Cigarette Smoke Impairs Endothelial Cell Prostacyclin Production

Jan Hendrik Reinders, Herm-Jan M. Brinkman, Jan A. van Mourik, and Philip G. de Groot

Production of prostacyclin by endothelial cells is considered to be important in rendering the vessel wall nonthrombogenic. Cigarette smoking is an important risk factor in the pathogenesis of atherosclerosis. Here we show that the incubation of cultured human endothelial cells with a cigarette smoke condensate impaired the basal prostacyclin release. Also, the enhanced release of prostacyclin provoked by phorbol myristate acetate was inhibited by cigarette smoke condensate. Furthermore, cigarette smoke condensate impaired the thrombin-induced prostacyclin production. The production of prostacyclin from exogenous arachidonate was not affected by cigarette smoke condensate, indicating that cigarette smoke condensate constituents exert their inhibitory properties on the level of arachidonate mobilization from cellular phospholipids, rather than on cyclooxygenase or prostaglandin synthetase. The effects noted for cigarette smoke condensate could not be attributed to the cigarette smoke constituents nicotine and cadmium. While inhibiting the endothelial cell prostacyclin production significantly, cigarette smoke condensate did not cause cell death or impairment of secretory function, as measured by the release of von Willebrand factor. This in vitro study shows that impairment of an endothelial cell function is related to a risk factor for atherosclerosis.

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Methods

Cell Culture

Human vascular endothelial cells were isolated from umbilical cord veins according to the method originated by Jaffe et al., with some minor modifications. Cells were cultured in plastic flasks precoated with fibronectin or on glass coverslips coated with glutaraldehyde-fixed gelatin (Merck, Darmstadt, FRG). Culture medium consisted of an equal mixture of RPMI 1640 and Medium 199 (Gibco, Paisley, UK), with 20% human serum (pool of 24 healthy donors) and antibiotics. When human serum was omitted from the medium, the following were added to the medium: 1% human serum albumin (Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, The Netherlands), insulin from bovine pancreas (10 μg/ml, Sigma, St. Louis, Missouri), and human transferrin (20 μg/ml, Sigma). The endothelial cells were identified by their typical characteristics, including the presence of von Willebrand factor; the cells in the experiments were in the third passage and the cultures had reached confluence.

Cigarette Smoke Condensate and Chemicals

Cigarette smoke condensate was obtained from the CIVO Food Analysis Institute, TNO Division of Nutrition and Food Research (Zeist, The Netherlands). The smoke condensate was prepared by smoking commercially available nonfilter cigarettes on a Borgwaldt smoking machine. Puffs of 35 ml in 2 seconds were taken once per minute until a 23 mm butt was left. The smoke was passed over a Cambridge glass fiber filter and this filter was extracted with dimethylsulfoxide (Merck). The smoking process was designed to imitate smoking by a person; the condensate contained the nongaseous smoke constituents that would have been inhaled by the smoker. The smoke generated by the machine represented so-called mainstream smoke. One cigarette yielded 20 mg of smoke condensate; the nicotine amounted to approximately 10% (wt/wt) of this smoke condensate. The cigarette smoke condensate in dimethylsulfoxide (stock solution = 8 mg/ml) was added to serum-free culture medium and filtered over a Millipore 0.22 μM filter.

Dimethylsulfoxide in the culture medium was included in all incubations at a concentration of 0.25% (vol/vol). Phorbol myristate acetate (PMA, Sigma) and ionophore A23187 (Boehringer, Mannheim, FRG) were dissolved in dimethylsulfoxide before being added to the culture medium. Nicotine (Serva, Heidelberg, FRG), cadmium chloride (Thomas Tyrer and Company, London, UK), and human thrombin (Sigma) were dissolved directly into the medium.

Prostacyclin

Prostacyclin in endothelial-cell-conditioned medium was measured as the hydrolysis product 6-keto-

Enzymes and von Willebrand Factor

Lactate dehydrogenase was measured according to the method of Bergmeyer and Berni; 6-hexosaminidase, by the method of De Groot et al.; von Willebrand factor by immunoradiometric assay according to the method of Ruggeri et al., with some modifications. Immunofluorescence staining for von Willebrand factor in endothelial cells was performed as described elsewhere with use of monoclonal antibody CLB-RAg-1 as indicator antibody.

