Prostacyclin Production by Endothelial Cells
Effects of Sera from Normal and Hyperlipidemic Subjects

Elena Tremoli, Eric A. Jaffe, Karen Tack Goldman, and Babette B. Weksler

The influence of hyperlipidemic sera on prostacyclin (PGI2) production by cultured endothelial cells was assessed by comparing sera from three types of hyperlipidemias with sera from normal subjects. Sera prepared from normal whole blood (WBS), platelet-rich plasma (PRPS), and platelet-poor plasma (PPPS) were also compared. Bovine aortic endothelial cells (BAEC) incubated with 25% WBS increased PGI2 synthesis significantly within 1 hour, with little further increase by 16 hours; human umbilical vein endothelial cells (HUEC) incubated with 25% WBS for 1 hour showed no elevation in PGI2, whereas PGI2 levels increased substantially after 16 hours. PPPS and PRPS stimulated PGI2 synthesis by BAEC equally at 1 hour. However, there was no rise in PGI2 after PPPS in HUEC; PGI2 rose after 16 hours with PRPS and rose further with WBS after 16 hours.

Since WBS best enhanced PGI2 production in human endothelial cells, it was chosen for comparison of the effects of hyperlipidemic and normolipidemic sera. PGI2 synthesis by HUEC significantly increased upon incubation with WBS from Types Mb and IV patients in comparison to WBS from Type Ila hypercholesterolemic patients or normal controls. In contrast, WBS from all these hyperlipidemic subjects stimulated PGI2 synthesis by BAEC similarly to WBS from controls. We conclude that incubation of human endothelial cells with WBS containing high levels of atherogenic lipoproteins does not reduce PGI2 formation by the cells. Moreover, the time course and the contribution of lipid, plasma, or cellular factors to PGI2 formation vary according to the cell type tested. Caution should be exercised in extrapolating results achieved with serum and cells from the same species to other settings.

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Endothelial cells produce prostacyclin (PGI2), a vasodilator and inhibitor of platelet aggregation.1,2 Several factors, including the availability of substrate arachidonic acid in membrane phospholipids, regulate the capacity of endothelial cells to produce PGI2.3 Enrichment of the cells with arachidonic acid increases their capacity to produce PGI2 after stimulation.3 Studies with separated lipoproteins have shown that high density lipoproteins (HDL) can provide arachidonic acid for PGI2 synthesis by porcine and human endothelial cells, while low density lipoproteins (LDL) apparently have a negligible effect under the same experimental conditions.4,5 In other studies, incubation of human umbilical cord endothelial cells (HUEC) with low density lipoproteins diminished PGI2 production,6 but this effect may have reflected oxidation of LDL during isolation.7 Since endothelial cells in vivo are not exposed to isolated lipoprotein fractions, and little information is available on the effect of hyperlipidemic sera on PGI2 production by endothelial cells, the influence of different serum lipoprotein patterns on PGI2 formation by cells requires further examination. Clarification of such effects takes on clinical relevance in view of recent evidence that vascular PGI2 may have an antiatherogenic effect both through regulation of cholesteryl ester deposition in vascular smooth muscle and through regulation of smooth muscle cell proliferation.8,9 The effects of different serum preparations on PGI2 formation by human endothelial cells have re-
ceived limited attention, although serum is generally necessary for growth of HUEC in culture. PGI₂ formation by porcine and bovine aortic endothelial cells (BAEC) is increased in the presence of both human plasma and serum, the stimulatory effect being greater for serum derived from whole blood.10,11 Isolated, activated platelets release nondialyzable substances that enhance PGI₂ formation by porcine endothelial cells.6 In addition, a dialyzable PGI₂-stimulating factor is formed during the activation of the intrinsic pathway of blood coagulation.12 These reports suggest the existence of at least two separate serum factors with PGI₂ stimulating activity.

In this study we evaluated the possibility that differences in the lipoprotein content of human sera affect PGI₂ synthesis by endothelial cells by comparing sera from patients with different types of hyperlipoproteinemias to sera from normal subjects. To determine what serum preparation was most active in influencing endothelial cell PGI₂ production, we also assessed the relative contributions of human serum prepared from whole blood, from platelet-rich plasma, and from platelet-poor plasma to the formation of PGI₂ by HUEC and BAEC.

