Photochemistry and Photobiology, Vol. 58, No. 5, pp. 661–669, 1993
Printed in the United States.

0031-8655/93 $05.00+0.00

RELAXATION OF VASCULAR SMOOTH MUSCLE INDUCED BY LOW-POWER LASER RADIATION

HINA CHAUDHRY, MARY LYNCH, KEVIN SCHOMACKER, REGINALD BIRNGRUBER, KENTON GREGORY and IRENE KOCHEVAR*
Wellman Laboratories of Photomedicine, Department of Dermatology, Massachusetts General Hospital, Harvard Medical School, Boston, MA 02114, USA

(*Received* 13 *April* 1993; *accepted* 16 *June* 1993)

Abstract—The relaxation of rabbit aorta rings induced by low-power laser radiation was investigated *in vitro* to determine the location of the chromophore(s) responsible for this response and evaluate possible mechanisms. An action spectrum for relaxation was measured on rabbit thoracic aorta rings precontracted with norepinephrine. The decrease in isometric tension was measured during exposure to laser light (351–625 nm) delivered *via* a fiber optic to a small spot on the adventitial surface. The shortest UV wavelength (351 nm) was 35-fold more effective than 390 nm and 1700-fold more effective than 460 nm. Ultraviolet wavelengths also produced greater maximum relaxation (0.40–0.45) than visible wavelengths (0.20–0.25), suggesting that photovasorelaxation involves more than one chromophore.

The adventitial layer was not necessary for photovasorelaxation, indicating that the light is absorbed by a chromophore in the medial layer. The same degree of relaxation was obtained on rings without adventitia when either one-half of the ring, or a small spot was irradiated indicating that communication between smooth muscle cells spreads a signal from the area illuminated to the entire ring.

The mechanism for photovasorelaxation was investigated using potential inhibitors. *N*-monomethyl-L-arginine and *N*-amino-L-arginine, inhibitors of nitric oxide synthase, did not alter photovasorelaxation nor did indomethacin, an inhibitor of cyclooxygenase, and zinc protoporphyrin, an inhibitor of heme oxygenase.

INTRODUCTION

Light-induced relaxation of vascular smooth muscle (photovasorelaxation) has been demonstrated *in vitro* and *in vivo* using UV and visible radiation from lamps and, more recently, from lasers. Photovasorelaxation induced by low-power light exposure in the absence of photosensitizing dyes was first reported by Furchgott and coworkers.[1,2] In these studies, the isometric tension of epinephrine-contracted vascular strips decreased within several seconds after the beginning of the light exposure and was reduced to <50% of the drug-induced tension upon continued exposure to light. Recontraction to the initial tension began within seconds after the light exposure was terminated. Photovasorelaxation has been shown to be independent of the presence of an intact endothelial layer.[3,5] A mechanism has been postulated involving a photochemical product of an endogenous chromophore that activates species capable of causing smooth muscle relaxation.

Current studies on photovasorelaxation produced by laser radiation are motivated by the possibility that laser radiation, delivered by a fiber optic, may be able to reverse vasospasm.[6,7] Three types of laser-induced vasorelaxation have been reported. Low-power radiation from a continuous wave (cw) visible laser produces reversible relaxation of blood vessels *in vitro* and *in vivo*.[7,8] This type of laser-induced vasorelaxation resembles the previously reported response to conventional low-power lamps.[1,2] When higher laser powers are used, the temperature of the tissue rises during the light exposure and irreversible, thermally induced relaxation and/or contraction are observed.[8] The third type involves pulsed, high-intensity laser radiation that elicits sustained vasorelaxation[9–12]; the mechanism for this response is not clear.

Exposure of blood vessels to low-power cw argon laser radiation (487–514 nm) *in vitro*[8] and *in vivo*[7] follows the same time course as that produced with conventional lamps. The most effective wavelengths were reported to be less than 350 nm in one study[2] and in the blue region of the spectrum (380–420 nm) in another study.[13] Knowledge of the action spectrum for vasorelaxation is important in order to identify the endogenous chromophore in blood vessels that initiates the photobiological reactions as well as for selection of the optimal wavelengths to use for possible clinical applications.

The studies reported here address the location of the chromophore in blood vessels that is responsible for initiating smooth muscle relaxation and possible mechanisms for this process. Studies were performed on aortic rings of rabbits to determine the relative effectiveness of UV and visible wavelengths delivered to a small area of the tissue, to determine the relationship of the size of the area irradiated to the response, and to evaluate inhibitors of possible pathways for photovasorelaxation.

MATERIALS AND METHODS

Preparation of aortic rings

New Zealand white rabbits weighing 3–5 kg were anesthetized with ketamine (50 mg/mL) and xylazine (100 mg/mL) given intramuscularly in a 2:1 ratio. The descending thoracic aorta was excised and placed immediately in cold Krebs bicarbonate buffer (118 m*M* NaCl, 4.7 m*M* KCl, 2.5 m*M* $CaCl_2$, 1.2 m*M* $MgSO_4$, 1.2 m*M* KH_2PO_4, 25 m*M* $NaHCO_3$, 11 m*M* glucose and 0.03 *M* versene). The vessel was cleaned of adherent fat and connective tissue and cut into rings 4–5 mm in length, avoiding side branches. Each aortic ring was mounted in a water-jacketed tissue suspension chamber containing

*To whom correspondence should be addressed.

Krebs buffer maintained at 37°C and continuously gassed with 95% O_2 and 5% CO_2. Each ring segment was mounted isometrically between a steel prong attached to the chamber assembly and a triangular steel wire connected to a force transducer (model FTO3, Grass Instruments, Quincy, MA).

Materials

Norpinephrine was from Winthrop Pharmaceuticals and stock solutions were prepared in Krebs buffer on the day of the experiment. *N*-monomethyl-L-arginine (NMMA) acetate salt was purchased from Sigma Chemical Co., and a 300 m*M* stock solution was prepared in Krebs buffer. *N*-nitro-L-arginine methyl ester (NAME) HCl from Sigma was prepared as a 10 m*M* stock in 0.01 *N* HCl. *N*-amino-L-arginine (NAA) HCl was a gift from Wellcome Research Laboratories, England and was prepared as a 35 m*M* stock in Krebs buffer. Zinc protoporphyrin IX from Aldrich was prepared as an 8 m*M* stock in 0.15 *N* NaOH, 81% ethanol. Indomethacin from Sigma was prepared as a 14 m*M* stock in absolute ethanol. Diethyldithiocarbamic acid from Aldrich Chemical Co. was prepared as a 35 m*M* stock in Krebs buffer. α-Furildoxime from Sigma was prepared as a 100 m*M* stock in 95% ethanol.