Other Materials

All reagents were of analytical grade and were obtained from Merck unless otherwise stated. Immunoreagents were from the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service. Statistical analysis was done by Students’ t test.

Results

Basal and Phorbol Myristate Acetate-Stimulated Prostacyclin Production

The accumulation of 6-keto-PGF1α (the hydrolysis product of prostacyclin) in endothelial cell-conditioned medium was hampered by cigarette smoke constituents in a dose-dependent manner. When cultured endothelial cells were incubated with cigarette smoke condensate, the 6-keto-PGF1α accumulation was reduced to 5% to 10% of control values at a concentration of 20 μg/ml cigarette smoke condensate (Figure 1). The phorbol ester PMA induced a 100-fold stimulation of endothelial cell prostacyclin production; the PMA-stimulated release of prostacyclin was significant after a 4-hour incubation period. Cigarette smoke condensate inhibited this PMA-stimulated prostacyclin release from the cultured endothelial cells (Figure 1). Significant impairment of the endothelial cell prostacyclin release had already occurred at a cigarette smoke condensate concentration of 2 μg/ml; this concentration is equivalent to

prostaglandin F1α (6-keto-PGF1α) by two means: 1) radioimmunoassay with a kit obtained from New England Nuclear (Boston, Massachusetts) and 2) high performance liquid chromatography (HPLC) with cell supernatants prepared for HPLC analysis by using a C-18 extraction column (Baker, Deerfield, The Netherlands) as described previously. HPLC was performed on a C-18 column (Beckman, Berkeley, California) by following the method of Terragno et al., with some modifications. To measure the prostacyclin synthesis from endogenous arachidonic acid, cells were prelabeled for 48 hours with 0.2 μCi/ml [3H]-arachidonate (specific activity, 100 mCi/μmol) in serum-free medium. Conversion of exogenous arachidonic acid into prostaglandins by endothelial cells was studied by incubating 2.10⁶ cells with [3H]-arachidonate (20 μM, 0.1 μCi/ml) in 10 ml RPMI 1640.
Figure 1. The influence of cigarette smoke condensate on the accumulation of 6-keto-PGF1α in conditioned medium of cultured endothelial cells. A. Cells were maintained in serum-free medium, and the 6-keto-PGF1α accumulation was measured in the supernates of cells incubated with condensate after 5 hours (○—○) and 24 hours (○—○). B. Cells were maintained in serum-free medium with 10 ng/ml phorbol myristate acetate (PMA) for 5 hours (■—■) and 24 hours (■—■). The 6-keto-PGF1α levels were measured by radioimmunoassay and are expressed as percentages of the controls (no exposure to condensate). The absolute amounts of 6-keto-PGF1α found in the control media were 0.7 and 2.4 ng/10⁶ cells at 5 hours and 24 hours, respectively. In PMA-treated cells, the amounts were 58.3 and 114.5 ng/10⁶ cells at 5 hours and 24 hours, respectively. The values are the means of three incubations; standard deviations are indicated.

Cigarette smoke condensate is an extremely complex mixture; at present the smoke constituents, nicotine and cadmium, have been implicated as atherogenic. 29 Nicotine at a concentration up to 20 μg/ml, a dose equivalent to 10 times the amount present in 20 μg/ml cigarette smoke condensate, did not exert an inhibitory effect on the accumulation of 6-keto-PGF1α in endothelial-cell-conditioned medium during a 24-hour incubation period (Figure 2). Nicotine also did not influence the PMA-stimulated prostacyclin release. When endothelial cells were incubated with Cd²⁺ ions at a concentration equivalent to 10 times the concentration present in 20 μg/ml cigarette smoke condensate, there was no effect on the endothelial cell prostacyclin production (Figure 2). In addition, when endothelial cells were incubated with both nicotine and cadmium at concentrations higher than those present in 20 μg/ml cigarette smoke condensate, no effect on the prostacyclin production was noted. Nicotine and Cd²⁺ ions were both cytotoxic, as judged by loss of cellular lactate dehydrogenase and β-hexosaminidase in the supernatant, at concentrations higher than 20 μg/ml and 500 ng/ml, respectively. The cigarette smoke condensate exerted a cytotoxic effect at a concentration of 50 μg/ml.

