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Lack of Correlation Between Myocardial Nitric Oxide and Cyclic Guanosine Monophosphate Content in Both Nitrate-Tolerant and -Nontolerant Rats

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ABSTRACT. We studied the effect of nitroglycerin (NTG) on cardiac nitric oxide (NO) and cyclic guanosine monophosphate (cGMP) content in nitrate-tolerant/nontolerant rats *in vivo*. The effect of the pharmacological blockade of endogenous NO synthesis and the effect of exogenous NO on cardiac cGMP were also examined. Rats were treated with 100 mg/kg of NTG and corresponding vehicle s.c. three times a day for 2.5 days to induce NTG-tolerance/nontolerance. Rats were then administered a single dose of s.c. 100 mg/kg of NTG to test the effect of NTG in tolerant/nontolerant states, respectively. Nontolerant rats treated with vehicle were controls, and nontolerant rats treated with the NO synthesis inhibitor NG-nitro-L-arginine (LNNA, 20 mg/kg) were negative controls. Another group of nontolerant rats treated i.v. with the direct NO donor sodium nitroprusside (SNP, 3 mg/kg) were positive controls. Cardiac NO assessed by electron spin resonance after *in vivo* spin-trapping increased 100-fold ($P < 0.05$) in the positive control, 10-fold ($P < 0.05$) in the NTG-tolerant group, and 4-fold ($P < 0.05$) in the single NTG group, when compared to controls. In the negative control group, NO was reduced to near the detection limit (four-fold reduction, $P < 0.05$). Cardiac cGMP measured by radioimmunoassay was increased significantly (two-fold, $P < 0.05$) only in the positive control group, and there were no differences among the other groups. This shows that: 1) *in vivo* cardiac bioconversion of NTG to NO is not impaired in nitrate tolerance; and 2) changes in cardiac NO content are not reflected by changes in cGMP content in nitrate-tolerant and -nontolerant rats. BIOCHEM PHARMACOL **56**;9:1139–1144, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. nitroglycerin; tolerance; NG-nitro-L-arginine; nitric oxide; cGMP; electron spin resonance

The anti-anginal effect of NTG¶ is believed to be based on the drug-induced decrease in preload and afterload, improvement of coronary collateral flow, dilation of stenotic coronary arteries, and inhibition of platelet aggregation [1, 2]. To exert these effects, NTG is considered a prodrug: it requires enzymatic bioconversion to NO, which, in turn, activates soluble guanylate cyclase, thereby increasing vascular cGMP content [3, 4]. Continuous administration of NTG or other organic nitrates leads to tolerance to their hemodynamic and clinical effects. The biochemical mechanism of the development of nitrate tolerance is rather controversial [2, 5]. Several mechanisms, such as neurohormonal activation counteracting NTG-mediated vasodilation, reduced conversion of NTG to NO, and attenuated increase in cGMP concentration, may be involved[2, 5].

Although numerous studies have looked at the effect of NTG on vascular tissue, very few have examined the effect of NTG on the heart. We have recently demonstrated that NTG exerts a direct myocardial anti-ischemic effect that is independent of any vascular effects of the drug. However, the underlying mechanism, which is also present in nitrate tolerance, has not yet been elucidated [6]. Cardiac conversion of NTG to NO and the possible elevation of cGMP may both account for the direct effect of NTG on the myocardium because both NO and cGMP were shown to protect the ischemic myocardium [7–9].

Therefore, the aim of the present study was to investigate whether NTG is converted to NO with a resulting elevation of cGMP in the heart of nitrate-tolerant and nontolerant rats *in vivo*, and whether cardiac NO content is mirrored by cardiac cGMP content.

MATERIALS AND METHODS

This investigation was approved by the local ethics committee, and conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH No 85-23, revised 1985).

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¶ *Abbreviations*: cGMP, cyclic guanosine monophosphate; DETC, diethyl-dithiocarbamate; ESR, electron spin resonance; LNNA, NG-nitro-L-arginine; NO, nitric oxide; NTG, nitroglycerin; and SNP, sodium nitroprusside.

