ROLE OF CYCLIC GMP OF CANINE VASCULAR SMOOTH MUSCLE IN RELAXATION BY ORGANIC NITRATES

TETSUO SHIBATA, M.D., KOUICHI OGAWA, M.D., TAKAYUKI ITO, M.D.
HIDEKAZU HASHIMOTO, M.D., HIROSHI NAKAGAWA, M.D.
AND TATSUO SATAKE, M.D.

The effects of organic nitrates on tone and tissue cyclic nucleotide levels were studied, using canine coronary, mesenteric and renal arteries, and femoral veins. Glyceryl trinitrate (GTN) relaxed all vascular tissues examined and increased tissue cyclic GMP (cGMP) levels in a concentration-dependent manner, but GTN induced no significant changes in cyclic AMP (cAMP) levels. An increase in cGMP levels induced by 10 μ M of GTN in coronary arteries was observed before the onset of relaxation. Methylene blue, an inhibitor of guanylate cyclase, inhibited the relaxant effect of GTN and decreased cGMP levels. In contrast, M & B 22,948, an inhibitor of cGMP phosphodiesterase, not only enhanced relaxation by GTN, but also increased cGMP levels. Other organic nitrates, pentaerythritol tetranitrate (PETN), nicorandil (NIC), and isosorbide dinitrate (ISDN), also relaxed coronary arteries and increased cGMP levels in a concentration-dependent manner. A significant correlation was observed between percentage increases in cGMP levels and percentage relaxation by 10 μ M of GTN, PETN, NIC, and ISDN ($r = 0.952$, $p < 0.001$). Plasma concentrations of 4 organic nitrates inversely correlated with percentage increases in cGMP levels by 10 μ M of these agents in coronary arteries ($r = -0.845$, $p < 0.001$). These results suggest that an increase in cGMP is responsible for relaxation in vascular smooth muscles by organic nitrates, and that therapeutic plasma concentrations may be estimated by the degree of increase in cGMP levels induced by their administration.

ORGANIC nitrates have been widely used for the therapy of angina pectoris or heart failure. However, the mechanism of vasodilative effects of organic nitrates has not been yet clarified. Recently, an increase in tissue cGMP levels induced by organic nitrates is thought to be responsible for the relaxation of vascular smooth muscles.¹⁻⁴ However, Diamond⁵ argued against a role of cyclic GMP (cGMP) in relaxation

in nonvascular smooth muscles. There are some reports that an increase in cGMP levels was observed after administration of organic nitrates in various types of smooth muscles,⁶⁻⁸ but few studies report changes in veins, upon which organic nitrates predominantly act.

Thus, it is important to clarify the relationship between relaxation of vascular smooth muscles and changes in tissue cyclic nucleotide levels induced by organic nitrates. For this purpose, mechanical experiments using isolated canine coronary, mesenteric and renal arteries, and femoral veins, and measurements of tissue cyclic nucleotide levels were designed to evaluate the

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The 2nd Department of Internal Medicine, Nagoya University School of Medicine, Nagoya, Japan

Mailing address: Kouichi Ogawa, M.D., The 2nd Department of Internal Medicine, Nagoya University School of Medicine, 65 Tsuruma-cho, Showa-ku, Nagoya 466, Japan

TABLE I MEAN VALUES OF THE APPARENT MEDIAN EFFECTIVE CONCENTRATION (EC_{50}) OF GTN AND TISSUE CYCLIC NUCLEOTIDE LEVELS INDUCED BY VARIOUS CONCENTRATIONS OF GTN IN CORONARY, MESENTERIC AND RENAL ARTERIES, AND FEMORAL VEINS