Thrombin-Stimulated Prostaglandin Production

Upon stimulation with thrombin, endothelial cells produce within minutes large amounts of prostaglandins, including prostacyclin. 32 The substrate for this stimulated prostaglandin production is arachidonate liberated from cellular phospholipids. When endothelial cells were treated with cigarette smoke condensate, cells were less responsive to thrombin in their release of prostaglandins (Figure 3). The amount of 6-keto-PGF1α recovered from the supernatant upon thrombin stimulation was 45% of that in the supernatant of nonexposed cells after cells were exposed to 20 μg/ml cigarette smoke condensate. The A23187-stimulated release of prostacyclin was reduced to 27% after exposure of the endothelial cells to 20 μg/ml cigarette smoke condensate (Figure 3). The pattern of prostaglandins produced was not altered by cigarette smoke condensate; the amounts of the major prostaglandins produced by the endothelial cell upon thrombin-stimulation were all reduced to the same extent by preincubation with cigarette smoke condensate.
Figure 2. The effect of cigarette smoke condensate, nicotine, and Cd\(^{2+}\) ions on the accumulation of 6-keto-PGF\(_{1\alpha}\) in conditioned medium of cultured endothelial cells. Cells were maintained in serum-free medium for 24 hours in the absence (open bars) or the presence (solid bars) of 10 ng/ml phorbol myristate acetate (PMA); amounts of 6-keto-PGF\(_{1\alpha}\) are expressed as percentages of controls (n = 3, ± SD). The amount of 6-keto-PGF\(_{1\alpha}\) accumulated in the supernates of controls was 4.3 ng/10\(^6\) cells; that in the supernates of PMA-treated cultures was 146.9 ng/10\(^6\) cells.

<table>
<thead>
<tr>
<th>Concentration (µg/ml)</th>
<th>6-keto-PGF(_{1\alpha}) (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cigarette smoke condensate</td>
<td>0, 1, 5, 10, 20, 25</td>
</tr>
<tr>
<td>Nicotine</td>
<td>0, 0.05, 0.5, 5, 50</td>
</tr>
<tr>
<td>Cd(^{2+})</td>
<td>0, 1, 5, 50</td>
</tr>
</tbody>
</table>

Figure 3. The effect of cigarette smoke condensate on the stimulated release of prostaglandins by cultured endothelial cells. A. Endothelial cells were exposed to condensate for 24 hours. Cells were washed twice with RPMI 1640 and subsequently incubated for 5 minutes with 2 U/ml thrombin (---), 10 µM ionophore A23187 (■), or no stimulus (○—○). The amounts of 6-keto PGF\(_{1\alpha}\) accumulated in the cell supernates during the 5-minute stimulation period were measured by radioimmunoassay and are the means of two incubations. B. The cells were prelabeled for 48 hours with \(^3\)H-arachidonate, incubated with 20 µg/ml condensate for 3 hours, and subsequently stimulated with 2 U/ml thrombin for 5 minutes. Control cells were not exposed to condensate. The supernates of the 5-minute incubations were subjected to high performance liquid chromatography. The amounts of prostaglandins are expressed as the radioactivity recovered in the respective fractions.
**Conversion of Exogenous Arachidonic Acid**

Endothelial cells can use exogenous arachidonate for the synthesis of prostaglandins. After pretreatment with cigarette smoke condensate, the capacity of endothelial cells to synthesize prostaglandins from the exogenous arachidonate was not affected (Figure 4).

