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Enzymatic generation of the NO/HNO-releasing IPA/NO anion at controlled rates in physiological media using β-galactosidase

Ryan J. Holland [a,*,1], Rika Paulisch [b,1], Zhao Cao [c], Larry K. Keefer [a], Joseph E. Saavedra [c], Sonia Donzelli [b]

[a] Drug Design Section, Chemical Biology Laboratory, National Cancer Institute at Frederick, Frederick, MD 21702, USA
[b] Department of Experimental Pharmacology and Toxicology, University Medical Center Hamburg-Eppendorf, Martinistr. 52, D-20246 Hamburg, Germany
[c] Basic Research Program, SAIC-Frederick, Frederick National Laboratory for Cancer Research, Frederick, MD 21702, USA

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ABSTRACT

We introduce a strategy for generating mixtures of nitric oxide (NO) and nitroxyl (HNO) at tunable rates in physiological media. The approach involves converting a spontaneously HNO/NO-generating ion to a caged (prodrug) form that is essentially stable in neutral media, but that can be activated for HNO/NO release by adding an enzyme capable of efficiently opening the cage to regenerate the ion. By judiciously choosing the enzyme, substrate, and reaction conditions, unwanted scavenging of the HNO and NO by the protein can be minimised and the catalytic efficiency of the enzyme can be maintained. We illustrate this approach with a proof-of-concept study wherein the prodrug is Gal-IPA/NO, a diazeniumdiolate of structure iPrHN–N(O)=NOR, with R = β-D-galactosyl. *Escherichia coli*-derived β-D-galactosidase at concentrations of 1.9–15 nM hydrolysed 56 μM substrate with half-lives of 140–19 min, respectively, producing the IPA/NO anion (iPrHN–N(O)=NO$^-$, half-life ~3 min), which in turn spontaneously hydrolysed to mixtures of HNO with NO. Using saturating substrate concentrations furnished IPA/NO generation rates that were directly proportional to enzyme concentration. Consistent with these data, the enzyme/substrate combination applied to ventricular myocytes isolated from wild-type mouse hearts resulted not only in a significant positive inotropic effect, but also rescued the cells from the negative inotropy, hypercontractions, and occasional cell death seen with the enzyme alone. This mechanism represents an alternate approach for achieving controlled fluxes of NO/HNO to investigate their biological actions.

Introduction

A variety of research tools are available for generating controlled fluxes of the multifaceted bioregulatory agent nitric oxide (NO) in physiological media, including a series of ionic and zwitterionic diazeniumdiolates whose reproducible half-lives for NO release range from 2 s to 20 h at pH 7.4 and 37 °C, depending on their structure. These agents have greatly aided the effort to characterise NO's biological activities over the last 20 years [1,2].

More recently, nitroxyl (HNO, the product of reducing NO by one electron) has been shown to possess intriguing biological properties, some of potential therapeutic significance [3–7]. However, there are several properties of HNO that have complicated attempts to study its chemistry and pharmacology. One problem is that it rapidly dimerises and self-destructs Eq. (1), meaning that it cannot be isolated in pure form. This problem can be circumvented by employing prodrug forms such as Angeli's salt (AS) [8,9], a well-established HNO prodrug that in our hands hydrolyses to a 93:7 mixture of HNO to NO in physiological buffer with a reliable half-life of ~3 min at physiological temperature and pH.

$$2HNO \rightarrow N_2O + H_2O \quad (1)$$

AS has provided many major insights into the chemical biology of HNO [9–13], but it has not been ideal for studying the effects of HNO fluxes much longer than the 3-min half-life of AS can provide. Recently, several alternate HNO prodrugs have been introduced that spontaneously hydrolyse to produce HNO in high yield with discrete half-lives of seconds to hours, depending on their structure [14–19]. These prodrugs have provided an increasingly valuable set of research tools as their respective by-product reactivity and toxicity profiles are elucidated.

