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NOTES

Pyocyanin from *Pseudomonas aeruginosa* Inhibits Prostacyclin Release from Endothelial Cells

JAY M. KAMATH, BRADLEY E. BRITIGAN, CHARLES D. COX, AND D. MICHAEL SHASBY*

Veterans Administration Hospital and the University of Iowa College of Medicine, Iowa City, Iowa 52242

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***Pseudomonas aeruginosa* pneumonia causes a vasculitis of small pulmonary arteries. While the fully developed lesion demonstrates vessel wall necrosis, the early lesion is remarkable for preservation of viable endothelium despite vessel wall invasion by bacteria. Pyocyanin, an exoproduct of *P. aeruginosa*, markedly inhibited prostacyclin production by pulmonary artery endothelial cells without causing cell lysis. Pyocyanin might alter vascular homeostasis in the absence of cytolysis.**

Pseudomonas aeruginosa pneumonia causes a distinctive vascular lesion which begins with *Pseudomonas* penetration of the wall of small pulmonary arteries and evolves into necrosis and hyalinization of the endothelial and muscular layers (4). When combined, two exoproducts of *P. aeruginosa*, pyocyanin and ferripyochelin, form cytolytic amounts of hydroxyl radical in the presence of endothelial cells, suggesting one possible mechanism for the necrosis (1, 1a). However, early *P. aeruginosa* pneumonia vascular lesions, as well as early systemic icthyma lesions, are remarkable for a paucity of histologically evident endothelial injury despite considerable bacterial invasion of the vessel wall (4, 12). Hence, it was of interest to identify noncytolytic effects of *P. aeruginosa* exoproducts on cells of the vessel wall. Release of prostaglandin I_2 (PGI_2) or PGE_2 is an important antithrombotic, antiinflammatory, and vasoregulatory activity of endothelium that is sensitive to redox potential (3, 5). Pyocyanin is a phenazine dye that is an effective electron acceptor that might interfere with redox-sensitive endothelial prostanoid production (1, 1a, 9).

Porcine pulmonary artery endothelial (PPAE) cells were cultured in 24-well plates (Costar, Cambridge, Mass.) until they were 2 days postconfluency, as previously described (1, 8). At the time of the experiment, the medium was aspirated and the cells were washed three times with phosphate-buffered saline, and then 250 μl of Hanks balanced salt solution was added to each well. Pyocyanin or vehicle (H_2O) was then added at various concentrations for 30 min. When the cells were to be stimulated with the calcium ionophore A23187 (1 μM in dimethyl sulfoxide) or arachidonic acid (in ethanol), either agonist (or vehicle) was added after the initial 30-min incubation, and the cells were incubated an additional 30 min in the presence of both pyocyanin and agonist. Crystalline pyocyanin was prepared by Charles Cox as previously reported (2).

PGI_2 was measured in the supernatant fluid by radioimmunoassay according to the manufacturer's directions (Advanced Magnetics Inc., Cambridge, Mass.). Briefly, the medium was acidified to pH 3.0 with HCl, extracted with 2 ml of ethyl acetate, and dried under nitrogen. The sample was then redissolved in the supplied phosphate buffer and mixed with antibody and ^{3}H-labeled standard, and the free (not antibody-bound) labeled standard was precipitated. Sample curves were constructed with standard 6-keto-PGF_1-alpha for each experiment, and counts from each sample were converted to picomoles on the basis of this curve. Cross-reactivity of the antibody is reported as 4% PGF_2-alpha, 2% PGF_1-alpha, 1.6% PGE_2, 1% PGE_1, 1% PGD_2, and 1% PGH_2 (5).

Unstimulated PPAE cells released only a small amount of PGI_2, and this was reduced by pyocyanin (Fig. 1). When PPAE cells were stimulated with A23187, they released more PGI_2, and this was also reduced by pyocyanin (Fig. 1).

Pyocyanin could decrease PGI_2 release by inhibiting the release of arachidonate from phospholipids or by inhibiting the synthesis of PGI_2 from arachidonate. When 50 μM arachidonate was added to the culture medium, PPAE cells produced more PGI_2. Pretreatment of PPAE cells with pyocyanin inhibited PPAE cell production of PGI_2 from arachidonate in a dose-dependent manner, and significant inhibition occurred with concentrations of pyocyanin as low as 1 to 5 μM (Fig. 2).