Methods

Endothelial Cell Monolayer Cultures

HUEC and BAEC were cultured as previously described.1,13 Cells were plated in 24-well (16 mm diameter) cluster plates (Costar, Cambridge, Massachusetts) and used at confluence. Experiments with HUEC were carried out at the second passage, with an average cell density of 90,000 cells/well; BAEC were used between passage 9 and 21; the cell density averaged 120,000 cells/well. Prostaglandin production by HUEC is stable from primary culture up to passage 4 in our laboratory, and prostaglandin production by BAEC is stable from primary culture up to passage 25 or more. These types of endothelial cells represent widely used and easily available cell types. Because HUEC and BAEC differ not only in species, maturity of donor source, and vascular origin (venous or arterial), direct comparisons of PGI₂ production were not made. HUEC were counted after being detached with 0.02% collagenase solution (Worthington Diagnostics, Freehold, New Jersey) containing 0.01% EDTA. BAEC were counted after being detached with 0.01% trypsin (Difco, Detroit, Michigan). Counts were performed using a Coulter Counter (Coulter Electronics, Hialeah, Florida).

Blood Collection

Venous blood was collected from 23 apparently healthy, normolipidemic subjects (12 males, 11 females, aged 24 to 51 years) and from 19 hyperlipoproteinemic subjects (8 males, 11 females, aged 31 to 57 years) selected from patients attending the E. Grossi Paoletti Center for the Study of Metabolic Diseases, Milan, Italy. Donors had normal complete blood counts. Nine of the patients had Type IIA hyperlipoproteinemia, six had Type IIB, and four had Type IV according to World Health Organization diagnostic criteria.14 No subject had taken any drug that affects arachidonic acid metabolism for at least 10 days before blood sampling. Lipid and lipoprotein levels were measured with standardized techniques.15 The serum lipid values of patients and controls are reported in Table 1.

Blood was collected from fasting subjects in plastic syringes, divided into two aliquots, and immediately transferred either to glass tubes for preparation of whole blood serum or to plastic tubes containing 3.8% sodium citrate (blood/citrate, 9:1, vol/vol) for preparation of platelet-rich plasma and platelet-poor plasma.

Serum Preparation

Serum Derived from Whole Blood (WBS)

Whole blood was allowed to clot in sterile glass tubes for 2 hours at 37°C and was then centrifuged at 700 g for 20 minutes at 4°C. The supernatant was immediately divided into aliquots and kept at −20°C until used.

<table>
<thead>
<tr>
<th>Group</th>
<th>Serum cholesterol (mg/dl)</th>
<th>Triglycerides (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total</td>
<td>LDL</td>
</tr>
<tr>
<td>Hyperlipidemic subjects (n = 19)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Type IIa (n = 9)</td>
<td>357 ± 16</td>
<td>283 ± 15</td>
</tr>
<tr>
<td>Type IIB (n = 6)</td>
<td>308 ± 18</td>
<td>184 ± 28</td>
</tr>
<tr>
<td>Type IV (n = 4)</td>
<td>283 ± 50</td>
<td>145 ± 24</td>
</tr>
<tr>
<td>Normal subjects (n = 23)</td>
<td>180 ± 29</td>
<td>110 ± 12</td>
</tr>
</tbody>
</table>

Results are expressed as means ± SEM.

LDL = low density lipoprotein; d = 1.006–1.063; VLDL = very low density lipoproteins; d < 1.006; HDL = high density lipoproteins; d = 1.021–1.063.
Serum Derived from Platelet-Rich Plasma (PRPS)

Citrated blood was centrifuged at 150 g and 20° C for 10 minutes. Platelet-rich plasma (PRP) was separated and platelet counts were performed using a Coulter Counter. The average platelet count was 350,000/µl. PRP was recalcified with CaCl₂ (25 mM) at 37° C for 2 hours. The clot was removed and the serum was centrifuged at 750 g (4° C) for 30 minutes. The supernatant was immediately divided into aliquots and kept at -20° C until used.

Serum Derived from Platelet-Poor Plasma (PPP)

Citrated whole blood was centrifuged at 750 g and 20° C for 20 minutes. The platelet-poor plasma (PPP) was separated, recalcified with CaCl₂ (25 mM) and incubated at 37° C for 2 hours. The clot was removed and the serum was processed as described above for PRPS. Platelet counts in (PPP) were less than 30,000/µl.

