Journal of Cardiovascular Pharmacology
14(Suppl. 11):S1–S5 © 1989 Raven Press, Ltd., New York

Molecular Aspects Underlying the Vasodilator Action of Molsidomine

Eike Noack and Martin Feelisch

Institute of Pharmacology, Heinrich-Heine-University, Düsseldorf, F.R.G.

Summary: Using different techniques, we measured the kinetics of nitric oxide (NO) liberation from SIN-1, the metabolite of molsidomine, and some related sydnonimines like its thiomorpholinyl analog, compound C 78-0698, and compared it under identical experimental conditions with its biological action at the guanylate cyclase (GC) site, taking this target enzyme as a suitable bioassay. There was a close relationship between half-maximal activation of GC and the velocity of NO release. The thiomorpholinyl analog was slightly more active in NO liberation than SIN-1 and activated the enzyme more rapidly. The kinetics of SIN-1A and SIN-1C formation, determined by high-performance liquid chromatography, could be accurately described by a Bateman equation. Oxyhemoglobin shifted the concentration–response curve of SIN-1 at the isolated soluble GC concentration to the right, whereas methemoglobin was without any effect. The results of our chemical and biochemical studies suggest that velocity and amount of NO formation are the only rate-limiting factors of guanylate cyclase activation by sydnonimines like SIN-1. NO, therefore, exclusively is the mediator of their pharmacodynamic action. In remarkable contrast to nitrate esters like glyceryl trinitrate or isosorbide dinitrate, NO liberation is not dependent on the interaction with thiol-containing compounds like cysteine. **Key Words:** NO liberation—Molsidomine—Degradation kinetics—Guanylate cyclase activation—Molecular mechanism of action.

For decades, organic nitrovasodilators such as glyceryl trinitrate or isosorbide dinitrate have represented the basic therapy for the management of coronary artery disease. With regard to their chemical structure, they are a heterogenous group of compounds that share a common metabolic pathway in that they activate the soluble form of guanylate cyclase in the smooth muscle cells of the vessel wall. The activation of this enzyme finally results in vasodilatation, preferentially at the level of the venous capacitance vessels.

Until recently, little was known about the basic molecular mechanisms by which organic nitrovasodilators exert their relaxing activity. With the recent introduction of rather specific and highly sensitive analytical methods for the detection of metabolites such as nitric oxide (NO) (1,2), and with the finding that endothelium-derived relaxing factor may be identical with NO (3,4), scientific interest has increased to further clarify their underlying mechanism of action. The results obtained substantially broaden our understanding and may offer a new basis for the development of more effective nitrovasodilators.

One of the most interesting classes of organic nitrocompounds is the sydnonimines. They are of special clinical interest because they are highly active in treating coronary artery disease and are nearly devoid of tolerance (i.e., the observation that long-term therapy with organic nitrates may rapidly become ineffective). The reason for this peculiar pharmacodynamic behavior must lie in differences in the molecular mechanism underlying the formation of the vasoactive metabolite (or metabolites) derived from these molecules.

Sydnonimines are unstable in aqueous solutions. They hydrolyze in a pH-dependent fashion by forming open-ring chemical compounds such as SIN-1A from SIN-1, which then decompose to NO and biologically inactive metabolites like SIN-1C (Fig. 1).

METHODS

Guanylate cyclase

A crude extract from rat liver was prepared according to Steurer and Schütz (5) and Ignarro et al. (6). The activity of soluble guanylate cyclase was determined in the high-speed supernatant by radioimmunoassay for cyclic GMP (Amersham/Buchler, Hannover, F.R.G.) according

Address correspondence and reprint requests to Prof. Dr. med. E. Noack at Institut für Pharmakologie, Heinrich-Heine Universität, Geb. 22.21, Moorenstr. 5, D-4000 Düsseldorf 1, Federal Republic of Germany.

