mathematical model which incorporates a limit as the number of rpms becomes arbitrarily large. A simple model is that production, $P$, of PG$_I_2$ is of the form: $P = P_\infty + K/rpm$, where $P_\infty$ is defined as production at an infinite stirring rate, and $K$ is a constant. Regression analysis of the baseline data for non-zero RPMs using this model leads to values for $P_\infty$ and $K$ of 2.9 ng/cm$^2$/min and −0.19 ng/cm$^2$/min$^2$, respectively. For the stimulated data, $P_\infty$ is 28.5 (3.9 times the unstirred production value), whereas $K$ is −1.85.

For non-group IV aortas, paired observations (at zero and 350 rpm for the same aorta) showed that stirring increased unstimulated production of 6-keto-PGF$_{1\alpha}$ 11 of 12 times ($2P < .01$ by the sign test). Similarly, stirring increased stimulated production 11 of 13 times ($2P < .05$).

Time dependence of PG$_I_2$ production. Figure 3 depicts stimulated production of 6-keto-PGF$_{1\alpha}$ as a function of time. For each of three group IV aortas at 0 rpm, the left panel shows that measured production reached a maximum sometime between the first and sixth minutes. For different segments of the same three aortas at 350 rpm, on the other hand, the right panel indicates that measured production peaked at some time within the first minute, with the subsequent decline having a half-time of approximately five minutes.

PG$_I_2$ production in the presence of thrombin. For each of the four aortas, production of 6-keto-PGF$_{1\alpha}$ during and following a two-minute exposure to 0.5 U/mL thrombin did not measurably increase over baseline. In fact, production values actually declined monotonically over time from baseline values (data not shown) in a manner identical to that seen when buffer was used as the “stimulating agent.” In terms of the sensitivity and precision of the assay, PG$_I_2$ production in the presence of thrombin was <1.0 ng/cm$^2$/min.

Endothelial removal by abrasion. Abrasion reduced basal PG$_I_2$ production for two aortas from 0.18 and 5.62 ng/cm$^2$/min to 0.16 and 3.41, respectively (average reduction of 25%). Stimulated production was reduced from 27.17 and 23.98 ng/cm$^2$/min to 4.01 and 5.18, respectively (average decrease of 82%).

By cytocentrifuge studies, chamber mounting without abrasion caused no measurable endothelial cell detachment for any of the four aortas examined. With abrasion (positive control), on the other hand, 8.0 ± 1.7 (mean ± SD) cells were seen per high-powered (450x) field.

Animal pretreatment. At thoracotomy, the great vessels of animals not pretreated with papaverine (groups I and III) appeared less distended than did the great vessels of rabbits.
of groups II and IV. Animals not treated with heparin (groups I and II) had clots in their thoracic aortas, whereas animals treated with heparin (groups III and IV) did not. All groups had occasional clots in abdominal aortas, especially distal to major branch points, and in the ostia of intercostal arteries. With stirring at 350 rpm, basal production of 6-keto-PGF$_{1\alpha}$ by aortic endothelium in the diffusion chamber was similar for all animal groups (Table 1). In contrast, stimulated production of 6-keto-PGF$_{1\alpha}$ in response to 25 $\mu$mol/L of Na arachidonate was greater for group IV aortas (combined papaverine and heparin pretreatment) than for groups I, II and III (Table 2). Ratios of group IV production to production for groups I, II, and III were 1.6, 2.0 and 2.8, respectively. Despite a suggestion that stimulated production with heparin pretreatment alone was reduced in comparison to saline controls, at the concentrations utilized neither papaverine nor heparin alone had any statistically significant effect on PG1$_2$ production (Table 2).

In the absence of stirring, pretreatment with papaverine and heparin had no significant effect on either basal or stimulated production of 6-keto-PGF$_{1\alpha}$ (Tables 1 and 2).

**Morphology.** Light microscopy and scanning electron microscopy showed confluent endothelium in both group I and group IV aortas. Transmission electron microscopy of group IV thoracic aortas confirmed the presence of ultrastructurally intact, confluent endothelium closely apposed to the subendothelium (Fig 4A). In contrast, group I thoracic aortas showed cytoplasmic disruption and extensive separation of endothelium from subendothelium with focal denudation (Fig 4B). These findings were confirmed in the abdominal aorta sections.