Vascular tissue	EC_{50} (μM)	Cyclic nucleotide levels (pmol/g.w.w.)	Concentration of glyceryl trinitrate (μM)				
			0	0.1	1	10	100
Coronary artery	2.4 ± 0.6	cAMP	113.1 ± 6.5	99.4 ± 6.0	103.1 ± 6.4	117.3 ± 8.4	122.1 ± 8.9
		cGMP	5.10 ± 0.43	6.16 ± 0.50	$8.14 \pm 0.56^{**}$	$12.17 \pm 0.60^{**}$	$16.96 \pm 0.96^{**}$
Mesenteric artery	7.9 ± 1.0	cAMP	200.0 ± 12.2	187.0 ± 11.3	183.7 ± 11.7	191.4 ± 13.9	211.1 ± 15.2
		cGMP	7.77 ± 0.55	$9.81 \pm 0.72^*$	$31.86 \pm 2.67^{**}$	$49.11 \pm 3.93^{**}$	$65.41 \pm 5.10^{**}$
Renal artery	14.8 ± 4.3^a	cAMP	221.7 ± 16.0	228.7 ± 20.3	208.3 ± 21.5	229.1 ± 27.4	204.3 ± 20.5
		cGMP	9.31 ± 0.61	$13.44 \pm 1.07^{**}$	$26.86 \pm 2.82^{**}$	$36.04 \pm 3.64^{**}$	$44.43 \pm 3.80^{**}$
Femoral vein	0.61 ± 0.12^a	cAMP	64.4 ± 3.0	63.4 ± 2.8	60.9 ± 2.3	67.3 ± 3.5	67.1 ± 3.1
		cGMP	4.99 ± 0.12	$7.19 \pm 0.66^{**}$	$9.11 \pm 0.78^{**}$	$11.16 \pm 0.81^{**}$	$16.69 \pm 0.90^{**}$

Results are expressed as mean $\pm$ SEM.

$n = 7$ in each experiment

a : Significantly different from the values with coronary arteries, $p < 0.05$.

$*$: Significantly different from controls, $p < 0.05$, ** : $p < 0.01$

Fig.1. Effects of glyceryl trinitrate on tone and tissue cGMP levels in coronary arteries. Results are expressed as mean $\pm$ SEM ($n = 7$).

effects of inhibitors related to the formation and destruction of cGMP.

MATERIALS AND METHODS

Mechanical experiments

Mongrel dogs (weighting 10–15 kg) were anesthetized with intravenous injections of sodium pentobarbital (25–30 mg/kg) and sacrificed by bleeding from the inferior vena cava. Coronary, superior mesenteric and renal arteries, and femoral veins were rapidly isolated and helically cut into strips (20–30 mm long, 2–3 mm wide). The strips were vertically mounted in an organ bath containing 30 ml Krebs-Henseleit (K-H) solution, which was maintained at 37°C and continuously bubbled with 95% O_2 –5% CO_2 . The K-H solution had the following composition (in mM): NaCl, 118; KCl, 4.7; $CaCl_2$, 2.55; $MgSO_4$, 1.18; KH_2PO_4 , 1.18; $NaHCO_3$, 24.88; glucose, 11.1. The pH of the solution was 7.40 ± 0.05 . The upper end of the strip was attached to the lever of a force-displacement transducer (TB 612-T, Nihonkoden, Co., Tokyo, Japan). The resting tension was adjusted to 1.5g for each strip. All the strips were allowed to equilibrate for two hours before the addition of any drugs, during which time the bathing media were replaced every 20–30 min. Isometric tension was displayed on a recorder (Model FBR-252A, TOA Electronics Co., Tokyo, Japan). The contractile response to 30 mM KCl was obtained first. After contractile response to 30 mM KCl stabilized, organic nitrates were added to the bathing media and cumulative concentration-response curves of the drugs (0.1, 1, 10, 100 μM) were obtained. The effects of organic nitrates were expressed as percentages of the preceding maximum response to 30 mM KCl (= 100%) and maximum relaxation achieved

Fig.2. Correlation between percentage increases in cGMP levels and percentage relaxation in coronary, mesenteric and renal arteries, and femoral veins by 0.1 to 100 μ M of glyceryl trinitrate. Each point represents the means of seven values. Significant correlations ($r = 0.905, 0.953, 0.965, 0.965, p < 0.001$) are seen between percentage increases in cGMP levels and percentage relaxation in all of the vascular tissues examined.

by addition of 100 μ M papaverine at the end of each experiment (= 0%). Concentration induced half maximum relaxation values (EC_{50}) were determined for vasodilators. Some coronary arterial strips had been treated for 30 min with either 10 μ M methylene blue, an inhibitor of guanylate cyclase, or 0.5 mM M & B 22,948, an inhibitor of cGMP phosphodiesterase, before the response to GTN was obtained. Responses to GTN were also examined in coronary arterial strips precontracted with 10 μ M serotonin.