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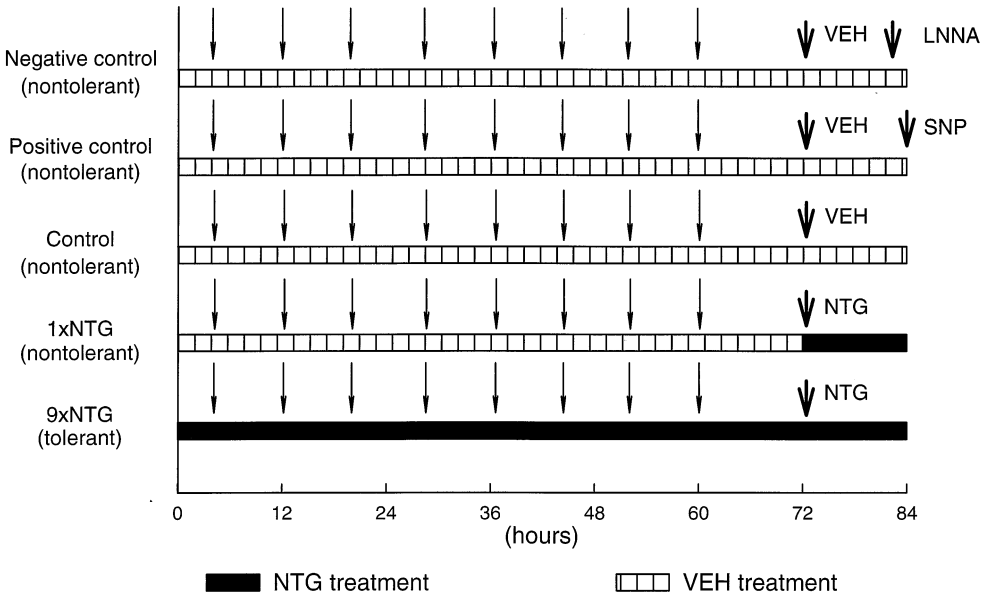

FIG. 1. Experimental protocol. To induce vascular tolerance/nontolerance to NTG, rats were treated s.c. with 100 mg/kg of NTG and/or its vehicle (VEH) three times a day for 2.5 days (eight injections, indicated by thin arrows). At the end of the third day, the first nontolerant group (negative control group) was given a ninth s.c. vehicle injection, and at the 4th day, 40 min before the isolation of the heart, 20 mg/kg of LNNA was given i.v. The second nontolerant group (positive control) treated with a ninth vehicle injection was given a single 3 mg/kg i.v. injection of SNP, 5 min before isolation of the heart. The third nontolerant group treated with a ninth vehicle injection at the end of the third day served as control. The fourth nontolerant group (1× NTG group) was given a single 100 mg/kg injection of NTG s.c. as the ninth injection at the end of the third day to test the effect of NTG in the nontolerant state. The tolerant group (9× NTG group) was given a ninth NTG injection (100 mg/kg) to test the effect of NTG in nitrate tolerance. Twelve hours after the last NTG/vehicle injections, hearts were isolated and cardiac NO and cGMP were analyzed.

Chemicals

NTG (EGIS Pharmaceuticals Co.) and/or its vehicle, lactose, were suspended in 100% propylene glycol. LNNA (Sigma-Aldrich Co.) was dissolved in physiological saline at 60° and cooled to room temperature before administration. SNP (Sigma-Aldrich Co.) was dissolved in physiological saline just before administration, and the solution was protected from light exposure. DETC, $FeSO_4$, and sodium citrate were dissolved in saline (Sigma-Aldrich Co.). Radioimmunoassay kits for cGMP were purchased from Amersham.

Animals and Treatments

Male Wistar rats (300–360 g) were assigned to 5 groups (N = 10 in each group, Fig. 1.). Five animals were used for cGMP measurements and five for NO determination in each group. To induce vascular tolerance/nontolerance to NTG [10] animals were treated s.c. with 100 mg/kg of NTG and/or its vehicle three times a day for 2.5 days (eight injections). At the end of the third day, the first nontolerant group (negative control group) was given a ninth s.c. vehicle injection, and to block endogenous NO synthesis, at the fourth day, 40 min before the isolation of the heart, 20 mg/kg of LNNA were given i.v. Our previous studies showed that LNNA exerted its maximal effect in the rat coronary vasculature approximately 40 min after administration [11].