Hence, the data are consistent with pyocyanin inhibiting the metabolism of arachidonic acid. However, pyocyanin might react with released PGI_2, as it does with nitric oxide (NO) and prevent the antibody from reacting with 6-keto-PGF_1-alpha (14). The addition of 10 μM pyocyanin to 100 pmol of 6-keto-PGF_1-alpha standard in Hanks balanced salt solution with 100 μM NADH (to reduce the pyocyanin in the absence of cells) for 30 min did not interfere with the ability of the antibody system to measure 6-keto-PGF_1-alpha (in the supernatant, 1,148 ± 36 cpm with pyocyanin and 1,093 ± 44 cpm without pyocyanin). Hence, pyocyanin did not interfere with the assay. Pyocyanin could also have complexed the A23187 and blocked stimulation of the cells. This, however, is unlikely because pyocyanin also blocked stimulation by arachidonic acid, which was in a greater-than-10-fold molar excess relative to effective concentrations of pyocyanin.

In our prior report the cytolytic effect of the combination of pyocyanin and ferripyochelin was attributed to hydroxyl radical formed by these two exoproducts in the presence of PPAE cells (1, 1a). However, the addition of superoxide dismutase (30 U/ml), catalase (500 U/ml), dimethylthiourea (50 mM), alpha-phenyl-*N*-*t*-butyl-nitrone (10 mM), or dimethyl sulfoxide (1%)

* Corresponding author. Mailing address: Department of Medicine, University of Iowa College of Medicine, Iowa City, IA 52242. Phone: (319) 356-4418. Fax: (319) 353-6406.

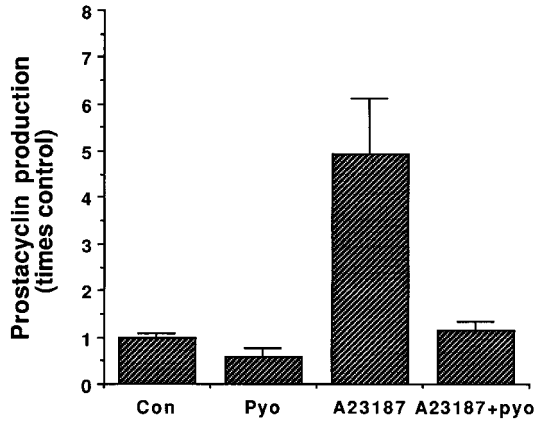

FIG. 1. Relative amounts of prostacyclin (measured as 6-keto-PGF_1-alpha) released from PPAE cell monolayers exposed to control medium (Con), 40 μM pyocyanin for 30 min (Pyo), 1 μM A23187 for 30 min (A23187), or 40 μM pyocyanin for 30 min and then 1 μM A23187 for an additional 30 min (A23187+pyo). Differences between control and pyocyanin and between A23187 and A23187 with pyocyanin were significant by Student's t test at a P value of <0.05. Unstimulated control cells released 26.5 ± 1.9 pmol/3 $\times 10^5$ cells. Control cells stimulated with A23187 released 131 ± 8.0 pmol/3 $\times 10^5$ cells.

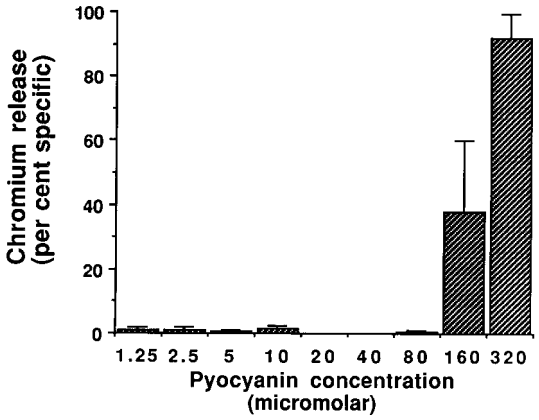

FIG. 3. Specific release of ^{51}Cr from PPAE cells exposed to pyocyanin for 4 h. ^{51}Cr release was increased at doses of ≥160 μM ($P < 0.05$ by analysis of variance). Specific release was calculated as (test well ^{51}Cr release − spontaneous ^{51}Cr release)/(maximum ^{51}Cr release − spontaneous ^{51}Cr release). Maximum release was achieved by adding 1% Triton X-100 to the medium. Control monolayers were incubated in Hanks balanced salt solution alone.

to the medium along with pyocyanin did not prevent inhibition of PGI_2 release from PPAE cells exposed to arachidonate.

