Endothelial Prostacyclin Production Is a Late Event in Granulocyte Migration Into Bovine Pulmonary Artery Intimal Explants

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Whether migration of granulocytes across pulmonary vascular endothelium in the absence of structural evidence of endothelial injury causes increased production of thromboxane or prostacyclin is not known. Using bovine pulmonary artery intimal explants mounted in Boyden chambers and homologous separated granulocytes, concentrations of thromboxane B2 and 6-keto-PGF1α in the upper-well fluid were measured by radioimmunoassay over a three-hour period under the following conditions: (1) granulocyte chemotaxis (zymosan-activated plasma in the lower well, granulocytes in the upper well); (2) unstimulated granulocyte migration (serum or plasma in the lower well, granulocytes in the upper well); (3) granulocyte activation without migration (zymosan-activated plasma and granulocytes in the upper well); (4) granulocyte chemotaxis in the absence of endothelium (identical to condition 1 above except that endothelium was scraped from the explant surface); and (5) explants incubated in the absence of granulocytes. Minimal increases in thromboxane B2 concentrations in upper-well fluid occurred under all conditions. In contrast, granulocyte chemotaxis was accompanied by large increases in concentrations of 6-keto-PGF1α evident by two hours of incubation and increasing markedly by three hours, to 524.3 ± 69.0 ng/mL (m ± SEM). Unstimulated migration of granulocytes toward serum or plasma and granulocyte activation without migration were accompanied, at three hours, by more modest increases in 6-keto-PGF1α (296.5 ± 46.4; 128.0 ± 38.8, and 236.7 ± 47.0 ng/mL, respectively) and, in the absence of granulocytes or in the absence of endothelium, only minimal increases in this prostacyclin metabolite occurred (137.2 ± 16.9 and 53.9 ± 12.6 ng/mL, respectively). The large rises in prostacyclin metabolite occurred at a time when the majority of granulocytes had migrated through the endothelial layer rather than during their adherence or transendothelial passage. We conclude that chemotaxis of granulocytes through pulmonary vascular endothelium causes endothelial production of large amounts of prostacyclin, but this occurs late in the chemotactic process, after granulocytes have transversed the endothelium.

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tive tissue down. The filters were 13 mm in diameter and had a pore diameter of 12 μm (Sartorius Filters, Hayward, Calif). The filters and explants were placed in chemotaxis chambers, endothelium uppermost. The lower well of the chamber contained either 20% homologous zymosan-activated plasma, 20% homologous heat-inactivated plasma, 20% homologous bovine plasma, or 10% fetal calf serum in medium 199. Since the results with heat-inactivated plasma were similar to those for plasma, only the latter are shown. Medium 199 with the addition of 10% fetal calf serum was added to the upper wells and the chambers were incubated at 37 °C for 60 minutes in 5% CO₂ in air to allow the endothelial cell layer to equilibrate prior to the start of experimentation.

Preparation of zymosan-activated plasma. Heparinized (20 U/mL) bovine blood was centrifuged and the plasma was removed and incubated with zymosan (10 mg/mL; Sigma Chemical Co, St Louis) at 37 °C for 45 minutes. After incubation, the zymosan was removed by centrifugation (2,000 rpm for 15 minutes) followed by Millipore filtration (0.45-μm pore size; Millipore Corp, Bedford, Mass). Some plasma was heat-inactivated by incubation at 60 °C for 45 minutes. This plasma was then centrifuged and filtered as outlined above.

Separation and labeling of bovine granulocytes. Granulocytes were isolated by a modification of the method of Weening et al. Heparinized blood was centrifuged and the buffy coat was removed and added to an equal volume of 6% dextran in Hanks’ balanced salt solution (HBSS). This suspension was漂浮 onto Histopaque-1077 (Sigma Chemical Co) and centrifuged. RBCs that passed through the gradient were lysed with ammonium chloride. The remaining granulocytes were washed twice in HBSS. Direct counts of granulocyte numbers were made using a hemocytometer. Wright’s stained smears of the preparations showed that the pellets contained >95% granulocytes, of which >95% were viable as judged by trypan blue dye exclusion.

