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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

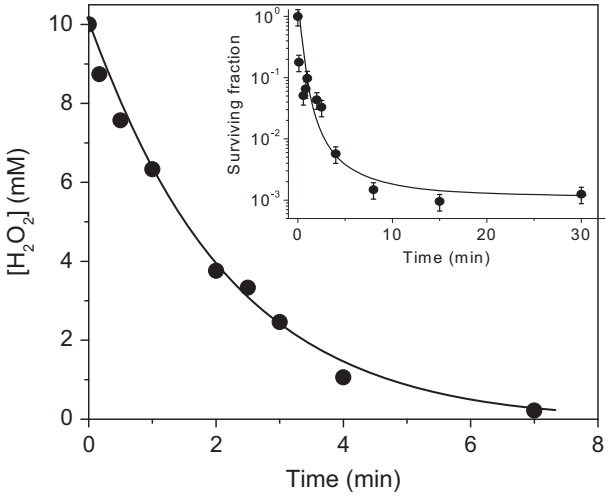

Fig. 1. Effect of high cell concentrations on the depletion of H_2O_2. Cultures of exponentially growing *B. subtilis* ($OD_{660}\sim 0.5$, 7×10^7 cells/mL) were challenged with 10 mM H_2O_2 in LB at 37 °C, and samples were taken for assaying residual H_2O_2 and for clonogenic assay (inset).

as compared to cells exposed to the same initial concentration of H_2O_2 but in the absence of ATZ (Fig. 2).

The implications are that in cell survival experiments involving high cell concentrations there is an exceedingly short time window in which the cells are actually exposed to oxidative stress. Therefore, we carried out our experiments using sufficiently low cell concentrations, avoiding significant oxidant depletion.

Exposure of the cells to NO alone caused a growth delay, but did not induce cell killing in agreement with previous reports [36,37]. The protective effect of NO was independent of the order of the exposure of the cells to NO and H_2O_2 as demonstrated in Fig. 3.

The lack of effect of the order of the addition of NO and the oxidant is also demonstrated when H_2O_2 was replaced by t-BuOOH, which was only slightly depleted even in the presence of high cell concentrations (Fig. 4).

When bolus addition of NO was replaced by GSNO or SNAP, which continuously and slowly release NO under visible illumination [48–50], both NO donors protected the cells (Fig. 5) independent of the order of their addition (Fig. 6). Photolysis of the cells exposed to GSNO or SNAP alone did not induce cell killing, indicating that any intermediates or end-products formed during the photolysis of these nitrosothiols are not toxic.

The HNO donor AS alone (0.05–1 mM) caused cell growth delay, but did not induce any cell killing in LB medium, in saline or in PBS. In the presence of H_2O_2 it synergistically potentiated the cell killing in LB medium (Fig. 7) as well as in saline or in PBS (data not shown).

It should be noted that despite systematic attempts to rigorously apply similar oxidative stress by a careful control of experimental conditions, the extent of the cell killing greatly varied between apparently similar experiments. In most experiments NO protected *B. subtilis* against extensive killing predominantly induced by high concentrations of H_2O_2. In some cases where the cells suffered only minor viability loss, NO demonstrated no protection or even potentiated cell killing. The poor reproducibility of H_2O_2-induced cell killing precluded drawing any clear correlation.

E. coli

E. coli cells deplete H_2O_2 at lower rates compared to those induced by *B. subtilis*. For instance, more than 75% oxidant is left when H_2O_2 at 4.4 mM is incubated with *E. coli* culture at ca. 10^7 cells/mL over 60 min. The HNO donor AS (0.05–1 mM) did not induce cell killing whether given alone or in combination with H_2O_2 in LB medium, in saline, or in PBS. Conversely, NO either added as a bolus or released by GSNO or SNAP enhanced the cell

