Delivery of Nitric Oxide Released from β-Gal-NONOate Activation by β-Galactosidase and Its Activity against *Escherichia coli*

Chang CHEN, Yan-Qiu SHI, Jing SONG, Qing-Sheng QI, Li GU,* and Peng George WANG*

State Key Laboratory of Microbial Technology (SKLMT), Shandong University; Jinan, Shandong 250100, China.
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β-Galactosyl-pyrrolidinyl diazeniumdiolates (β-Gal-NONOate) is a new site-specific nitric oxide (NO)-releasing compound, which releases NO once activated by β-galactosidase. In the present experiment, we used β-Gal-NONOate as a bactericidal reagent to investigate its effectiveness of NO releasing. Through the evaluation of intracellular NO level and the comparison of survival of *E. coli* transformed with *lacZ* gene but treated with β-Gal-NONOate and NONOate, respectively, it's evident that β-Gal-NONOate had a higher bactericidal activity than conventional NONOate. While either β-Gal-NONOate- or NONOate-treated *E. coli* without transferred *lacZ* gene showed low bactericidal activity. The results revealed that β-Gal-NONOate was a potentially efficient NO donor, which took on a novel and promising approach to deliver NO into cells.

Key words nitric oxide; NO donor; β-galactosyl-pyrrolidinyl diazeniumdiolates (β-Gal-NONOate); site-specific delivery; bactericidal activity

One of the most surprising and exciting developments in recent biological research is the discovery of the physiological and pathophysiological roles of nitric oxide (NO).[1—5] Nitric oxide, a free radical gas, is a multifunctional and widespread biological messenger molecule. It shows a broad range of biological activities including the regulation of vascular tone, blood flow, neurotransmission, signal transduction, immunomodulation, cellular redox status, hepatocellular apoptosis and anti-microbial defense.[6] The mechanism of microorganism killing of NO can be concluded as following: either it reacts with Fe-prosthetic groups of the mitochondrial enzymes or alternatively, NO reacts with superoxide anion O_2^- to form peroxynitrite. Its synthesis *in vivo* is catalyzed by neuronal, inducible, and endothelial isoforms of nitric oxide synthase (NOS) using L-arginine as substrate.[7] Because of numerous pharmacological benefits of NO, applications of NO releasing compounds have been increasingly reported. It is known that NO has a very short half-life (less than 10 s), and the effects of NO depend on both the source of NO (neuronal, glial, extracellular, exogenous) and the timing of NO generation.[8] NO is usually released from NO donors, which are organic compounds that produce reactive nitrogen species (RNS) *in vivo* or *in vitro* by a variety of mechanisms including decomposition, oxidation, or reduction.[9] Recently, we synthesized a novel NO releasing compound, β-galactosyl-pyrrolidinyl diazeniumdiolates (β-Gal-NONOate).[10] In this study, we used this compound to assay its bactericidal activity against microorganism, *E. coli*, and demonstrated a novel drug delivery route into cells (Fig. 1).

MATERIALS AND METHODS

Strains and Media *Escherichia coli* strains in the antibacterial activity assay were *E. coli* DH5α (kept in our lab). *E. coli* DH5α (pUC18) (*LacZ*+) was kindly provided by Professor Min Xiao (SKLMT, Shandong University, China). Culture medium was LB medium containing 5 g yeast extract (Merck, Germany), 10 g NaCl and 10 g tryptone (Oxoid Ltd., Basingstoke, Hampshire, England) per liter of distilled water. Solid medium used in this experiment was LB medium with 15 g agar (Amreso Biosharp, U.S.A.) added. Plate assays were performed by diluting overnight cultures into a series of concentrations and pouring 10 μl of each onto different plates, and then incubated overnight at 37 °C.

Chemicals NONOate (pyrrolidinyl diazeniumdiolates) and β-Gal-NONOate (β-galactosyl-pyrrolidinyl diazeniumdiolates) synthesized in our lab[10] were dissolved in PBS solution.

