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

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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 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), unstirulated and arachidonate-stimulated 6-keto-PGF₁α increased with stirring rate toward limits of 2.9 and 28.5 ng/cm²/min, respectively. Unstimulated arachidonate-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 combination 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 up to four prescribed areas (each 0.5 cm²) of endothelial cells 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 x 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 (Strobotac, Type 1531-A, General Radio, Concord, Mass). Following a five-minute warm-up period, there was approximate linearity between rpm and stirrer setting over the range of rpm from 160 to 700. Reproducibility of a
calculated in vivo concentration (5 x 10^-mol/L) which approxi-
cellular water by the time of killing, the above papaverine dose,
similar to a therapeutic human dose on a per weight basis, led to a
same doses. Under the assumption of uniform distribution in extra-
given rpm was approximately 3% at 350 rpm, the variability
vascular resistance was first observed at 10 mol/L of papaverine.
vascular resistance for perfusate papaverine concentrations over the
osmolarity under constant flow, and changes in aortic pressure were
ear vein 2.5 and 2.0 minutes, respectively, before the animals were
mated that used in the initial (collection) buffer.
was adopted for the present work as a concentration to
mol/L range from 0 to 10 mol/L. A significant reduction (20%) in
used to estimate changes in vascular resistance. The net change in
lated rabbit hearts were subjected to step changes in perfusate
based on earlier experiments in which Langendorff-perfused, iso-
left for 40 seconds. Baseline
PGI2 production and stirring. For group IV aortas, a wire loop was used to abrade
endothelium in one of two adjacent wells for 40 seconds. Baseline
PGI2 production. As many as four 0.5-cm² areas could be studied
for 6-keto-PGF1α. Buffer containing 25 μmol/L Na arachidon-
unstimulated (baseline) PGI2 production using RIA for 6-keto-PGF1α. Buffer containing 25 μmol/L Na arachidon-
ated for determination of unstimulated (baseline) PGI2 production using
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 arachidon-
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 arachidon-
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
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 follow-
ing 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
positive controls. All buffer samples, diluted with an equal volume of
all animals were killed by an overdose of Na pentobarbital (60 mg/kg, IV). For all groups, excised thoracic
aortas were collected into cold (4 °C) buffer: 10 mmol/L of Heps-
buffered normal saline with 11.1 mmol/L of dextrose, 4.0 mmol/L of
KCl, 1.8 mmol/L of calcium chloride, and 1.0 mmol/L of
magnesium chloride. For group II and IV aortas, 80 μmol/L of
papaverine HCl was added to the buffer. After thoracotomy, group
II and IV animals also received 7 mL of buffer plus papaverine by
intracardiac infusion. Aortas of all animals were studied within 30
minutes of collection.
The concentration of papaverine that was added to the buffer was
based on earlier experiments in which Langendorff-perfused, iso-
lated rabbit hearts were subjected to step changes in perfusate
osmolarity under constant flow, and changes in aortic pressure were
used to estimate changes in vascular resistance. The net change in
aortic pressure was used to estimate the fractional change in
vascular resistance for perfusate papaverine concentrations over the
range from 0 to 10^-4 mol/L. A significant reduction (20%) in
vascular resistance was first observed at 10^-3 mol/L of papaverine.
Consequently, a papaverine concentration between 10^-5 and 10^-4
mol/L was adopted for the present work as a concentration to
promote vascular relaxation.
Experimental procedure. Thoracic aortas were carefully dis-
sected free of extraneous connective tissue, opened along the poste-
rior aspect between the rows of intercostal osta, 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 ± 40 mm Hg (mean ± SD). Magnetic stirring rate
was adjusted to a preselected value: 0, 160, 350, or 650 rpm for group
IV aortas; 0 or 350 rpm for aortas from the other three groups.
Papaverine was absent from all buffer used subsequent to mounting

Fig 1. Stirring chamber (7.9 x 2.6 x 3.2 cm), opened. Left portion is scored for convenience of tissue alignment; right portion contains four buffer wells, accessed from top, and shows thumb screws in place.
Factors of 3.5 and 3.7, respectively. Although the SDs of baseline and stimulated production values increased with rpm, coefficients of variation did not: SDs were proportional to the production values themselves. In the case of stimulated production, for instance, coefficients of variation at 0, 160, 350, and 650 rpm were 0.53, 0.26, 0.50, and 0.49, respectively.

Because production of baseline and stimulated PGF\textsubscript{2\alpha} 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 rpm becomes arbitrarily large. A simple model is that production, \( P \), of PGF\textsubscript{2\alpha} is of the form: \( P = P_0 + K/\text{rpm} \), where \( P_0 \) is defined as production at an infinite stirring rate, and \( K \) is a constant. Regression analysis of the baseline data for non-zero rpm using this model leads to values for \( P_0 \) and \( K \) of 2.9 ng/cm\textsuperscript{2}/min and \(-0.19\) ng/cm\textsuperscript{2}/min\textsuperscript{2}, respectively. For the stimulated data, \( P_0 \) 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 unstirred production of 6-keto-PGF\textsubscript{1\alpha} 11 of 12 times (2\( P \) < .01 by the sign test). Similarly, stirring increased stimulated production 11 of 13 times (2\( P \) < .05).