Radioimmunoassay for 6-keto-PGF₁α and TxB₂. After appropriate dilutions, 6-keto-PGF₁α and TxB₂ in the aliquots taken from the upper well of the chemotaxis chambers were determined by radioimmunoassay using 6-keto-PGF₁α(121) histamine and TxB₂-(121) histamine as tracers. The specific activity of these tracers is ≥2,500 μCi/μg. Each assay was carried out in triplicate. The antibodies to 6-keto-PGF₁α bovine serum albumin (BSA) and TxB₂ BSA were produced as recently described. The detection limits are 0.4 and 4.0 pg and the B/Bo at 50% displacement is 8 and 22 pg for 6-keto-PGF₁α and TxB₂ respectively. The cross-reactivity of the antiserum to 6-keto-PGF₁α is 6.8% dinor-6-keto-PGF₁α; 5.5% PGF₂α; 0.2% 6,15-diketo-13,14-dihydro-PGF₁α; 0.12% 4,13-diketo-11,12-dihydroprostanoic acid; 0.01% PGE₂; 0.009% 6-keto-PGE₁; and 0.006% TXB₂; that of the antiserum to TxB₂ is 0.39% PGD₂; 0.05% PGE₂; 0.067% PGG₂; 56% dinor-TXB₂; 0.12% 6-keto-PGF₁α; 0.23% 6-keto-13,14-dihydro-PGF₁α; 0.03% 6,15-diketo-PGF₁α; 0.009% 6,15-diketo-13,14-dihydro-PGF₁α; and 0.009% 6,15-diketo-13,14-dihydro-PGF₁α.

Experimental protocols. After equilibration, the medium in the upper wells and the chambers were incubated at 37 °C for 60 minutes. This incubation was then continued by marked accumulation of the prostacyclin metabolite, 6-keto-PGF₁α, in the upper well of chemotaxis chambers (Fig 1). When zymosan-activated plasma was placed in the lower well and granulocytes in the upper well of the chamber, accumulation of 6-keto-PGF₁α was modest over the first 30 minutes but its accumulation became striking with longer incubation periods, reaching a mean value of 524.3 ± 69.0 ng/mL (± SEM) at three hours (Fig 1). Unstimulated or random migration toward either serum or plasma in the lower well was associated with more modest increases in 6-keto-PGF₁α, the levels being strikingly and significantly less after three hours’ incubation than seen with granulocyte chemotaxis (serum, 296.5 ± 46.4 ng/mL; plasma, 128.0 ± 38.6 ng/mL). When granulocytes were stimulated but migration was inhibited (zymosan-activated plasma and granulocytes in the upper well), the rate of 6-keto-PGF₁α accumulation was similar to that seen with random migration (three hours, 236.7 ± 47.0 ng/mL) (Fig 2). Prostacyclin production by the intimal explant in the absence of granulocytes was minimal over the three-hour period of incubation.
whether fetal calf serum (three hours, 118.2 ± 11.7 ng/mL) or zymosan-activated plasma (three hours, 169.7 ± 18.9 ng/mL) was in the lower well. Since 6-keto-PGF₁α concentrations were not significantly different between the groups, the combined data from both of these groups is shown in Fig 1.

When granulocytes were incubated either alone or in the presence of 20% zymosan-activated plasma, even after three hours of incubation, accumulation of either 6-keto-PGF₁α and TxB₂ in the supernatant was <0.05 ng/mL.

To ascertain whether the endothelial cells per se were the source of the increased levels of prostacyclin, intimal explants whose endothelial layer had been removed with a scalpel blade prior to experimentation were studied under conditions of granulocyte chemotaxis (zymosan-activated plasma in the lower well and granulocytes in the upper well). Accumulation of 6-keto-PGF₁α was minimal over the three hours of incubation (53.9 ± 12.6 ng/mL) (Fig 2).

