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Possible Role of Nitric Oxide in Prevention of Atherosclerosis: Photo-induced adequate nitric oxide (PIANO)-mediated relaxation involves cyclic GMP increment*

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ABSTRACT

Our purpose was to know whether photo-induced adequate nitric oxide (PIANO)-mediated relaxation of rat aorta is involved in cyclic GMP increment as well as inhibition of phosphatidylinositide hydrolysis due to phenylephrine (PE). Isometric tension was measured in vitro in response to either agents that modulate NO production or release NO by photolysis of photosensitizing agents in rat aorta that had been contracted with PE submaximally. PIANO-mediated relaxation was accompanied by increment of cyclic GMP, which was dependent on the intensity and duration of light exposure and concentration of photosensitizers. Phosphatidylinositide (PI) turnover augmented by PE was significantly inhibited by PIANO. These findings indiate that cGMP increment is responsible for PIANO-mediated relaxation and which may account for the inhibition of PI turnover due to α-adrenergic receptor stimulation.

Key Words: Photorelaxation, cGMP, Phosphoinositide, Rat aorta

INTRODUCTION

Nitric oxide, NO, is a prominent vascular and neuronal messenger molecule first demonstrated as a chemical responsible for endothelium-derived relaxing factor activity (Furchgott and Zawasdzski, 1980; Garthwaite *et al.*, 1989; O'Dell *et al.*, 1991; Shibuki and Okada, 1991; Bredt and Snyder, 1992). By discovering nitric oxide synthase, L-arginine/NO pathway seems to be an important and ubiquitous effector system that plays a significant role in the regulation of a diverse set of mammalian physiological processes (Moncada *et al.*, 1991; Rajfer *et al.*, 1992). Photolysis of NO or NO_2-carrying molecules is thought to liberate NO, which was termed as photo-induced adequate nitric oxide (PIANO) and is effective method to investigate the role of NO in vascular and nonvascular smooth muscle (Chang *et al.*, 1993a; Chung and Chang, 1994). We exploited streptozotocin(STZ) as source of exogenously supplied NO by UV irradiation (Chang *et al.*, 1993a; Turk *et al.*, 1993; Kwon *et al.*, 1994). The characteristics of PIANO, however, are not fully characterized. The purpose of the present study, therefore, was to know whether 1) PIANO is responsible for the re-

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laxation of vascular smooth muscle, via NO/cGMP pathway, and 2) NO may account for the inhibition of phosphatidylinositide turnover due to α-adrenergic receptor stimulation.

MATERIALS AND METHODS

Tissue preparations

Male Wistar rats (15 to 18 weeks, 350~400 g) were sacrificed by stunning and the thoracic aorta removed. The tissues were cleaned of adhering fat and connective tissue, and thoracic aortic rings (3~4 mm) were prepared. The vascular endothelium was mechanically removed by rubbing gently with wooden stick and great care was taken not to damage the endothelium when endothelium intact preparations were needed.

Materials

Phenylephrine HCL, N^{w}-nitro-L-arginine methyl ester (L-NAME), N^{w}-nitro-D-arginine methyl ester (D-NAME), N-monomethy-L arginine (L-NMMA) were purchased from Sigma Chemical Co (St. Louis MO). Cyclic GMP Kit and [^{3}H] myoinositol were from Amersham (U.K).

Measurement of photorelaxation

Each ring was mounted in a 10 ml water jacketed muscle chamber containing 37℃ modified Krebs-Ringer bicarbonate solution which was gassed with 95% O_2-5% CO_2 and had the following composition (mM): NaCl (136.9), KCl (5.4), $MgCl_2$ (1.0), $NaHCO_3$ (23.8), $CaCl_2$ (1.5), glucose (5.5) and EDTA (0.03). The rings were equilibrated at 1 g tension for more than 90 min, with washing at 20 min intervals, prior to drug addition. Aortic ring preparations were brought to an approximate EC 70 to EC 80% of contraction with phenylephrine (PE). After reaching a plateau of contraction, rings were exposed to UV light (1~60 sec) using a long wavelength UV lamp (366 nm, Mineralight UV GL 58, San Gabriel, CA) in the presence or absence of test substances.