Sources of UV and visible radiation

Lasers. The 334, 351 and 364 nm UV lines of a cw argon ion laser (Innova 100, Coherent Inc.), spatially separated with an extracavity prism, were used. The combined UV lines were also used to pump a dye laser (CR-599, Coherent Inc.) containing either Exalite 392 (380–400 nm) or stilbene 420 (420–480 nm). In addition, the 514 nm argon line was separated with an intracavity prism and used as such or used to pump either rhodamine 560 (540–570 nm) or DCM (630 nm). All laser dyes were obtained from Exciton Chemical Co. To remove UV plasma lines, a Corning 3-71 longpass filter was used with 514 nm radiation. The laser output was coupled to a 200 μm diameter, 0.257 NA fiber using a 1 inch focal length quartz lens. The laser power was altered by inserting calibrated neutral density filters in the laser beam before the optical fiber. The power delivered was measured before irradiation using a calibrated thermopile (model 210, Coherent Inc.) for powers greater than 10 mW; for powers less than 10 mW, a calibrated photodiode (560B Lite Mike, Salem, MA) was used.

For most laser experiments, the optical fiber was threaded through a glass delivery arm that permitted precise alignment of the fiber tip 1 mm in front of the adventitial surface of the ring by means of a translator stage assembly. The area illuminated was 0.0028 cm^2.

For experiments in which uniform irradiation of one entire side of an arterial ring was desired a beam expander was used. In the beam expander, a 1 mm diameter optical fiber was placed at the focal point of a 5 mm diameter sapphire sphere and the beam was turned 90° with a prism to irradiate the adventitial surface of the arterial ring at right angles. The beam was 9 mm in diameter at the distance of the arterial ring surface. The area of the arterial ring (approximately 0.4 × 0.5 cm) fit within the illuminated area. The power was measured with a thermopile (model E4, Eppley Laboratories, Newport, RI).

Lamps. A 1 kW xenon lamp with a 0.2 m monochromator (model 1681, Spex Industries) was used for large beam diameter 351 and 390 nm radiation (11 nm bandwidth). The exiting light was focused into the beam-expanding device described above. For studies of inhibitors or enhancers of photovasorelaxation, a 75 W xenon lamp (model A1010, Photon Technology International, S. Brunswick, NJ) filtered with a 350 nm bandpass filter (Corning 3-37) was used with calibrated neutral density filters. The filtered beam was focused and launched either into the beam-expanding device or into the 200 μm fiber for small spot size irradiations.

Irradiation of aortic rings

The mounted rings were brought to a resting tension of 6.0 g over a period of 1 h before an intermediate level of contraction (4–6 g above resting tension) was induced using 30–50 n*M* norepinephrine. The light was delivered to one side of the adventitial surface of the aorta ring. An arterial ring was usually treated to a series of five to seven exposures of increasing power, P. For small spot size irradiations, the fiber tip was moved to a fresh site for each irradiation. The irradiation was continued until a constant level of relaxation was attained except when high powers were used where a constant level was not achieved (see Results). After each exposure, the ring was allowed to recontract before the next irradiation. This procedure was followed for at least three arterial segments for each experiment.

Data collection and analysis

Isometric tension was continuously recorded during an experiment. Output from each force transducer were fed to low-level DC amplifiers (model 7P122E, Grass Instruments, Quincy, MA). For action spectra and irradiation area studies, the amplified signals were acquired using MacInstruments Data Acquisition Software and interface board (GW Instruments, Somerville, MA). The initial rates of relaxation and percent steady-state relaxation were calculated. For inhibitor studies, the amplified signals were fed to an analog to digital converter interface board (Labmaster DMA, Scientific Solutions, Solon, OH) installed in a 8086 computer. A shutter driver (model SD-10, Vincent Associates, Rochester, NY) was controlled by the interface board to keep the irradiation times in synchronization with the timed measurements (<0.1 s resolution). All force transducer measurements were calibrated against a 10 g weight, converting voltage levels to grams. The resting tension was subtracted from the data before analysis.

Removal of adventitia

A 1 mL pipette was inserted into the lumen of one-half of an aorta and, under a dissecting microscope, a shallow incision was made along the longitudinal axis of the vessel. The adventital layer was gently eased away from the medial layer. Light microscopic examination (hematoxylin and eosin stain) demonstrated that the adventitia had been removed along with the external elastic lamina, but the layer of smooth muscle was intact, as was the internal elastic lamina. There was evidence of partial removal of the endothelial layer. The tissue was then cut into 5 mm rings. The other half of the aorta was used as a control. Rings without adventitia stretched more easily and only reached 3 g baseline tension. They also required about four-fold more norepinephrine to produce sufficient contraction for measurements.

Studies of potential inhibitors of photovasorelaxation

Evaluations of potential inhibitors of photovasorelaxation were performed by comparing the light-induced response of aorta rings treated with inhibitor plus vehicle *versus* the response in rings treated with vehicle alone. Vascular rings were incubated with potential inhibitors for at least 15 min prior to irradiation. These studies employed the 75 W xenon lamp filtered to deliver 350 ± 10 nm light to the entire side of an arterial ring.

Statistical analysis

Student's unpaired *t*-test two-tailed was used to test for differences between groups.

RESULTS

Kinetics of photovasorelaxation

The change isometric tension in isolated rabbit aorta rings was measured as a function of irradiation time. The photovasorelaxation produced by exposure to UV and visible laser radiation followed a similar pattern at most wavelengths and powers (Fig. 1). The baseline tension, T_o, induced by norepinephrine is the total tension on the ring minus about 6 g of resting tension produced by stretching the ring. The value of T_o decreased upon light exposure. The relaxation, R, is defined as:

$$R = \Delta T/T_o \quad (1)$$

where ΔT is the difference between the baseline tension, T_o, and the tension above the resting tension at any time during