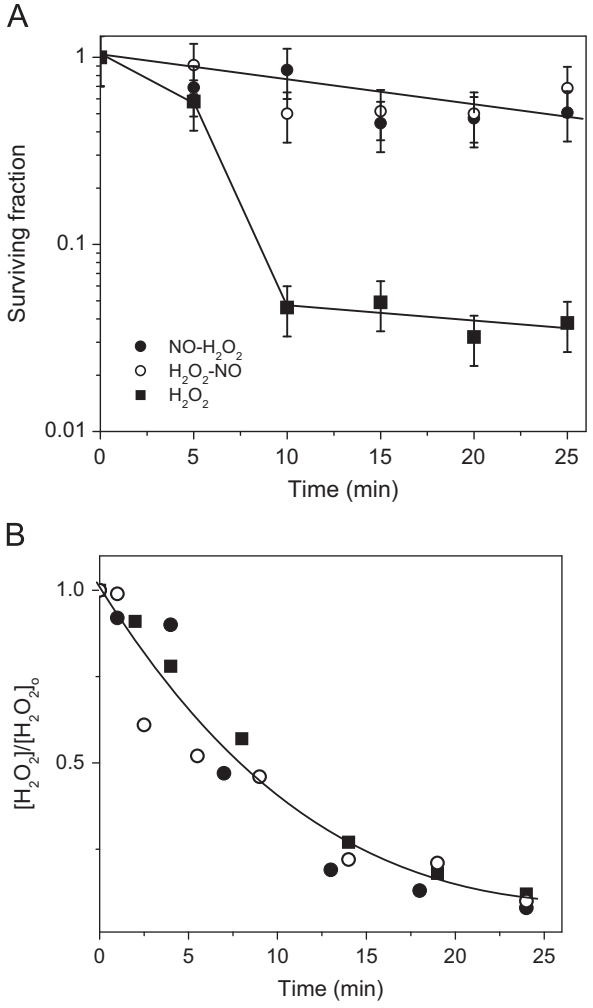

Fig. 3. Effect of NO on survival of *B. subtilis* at low concentrations exposed to H_2O_2 (A) and on H_2O_2 depletion (B). Cultures of exponentially growing *B. subtilis* (OD_{660} ~ 0.03, 6×10^6 cells/mL) in LB at 37 °C were challenged with 4 mM H_2O_2 alone (■) or with 90 μM NO added 10 s before (●) or after (○) the challenge with the oxidant.

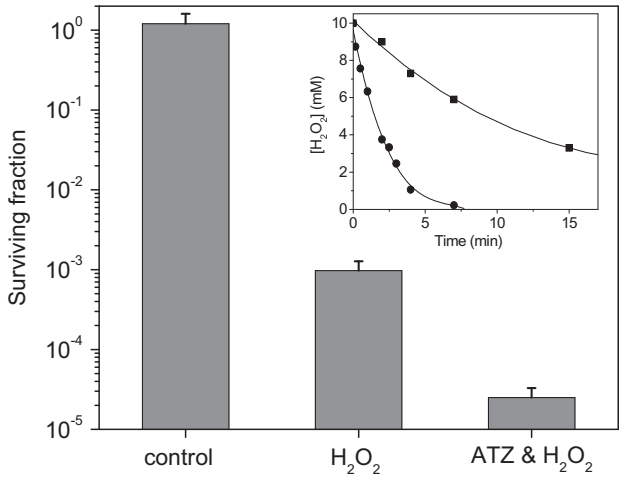

Fig. 2. Effect of ATZ on survival of *B. subtilis* exposed to H_2O_2 and on oxidant depletion. Cultures of exponentially growing *B. subtilis* (OD_{660} ~ 0.5, 7×10^7 cells/mL) in LB at 37 °C were challenged for 15 min with 10 mM H_2O_2 alone and in the presence of 10 mM ATZ. The inset of the figure shows the kinetics of H_2O_2 depletion in the absence (circle) and presence of ATZ (square).