Determination of cyclic GMP

Cyclic GMP levels were measured in aortic smooth muscle that had been equilibrated under tension. Strips were quickly frozen with the aid of brass clamps precooled in liquid nitrogen after exposing UV light at the indicated time in the presence of photosensitizers. Samples were extracted and assayed for cGMP by radioimmunoassay as described (Chang *et al.*, 1992).

Inositol Phosphate studies

The accumulation of [^{3}H]inositol phosphates in aortic preparations was determined according to Chang *et al.*, (1993). Briefly, aortic rings were equilibrated for 45 min in Krebs solution, incubated in a solution containing 24 μCi/ml of myo-[^{3}H]inositol for 4 hr at 37℃, and washed 3 times at cold Krebs solution. After this, tissues were treated with 10 μM PE, in the presence of LiCl (10 mM), in the presence or absence of STZ under irradiation, and the incubation was allowed to continue for 60 min. The reaction was terminated by freezing tissues with clamps precooled in liquid nitrogen. The tissues were then homogenized in 1 ml of 10% TCA, centrifuged and the supernatants extracted with ether (5×2 ml). The supernatants were diluted with 5 ml of water and loaded onto anion exchange columns (650 mg; Bio-Rad AG1X-8, formate form) and the columns were washed with 60 ml of water in order to remove [^{3}H] inositol. Glycerolphosphoinositol and inositol phosphates were then eluted sequentially with 16 ml of 50 mM ammonium formate-5mM sodium tetraborate and 0.1 M formic acid containing 0.2 M ammonium formate, respectively (Berridge *et al.*, 1982).

Analysis of data and statistics

The results were expressed as the mean±SEM. The n values represent the number of experiments. Statistical evaluation was made using Student t-test. Probabilities of less than 5% ($p<0.05$) were considered significant. Drug concentrations are expressed as final negative log molar concentrations.

RESULTS

Effects of ACh and the PIANO on cyclic GMP

Figure 1 shows that an increase in cyclic GMP occurs in aortic preparations during endothelium-

independent photorelaxation as well as during endothelium dependent relaxation by ACh. The onset of the rise in cGMP precedes the onset of relaxation in case of photorelaxation, just as has been demonstrated by ACh (Furchgott *et al.*, 1984). In case of PIANO-mediated relaxation, cyclic GMP was already elevated significantly at 2 s after the start of UV irradiation (382±45 vs. 66± 13 pmol g^{-1} protein for zero-time paired rings, n= 4). The increase reached a maximum within 15s in the case of both photorelaxation and PIANO-induced relaxation.

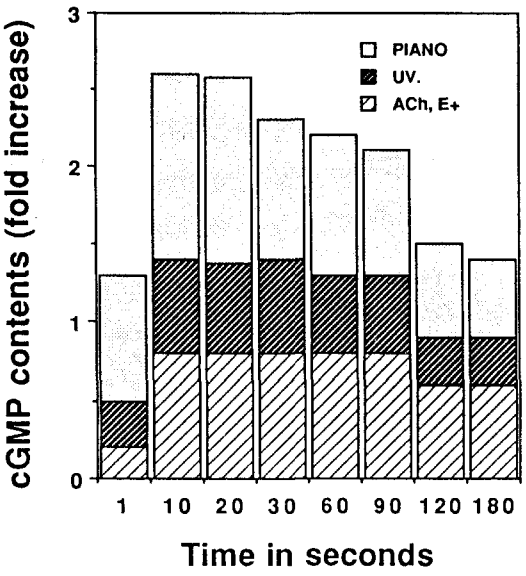

Fig. 1. Effects of acetylcholine (Ach, 1μM), UV light and photolysis of streptozotocin (STZ, 1 μM; by PIANO) on cyclic GMP contents in rat aorta. Data represent fold increase in time-course of change of cyclic GMP.

Effects of zaprinast and methylene blue on the PIANO-mediated relaxation

Zaprinast, an inhibitor of cGMP specific phosphodiesterase, increased both the magnitude of relaxation and cyclic GMP levels due to PIANO generation system (Figure 2 and Table 1). We have already reported that methylene blue (MB) is an inhibitor of PIANO-mediated relaxation in isolated rabbit corpus cavernosum (Chung and Chang, 1994). When MB was added during relaxation by ACh, it reversed the relaxation within a minutes (data not shown). It can be seen in Table 1 that control levels of cyclic GMP in the endothelium-denuded rings is only about one-half to one-third of the control level in endothelium-containing rings.