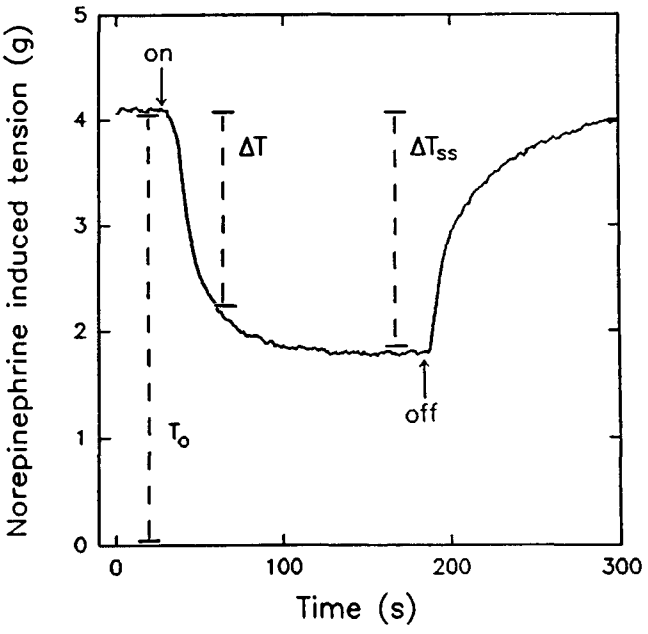

Figure 1. Change in norepinephrine-induced isometric tension, T, produced by laser irradiation of a rabbit aortic ring. Laser radiation at 351 nm was delivered to a 0.0028 cm^2 area on the adventitial surface of the ring. Irradiation with 0.050 mW began at time = 30 s and ended at 180 s. T_0 is the baseline tension induced by norepinephrine above the 6 g of tension produced by stretching the ring. ΔT equals T_0 minus the tension at any time during the experiment, and ΔT_{ss} is T_0 minus the tension when a steady-state tension is maintained.

the light exposure (Fig. 1). Relaxation from T_0 to a steady-state tension occurred in less than 3 min. The steady-state relaxation, R_{ss}, is

$$R_{ss} = \Delta T_{ss}/T_0 \quad (2)$$

where ΔT_{ss} is the difference between T_0 and the tension when a steady state is reached. Relaxation rates, dT/dt, were calculated from the linear portion of tension *versus* time curves such as shown in Fig. 1 between 40 and 60 s.

When the laser exposure was terminated, contraction began immediately. In 3–4 min the recontraction was complete. This pattern of light-induced relaxation and recontraction to T_0 was observed when low laser powers (P < 300 mW) were used and when lamp irradiation at 350 nm was used.

Dependence of photovasorelaxation on power

In contrast to most photobiological responses, the extent of photovasorelaxation depends on the rate of photon delivery rather than the total number of photons observed. The relationship of R_{ss} and power at wavelengths (351–460 nm) where relatively low powers produced maximum relaxation (P < 300 mW) showed similar patterns. At powers producing R_{ss} less than about 0.30, the values for R_{ss} are proportional to the incident power as shown for exposure of rings to 351 nm radiation in Fig. 2A. At higher power levels the increment of R_{ss} per increment of P decreased until R_{ss} reached a maximum, R_{max}. At the longest wavelengths (514–630 nm) where high powers were required, R_{ss} initially increased with power and then decreased (Fig. 3). The R_{max} was taken as the highest

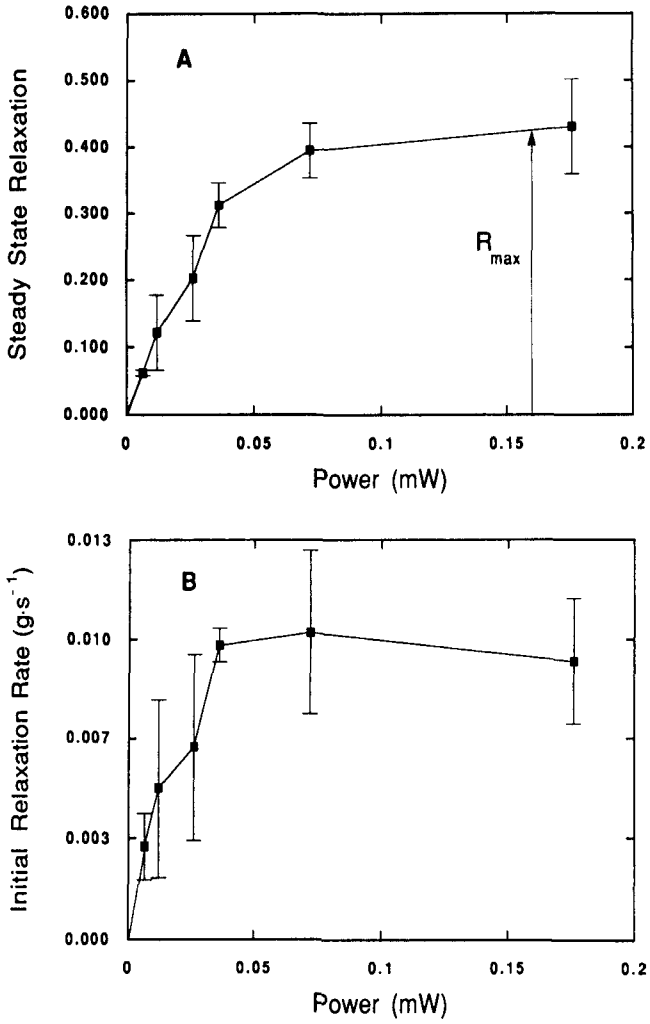

Figure 2. Relaxation of norepinephrine-induced tension in rabbit aortic rings using 351 nm laser radiation. (A) Relationship between light-induced steady-state relaxation, R_{ss}, and laser power. (B) Relationship between the light-induced relaxation rate, dT/dt, and laser power. Laser radiation was delivered to a 0.0028 cm^2 area of the adventitial surface. Error bars show the SEM from experiments on at least three aorta rings.

R_{ss} value obtained in these experiments. At these wavelengths, the high powers produced partially irreversible relaxation most likely due to thermal damage to the area irradiated and adjacent tissue. The rise in temperature accompanying cw laser 514 nm exposure has been measured.[8]

The relaxation rate, dT/dt, increased with the laser power incident on an aorta ring at low powers. Figure 2B shows dT/dt *versus* incident power for irradiation at 351 nm. The value of dT/dt increased with increasing laser power up to about 0.04 mW after which it remained approximately constant. A similar pattern was obtained for the other wavelengths examined (364, 375, 390, 400 nm). The values for dT/dt showed more variability between rings than the values for R_{ss}.

