Effect of Photodynamic Therapy on the Endothelium-dependent Relaxation of Isolated Rat Aortas

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ABSTRACT

The early vascular effects of photodynamic therapy (PDT) include transient vasoconstriction and platelet aggregation. Since endothelium-derived relaxing factor (EDRF) is a potent vasodilator and inhibitor of platelet aggregation, we questioned whether PDT impairs the production of EDRF.

To study this possible effect of PDT, endothelium-dependent relaxations of thoracic aortas obtained from male Wistar rats were determined. The aortic rings were connected to a isometric force transducer, exposed to various doses of Photofrin porfimer sodium (Photofrin) (0.1-1.0 µg/ml), and illuminated with red light (wavelength > 610 nm, 14.6 ± 1.5 mW/cm²) for different time periods (5-25 min). Endothelium-dependent relaxation was induced by acetylcholine in precontracted aortic rings. This EDRF-mediated relaxation was decreased after PDT in a light dose- and drug dose-dependent manner. Light microscopic examination did not show loss of endothelial cells. Similar results were obtained with rat aortas exposed to Photofrin in vivo and illuminated in vivo. Direct smooth muscle relaxation induced with sodium nitroprusside was not impaired, showing that PDT did not reduce the ability of smooth muscles to relax. No effect on the contractile responses was found either.

We conclude that PDT impairs the production or release of EDRF by the endothelium. This could play an important role in the initial events occurring in vivo during and after PDT.

INTRODUCTION

PDT is a promising form of cancer treatment, which is under investigation in several clinical trials in Europe, the United States, and Japan (1). PDT involves the administration of a photosensitizer, followed 24-78 h later by illumination of the tumor area with laser light of a suitable wavelength. The photosensitizers are generally porphyrin-like substances; Photofrin is the most investigated. Illumination leads to excitation of the photosensitizer retained in the tissue, with subsequent formation of singlet oxygen (¹O₂) through a type II reaction with molecular oxygen (2, 3). Singlet oxygen is the main species in PDT-related cytotoxicity (4) disrupting plasma membranes, mitochondria, and lysosomes of cells (5).

Besides direct tumor cell kill, prominent vascular effects in the tumor area occur during and after PDT. Transient vasoconstriction, reduction of blood flow, platelet aggregation, and leukocyte adhesion have been observed within minutes after the beginning of PDT (6-10). With time thrombus formation, hemorrhage, extravasation of red blood cells, disruption of the vasculature, and complete stasis occur, leading to oxygen and nutrient deprivation of the tumor with consequent tumor regression (7, 8, 11). Several investigators have shown that the vascular effects of PDT are indispensable for tumor necrosis (7, 11, 12).

Although many investigations have been performed on the vascular effects of PDT in vivo, the underlying mechanisms and sequence of events are not fully elucidated. Because of the local character and the brief time sequence in which vessel events occur, it is likely that PDT exerts its effects on the vasculature through alterations in or very near the vessels (6, 8). There is evidence that cyclooxygenase products play a role (13, 14).

In the present study we focus on direct PDT effects on the endothelium. The endothelium, which forms a continuous monolayer enveloping the circulating blood, is a very important metabolic and endocrine organ (15). One of the vasoactive factors produced by the endothelium is the EDRF, a nitric oxide-related species. EDRF is a strong vasodilator and potent inhibitor of platelet and leukocyte adhesion (16, 17). Consequently, impairment of EDRF production will lead to vasospasm and to aggregation of platelets. Indeed, in the cerebral microcirculation it has been shown that even minimal endothelial injury converts acetylcholine-induced relaxation of brain arterioles to constriction and increases platelet adhesion (18-20).

Considering this, we hypothesized that the observed early vascular effects of PDT could be due partly to impairment of EDRF production. The present investigation was performed in order to study the effect of PDT on endothelium-dependent responses in isolated vessels.

MATERIALS AND METHODS

Photosensitizer. Photofrin in NaCl solution (2.5 mg/ml) was obtained from Photomedica, Inc. (Raritan, NJ). This compound is hematoporphyrin derivate, enriched in active material. Photofrin was stored at ~20°C until use.