To achieve an extremely high cardiac NO concentration, the second nontolerant group (positive control) treated with a ninth vehicle injection was given a single 3 mg/kg i.v. bolus injection of a nonenzymatic releaser of NO, SNP, 5 min before the isolation of the heart. The third nontolerant group treated with a ninth vehicle injection at the end of the third day served as controls. To test the effect of NTG in the nontolerant state (1× NTG group), the fourth nontolerant group was given a single 100 mg/kg injection of NTG s.c. as the ninth injection at the end of the third day. To test the effect of NTG in nitrate tolerance (9 × NTG group), the tolerant group was given a ninth NTG injection (100 mg/kg). At the fourth day (12 h after the last NTG/vehicle injections), rats were anesthetized with diethylether, their hearts were isolated and, cardiac NO and cGMP content were analyzed from all groups as described below.

Confirmation of Vascular Nitrate Tolerance

Development of vascular tolerance to NTG was confirmed by testing endothelium-free, thoracic, aortic rings for isometric tension as described previously [6,12]. Rings of 4 mm in length were precontracted with an EC_{50} concentration of norepinephrine in addition to a resting tension of 20 mN. The rings were then exposed to cumulative NTG concentrations in half-log increments. NTG concentrations required to produce half-maximal relaxation were 0.078 ±

0.011 μM in nontolerant rings versus 1.58 ± 0.23 μM ($P < 0.05$, N = 5 in both groups) in tolerant ones.

Measurement of Cardiac NO

NO was assayed by an *in vivo* spin-trapping method followed by ESR analysis of left ventricular tissue samples as described previously [8,13,14]. Briefly, the spin-trap DETC (200 mg/kg), 50 mg/kg of $FeSO_4$, and 200 mg/kg of sodium citrate were slowly administered i.v. into the femoral vein under ether anesthesia to all groups (N = 5 in each group). Five minutes after the DETC, $FeSO_4$, and citrate treatment, the hearts were isolated and perfused in Langendorff mode for 30 sec to remove coronary blood, then 100 mg of tissue samples from the left ventricles were placed into quartz tubes and frozen in liquid nitrogen until assayed for ESR spectra of NO-Fe^{2+}-$(DETC)_2$ complex. To obtain the background spectra of $Cu^{2+}(DETC)_2$ complex, animals were given 200 mg/kg of DETC only. ESR spectra were recorded with a Bruker ECS106 (Rheinstetten, Germany) spectrometer operating at X band with 100 kHz modulation frequency at a temperature of 160 K, using 10 mW of microwave power to avoid saturation. Scans were traced with 2.85 G modulation amplitude, 340 G sweep width, and 3356 G central field. After subtracting the background signal of $Cu^{2+}(DETC)_2$, analysis of NO content was performed with double integration of all spectra. The detection limit of NO by this ESR method, which is absolutely specific for NO free radical, is 0.05 nM/g [15]. However, precise quantification of ESR detection of NO in biologic samples is not reliable because the spin-trap Fe^{2+}-$(DETC)_3$ complex is lipophile, and introducing a known quantity of NO/NO donor into the lipid phase of biologic samples is problematic [16]. Therefore, NO content was expressed in arbitrary units showing the ratio of trapped NO in the different groups.

Determination of Cardiac cGMP

To determine cardiac cGMP content in separate experiments, hearts from all groups (N = 5 in each group) were excised under ether anesthesia and perfused in Langendorff mode for 30 sec to remove blood. Left ventricular tissue mass was then frozen by means of a Wollenberger clamp prechilled in liquid nitrogen. Samples were homogenized and centrifuged and the supernatants were extracted three times in water-saturated diethylether, evaporated, and assayed for cGMP by radioimmunoassay, as described previously [7,12].

Statistics

Data were expressed as means ± SEM and analyzed with one-way ANOVA. If a statistically significant difference was established ($P < 0.05$), a modified *t*-test that was corrected for simultaneous multiple comparisons according to the Bonferroni method was applied using SigmaStat 2.0 software [17].