Growth and Enzyme Activity Curve of Bacteria Isopropyl-β-D-thiogalactoside (IPTG, 1 mM) induced and non-induced *E. coli* DH5α (pUC18) and *E. coli* DH5α were incubated in LB culture medium, respectively, for 18 h at 37 °C with the initial density of 10^7 c.f.u./ml. During this process, the plate assay was performed through extraction of culture every one hour and calculated the colony forming units (c.f.u.) later. Thus the bacterial growth curves were made and the maximum concentration of bacteria was determined. At the same time, the β-galactosidase activity of *E. coli* DH5α (pUC18) was detected by ELISA reader (420 nm, SPECTRO MAX 190, Molecular device, U.S.A.) according to the reported protocol.[11]

Effect of NO Donor on Bacterial Survival NONOate (0.1 mM) and β-Gal-NONOate (0.1 mM) was added to LB culture medium, respectively, at the time bacteria reached the maximum concentration. The reaction mixture was cultured for 8 h at 37 °C. After that, c.f.u. was counted by plate assay for quantification of bacterial activity.

Measurement of Intracellular NO Level The amount

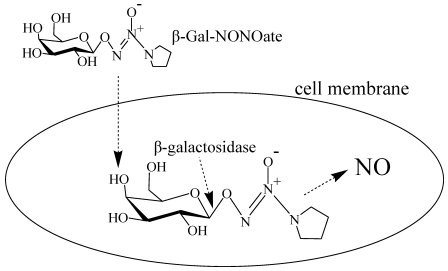

Fig. 1. Postulated Pathway of NO Release from β-Gal-NONOate Inside Cells

* To whom correspondence should be addressed. e-mail: yehe.gl@sdu.edu.cn; pwang@sdu.edu.cn

of NO released from NONOate or β-Gal-NONOate inside cells can be evaluated by measuring the nitrite concentration of cell lysate with Griess Assay.[12] Bacteria were harvested from culture medium by centrifugation after 1 h of 0.1 mM NONOate or β-Gal-NONOate treatment. The cell lysate was obtained by sonication and centrifugation. Each sample containing 80 μl culture supernatant supplemented with 20 μl nitrate reductase and enzyme co-factor mixture solution (R&D, U.S.A.) was incubated for 10 min. Then 100 μl Griess reagent (1% sulfanilamide mixed with 0.1% naphthylethylenediamine dihydrochloride in 5% phosphoric acid) was added. After 10-min-incubation at room temperature, the absorbance was determined at 540 nm with an ELISA reader (SPECTRO MAX 190, Molecular device, U.S.A.). Nitrite concentrations were then calculated using a standard curve generated with a serial dilution of sodium nitrite from 0.5 to 100 μM.

Statistical Analysis All the experiments were conducted at least three times. Results are expressed as means±S.D. Statistical significance was determined by Student's *t*-test and the differences were regarded as significant for $p<0.05$.

RESULTS

Analysis of Bacteria Growth Curve and Enzyme Activity Curve IPTG (1 mM) was added when OD_{600} reached 0.4 at the time of 3 h after incubation. The growth curves of the three differently pretreated bacteria had the similar growth trend under the same cultivation condition, and all of them reached the maximum density approximately at 11 h. Enzyme activity curve showed that the maximum β-galactosidase activity appeared at 9 h after incubation. In contrast, *E. coli* DH5α (pUC18), non-induced by IPTG, had a low enzyme activity without much fluctuation in its curve.

Effects of NO Donors on the Survival of Bacteria The survival of differently treated *E. coli* under the same cultivation condition was shown in Fig. 2. In order to compare the effects of two different NO donors on the survival, *E. coli* DH5α (pUC18) was treated with NONOate (0.1 mM) and β-Gal-NONOate (0.1 mM), respectively, at the time of 11 h. Under the normal growth condition where lactose is not used for nutrients, *E. coli* DH5α (pUC18) possesses very low level of intracellular β-galactosidase. The *lacZ* operon in the *E. coli* strain can be induced by IPTG (1 mM) to express a high level of β-galactosidase. Thus, a large amount of NO is released when β-Gal-NONOate is hydrolyzed by intracellular β-galactosidase. In contrast, non-induced *E. coli* DH5α (pUC18) has much less NO release. Therefore, the survival of induced *E. coli* DH5α (pUC18) with β-Gal-NONOate treatment was conspicuously lower (22.4%) than that of non-induced (89.2%) ($p<0.01$). On the other hand, in the presence of IPTG, the discrepancy between the survival of NONOate-treated (87.3%) and β-Gal-NONOate-treated *E. coli* DH5α (pUC18) (22.4%) was great ($p<0.01$), which was assumed to be the effects of different amounts of NO generated inside cells.

