Production of Endothelin by Cultured Human Endothelial Cells Following Exposure to Nicotine or Caffeine

William O. Lee and Stephen M. Wright

This study evaluated endothelin production by endothelial cells after exposure to nicotine or caffeine. Vasoconstrictive properties have been attributed to both nicotine and caffeine. The presence of endothelin, a potent vasoconstrictor itself, was determined using a radioimmunoassay. The optimal stimulatory doses for nicotine and caffeine were determined to be 1.0 μmol/L and 1.0 mmol/L, respectively. When endothelin production was evaluated over time after exposure to the optimal dose of each agent, it was determined that nicotine stimulated maximum endothelin production within 5 minutes. Caffeine failed to cause a distinct peak of endothelin production within 20 minutes. These results suggest that nicotine may have a possible acute and short-lived effect on the vasoconstrictive response associated with endothelin, while caffeine-induced endothelin release may require more long-term exposure.

GREATER THAN 10% of the population of the United States suffers from hypertension.[1] Despite this high incidence of hypertension, its etiology in many cases is unknown. Biologically active compounds in association with the pathophysiology of hypertension may play an important role in understanding the causes of hypertension.

Endothelin is a potent vasoconstrictor peptide produced by endothelial cells.[2] It has been suggested that endothelin acts directly on membrane ion channels. The vasoconstrictive response caused by endothelin is mediated by an increase in the intracellular concentration of calcium via influx of extracellular calcium through the plasma membrane channels and/or mobilization of intracellular calcium by phospholipase C–stimulated inositol triphosphate formation.[3] Endothelial cells have been demonstrated to release endothelin after exposure to various biological agents such as thrombin,[4] interleukin-1,[5] and angiotensin II,[6] which are responsible for hemostasis and vascular tone.

Nicotine causes vasoconstriction both in vivo[7] and in vitro.[8] Elevated endothelin levels in human plasma have been reported after cigarette-smoking.[9] Caffeine has been reported to result in an endothelium-dependent contraction in canine mesenteric arteries; however, this contraction was reportedly not solely due to endothelin release.[10]

Thrombin was used in the current study as a model stimulatory compound for endothelin production by cultured human endothelial cells. Following verification of endothelin release, this study first evaluated the stimulatory effects of nicotine or caffeine on human endothelial cells. After determination of the optimal stimulatory dose of nicotine or caffeine, endothelin release was evaluated within the first 20 minutes of exposure.

MATERIALS AND METHODS

Cell Culture

The endothelial cell line ECV304 (American Type Culture Collection, Rockville, MD) was used. These established cells originated from the vein of a normal human umbilical cord. Cells were grown at 37°C in medium 199 (Sigma Chemical, St Louis, MO) supplemented with 10% fetal bovine serum (GIBCO, Grand Island, NY) in 25-cm^2 flasks (Corning, Cambridge, MA). Trypsin/EDTA (Sigma) was used to detach the cells, and 1:10 dilutions were made. Cells were subcultured every 7 to 10 days after a complete monolayer formed. Experimentation was commenced when the cells achieved a confluent monolayer. To ensure equivalent numbers of cells for all experiments, cells were counted and viability was determined after exposure to each compound investigated. Levels of endothelin were normalized for expression as picograms per 6×10^6 cells.

Optimal Dose Determination

The minimum dose resulting in optimum endothelin release was determined for the stimulatory agents thrombin, nicotine, and caffeine (all from Sigma). The medium from flasks containing a cell monolayer was decanted, and the cells were washed twice with phosphate-buffered saline. Medium 199 was added with varying concentrations of each agent to a total volume of 1 mL. After exposure for 1 hour, the medium was removed and frozen at −70°C until high-performance liquid chromatography (HPLC) and radioimmunoassay. Each experiment was performed in triplicate.