Comparison of Fig. 2A,B indicates that R_{ss} and dT/dt show similar relationships to power when the irradiation is at 351 nm. The same types of plots were made at 364, 375, 390 and

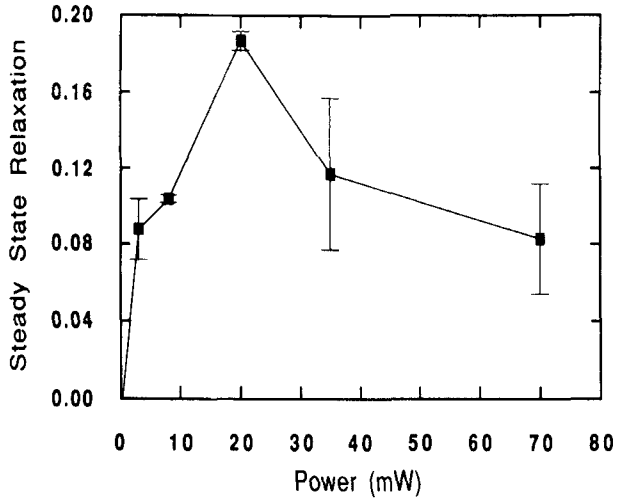

Figure 3. Relationship between the steady-state relaxation, R_{ss}, and laser power using 514 nm radiation. Relaxation was induced in norepinephrine-contracted rabbit aorta rings using laser radiation delivered to a 0.0028 cm^2 area of the adventitial surface. Error bars show the SEM from experiments on at least three aorta rings.

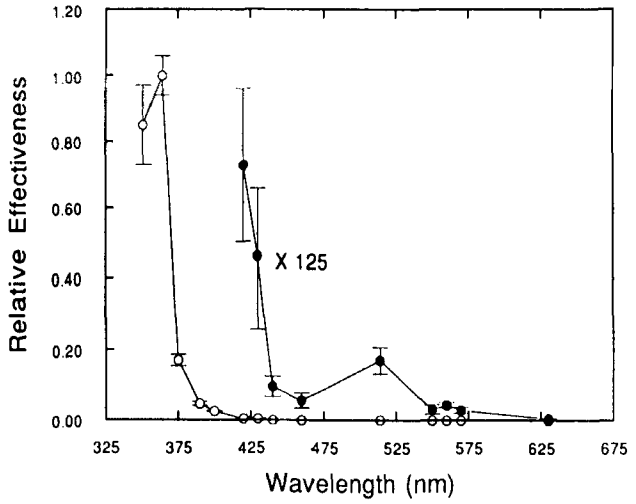

Figure 5. Action spectrum for photovasorelaxation of norepinephrine-contracted rabbit aorta rings using low-power laser radiation. The initial slopes of plots of steady-state relaxation, R_{ss}, *versus* rate of photon delivery, k_p, are the measure of effectiveness, S_o. Values of S_o have been normalized to unity at 364 nm. Laser radiation was delivered to a 0.0028 cm^2 area of the adventitial surface. Error bars show the SEM from experiments on at least three aorta rings.

400 nm. At each wavelength, there was an initial linear relationship and then a plateau. To demonstrate this relationship, the slopes of the linear portion of the plots (low-power slopes) such as those in Fig. 2A,B were compared and were plotted *versus* wavelength. The values for the low-power slopes were normalized at 364 nm to facilitate comparison, and the results are shown in Fig. 4. The slopes at low power (for both relaxation rate or steady-state relaxation) were higher at the lower wavelengths, indicating that these wavelengths are more effective. At all wavelengths, the relative values for the two slopes are the same within experimental error ($P > 0.15$).

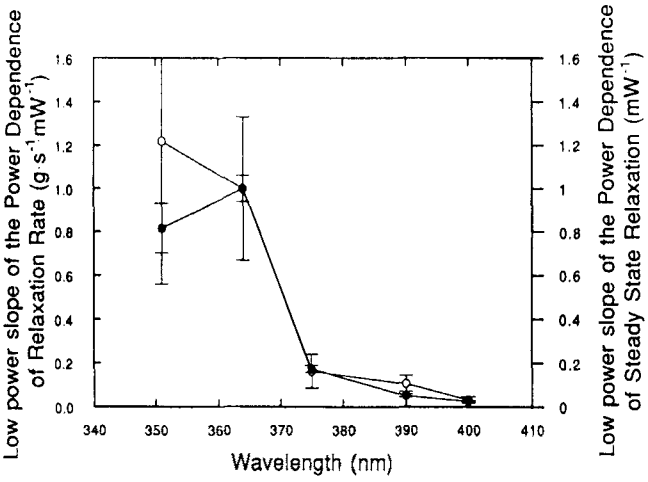

Figure 4. Comparison of the power dependence of relaxation rate with the power dependence of steady-state relaxation at five wavelengths. The slopes at low powers of plots of relaxation rate, dT/dt, *versus* power are shown as filled circles. The slopes at low powers of plots of steady-state relaxation, R_{ss}, *versus* power are shown as open circles. Rabbit aorta rings contracted with norepinephrine were exposed to laser radiation on a 0.0028 cm^2 area of the adventitial surface. Error bars show the SEM from experiments on at least three aorta rings.

Action spectrum for photovasorelaxation

Light-induced vasorelaxation was investigated at wavelengths between 351 and 625 nm. The power required to decrease the norepinephrine-induced tension varied greatly over this wavelength range. The relative decrease in steady-state tension at each wavelength as a function of rate of photon delivery, k_p, can be obtained from the R_{ss} *versus* power plots such as shown in Fig. 2A for 351 nm. The slopes of plots of R_{ss} *versus* k_p at low k_p are linear. The slope at low k_p, S_o, is defined as the effectiveness of that wavelength in accordance with the treatment originally proposed by Furchgott *et al.*[2]:

$$\text{Effectiveness} = (dR_{ss}/dk_p)_{k_p \to 0} = S_o. \quad (3)$$

In this treatment, S_o at each wavelength is obtained by assuming that the R_{ss} values are a hyperbolic function of the incident k_p, that is, the data take the following form:

$$R_{SS} = \frac{(R_{max} \times k_p)}{(C + k_p)} \quad (4)$$

where C is a constant with units of photon/s. This assumption is consistent with the model for photovasorelaxation discussed below. Equation 4 can be rearranged to:

$$(R_{SS})^{-1} = \frac{(R_{max})^{-1} + C}{(R_{max} \times k_p)}. \quad (5)$$

Plotting $(R_{ss})^{-1}$ *versus* k_p^{-1} yields linear plots with intercepts of R_{max}^{-1}. More importantly, the reciprocal of the slope of these plots can be shown to be equal to S_o. The slopes from linear regression analysis of these double-reciprocal plots gave values of S_o that were used to construct an action spectrum for photovasorelaxation that shows the relationship between wavelength and effectiveness for producing photovasorelaxation. The spectrum shown in Fig. 5 has been normalized to unity at 364 nm.

