N-t-Butyl Hydroxylamine, a Hydrolysis Product of \( \alpha \)-Phenyl-N-t-butyl Nitrone, Is More Potent in Delaying Senescence in Human Lung Fibroblasts*

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\( \alpha \)-Phenyl-N-t-butyl nitrone (PBN), a spin trap, scavenges hydroxyl radicals, protects tissues from oxidative injury, and delays senescence of both normal human lung fibroblasts (IMR90) and senescence-accelerated mice. N-t-butyl hydroxylamine and benzaldehyde are the breakdown products of PBN. N-t-Butyl hydroxylamine delays senescence of IMR90 cells at concentrations as low as 10 \( \mu \)M compared with 200 \( \mu \)M PBN to produce a similar effect, suggesting that N-t-butyl hydroxylamine is the active form of PBN. N-Benzyl hydroxylamine and N-methyl hydroxylamine compounds unrelated to PBN were also effective in delaying senescence, suggesting the active functional group is the N-hydroxylamine. All the N-hydroxylamines tested significantly decreased the endogenous production of oxidants, as measured by the oxidation of 2,7′-dichlorodihydrofluorescein and the increase in the GSH/GSSG ratio. The acceleration of senescence induced by hydrogen peroxide is reversed by the N-hydroxylamines. DNA damage, as determined by the level of apurinic/apyrimidinic sites, also decreased significantly following treatment with N-hydroxylamines. The N-hydroxylamines appear to be effective through mitochondria; they delay age-dependent changes in mitochondria as measured by accumulation of rhodamine-123, they prevent reduction of cytochrome \( \text{CFe}^{3+} \) by superoxide radical, and they reverse an age-dependent decay of mitochondrial aconitase, suggesting they react with the superoxide radical.

\[ \text{N-t-Butyl hydroxylamine} \]

\( \alpha \)-Phenyl-N-t-butyl nitrone (PBN)\( ^3 \) is one of the most widely used spin-trapping agents for investigating the existence of free radicals in biological systems. PBN reverses the age-related oxidative changes in the brains of old gerbils (1, 2) and delays senescence in senescence-accelerated mice (3) and in normal mice (4). PBN also delays senescence in the normal human lung fibroblast cell line IMR90 (5). In addition, PBN reverses mitochondrial decay in the liver of old rats (6) and exerts a neuroprotective effect in gerbils (1, 7) and rats (8, 9) after oxidative damage from ischemia/reperfusion injury. The mechanism underlying the biological activity of PBN is still controversial. However, PBN is a well-known scavenger of radical species, although a variety of other well-known spin traps or antioxidants do not mimic its anti-senescent activity in IMR90.\( ^2 \) PBN at relatively high concentrations reduces the production of hydrogen peroxide in mitochondrial preparations of cerebral cortex (10) and therefore may exert similar properties \( \text{in vivo} \). This suggests that PBN possesses special properties that do not exist in other spin traps or antioxidants.

In the course of our study of the effect of PBN on IMR90 cells, we observed that old solutions were more effective than fresh solutions in delaying senescence of IMR90 cells. This raised the question about the interaction of the decomposition products of PBN with IMR90 cells. This encouraged us to test the anti-senescent effect of the PBN decomposition products, N-t-butyl hydroxylamine and benzaldehyde (Scheme 1) on IMR90 cells. PBN (or PBN/OH) has been reported to decompose to N-t-butyl hydroxylamine or N-t-butyl hydronitroxide and benzaldehyde (11–13). PBN as purchased often contains N-t-butyl hydroxylamine (14). Benzaldehyde is both mutagenic (15) and carcinogenic (16). N-t-Butyl hydroxylamine is a primary hydroxylamine that can be oxidized under certain conditions (such as with UV or Fe\(^{3+}\)) to N-t-butylaminoxy (also referred as N-t-butyl hydronitroxide (10–12). N-t-Butylaminoxy and the corresponding N-hydroxylamine (Scheme 1) are primary amines and are, thus, different from the well-known cyclic nitroxides/cyclic hydroxylamines (see references herein). The antioxidant and protective features of some cyclic nitroxides/cyclic hydroxylamines are known. Probably the most important feature in this regard is their ability to catalyze superoxide radical dismutation to form \( \text{H}_2\text{O}_2 \) (17–21). In vitro cyclic nitroxides can either be oxidized to o xo-ammonium cation or reduced to the corresponding hydroxylamine by superoxide radical, depending on the type of cyclic nitroxide. Thus cyclic hydroxylamine or the corresponding oxo-ammonium cation are intermediates during the dismutation of superoxide radical by nitroxide. Interestingly, the oxo-ammonium cation species is reduced to the corresponding cyclic hydroxylamine by the cellular reductant NADH, which suggests that cyclic hydroxylamine can be the dominant form inside the cells. In addition the cyclic nitroxide species can undergo oneelectron reduction to the corresponding cyclic hydroxylamine, a reaction proposed to be mediated by