Exposure Over Time

After the optimum stimulatory dose for each compound was determined, cells were exposed to the dose for 5, 10, 15, and 20 minutes. After exposure for the allotted time, the medium was removed and frozen at −70°C until HPLC and radioimmunoassay. Each experiment was performed in triplicate.

Cell Viability

Cells were counted in a hemacytometer, and viability was determined by trypan blue exclusion. Three counts were performed for each concentration of nicotine or caffeine and each exposure time.

HPLC and Radioimmunoassay

Samples in 10-μL volumes were separated by a Vydac (Cole-Parmer Instrument, Vernon Hills, NJ) RP 25-cm × 4.6-mm C-18 protein peptide column with a particle size of 5 μm using an acetonitrile gradient as previously described.[11] The eluted endothelin fraction was stabilized with endothelial cell buffer solution from the radioimmunoassay kit (Dupont, Wilmington, DE). The procedure was performed according to the manufacturer's instructions.

From the Department of Biology, Middle Tennessee State University, Murfreesboro, TN; and Universidad Autonoma de Guadalajara, Guadalajara, Mexico.

Submitted May 19, 1998; accepted January 31, 1999.

Address reprint requests to Stephen M. Wright, PhD, Department of Biology, Middle Tennessee State University, Murfreesboro, TN 37132.

0026-0495/99/4807-0006$10.00/0

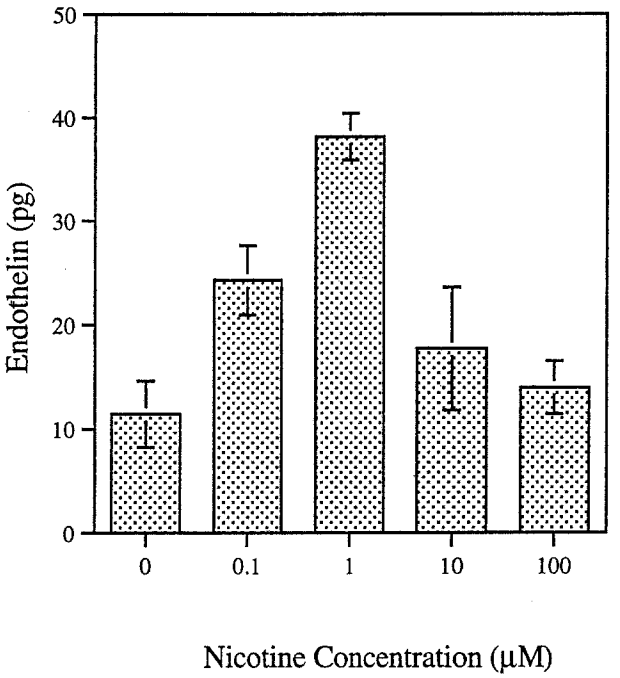

Fig 1. Endothelin production following exposure to nicotine. Cells were exposed to 0.1, 1.0, 10.0, and 100.0 μmol/L nicotine for 1 hour. After elution by HPLC, the concentration of endothelin was determined by radioimmunoassay.