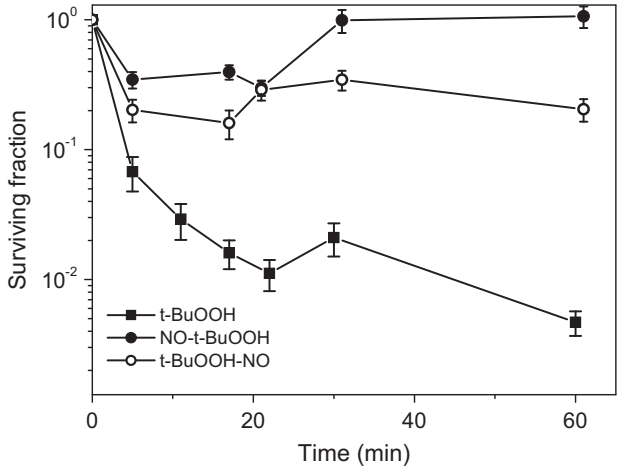

Fig. 4. Effect of NO on survival of *B. subtilis* exposed to t-BuOOH. Cultures of exponentially growing *B. subtilis* (OD_{660} ~ 0.5, 5×10^7 cells/mL) in LB at 37 °C were challenged with 1.5 mM t-BuOOH alone (■) or with 60 μM NO added 10 s before (●) or after (○) the challenge with the oxidant. The consumption of the peroxide within 60 min was less than 30% in the absence and presence of NO.

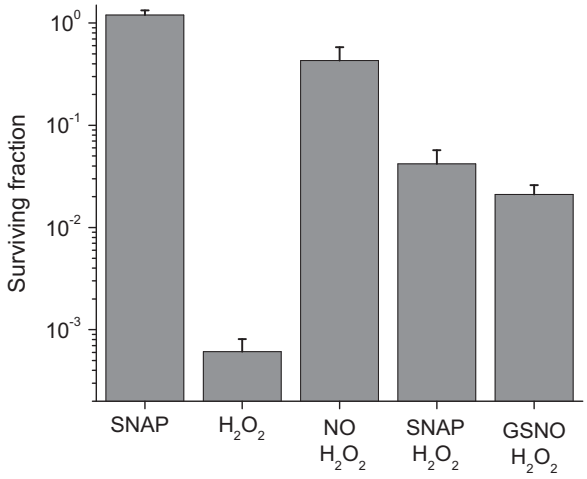

Fig. 5. Effect of NO and NO donors on survival of *B. subtilis* exposed to H_2O_2. Cultures of exponentially growing *B. subtilis* (OD_{660} ~ 0.013, 3.5×10^6 cells/mL) in LB at 37 °C were challenged for 20 min with 10 mM H_2O_2 alone or in combination with NO, either adding 40 μM NO 10 s before H_2O_2 or illuminating with visible light 0.79 mM SNAP and 0.78 mM GSNO starting 10 s before H_2O_2 addition. The rates of nitrite accumulation during the experiment were 0.38 ± 0.05 and 0.34 ± 0.03 μM/min for GSNO and SNAP, respectively.