**DISCUSSION**

**Effects of controlled stirring.** Controlled stirring of the buffer adjacent to the endothelial surface of Group IV rabbit aortic segments mounted in the chamber markedly increased measurable levels of basal and stimulated 6-keto-PGF$_{1\alpha}$ at stirring rates of $\geq$160 rpm. The limit suggested at higher rates by the manner of increase in 6-keto-PGF$_{1\alpha}$ is consistent with the unstirred fluid layer hypothesis in that 6-keto-PGF$_{1\alpha}$ values should approach a limit asymptotically as stirring rate is increased. Unstirred layer phenomena, well known to chemical engineers and physiologists, are important in evaluating prostaglandin production at a tissue–fluid interface, whether by intact vessels in vitro or by cells in tissue culture. Given the theoretically hours-long diffusion times required for 6-keto-PGF$_{1\alpha}$ to approach a uniform concentration distribution in the diffusion chamber, stirring might have led to an increase measuring orders of magnitude. The smaller observed effect (up to 3.7-fold) likely reflected the presence even at 0 rpm of some degree of uncontrolled agitation, whether from pipetting, room vibrations, or natural convection (fluid motion in response to small-scale temperature gradients).

Additional benefits of stirring are likely to include more efficient delivery of exogenous substrate (Na arachidonate), improved oxygen delivery (for the same in-bulk oxygen tension of buffer), and greater uniformity of temperature. Sodium arachidonate, theoretically, also has an hours-long diffusion time to approach a uniform concentration distribution, one providing maximal concentrations in the fluid layers immediately adjacent to the endothelium. In the absence of stirring oxygen tensions should also be relatively lower and temperature should be relatively higher in the unstirred fluid layers. Such conditions predispose endothelium to greater PG1$_2$ production through hypoxia and promote mixing through natural convection. As such, they constitute additional explanations for the fact that the increase in 6-keto-PGF$_{1\alpha}$ values was not even greater in this in vitro system.

An alternative possibility that shear stress associated with stirring directly enhanced baseline and stimulated production of PG1$_2$ by endothelial cells is considered unlikely. We have observed that shear stresses of $\geq$2.7 dynes/cm$^2$ are required in order to yield peak production rates of PG1$_2$ (1.0 to 2.0 ng/cm$^2$/min) by monolayers of cultured endothelial cells comparable to the baseline production rates of the present work. Shear stresses in the present study were at

<table>
<thead>
<tr>
<th>Animal Pretreatment</th>
<th>RPM</th>
<th>I Saline</th>
<th>II Papaverine</th>
<th>III Heparin</th>
<th>IV Papaverine + Heparin</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td></td>
<td>0.9 $\pm$ 0.2 (7)</td>
<td>ND</td>
<td>1.4 $\pm$ 0.3 (6)</td>
<td>0.9 $\pm$ 0.2 (10)</td>
</tr>
<tr>
<td>350</td>
<td></td>
<td>2.4 $\pm$ 0.9 (8)</td>
<td>1.8 $\pm$ 0.4 (7)</td>
<td>2.3 $\pm$ 0.6 (6)</td>
<td>2.2 $\pm$ 0.4 (16)*</td>
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*2P $<$ 0.05 when compared to 0 RPM group IV values.

### Table 2. Stimulated (25 $\mu$g Na Arachidonate) 6-Keto PGF$_{1\alpha}$ (mean $\pm$ SE, N), ng/cm$^2$ x min

<table>
<thead>
<tr>
<th>Animal Pretreatment</th>
<th>RPM</th>
<th>I Saline</th>
<th>II Papaverine</th>
<th>III Heparin</th>
<th>IV Papaverine + Heparin</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td></td>
<td>7.2 $\pm$ 1.4 (7)</td>
<td>ND</td>
<td>5.0 $\pm$ 0.9 (6)</td>
<td>7.2 $\pm$ 1.0 (11)</td>
</tr>
<tr>
<td>350</td>
<td></td>
<td>14.0 $\pm$ 2.5 (9)</td>
<td>11.1 $\pm$ 2.4 (7)</td>
<td>8.0 $\pm$ 2.3 (6)</td>
<td>22.6 $\pm$ 2.3 (16)*</td>
</tr>
</tbody>
</table>

*2P $<$ 0.001 when compared to 0 RPM group IV values; 2P $<$ 0.05, 0.01, and 0.005 when compared to 350 RPM values for groups I, II and III, respectively.
These workers found peak production to occur at four minutes at 1.0 mL/min, but at two minutes at 20 mL/min. This is in agreement with principles of convective mass transfer, in which a greater flow rate reduces the thickness of unstirred diffusional layers.

Influence of papaverine and heparin. Our data obtained in the presence of stirring (Tables 1 and 2), indicate that treatment with the combination of papaverine and heparin before the animals are killed results in a 1.6-fold augmentation of stimulated (25 μmol/L of Na arachidonate) production of PG12 by rabbit aorta in vitro over saline controls. At the drug concentrations used, neither drug alone yielded a significant change in this production. This synergistic effect of papaverine and heparin suggests a need to protect the vessel wall from effects of sacrifice and excision not prevented by either drug alone.