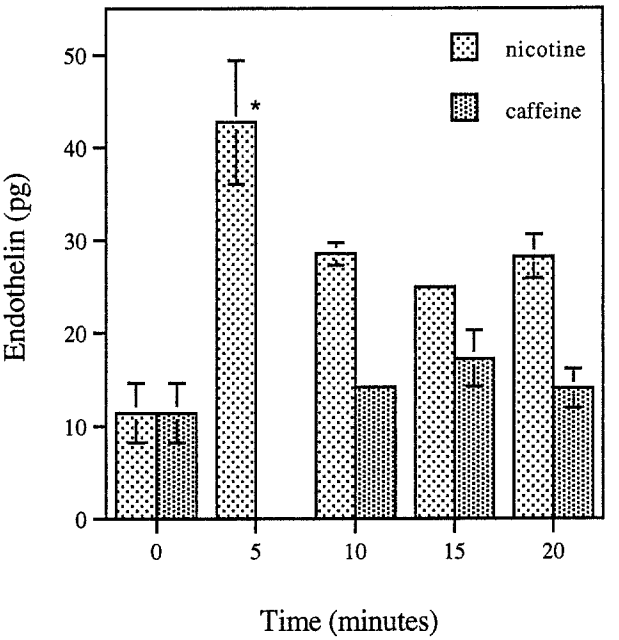

Fig 2. Endothelin production over time using optimal doses of nicotine or caffeine. Cells were exposed to 1.0 μmol/L nicotine or 1.0 mmol/L caffeine over 20 minutes. Endothelin production after exposure to caffeine for 5 minutes was not measured. *$P < .05$ *v* baseline endothelin levels.

Statistical Analysis

A Kruskal-Wallis ANOVA was performed for cell viability, determination of optimal dose, and exposure over time for both nicotine and caffeine. Values are reported as the mean ± SEM.

RESULTS

Thrombin was chosen as a model stimulatory agent to ensure that ECV304 cells responded to an established inducer of endothelin. The endothelin fraction eluted at approximately 45% solvent concentration during HPLC separation. The lowest dose of thrombin that resulted in maximal production of endothelin (>120 pg endothelin/6 × 10^6 cells) was 2.0 U/mL. Peak levels of endothelin release occurred at 15 minutes after exposure to 2.0 U thrombin (data not shown).

Nicotine at a concentration of 1.0 μmol/L was determined to cause a peak release of 38.13 ± 2.29 pg endothelin/6 × 10^6 cells (Fig 1). This amount of endothelin was approximately threefold the baseline level. Higher concentrations of nicotine did not result in increased levels of endothelin, and 1.0 μmol/L nicotine was used as the lowest concentration to achieve maximum endothelin production. At nicotine concentrations of 0, 0.1, 1.0, and 10 μmol/L, cell viability remained between 92% and 95%. At a nicotine concentration of 100 μmol/L, cell viability decreased to 84%, but this does not represent a significant difference (Table 1). To measure endothelin release over time, cells were exposed to 1.0 μmol/L nicotine. Figure 2 shows that maximum levels of endothelin were evident at 5 minutes postexposure. This level of endothelin, 42.69 ± 6.72 pg, represents a significant difference from the baseline levels. There were no significant differences in cell viability at any of the times evaluated (Table 2).

Caffeine concentrations of 1.0 and 10.0 mmol/L caused an approximately threefold increase in endothelin levels over baseline (Fig 3). Since there was no significant difference in endothelin detection by 1.0 or 10.0 mmol/L caffeine, the lower concentration of 1.0 mmol/L was used as the stimulatory dose over time. No significant differences were noted between baseline endothelin levels and the levels found 20 minutes postexposure to 1.0 mmol/L caffeine (Fig 2). Cell viability

Table 1. Viability of ECV304 Cells After Exposure to Different Concentrations of Nicotine for 1 Hour

Nicotine (μmol/L)	Cell Viability (%)
0.0	95.67 ± 1.20
0.1	92.33 ± 5.17
1.0	93.33 ± 1.17
10.0	95.00 ± 1.15
100.0	84.33 ± 5.21

NOTE. Results are the mean ± SEM.

Table 2. Viability of ECV304 Cells Exposed to 1.0 μmol/L Nicotine for Different Times

Exposure Time (min)	Cell Viability (%)
5	94.33 ± 0.88
10	78.33 ± 6.17
15	87.00 ± 3.21
20	88.00 ± 2.08

NOTE. Results are the mean ± SEM.