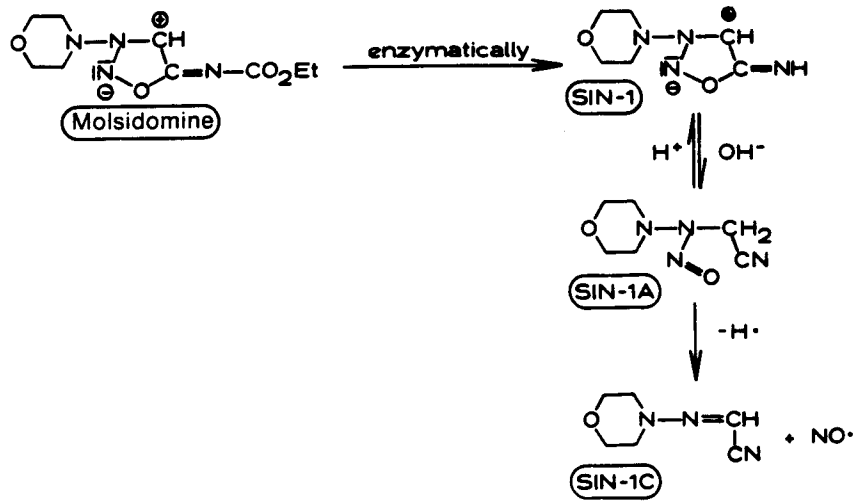

FIG. 1. Degradation pathway of SIN-1 in vitro to SIN-1C and NO.

to Kimura et al. (7). Typical assay conditions were 50 m*M* phosphate buffer (pH 7.40), 4 m*M* $MgCl_2$, 1 m*M* GTP, 30 μg of protein, incubation time of 15 min, and 37 ± 0.05°C. For the kinetic measurements, the incubation was started by the addition of the sydnonimine, and in all other cases by the addition of the enzyme extract. The final incubation volume was 3.5 ml. At time intervals of 60 s, 100-μl samples were taken from the incubate. The reaction was quickly stopped by addition to ice-cold Na_2EDTA buffer and the content of cyclic GMP estimated.

Nitric oxide

The formation of nitric oxide was monitored by using an indirect difference-spectrophotometric method based on the NO-induced oxidation of oxyhemoglobin to methemoglobin (1,8,9), which was validated by chemiluminescence (2). All measurements were performed in 50 m*M* phosphate buffer, pH 7.70 at 37 ± 0.05°C. Values are expressed as initial kinetics calculated from the slope of the extinction vs. time gradient obtained after the lag phase, which is due to hydrolysis of the sydnonimine compounds to their respective A forms.

Determination of molsidomine metabolites

Metabolites generated by the in vitro decomposition of SIN-1 and related compounds were determined simultaneously by high-performance liquid chromatography (HPLC). Incubations were carried out in 50 m*M* phosphate buffer, pH 7.40 at 37°C. Measurements were performed on a Superspher RP-8 column (25 cm × 4 mm) with a mobile phase comprised of aqueous 0.01*M* NH_4 acetate buffer, pH 3.2, acetonitrile, and methanol (90:7.5:2.5) at a flow rate of 1 ml/min. Detection wavelength was recorded at 290, 276, and 237 nm. Calibration was performed every day with freshly prepared aqueous standard solutions. The retention times for molsidomine metabolites were as follows: SIN-1, 5.3 min; SIN-1A, 8.5 min; SIN-1C, 9.1 min.

Materials

All sydnonimines and related *N*-nitroso compounds were from Cassella AG (Frankfurt, F.R.G.). All compounds were stored dry, cool, and protected from light prior to use. SIN-1A was stored under nitrogen at −80°C. Glyceryl trinitrate and glyceryl-1-mononitrate were from Schwarz Pharma AG (Monheim, F.R.G.). Aqueous solutions of all compounds were prepared freshly before the measurements were made. Bovine hemoglobin was purchased from Sigma (Taufkirchen, F.R.G.). All other chemicals were of the highest grade available.

Statistics

Data are expressed as means ± SEM. Calculation of the concentrations that induce half-maximal enzyme stimulation of soluble guanylate cyclase (EC_{50} values) were assessed by using the logit transformation according to Hafner et al. (10).

RESULTS

The formation of metabolites from various sydnonimines and the degree of activation of guanylate cyclase by these compounds in vitro were compared with special attention for SIN-1 and its thiomorpholinyl analog, compound C 78-0698. Their metabolic fate was compared directly under identical experimental conditions. Figure 2 shows the chemical structure of the compounds used.

From previous results, it is obvious that the sydnonimine derivatives and especially SIN-1 are on a molecular basis substantially more active in stimulating soluble guanylate cyclase than the classic nitro compounds such as glyceryl trinitrate. Table 1 shows the EC_{50} values, which we obtained for half-maximal activation of the enzyme.

In contrast to the organic nitrate esters, enzymatic activation was independent of the presence of special thiol-containing compounds such as cysteine. The addition of 5 m*M* L-cysteine induced a weak rightward shift of the concentration–response curve to SIN-1 (Fig. 3).