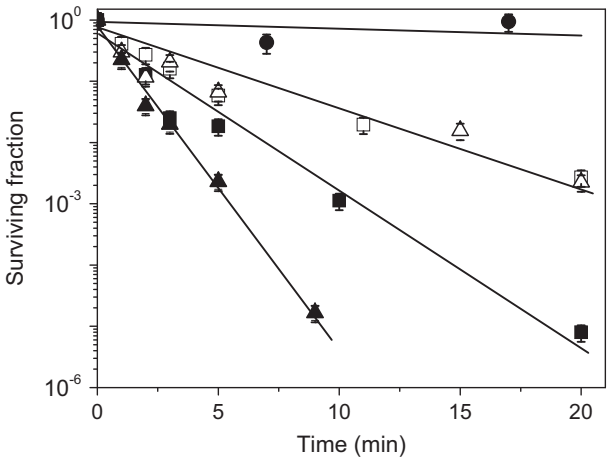

Fig. 7. Synergic pro-oxidative effect of AS on *B. subtilis* subjected to oxidative stress. Cultures of exponentially growing *B. subtilis* (OD_{660} ~ 0.012, 6×10^6 cells/mL) in LB at 37 °C were challenged with 10 mM H_2O_2 (□), 15 mM H_2O_2 (△), 0.2 mM AS (●), 10 mM H_2O_2 and 0.2 mM AS (■), and 15 mM H_2O_2 and 0.2 mM AS (▲). AS was added 10 s before H_2O_2 addition. The rate of the decomposition 0.2 mM AS in 40 mM phosphate buffer at pH 7.4 and 37 °C was determined spectrophotometrically at 240 nm to be $(1.9 \pm 0.1) \times 10^{-3}$ s^{-1} (i.e., half-life ca. 6 min).

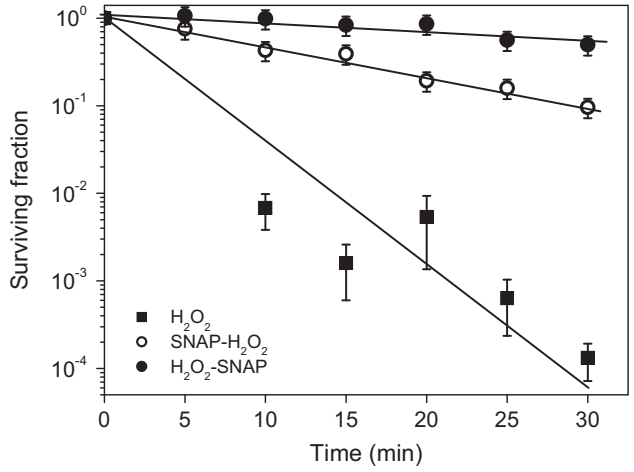

Fig. 6. SNAP protects *B. subtilis* from H_2O_2 independent of the order of their delivery. Cultures of exponentially growing *B. subtilis* (OD_{660} ~ 0.01, 1.5×10^6 cells/mL) in LB at 37 °C were challenged with 4 mM H_2O_2 alone (■) or in combination with 1 mM SNAP illuminated with visible light 20 s before (○) and after (●) H_2O_2 addition.

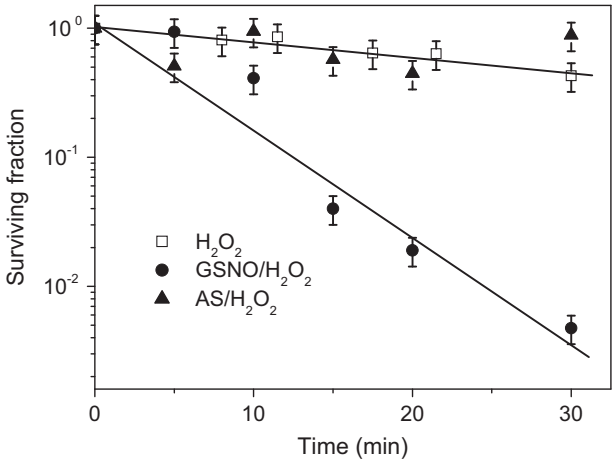

Fig. 8. Effects of GSNO and AS on *E. coli* subjected to oxidative stress. Cultures of exponentially growing *E. coli* (OD_{660} ~ 0.01, 3×10^6 cells/mL) in LB at 37 °C were challenged with 4 mM H_2O_2 alone (□) and in combination with either 0.1 mM AS added 10 s before H_2O_2 addition (▲) or 1 mM GSNO illuminated with visible light starting 10 s before H_2O_2 addition (●). The rate of nitrite accumulation induced by GSNO was 0.63 ± 0.05 μM/min. The rate of AS decomposition was determined to be $(1.9 \pm 0.1) \times 10^{-3}$ s^{-1} (i.e., half-life ca. 6 min) by following the decay of 0.2 mM AS at 240 nm in 40 mM phosphate buffer at pH 7.4 and 37 °C.

killing induced by H_2O_2 (Fig. 8) as previously reported for NON-Oates [28,29,32,37,40–42]. Photolysis of either GSNO or SNAP alone caused a growth delay, but did not induce cell killing, indicating that no intermediate or product formed during the photolysis of these *S*-nitrosothiols is toxic to the cells.