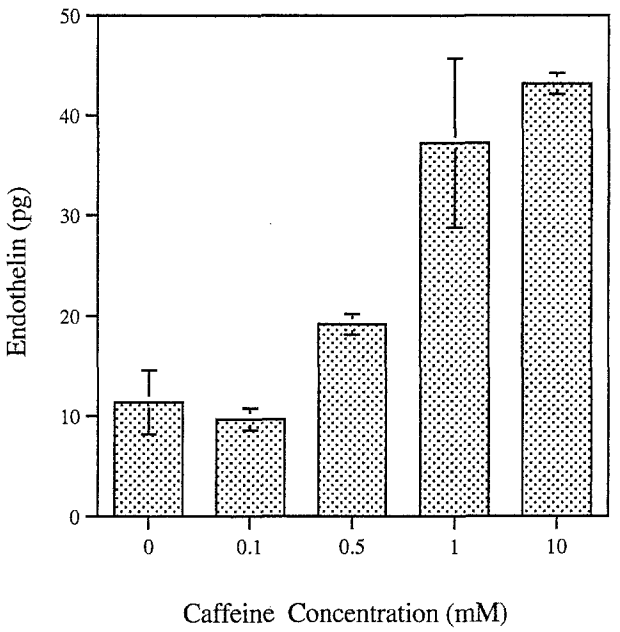

Fig 3. Endothelin production following exposure to caffeine. Cells were exposed to 0.1, 0.5, 1.0, and 10.0 mmol/L caffeine for 1 hour. After elution by HPLC, the concentration of endothelin was determined by radioimmunoassay.

remained between 95% and 97% for all concentrations of caffeine tested and for all times measured.

DISCUSSION

The ECV304 cells used in this study, first described in 1990,[12] provide a convenient cell line for investigations of endothelin production, obviating the need for preparation of cells from blood vessels. To ensure that ECV304 cells produced endothelin, the model stimulatory compound thrombin was used. The profile of thrombin doses used in this study agrees with the optimal stimulatory thrombin doses previously reported.[4]

This investigation determined the nicotine concentration responsible for maximal endothelin production to be 1.0 μmol/L (Fig 1). This concentration is comparable to plasma levels of nicotine present in smokers.[13,14] Elevated endothelin levels have been reported in individuals who smoke high-tar cigarettes containing 1.6 mg nicotine, although smoking low-tar cigarettes (0.1 mg nicotine) did not result in significant endothelin production.[9] In the present study, stimulatory doses of nicotine greater than the plasma levels present in smokers did not increase endothelin production. In fact, a decrease in ECV304 cell viability was noted when nicotine was used at 100 μmol/L (Table 1). The effect of nicotine at higher concentrations also was reported to be toxic to bovine pulmonary endothelial cells.[15]

While levels at least twofold higher than baseline endothelin levels were observed at 10, 15, and 20 minutes after exposure to 1.0 μmol/L nicotine, the most dramatic endothelin release occurred early, at 5 minutes (Fig 2). Lower levels of endothelin at other times may be related to the cytotoxic effects of nicotine on cells after prolonged exposure.

Exposure of ECV304 cells to caffeine did result in increased levels of endothelin; however, there was no significant increase over baseline levels during the 20-minute period measured (Fig 2). Plasma caffeine levels have been previously reported,[16] and for this study, the caffeine doses required to produce measurable elevations in endothelin were approximately 40 times higher than the normal plasma caffeine levels found after drinking a cup of coffee. It may be that caffeine exposure in the form of caffeinated beverages affords no immediate effect on the vasculature, as measured by endothelin production.

It is important to note that this in vitro system may not be an accurate reflection of all pathophysiologic factors involved in vivo. The culturing process alone introduces an artificial state. Natural inhibitors of endothelin may be missing or culture conditions may stimulate endothelin release. Endothelin itself has been reported to stimulate its own synthesis in cultured human umbilical cord endothelial cells.[17] The endogenous vasodilator atrial natriuretic peptide has been shown to have an inhibitory effect on endothelin production by cultured human endothelial cells.[18]

This study suggests another possible health concern for individuals who use tobacco products, by linking the vasoconstrictor endothelin to nicotine. This connection suggests that nicotine and endothelin could play an acute role in the hypertensive process leading to or complicating cardiovascular disease.