Here we describe a system that delivers both HNO and NO simultaneously over an infinitely tunable range of half-lives. It involves the use of stable prodrugs that in presence of an activating enzyme undergo two-step hydrolysis releasing both potent

Abbreviations: NO, nitric oxide; IPA/NO, sodium 1-(isopropylamino)diazen-1-ium-1,2-diolate; HNO, nitroxyl.

* Corresponding author. Fax: +1 301 846 5946.
E-mail address: hollandrj@mail.nih.gov (R.J. Holland).

[1] These authors contributed equally to this work.

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bioregulators, and any downstream cross talk products, at rates that can be precisely programmed.

Materials and methods

Chemicals

Liberase TM (Thermolysin medium) Research Grade Enzyme was obtained from Roche Diagnostics (Mannheim). Fetal calf serum (FCS) was purchased from Invitrogen (Molecular Probes™, Karlsruhe). All other chemicals used in the experiment (including β-galactosidase from *Escherichia coli*) were acquired from Sigma to Aldrich (St. Louis, MO, USA), except $CaCl_2$ (Merck, Darmstadt).

O^2-(2,3,4,6-tetra-O-acetyl-β-D-galactopyranosyl) 1-(N-isopropylamino)diazen-1-ium-1,2-diolate

A solution of 2.64 g (0.0064 mol) of acetobromo-α-D-galactose in 100 mL of acetone was cooled to 0 °C. A solution of 1.06 g (0.0075 mol) of IPA/NO in 50 mL of cold (5 °C) 5% aqueous sodium bicarbonate was placed in a dropping funnel equipped with an ice-jacket and added dropwise to the acetone solution. The reaction mixture was allowed to warm up to room temperature gradually and stirred for 48 h. The acetone was then removed on a rotary evaporator and the remaining aqueous portion was extracted with dichloromethane. The organic layer was separated, dried over sodium sulfate, filtered through magnesium sulfate and concentrated under vacuum to give 1.22 g of a syrup. The crude product was chromatographed on an 80-g silica gel pre-packed column and eluted with dichloromethane:ethyl acetate to give 370 mg (13%) of O^2-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl) 1-(*N*-isopropylamino)diazen-1-ium-1,2-diolate as a crystalline solid; several preparations following this procedure produced a total of 1.39 g of material: mp 36–8 °C; UV (ethanol) λ_{max} (ε) 238 nm (6.9 mM^{-1} cm^{-1}); ^1H NMR ($CDCl_3$) δ 1.19 (d, 6H) J = 6.25 Hz, 1.99 (s, 3H), 2.05 (s, 3H), 2.05 (s, 6H), 2.16 (s, 3H), 3.97–4.01 (m, 1H), 4.11–4.18 (m, 2H), 5.07–5.14 (m, 2H), 5.42–5.43 (m, 1H), 5.48–5.52 (m, 1H), 6.16 (d, 1H) J = 8.99 Hz; ^{13}C NMR ($CDCl_3$) δ 20.40, 20.42, 20.49, 20.58, 20.61, 49.20, 61.06, 66.63, 66.78, 70.91, 71.44, 100.84, 169.02, 170.02, 170.11, 170.31. HRMS (ESI) m/z calculated for $C_{17}H_{27}N_3O_{11}$ $(M+H)^+$ 450.1784, found 450.17131, Δppm = 1.04.

O^2-β-D-Galactopyranosyl) 1-(N-isopropylamino)diazen-1-ium-1,2-diolate (Gal-IPA/NO)

To a solution of 712 mg (1.59 mmol) of the above tetraacetate in 75 mL of methanol was added 80 μL of 25% sodium methoxide in methanol; the mixture was stirred for 2 h at room temperature. To the solution was added 2 g of washed Amberlist 15-H^+; the mixture was swirled for a few minutes, filtered, and evaporated under vacuum to give 568 mg of product as a white glassy substance that became crystalline on storage at −10 °C: mp 54–56 °C; UV (PBS) λ_{max} (ε) 237 nm (7.2 mM^{-1} cm^{-1}); ^1H NMR (CD_3OD) δ 1.05 (d, 6H) J = 6.3 Hz, 3.45–3.47 (m, 2H), 3.67–3.70 (m, 3H), 3.78–3.83 (m, 3H), 4.81 (d, 1H) J = 9.2 Hz; ^{13}C NMR (CD_3OD) δ 18.74, 60.83, 68.56, 69.04, 73.38, 75.89, 103.86. HRMS (ESI) m/z calculated for $C_9H_{20}N_3O_7$ $(M+H)^+$ 182.12958, found 182.12881, Δppm = 4.2.