Determination of tissue cyclic nucleotide levels

Vascular smooth muscle tissues (60–80 mg wet wt.) were prepared for the determination of cyclic nucleotide levels. Smooth muscle tissues were excised from the same dog that was used in the mechanical experiment. Tissues were kept at 37°C for 120 min in the aerated K-H solution, and then kept for 20 min in the solution containing 30 mM KCl, as described in the mechanical experiment and served as control. To observe

the effects of organic nitrates, various concentrations of these agents were added respectively for the final 10 min of the 20-min incubation period, when the full drug effect was developed. Changes of cyclic nucleotide levels in time course study were also determined with the tissues excised from the same animal used in the mechanical experiment. Tissues were allowed to stabilize for 30 min after addition of methylene blue or M & B 22,948 before GTN was added. After incubation or addition of the drugs, the tissue was frozen with liquid nitrogen and homogenized in 6% trichloroacetic acid. The tissue was centrifuged at 1,500g, 4°C. Supernatant fraction was extracted three times with watersaturated ether. Following succinization, tissue cyclic nucleotide levels were radioimmunoassayed⁹ using cAMP and cGMP assay kit (Yamasa Shoyu Co. Ltd., Chiba, Japan).

Materials

Methylene blue and 5-hydroxytryptamine

Fig.3. Time course of relaxation and cGMP accumulation in coronary arteries by 10 μ M of glyceryl trinitrate. Results are expressed as mean $\pm$ SEM, $n = 6$ and 7 in relaxation and cGMP experiments, respectively. **, Significantly different from controls, $p < 0.01$.

(Serotonin) were purchased from Sigma. Glyceryl trinitrate (GTN) was a gift from Nihonkayaku Co., Tokyo. Pentaerythritol tetranitrate (PETN) was a gift of Shionogi Co., Osaka. N-(2-hydroxyethyl)-nicotinamide nitrate (Nicorandil) was a gift of Chugai Co., Tokyo. Isosorbide dinitrate (ISDN) was obtained from Toaeiyo Co., Tokyo. 2-O-propoxyphenyl-8-azaprin-6-one (M & B 22,948) was a gift from May & Baker Ltd., Dagenham, Essex, GB.

Statistical analysis

Results are expressed as mean $\pm$ SEM. Statistical analysis of results was made by analysis of variance and unpaired t test. Differences were regarded as significant when $p < 0.05$.

RESULTS

Effects of GTN on potassium-induced contraction and cyclic nucleotide levels

Table I shows the effects of GTN upon contractions induced by 30 mM KCl and upon tissue cyclic nucleotide levels obtained after GTN administration to coronary, mesenteric and renal arteries, and femoral veins. EC_{50} values of GTN

in coronary arteries were significantly less than those in mesenteric and renal arteries, and significantly greater than those in femoral veins. EC_{50} values of GTN in coronary arteries were less than those in mesenteric arteries, but the differences were not significant. Tissue cGMP levels were significantly increased over control values by administration of more than 1 μ M of GTN in coronary arteries and more than 0.1 μ M of GTN in mesenteric, renal arteries and femoral veins. However, GTN induced no significant changes in cAMP levels in all vascular tissues examined. GTN effectively relaxed coronary arterial strips, which had been contracted with 10 μ M of serotonin (EC_{50} : $0.11 \pm 0.04 \mu$ M) and significantly increased cGMP levels but GTN induced no significant changes in cAMP levels.

Figure 1 shows the percentage relaxation of GTN on coronary arteries contracted with 30 mM KCl and percentage increases in tissue cyclic nucleotide levels. GTN relaxed coronary arterial strips and increased cGMP levels in a concentration-dependent manner.

Correlation between relaxation and increase in cGMP levels

Fig.4A. Effects of glyceryl trinitrate (GTN) on tone in coronary arteries in the presence or the absence of methylene blue (MB) or M & B 22,948. Relaxation by GTN ($n = 7$) was significantly inhibited in the presence of $10 \mu\text{M}$ of MB ($n = 6$) and significantly enhanced in the presence of 0.5 mM of M & B 22,948 ($n = 6$). Results are expressed as mean $\pm$ SEM.