\( \text{H. Atamma, A. Paler-Martinez, and B. N. Ames, unpublished observation.} \)
mitochondrial coenzyme Q and ascorbic acid (21–23). Mitochondrial cytochrome c oxidase can also oxidize the cyclic hydroxylamine to the corresponding nitroxide (24). Thus, it appears that mitochondria can contribute to the cycling of cyclic nitroxides/cyclic hydroxylamines, which in turn can facilitate dismutation of superoxide radical to H₂O₂. The N-t-butyl hydroxylamine and the other N-hydroxylamines tested in this study are primary N-hydroxylamines that have not been previously examined as antioxidants, although in comparison with the cyclic hydroxylamines described above, the effect of PBN on life span was tested as described by Atamna and Ames. Briefly, IMR90 cells were grown in 100-mm Corning tissue culture dishes containing 10 ml of Dulbecco’s modified Eagle’s medium supplemented with 10% (V/V) fetal bovine serum (Hyclone). Stock cultures were each split into two additional groups and then either 1) treated with 20 or 30 μM H₂O₂ or 2) left untreated, with H₂O₂.

### EXPERIMENTAL PROCEDURES

#### Materials—N-t-Butyl hydroxylamine, N-benzyl hydroxylamine, N-methyl hydroxylamine (and the corresponding O-hydroxylamines), nitroso-tert-butane, 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO), 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl (4-OH-TEMPO), 3-carbamoyl2,5,5-tetramethylpyrroldin-1-oxyl (3-CP), octanesulfonic acid, and methanesulfonic acid were purchased from Aldrich. Cytochrome c, Rho123 and xanthine, NADP, fluorocitrate, isocitrate dehydrogenase, and PBN were from Sigma. Xanthine oxidase was from Roche Molecular Biochemicals. Aldehyde-reactive probe was from Dojindo (Kumamoto, Japan). DCFP was from Molecular Probes (Eugene, OR). N,N-bis(3,3’-dimethylamino) propylamine)-3,4,9,10-perylene-3-carboxamyl-tetracarboxamide-biocryl-diimide (DAPER) was from Pierce. The ABC kit was from Vector labs (Burlington, CA). The DNA isolation QIAamp kit was from QIagen (Valencia, CA).

#### Oxidants and Mitochondrial Membrane Potential in IMR90 Cells by FACs—IMR90 cells were trypsinized and resuspended in complete Dulbecco’s modified Eagle’s medium. For each condition, two tubes were prepared with 1 × 10⁶ cells each. Tubes were then spun at 250 × g for 10 min at room temperature, and supernatant was replaced with 1 ml of Hank’s balanced salt solution without Ca²⁺ or Mg²⁺. Rho123 (20 μM of 525 μM stock; 10.5 μM final concentration) was added to one tube, and DCFH (20 μM of 1.25 mM stock; 25 μM final concentration) was added to the other tube. Cells were then incubated in the dark in a water bath at 37 °C for 30 min followed by cell resuspension and centrifugation at 250 × g for 10 min at room temperature. The supernatant (500 μl) was removed from each tube, and the cells were resuspended in the remaining 500 μl before FACs analysis on a FACSort analyzer (Becton Dickinson, San Jose, CA). Cell Quest was used for data acquisition and analysis. The data are reported as the mean of the channel of the fluorescence histogram obtained. Fluorescence output was calibrated with LinearFlow Green Flow cytometry intensity calibration particles. (Molecular Probes, Eugene, OR).
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manufacturer but with avidin-HRP concentrations diluted 1:3 and the incubation volumes scaled down to 1 ml. The DNA-avidin-HRP complex (DNA-HRP) was separated from unbound avidin-HRP by gently mixing 65 μl of 1 mM DAPER with the DNA and incubated at room temperature for 5 min (the mole ratio of DAPER/NTPT should be 66). The DNA-DAPER precipitate was then collected by centrifugation for 5 min at 12,500 × g and washed twice with 1.5 ml of 0.17 mM NaCl, 20 mM Tris, 0.25% Tween 20, 1% bovine serum albumin, pH 8. The precipitate of DNA-HRP was suspended in 500 μl of ice-cold 50 mM sodium citrate, pH 5.3 and sonicated at output 1–2 watts for 5 s (Sonifier Cell Disruptor, model W185D, Branson) and cooled immediately. HRP activity was measured as an indicator of AP sites in DNA-HRP by using the chromogenic ImmunoPure TMB or the fluorogenic QuantaBlu substrate kits.