By high-pressure liquid chromatography, we measured quantitatively the degradation of 500 μ*M* SIN-1 in an aqueous solution to its metabolites SIN-1A and SIN-1C. SIN-1 was converted to SIN-1A with a half-time of 126 min, while SIN-1C was formed three to four times more quickly with a half-

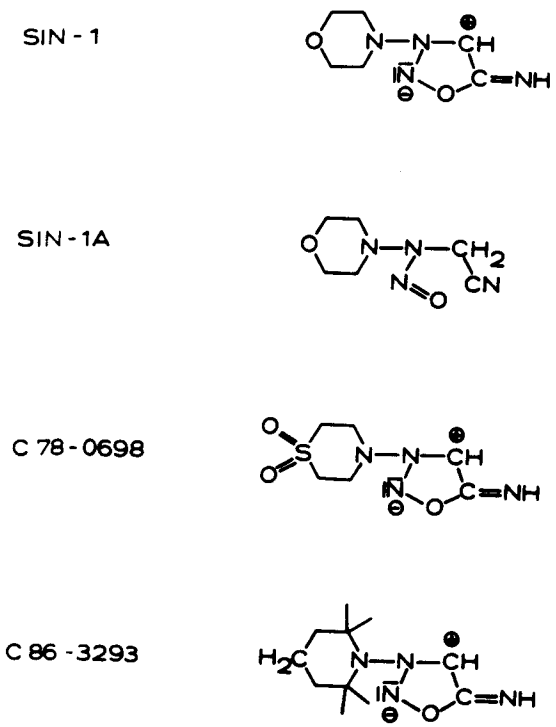

FIG. 2. Chemical structure of the sydnonimines tested [SIN-1: 3-morpholinosydnonimine-*N*-ethylcarbamide; SIN-1A: *N*-morpholino-*N*-nitrosoaminoacetonitrile; C 78-0698: 3-(thiomorpholino-S,S-dioxide)sydnonimine hydrochloride; C 86-3293: 3-(2,2,6,6-tetramethylpiperidino)sydnonimine hydrochloride].

time of 38 min (Fig. 4). The kinetics of the formation and cleavage of SIN-1A could be described by a Bateman equation, taking into account the following parameters:

$$C = [(A \cdot k_1)/(k_1 - k_2)]\,(e^{-k_2 \cdot t} - e^{-k_1 \cdot t})$$

where $A = 172.4$, $k_1 = 1.071 \times 10^{-2}$, and $k_2 = 8.778 \times 10^{-3}$.

Additional experiments with the thiomorpholinyl analog of SIN-1, i.e., compound C 78-0698, showed that the A form was more rapidly formed than with SIN-1, but it was considerably more stable. However, an equimolar concentration not only was slightly more active on guanylate cyclase (Table 1), but also quicker in activating the enzyme. The time dependence of the activation of guanylate cyclase by both sydnonimines (final concentration of 1 μM) was measured (Fig. 5). C 78-0698 was more rapid in activating the enzyme than SIN-1. The insufficient resolution of the kinetic process did not permit a more detailed determination of the half-time for the activation of guanylate cyclase. Additional experiments, at which the initial velocity of NO liberation from the sydnonimines was measured (Table 2), confirmed that the thiomorpholinyl analog is more active than SIN-1. Thus, although the open-ring A form of the thioderivative was more stable than that of SIN-1A, the total amount of NO it liberated was higher.

TABLE 1. *Concentrations (μM) of various sydnonimines, glyceryl trinitrate (GTN), isosorbide dinitrate (ISDN), and isosorbide-5-mononitrate (IS-5-N) causing half-maximal activation of isolated soluble guanylate cyclase (EC_{50})*

Compound	EC_{50} (μM)
SIN-1A	0.36
C 78-0698	0.62
SIN-1	1.23
C 86-3293	460.00
GTN	79.00
ISDN	290.00
IS-5-N	940.00

The calculation of EC_{50} values was performed from complete concentration–response curves by logit transformation (10).

The concentration-dependent activation of guanylate cyclase by SIN-1 was determined in the presence and absence of 10 μM oxygenated hemoglobin. Enzyme stimulation was completely inhibited when oxyhemoglobin was present ($n = 3$). By contrast, 10 μM methemoglobin only caused a minimal rightward shift of the concentration–response curve.