**Accumulation of TxB₂.** Chemotaxis and/or random migration of granulocytes across an intact endothelial layer did not cause a significant increase in TxB₂ accumulation in the upper well of the chamber (Fig 3). In the absence of granulocytes and under conditions in which granulocyte migration was inhibited, accumulation of TxB₂ was also minimal. The combined data for serum (three hours, 6.0 ± 2.7 ng/mL) and for zymosan-activated plasma in the lower well (three hours, 4.0 ± 0.4 ng/mL) is shown in Fig 3.

**DISCUSSION**

Using both scanning and transmission electron microscopy, we have shown previously that the endothelial layer of bovine pulmonary artery intimal explants is continuous and "tight" junctions between endothelial cells are maintained. Additionally, we have shown, using such explants mounted in chemotaxis chambers and ³²Cr-labeled granulocytes, that granulocyte chemotaxis across an endothelial cell layer reached a plateau between the second and third hours of incubation, when approximately 50% of the label was in the explant. Unstimulated or random granulocyte migration was lower and continued to increase to approximately 35% over a three-hour incubation period. Inhibition of granulocyte migration (granulocytes and zymosan-activated plasma in the upper well) revealed a plateau from one hour, when approximately 20% of the label was in the explant. Removal of the endothelium by gentle scraping resulted in a rate of granulocyte chemotaxis similar to that seen into the intact explant.

Granulocyte chemotaxis and prostacyclin production. The present study followed the time course of both prostacyclin and thromboxane production during granulocyte chemotaxis toward zymosan-activated plasma and random migration across an intact endothelial cell layer of pulmonary artery intimal explants. Granulocyte chemotaxis and, to a lesser extent, unstimulated granulocyte migration led to striking accumulations of prostacyclin, but not thromboxane, in the upper well of the chemotaxis chamber after two to three hours of incubation. Under conditions in which
granulocytes were stimulated but migration was inhibited by placing zymosan-activated plasma in the upper well, prostacyclin production was similar to that seen with random migration. In the absence of granulocytes (ie, with the explant alone) only a small accumulation of prostacyclin was seen over the three-hour period of study. In the absence of endothelium, but in the presence of granulocyte migration, prostacyclin accumulation was minimal. These findings indicate that passage of granulocytes across the endothelial cell layer is followed by a marked increase in prostacyclin accumulation and that the endothelial cells are the major source of prostacyclin.

Under conditions of granulocyte chemotaxis, the present study has shown a marked increase in prostacyclin production occurring between the second and third hours of incubation, a time at which we have shown previously, using $^{51}$Cr-labeled granulocytes, that chemotaxis of granulocytes toward zymosan-activated plasma has reached a plateau, and additionally, by electron microscopy, that the majority of granulocytes are through the endothelial cell layer and its basal lamina. Thus, increased production of prostacyclin is a late event in granulocyte chemotaxis across an endothelial layer, occurring mainly after migration has occurred. This finding correlates well with our observations in sheep given endotoxin. In these experiments, migration of granulocytes into the interstitium occurs as early as 30 minutes following the start of endotoxin infusion and prior to the peak increase in prostacyclin concentrations in lung lymph, which occurs between 60 and 90 minutes.

Zymosan-activated plasma is known to cause activation of granulocytes both in vivo and in vitro. Our results with zymosan-activated plasma in the upper well show that granulocyte activation and interaction of activated granulocytes with the endothelial surface does cause a moderate increase in prostacyclin production, but the increase is strikingly higher when chemotaxis of granulocytes occurs. Thus, maximum generation of prostacyclin requires that granulocytes migrate through an endothelial cell layer, since adherence of activated granulocytes to the endothelial surface is a less potent stimulus for prostacyclin production than chemotaxis and maximum concentrations of prostacyclin occur late in the chemotactic process.