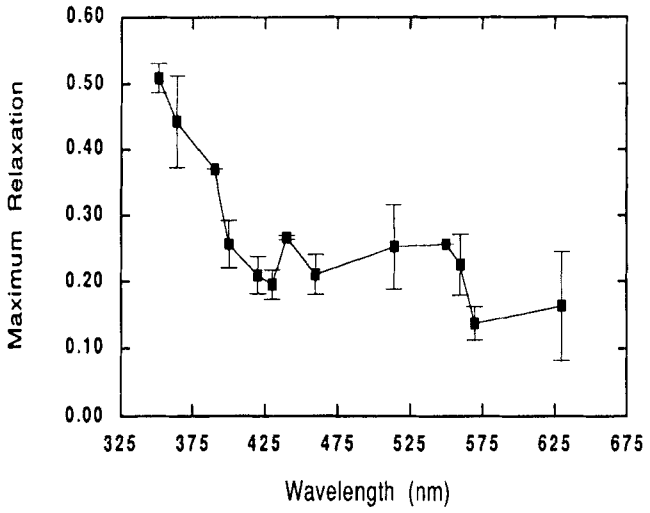

Figure 6. Relationship between maximum relaxation, R_{max}, and wavelength. Norepinephrine-contracted rabbit aorta rings were exposed to laser radiation on a 0.0028 cm^2 area of the adventitial surface. The R_{max} is the greatest relaxation produced at high laser power at each wavelength. Error bars show the SEM from experiments on at least three aorta rings.

Wavelengths in the UV region produced vasorelaxation most efficiently; the values for 351 and 364 nm were not significantly different ($P = 0.5$). At 351 nm, S_o was 7.4×10^{11} R/photon incident on the aorta ring. The effectiveness decreased rapidly as the wavelength increased; radiation at 351 nm was approximately 35-fold more effective than 390 nm radiation and 1700-fold more effective than 460 nm radiation.

Wavelength dependence of the maximum relaxation

Values for the maximum percent relaxation, R_{max}, were obtained from plots such as those shown in Fig. 2A. The values of R_{max} varied with the irradiation wavelength as shown in Fig. 6. The greatest R_{max} values (0.40–0.50) were obtained using the shortest UV radiation. A constant R_{max} (approximately 0.20–0.25) was observed for 390–560 nm radiation, and R_{max} decreased at the longest wavelengths (570 and 630 nm). Although R_{max} decreased at longer wavelengths, the decrease was not as great as observed for relaxation effectiveness, S_o, at the same wavelength range (Fig. 5). Radiation at 351 nm was approximately 1.2-fold more effective than 390 nm radiation and 2-fold more effective than 460 nm radiation. Radiation at 630 nm was 1.3-fold less effective than 460 nm radiation. Thus, even though S_o decreased dramatically at longer wavelengths, the R_{max} decreased by less than a factor of four.

Light-induced vasorelaxation as a function of area irradiated

The vasorelaxation produced by exposing one-half of an aortic ring uniformly to light was compared to that produced when less than 2% of the area was exposed to the same number of photons. The area of one-half of an aorta ring was approximately 0.20 cm^2, whereas the fiber optic delivered laser radiation to an area calculated to be only 0.0028 cm^2. The decrease in tension produced by irradiating one-half of an aorta ring or a small spot was measured at three wavelengths in different regions of the action spectrum (351, 390 and 514 nm). The maximum powers used were 0.18 mW (351 nm), 10 mW (390 nm) and 40 mW (514 nm). The intensities for the small area irradiation were over 70 times those for the large area irradiation since the same power was used at each spot size. The initial slopes, S_o, of the R_{ss} *versus* P plots and R_{max} values are shown in Table 1. At 351 and 514 nm, S_o and R_{max} were independent of the size of the area irradiated. At 390 nm, irradiation of the larger area produced more efficient relaxation by a factor of about four, which is much smaller than the 70-fold difference in areas irradiated.

These results indicate that there is efficient transmission of a signal from one spot to the entire aorta ring. Two mechanisms for this signal are communication *via* the gap junctions of smooth muscle cells or *via* the nerves in the adventitia. If the transmission occurs in the adventitia, a significant dependence on area irradiated should occur when rings are irradiated without adventitia. As shown in Table 2, the relaxation produced by irradiation of rings without adventitia on a small spot at 350 nm ($R = 7.6 \pm 6.2$) were only about two-fold less than that obtained by irradiating a large spot ($R = 16.0 \pm 7.6$), indicating that the light-induced signal is transmitted predominantly by the medial layer. This is supported by the observation that when rings with and without adventitia are irradiated in a small spot the relaxation is the same (Table 2).

Table 1. Comparison of vasorelaxation produced in rabbit aorta rings by large and small area irradiations

Wavelength, nm	Area irradiated	S_o (mW^{-1})*	R_{max}*
351	Small	1100 ± 280 (6)	42 ± 7 (3)
	Large	1100 ± 380 (6)	37 ± 2 (2)
390	Small	68 ± 15 (6)	48 ± 10 (3)
	Large	263 ± 123 (8)†	‡
514	Small	2.5 ± 0.9 (5)	18 (1)
	Large	3.2 ± 0.8 (8)	21 ± 2 (2)

*Numbers in parentheses are the number of rings measured. The number of rings is lower for R_{max} than S_o because powers were not high enough in all experiments to reach maximum relaxation.
†$P < 0.005$ compared to small area irradiation at 390 nm.
‡Insufficient powers used to reach R_{max}.

Table 2. Effect of area illuminated and of removing adventitial layer on photovasorelaxation*

Adventitia	Area irradiated	R†
Absent	Large	16.0 ± 7.6
Absent	Small	7.6 ± 6.2
Present	Large	15.5 ± 5.1‡
Present	Small	16.2 ± 2.7

*Irradiation at 350 ± 10 nm using filtered xenon lamp, P = 0.032 mW.
†Mean value ± standard deviation.
‡$P > 0.25$ compared to large area irradiation in absence of adventitia and compared to small area irradiation in the presence of adventitia.