Analysis for NO

Chemiluminescence detection and quantification of NO evolving from the reactions were conducted using a Sievers 280i nitric oxide analyser (NOA). A pH 7.4 solution of 0.1 M phosphate buffer with 50 μM diethylenetriamine pentaacetic acid (DTPA) containing β-galactosidase at 37 °C was sparged with inert gas until a steady detector response was established. IPA/NO or Gal-IPA/NO were added to a final concentration of 56 μM and the NO release profile was followed over time after injection. The resulting curve was integrated to quantify the amount of NO released/mol of compound.

Griess assay test for nitrite detection

Substrate/enzyme reactions were allowed to proceed to completion in the absence of purging. We then added 100 μL of Griess reagent, 300 μL of sample and 2.6 mL of deionised water together in a spectrophotometer cuvette. We incubated the mixture for 30 min at room temperature and prepared a reference sample by mixing 100 μL of Griess reagent and 2.9 mL of deionised water. The absorbance of the nitrite-containing sample at 548 nm relative to the reference sample was converted to nitrite concentrations using a calibration curve.

N_2O measurements by gas chromatography

Reactions were run according to the conditions stated above. The gas chromatography was performed on a Shimadzu GC-2014 with an electron capture detector, equipped with ^{63}Ni 370 MBq source. A Restek ShinCarbon 80/100 packed column (2 m × 2.0 mm ID) was used with helium as carrier gas. The GC operation conditions were as follows: injector and detector temperatures were at 250 °C, oven temperature was programmed from 90 to 200 °C at 20 °C/min and held at 200 °C for 1.1 min. Helium flow was 30 mL/min and nitrogen was used as makeup gas at 2 mL/min.

Kinetic studies

Kinetic experiments were performed at 37 °C using a standard UV–visible spectrophotometer. Reactions were initiated by addition of substrate after the buffer and enzyme reached thermal equilibrium. Typical substrate concentrations were 56 μM with a β-galactosidase concentration range of 2–15 nM in 0.1 M phosphate buffer, pH 7.4, containing 50 μM diethylenetriamine pentaacetic acid (DTPA). In each experiment the data were analysed at 247 nm and the rate was derived by fitting the data to an exponential curve typical for first order processes.

Analysis for amine and alcohol products

β-Galactosidase was stirred in 1 mL of 0.1 M phosphate buffer, pH 7.4, containing 50 μM diethylenetriamine pentaacetic acid (DTPA), at 37 °C in a sealed cuvette. To this solution was added 12 mg of Gal-IPA/NO for a final concentration of 6.6 μM. The decomposition of Gal-IPA/NO was followed by UV/vis spectroscopy. Upon completion the solution was cooled to 4 °C prior to opening the cuvette. It was then filtered through a YM-3 microcon centrifugation filter and added to an NMR tube with 10% D_2O. Spectra were run on a Varian Inova 400 MHz NMR with a Dell Precision 390 workstation. The samples were run at 37 °C and water suppression was achieved by using the preset pulse sequence.

Animals

Mice (3–4 months of age) from a black Swiss background were used in this study. Animal care and experimental procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals (NIH Pub. No. 85-23, revised 1996) and were approved by the governmental review board in Hamburg (G21/1-46/04).