Effects of PIANO on PE-induced [^{3}H]inositol phosphate formation

The effects of PE on the [^{3}H]inositol phosphate accumulation in thoracic aorta from control and PIANO activated rats are illustrated in figure 3. The accumulation of [^{3}H]inositol phosphate in response to PE was significantly greater in control aorta than the PIANO-activated ones.

DISCUSSION

At present there are few report concerning the

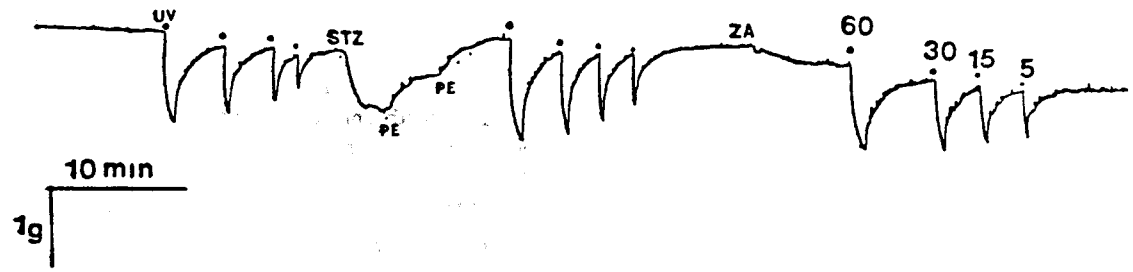

Fig. 2. Potentiation effect of zaprinast(ZA) on PIANO-mediated relaxation in rat aorta. Streptozotocin (STZ, 1μM) was utilized as photosensitizer. Zaprinast potentiated the PIANO-mediated relaxation by delaying the recovery time which results in increment of area of relaxation curve.

Table 1. Effects of zaprinast on ACh and the PIANO-mediated relaxation and cyclic GMP in rat aorta

Treatment	Cyclic GMP (pmol/g)	Relaxation (%)
None (control)	121±11(4)	–
ACh (0.1μM, 60s)	956±87(5)*	64±5.7
ACh (0.1μM)+zaprinast (0.3μM), 60s	1359±143(3)**	96±5.2
None (control)	247±25(3)	–
STZ (1μM)	420±31(4)*	36±4.2
STZ(1μM)+zaprinast (0.3μM), 60s	2668±253(4)**	98±1.3

Each value is mean±SE. Number of aortic ring preparations are indicated in parentheses from 3 to 5 different rats. *P<0.05; **P<0.01

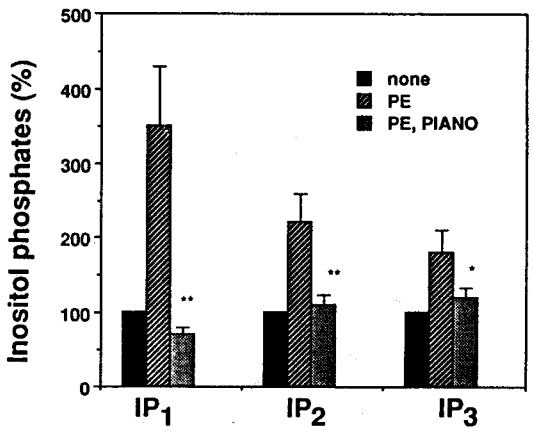

Fig. 3. Effects of PIANO generation system on phosphatidylinositide hydrolysis. Data represent mean±SE of 3 experiments. *P<0.05; **<0.01