Location of the chromophore for photovasorelaxation

In all of the experiments reported here the aorta rings were irradiated on the adventitial surface. Since previous reports[3-5] indicated that endothelial cells were not required for photovasorelaxation, the chromophore for this response must lie in either the medial or adventitial layers. To distinguish these two possibilities, the adventitia was removed from one-half of the length of an aorta, and the relaxation of segments with and without adventitia was compared when one-half of the ring (large area) was irradiated. As shown in Table 2, the absence of adventitia did not reduce the relaxation (R = 15.5 *vs* 16), indicating that the chromophore for photovasorelaxation is in the medial layer.

Effects of potential inhibitors and enhancers of photovasorelaxation

In order to determine whether or not nitric oxide synthase (NOS) participates in photovasorelaxation, the effect of arginine analogues that inhibit NOS was investigated. Three arginine analogues were used, NMMA, NAME and NAA. The concentrations chosen enhanced the norepinephrine-induced contraction of the aorta rings thereby demonstrating that the concentrations were sufficient to inhibit NOS. The relaxation produced by exposing a ring to 350 ± 10 nm radiation in the absence of NOS inhibitor was compared to that produced by the same power in the presence of varying concentrations of inhibitor. The results are shown in Table 3. Concentrations of NMMA between 30 and 300 μM did not alter the UV-induced relaxation. Similarly, 30 and 100 μM NAA did not inhibit photovasorelaxation. In contrast, 100 μM NAME increased the relaxation produced by UV radiation by 41%.

Indomethacin, a cyclooxygenase inhibitor, increased the light-induced relaxation by 37%. The effect of zinc protoporphyrin IX, an inhibitor of heme oxygenase, on photovasorelaxation was investigated. A concentration of 0.95 μM did not alter photovasorelaxation but 1.9 μM increased the relaxation by 31%.

Table 3. Effect of potential inhibitors of photovasorelaxation induced by 350 ± 10 nm radiation

Inhibitor	Percent change in R*
NMMA	
30 μM	+3 ± 3†
100 μM	+10 ± 8†
300 μM	+11 ± 18†
NAME	
100 μM	+41 ± 17‡
NAA	
50 μM	+8 ± 10‡
100 μM	+21 ± 11‡
Indomethacin	
7 μM	+37 ± 7‡
Zn protoporphyrin	
0.95 μM	+7 ± 2†
1.9 μM	+31 ± 4†

* $\frac{(\text{R with inhibitor} - \text{R without inhibitor})100}{\text{R without inhibitor}}$.

†Mean ± range for two independent measurements.
‡Mean ± SEM.

DISCUSSION

Although light-induced relaxation of blood vessels in the absence of photosensitizing drugs was first reported decades ago, the photobiologic mechanism for this phenomenon is still unknown. Proposed mechanisms must be consistent with the findings that showed that photovasorelaxation produced by low-power light is reversible[2] and does not require the presence of endothelial cells.[3-5] An important observation made by several groups is that the level of cyclic guanosine monophosphate (cGMP) increases in blood vessels exposed to UV or blue light *in vitro*[3,5] and that the level of cGMP correlated with the degree of photovasorelaxation.[3] These results indicate that light absorption by chromophores in blood vessels leads, directly or indirectly, to the activation of guanylate cyclase or the inhibition of a cGMP phosphodiesterase.

Mechanisms can be considered in the context of the kinetic model proposed by Furchgott *et al.*[2] In this model, the chromophore, C, absorbs a photon to form a short-lived excited state, C* (Eq. 6). The rate of formation

$$C \underset{k_d}{\overset{k_a}{\longrightarrow}} C^* \qquad (6)$$

of C* depends on the rate of photon absorption at that wavelength, k_a. Excited singlet and triplet states typically revert to the starting molecule with rate constants, k_d, of 10^6–10^9 s^{-1}. Thus, a steady state population of C* is established within a few microseconds of the beginning of the irradiation. C*, in a modification of the Furchgott model, reacts with another molecule, B, to produce an activated molecule, B** (Eq. 7).

$$C^* + B \xrightarrow{k_b} C + B^{**}. \qquad (7)$$

The concentration of B** is assumed in this model to be proportional to the loss of tension. B** may be an activated enzyme, such as guanylate cyclase, or a species that activates guanylate cyclase. B** is deactivated to B with a rate constant of k_r (Eq. 8), which

$$B^{**} \xrightarrow{k_r} B \qquad (8)$$

accounts for the increase in tension when the light exposure is terminated. This mechanism accounts for the observation that photovasorelaxation is proportional to the rate of photon delivery.

Because both the relaxation rate, dT/dt, and steady-state relaxation, R_{ss}, show the same relationship to power (Fig. 4), it appears that both are governed by the steady-state concentration of B**. This indicates that the steady-state concentration for B** must be established rapidly, within a few seconds of the beginning of the light exposure.

The action spectrum reported here (Fig. 5) was obtained by exposing less than 2% of each rabbit aorta ring on the adventitial surface to low-power laser radiation. It is very similar to the action spectrum reported by Furchgott *et al.*

using a low-power lamp source with a monochromator and exposing a large area of the intimal surface of aorta strips.[2] In both cases wavelengths between 351 and 375 nm were much more effective than longer wavelengths.

These action spectra are not compatible with the absorption spectra of common chromophores in biological tissue. Although the magnitude of the effect varies greatly, all wavelengths between 351 and 630 nm produced relaxation, indicating that chromophore(s) responsible for initiating the cascade of events leading to relaxation also absorb over this entire wavelength region. Ultraviolet-absorbing chromophores such as protein and NADH do not have absorption spectra extending over the entire UV and visible ranges. Other chromophores, such as porphyrins, absorb throughout the UV and visible range but exhibit distinct absorption maxima. One possible explanation is that two, or more, chromophores are responsible for photovasorelaxation and that they absorb in overlapping wavelength ranges. This explanation is consistent with the differences in the R_{max} between the UV and visible portions of the spectrum. As shown in Fig. 6, the R_{max} in the visible range is about 0.20–0.25, whereas below 390 nm it increases to 0.35–0.50. If a single chromophore were responsible for photovasorelaxation throughout the entire spectrum, the same R_{max} would be expected since the response should be proportional only to the number of photons absorbed. The results shown in Fig. 6 indicate that one chromophore may be active in the shorter UV range and another at wavelengths of 390 nm and above.