Sarcomere shortening and Ca^{2+} transients measurements in isolated ventricular myocytes

Ventricular myocytes were isolated as previously described [20]. The isolated myocytes were preincubated in IonOptix solution (in mmol/L: 135 NaCl, 4.7 KCl, 0.6 KH_2PO_4, 0.6 Na_2HPO_4, 1.2 $MgSO_4$, 1.25 $CaCl_2$, 20 glucose, 10 hepes, pH 7.46), containing 1 μmol/L Fura-2-AM for 30 min. Sarcomere shortening and Ca^{2+} transients were simultaneously assessed on field stimulation (1 Hz with 4-ms duration, 15 V) using a video-based sarcomere length detection system (IonOptix Corporation) maintained at 37 °C by a heater (IonOptix) attached under the chamber.

Statistical analysis

Paired Student *t*-test was used to compare sarcomere length shortening before and after treatment. Results are presented as mean ± SEM, with n indicating the number of myocytes for each experiment. Differences between sarcomere shortening before and after treatment were considered significant when $p < 0.05$. Analysis of the data and plotting of the figures were done using Graphpad Prism 5.0 software.

Results

Prodrug synthesis

Theoretically, the half-life of AS could be extended by modifying its structure as shown in Eq. (2) to produce derivatives whose R groups can be programmed for cleavage under controlled conditions to regenerate the AS anion at the desired rate. Unfortunately, attempts to prepare such protected derivatives of Angeli's salt have to our knowledge been uniformly disappointing. Even if AS could be doubly O-derivatised to form RO–N(O) = NOR as shown, it would presumably need two different R–O bond-breaking steps to revert to the desired dianion, and the intermediate half-derivatised species may fragment spontaneously via some unpredicted pathway leading to products other than Angeli's anion.

$$Na_2N_2O_3 + 2RX \rightarrow RO{-}N(O){=}N{-}OR + 2NaX \quad (2)$$

Accordingly, we have chosen to develop this approach using IPA/NO, a diazeniumdiolate anion that hydrolyses to a mixture of NO and HNO in physiological buffer (Fig. 1) with a half-life similar to that of Angeli's salt [21,22]. Although hydrolysis of IPA/NO at physiological pH yields a lower HNO/NO ratio among the products (Fig. 1), the two anions nevertheless compare and contrast favorably with each other in some important ways. Of special importance in the present connection, IPA/NO can be easily O-derivatised by reaction with a diversity of protecting groups [23], each of which can be envisioned to cleave under activating conditions to free the IPA/NO anion *in situ*.

After preliminary consideration of numerous prodrug candidates, we chose Gal-IPA/NO as substrate to investigate HNO release from an enzymatic reaction. Gal-IPA/NO was synthesized by reacting IPA/NO sodium salt with tetraacetylated galactosyl bromide. This intermediate product was exposed to methanolic sodium methoxide to remove the acetyl groups, as summarised in Fig. 2A. The synthesized Gal-IPA/NO hydrolysed only slowly in 0.1 M phosphate at pH 7.4 and 37 °C, with a half-life of greater than two weeks.

Hydrolysis of Gal-IPA/NO is first order in enzyme under saturating conditions

We found that β-D-galactosidase isolated from *E. coli* very efficiently cleaved Gal-IPA/NO at pH 7.4 and 37 °C (Fig. 2B). The reaction, as followed by HPLC, was cleanly first order in enzyme at a saturating substrate concentration of 56 μM with enzyme in the 1.9–15 μM range (see Fig. 3).

Enzyme integrity and catalytic efficiency were not affected by exposure to Gal-IPA/NO and its hydrolysis products

We checked for possible suicide inhibition of the galactosidase in these experiments. No evidence for this unwanted effect was found. The hydrolysis of 56 μM Gal-IPA/NO by 15 μM enzyme was cleanly first order through five half-lives, with no evidence of impaired activity at the later time points (Fig. 4, left-most trace). The reaction was twice repeated, each time by restoring the substrate concentration to 56 μM in the same solution in the same cuvette, with the same observed rate constant of $1.5 \times 10^{-3}\ s^{-1}$ (Fig. 4) for all three reactions. There may have been a slight trend toward decreasing rate in run 3 ($1.50 \times 10^{-3}\ s^{-1}$ vs. $1.52 \times 10^{-3}\ s^{-1}$ for the first two), and some ill-defined background absorbance began to accumulate over time, but we did not see a significant drop in catalytic efficiency through the first 11,200