From in vitro studies, it is known that the major source of prostacyclin is the endothelial cell. Harlan and Callahan have recently shown that granulocytes stimulated with phorbol myristate acetate cause cultured bovine endothelial cells to release prostacyclin and that this release is H$_2$O$_2$-dependent. Our findings also show that stimulated granulocytes cause increased production of prostacyclin by the endothelial cell. However, following granulocyte migration through the endothelial cell layer (chemotaxis), prostacyclin production is markedly enhanced. Thus, our studies indicate that for such large amounts of prostacyclin to be released, granulocytes must achieve an abluminal position.

In vitro experiments have shown previously that both the aortic and pulmonary artery walls stripped of endothelium can produce modest amounts of prostacyclin. Thus, it is possible that the endothelial cell is not the sole source of prostacyclin but that some other cell type in the subendothelial or arterial media contributes to its production. Additionally, it has been demonstrated, using immunofluorescence staining, that levels of prostacyclin synthase are similar throughout the entire intima and media of vessel walls. However, our results with scraped explants strongly suggest that the increased levels of prostacyclin in these studies were derived principally from endothelial cells. Furthermore, since the amount of prostacyclin that accumulated was greater following chemotaxis than following random migration, the number of granulocytes that obtain an abluminal position may determine the amount of prostacyclin produced.

Mechanism of increased prostacyclin production. The mechanism for increased production of prostacyclin is not certain. Endothelial cells grown in culture when stimulated by thrombin, bradykinin, and histamine produce increased amounts of prostacyclin. It is possible, but not likely, that such mediators were present in our preparations. The increase in prostacyclin production in the present study far exceeds the values reported in those earlier studies even when the endothelial cell number (the endothelial cell number on the exposed surface of an intimal explant of 8 mm diameter is approximately 500,000 cells) is taken into account. Additionally, the timing of the marked increase in prostacyclin metabolite is much later than that reported in those studies. Thus, presence of mediators that stimulate prostacyclin production could contribute to, but would not seem to account entirely for, the increased prostacyclin production seen following granulocyte chemotaxis. The present data also confirm the findings of Goldsmith et al who demonstrated that prostacyclin production by ex vivo arterial segments is markedly greater than that of the endothelial monolayers.

The relative lateness of the increase in prostacyclin seen in the present study may be explained by the availability of arachidonate. It may be that granulocytes caused release of arachidonate either from themselves or from other cells when they burrowed through the endothelium, subendothelium or media of the explants that were utilized by the abluminal aspect of the endothelium. Alternatively, it may be that granulocytes caused release of arachidonate from endothelial cell membranes, but only on contact with the abluminal surface of endothelial cells. Under normal in vivo conditions, a glycosylxy covers the luminal surface of endothelial cells and could act as a barrier to granulocyte-endothelial interactions, whereas a glycosylxy is not found on the abluminal surface and thus interactions that trigger arachidonate release could occur more readily.

The consequences of the increase in prostacyclin production are also obscure. Migration of granulocytes through an endothelial layer occurs in the absence of structural evidence of endothelial cell damage but may still cause subtle perturbations in endothelial metabolism and, certainly, changes in cell shape. Release of the vasodilator prostacyclin, after migration has occurred, could hasten the return of the endothelial layer to its normal configuration. Alternatively, the principal physiologic effect of prostacyclin release could be an effect on microvessels distal to the site of prostacyclin production.
Granulocyte chemotaxis and thromboxane production. The results of the present study show little increase in thromboxane production during either chemotaxis or random migration of granulocytes. This is not unexpected, since both the granulocyte and the endothelial cell have been shown to synthesize only small amounts of thromboxane. The major source of thromboxane is the platelet and the small increases shown here perhaps reflect the presence of platelets in the granulocyte preparation. Platelets are a common contaminant of separated granulocytes and in our preparations were usually present in a 1:1 or 2:1 ratio with granulocytes (5,000 to 10,000 platelets per cubic millimeter).

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