RESULTS

In the positive control group, an i.v. bolus dose (3 mg/kg) of the spontaneous NO releaser, SNP, resulted in a 100-fold increase in cardiac NO content and induced a relatively minor (two-fold), but statistically significant, elevation of cardiac cGMP concentration as compared to the control group (Figs. 2 and 3). In the NTG-tolerant group, a 10-fold increase in cardiac NO content was observed without a statistically significant elevation of cardiac cGMP concentration (0.4-fold increase). In the nontolerant single NTG group, cardiac NO was significantly elevated (4-fold); however, the cardiac cGMP concentration was not changed. The NO signal was significantly higher in the tolerant (9 × NTG) group than in the single NTG group (Figs. 2 and 3). In the negative control group, LNNA significantly reduced cardiac NO signal intensity to near its detection limit, whereas cardiac cGMP content was not affected.

DISCUSSION

In this study, we investigated the effect of NTG on cardiac NO and cGMP content in NTG-tolerant and nontolerant rats and examined if changes in cardiac NO content are reflected by cardiac cGMP content. This is the first demonstration that even extreme changes in cardiac NO content have a minor influence on cardiac cGMP levels in NTG-tolerant or nontolerant rats (Fig. 3). Another important finding, which supports that of Laursen *et al.* [18], is that cardiac bioconversion of NTG to NO is not impaired in vascular tolerance to NTG *in vivo*. Our study also suggests that cardiac cGMP is not a good indicator of changes in cardiac NO content; therefore, a direct measurement of cardiac NO cannot be avoided.

Earlier studies proposed that the development of vascular tolerance to NTG was based on reduced conversion of NTG to NO; however, direct measurement of NO was not attempted in these studies [2, 5, 19]. The recent study by Laursen *et al.* [18] using the *in vivo* spin-trapping method followed by ESR analysis showed that nitrate tolerance was not associated with reduced conversion of NTG to NO. In accordance, our present study demonstrates that cardiac NO content is significantly higher in the tolerant group as compared to the nontolerant control or single NTG group, which excludes the possibility of reduced formation of NO from NTG in NTG tolerance. Moreover, the finding that the NO signal is significantly higher in the tolerant group than in the single NTG group may show an enhanced cardiac bioconversion of NTG to NO or a cardiac accumulation of NTG in nitrate tolerance. Accordingly, an accumulation of tissue NTG during tolerance development was shown by Torfgard et *al.* [20]