Effect of aceto-HX on bacterial cells subjected to oxidative stress

Aceto-HX alone (0.5–4 mM) did not induce cell killing in both cells, but potentiated cell killing induced by H_2O_2 and $MbFe^{III}$ in a dose-dependent manner (Figs. 9 and 10).

Nitrite accumulates in these cell cultures, and its initial rate of accumulation increases on increasing [$MbFe^{III}$] or [aceto-HX], and is hardly affected by [H_2O_2]=2–10 mM, e.g., 0.89 ± 0.08 μM/min in cell culture containing 4 mM H_2O_2, 5 μM $MbFe^{III}$, and 1 mM aceto-HX. Aceto-HX also potentiates the cell killing induced by H_2O_2 alone (Figs. 9 and 10), but in this case nitrite is not accumulated. This synergic effect might involve the metal-chelating properties of hydroxamic acids since Tempol, which has been previously demonstrated to protect from metal-mediated oxidative stress [57,58], inhibited the H_2O_2-induced cell killing (Fig. 11).

Discussion

Oxidation of aceto-HX by radiolytically borne radicals and by the $MbFe^{III}/H_2O_2$ reactions system initially gives rise to HNO, which in the latter system is partially oxidized to NO [11,12]. Aceto-HX, which is a HNO donor, might be considered as a NO donor if HNO conversion into NO is more efficient than its reactions with other biological targets such as thiols and metallo-proteins [16]. While NO effects have been primarily studied using *E. coli* and *B. subtilis*, those of HNO have not been studied on bacterial cells. A previous publication reported a dramatic protective effect of NO added shortly before the exposure of high concentrations of *B. subtilis* to 10 mM H_2O_2, and concluded that this phenomenon is general for

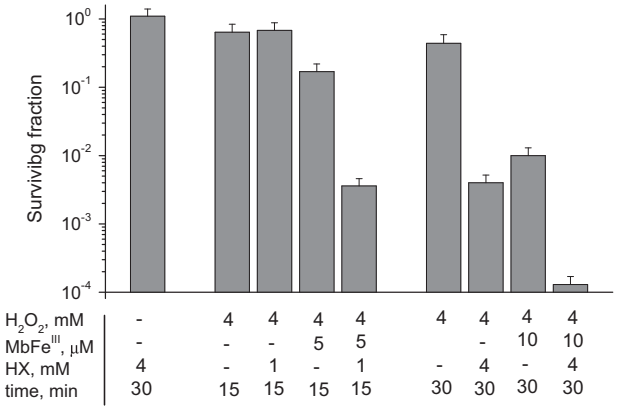

Fig. 9. Synergic pro-oxidative effect of aceto-HX on *E. coli* subjected to oxidative stress. Cultures of exponentially growing *E. coli* (OD_{660}=0.013, 2×10^7 cells/mL) in LB at 37 °C were challenged with mixtures of H_2O_2, $MbFe^{III}$, and aceto-HX for 15 or 30 min as indicated below the columns. The exposure time was measured once H_2O_2 was added to the reaction mixture.

