Commentary

After 130 years, the molecular mechanism of action of nitroglycerin is revealed

Louis J. Ignarro*

Department of Molecular and Medical Pharmacology, David Geffen School of Medicine, University of California, Los Angeles, CA 90095

Nitroglycerin, which was originally synthesized by Ascanio Sobrero, was used by Alfred Nobel to manufacture dynamite. It was in Nobel's dynamite factories in the late 1860s that the antianginal effect of nitroglycerin was discovered. Two interesting observations were made. First, factory workers on Monday mornings often complained of headaches that disappeared over the weekends. Second, factory workers suffering from angina pectoris or heart failure often experienced relief from chest pain during the work week, but which recurred on weekends. Both effects were attributed to the vasodilator action of nitroglycerin, which quickly became apparent to the physicians and physiologists in local communities. But what was the mechanism of this vasodilator action of the most powerful explosive chemical discovered in the nineteenth century? The answer to this question was not to come for another century. In the late 1970s and early 1980s, the vasodilator effect of nitroglycerin was discovered to be caused by nitric oxide (NO), which was apparently generated from nitroglycerin in vascular smooth muscle (1–4). These early observations on NO culminated less than 10 years later, in 1986, with the discovery that mammalian cells synthesize NO (5). In 1998, about 130 years after Alfred Nobel's invention of dynamite and the first observed clinical benefit of nitroglycerin, the Nobel Prize in Physiology or Medicine was awarded for "Nitric Oxide as a Signaling Molecule in the Cardiovascular System". Despite these achievements, the precise molecular mechanism by which NO is generated from nitroglycerin remained elusive until the work of Chen *et al.* (6), reported in this issue of PNAS.

Previous studies showed that the bioactivation of nitroglycerin somehow involved thiols or sulfhydryl-containing compounds, and that NO or NO-containing compounds constituted the biologically active species (1–5, 7). The earliest studies suggested that an interaction between nitroglycerin and sulfhydryl (-SH)-containing cellular receptors was necessary for vascular smooth muscle relaxation to occur and that repeated administration of nitroglycerin caused sulfhydryl depletion (via oxidation) and consequent tolerance to further vasodilation (7–9). Subsequent studies addressing the activation of cytosolic guanylate cyclase by organic nitrate esters (nitroglycerin), organic nitrite esters (isoamyl nitrite), and nitroso compounds revealed that a chemical reaction occurred between the nitro compound and a thiol to generate an intermediate *S*-nitrosothiol, which then decomposed with the liberation of NO (3). Tolerance to nitroglycerin was explained simply by thiol utilization and depletion in the presence of excess nitroglycerin, thereby resulting in deficient production of *S*-nitrosothiol and NO. This working hypothesis was supported by animal and clinical studies showing that the administration of relatively large doses of cysteine or *N*-acetylcysteine could prevent or reverse the tolerance to the vasodilator action of repeated administration of nitroglycerin (see ref. 5). There were many unanswered questions associated with these earlier studies, however. The molecular mechanism of the interaction between nitroglycerin and thiol to generate *S*-nitrosothiol and NO remained an enigma. Moreover, the basis of the earlier hypotheses was activation of cytosolic guanylate cyclase in enzyme reaction mixtures and not vascular smooth muscle relaxation (3). Isolated enzyme reaction mixtures or broken cell preparations are very different from intact cells or tissues. The early work with cellular extracts did not address the likely possibility that the reaction between nitroglycerin and thiol might be enzymatic in nature. In fact, the evidence was in favor of a nonenzymatic chemical reaction (3). Subsequent studies suggested that one or more enzymatic mechanisms might be responsible for the bioactivation of nitroglycerin (10–16). However, none of these enzyme systems could catalyze the selective formation of 1,2-glyceryl dinitrate from nitroglycerin and no correlation could be found between tolerance to nitroglycerin action and tolerance to enzyme activities. The article by Chen *et al.* (6) uncovers the role of mitochondrial aldehyde dehydrogenase, which specifically generates 1,2-glyceryl dinitrate from nitroglycerin, in the bioactivation of nitroglycerin to elicit vasorelaxation and in the development of tolerance to nitroglycerin.