Analysis of Intracellular NO Level Nitrite concentrations of bacteria were determined as a measure of NO level with Griess Assay (Fig. 3). NO level of induced *E. coli* DH5α (pUC18) in the presence of β-Gal-NONOate was apparently higher ($p<0.01$) than that in the presence of NONOate. While NO level of non-induced *E. coli* DH5α (pUC18) treated with β-Gal-NONOate was lower ($p<0.01$) than that of induced, which indicated that intracellular NO level of β-Gal-NONOate was β-galactosidase-dependent. It was also shown that intracellular NO levels of NONOate-treated bacteria were similar, which testified its independence of β-galactosidase and resulted in its moderate bactericidal activity. These results, which corresponded with the bactericidal activity assay, suggested that bacteria with higher intracellular NO level exhibited more powerful cytotoxicity and *vice versa*.

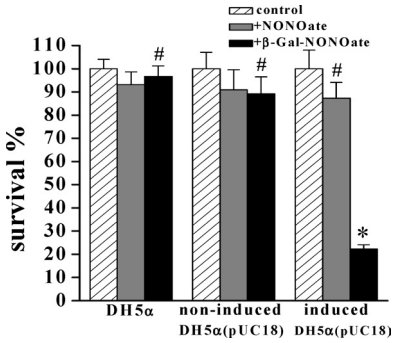

Fig. 2. Effects of NO Donor on the Survival of Bacteria

E. coli was grown in LB medium and induced with 1 mM IPTG. Survival % was calculated from the colony forming units after NO donors (0.1 mM) treatment (+NONOate; +β-Gal-NONOate) against control (without any prodrug 100% survival). The results are expressed as means±S.D. calculated from three separate experiments. * $p<0.01$ *vs.* #.

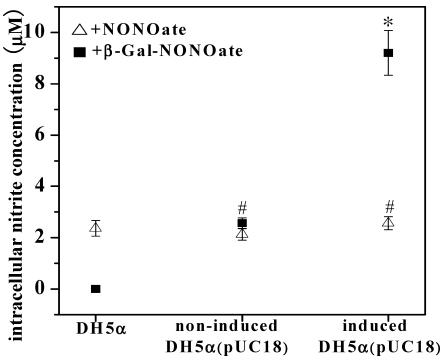

Fig. 3. Intracellular NO Level of Bacteria

Intracellular nitrite concentration was detected after 1 h of 0.1 mM β-Gal-NONOate or NONOate treatment. The results are expressed as means±S.D. calculated from three separate experiments. * $p<0.01$ *vs.* #.

DISCUSSION

NO is a critical molecule in host defense against infection. NO production is a necessary component of non-specific defense mechanism against several pathogens including bacteria, viruses, fungi and parasites.[13,14] However, NO has an extremely short half-life, so it is difficult to utilize this active small molecule in therapy and research. Thus, a variety of NO donor prodrugs have been developed, for example, sodium nitroprusside (SNP), hydroxyurea and synthetic hydroxyurea derivatives, NONOates. But a majority of conventional NO donors are hampered by lack of specificity, resulting in numerous side-effects, and the lack of adequate and localized means of NO delivery. Therefore, there is an urgent need to design a new NO donor for drug targeting and NO

releasing, which delivers drugs directly to the site of action at a controllable dose. β-Gal-NONOate is a promising NO releasing compound which can be utilized in therapy and research. Diazeniumdiolates (formerly known as NONOates) are salts containing the anionic $[N(O)NO]^-$ structural unit, and they can spontaneously release NO under physiological conditions with a range of half-lives from a few seconds to several days. Since the most characteristic property of NONOates is their spontaneous decomposition in aqueous solution with the generation of pure NO radical, in order to target a specific site with those compounds, careful protection at the terminal oxygen is necessary.[15—17] β-Gal-NONOate, a new NO donor with galactosylated protection at the terminal oxygen, is a β-galactosidase-dependent NO donor with many favorable properties, such as improved stability under physiological conditions, preferable water solubility and superior transmembrane property.[10] Our design takes advantage of a commonly used reporter or marker gene techniques to transform the cell of interest with a reporter gene (*e.g. E. coli lacZ* gene), and then feed the cell with the designed synthetic probe (β-Gal-NONOate in our study). The bacterial β-galactosidase activity can be readily measured. Thus when β-Gal-NONOate is transferred into the cell that has been transformed with *lacZ* gene, β-Gal-NONOate will be hydrolyzed inside the cell and release the NONOate to exhibit its biological effects.

