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Original Contribution

Synergistic activity of acetohydroxamic acid on prokaryotes under oxidative stress: The role of reactive nitrogen species

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ABSTRACT

One-electron oxidation of acetohydroxamic acid (aceto-HX) initially gives rise to nitroxyl (HNO), which can be further oxidized to nitric oxide (NO) or react with potential biological targets such as thiols and metallo-proteins. The distinction between the effects of NO and HNO *in vivo* is masked by the reversible redox exchange between the two congeners and by the Janus-faced behavior of NO and HNO. The present study examines the ability of aceto-HX to serve as an HNO donor or an NO donor when added to *Escherichia coli* and *Bacillus subtilis* subjected to oxidative stress by comparing its effects to those of NO and commonly used NO and HNO donors. The results demonstrate that: (i) the effects of NO and HNO on the viability of prokaryotes exposed to H_2O_2 depend on the type of the bacterial cell; (ii) NO synergistically enhances H_2O_2-induced killing of *E. coli*, but protects *B. subtilis* depending on the extent of cell killing by H_2O_2; (iii) the HNO donor Angeli's salt alone has no effect on the viability of the cells; (iv) Angeli's salt synergistically enhances H_2O_2-induced killing of *B. subtilis*, but not of *E. coli*; (v) aceto-HX alone (1–4 mM) has no effect on the viability of the cells; (vi) aceto-HX enhances the killing of both cells induced by H_2O_2 and metmyoglobin, which may be attributed in the case of *B. subtilis* to the formation of HNO and to further oxidation of HNO to NO in the case of *E. coli*; (vii) the synergistic activity of aceto-HX on the killing of both cells induced by H_2O_2 alone does not involve reactive nitrogen species. The effect of aceto-HX on prokaryotes under oxidative stress is opposite to that of other hydroxamic acids on mammalian cells.

Introduction

Hydroxamic acids (RC(O)NHOH, HXs) are important compounds used in the clinic [1,2]. Their therapeutic activities may be explained by their ability to bind metal ions, thus treating metal poisoning [3,4], and inhibiting metallo-enzymes as in the treatment of cutaneous T cell lymphoma by suberoylanilide hydroxamic acid (SAHA, Vorinostat) [5,6]. In addition, their physiological effects are attributed to their capacity to generate nitric oxide (NO) and/or its reduced form HNO (nitroxyl, azanone) [7–12]. NO and HNO play diverse roles in physiological and pathophysiological processes [13–16]. The distinction between their effects is difficult due to the redox exchange between the two congeners and by the Janus-faced behavior of NO and HNO [17–24]. Opposing effects of NO have been observed in nearly every area of its research, which were mostly ascribed to differences in its tissue level, or rates and duration of its formation [21,23,25].

Recently, we have demonstrated that oxidation of acetohydroxamic acid (aceto-HX) by radiolytically borne radicals and by the metmyoglobin ($MbFe^{III}$) and H_2O_2 reactions system initially gives rise to HNO, which in the latter system is partially oxidized to NO by compound II ($MbFe^{IV}$) [11,12]. Hence, aceto-HX might be considered as a NO donor if HNO oxidation to NO is more efficient than its reaction with other biological targets such as thiols and metallo-proteins [16].

The present work examined the ability of aceto-HX to serve as an HNO donor or an NO donor on prokaryotes under oxidative stress by comparing its effects to those of authentic NO and commonly used NO and HNO donors. The effect of HNO on prokaryotes subjected to oxidative stress has never been studied. The only reported work is on mammalian cells (MCF-7) where the HNO donor Angeli's salt and H_2O_2 displayed synergistic cytotoxic effects [26]. NO itself demonstrates opposite effects on cells subjected to oxidative stress [19,22,27–39]. NO predominately protects

Abbreviations: aceto-HX, acetohydroxamic acid; AS, 3-amino-1,2,4-triazole, Angeli's salt; ATZ, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonate, $ABTS^{2-}$; DTNB, 5-5′-dithio-bis-(2-nitrobenzoic acid); BSA, bovine serum albumin; GSH, glutathione; $MbFe^{IV}$=O, ferryl myoglobin; HX, hydroxamic acid; Tempol, 4-hydroxy-2,2,6,6-tetramethylpiperidin-1-oxyl; $MbFe^{III}$, metmyoglobin; PB, phosphate buffer; PBS, phosphate buffer saline; SNAP, *S*-nitroso-*N*- acetylpenicillamine; GSNO, *S*-nitroso-GSH; SNP, sodium nitroprusside; SAHA, suberoylanilide hydroxamic acid; t-BuOOH, *tert*-butyl-hydroperoxide.