The background control was established by performing a parallel analysis on calf thymus DNA. The standard curve for AP sites was constructed with 100 ng of DNA containing a known amount of uracil suspended in 50 μl of 10 mM Na2HPO4, pH 7.5. The standard DNA was incubated with 25 μM spermine for 3 min and then with 3 units of uracil-DNA N'-glycosylase for 20 min at 37 °C to catalyze the removal of uracil residues and generate AP sites. The resulting “AP-enriched” DNA was incubated with 3 mM ARP for 45 min at 37 °C. The standard DNA-ARP adducts were isolated from unbound ARP by QIAamp columns (without the protease step) and quantified. The number of AP sites was corrected for the loss of DNA during isolation (10–20% loss). The biotinylated DNA was incubated with avidin-HRP and processed as above.

Reduction of cyt CFeIII by Superoxide Radical—Superoxide radical was generated by the reaction of xanthine (120 μM) with xanthine oxidase (0.06 units). The reaction was performed at 25 °C in a final volume of 1 ml of PBS containing 40 μM cyt CFeIII. The reaction was started by the addition of the substrate xanthine. N-Hydroxylamines were added just before the addition of xanthine. The initial rate of reduction of cyt CFeIII was determined based on the linear change in absorbance at 550 nm.

To test the effect of N-hydroxylamines on the spontaneous oxidation of cytochrome c, a complete reduction of cyt CFeIII was achieved by incubating the xanthine/xanthine oxidase system for 4–5 min at 25 °C. Auto-oxidation of cyt CFeIII is associated with a decrease in absorbance at 550 nm. Reduced cytochrome c was incubated at 25 °C with or without 2 or 3 mM N-hydroxylamines, and the auto-oxidation was followed by spectrophotometer. The rate of reduction of cytochrome c by different concentrations of each N-hydroxylamine was measured by the increase in absorbance at 550 nm.

Measurement of Cellular Levels of GSH and GSSG in IMR90 Cells—Cultivated IMR90 cells (≈3 × 106) were washed once in cold PBS and resuspended in 200 μl of ice-cold methanol-sulfonic acid (0.2 mM methanesulfonic acid, 0.5 mM DTPA) and allowed to stand for 10 min at room temperature. Denatured proteins were removed by centrifugation, and the supernatant was filtered with 30,000 Mf cut-off Ultrafree filters (Millipore) before injection. Fifty microliters were injected and separated on a HPLC column (β-μm 0.46 × 15-cm Supelcoil LC18-DB, Supelco, Bellefonte, PA) with a flow rate of 1 ml/min using a mobile phase consisting of 25 mM NaH2PO4, 5 mM octane sulfonic acid, and 2% acetonitrile adjusted to pH 2.7 with phosphoric acid (27). An ESA model 5100A Coulchom detector, 5020 guard cell, and model 5010 analytical cell combination was used for analysis. Oxidation potentials of 900, 400, and 880 mV were used for guard cell and electrodes 1 and 2, respectively. Full-scale output was 10 μA, and peak areas were compared using commercial GSH and GSSG as standards.