It is widely accepted that, in smooth muscle cells,

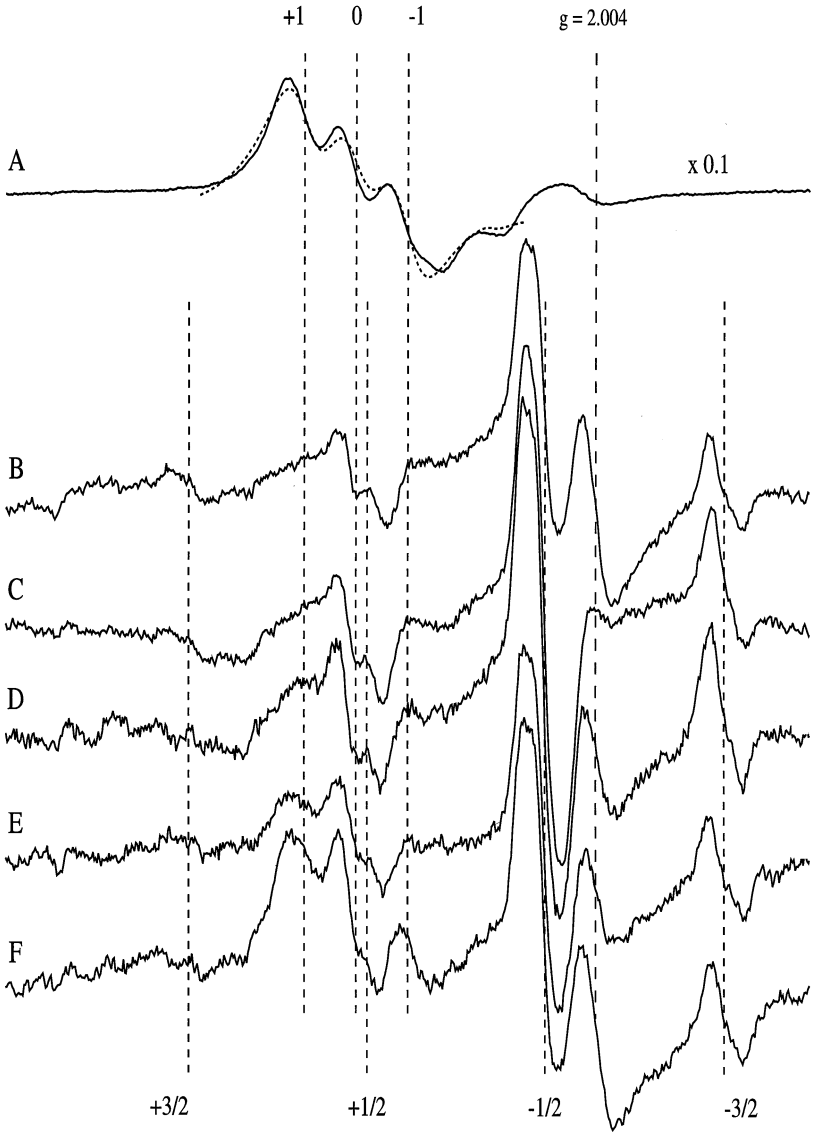

FIG. 2. Representative ESR spectra of NO-Fe^{2+}-$(DETC)_2$ complex in left ventricular tissue samples of rat hearts from the five different experimental groups and demonstration of the background signal. (Curve A): solid line, positive control (3 mg/kg of SNP); dotted line, fitted curve; ×0.1, reduced gain compared to curves B-F. (Curve B): background spectrum of $Cu(DETC)_2$. (Curve C): negative control (LNNA treatment). (Curve D): control (nontolerant). (Curve E): single NTG. (Curve F): NTG-tolerant. +1 0–1: hyperfine splitting of NO-Fe^{2+}-(DETC) triplet; +3/2 +1/2–1/2–3/2: hyperfine splitting of $Cu(DETC)_2$. ESR parameters: X band, 100 kHz modulation frequency, 160 K, 10 mW microwave power, 2.85 G modulation amplitude, 340 G sweep width, and 3356 G central field.

endogenous or exogenous NO activates soluble guanylate cyclase resulting in the production of cGMP [3]. Because the cost and availability of a direct measurement of NO limit its widespread use, cGMP concentration is used in many studies as an indirect indicator of NO production [21, 22]. However, several studies have shown that NTG has a minor effect on cardiac cGMP synthesis, which was mainly explained by a reduced conversion of NTG to NO [23–25]. Our present study revealed, however, that in the heart, NTG is metabolized to NO in both NTG-tolerant and nontolerant animals. And in our study, a 10-fold increase in cardiac NO content resulted in a slight elevation in the cardiac cGMP level, and a 100-fold increase in NO induced by a bolus injection of a high pharmacological dose of SNP, which releases NO spontaneously in aqueous solution, resulted in a minor, two-fold increase in cardiac cGMP. Forty minutes after treatment with the nonselective NO synthase inhibitor LNNA (20 mg/kg), cardiac NO was reduced to the detection limit, while the cardiac cGMP level was not decreased. These findings suggest that in the rat heart—irrespective of the development of nitrate tolerance—NO makes a minor contribution to the regulation of cGMP metabolism and that the NO-cGMP coupling is far less clear than in vascular tissue. Therefore, cardiac cGMP is not a good indicator of physiological changes in cardiac NO.

A limitation of our study is that, due to technical considerations, *in vivo* determination of NO and cGMP could be performed only in whole cardiac tissue mass; therefore, endothelial, myocardial, and neural conversion of NTG to NO all contributed to the total cardiac NO and cGMP content. Nevertheless, because left ventricular tissue mass consists of more than 90% myocardial cells, total cardiac NO and cGMP refer to myocardial NO and cGMP content with a good approximation, unless the contributions of the different tissues were profoundly different.