ACKNOWLEDGMENT

We thank Dr Raymond Hakim for assistance with radioimmunoassays performed in his laboratory at Vanderbilt University School of Medicine.

REFERENCES

1. Berkow R, Fletcher AJ: The Merck Manual of Diagnosis and Therapy. Rahway, NJ, Merck, 1987

2. Yanagisawa M, Kurihara H, Kimura S, et al: A novel potent vasoconstrictor peptide produced by vascular endothelial cells. Nature 332:411-415, 1988

3. Shultz P, Knauss T, Meme P: Endothelin stimulates phospholipase C, Na^+/H^+ exchange, c-*fos* expression, and mitogenesis in rat mesangial cells. J Clin Invest 83:708-712, 1989

4. Fukunaga M, Ochi F, Yokoyama K, et al: Stimulatory effect of thrombin on endothelin-1 production in isolated glomeruli and cultured mesangial cells of rats. J Cardiovasc Pharmacol 17:S411-S413, 1991 (suppl)

5. Yoshizuni M, Kurihara H, Morita T, et al: Interleukin-1 increases the production of endothelin-1 by cultured endothelial cells. Biochem Biophys Res Commun 66:324-329, 1990

6. Scott-Burden T, Resink T, Hahn AW, et al: Induction of endothelin secretion by angiotensin II: Effects of growth and synthetic activity of vascular smooth muscle cells. J Cardiovasc Pharmacol 17:S96-S100, 1991 (suppl 7)

7. Kitamura S: Effects of cigarette smoking on metabolic events in the lung. Environ Health Perspect 72:283-296, 1987

8. Holden WE, Kishiyama S, Dong SP, et al: Endothelium-dependent effects of cigarette smoke components on tone of porcine

intrapulmonary arteries in vitro. Toxicol Appl Pharmacol 104:191-199, 1990

9. Haak T, Jungmann E, Raab C, et al: Elevated endothelin-1 levels after smoking. Metabolism 43:267-269, 1994

10. Jino H, Usui H, Temma S, et al: An endothelium-dependent contraction in canine mesenteric artery caused by caffeine. Br J Pharmacol 111:435-438, 1994

11. Fyhrquist F, Saijonmaa O, Metsarinne K, et al: Raised plasma endothelin-1 concentration following cold pressor test. Biochem Biophys Res Commun 169:217-221, 1990

12. Takahashi K, Sawasaki Y, Hata J, et al: Spontaneous transformation and immortalization of human endothelial cells. In Vitro Cell Dev Biol 25:265-274, 1990

13. Sutton SR, Russell M, Iyer R, et al: Relationship between cigarette yields, puff patterns and smoke intake: Evidence for tar compensation. BMJ 285:600-603, 1982

14. Russell M, Jarvis MJ, Feyerabend C, et al: Nasal nicotine solution: A potential aid to giving up smoking. BMJ 286:263-284, 1983

15. Suzuki N, Ishii Y, Kitamura S: Effects of nicotine on production of endothelin and eicosanoid by bovine pulmonary artery endothelial cells. Prostaglandins Leukot Essent Fatty Acids 50:193-197, 1994

16. Jacobson AF, Cerqueira MD, Raisys V, et al: Serum caffeine levels after 24 hours of caffeine abstention: Observations on clinical patients undergoing myocardial perfusion imaging with dipyridamole or adenosine. Eur J Nucl Med 21:23-26, 1994

17. Saijonmaa O, Nyman T, Fyhrquist F: Endothelin-1 stimulates its own synthesis in human endothelial cells. Biochem Biophys Res Commun 188:286-291, 1992

18. Saijonmaa O, Ristimaki A, Fyhrquist F: Atrial natriuretic peptide, nitroglycerine and nitroprusside reduce basal and stimulated endothelin production from cultured endothelial cells. Biochem Biophys Res Commun 173:514-520, 1990