Chemicals. U-46619, a prostaglandin analogue, was obtained from Cayman Chemical Co., Inc. (Ann Arbor, MI); acetylcholine chloride was from Dispersa A.G. (Hettingen, Switzerland); sodium nitroprusside was from the British Drug House, Ltd. (Poole, England); indomethacin was from Sigma Chemical Co. (St. Louis, MO); and histamine was from Aldrich Chemie (Brussels, Belgium). All other chemicals used were of analytical grade.

Isometric Force Recording on Rat Aortas. Rat thoracic aortas were isolated from adult male Wistar rats (250-300 g) anesthetized with ether. Aortas were cleaned of excessive fat and connective tissue while kept in cold buffer. Rings of about 4-mm length were cut and suspended between two stainless steel wired hooks; the wires were attached to the bottom of an 8-ml organ bath and to a force transducer (52-9529; Harvard Apparatus, Inc.) for continuous isometric tension monitoring. The bath contained a Krebs-Ringer-bicarbonate buffer (in iml: 118 NaCl, 4.7 KCl, 1.5 CaCl₂, 25 NaHCO₃, 1.1 MgSO₄, 1.2 KH₂PO₄, 5.6 glucose) maintained at 37°C and aerated with 95% O₂/5% CO₂ (pH 7.4).

The suspended aortic rings were progressively stretched for 30-60 min until a basal tension of 2.5 g was reached. After stabilization, aortic rings were given 30 ms KCl and 50 ms U-46619 successively to test for normal smooth muscle contraction.

To study relaxation, the rings were contracted with 10-25 ms U-46619 to approximately 80% of the maximal contractile response to this agent. Next, endothelium-dependent relaxation was induced with ACh in the presence of indomethacin, a cyclooxygenase inhibitor, to prevent the release of prostanooids. Under these conditions, ACh triggers the production of EDRF, and thus ACH-dependent relaxation confirms the presence of functional endothelium (21). PDT experiments were then carried out.

PDT Treatment: Drug and Light Exposure. Aortic rings were incubated in vitro with various concentrations of Photofrin (0.1 to 1.0 µg/ml) in the organ bath for 15 min and then washed three times with buffer. Aortas treated in vivo
with Photofrin were obtained from rats which were given i.p. injections of 10 mg Photofrin/kg body weight. After keeping the animals in the dark for 24 h, the aortas were isolated and connected to the force transducer as described above.

Red light was delivered for 5 to 25 min by a 500-W slide projector with a cutoff filter (<610 nm, no. 59512; Oriel Co., Stratford, CT). The average fluence rate was measured in the organ bath with an isotropic light detector, calibrated against radiometer UDT 371 (United Detector Technology), and amounted to 14.6 ± 1.5 mW/cm²

Illumination was provided during basal tension. During the experiments the aortic rings were shielded from ambient light. At the time of illumination no Photofrin was present in the buffer.

Measurements of Tissue Porphyrin Levels. Porphyrin levels were determined according to the method of Star et al. (7). In short, aortic rings were minced and porphyrins were hydrolyzed and extracted in 0.1 M NaOH/2% sodium dodecyl sulfate at 100°C during 15 min. After centrifugation, fluorescence intensity in the supernatant was measured in a fluorescence spectrophotometer (Perkin-Elmer MFP-3) at an excitation wavelength of 403 nm and an emission wavelength of 625 nm. Fluorescence peaks were compared to appropriate standards of known concentrations of Photofrin in 0.1 M NaOH/2% sodium dodecyl sulfate. Amounts of porphyrin are expressed as ng/mg wet weight.

Light Microscopy. After PDT the lumen of the aortic rings was filled with warm liquid gelatin solution (10%, 60°C). When the gelatin had become solid the specimens were fixed with 1% glutaraldehyde and 4% formaline in phosphate buffer for 24 h, dehydrated, and embedded in paraffin. Transversal sections (5 μm) stained with hematoxylin/eosin were examined by light microscopy.