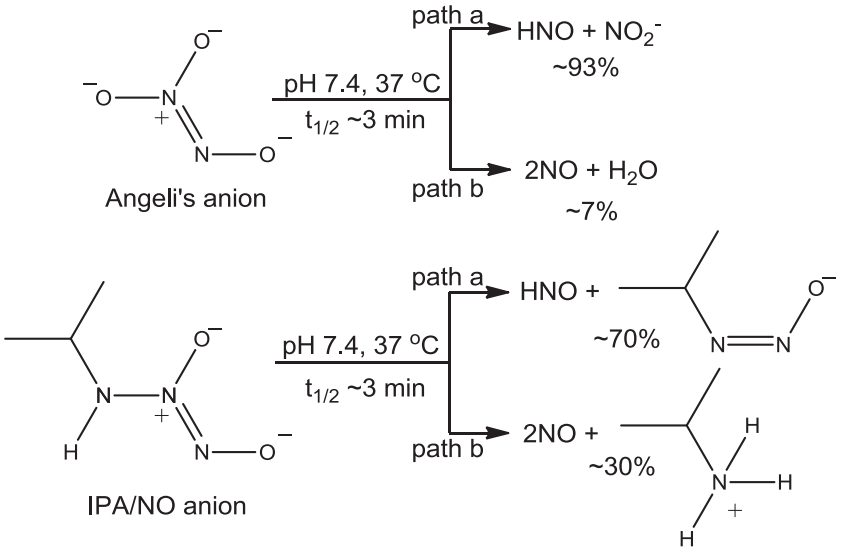

Fig. 1. Similarities and differences between Angeli's salt and IPA/NO: hydrolysis rates and product profiles. Note that each of these ions hydrolyses via two mechanisms running in parallel: HNO-producing path a and NO-generating path b.

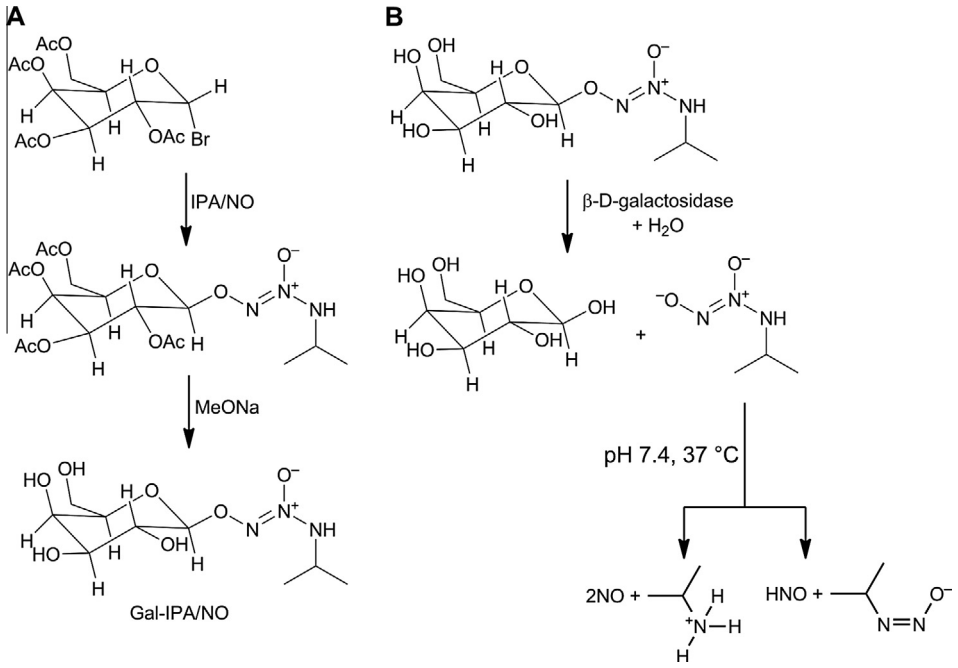

Fig. 2. (A) Synthesis of Gal-IPA/NO. (B) Enzymatic hydrolysis of Gal-IPA/NO with β-D-galactosidase.