**Release of von Willebrand Factor**

The endothelial cell synthesizes and secretes, besides prostacyclin, a variety of secretory products, including von Willebrand factor. In cultured endothelial cells, the von Willebrand factor is released continuously in the medium, incorporated into the extracellular matrix, and stored intracellularly. When the endothelial cell is treated with thrombin, PMA, or ionophore A23187, the von Willebrand factor is secreted into the medium. The toxic effects of cigarette smoke condensate constituents, which are exerted on endothelial cell prostaglandin production, might also influence the synthesis and intracellular routing of von Willebrand factor. The release into the medium of von Willebrand factor by cultured endothelial cells was not altered by incubating the cells with cigarette smoke condensate (Figure 5). Also the PMA-stimulated release of von Willebrand factor was not hampered by cigarette smoke condensate. The von Willebrand protein levels in the cell supernatants after 1 hour of incubation with PMA, thrombin, or ionophore A23187 were not altered by the previous exposure of the cells to cigarette smoke condensate for 1, 4 or 24 hours (not shown). Immunofluorescence studies on cultured endothelial cells to visualize the subcellular localization of von Willebrand factor demonstrated the presence of von Willebrand factor in rod-shaped organelles, presumably identical to Weibel-Palade bodies; cigarette smoke condensate did not seriously alter the subcellular localization of von Willebrand factor and the secretion of this protein from these organelles, following incubation of the cells with PMA (Figure 6).

![Figure 4](image_url)  
**Figure 4.** The effect of cigarette smoke condensate on the conversion of exogenous arachidonate by cultured endothelial cells. Cells were exposed to 20 μg/ml cigarette smoke condensate for 24 hours. Cells were washed twice with RPMI 1640 and incubated for 15 minutes with 20 μM [3H]-arachidonate in RPMI 1640. Prostaglandins that accumulated in the cell supernatants were analyzed by high performance liquid chromatography; the amounts of prostaglandins are expressed as radioactivity recovered. During exposure to the condensate, the endothelial cells accumulated 47% ± 8% of 6-keto-PGF1α compared to nonexposed cells (n = 4). The results shown here are representative for three experiments.

![Figure 5](image_url)  
**Figure 5.** The effect of cigarette smoke condensate on the von Willebrand factor accumulation in conditioned medium of cultured endothelial cells. The cells were incubated with (○—○) and without (●—●) 10 ng/ml phorbol myristate acetate (PMA) in serum-free medium. The von Willebrand factor accumulation was assessed as the von Willebrand antigen (protein) after 24 hours and 96 hours of incubation (n = 3, ± SD).
**Cell Viability**

The influence of cigarette smoke condensate on cell viability was evaluated in order to learn whether the observed impairment of the prostacyclin production was due to general cell toxicity. Cell viability was evaluated in confluent monolayers of endothelial cells by measuring the release of cytosolic lactate dehydrogenase and the lysosomal enzyme β-hexosaminidase into the conditioned medium and by determining the percentage of cells that did not exclude trypan blue following a 24-hour exposure to cigarette smoke condensate. Cigarette smoke condensate at concentrations up to 20 μg/ml did not enhance the release of cellular enzymes or the decrease of the viability of the cells over controls (Table 1). At a concentration of 50 μg/ml of cigarette smoke condensate, cytotoxicity was observed.

**Table 1. Viability of Endothelial Cells Exposed to Cigarette Smoke Condensate**

<table>
<thead>
<tr>
<th>CSC (μg/ml)</th>
<th>Dead cells (%) total at 24 hrs</th>
<th>LDH in supernatant (%) total</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>14.4±3.5</td>
<td>6.7±2.3</td>
</tr>
<tr>
<td>5</td>
<td>13.2±5.4</td>
<td>8.0±0.5</td>
</tr>
<tr>
<td>10</td>
<td>12.9±5.1</td>
<td>7.6±2.1</td>
</tr>
<tr>
<td>20</td>
<td>13.9±1.7</td>
<td>7.5±0.7</td>
</tr>
</tbody>
</table>

Endothelial cells were maintained on serum-free medium, exposed to cigarette smoke condensate (CSC), and harvested by trypsinization after 24 hours, including floating cells in the supernatant. Viability was evaluated by trypan blue exclusion; the percentage of dead cells ± so (n = 3) is presented. The activity of lactate dehydrogenase (LDH) in supernatants of the cells is expressed as a percentage of the total present in cells and supernatants.
proximately 30% of the cells detached from the substrate after a 24-hour incubation period; this is twice as much as in the controls. The morphology of the endothelial cells, as judged by light microscopy, was not affected by 20 \( \mu \text{g/ml} \) cigarette smoke condensate (Figure 7).