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eukaryotes from H_2O_2 and alkyl peroxide [22,27,30,33–35], protects *Bacillus subtilis* and *Neisseria meningitides* against H_2O_2 cytotoxicity [36,37], but enhances the killing of *Escherichia coli* [28,29,32,37,40–42]. Moreover, NO protected *Staphylococcus aureus* exposed to 370 mM H_2O_2 [36], but enhanced the killing when the cells were exposed to 10 mM H_2O_2 [43]. Here, we studied the effects of NO, *S*-nitrosothiols and Angeli's salt on H_2O_2-induced killing of *B. subtilis* and *E. coli* and compared their effects to those of aceto-HX on cells exposed to H_2O_2 and $MbFe^{III}$.

Materials and methods

Chemicals

Aceto-HX, glutathione (GSH), *N*-acetylpenicillamine, cysteine, bovine serum albumin (BSA), myoglobin from horse heart, 3-amino-1,2,4-triazole (ATZ), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonate) ($ABTS^{2-}$), bovine serum albumin (BSA), 5-5′-dithio-bis-(2-nitrobenzoic acid) (DTNB), sodium nitroprusside (SNP), 4-hydroxy-2,2,6,6-tetramethylpiperidin-1-oxyl (Tempol), *tert*-butyl-hydroperoxide (t-BuOOH), and Griess reagent were purchased from Sigma-Aldrich (St. Louis, MO, USA). Catalase was purchased from Boehringer Biochemicals. Sephadex G-25 for gel-filtration chromatography was purchased from Pharmacia (Uppsala, Sweden). $MbFe^{III}$ was prepared by adding an excess of ferricyanide to myoglobin in 5-50 mM phosphate buffer (PB) at pH 7 followed by chromatographic separation through a Sephadex G-25 column. The concentrations of $MbFe^{III}$ were determined spectrophotometrically using $\varepsilon_{408}=188\ mM^{-1}\ cm^{-1}$ [44]. Angeli's salt (AS) was purchased from Cayman Chemicals Co. Stock solutions of AS were prepared in 10 mM NaOH and the concentration was determined by the absorbance at 248 nm ($\varepsilon=8300\ M^{-1}\ cm^{-1}$) [45]. NO was purchased from Matheson Gas Products and was purified by passing the gas through a series of traps containing deaerated 50% w/v NaOH and purified water in this order. Stock solutions of NO solutions were prepared in gas tight syringes containing 10 mM PB, pH 6.8, and the concentration of NO was determined immediately before use employing a spectroscopic assay with $ABTS^{2-}$ as a reductant ($\varepsilon_{660}=12000\ M^{-1}\ cm^{-1}$ and 60% yield [46]). *S*-Nitrosothiols were prepared daily by mixing equimolar concentrations of the thiol with HNO_2 in 0.1 N H_2SO_4 stored in an ice bath. The concentration of *S*-nitroso-GSH (GSNO) was determined spectrophotometrically at 336 nm ($\varepsilon_{336}=770\ M^{-1}\ cm^{-1}$) and that of *S*-nitroso-*N*-acetylpenicillamine (SNAP) at 340 nm ($\varepsilon_{340}=815\ M^{-1}\ cm^{-1}$) [47]. Visible light was used to release NO from GSNO or SNAP [48–50], and the rate of its release was evaluated by determining the accumulation rate of nitrite. Nitrite concentration was assayed with the Griess reagent. The absorption at 540 nm was read 15 min after mixing the sample with the reagent. Calibration curves were prepared using known concentrations of nitrite. The concentrations of H_2O_2 and t-BuOOH were assayed iodometrically at 352 nm ($\varepsilon=25{,}800\ M^{-1}\ cm^{-1}$) [51]. In view of the relatively slow oxidation of iodide by t-BuOOH, the buildup of I_3^- was followed at 352 nm until a plateau value was reached.