Incubation Procedures

Confluent cell monolayers were carefully washed twice with the following mediums: Medium 199 with Earle’s salts, L-glutamine, and 25 mM Heps (Gibco Laboratories, Grand Island, New York) for HUEC; and RPMI 1640 with 25 mM Heps, (M.A. Bioproducts, Walkersville, Maryland) for BAEC. In each experiment, the serum was kept at 37° C for 1 hour to inactivate any residual thrombin and was filtered immediately before use through 0.22 µm millipore filters (Millipore Corporation, Bedford, Maryland). Inactivation of thrombin was monitored using fibrinogen clotting times. The serum was added to the medium immediately before use.

Serum-free medium (SFM) alone (0.5 ml) or medium plus serum was added to each cell monolayer. Incubations were carried out in duplicate or triplicate. After the incubation fluid was added, cell cultures were agitated as little as possible to prevent any shear-induced PGI₂ production. Reagent blank samples were 0.5 ml SFM or 0.5 ml SFM plus the corresponding serum. At the end of incubation, the culture supernatants were carefully removed and processed for 6-keto-PGF₁α determination. Fresh medium (0.5 ml) was then added to cell monolayers and the cells were inspected by phase-contrast microscopy. In some experiments, we also determined the capacity of the cells to respond to other stimuli after incubation with serum. For this purpose, endothelial cell monolayers were washed twice with 0.5 ml 0.01 M prewarmed Hepes buffer (10 mM Hepes, 137 mM NaCl, 5 mM KCl, 1.8 mM CaCl₂, 1 mM MgCl₂, and 5.5 mM glucose; pH 7.4 at 37° C). Hepes buffer (0.5 ml) was added and the cells were incubated for 5 minutes at 37° C. After removing the supernatant, 0.5 ml Hepes buffer was added for 1 minute, followed by incubation of the monolayers with 25 µM arachidonic acid (Nucheck Corporation, Elysian, Minnesota) for 5 minutes. The supernatants (Hepes buffer, or Hepes buffer plus sodium arachidonate) were removed and processed for measurement of 6-keto-PGF₁α.

Determination of 6-keto-PGF₁α

We measured 6-keto-PGF₁α by radioimmunoassay (RIA) as previously described. Because there was human serum in most samples, we used a double antibody technique with goat antirabbit immunoglobulin (IgG) bound to beads (Immunobead Second Antibody, Bio-Rad Laboratories, Richmond, California) following the procedure described for thromboxane B₂ assay. Samples to be assayed were thawed and incubated at 37° C for 2 hours before RIA. Dilutions with Hepes buffer were made at the time of the assay. Results are expressed as ng/ml of 6-keto-PGF₁α in each sample.

Statistical Analysis

Statistical analysis was performed on a TI-59 programmable calculator (Texas Instruments), using prepared programs for the t test and two-or three-way analyses of variance. All results are expressed as mean ± SD.

Results

To determine the optimal system for evaluating the influence of hyperlipidemic serum on the production of PGI₂ by cultured endothelial cells, a series of experiments was performed to establish the effects of time, serum concentration, and manner of serum preparation on PGI₂ production by HUEC and BAEC.

PGI₂ Formation by BAEC and HUEC Monolayers with WBS from Normal Subjects

PGI₂ levels in cell monolayer cultures incubated with SFM increased with time, rising from 0.3 ng/ml at 0 time for both cell types to 1.1 ng/ml and 3.9 ng/ml at 16 hours for BAEC and HUEC respectively. Cell counts after a 16-hour incubation with sera were the same as basal values.

In preliminary experiments, it was demonstrated that BAEC incubated with medium plus WBS from normal subjects produced significantly higher amounts of PGI₂ than cells incubated with medium alone. Serially increasing dilutions of WBS were prepared in medium and 0.5 ml aliquots of each dilution of serum-supplemented medium were placed over BAEC monolayers. A concentration-dependent increase in 6-keto-PGF₁α was observed. The dose-response curve for BAEC is represented in Figure 1. A similar curve with a different time course was obtained when human WBS was incubated with HUEC (data not shown). A 25% concentration of WBS giving maximal increase in 6-keto-PGF₁α for HUEC and BAEC was selected for use in all further studies.