Chen *et al.* (6) used several ingenious approaches to elucidate the enzymatic mechanism of bioactivation of nitroglycerin: a source of large numbers of cells so that the lack of starting material would not be a limiting factor. By using mouse macrophages grown in cell culture, physiologically relevant, relatively low concentrations of nitroglycerin (0.1 μM) were shown to generate 1,2-glyceryl dinitrate through the catalytic action of an enzyme that was virtually identical to mouse mitochondrial aldehyde dehydrogenase. Mitochondrial aldehyde dehydrogenase purified from bovine liver showed identical catalytic properties to the mouse enzyme. Inhibitors of aldehyde dehydrogenase, such as cyanamide and chloral hydrate, blocked the formation of 1,2-glyceryl dinitrate from nitroglycerin. Aldehyde dehydrogenase possesses esterase activity (17) in addition to the classical NAD^+-dependent dehydrogenation activity, and the catalytic action on nitroglycerin was analogous to its esterase activity, with the important exception that nitrite (NO_2^-) rather than nitrate (NO_3^-) was a product of the enzymatic reaction. Thus, these observations were in agreement with the earliest biological findings that nitroglycerin is metabolized by tissues to inorganic nitrite or NO_2^- (3–5, 7–9). The classical sulfhydryl requirement for vascular smooth muscle relaxation by nitroglycerin (7) was explained as a chemical reaction between nitroglycerin and thiol sulfhydryl group to generate an intermediate *S*-nitrosothiol species, which then decomposed with the liberation of NO (3). Other explanations and hypotheses were offered, but none of them could be replicated or confirmed across different tissues (18–19). Therefore, the selective conver-

See companion article on page 8306.

*E-mail: lignarro@mednet.ucla.edu.

www.pnas.org/cgi/doi/10.1073/pnas.132271799

sion of 1,2,3-glyceryl trinitrate (nitroglycerin) to 1,2-glyceryl dinitrate plus nitrite, together with the dependence on a reducing thiol cofactor, made mitochondrial aldehyde dehydrogenase a compelling choice for the elusive enzyme pathway responsible for nitroglycerin bioactivation in vascular smooth muscle.

Repeated and prolonged administration of nitroglycerin and other organic nitrate esters causes the development of tolerance or desensitization of vascular smooth muscle to further vasorelaxation by nitroglycerin. This phenomenon has become a serious limitation to the chronic use of organic nitrate esters to treat angina pectoris. Understanding the molecular mechanisms associated with the development of ''nitroglycerin tolerance'' would undoubtedly lead to the discovery either of ways to avoid tolerance or of new NO-generating drugs that do not cause tolerance. The studies of Chen *et al.* (6) demonstrate that in vascular tissue made tolerant to the vasorelaxant effect of nitroglycerin, a comparable decrease occurs in both mitochondrial dehydrogenase activity and tissue cGMP accumulation. Consistent with this observation is the report that aldehyde dehydrogenase activity is markedly inhibited in patients undergoing chronic administration of nitroglycerin and other organic nitrate esters (20). These findings also are consistent with previous reports that nitroglycerin tolerance in patients can sometimes be overcome by administration of *N*-acetylcysteine (5, 21).

The authors reveal that mitochondrial aldehyde dehydrogenase functions also as a nitroglycerin reductase, where nitroglycerin acts as a substrate for the enzyme's reductase activity. As illustrated in Scheme 1 of the article (6), the authors suggest that nitroglycerin binds to one of the two cysteine sulfhydryl groups adjacent to the active-site thiol to form a thionitrite-enzyme complex intermediate plus the product 1,2-glyceryl dinitrate. Then, presumably, the NO_2^- is released from the thionitrite intermediate and is reduced to NO. These observations are analogous to the earlier views of a ''thiol receptor model,'' where nitroglycerin bioactivation required the presence of thiols, NO_2^- was generated as an intermediate (in the production of NO), and tolerance was explained as thiol depletion (3–5, 7–9). Since nitroglycerin was discovered to elicit vascular smooth muscle relaxation via mechanisms involving conversion to NO and stimulation of cGMP production, experiments were conducted to ascertain exactly how nitroglycerin and NO activate cytosolic guanylate cyclase (22). Using unpurified sources of enzyme, NO activated guanylate cyclase in the absence of further additions, whereas nitroglycerin required the addition of cysteine to cause the enzyme activation. Additional experiments revealed that nitroglycerin can undergo chemical reactions with cysteine to form NO_2^- and *S*-nitrosocysteine (3). Several *S*-nitrosothiols, including *S*-nitrosocysteine, were synthesized and found to activate guanylate cyclase in the absence of further additions. No other thiol (including DTT or glutathione) or reducing agent could substitute for cysteine in enabling nitroglycerin to activate guanylate cyclase. Interestingly, the reaction between nitroglycerin and cysteine to form NO_2^- occurred best at pH 9.6, which is near the pH optimum of mitochondrial aldehyde dehydrogenase. However, the pH-dependent chemical reaction between nitroglycerin and cysteine occurred in the absence of any aldehyde dehydrogenase or in the absence of any tissue extract, for that matter. Therefore, this reaction was a nonenzymatic reaction that was responsible for the activation of guanylate cyclase by nitroglycerin. These earlier observations taken together with the recent findings of Chen *et al.* (6) indicate that both enzymatic and nonenzymatic mechanisms may play roles in the bioactivation of nitroglycerin.