An action spectrum for photovasorelaxation that showed a maximum between 360 and 400 nm has been reported.[13] These data were interpreted as being due to direct photoactivation of guanylate cyclase, which contains a heme group absorbing at 430 nm.[14] However, the absorption maximum for guanylate cyclase at 430 nm does not match the action spectrum very well. In addition, the relaxation and cGMP measurements were made using a single intensity of light at all wavelengths, which is a much less accurate, and possibly incorrect, method for determining an action spectrum compared to measuring a dose–response relationship.[15] Also, as noted previously,[5] measurements for this action spectrum were made using light passed through Lucite (polymethylmethacrylate), which blocks shorter UV wavelengths. Thus, activity of shorter UV wavelengths could not be measured using that apparatus leading to an apparent maximum at 360–400 nm.

Although the chromophore(s) for photovasorelaxation have not been identified, our results indicate that the chromophore(s) are in the medial layer of aorta rings since removal of the adventitia did not decrease R when a large area of the adventitial surface was irradiated (Table 2). Because previous studies had shown that the endothelial layer was not required for photovasorelaxation,[3–5] it appears that the chromophore(s) are in the medial layer.

The action spectrum for photovasorelaxation reported here was obtained using a laser-fiber optic that directly illuminated less than 1% of the vessel wall. Previous action spectra studies had exposed at least one-half of an arterial ring.[2,13] Our data indicate that the size of the area irradiated does not have a large influence on R. Our results correlate well with those of Furchgott *et al.*[2] where 0.05 mW produced R = 0.30 at 350 nm. In our study using a fiber optic to deliver the light to the adventitial surface, 0.035 mW produced R = 0.30. These results indicate that the same fundamental photobiological mechanism is responsible for photovasorelaxation whether the light is delivered to a small or large area of the tissue.

A direct comparison of irradiating large and small areas clearly demonstrated that the magnitude of the response was related to the power (energy/time) and not to the irradiance (power/area/time). Despite an approximately 70-fold higher irradiance when the small area was exposed, the effectiveness, S_0, and the maximum relaxation were approximately the same for large and small irradiation areas (Table 1). These results indicate that the chromophore, C, is present in high enough concentration that the higher irradiance does not deplete it. This was one of the assumptions in the kinetic model.[2]

These results also suggest that a signal is generated in proportion to the number of photons absorbed in the small area and the signal is transmitted with surprising efficiency to the entire ring. Because only about 1% of the ring is exposed by irradiating with the fiber optic, decreases in T_0 of up to 50% would not be expected if only the smooth muscle fibers in that small area responded.

Spreading of the signal produced by initial photochemical events may occur *via* gap junctions between smooth muscle cells or by the nerves in the adventitia. Our results showed that removing the adventitia decreased R slightly, if at all, when a small spot was irradiated at 350 nm. Thus, spreading of the signal does not depend on the presence of adventitia. It appears that most of the signal is transmitted *via* gap junctions between smooth muscle cells.

An alternative explanation is that the incident light is scattered throughout the ring from the small area illuminated thus abrogating difference in light delivery between large and small spots. From the scattering and absorption coefficients of human aorta reported at 350 nm,[16] we calculate that >95% of the incident photons are absorbed within 0.3 mm of the surface irradiated. Also, for radially diffuse light, >90% of the incident photons lie in a 1.5 mm diameter spot. This corresponds to an area irradiated of 0.018 cm^2, which is still a factor of 10-fold smaller than one side of the ring. Thus, light scattering in the tissue does not account for the similarity of R obtained by irradiating large and small areas.

The most intriguing information provided by the study by Karlsson *et al.*[3] is the correlation between photovasorelaxation and cGMP levels. As discussed above, direct light absorption by the heme of guanylate cyclase is not the mechanism for the increase in cGMP. An alternative mechanism is the photoactivation of a species that subsequently activates guanylate cyclase. We investigated whether or not NOS might be the photoactivatable enzyme for photovasorelaxation since nitric oxide (NO) activates guanylate cyclase by binding to its heme group. Nitric oxide synthase is a likely candidate as a chromophore for photovasorelaxation induced by 350 nm radiation because its activity requires two cofactors that absorb in the UV range, NADPH (340 nm) and tetrahydrobiopterin (370 nm).[17] Nitric oxide synthase is inducible in smooth muscle cells[18–20] and NO is released by vascular smooth muscle cells *in vitro*.[21]

Two NOS inhibitors, NMMA and NAA, did not alter the UV-induced relaxation of aorta rings, indicating that NOS

was not being activated either directly or indirectly by UV radiation. Because cGMP increases during photovasorelaxation,[3,5] the light-activated process appears to create species other than NO that act on guanylate cyclase.

Unexpectedly, NAME enhanced photovasorelaxation. This response is most likely caused by light-induced cleavage of the nitro group from NAME to form NO, which causes vasorelaxation. Light-induced cleavage of nitro groups has been demonstrated previously for nitrate[22] and BAY 8644.[23] This result suggests that care should be taken when NAME is used to avoid exposure to UV light since NO-induced effects may be seen.

Several species appear to activate guanylate cyclase in addition to NO, including carbon monoxide[24,25] and oxygen free radicals.[26] In order to determine whether activation of guanylate cyclase might involve light-initiated production of CO instead of NO, an inhibitor of heme oxygenase, Zn protoporphyrin, was evaluated. The concentration of Zn protoporphyrin used, 1.9 μM, has been shown to inhibit *in vitro* preparations of heme oxygenase from intestine, liver and spleen of rats.[27] However, Zn protoporphyrin did not inhibit light-induced vasorelaxation (Table 3), indicating that photoactivation of heme oxygenase is not involved in the mechanism. Indeed, Zn protoporphyrin enhanced photovasorelaxation, although the mechanism for this effect is unclear.

Indomethacin, a cyclooxygenase inhibitor that should decrease production of prostacyclin, was also ineffective as an inhibitor of photovasorelaxation (Table 3). The potentiation of light-induced relaxation by indomethacin may be caused by decreased prostaglandin production required for vasoconstriction.

In summary, this investigation has demonstrated that short-wavelength UV is more effective than visible light for photovasorelaxation when a small area of the adventitial surface is exposed to light and that photovasorelaxation may involve more than one chromophore. Our results indicate that the chromophore is located in the medial layer and that a light-initiated signal is largely transmitted between smooth muscle cells, presumably *via* gap junctions. Participation of several possible species that may be produced upon UV exposure of tissue was not supported.

Acknowledgements—We gratefully acknowledge discussions with Drs. John Cooke and Scott A. Waldman and the technical assistance of Joseph Bouvier and Thomas Haw.