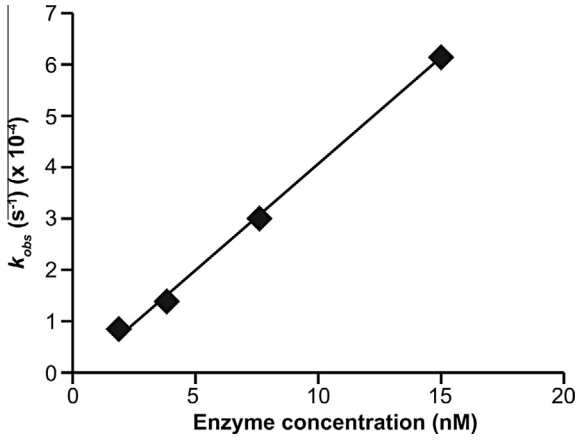

Fig. 3. The rate of IPA/NO release from 56 μM Gal-IPA/NO proved directly proportional to enzyme concentration under conditions suitable for studying the chemical biology of HNO/NO mixtures (37 °C in 0.1 M phosphate buffer, pH 7.4, containing 50 μM diethylenetriamine pentaacetic acid). $y = 4 \times 10^{-5} \times +6 \times 10^{-6}$. $R^2 = 0.9985$.

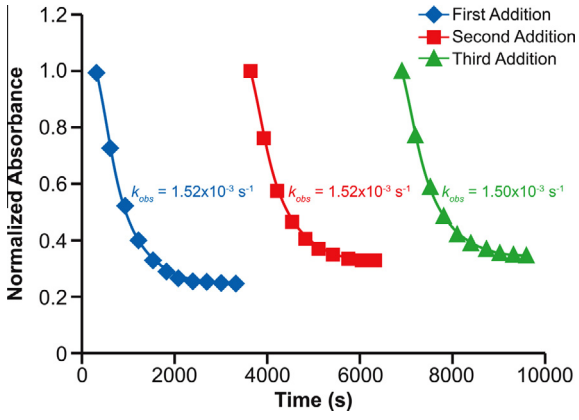

Fig. 4. Effect of repeated substrate additions on catalytic efficiency. A phosphate buffer solution (0.1 M, pH 7.4) containing 50 μM diethylenetriamine pentaacetic acid and 15 μM β-galactosidase was placed in a cuvette held at 37 °C. A concentrated solution of Gal-IPA/NO was added with stirring to give an initial concentration of 56 μM and loss of absorbance at 247 nm was followed as a function of time. The data were normalised to the absorbance at t_0, yielding the blue trace shown at the left. When substrate hydrolysis was effectively complete, Gal-IPA/NO was added to the same cuvette to again give an initial concentration of 56 μM, leading to the red trace. The process was repeated one more time, leading to the green curve. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

turnovers. Additionally, there was no detectable change in the enzyme's high resolution mass spectrum after a round of catalysis.

Product distribution was similar to that of IPA/NO anion hydrolysis

Gases formed were analysed by gas chromatography for N_2O as a measure of HNO yield and, separately, conducted into a chemiluminescence analyser to quantify NO. Nitrite was assayed using the Griess procedure. Organic products of Gal-IPA/NO hydrolysis were estimated by NMR. Isopropylamine is produced through NO-yielding path b of Fig. 1 (bottom reaction), while isopropanol is concurrently produced through the protonation, dehydration, and hydrolysis of the diazoate ion generated via path a. The data of Table 1 confirm the similarity of product profiles on hydrolyzing IPA/NO vs. its galactosylated derivative.