Several key questions arise from these findings. First, what is the role and precise effects of NAD^+ in catalyzing the apparent organic nitrate ester reductase activity of mitochondrial aldehyde dehydrogenase? Second, what is the influence of NAD^+ on the kinetic parameters associated with the organic nitrate reductase activity? Third, because glutathione does not ''reactivate'' the enzyme *in vitro*, what thiol is responsible for reactivation *in vivo*? Fourth, because nitroglycerin is well known to be a more potent and effective venodilator than arteriodilator, what are the differences in distribution and activities of mitochondrial aldehyde dehydrogenase in venous vs. arterial smooth muscle? Answers to these important questions will enable an even deeper understanding of the mechanism of nitroglycerin bioactivation.

The study of Chen *et al.* (6) teaches us that mitochondrial aldehyde dehydrogenase is at least partially responsible for the bioactivation of nitroglycerin and is likely to be the target of nitroglycerin tolerance. Moreover, by understanding the molecular mechanism of nitroglycerin bioactivation and tolerance, it may now be possible to design and develop novel nitrovasodilator drugs that do not cause tolerance. One approach might be to develop drugs that do not engage mitochondrial aldehyde dehydrogenase for the generation of NO. Ideally, the most appropriate kind of NO-donor drug might be one that is targeted to an enzyme that is selectively distributed to the vascular smooth muscle and acts as a substrate with only limited capacity to inhibit catalytic activity. Such a drug would be an effective vasodilator that could be used in combination with other drugs for the symptomatic treatment of hypertension. To be useful for the symptomatic treatment of angina pectoris, however, the drug would need to be targeted more to venous than arterial smooth muscle. Despite the desire to avoid tolerance, it may be a difficult task, indeed, to come up with an overall better antianginal drug than the 130-year-old nitroglycerin.

1. Arnold, W. P., Mittal, C. K., Katsuki, S. & Murad, F. (1977) *Proc. Natl. Acad. Sci. USA* **74,** 3203–3207.
2. Murad, F., Mittal, C. K., Arnold, W. P., Katsuki, S. & Kimura, H. (1978) *Adv. Cyclic Nucleotide Res.* **9,** 145–158.
3. Ignarro, L. J., Lippton, H., Edwards, J. C., Baricos, W. H., Hyman, A. L., Kadowitz, P. J. & Gruetter, C. A. (1981) *J. Pharmacol. Exp. Ther.* **218,** 739–749.
4. Ignarro, L. J., Gruetter, C. A., Hyman, A. L. & Kadowitz, P. J. (1984) in *Dopamine Receptor Agonists*, eds. Poste, G. & Crooke, S. T. (Plenum, New York), pp. 259–288.
5. Ignarro, L. J. (1989) *Circ. Res.* **65,** 1–21.
6. Chen, Z., Zhang, J. & Stamler, J. S. (2002) *Proc. Natl. Acad. Sci. USA* **99,** 8306–8311.
7. Needleman, P., Jakschik, B. & Johnson, E. M., Jr. (1973) *J. Pharmacol. Exp. Ther.* **187,** 324–331.
8. Needleman, P. (1976) *Annu. Rev. Pharmacol.* **16,** 81–93.
9. Needleman, P. & Johnson, E. M. (1973) *J. Pharmacol. Exp. Ther.* **184,** 709–715.
10. Tsuchida, S., Maki, T. & Sato, K. (1990) *J. Biol. Chem.* **265,** 7150–7157.
11. Yeates, R. A., Schmid, M. & Leitold, M. (1989) *Biochem. Pharmacol.* **38,** 1749–1753.
12. Millar, T. M., Stevens, C. R., Benjamin, N., Eisenthal, R., Harrison, R. & Blake, D. R. (1998) *FEBS Lett.* **427,** 225–228.
13. McDonald, B. J. & Bennett, B. M. (1990) *Can. J. Physiol. Pharmacol.* **68,** 1552–1557.
14. McDonald, B. J. & Bennett, B. M. (1993) *Biochem. Pharmacol.* **45,** 268–270.
15. Seth, P. & Fung, H. L. (1993) *Biochem. Pharmacol.* **46,** 1481–1486.
16. McGuire, J. J., Anderson, D. J., McDonald, B. J., Narayanasami, R. & Bennett, B. M. (1998) *Biochem. Pharmacol.* **56,** 881–893.
17. Mukerjee, N. & Pietruszko, R. (1994) *J. Biol. Chem.* **269,** 21664–21669.
18. Boesgaard, S., Aldershville, J., Poulsen, H. E., Loft, S., Anderson, M. E. & Meister, A. (1994) *Circ. Res.* **74,** 115–120.
19. Munzel, T., Sayegh, H., Freeman, B. A., Tarpey, M. M. & Harrison, D. G. (1995) *J. Clin. Invest.* **95,** 187–194.
20. Towell, J., Garthwaite, T. & Wang, R. (1985) *Alcohol Clin. Exp. Res.* **9,** 438–442.
21. Sage, P. R., de la Lande, I. S., Stafford, I., Bennett, C. L., Phillipov, G., Stubberfield, J. & Horowitz, J. D. (2000) *Circulation* **102,** 2810–2815.
22. Ignarro, L. J. & Gruetter, C. A. (1980) *Biochim. Biophys. Acta.* **631,** 221–231.