Our prior experiments had demonstrated very little cytotoxicity of pyocyanin alone (1, 1a). When ^{51}Cr-labeled PPAE cells were exposed to pyocyanin, specific chromium release from the cells did not increase until the concentration of pyocyanin was ≥160 μM (Fig. 3).

PGI_2 is released from endothelial cells, and it modulates inflammatory and thrombotic pathways and vascular tone and even reduces the changes in vascular permeability following ischemia-reperfusion in some vascular beds (3, 8, 13). Pyocyanin is an effective electron acceptor capable of altering cell redox potential (1, 9). Because prostaglandin synthase is sensitive to redox tone, it was possible that pyocyanin, which by itself is not cytolytic in low concentrations, would alter prostaglandin synthesis. Indeed, at concentrations as low as 5 μM, pyocyanin significantly inhibited endothelial PGI_2 release. At similar concentrations pyocyanin reduced prostaglandin release from platelets (10). However, in contrast to our observations, the inhibition of platelet prostaglandin release was reversed by scavengers of oxygen radicals. The reason for the difference in the sensitivity to scavengers of oxygen radicals is not evident; our experiments tested a wide variety of both intracellular and extracellular scavengers multiple times with no effect.

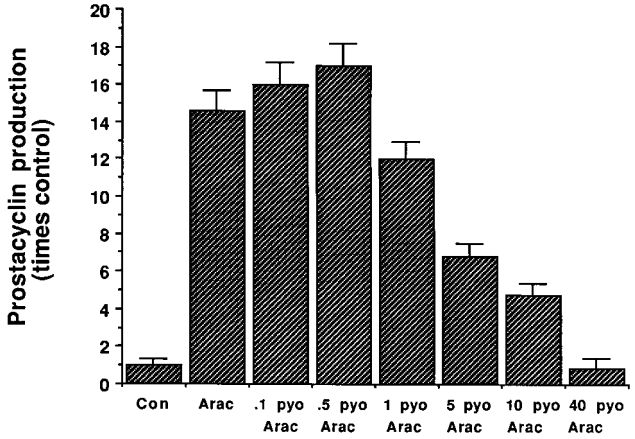

FIG. 2. Relative amounts of prostacyclin (measured as 6-keto-PGF_1-alpha) released from PPAE cell monolayers exposed to control medium (Con), 50 μM arachidonic acid (Arac), or 50 μM arachidonic acid with increasing concentrations of pyocyanin (pyo) from 0.1 to 40 μM. Control monolayers released 22.5 ± 1.5 pmol/3 $\times 10^5$ cells. Control cells with arachidonic acid released 174 ± 13 pmol/3 $\times 10^5$ cells. Differences among arachidonic acid alone and arachidonic acid groups with pyocyanin were significant ($P < 0.05$ by analysis of variance) at pyocyanin concentrations of ≥1 μM.

There are some important limits to the implications of our experiments that are inherent in our model. While the organisms must traverse the endothelium to enter the vessel wall during intravascular dissemination, the large numbers of organisms that accumulate in the more peripheral vessel wall of medium-sized arteries and veins might be separated from the endothelium by several layers of mesenchymal cells. However, in small pulmonary arteries and veins with only one or two layers of smooth muscle, the organisms were very close to the abluminal endothelial surface (4). It is difficult to estimate what concentrations of pyocyanin would be present in the immediate vicinity of the abluminal surfaces of these cells. Pyocyanin is very small (molecular weight, 210) and would likely diffuse rapidly between mesenchymal cells. In the sputa of bronchiectasis patients, pyocyanin concentrations reach 100 μM (6). In our experiments, concentrations of 5 μM caused 50% inhibition of prostacyclin release. We cannot be certain that these concentrations would be achieved in situ.

Nitric oxide released from endothelium, like PGI_2, plays important vasoregulatory and anti-inflammatory roles (7, 14). Pyocyanin also inactivates the vasoregulatory effects of nitric oxide (14). It is not certain how the inhibiting effects of pyocyanin on these important modulators of vascular tone and inflammation contribute to the characteristic *Pseudomonas* vascular lesion, but inhibition of both mechanisms would likely limit both the control of vascular tone and the local mechanisms that limit the inflammatory response (3, 7, 11). Loss of these normal homeostatic mechanisms may contribute to the lesions observed.

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