RESULTS

N-t-Butyl hydroxylamines and Other N-Hydroxylamines Delay Senescence of IMR90 Cells—N-t-Butyl hydroxylamine, N-benzyl hydroxylamine, N-methyl hydroxylamine (N-hydroxylamines, Scheme I) at 100 μM (added once per 7 days) delay senescence of IMR90 cells by at least 17–20 PDLs (Fig. 1). The concentration of PBN required to achieve a similar gain in PDLs is eight times higher than N-hydroxylamines (Table I and Ref. 5). The minimal concentration of N-hydroxylamines required to achieve a gain of 5–7 PDLs above the untreated control was 20 times lower than that for PBN (200 μM) to achieve 2–3 PDLs (Table I and Ref. 5). For each of the three N-hydroxylamines, when they were added to IMR90 cells every 3 days at 25 μM, they were twice as efficient as 100 μM every 7 days (data not shown). None of the N-hydroxylamines tested were toxic at the concentration applied to the cells, as measured by PDL, whereas benzaldehyde, the co-product of PBN hydrolysis, was without effect or toxic at high concentrations (data not shown). All the N-hydroxylamines at the concentrations tested were much more effective than PBN in delaying senescence. The N-hydroxylamines studied appear to be equally efficient in delaying senescence, with a variation only when cells were close to senescence (late PDLs, Fig. 1A). We have developed a new HPLC-electrochemical detection method for N-hydroxylamines. We find, using this method, that N-t-buty1 hydroxylamine rapidly enters cells and reaches saturating concentrations after 1–2 min (55–65 nmol/mg of protein (12 × 106 cells)), which is approximately 5-fold the extracellular concentration, 1 μmol (3).

A simultaneous treatment of the cells with all three of the N-hydroxylamines (30 μM each) yielded results similar to single treatments, delaying senescence by 14–17 PDLs. In contrast, at concentrations equivalent to the N-hydroxylamines, the isomeric O-hydroxylamines were either without effect (O-t-buty1 hydroxylamine) or induced a small decline in the final PDL (O-benzyl hydroxylamine and O-methyl hydroxylamine) (Fig. 1B).
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IMR90 cells were treated with N-hydroxylamines beginning at PDL 24–27. Every week the gain in PDLs was calculated and DCFH, and Rho123 accumulation was determined by FACS analysis. The PDL-dependent change for each parameter was calculated using linear regression for data obtained of cells cultured between 26–50 PDLs (at least 8–10 points for each treatment). Data from a representative experiment is shown. AFU, arbitrary fluorescence units. NMHA, N-methyl hydroxylamine; NBHA, N-benzyl hydroxylamine; NtBHA, N-t-butyl hydroxylamine.

**TABLE II**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>DCFH, AFU/ PDL</th>
<th>Rho123, AFU/PDL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control, PBS</td>
<td>2.18 (0.73) 31.7 (0.67)</td>
<td>0.27 (0.32) 9.3 (0.85)</td>
</tr>
<tr>
<td>NBHA (100 μM)</td>
<td>0.11 (0.08) 9.7 (0.56)</td>
<td>0.45 (0.3) 15.3 (0.56)</td>
</tr>
</tbody>
</table>

**FIG. 2.** Accumulation of Rho123 over the life span of IMR90 cells. IMR90 cells (1 × 10⁵ cells) were sampled for Rho123 accumulation once per week from PDL 25 until reaching senescence at PDL ~ 55. Cells were incubated for 30 min in the dark with 10 μM Rho123, and fluorescence was measured using a FACSort. Data are expressed as the mean channel of the fluorescence histogram obtained. Data are from a representative experiment.

We tested the ability of the cyclic nitroxides TEMPO, 4-OH-TEMPO, and 3-CP to delay senescence of IMR90 cells. None of these nitroxides delayed senescence at 25 μM; moreover, a decline of about 8–9 PDLs was observed at 100 μM. Also, we tested nitroso-tert-butane (tNB), a potential generator of nitric oxide (NO), for its effect on the life span of IMR90 cells. We found that tNB is toxic at 50 μM and has no effect on the cells at concentration of 10 μM.

**N-t-Butyl Hydroxylamines and Other N-Hydroxylamines Delay Senescent-dependent Change in mitochondria—Senescence-dependent change in mitochondria of IMR90 cells was estimated by Rho123. The Rho123 fluorescence that accumulated in the cells was measured weekly by FACS for a total of at least 8 weeks and plotted against the current PDL (i.e. age of the cells). The age-dependent incorporation of Rho123 in IMR90 is biphasic (Fig. 2). This is characterized by a slow and linear increase at early PDLs, followed by a shorter and steeper phase at late PDLs. Linear regression analysis was used to calculate the rate of Rho123 accumulation as a function of PDL (Table II). The regression analysis was based on early PDLs only; late PDLs were not included in the analysis (Fig. 2). The increment in accumulation of Rho123 indicates a senescence-dependent change in the mitochondria of IMR90 cells as they become senescent. N-Hydroxylamines delay these changes in mitochondria, and the rate of Rho123 accumulation as a function of senescence decreased by 70, 69, and 52% for N-t-butyl hydroxylamine, N-benzyl hydroxylamine, and N-methyl hydroxylamine, respectively (Table II).