DISCUSSION

Previous experiments in which NO liberation was measured directly and continuously from chemically different organic nitrovasodilators such as gly-

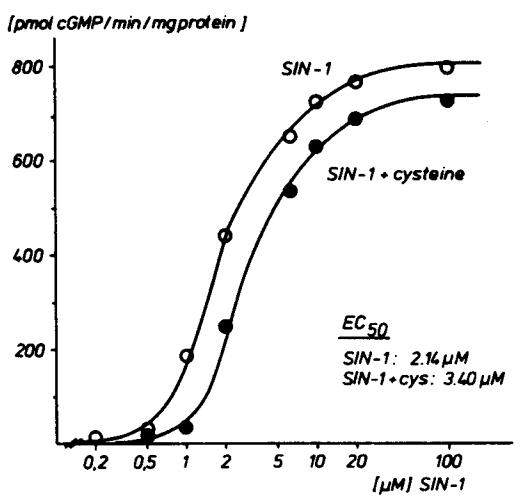

FIG. 3. Concentration-dependent activation of isolated soluble guanylate cyclase by SIN-1 in the presence and absence of 5 m*M* L-cysteine (cys; $n = 3$). cGMP = cyclic guanosine-5'-monophosphate.

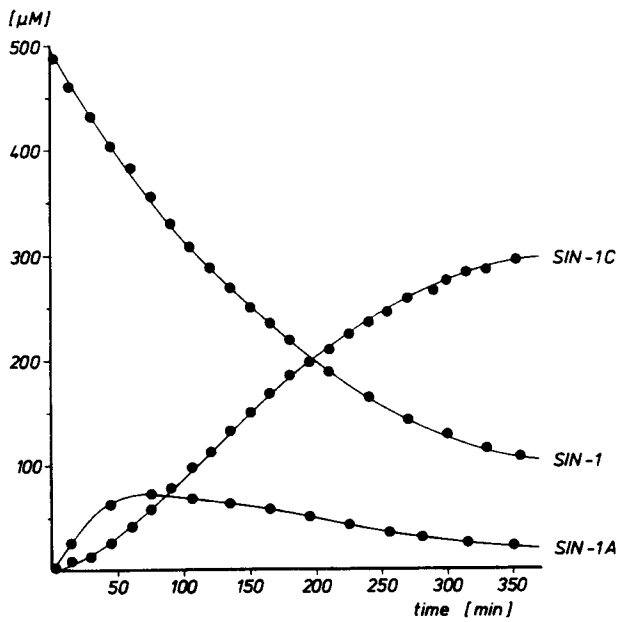

FIG. 4. Time-dependent formation (in μ*M*) of various metabolites from SIN-1 in buffered aqueous solution (pH 7.40 and 20°C) measured by high-pressure liquid chromatography. The initial concentration of SIN-1 was 500 μ*M*. Representative tracing of three separate experiments.

ceryl trinitrate, isosorbide dinitrate, amyl nitrite, nitrosothiols, furoxans, or sodium nitroprusside (11) demonstrate that NO is the exclusive, common mediator that induces the activation of soluble guanylate cyclase. The exact molecular mechanism underlying the action of the sydnonimines and especially the therapeutically, widely used molsi-

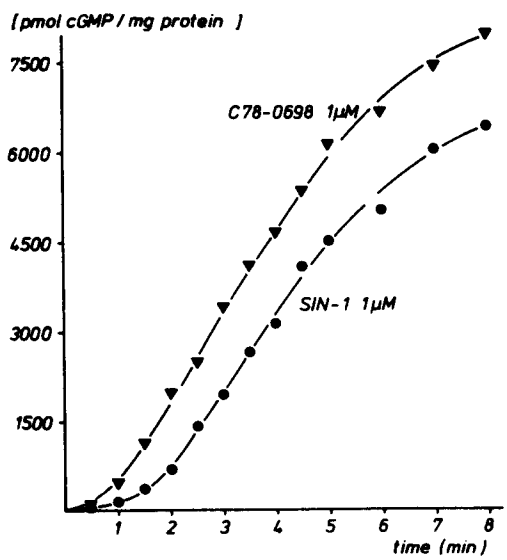

FIG. 5. Time-dependent activation of isolated soluble guanylate cyclase by 1 μ*M* SIN-1 or C 78-0698 (pH 7.40, 37°C, *n* = 3). cGMP = cyclic guanosine-5'-monophosphate.