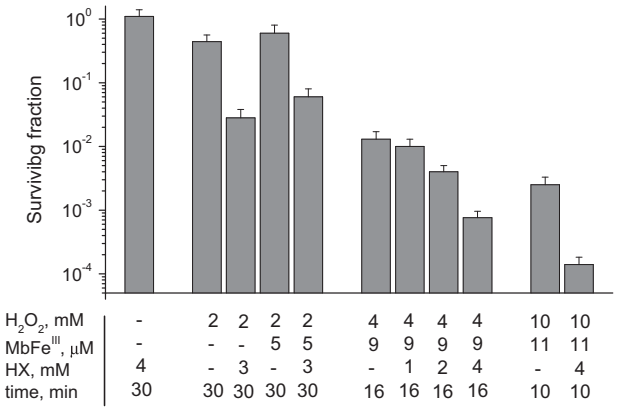

Fig. 10. Synergic pro-oxidative effect of aceto-HX on *B. subtilis* subjected to oxidative stress. Cultures of exponentially growing *B. subtilis* (OD_{660} ~ 0.012, 5×10^6 cells/mL) in LB at 37 °C were challenged with mixtures of H_2O_2, $MbFe^{III}$, and aceto-HX for different periods of time as indicated below the columns. The exposure time was measured once H_2O_2 was added to the reaction mixture.

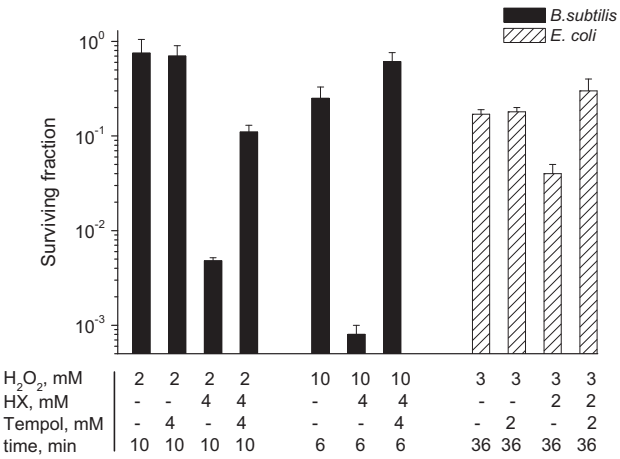

Fig. 11. Effect of Tempol on the synergic pro-oxidative effect of aceto-HX on cells exposed to H_2O_2. Cultures of exponentially growing cells (~ 5×10^6 cells/mL) in LB at 37 °C were challenged with mixtures of H_2O_2, aceto-HX, and Tempol for different periods of time as indicated below the columns.

prokaryotes [35]. However, this report has overlooked previous studies demonstrating the synergic effect of NO on *E. coli* [28,29,32,37,40–42]. The present work examined the effect of NO, NO donors, HNO donor, and aceto-HX on *E. coli* and *B. subtilis* subjected to oxidative stress to determine whether aceto-HX acts as an HNO donor or an NO donor.

Our results indicate that in the absence of oxidative stress neither NO nor HNO induce bactericidal effects. These observations imply that nitrite, which is the product of NO reaction with O_2 and of AS decomposition, is not involved in killing of prokaryotes. Also under oxidative stress AS does not potentiate killing of *E. coli*, indicating that nitrite is not toxic also in the presence of H_2O_2. This is also the case with *B. subtilis* where it has been shown that nitrite has no effect on the cells exposed to H_2O_2 [36,37]. There is a clear distinction between the effects of NO and HNO on H_2O_2-induced killing of *E. coli* where only NO, but not HNO, demonstrates a synergistic pro-oxidative effect. NO greatly inhibits extensive killing of *B. subtilis* by H_2O_2 while HNO demonstrates a synergistic pro-oxidative effect. The dependence of NO effect on the extent of cell injury, which was observed for *S. aureus* cells exposed to 370 mM H_2O_2 [36] vs 10 mM H_2O_2 [43], is intriguing and deserves further elucidation.

Aceto-HX (pK_a=9 [11,59]) is cell permeable and an efficient metal chelator [60]. Since it enhances H_2O_2-induced cell killing, but does not modify cell viability in the absence of oxidative stress, its synergic pro-oxidative activity should not be attributed to inhibition of essential metallo-enzymes. Instead, the synergic effect of aceto-HX on the killing of cells exposed to H_2O_2/$MbFe^{III}$ might be ascribed to the formation of reactive nitrogen species. In this case aceto-HX undergoes a one-electron oxidation to yield initially the respective transient nitroxide radical [11,12].

$$CH_3C(O)NHOH \underset{-H^+}{\overset{-e^-}{\rightleftharpoons}} H_2O + CH_3C(O)NHO^{\bullet} \quad (1)$$

Our recent work has demonstrated that the transient nitroxide radical generates HNO through the hydrolysis of $CH_3C(O)N{=}O$ formed via the dismutation of the nitroxide radical or its oxidation by compound II ($MbFe^{IV}$) [12].