Although we observed that 25% WBS from normal subjects added to SFM significantly increased PGI₂ production by both BAEC and HUEC, there was a
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Figure 1. Production of 6-keto-PGF₁α by bovine aortic endothelial cells (BAEC) after 1 hour of incubation with serially increasing concentrations of whole blood serum (WBS) from normal subjects. Data represent triplicate samples in three separate 24-well plates of BAEC. Variability averaged ± 10%.

Figure 2. Production of 6-keto-PGF₁α by bovine aortic endothelial cells (BAEC) (Panel A) and human umbilical endothelial cells (HUEC) (Panel B) after incubation for 0, 1, 2, 4, 6, and 16 hours with serum-free medium and without 25% whole blood serum (WBS) from normal subjects. In Panel A, * indicates that \( p < 0.001 \). In Panel B, * indicates that \( p < 0.005 \); ** indicates that \( p < 0.001 \). Results represent triplicate determinations in a total of 14 separate plates of endothelial cells.

great difference between BAEC and HUEC in the time required to achieve maximal PGI₂ synthesis. At 1 hour, PGI₂ production by BAEC incubated in medium containing 25% WBS was markedly increased (28.6 ± 4.9 ng/ml 6-keto-PGF₁α for BAEC incubated with medium containing 25% WBS, versus 0.87 ± 0.2 ng/ml for cells with medium alone \( (p < 0.001) \). Incubation for a longer time did not result in any further increase in 6-keto-PGF₁α (Figure 2A).

Whole blood serum preparations from a few normal donors (4/23 donors) consistently had no effect on PGI₂ formation in BAEC monolayers; after addition of serum, the PGI₂ levels were the same as after incubation with SFM. The data obtained with these nonstimulatory sera are not included in the present study of BAEC.

In contrast, human serum added to HUEC had a much slower effect on PGI₂ synthesis than when serum was added to BAEC. When 25% WBS from normal subjects was added to medium, PGI₂ formation by HUEC was unaffected even after 6 hours of incubation. A significant increase in 6-keto-PGF₁α levels was detected only after HUEC were incubated for 16 hours with medium containing 25% WBS (19.7 ± 2.6 ng/ml 6-keto-PGF₁α levels in medium plus 25% WBS; 3.8 ± 0.33 ng/ml 6-keto-PGF₁α levels in medium alone) (Figure 2B). Cell counts after 16-hour incubations with or without serum were the same as initial cell counts for both cell types. Higher concentrations of WBS (up to 50%) resulted in similar levels of stimulation of PGI₂ synthesis by HUEC, but no change in the time course of stimulation.

It was of interest that all the individual WBS preparations from normal subjects, even those unable to induce changes with BAEC, augmented PGI₂ production by HUEC after 16-hour incubations.

WBS preparations from different subjects showed a threefold variation in their effect on PGI₂ production by endothelial cells. Under our experimental conditions, 6-keto-PGF₁α levels after 16-hour incubations in the presence of serum ranged from 19.5 to 62.0 ng/ml for BAEC and from 9.1 to 28.9 ng/ml for HUEC. The observed levels of PGI₂ did not correlate with the age or gender of the serum donor. Platelet count affected the result as shown below.

Effect of PRPS and PPPS on PGI₂ Production by BAEC

Figure 3 shows the results from incubations of BAEC monolayers with mediums containing 25% PRPS, PPPS, or WBS. After a 1-hour incubation, BAEC incubated with 25% PPPS or PRPS synthesized over 20 times more 6-keto-PGF₁α than cells incubated with medium alone. The addition of 25% WBS to medium also stimulated 6-keto-PGF₁α production by BAEC, although to a lesser degree. After a 16-hour incubation, the levels of 6-keto-PGF₁α in cultures incubated with either 25% PRPS or PPPS were further increased compared to levels found at 1 hour, whereas the effect of WBS was not changed.
Effect of PRPS and PPPS on PGI₂ Production by HUEC

After incubation of HUEC for 1 hour with 25% PPPS, PRPS, or WBS, 6-keto-PGF₁α levels were comparable to those measured in HUEC monolayers incubated in medium alone (Figure 4). After incubation of HUEC for 16 hours, the levels of 6-keto-PGF₁α in all cultures supplemented with serum were significantly greater than at 1 hour. However, levels in samples incubated with 25% PPPS were the same as those in samples incubated without serum for the same period of time. In contrast, significantly higher amounts of 6-keto-PGF₁α were measured in cultures incubated with 25% PRPS than in those incubated with 25% PPPS. Cells incubated with 25% WBS synthesized even more 6-keto-PGF₁α compared to cells incubated with PRPS.