In our experiments, the intracellular NO level was considered to exert direct influence on the bactericidal activity of NO donors. When β-Gal-NONOate was applied to induced and non-induced *E. coli*, it reduced the colony forming units of the induced bacteria by about 77.6%, while it showed less inhibitory effects on the non-induced bacteria. This indicated that β-galactosidase was required to release NO inside the *E. coli* cells to lead to an effective dose for cytotoxicity. This result was in sharp contrast to the result from Brunelli *et al.*[18] They reported that the exposure of *E. coli* to 1 mM NO did not decrease the viability of the bacteria. Now we can argue that this was possibly due to the limited intracellular concentration of NO. In this experiment, induced *E. coli* DH5α (pUC18) treated with NONOate and β-Gal-NONOate respectively, have notable contrast in their survivals (87.3% to 22.4%) and intracellular NO levels. This can be well explained through the different mechanisms of prodrug delivery. NONOate, once dissolved, releases NO so fast in the solution that it has no time to penetrate into the cells to function at an effective dose. Since the half-life of NO is less than 10 s, the use of regular NONOate in bulk solution cannot maintain an effective concentration of NO inside the cell (as was shown in Fig. 3). As for β-Gal-NONOate, owning to its favorable stability under physiological conditions and that it can be preferentially transported into cell *via* sugar transporter on the cell membrane,[19] it is more potential to achieve an effective NO concentration inside cell.

Here the advantage of β-Gal-NONOate was obvious since it could release large amounts of NO and thus reach an effective concentration quickly after it was hydrolyzed by intracellular β-galactosidase. Thereby, it is evident that β-Gal-NONOate is more effective and site-specific, which makes it a promising prodrug used in therapy and research.

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REFERENCES

1) Ignarro L. J., *Angew. Chem. Int. Ed.*, **38**, 1882—1892 (1999).
2) Furchgott R. F., *Angew. Chem. Int. Ed.*, **38**, 1870—1880 (1999).
3) Pfeiffer S., Mayer B., Hemmens B., *Angew. Chem. Int. Ed.*, **38**, 1714—1731 (1999).
4) Felman P. L., Griffith O. W., Stuehr D. J., *Chem. Eng. News*, **20**, 26—38 (1993).
5) Bredt D. S., Snyder S. H., *Annu. Rev. Biochem.*, **63**, 175—195 (1994).
6) Liu J., Waalkes M. P., *Toxicology*, **208**, 289—297 (2005).
7) Weller R., *Clin. Sci.* (London), **105**, 533—535 (2003).
8) Serra P. A., Rocchitta G., Delogu M. R., Migheli R., Taras M. G., Mura M. P., Esposito G., Miele E., Desole M. S., Miele M., *J. Neurochem.*, **86**, 1403—1413 (2003).
9) Wang P. G., Xian M., Tang X. P., Wu X. J., Wen Z., Cai T. W., Janczuk A. J., *Chem. Rev.*, **102**, 1091—1134 (2002).
10) Wu X. J., Tang X. P., Xian M., Wang P. G., *Tetrahedron Lett.*, **42**, 3779—3782 (2001).
11) Sambrook J., Fritsch E. F., Maniatis T., "Molecular Cloning," A Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989, pp. 808—809.
12) Green L. C., Wagner D. A., Glogowski J., Skipper P. L., Wishnok J. S., Tannenbaum S. R., *Anal. Biochem.*, **126**, 131—138 (1982).
13) Schmidt F., Ungureanu I., Duval R., Pompon A., Monneret C., *Eur. J. Org. Chem.*, **2001**, 2129—2134 (2001).
14) Desbene S., Van H. D., Michel S., Tillequin F., Koch M., Schmidt F., Florent J. C., Monneret C., Straub R., Czech J., Gerken M., Bosslet K., *Anticancer Drug Des.*, **14**, 93—106 (1999).
15) Radomski M. W., Moncada S., "Clinical Relevance of Nitric Oxide in the Cardiovascular System," ed. by Moncada S., Higgs E. A., Berrazueta E. R., Edicomplet, Madrid, Spain, 1991, pp. 45—56.
16) Battinelli E. M., Loscalzo J., "Nitric Oxide Cardiovascular System," ed. by Loscalzo J., Vita J. A., Humana Press Inc., Totowa, NJ, 2000, pp. 123—138.
17) Brennan P. A., *Eur. J. Surg. Oncol.*, **26**, 434—437 (2000).
18) Brunelli L., Crow J. P., Beckman J. S., *Arch. Biochem. Biophys.*, **316**, 327—334 (1995).
19) Saier M. H., Jr., *Mol. Microbiol.*, **35**, 699—710 (2000).