Papaverine in combination with heparin has recently been used to protect aortic and venous endothelium from injury and detachment accompanying vessel excision for grafting. Haudenschild et al have reported that sheath cell separation and exposure of subendothelium in untreated, excised rabbit carotid arteries examined by light and electron microscopy. The endothelium of such vessels was better preserved following vessel perfusion with heparinized (4 U/mL) solutions containing papaverine HCl (0.53 and 1.58 x 10^-7 mol/L) prior to excision. Similar observations have been made with respect to vein grafts from dogs and human saphenous veins.

Papaverine is a well-recognized, nonspecific smooth muscle vasodilator that inhibits a cyclic nucleotide phosphodiesterase found in many tissues, resulting in elevation of the intracellular concentration of cyclic adenosine 3',5'-monophosphate (cyclic AMP). Used at a concentration that relaxed vascular smooth muscle, alone or in combination with heparin, papaverine did not affect basal production of PG12 by rabbit aorta in vitro.

By blocking thrombin generation, heparin may prevent both direct and platelet-mediated injury to rabbit aortic endothelium caused by thrombin and characterized by disruption of the superficial fibrillary elastin layers, endotheial cell separation, and contraction. Thrombin has been demonstrated to be a potent vasoconstrictor of both isolated dog cerebral arteries and ischemic dog coronary arteries. Buchanan et al have reported that thrombin generation can lead to uncontrolled PG12 production by segments of whole rabbit aorta studied in vitro, production which can be prevented by administration of heparin to animals before they are killed. However, in our studies, production of PG12 did not increase over basal values when rabbit thoracic aorta was exposed to human thrombin.

Heparin, on the other hand, may have vessel wall effects independent of its known interaction with thrombin. Dog coronary artery strips, for example, undergo a dose-related PG12-related relaxation upon exposure to heparin. In the absence of stirring, we found no significant effect of pretreatment on unstimulated or stimulated PG12 production including pretreatment with the combination of papaverine and heparin (Tables 1 and 2). That combined papaverine and heparin pretreatment augments PG12 production only in the presence of stirring likely results from the fact that stirring...

**Fig 4.** (A) Transmission electron micrograph of group IV thoracic aorta showing intact endothelium (E) closely apposed to the subendothelium directly above the internal elastic lamina (IEL). A medial smooth muscle cell (SMC) is present below the internal elastic lamina. Scale bar is 1 μm. (B) Transmission electron micrograph of group I thoracic aorta showing cytoplasmic disruption (arrows) of an endothelial cell (E) with separation (arrow heads) from subendothelium and internal elastic lamina (IEL). Scale bar is 1 μm.
allows more accurate measurement of PGI₂ production rates.

In the absence of combined papaverine and heparin pretreatment, on the other hand, stirring still significantly enhanced both unstimulated and stimulated PGI₂ production.

**Endothelial removal by abrasion.** The studies involving endothelial abrasion show that endothelial cells present after vessel mounting in the chamber were indeed the source of most of the stimulated PGI₂ production. That portion of the basal production not eliminated by abrasion may have been due to some combination of residual endothelial cells and exposed smooth muscle cells.

**Morphology.** The morphological observations by transmission electron microscopy of preserved endothelial ultrastructure, and preserved apposition of confluent endothelium to subendothelium with pretreatment with the combination of papaverine and heparin but not with saline, is consistent with earlier observations by light and electron microscopy.¹⁶,¹⁷ Our observations raise the possibility that endothelial cyclooxygenase and PGI₂ synthetase, critical enzymes in PGI₂ production, may also have been better preserved.

**APPENDIX**

*Estimation of level of shear stress on aortic endothelium within the diffusion cell.* The minimum spacing, L, between the aortic endothelial surface and the tip of the stirring bar is fixed, by the chamber's circular depression for each stirring bar, at 0.2 cm. The maximum shear stress (fluid friction), τ, on the endothelial surface, is, therefore,

\[ \tau = \nu \left( \frac{V}{L} \right), \]

where \( \nu \) is buffer viscosity and \( V \) is the velocity of the stirring bar tip. The velocity \( V \) is given by

\[ V = \omega A, \]

where \( \omega \) is angular velocity in radians per second, and \( A \) is the stirring bar half-length. For \( \omega = 36.6 \) radians per second (350 rpm), \( A = 0.25 \) cm, and \( \nu = 0.01 \) g/cm/s, one obtains

\[ V = 9.2 \text{ cm/s}, \]

\[ \tau = 0.09 \text{ dynes/cm}^2. \]

**ACKNOWLEDGMENT**

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