**N-t-Butyl Hydroxylamines and Other N-Hydroxylamines Decrease Formation of Oxidants and Oxidative DNA Damage in IMR90 Cells**—The level of oxidants was measured each week by estimating the oxidation of DCFH (28) in the living cells. Measurements of fluorescence of oxidized DCFH were made weekly by FACS for total of at least 8 weeks and plotted against the current PDL (i.e. age of the cells), and a biphasic curve similar to that seen with Rho123 fluorescence was observed with DCFH fluorescence (data not shown). A linear regression analysis was used to calculate the initial linear rate of DCFH oxidation as a function of PDL (Table II). The regression analysis was based on early PDLs only. Late PDLs were not included in the analysis. IMR90 cells treated continuously with N-hydroxylamines exhibit a slower rate of formation of oxidants compared with control cells. The percent of decrease in the rate of oxidant formation from control are 88, 95, and 79% for N-t-butyl hydroxylamine, N-benzyl hydroxylamine, and N-methyl hydroxylamine, respectively (Table II). The level of AP sites in DNA can be used as a measure of the level of oxidative damage. IMR90 cells treated simultaneously with the three N-hydroxylamines (30 μM each) showed a 52% reduction in AP sites compared with control cells treated with DCFH (Table II).

**N-Hydroxylamines Increase the GSH/GSSG Ratio in IMR90 Cells**—The three N-hydroxylamines tested improved the glutathione status in IMR90 cells. The GSH/GSSG ratio increased by 75, 90.4, and 94% for N-t-butyl hydroxylamine, N-benzyl hydroxylamine, and N-methyl hydroxylamine, respectively (Table III). The GSH/GSSG ratio increased because of a decrease in the level of GSSG in treated cells compared with untreated cells. No change in the level of GSH was observed between the treated and control groups (Table III). When the cells were treated simultaneously with the three N-hydroxylamines a similar effect on GSH metabolism was observed. IMR90 Cells Treated With N-t-Butyl Hydroxylamine and N-Benzyl Hydroxylamine Are Resistant to Hydrogen Peroxide—Hydrogen peroxide, applied at low concentrations (20 or 30 μM in fresh medium) once a week to control IMR90 cells, accelerated senescence (Fig. 5). The H₂O₂-induced senescence was attenuated when these cells were continuously treated with N-t-butyl hydroxylamine, N-benzyl hydroxylamine, or both compounds + N-methyl hydroxylamine (Fig. 5).

**N-Hydroxylamines Inhibit Reduction of cyt C FeIII by Superoxide Radical**—N-Hydroxylamines at relatively high concentrations (5–10 mM) were able to inhibit the reduction of cyt CFeIII by xanthine/xanthine oxidase, a system that generates superoxide radical (Fig. 6). The catalytic activity of the enzyme xanthine oxidase was not inhibited by N-hydroxylamines, as judged from the rate of formation of uric acid (the co-product with O₂⁻) in the presence or absence of N-hydroxylamines (data not shown). Moreover, N-hydroxylamines prevented autooxidation of cyt CFeIII (Fig. 7A). N-Hydroxylamines were able to reduce cyt CFeIII directly to cyt CFeII, which explains their ability to delay the oxidation of reduced cyt CFeII (Fig. 7B).
in the activity of aconitase.

sites were determined as described under “Experimental Procedures.”

separated from the cells by centrifugation, DNA was isolated, and AP

NBHA, N6 measurements for each treatment). NtBHA, N6-hydroxylamines, Data are the mean ± S.D. of one representive experiment.