In summary, our present results revealed that changes in cardiac cGMP do not reflect changes in cardiac NO content in the rat and that the cardiac bioconversion of NTG to NO is not impaired in nitrate tolerance.

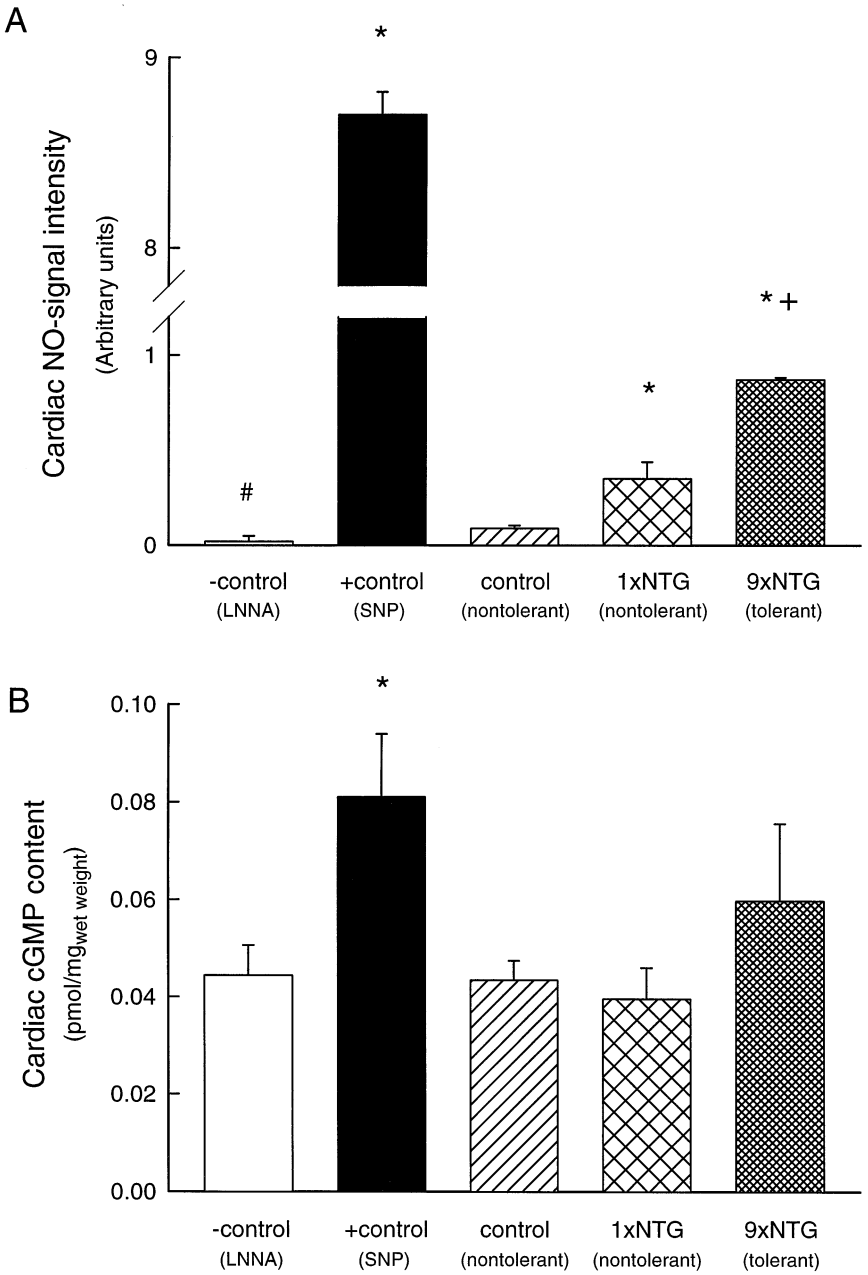

FIG. 3. Cardiac NO (A) and cGMP (B) content in negative control (nontolerant with LNNA treatment), positive control (nontolerant with SNP treatment), control (nontolerant), 1× NTG (nontolerant treated with a single dose of NTG), and NTG-tolerant group treated with NTG three times a day for 3 days (9× NTG group). */#($P < 0.05$) show significant increase/decrease compared to control, $^{+}$($P < 0.05$) show significant increase compared to 1× NTG.

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