Statistics. All data are presented as means ± SEM. Light dose- and drug dose-dependent response relationships were assessed by multiple linear regression using the Stata release 2.0 (Computing Resource Center, Los Angeles, CA). The coefficient of correlation (R²) and probability P (significance) were determined.

The relaxation and contraction curves were fitted to a three-parameter logistic function and −log EC₅₀, the slope, and the maximum of each curve were calculated (22). These values are presented as means ± SD and analyzed if appropriate on the basis of Student's two-tailed test or by one-way analysis of variance. A P value of <0.05 was considered significant.

RESULTS

PDT Effect on Endothelium-dependent Relaxation. To investigate the effect of Photofrin plus light (i.e., PDT) on the production or release of EDRF, the effect of this treatment on basal tension was studied first. Exposure of control aortas to red light (up to 60 min) did not alter the basal tension of 2.5 g. Basal tension of Photofrin-treated aortas during and after illumination also did not change.

Next, the EDRF-dependent relaxation responses to cumulative doses of ACh of Photofrin-treated aortic rings, precontracted with 10−25 μM U-46619, were compared before and after illumination. Relaxation was expressed as a percentage of the maximal response to ACh.

The results show that treatment of the rings with 0.25 μg/ml Photofrin in the dark did not influence the dose-dependent response to ACh compared to the relaxation before Photofrin exposure (Fig. 1). In contrast, illumination of those Photofrin-treated aortas with red light (10 min, 14.6 ± 1.5 mW/cm²) did cause a significant decrease of relaxation in response to ACh (Fig. 1). While the −log EC₅₀ and the slope of the relaxation curves of Photofrin-treated aortas did not differ before and after illumination (−log EC₅₀: 7.0 ± 0.3 and 6.8 ± 0.5, not significant; slope: 1.6 ± 0.8 and 1.0 ± 0.5, respectively, not significant), the maximal response to ACh was significantly decreased after light treatment to 32 ± 8.2% (P = 0.003). This effect of light on the relaxation was irreversible; no recovery of function was found during a maximum observation period of 3 h.

To examine whether the effect of PDT on the endothelium-dependent relaxation was light dose- and/or drug dose-dependent, we performed PDT with either a fixed drug dose and various illumination time periods or a fixed illumination time and various drug doses. Illumination of Photofrin-treated aorta rings (0.25 μg/ml) for different time periods caused a significant decrease of ACh-induced relaxation in a light dose-dependent manner (R² = 0.60, P = 0.001; Fig. 2).

Using different drug doses and an illumination time of 5 min, a decrease in response to ACh was found that was Photofrin dose-dependent (R² = 0.85, P < 0.001; Fig. 3).

In Vivo Treatment with Photofrin. To examine possible differences between the administration of Photofrin to aortas in vitro and exposure to the drug in vivo, rats were given 10 mg Photofrin/kg body weight i.p. 24 h before the isolation of the aorta. Illumination of those aortas resulted in a light dose-dependent decrease of ACh-induced relaxation (R² = 0.56, P = 0.004; Fig. 4). These results were similar to those obtained with aortas exposed in vitro to 0.1 μg/ml Photofrin.

Porphyrin Levels in Aorta Rings. To investigate whether the drug dose-dependent effect of PDT on the ACh response was directly related to the amount of Photofrin in the aorta and to determine
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Fig. 3. Drug dose-dependent effect of PDT on the relaxation to ACh. The relaxation of PDT-treated aortas (various doses of Photofrin; 5 min of red light) in response to 1.0 \( \mu \)M ACh was determined and expressed as a percentage of the relaxation before PDT. Each point represents the mean ± SEM of at least three separate experiments.

Fig. 4. Effect of illumination on the endothelium-dependent relaxation of aortas from rats given Photofrin in vivo. The relaxation in response to 1.0 \( \mu \)M ACh was determined in aortas obtained from rats given 10 mg Photofrin/kg i.p. 24 h before and after illumination in vitro for various time periods. The relaxation response is expressed as a percentage of the relaxation before illumination. Each point represents the mean ± SEM of at least three separate experiments.