Cell cultures

B. subtilis PY79 and *E. coli* 25922 were cultured aerobically in Luria-Bertani (LB) medium adjusted to pH 7 by 40 mM PB in a vigorously shaking incubator at 37 °C. Cells were diluted 1:100 in fresh LB and grown aerated at 37 °C until $OD_{660}\sim 0.5$. In some experiments the cells were diluted in LB or 1:100 in saline (0.9% NaCl) or phosphate buffered saline (PBS, 40 mM PB, 0.65% NaCl) to the desired cell concentration and challenged with various substrates. Cells cultures were sampled at various time points, diluted in sterile water containing 60 U/mL catalase to remove residual H_2O_2, plated in triplicates on LB agar, and incubated overnight at 37 °C (*E. coli*) or 30 °C (*B. subtilis*) for clonogenic assay. All experiments were repeated at least 3 times and each survival curve represents a typical experiment.

Analysis of thiols in LB medium

HNO readily reacts with thiols [16], and therefore it is essential to determine the potential contamination of LB medium with thiols. No traces of thiols were detected using Ellman's reagent (DTNB) [52,53] in LB medium whereas thiols were readily detectable when the LB medium was deliberately contaminated with 10 μM cysteine or 0.5 mM BSA. We also examined any accumulation of nitrite in the LB medium containing 4 mM SNP in the dark, which is extremely sensitive to the presence of thiols [54,55]. Nitrite was not accumulated unless we deliberately contaminated the LB medium with GSH or cysteine.

Results

Effects of NO and HNO on bacterial cells subjected to oxidative stress

NO demonstrates opposing effects on *E. coli* and *B. subtilis* exposed to peroxides [28,29,32,37,40–42] whereas the effect of HNO on prokaryotes has not been studied. These bacterial cells were selected as model systems for studying the ability of aceto-HX to serve as an HNO donor or an NO donor by performing comparative studies utilizing authentic NO, GSNO, SNAP, and Angeli's salt.

B. subtilis

A previous study of the effect of NO on *B. subtilis* subjected to oxidative stress involved high cell concentrations ($OD_{660}=0.5$) treated with 10 mM H_2O_2 [36]. Under such conditions the cells were protected from oxidative stress only when exposed to NO shortly before the addition of H_2O_2 [36]. We show that at such high cell concentrations the oxidant is diminished to subtoxic levels within less than 4 min, thus terminating the oxidative stress (Fig. 1).

When the cells were grown in the presence of 10 mM ATZ, which irreversibly inhibits catalase [56], the depletion of H_2O_2 was slowed down prolonging the time window of the oxidative damage, thereby increasing the duration and rate of cell killing

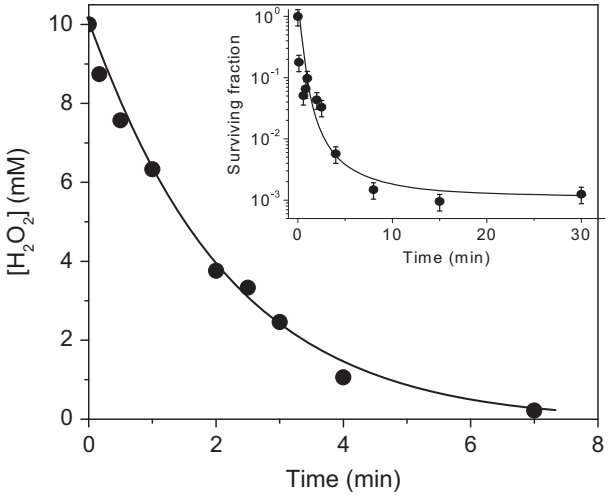

Fig. 1. Effect of high cell concentrations on the depletion of H_2O_2. Cultures of exponentially growing *B. subtilis* ($OD_{660}\sim 0.5$, 7×10^7 cells/mL) were challenged with 10 mM H_2O_2 in LB at 37 °C, and samples were taken for assaying residual H_2O_2 and for clonogenic assay (inset).