The results of the current study show that AS exhibits a synergistic pro-oxidative effect when added to *B. subtilis* cells exposed to H_2O_2 similar to the effect of aceto-HX on the cell killing induced by H_2O_2/$MbFe^{III}$, indicating that in this case aceto-HX acts as a HNO donor. Testing this conclusion using thiols as HNO scavengers [16] is impossible because thiols are readily oxidized by compounds I and II [61]; i.e., elimination of the synergistic activity of aceto-HX would result from the competition of thiols with aceto-HX for the oxidizing species rather than from HNO scavenging. The use of cyclic nitroxides or other one-electron oxidants as HNO scavengers is further complicated by oxidation of HNO to NO [62,63]. In the case of *E. coli*, aceto-HX demonstrates a synergistic pro-oxidative effect, thus resembling the effect of NO donors. A substantiation for this conclusion using specific NO scavengers, e.g., nitronyl nitroxides, is complicated since these nitroxides also oxidize HNO to NO [64]. Whether aceto-HX acts as an HNO donor or an NO donor is determined by the competition between HNO oxidation to NO and its reaction with thiols and heme proteins [16], which could depend on the type of the bacteria.

The synergic pro-oxidative effect of aceto-HX on cell killing induced by H_2O_2 alone cannot be ascribed to the formation of reactive nitrogen species; i.e., H_2O_2 does not oxidize directly the hydroxamate moiety and in this system nitrite is not accumulated. The mechanism underlying the pro-oxidative activity of aceto-HX is not fully understood. Aceto-HX is an efficient metal chelator, which does not necessarily imply cytoprotection from metal-catalyzed oxidative stress. Metal chelators, which do not render transition metal redox inactive, but rather increase their solubility and availability for binding to critical cellular targets, actually potentiate biological injury. Since aceto-HX enhanced H_2O_2-induced cell killing, we assume that it acts by releasing redox-inactive metals from cellular stores, rending them redox active, thus enhancing bactericidal effects via the Fenton-type

reaction. Tempol has several biological effects including oxidation of reduced metals thus preemptying the Fenton-like reaction [57,58]. Our results demonstrate that Tempol inhibit the potentiation by aceto-HX of H_2O_2-induced cell killing (Fig. 11), and this protective effect supports our assumption. The full elucidation of this pro-oxidative synergic activity is beyond the scope of this study and is currently under investigation.

Aceto-HX synergistically enhances the bactericidal effect of H_2O_2 with and without $MbFe^{III}$ through more than a single mechanism. Such a synergy is intriguing since SAHA and its structural analog Trichostatin A provided protection against H_2O_2-induced killing of mammalian cells [10].

Conclusions

The results obtained from the present study can be summarized as follows: (i) the effects of NO and HNO on prokaryotes under oxidative stress depend on the type of the bacterial cell; (ii) NO synergistically enhances H_2O_2-induced killing of *E. coli*, and protect *B. subtilis* depending on the extent of cell killing by the oxidant; (iii) HNO has no effect on the viability of the cells; (iv) HNO does not modify the killing of *E. coli* subjected to oxidative stress, but synergistically enhances H_2O_2-induced killing of *B. subtilis*; (v) aceto-HX alone has no effect on the viability of both cells; (vi) aceto-HX demonstrates synergic pro-oxidative effects on both cells exposed to H_2O_2 and $MbFe^{III}$, 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) aceto-HX-induced synergy in the presence of H_2O_2 alone does not involve reactive nitrogen species.

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