Effect of varying IPA/NO generation rate on myocyte contractility

IPA/NO has been shown to possess positive inotropism, i.e., the ability to strengthen the beating of isolated cardiac myocytes, an effect to be expected of prodrugs that generate HNO [21]. Here, we report that Gal-IPA/NO is hydrolysed by β-D-galactosidase to generate free IPA/NO anion and thence HNO in admixture with NO. To apply this chemistry to a biological system, we chose isolated cardiac myocytes, a well known and characterised target of HNO. Isolated cardiac myocytes treated simultaneously with Gal-IPA/NO (56 μM) and β-D-galactosidase (15 μM) at a constant temperature of 37 °C showed initially a significant increase in

Table 1
Products (% of theoretical) of exhaustively hydrolyzing IPA/NO vs. Gal-IPA/NO in presence or absence of β-D-galactosidase at pH 7.4 and 37 °C in the presence of 50 μM diethylenetriamine pentaacetic acid.[a]

Analyte	Gal-IPA/NO + enzyme	IPA/NO, no enzyme	IPA/NO + enzyme
N_2O	60 ± 1.6	68 ± 1.1	64 ± 0.5
NO	29 ± 2.1	30 ± 0.3	32 ± 0.6
Nitrite	9.3 ± 1.1	2.7 ± 0.3	3.9 ± 0.5
Isopropylamine	~30	–	–
Isopropanol	~70	–	–

[a] Values given are means ± standard deviations for three replicates each. "% Of theoretical" refers to the number of moles measured for a given analyte divided by the number of moles that would have been seen in the absence of competing pathways (e.g., the parallel hydrolysis mechanisms represented by paths a and b in Fig. 1).

sarcomere shortening (maximum change versus baseline, from 8.3% to 11.1%, net change of 2.8%) that was completely reversed 20 min later (final condition, from 11.1% to 5.5%, Fig. 5). Next, we inactivated half of the enzyme by incubating it at 70 °C for 10 min and treated the myocytes as above. As expected, the maximum increase on sarcomere shortening was only about two thirds that seen with the fully activated enzyme (6.6% increasing to 8.3%, or an increase of 1.7% compared to 2.8%). The increase in this case did not reach statistical significance, though the trend was clear (Fig. 5).

Surprisingly, β-D-galactosidase alone induced toxicity in the myocytes. In this case, a significant immediate drop in sarcomere shortening relative to baseline was observed (from 7.4% to 3.3%), followed by a gradual but incomplete recovery at the end of the 20-min observation period. This toxic insult was prevented by the simultaneous treatment of the myocytes with Gal-IPA/NO and β-D-galactosidase (Fig. 5). At first glance this immediate response to treatment was hard to rationalise as the kinetic studies indicate that HNO and NO should not be present in significant quantities at such short time points. What then is responsible for preventing the initial negative inotropism? We speculate that this observation may be due to an ability of the cells to metabolise Gal-IPA/NO by pathways independent of the exogenous β-D-galactosidase. Support for this hypothesis was found in the Gal-IPA/NO control (Fig. 5). When isolated cardiac myocytes were exposed to Gal-IPA/NO alone, a slight increase in sarcomere shortening was observed. This result is consistent with the view that Gal-IPA/NO is, to some extent, metabolised by endogenous enzymes upon treatment of the myocytes.

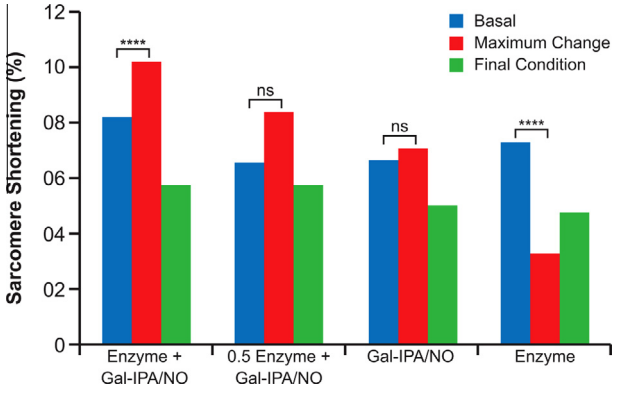

Fig. 5. Effect of Gal-IPA/NO and β-D-galactosidase on myocyte shortening in isolated cardiac myocytes. The basal condition levels were established within two to ten minutes after myocyte selection and mounting. The final condition bars reflect the average contractility at the 20-min time point. The middle bar provides the point of maximum myocyte length observed under the conditions described. The number of myocytes used for each experiment was 20, 7, 10, and 12 for each *x*-axis entry, respectively. ****$p < 0.0001$ vs. basal, ns = not significant ($p > 0.05$).