Figure 2 shows the correlation between percentage increases in cGMP levels and percentage relaxation induced by GTN in coronary, mesenteric and renal arteries, and femoral veins. Percentage increases in cGMP levels by GTN significantly correlated with percentage relaxation in coronary, mesenteric and renal arteries, and femoral veins, respectively ($r = 0.953, 0.965, 0.965, 0.905, n = 28, p < 0.001$). Although increases in cGMP levels by GTN were smaller in coronary arteries and femoral veins than in mesenteric and renal arteries, percentage relaxation in coronary arteries and femoral veins was greater than in mesenteric and renal arteries.

Time course of relaxation and cGMP accumulation

Figure 3 shows the time course of relaxation and cGMP levels in coronary arteries induced by $10 \mu\text{M}$ of GTN. Tissue cGMP levels were significantly increased (twice the control values) 15 sec after administration of GTN, and the

Fig.4B. Effects of GTN on tissue cGMP (hatched columns) levels in coronary arteries in the presence or in the absence of MB or M & B 22,948. Tissue cGMP levels obtained by $10 \mu\text{M}$ of GTN (on the left) significantly decreased in the presence of $10 \mu\text{M}$ of MB (in the middle) and significantly increased in the presence of 0.5 mM of M & B 22,948 (on the right). Results were expressed as mean $\pm$ SEM ($n = 6$).

increase lasted for 15 min. On the contrary, relaxation was $14.8 \pm 3.0\%$ at 30 sec and $83.3 \pm 3.6\%$ at 15 min after administration of GTN. An increase in cGMP levels preceded relaxation.

Effects of inhibitors on relaxation and cyclic nucleotide levels

Figure 4A shows effects of methylene blue (MB), an inhibitor of guanylate cyclase, and of M & B 22,948, an inhibitor of cGMP phosphodiesterase, on relaxation in coronary arteries induced by GTN. Relaxation by GTN was significantly enhanced by 0.5 mM of M & B 22,948 and significantly inhibited by $10 \mu\text{M}$ of MB.

Figure 4B shows effects of MB and M & B 22,948 on cGMP levels in coronary arteries induced by $10 \mu\text{M}$ of GTN. Tissue cGMP levels

TABLE II MEAN VALUES OF THE APPARENT MEDIAN EFFECTIVE CONCENTRATION (EC_{50}) OF PETN, NIC, AND ISDN AND TISSUE CYCLIC NUCLEOTIDE LEVELS INDUCED BY VARIOUS CONCENTRATIONS OF THESE AGENTS IN CORONARY ARTERIES

Drugs	$EC_{50}(\mu M)$	Cyclic nucleotide levels (pmol/g.w.w.)	Concentrations of drugs (μM)				
			0	0.1	1	10	100
Pentaerythritol tetranitrate	15.4 $\pm$ 3.1	cAMP	101.9 $\pm$ 8.2	96.3 $\pm$ 7.4	104.0 $\pm$ 8.3	106.1 $\pm$ 9.6	95.1 $\pm$ 7.0
		cGMP	5.24 $\pm$ 0.23	5.87 $\pm$ 0.27	6.79 $\pm$ 0.36*	9.19 $\pm$ 0.52**	14.73 $\pm$ 0.83**
Nicorandil	39.1 $\pm$ 6.2	cAMP	100.0 $\pm$ 7.6	98.3 $\pm$ 7.1	104.7 $\pm$ 8.0	94.4 $\pm$ 7.0	96.6 $\pm$ 7.4
		cGMP	5.27 $\pm$ 0.26	5.63 $\pm$ 0.31	6.39 $\pm$ 0.34*	7.43 $\pm$ 0.40**	11.30 $\pm$ 0.70**
Isosorbide dinitrate	98.6 $\pm$ 13.3	cAMP	102.4 $\pm$ 7.7	98.6 $\pm$ 7.1	101.4 $\pm$ 8.2	105.4 $\pm$ 9.2	103.4 $\pm$ 9.0
		cGMP	5.14 $\pm$ 0.25	5.20 $\pm$ 0.22	5.54 $\pm$ 0.38	6.04 $\pm$ 0.44	9.99 $\pm$ 0.81**

Results are expressed as mean $\pm$ SEM

n = 7 in each experiment

*: Significantly different from controls, $p < 0.05$, **: $p < 0.01$

Fig.5A. Correlation between percentage increases in cGMP levels and percentage relaxation in coronary arteries by 10 μM of GTN, PETN, NIC, and ISDN. A significant correlation was observed between them ($r = 0.952$, $p < 0.001$).