Whether in vivo administration of Photofrin led to comparable retention of the drug, porphyrin levels in the treated aortas were determined. As shown in Table 1, in vitro exposure to Photofrin showed a dose-dependent increase of porphyrin content in the tissue \((R^2 = 0.76, P = 0.001)\). Apparently, the dose-dependent character of the decreased relaxation after PTD is due to a drug dose-dependent retention of Photofrin in the tissue. In vivo treatment with Photofrin \(10 \text{ mg/kg body weight}\) led to a level of 3.4 ± 0.7 ng porphyrin/mg wet tissue in the aorta, which is of a magnitude similar to that obtained by in vitro treatment with 0.1 \( \mu \)g/ml Photofrin (Table 1).

**Microscopic Study.** The decreased ACh response after PDT could be due to the loss of endothelial cells. Therefore, PDT-treated aorta rings were examined by light microscopy. A normal continuous layer of endothelial cells lining the vessel wall of PDT-treated aorta rings was found (Fig. 5).

**PDT Effect on Smooth Muscle Relaxation.** The impairment of the endothelium-dependent relaxation could be caused by a reduced ability of the smooth muscles to relax and/or a reduced ability to respond to EDRF after PDT. To investigate this, we determined the endothelium-independent smooth muscle relaxation of precontracted aortic rings to the nitric oxide-generating agent sodium nitroprusside before and after PDT treatment. For untreated rings a dose-dependent relaxation curve was found with a \(-\log EC_{50}\) of 7.5 ± 0.2 and a slope of 1.2 ± 0.2 (Fig. 6).

Treatment of the rings with 1.0 \( \mu \)g/ml Photofrin, either shielded from light or followed by 10 min of illumination, did not lead to significant changes in values for maximum, slope, and \(-\log EC_{50}\) of the dose-response curve for nitroprusside. PDT did not decrease the smooth muscle relaxation to nitroprusside.

**PDT Effect on Smooth Muscle Contraction.** Another possibility is that PDT stimulates the contraction induced by U-46619, which could interfere with the ACh-responses. Therefore, the contractions by U-46619 were examined in normal and PDT-treated aortic rings. Untreated specimens showed a dose-dependent contraction in response to U-46619 with a maximum of 3.3 ± 0.6 g above the basal

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**Table 1** Levels of porphyrins in aortic tissue after exposure to Photofrin

Data are the means ± SEM of porphyrin levels determined in aortic pieces which had been treated with different concentrations of Photofrin for 15 min in vitro or for 24 h in vivo. Measurements were performed in triplicate according to the method of Star et al. (7). Regression analysis showed a statistically significant relationship between incubation dose and porphyrin content \((R^2 = 0.76, P = 0.001)\).

<table>
<thead>
<tr>
<th>Photofrin administration (µg/ml in organ bath)</th>
<th>Porphyrins in aortic rings (ng/mg wet tissue)</th>
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<tbody>
<tr>
<td>0.0</td>
<td>1.9 ± 0.2</td>
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<tr>
<td>0.10</td>
<td>3.0 ± 0.6</td>
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<tr>
<td>0.25</td>
<td>4.9 ± 1.0</td>
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<td>0.50</td>
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<td>1.0</td>
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![Fig. 5. Transverse section of a PDT-treated aorta (1 µg/ml Photofrin, 10 min of red light). Note the continuous endothelial cell lining and the intact medial smooth muscle layer. Hematoxylin/azofloxin; Zeiss light microscope; magnification: A, × 400; B, × 1000.](cancerres.aacrjournals.org)
tension of 2.5 g, a $-\log \text{EC}_{50}$ of 8.4 ± 0.05, and a slope of 3.0 ± 0.8 (Fig. 7). Incubation of the rings with 1.0 μg/ml Photofrin either shielded from light or followed by 10 min of illumination, did not induce significant changes in the characteristics of the dose-dependent U-46619 response.

**DISCUSSION**

The major finding of the present study was that PDT impaired the endothelium-dependent relaxation of vascular smooth muscle.