effect of photolysis-induced NO on cyclic GMP levels in vascular smooth muscle. Moreover, reports about the effect of PIANO on phosphatidylinositide turnover are nearly absent. In the present study we clearly demonstrated that NO increases cyclic GMP levels as it is manifested by EDRF in endothelium-dependent relaxation in various smooth muscles. NO donors are thought to cause relaxation of smooth muscle by liberating NO. The PIANO system is effective method to investigate the role of NO in vascular and nonvascular smooth muscles (Chen and Gillis, 1993; Chang *et al.*, 1993a, 1993b; Chung and Chang 1994) and has strong point in that NO generation can be easily controlled simply by turning off or on the light. In the present study, PIANO by photosensitizer (STZ, L-NAME etc) potentiated substantial UV-induced relaxation of the rat aorta. The fact that NO mediates photorelaxation was already reported by the findings that pyrogallol(PYR) did prevent and super oxide dismutase (SOD) potentiated the PIANO-mediated relaxation (Chang *et al.*, 1993a). Since PYR was known to generate superoxide anion (Moncada *et al.*, 1986; Chang *et al.*, 1993b) which inactivates NO (Rubanyi and Vanheutte, 1986; Chang *et al.*, 1993a). This finding indicates that NO, whether it is derived from receptor-mediated (EDRF) or from other ways such as through PIANO system, is common pathway for the vascular smooth muscle relaxation and involves activation of guanylate cyclase.

The most striking aspect of these studies is that the onset of the increase in cyclic GMP was found to procede the onset of relaxation. At the present time it is not known this increase is result directly from the photoactivation of guanylate cyclase itself or from a product of some other molecule within the smooth muscle cells. On the other hand, MB, guanylate cyclase inhibitor, inhibited PIANO-mediated relaxation and increment of cGMP. Furthermore zaprinast, an inhibitor of cGMP specific phosphodiesterase, increased both the magnitude of relaxtion and cGMP levels due to PIANO generating system.

It is generally accepted that PI turnover plays a significant role in signal transduction in a variety of cellular events including contraction of vascular smooth muscle (Legan, 1989). There is little information investigating the relationship between

the PIANO production and PI turnover in isolated vessels. The endothelium by itself has been reported to modulate PI turnover in vascular smooth muscle by cyclic GMP (Rapoport *et al.*, 1983; Rapoport, 1986; Legan, 1989). Cyclic nucleotides also can modify *a*-agonist-induced PI turnover in vascular smooth muscle. We found that NE-induced PI hydrolysis was inhibited by cyclic AMP in rat aorta (Ahn *et al.*, 1992). Others also reported that cyclic GMP inhibited PI turnover in vascular smooth muscle (Hirata *et al.*, 1990; Lang and Lewis, 1991). In the present study PIANO increased cyclic GMP, which in turn may inhibit the enhanced PI hydrolysis by PE.

In conclusion, NO released through the PIANO system is responsible for relaxation of the rat aorta via guanylate cyclase activation which gives rise to increment of cyclic GMP and inhibition of PI hydrolysis due to PE. The physiological significance of the PIANO is still not known, however, it may add another powerful tool for investigation of NO-mediated biological responses.

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=국문초록=

동맥경화 예방과 치료를 위한 연구시도: Nitric Oxide의 역활 —광 유도 nitric oxide(PIANO)의 혈관이완에 따른 cyclic GMP의 증가

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본 연구의 목적은 광 유도에 의한 nitric oxide (PIANO)유리가 혈관이완에 대해 cyclic GMP (cGMP)가 관여하는 지의 여부와 아울러 α-수용체를 통한 수축에 PIANO가 어떻게 작용하는 지를 파악하고자 하였다. In vitro 실험에서 흰쥐의 대동맥을 준 최고농도의 phenylephrine (PE)으로 수축시킨 후 nitric oxide 생성을 변화시키는 약물이나 광민감성 (photosensitizing) 약물에 대한 반응을 등장력 변화로 기록하였다. PIANO에 의한 혈관이완은 광노출 강도와 기간 및 광민감성 약물농도에 비례하여 증가하였고 cGMP의 증가를 수반하였다. PE에 의해 증대되는 phosphatidylinositide(PI) 전환은 PIANO에 의해 억제되었다. 이상의 결과는 cGMP의 증가로 인해 PIANO에 의한 혈관이완이 일어나며 α-아드레날성 수용체 자극에 의한 PI 전환의 억제현상은 cGMP 증가의 결과로 생각할 수 있다. 결론적으로 PIANO에 의한 혈관이완은 cGMP의 증가로 인함을 확인할 수 있었다.