REFERENCES

1. Furchgott, R. F. (1955) The pharmacology of vascular smooth muscle. *Pharmacol. Rev.* **7**, 183–265.
2. Furchgott, R. F., S. J. Ehrreich and E. Greenblatt (1961) The photoactivated relaxation of smooth muscle of rabbit aorta. *J. Gen. Physiol.* **44**, 499–519.
3. Karlsson, J. O. G., K. L. Axelsson and R. G. G. Andersson (1984) Effects of ultraviolet radiation of the tension and the cyclic GMP level of bovine mesenteric arteries. *Life Sci.* **34**, 1555–1563.
4. Lincoln, T. M., J. Laks and R. M. Johnson (1985) Ultraviolet radiation-induced decreases in tension and phosphorylase a formation in rat aorta. *J. Cyclic Nucleotide Protein Phosphorylation Res.* **10**, 525–533.
5. Furchgott, R. F. and D. Jothianandan (1991) Endothelium-dependent and -independent vasodilation involving cyclic GMP: relaxation induced by nitric oxide, carbon monoxide and light. *Blood Vessels* **28**, 52–61.
6. Gal, D., S. K. Chokshi, M. Mosseri, R. H. Clarke and J. M. Isner (1992) Percutaneous delivery of low-level laser energy reverses histamine-induced spasm in atherosclerotic Yucatan microswine. *Circulation* **86**, 756–768.
7. Gourgouliatos, Z. F., A. J. Welch, K. R. Diller and S. J. Aggarwal (1990) Laser-irradiation-induced relaxation of blood vessels *in vivo*. *Lasers Surg. Med.* **10**, 524–532.
8. Steg, P. G., D. Gal, A. J. Rongione, S. T. DeJesus, R. H. Clarke and J. M. Isner (1988) Effect of an argon laser irradiation on rabbit aortic smooth muscle: evidence for endothelium-independent contraction and relaxation. *Cardiovasc. Res.* **22**, 747–753.
9. Gregory, K. W., B. O. Berdus, P. C. Grandaw and R. R. Anderson (1988) Pulsed laser-induced vasodilation and vasospasm. *Circulation* **74**(Suppl. 2), 295.
10. Steg, P. G., A. J. Rongione, S. T. DeJesus, R. H. Clarke and J. M. Isner (1989) Pulsed ultraviolet laser irradiation produces endothelium-independent relaxation of vascular smooth muscle. *Circulation* **80**, 189–197.
11. Teramura, A., R. Macfarlane, C. J. Owen, R. de la Torre, K. W. Gregory, R. Birngruber, J. A. Parrish, J. W. Peterson and N. T. Zervas (1991) Application of the 1-μsec pulsed-dye laser to the treatment of experimental cerebral vasospasm. *J. Neurosurg.* **75**, 271–276.
12. Macfarlane, R., A. Teramura, C. J. Owen, S. Chase, R. de la Torre, K. W. Gregory, J. W. Peterson, R. Birngruber, J. A. Parrish and N. T. Zervas (1991) Treatment of vasospasm with a 480-nm pulsed-dye laser. *J. Neurosurg.* **75**, 613–622.
13. Karlsson, J. O. G., K. Axelsson, H. Elwing and R. G. G. Andersson (1986) Action spectra of photoactivated cyclic GMP metabolism and relaxation in bovine mesenteric artery. *J. Cyclic Nucleotide Protein Phosphorylation Res.* **11**, 155–166.
14. Gerzer, R., E. Böhme, F. Hofmann and G. Schultz (1981) Soluble guanylate cyclase purified from bovine lung contains heme and copper. *Fed. Eur. Biochem. Soc.* **132**, 71–74.
15. Jagger, J. (1967) *Introduction to Research in Photobiology*, pp. 80–88. Prentice-Hall, Englewood Cliffs, NJ.
16. Keijzer, M., R. R. Richards-Kortum, S. L. Jacques and M. S. Feld (1989) Flourescence spectroscopy of turbid media: autofluorescence of the human aorta. *Appl. Optics* **28**, 4286–4292.
17. Kwon, N. S., C. F. Nathan and D. J. Stevhr (1989) Reduced biopterin as a cofactor in the generation of nitrogen oxides by murine macrophages. *J. Biol. Chem.* **264**, 20496–20502.
18. Busse, R. and A. Mulsch (1990) Induction of nitric oxide synthase by cytokines in vascular smooth muscle cells. *FEBS Lett.* **275**, 87–90.
19. Schini, V. B., D. C. Junquero, T. Scott-Burden and P. M. Vanhoutte (1991) Interleukin-1β induces the production of an L-arginine-derived relaxing factor from cultured smooth muscle cells from rat aorta. *Biochem. Biophys. Res. Commun.* **176**, 114–121.
20. Beasley, D., J. H. Schwartz and B. M. Brenner (1991) Interleukin 1 induces prolonged L-arginine dependent cyclic guanosine monophosphate and nitrite production in rat vascular smooth muscle cells. *JCI* **87**, 602–608.
21. Bernhardt, J., M. R. Tschudi, Y. Dohi, I. Gut, B. Urwyler, F. R. Bühler and T. F. Lüscher (1991) Release of nitric oxide from human vascular smooth muscle cells. *Biochem. Biophys. Res. Commun.* **180**, 907–912.
22. Matsunaga, K. and R. F. Furchgott (1989) Interactions of light and sodium nitrite in producing relaxation of rabbit aorta. *J. Pharmacol. Exp. Ther.* **248**, 687–695.
23. Mikkelsen, E., S. Kazda and N. C. B. Nyborg (1985) Effects of light and Bay K8644, a new 1,4-dihydropyridine, on mechanical responses of rat thoracic aorta. *Acta Pharmacol. Toxicol.* **56**, 126–132.
24. Brune, B., K.-U. Schmidt and V. Ullrich (1990) Activation of soluble guanylate cyclase by carbon monoxide and inhibition by superoxide anion. *Eur. J. Biochem.* **192**, 683–688.
25. Snyder, S. H. (1992) Nitric oxide: first in a new class of neurotransmitters? *Science* **257**, 494–496.

26. Mittal, C. K. and F. Murad (1977) Activation of guanylate cyclase by superoxide dismutase and hydroxyl radical: a physiological regulator of guanosine 3'5'monophosphate formation. *Proc. Natl. Acad. Sci. USA* **74**, 4360–4364.

27. Vreman, H. J., M. J. Gillman and D. K. Stevenson (1989) *In vitro* inhibition of adult rat intestinal heme oxygenase by metalloporphyrins. *Pediatr. Res.* **26**, 362–365.