TABLE 2. *Velocity of initial nitric oxide liberation from four sydnonimine analogs determined by difference spectrophotometry at pH 7.70, 37°C (n = 3)*

Compound	NO liberation (μ*M*/min)
SIN-1	2.39 ± 0.18
C 78-0698	3.81 ± 0.21
SIN-1A	9.69 ± 0.32
C 86-3293	0.13 ± 0.03

domine is speculative. We were interested in knowing whether these open-ring metabolites or the nitric oxide formed as a consequence of further cleavage of these compounds, are representative of the biologically active form that ultimately activates the enzyme guanylate cyclase. Therefore, the present series of experiments was performed.

We analyzed in detail the kinetics of the degradation of these compounds in aqueous solution and the concomitant activation pattern of soluble guanylate cyclase under identical experimental conditions. We observed that the sydnonimine derivative SIN-1, the in vivo metabolite of the prodrug molsidomine (12), and chemically related compounds like the thiomorpholinyl analog C 78-0698 and the methylated analog compound C 86-3293 are hydrolyzed spontaneously in a pH-dependent manner in aqueous solution. Their biological activity (their potency to stimulate guanylate cyclase) correlates directly with the velocity of NO formation in response to the degradation of the open-ring A compounds. The quicker and stronger activation of soluble guanylate cyclase by C 78-0698 in comparison to that by SIN-1 clearly contradicts the opinion that the open-ring configuration of the sydnonimines themselves is the activating principal of the target enzyme (13), because its A form is much more stable than that of SIN-1. This is due to the comparably much higher concentration of the intermediate compound. The same holds true for SIN-1A, which in a comparable concentration liberates more NO than its precursor compound. The close correlation between enzyme stimulation and the rate of NO formation from sydnonimines and organic nitrates (Fig. 6) confirms our finding that it is not the concentration of the open-ring form of the respective sydnonimine but the rate of NO liberation that is responsible for biological activity.

The stimulatory effect on guanylate cyclase is totally inhibited in the presence of small concentrations of oxyhemoglobin, which may most likely be explained by a rapid interaction of the NO formed with oxyhemoglobin, so that NO is no longer able to activate the enzyme effectively. Again, therefore, it seems rather unlikely that the metabolite of SIN-

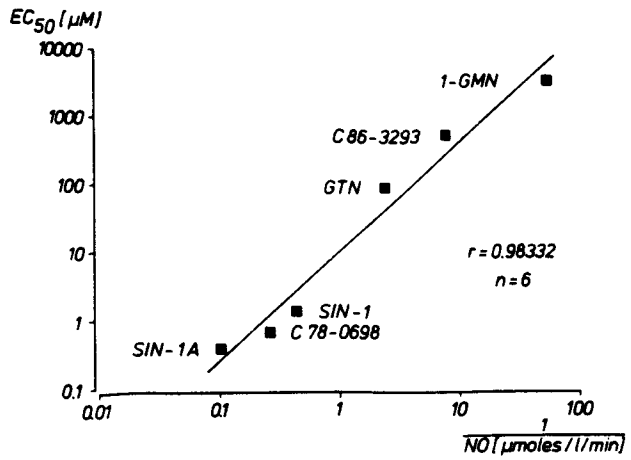

FIG. 6. Correlation between half-maximal activation of soluble guanylate cyclase (EC_{50}) and the inverse rate of NO liberation [$(\mu M\ min)^{-1}$] by various sydnonimines in comparison to glyceryl trinitrate (GTN) and glyceryl-1-mononitrate (1-GMN).

1, the SIN-1A compound, will interact itself with the guanylate cyclase, for this should not be influenced by oxyhemoglobin. This is furthermore confirmed by control experiments including methemoglobin, which does not inactivate liberated NO. The weak shift to the right of the concentration–response curve to SIN-1 may be explained by the presence of small amounts of oxygenated hemoglobin still remaining in the methemoglobin preparation. Collectively, these data strongly suggest that NO, but no other intermediate metabolites of the sydnonimines, is responsible for the pharmacodynamic action of these compounds. In addition, NO liberation from sydnonimines is completely independent of the catalytic interaction with thiol-containing compounds such as cysteine, as previously reported (1,11). This may explain why these compounds, in contrast to classical nitrovasodilators such as glyceryl trinitrate or isosorbide dinitrate, do not evoke tolerance, making their therapeutic use easier and safer.