**Discussion**

For several decades, it has been known that cigarette smoking is a risk factor in the pathogenesis of atherosclerosis.\(^5\)\(^-\)\(^11\)\(^,\)\(^37\) However, the smoke constituents responsible and the precise mechanism by which they exert their devastating effects on the blood vessel wall are largely unknown. Ultrastructural studies of human umbilical and placental vasculature revealed a demonstrable effect of maternal smoking on the integrity of the endothelium.\(^12\)\(^,\)\(^13\) These ultrastructural observations were affirmed by animal studies; smoking caused endothelial cell injury.\(^14\)\(^,\)\(^15\) It was reasoned that cigarette smoke constituents interfered with endothelial cell metabolism. The study of Pittilo et al.\(^15\) indicated that exposure of rats to cigarette smoke inhibited the aortic prostacyclin production. We examined the effect of cigarette smoke condensate on cultured vascular endothelial cells. In particular, we studied the effect on the synthesis of prostacyclin and the secretion of von Willebrand factor, markers of the endothelial function. The cigarette smoke condensate contained all smoke constituents retained on a glass filter and extracted from this glass filter with dimethylsulfoxide; it included most of the cigarette's nicotine, but not the gaseous components like carbon monoxide.

It is shown here that cigarette smoke condensate is a potent inhibitor of endothelial cell prostacyclin synthesis (Figure 1). Both the basal release and the production of prostacyclin as the result of PMA stimulation are hampered. The thrombin-stimulated production of prostacyclin and other prostaglandins is also inhibited (Figure 3).

The conversion of exogenous arachidonate by the cells is not affected by preincubation with the cigarette smoke condensate (Figure 4), whereas the thrombin-stimulated release of prostaglandins is hampered seriously (Figure 3). It is, therefore, unlikely that prostacyclin production is hampered by inhibition of cyclooxygenase or prostaglandin synthetase. Apparently, the cigarette smoke constituents inhibit endothelial prostaglandin production by preventing the liberation of arachidonate from cellular phospholipids.

The capacity of cigarette smoke condensate to inhibit the basal and PMA-stimulated accumulation of 6-keto-PGF\(_1\alpha\) in endothelial cell culture medium during a 24-hour period was less than after 5 hours (Figure 1). Part of the thrombin- and ionophore-stimulated prostacyclin production was resistant to cigarette smoke condensate pretreatment of the cells (Figure 3). These two findings might imply that the effects of cigarette smoke condensate on the endothelial cell prostacyclin production are reversible or that there are two endothelial cell prostacyclin-producing systems\(^36\) that differ in susceptibility to cigarette smoke condensate.

The active principle and precise mode of action of the cigarette smoke condensate are not clear at present. Inhibition of the prostaglandin production is not associated with cell death (Table 1). Furthermore, endothelial cell secretion of the von Willebrand factor was not influenced by the cigarette smoke condensate.
condensate; the basal secretion into the medium as well as the stimulus-induced release from intracellular storage sites were unaffected (Figures 5 and 6). Also the cell morphology as observed in phase-contrast microscopy did not show any differences between cigarette smoke condensate exposed and control cells (Figure 7). Nicotine inhibits the release of prostacyclin by the vasculature in vivo. Furthermore, nicotine has caused ultrastructural changes in the aortic endothelium of rabbits and at concentrations higher than 15 μg/ml nicotine, has had some effect on the morphology of cultured bovine aortic endothelial cells. It has been suggested that cadmium is an atherogenic factor. Here we show that the effects noted for cigarette smoke condensate could not be attributed to either nicotine or cadmium alone or to a combination of nicotine and cadmium (Figure 2).

Endothelial cell prostacyclin production is considered important in controlling the thrombus size at sites of vascular injury. Limiting platelet activation at sites of vascular injury might help prevent atherosclerotic processes. Cigarette smoke constituents interfered with endothelial cell prostacyclin production. This might explain why cigarette smoking induces atherosclerosis.

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