(11.20 $\pm$ 0.72 pmol/g.w.w.) obtained by 10 μM of GTN decreased to 4.55 $\pm$ 0.39 pmol/g.w.w. by pretreatment with 10 μM of MB, and increased to 18.58 $\pm$ 1.25 pmol/g.w.w. by pretreatment with 0.5 mM of M & B 22,948. Relaxation by GTN was enhanced in accordance with increase in cGMP levels, and was inhibited in accordance with decrease in cGMP levels.

Effects of other organic nitrates on potassium-induced contraction and cyclic nucleotide levels

Table II shows the effects of PETN, NIC, and ISDN on contraction induced by 30 mM KCl and tissue cyclic nucleotide levels obtained after addition of these agents in coronary arteries. EC_{50} values of ISDN, NIC, PETN (Table II), and GTN (Table I) became smaller in turn. Tissue

Fig.5B. Correlation between plasma concentrations of organic nitrates and percentage increases in cGMP levels in coronary arteries by 10 μ M of GTN, PETN, NIC, and ISDN. Plasma concentrations of 4 organic nitrates inversely correlated with percentage increases in cGMP levels ($r = -0.845$, $p < 0.001$).

cGMP levels were significantly increased over control values by the addition of more than 1 μ M of PETN and NIC, and 100 μ M of ISDN in coronary arteries. But PETN, NIC, and ISDN induced no significant changes in cAMP levels in coronary arteries.

One concentration study with organic nitrates

Figure 5A shows the correlation between percentage increases in cGMP levels and percentage relaxation by 10 μ M of GTN, PETN, NIC, and ISDN, in coronary arteries. A significant correlation was obtained between percentage increases and percentage relaxation induced by 10 μ M of 4 organic nitrates ($r = 0.952$, $p < 0.001$).

Correlation between plasma concentrations of organic nitrates and increase in cGMP levels

Figure 5B indicates the correlation between therapeutic plasma concentrations of organic nitrates (GTN: 0.3 mg sublingual dose,¹⁰ PETN: 10 mg oral dose,¹¹ NIC: 10 mg oral dose,¹² ISDN: 25 mg oral dose¹³) and percent increase in cGMP levels by 10 μ M of 4 organic nitrates in coronary arteries. Plasma concentrations were expressed

as nM instead of ng/ml to exclude the influence of different molecular weights of 4 organic nitrates. Plasma concentrations inversely correlated with percent increase in cGMP levels in coronary arteries ($r = -0.845$, $p < 0.001$). This means that an organic nitrate, which induces a large increase in cGMP levels, requires fewer therapeutic plasma concentrations.

DISCUSSION

Although the mechanism of relaxant effects of nitrates compounds has not been completely clarified, there have been some reports of increase in tissue cGMP levels after administration of organic nitrates.¹⁻⁴ This suggests that an increase in cGMP levels is responsible for relaxation in vascular smooth muscles by organic nitrates. However, there have been other reports of no observable correlation between relaxation and increase in cGMP levels in non-vascular smooth muscles.^{5,14}

Our results demonstrated that GTN, a typical organic nitrate, induced both relaxation and an increase in cGMP levels in a concentration-dependent manner not only in coronary, mesen-

teric and renal arteries, but also in femoral veins. High correlations were observed between percentage increase in cGMP levels and relaxation by GTN in coronary, mesenteric and renal arteries, and femoral veins, respectively ($r = 0.953, 0.965, 0.965, 0.905, p < 0.001$). The time course study shown in Fig. 3 showed that an increase in cGMP levels by GTN preceded relaxation. Although the determination of cGMP level was performed with tissues excised from the same dog which was used in the mechanical experiment in our experiment, Gruetter et al.³ evaluated simultaneous measurement of isometric force and cGMP levels in the same arterial strips at time intervals as short as 5 sec after the addition of relaxants and indicated that cGMP levels induced by $1 \mu\text{M}$ of GTN were significantly increased above control levels at 5, 10, and 15 sec, before the onset of relaxation in response to GTN. This finding is in accordance with our results. Increase in cGMP levels by M & B 22,948, an inhibitor of cGMP phosphodiesterase, was closely related to enhancement of relaxation by GTN. On the contrary, a decrease in cGMP level by MB, an inhibitor of guanylate cyclase, was closely connected to inhibition of relaxation by GTN. These findings suggest that vasodilator effects of organic nitrates occur through the intermediary of an increase in cGMP levels. Although an increase in cGMP levels by GTN is correlated with relaxation in coronary arteries contracted with 30 mM of KCl, a high correlation was also observed between them in coronary arteries contracted with $10 \mu\text{M}$ of serotonin ($r = 0.924, p < 0.001$). Endoh and Taira¹⁵ discovered a correlation between increase in cGMP levels and relaxation by nicorandil in canine mesenteric arteries contracted with norepinephrine. These correlations between increase in cGMP levels and relaxation by organic nitrates were observed independently in spite of the different way of contraction.