**Time dependence of PGF\textsubscript{2\alpha} production.** Figure 3 depicts stimulated production of 6-keto-PGF\textsubscript{1\alpha} as a function of time. For each of the four aortas, production of 6-keto-PGF\textsubscript{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, PGF\textsubscript{2\alpha} production in the presence of thrombin was <1.0 ng/cm\textsuperscript{2}/min.

**Endothelial removal by abrasion.** Abrasion reduced basal PGF\textsubscript{2\alpha} production for two aortas from 0.18 and 5.62 ng/cm\textsuperscript{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\textsuperscript{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 (450×) 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.
respectively. 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 µmol/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 PGI$_2$ 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 I 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 rates of basal and stimulated 6-keto-PGF$_{1\alpha}$ at stirring rates of ≥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$^9$ and physiologists,$^{10}$ 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 PGI$_2$ 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 basal and stimulated production of PGI$_2$ by endothelial cells is considered unlikely. We have observed that shear stresses of ≥2.7 dynes/cm$^2$ are required in order to yield peak production rates of PGI$_2$ (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.$^{12,13}$ Shear stresses in the present study were at

| Table 1. Unstimulated 6-Keto PGF$_{1\alpha}$ (mean ± SE, N), ng/cm$^2$ x min |
|-----------------------|----------------|----------------|----------------|
| RPM  | I Saline | II Papaverine | III Heparin | IV Papaverine + Heparin |
| 0    | 0.9 ± 0.2 (7) | ND | 1.4 ± 0.3 (6) | 0.9 ± 0.2 (10) |
| 350  | 2.4 ± 0.9 (8) | 1.8 ± 0.4 (7) | 2.3 ± 0.6 (6) | 2.2 ± 0.4 (16)* |

*2P < 0.05 when compared to 0 RPM group IV values.

| Table 2. Stimulated (25 µmol Na Arachidonate) 6-Keto PGF$_{1\alpha}$ (mean ± SE, N), ng/cm$^2$ x min |
|-----------------------|----------------|----------------|----------------|
| RPM  | I Saline | II Papaverine | III Heparin | IV Papaverine + Heparin |
| 0    | 7.2 ± 1.4 (7) | ND | 5.0 ± 0.9 (6) | 7.2 ± 1.0 (11) |
| 350  | 14.0 ± 2.5 (9) | 11.1 ± 2.4 (7) | 8.0 ± 2.3 (6) | 22.6 ± 2.3 (16)* |

*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.15

**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 augmenta-
tion of stimulated (25 μmol/L of Na arachidonate) produc-
tion of PGI2 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 pre-
vented 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 al16 have reported significant endothelial
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 HCI (0.53 and
1.58 × 10−7 mol/L) prior to excision. Similar observations
have been made with respect to vein grafts from dogs18,17 and
human saphenous veins.16

Papaverine is a well-recognized, nonspecific smooth mus-
cle vasodilator that inhibits a cyclic nucleotide phosphodies-
terase 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
PGI2 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 dis-
ruption of the superficial fibrillary elastin layers,4 endothelial
cell separation,7 and contraction.10,11 Thrombin has been
demonstrated to be a potent vasoconstrictor of both isolated
dog cerebral arteries13 and ischemic dog coronary arteries.13
Buchanan et al12 have reported that thrombin generation can
lead to uncontrolled PGI2 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 PGI2
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
concentration of PGI2 by rabbit aorta in vitro.

In the absence of stirring, we found no significant effect of
pretreatment on unstimulated or stimulated PGI2 production
including pretreatment with the combination of papaverine
and heparin (Tables 1 and 2). That combined papaverine and
heparin pretreatment augments PGI2 production only in the
presence of stirring likely results from the fact that stirring

most on the order of 0.1 dyne/cm² (Appendix). Moreover,
nearly doubling the stirring rate (from 350 to 650 rpm) did
not lead to a further significant increase in PGI2 production.
Our data12,13 suggest that one might expect such an increase
only for shear stresses of the order of 1.0 dyne/cm² or
greater.

Correction of unstirred diffusional layers demonstrates that
the true maximal production rate of 6-keto-PGF1α is
reached rapidly, long before the artifactual maximum at six
minutes in the unstirred system. Not only does the measured
production rate rise much more rapidly, peaking by one
minute, but it also declines more rapidly (Fig 3). Conse-
quentially, the values of Fig 2 may be interpreted to be “peak”
values in time, at least for the two highest RPMs. The areas
under the curves of the right panel of Fig 3 average 60% more
than the corresponding areas of the left panel. This simply
reflects the fact that controlled stirring allows earlier detec-
tion of the 6-keto-PGF1α produced during the 16 minutes
depicted. Recently, Kent et al14 noted but could not explain a
flow-rate dependence of peak PGI2 production by rabbit
aorta perfused with buffer containing Na arachidonate.
These workers found peak production to occur at four
allows more accurate measurement of PGI₂ production rates.

In the absence of combined papaverine and heparin pre-
treatment, on the other hand, stirring still significantly
enhanced both unstimulated and stimulated PGI₂ produc-
tion.

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 trans-
mission electron microscopy of preserved endothelial ultra-
structure, 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 micros-
copy. Our observations raise the possibility that endothe-
lial cyclooxygenase and PGI₂ synthetase, critical enzymes in
PGI₂ production, may also have been better preserved.

APPENDIX

Estimation of level of shear stress on aortic endothe-
ilum 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
traction), τ, on the endothelial surface, is, therefore,

\[ \tau = \nu \frac{V}{L}, \]

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

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

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

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