The sydnonimines, in particular molsidomine, are prodrugs that release NO, which presumably is the endogenous "endothelium-derived relaxing factor" generated by the endothelium and involved in the control of vascular tone (14,15). A reduction in the synthesis of NO may occur if vascular damage takes place during the process of atherogenesis, for instance in the coronary arteries. In this case, the therapeutic application of endothelium-independent NO-liberating compounds such as molsidomine can be useful locally as substitutes for the NO deficiency in the vascular wall, preventing not only local vasoconstriction but also augmented adhesion and aggregation of platelets by stimulating their soluble guanylate cyclase (16).

Acknowledgment: We thank Mrs. I. Beier for skillful technical assistance. This work was supported in part by the Deutsche Forschungsgemeinschaft (SFB 242, Coronary Heart Disease, Teilprojekt C3 Noack, Düsseldorf).

REFERENCES

1. Feelisch M, Noack E. Correlation between nitric oxide formation during degradation of organic nitrates and activation of guanylate cyclase. *Eur J Pharmacol* 1987;139:19–30.
2. Feelisch M, Noack E. Nitric oxide formation from nitrovasodilators occurs independently of hemoglobin or nonheme iron. *Eur J Pharmacol* 1987;142:465–9.
3. Moncada, S, Palmer RMJ, Higgs EA. The discovery of nitric oxide as the endogenous nitrovasodilator. *Hypertension* 1988;12:365–72.
4. Kelm M, Feelisch M, Spahr R, Piper HM, Noack E, Schrader J. Quantitative and kinetic characterization of nitric oxide and EDRF from cultured endothelial cells. *Biochem Biophys Res Commun* 1988;154:236–44.
5. Steurer G, Schütz W. Guanylate cyclase stimulation by nitrocompounds is dependent on free Ca. *Experientia* 1984;40:970–1.
6. Ignarro LJ, Kadowitz PJ, Baricos WH. Evidence that regulation of hepatic guanylate cyclase activity involves interactions between catalytic site SH-groups and both substrate and activator. *Arch Biochem Biophys* 1981;208:75–86.
7. Kimura H, Mittal CK, Murad F. Activation of guanylate cyclase from rat liver and other tissues by sodium azide. *J Biol Chem* 1975;250:8016–8022.
8. Doyle MP, Hoekstra JW. Oxidation of nitrogen oxides by bound dioxygen in hemoproteins. *J Inorg Biochem* 1981;14:351–8.
9. Haussmann H-J, Werringloer J. Mechanism and control of the denitrosation of *N*-nitrosodimethylamine. In: Bartsch H, O'Neill IK, Schulte-Herman R, eds. *Relevance of N-nitros compounds to human cancer: exposure and mechanisms*, Vol. 84. Lyon: IARC Publ., 1987:109–12.
10. Hafner D, Heinen E, Noack E. Mathematical analysis of concentration–response relationship. Method for the evaluation of the EC_{50} and the number of binding sites per receptor molecule using the logit transformation. *Arzneimittelforschung* 1977;27:1871–3.
11. Feelisch M, Noack E. Chemical pathways proposed for the in vitro metabolism of nitrovasodilators and their conversion into vasoactive species. In: *Heart failure—mechanisms and management*. Berlin: Springer Verlag (in press).
12. Tanayama S, Nakai Y, Fujita T, Suzuoki Z. Biotransformation of molsidomine (N-ethoxycarbonylmorpholinosydnonimine), a new anti-anginal agent in rats. *Xenobiotica* 1974;4:175–91.
13. Böhme E, Spies C, Grossmann G. Wirksamer Metabolit von Molsidomin und Stimulation der cGMP-Bildung durch Sydnonimine. In: Bassenge E, Schmutzler H, eds. *Molsidomin: Neue Aspekte zur Therapie der ischämischen Herzerkrankung*. München: Urban & Schwarzenberg, 1982:37–44.
14. Kelm M, Schrader J. Nitric oxide release from the isolated guinea pig heart. *Eur J Pharmacol* 1988;155:317–21.
15. Rees DD, Palmer RMJ, Moncada S. Role of the endothelium-derived nitric oxide in the regulation of blood pressure. *Proc Natl Acad Sci USA* 1989;86:3375–8.
16. Nishikawa M, Kanamori M, Hidaka H. Inhibition of platelet aggregation and stimulation of guanylate cyclase by an antianginal agent molsidomine and its metabolites. *J Pharmacol Exp Ther* 1982;220:183–90.