Discussion

Starting with the pioneering work of Nagasawa and colleagues, investigators have developed various enzyme/prodrug methods for generating nitroxyl to study its biological effects [19,24–26]. Here we show how one such prodrug can be used for reliably tuning the reaction rate.

Design considerations

Several factors were considered in developing this strategy for programming the rate of IPA/NO generation in solution by converting it to prodrug form and varying the concentration of a metabolizing enzyme. Ideally, the caged IPA/NO donor molecule would be totally stable in the reaction medium, fragmenting to initiate IPA/NO generation only when the enzyme is added. The enzyme should be unreactive with HNO, NO, and the other products of metabolism; any reaction of HNO or NO with the protein would artifactually diminish the amount available and potentially also compromise the catalytic efficiency of the enzyme. Accordingly, the catalytic efficiency of the enzyme should be as high as possible, presenting a smaller protein target for this unwanted loss of HNO/NO to the system. After considering a variety of enzyme/substrate combinations, we chose β-galactosidase from *E. coli* with galactosylated IPA/NO (Gal-IPA/NO) as substrate for these initial proof-of-concept studies.

Solution chemistry

As shown in Fig. 3, the rate at which 56 μM Gal-IPA/NO was hydrolysed proved linearly dependent on enzyme concentration over the range 15–1.9 μM, furnishing reproducible half-lives for IPA/NO release of 19–140 min. Quantitative yields of HNO/NO were estimated and found to be in ratios consistent with previous studies of IPA/NO in our hands. There was no evidence for destructive interaction between enzyme and substrate; the rate constant remained essentially undiminished through 11,200 turnovers and peaks suggestive of adduct formation were absent from the mass spectrum of the reisolated enzyme.

Proof of concept. Use of this approach in chemical biology applications

IPA/NO has been shown to exhibit positive inotropism, i.e., to strengthen the beating of isolated cardiac myocytes, an effect to be expected of prodrugs of HNO [21]. The present results provide a way to extend the time course of IPA/NO's normal 3-min half-life for co-generating HNO and NO by converting the anion to a hydrolytically stable derivative, Gal-IPA/NO, that can generate free IPA/NO anion into the medium at precisely tunable rates in a reaction that is first order in β-D-galactosidase at saturating substrate concentration. When this prodrug/enzyme mixture was added simultaneously to a chamber containing isolated cardiac myocytes

undergoing electrically stimulated contraction, the intensity of sarcomere shortening was about 50% greater when the amount of active (non-denatured) enzyme was doubled, and the enzyme/prodrug combination largely abrogated the cellular toxicity seen with one of the enzyme batches we used. It is worth noting that the positive inotropy observed in this experiment would likely have been even more positive if the enzyme batch we used had not been toxic.

We take these results as a proof-of-concept study suggesting the utility of such approaches for co-generating NO and HNO at controllable rates for characterizing the chemical biology of mixed fluxes of these two bioeffector molecules. Additionally, this approach could be used to provide insight into the effects of the highly reactive $N_2O_2^-$ radical anion, which is formed by direct reaction of NO with HNO. By simultaneously generating NO and HNO at known rates and applying the published [27,28] rate constants for formation of $N_2O_2^-$ and its subsequent rapid reaction with NO to generate the $N_3O_3^-$ ion, the momentary fluxes of $N_2O_2^-$ and $N_3O_3^-$ should be calculable. Disproportionation of the latter species would lead to equimolar amounts of N_2O and nitrite ion, both of which are observed in the reactions studied here (Table 1).

Acknowledgments

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