An increase in cGMP level by organic nitrates was also observed in an *in vivo* study. Kobayashi and Ogawa¹⁶ showed a significant increase in cGMP levels in canine coronary arteries by GTN and ISDN.

As to the mechanism of increase in cGMP levels by nitrates, Ignarro and Gruetter¹⁷ demonstrated that S-nitrosothiols, a product of nitrate and SH group, stimulated guanylate cyclase to increase cGMP levels. However, it is unknown how cGMP increased by organic nitrates acts on

and relaxes vascular smooth muscle. Zsoter et al.¹⁸ demonstrated that the efflux of ^{45}Ca was enhanced from rabbit vessels exposed to sodium nitroprusside. On the contrary, Harder et al.¹⁹ showed that relaxant effects of GTN were related to the inhibition of Ca^{2+} inward current. Recently, Itoh et al.²⁰ has shown that GTN increases the amount of cGMP and that the main effect of cGMP is activation of Ca extrusion, thus reducing the amount of Ca stored in the cell. Consequently cGMP reduces the free Ca in the myoplasm and promotes relaxation.

In the present study, smaller increases in cGMP levels by GTN in coronary arteries and femoral veins induced marked relaxation, although greater increases in cGMP levels in mesenteric and renal arteries induced less relaxation than in coronary arteries and femoral veins. Although the reason for this is not clear, it may be explained by the different activities of cGMP-dependent protein kinase²¹ and/or cGMP compartment in various types of vascular smooth muscles.

One concentration response study (Fig. 5A) showed that a percent increase in cGMP levels by $10 \mu\text{M}$ of 4 organic nitrates (GTN, PETN, NIC, and ISDN) significantly correlated with percent relaxation in coronary arteries ($r = 0.952, p < 0.001$). NIC, a new potent dilator, which has a terminal nitrate group, resembles GTN in its hemodynamic actions. But, NIC is postulated to have two independent mechanism of vasodilation.²²⁻²⁴ One is a hyperpolarizing effect of NIC in several kinds of arteries, which was ascribed to an augmentation of potassium permeability of the surface membrane. The other is an inhibitory action of calcium-induced calcium release from the intracellular store-sites as seen in relaxation of caffeine-contraction by NIC. Holzmann⁴ indicated that NIC at concentrations producing dose-dependent relaxation up to 94% ($0.47\text{--}473 \mu\text{M}$) similar raised cGMP levels in the strips, and that this effect preceded the mechanical response. The inhibition of calcium-induced calcium release by NIC is assumed to be related to the increase in cGMP levels observed after administration of NIC. Although the mechanism of relaxant effects of NIC differs from the other organic nitrates, a high correlation was observed between percent increases and percent relaxation induced by $10 \mu\text{M}$ of 4 organic nitrates. The reason for this is not clear, but the hyperpolarizing effect of NIC was not probably present in our experiment, because

the mechanical experiment was performed with the solution containing high potassium, i.e. 30 mM KCl. Furukawa et al.²³ indicated that lower concentrations of (K)₀ hyperpolarized the membrane of the large porcine coronary artery to a great extent in the presence of 2-nicotinamidoethyl nitrate (2-NN), whereas in the presence of over 23.6 mM (K)₀, hyperpolarization did not occur in the presence of 10⁻⁴ M 2-NN.

The present results indicate that an increase in cGMP levels by 4 organic nitrates is positively correlated with percentage relaxation and inversely correlated with plasma concentrations of organic nitrates (Fig. 5B, $r = -0.845$, $p < 0.001$). This means it may be possible to estimate plasma concentrations of an organic nitrate to relieve angina pectoris by the drug's effects in inducing an increase in cGMP levels.

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