Incubations of BAEC and HUEC with 25% WBS from Normal and Hyperlipoproteinemic Subjects.

We expected that sera with abnormal lipoprotein patterns would affect endothelial cell PGI₂ production because isolated lipoprotein fractions have such effects. The response of endothelial cells to WBS from hyperlipoproteinemic subjects was compared to the response to WBS from normal lipidemic subjects. Preliminary studies showed that the dose-response and time-response curves for WBS from hyperlipoproteinemic subjects were similar to those for WBS from normal subjects. Therefore, we selected 25% serum and incubation times of 1 and 16 hours for further study. BAEC incubated with medium containing 25% WBS prepared from blood of normal subjects and of hyperlipoproteinemic patients showed similar stimulation of PGI₂ after both 1 and 16 hours of incubation (Figure 5).

One interesting exception to this pattern was observed, however. Among the sera tested from normal and hyperlipidemic patients, a few individual preparations of WBS failed to increase 6-keto-PGF₁α levels in BAEC monolayers. Two WBS preparations from patients with Type IV hyperlipoproteinemia were "nonresponders." Two other WBS preparations from Type IV patients gave comparable results to those found with normal subjects. In contrast, all these sera stimulated 6-keto-PGF₁α in HUEC monolayers. Mixing experiments indicated that the nonresponder sera were not inhibitory (data not shown).
Monolayers of HUEC incubated for 16 hours with 25% WBS from Type Ila patients produced similar amounts of 6-keto-PGF\textsubscript{1α} as did HUEC incubated with WBS from normal subjects. In contrast to the findings with BAEC, the levels of 6-keto-PGF\textsubscript{1α} produced in HUEC incubated under the same conditions with 25% WBS from Type Iib and Type IV subjects were significantly higher than levels of 6-keto-PGF\textsubscript{1α} obtained with WBS from normal and from Type Ila subjects (Figure 6).

### Discussion

PGI\textsubscript{2} is the most powerful endogenous inhibitor of platelet aggregation and is a potent vasodilator. Under physiological conditions, the synthesis of this prostaglandin by vascular tissue is modulated by factors present in plasma and in serum. Patients with hemolytic uremic syndrome and related disorders, such as thrombotic thrombocytopenic purpura, lack of plasma factor that stimulates PGI\textsubscript{2} synthesis. Plasma from a patient with "lupus anticoagulant" inhibited PGI\textsubscript{2} formation by vascular tissue. Reductions or increases in PGI\textsubscript{2} production have been reported in patients with metabolic diseases such as diabetes and hyperlipidemia and in patients with documented atherosclerotic disease. Alterations in the capacity of plasma and serum to affect PGI\textsubscript{2} synthesis may contribute to the occurrence of thrombosis and to the development of atherosclerosis.

The first objective of our study was to compare the effects of human serum on PGI\textsubscript{2} production by HUEC and BAEC under defined conditions. Our data clearly indicate that the time course of increased PGI\textsubscript{2} production by HUEC and BAEC upon incubation with WBS is very different, although the dose-response patterns are similar. We suggest that the rate of uptake of arachidonate from serum might differ between HUEC and BAEC, since increases in PGI\textsubscript{2} production by BAEC were rapid. In the presence of excess free arachidonate, both HUEC and BAEC showed immediate PGI\textsubscript{2} responses. A rapid increase in PGI\textsubscript{2} synthesis during incubations of BAEC with fetal calf serum, as well as with human serum, was observed, suggesting that species differences in sera, or interspecies immunologic differences do not explain the response patterns. Several human sera were not stimulatory to bovine cells, whereas all human sera tested stimulated human cells.

We next explored the contribution of cellular factors released into serum during blood coagulation by comparing sera prepared from whole clotted blood (WBS) with sera prepared from PRP or PPP. The main cellular component in PRP derives from released platelet factors, whereas WBS may contain factors released from leucocytes, platelets, and red blood cells, or from their interactions.