Exposure of aortic rings to Photofrin for 15 min followed by illumination with red light (14.6 ± 1.5 mW/cm²) led to a light dose- and drug dose-dependent reduction of the EDRF-mediated relaxation response (Figs. 2 and 3). The relationship between the reduction in relaxation and the concentration of Photofrin in the organ bath coincided with the amount of drug in the vessels. In vivo exposure of rats to Photofrin and ex vivo illumination showed comparable decreases in the responses to ACh.

To examine the possibility that PDT has affected the function of the smooth muscle cells, causing a reduced ability to relax and/or to respond to EDRF, we studied the endothelium-independent smooth muscle relaxation by sodium nitroprusside. NO is released from nitroprusside in the cytosol of the smooth muscle cell and activates soluble guanylate cyclase, leading to the formation of cyclic GMP and eventually to relaxation (15). We found that PDT did not decrease the responses to sodium nitroprusside. Apparently the activation pathway of NO in the smooth muscles is not disturbed. The contractile responses of the smooth muscles to U-46619 were also not affected. Furthermore, we did not find a direct PDT-dependent effect on the smooth muscle basal tone. Others (23) have reported that PDT did have such an effect on in vitro vessels, i.e., spontaneous endothelium-independent contraction. This might be due to the higher photosensitizer concentrations these investigators used (30 μg/ml hematoporphyrin derivative). In the present study PDT impaired the relaxation in response to ACh, which occurs via an endothelial membrane receptor-mediated production and release of NO (EDRF) (21), without affecting direct smooth muscle relaxation or contraction. Therefore, we conclude that PDT inhibited the production or release of EDRF by the endothelium.

How PDT has led to impairment of EDRF production or release remains to be elucidated. This effect of PDT was not specific for the muscarinic receptor-mediated response (i.e., ACh response) since a similar reduction of relaxation was found in response to histamine, which induces EDRF production through the histaminergic receptor (data not shown). Several in vivo studies have reported that injury to endothelial cells (9, 24–27) does occur after PDT but not until 1 to 6 h after completion of PDT. This in vivo damage could be caused by thrombus formation and cessation of blood flow. We found a continuous endothelial cell layer after PDT in vitro. The impaired EDRF response might therefore be caused by a PDT-dependent effect on membrane receptors, signal transduction systems, or the EDRF synthetase of the endothelial cells. In fact, damage to proteins leading to inter- and intramolecular cross-linking and impairment of the active and passive ion transport after PDT have been reported (28, 29).

In addition to EDRF, endothelial cells produce prostacyclin, which also has relaxing and antiaggregating properties. An in vivo study in rats (14) showed that PDT did not change the level of prostacyclin in the circulation, but the level of thromboxane did increase significantly after PDT. Activated platelets are the major source of thromboxane. This vasoconstrictor causes severe contraction if its effect is not opposed by relaxators like EDRF (15). It seems unlikely that PDT activates platelets directly, because exposure of platelets to Photofrin and light in vitro leads to a very rapid, drug dose-dependent inhibition of aggregation (30). However, local impairment of EDRF production in vivo facilitates the adhesion and subsequent aggregation of platelets to the endothelium (18–20). It is conceivable that the photosensitized release of von Willebrand factor multimers originating from the Weibel-Palade bodies of the endothelium (31) provides the necessary anchors for those platelets to adhere to the endothelium. Furthermore, exposure of the flowing blood to the subendothelium by a transient change in shape of the endothelial cells may render the vessel wall thrombogenic as well. PDT may mediate this process by its ability to induce microtubule depolymerization in the endothelial cells (32). During PDT the number of leukocytes that adhere to the vascular wall increased as well (10). Since this event is not prostanoiid dependent, the increased adherence might be due to an impaired release of EDRF, as shown in the present study.

Taken together, the underlying mechanisms of the vascular changes after PDT in vivo are complex. In addition, the organization and architecture of tumor vasculature changes in time and space (33). This means that tumor vessels would not necessarily respond to PDT as the
normal vessels studied here. However, a PDT-mediated decrease in the production of EDRF could very well be one of the earliest events resulting in constriction of the vessels that are composed of smooth muscle cells.

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REFERENCES

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