Serum derived from either PPP or PRP stimulated PGI\textsubscript{2} formation in BAEC equally, suggesting that factors released from human platelets during coagulation do not augment formation of PGI\textsubscript{2} by BAEC. PGI\textsubscript{2} production in the presence of PPS or PRPS
increased similarly over time, with a fourfold increase at 16 hours (Figure 3). In contrast, BAEC incubated with WBS exhibited maximal stimulation at 1 hour with no significant further increase in PGI$_2$ during continued incubation. Thus, there may be a factor released from leukocytes that induces a prompt stimulation of PGI$_2$ synthesis in BAEC. Cell counts of the endothelial monolayers showed no appreciable difference in cell numbers at any incubation time, suggesting that an effect on cell proliferation was not involved.

The results of incubating HUEC with sera derived from PPP, PRP, or whole blood were markedly different from the results of incubating BAEC with these same sera. None of these serum preparations stimulated PGI$_2$ production by HUEC after a 1-hour incubation. After 16 hours of incubation, PGI$_2$ levels in cultures incubated with PRPS were threefold higher than PGI$_2$ levels in cultures incubated with SFM or with PPPS. A fivefold increase in PGI$_2$ levels over those in controls was found in cultures incubated with WBS, suggesting that both platelet and leukocyte factors are involved. In these experiments, PGI$_2$ levels in cultures incubated with PPPS were not different from those measured in cultures incubated with SFM. However, HUEC incubated in the absence of serum may show some injury-induced PGI$_2$ formation. Therefore, this experimental design, using a serum-free control culture, might mask a modest increase in PGI$_2$ formation induced by PPPS.

It has been reported that LDL and HDL influence endothelial cell PGI$_2$ production; HDL stimulates production and LDL inhibits or has no effect on production. In addition to possible effects on PGI$_2$ synthesis, LDL has been shown to injure endothelial cells and to inhibit endothelial cell migration. Conversely, incubation of endothelial cells with HDL may protect against LDL-induced cell injury and may augment PGI$_2$ formation by cells. Our results indicate that hyperlipidemic sera obtained from Type Ila (increased LDL), Type IIb (increased LDL and VLDL), and Type IV (increased VLDL) patients had the same effects on PGI$_2$ formation by BAEC as did sera from normal subjects. In HUEC, PGI$_2$ production was increased by sera from Types IIb and IV hyperlipidemic subjects more than by serum from normal subjects, but the stimulatory effect of serum from Type Ila hyperlipidemias was no greater than that of normal serum. Thus, sera with high triglyceride levels (VLDL are rich in triglycerides) gave the highest stimulation of PGI$_2$ in HUEC. These results indicate that the effects of isolated lipoproteins on endothelial cells are not similar to the effects of whole serum. The concomitant presence of the different lipoprotein classes, as in serum, both at normal and pathologic concentrations, might result in reduction or even reversal of their individual effects.

Two possible interpretations of these observations may be made: 1) donation to confluent endothelial cells of arachidonic acid carried by separated HDL is not the major determinant of the stimulatory effect of whole serum; or 2) moderate levels of HDL are sufficient for maximal stimulation even in the presence of high LDL. The effects of sera with elevated HDL levels were not tested in these experiments. Subconfluent endothelial cells may display different responses.

The positive relation between PGI$_2$ production and triglyceride levels (i.e., in Types IIb and IV hyperlipoproteinemic sera) rather than either LDL or HDL cholesterol is difficult to explain. In cultures incubated with these different hyperlipidemic sera, the cell counts, morphology, PGI$_2$ production during culture, and response to arachidonate stimulation all remained normal, suggesting no injurious effect of serum treatment. However, incubation of cells with hypertriglycerolemic VLDL reportedly affects endothelial cell viability.

In conclusion, our study demonstrates that: 1) the time course for PGI$_2$ stimulation in the presence of serum and the contribution of plasma or cellular factors to PGI$_2$ formation vary according to the type of endothelial cell considered (BAEC and HUEC); and 2) incubations of endothelial cell monolayers with serum containing high levels of atherogenic lipoproteins (LDL and VLDL) do not reduce PGI$_2$ formation by these cells but, in fact, often stimulate PGI$_2$ production.

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