FIG. 4. N-Hydroxylamines prevent the age-dependent decline in the activity of aconitase. IMR90 cells (5–4 × 10⁶) were harvested and washed with ice-cold PBS. The cells were suspended in ice-cold 50 mM Tris, pH 7.4, 0.6 mM MnCl₂, 20 μM fluorocitrate. Cells were disrupted by three short (2–3 s) cycles of sonication separated by a 1-min incubation in ice. The cellular lysate was spun at 12000 × g for 5 min, and the supernatant was used for protein quantification and the aconitase assay as described under “Experimental Procedures.” NMHA, N-methyl hydroxylamine; NBHA, N-benzyl hydroxylamine; NtBHA, N-t-butyl hydroxylamine. Data are the mean ± S.D. of one representative experiment.

TABLE III

The status of GSH, GSSG, and GSH/GSSG in IMR90 cells treated with N-hydroxylamines

IMR90 cells at PDL 24–27 were cultivated with N-hydroxylamines, and every week the gain in PDL was calculated. About 3 × 10⁶ cells were used to determine GSH and GSSG by HPLC-electrochemical detection. The range of PDLs included between 26 and 50 PDLs (at least 6 measurements for each treatment). NtBHA, N-t-butyl hydroxylamine; NBHA, N-benzyl hydroxylamine; NMHA, N-methyl hydroxylamine.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>GSH (nmol/mg)</th>
<th>GSSG (nmol/mg)</th>
<th>GSH/GSSG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control, PBS</td>
<td>54.4 ± 5.2</td>
<td>0.77 ± 0.06</td>
<td>70.6</td>
</tr>
<tr>
<td>NtBHA (100 μM)</td>
<td>49.5 ± 5.6</td>
<td>0.36 ± 0.045</td>
<td>132.8</td>
</tr>
<tr>
<td>NBHA (100 μM)</td>
<td>48.4 ± 3.4</td>
<td>0.36 ± 0.015</td>
<td>134.4</td>
</tr>
<tr>
<td>NMHA (100 μM)</td>
<td>52.15 ± 6.7</td>
<td>0.38 ± 0.05</td>
<td>137.2</td>
</tr>
</tbody>
</table>

* P < 0.01.

FIG. 5. N-Hydroxylamines protect IMR90 cells from the toxicity of hydrogen peroxide. IMR90 cells were seeded at an initial density of 0.5 × 10⁶/dish and grown for a week in the presence of N-hydroxylamines before the start of hydrogen peroxide treatment as described under “Experimental Procedures.” Every week the cells were harvested, counted, and seeded with or without N-hydroxylamines and/or hydrogen peroxide. □, control; ▼, 20 μM H₂O₂; ●, H₂O₂ + N-t-butyl hydroxylamine; ▲, H₂O₂ + N-benzyl hydroxylamine. Inset: ■, control; ▼, 30 μM H₂O₂; ○, H₂O₂ + three N-hydroxylamines.

FIG. 6. N-Hydroxylamines inhibit the reduction of cyt C⁹⁺⁺⁺⁺ by superoxide radical. Superoxide radical was generated by a xanthine/xanthine oxidase system, and cytochrome c reduction was followed at 550 nm. N-Hydroxylamines were added immediately before starting of the enzymatic reaction by the addition of the substrate xanthine. Inhibition of the superoxide-dependent reduction of cytochrome c was estimated by the difference in the rates of its reduction in the absence and presence of the N-hydroxylamines (5–10 μM). □, N-t-butyl hydroxylamine; ●, N-benzyl hydroxylamine; ▲, N-methyl hydroxylamine.

One of the two breakdown products of PBN or PBN/OH hydrolysis, N-t-butyl hydroxylamine, but not the other product, benzoaldehyde, delays the replicative senescence of human lung fibroblasts at concentrations at least 20 times lower than PBN. Other N-hydroxylamines tested (not related to PBN, i.e. N-benzyl hydroxylamine and N-methyl hydroxylamine, Scheme 1) were also able to delay the senescence of IMR90 cells. Thus, it appears that the N-hydroxylamine functional group is responsible for the biological activity of the three compounds tested. Although PBN is a spin trap and an antioxidant, none of the well known antioxidants studied (ascorbic acid, vitamin E, catalase, 3-CP, 4-OH-TEMPO, and TEMPO) can delay senescence of IMR90 cells as does PBN. These results suggest that the effect of PBN on IMR90 cells is due to the N-t-butyl hy-
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To gain more insight into the effect of N-hydroxylamines on cells, we assessed the status of different cellular parameters in cells that have been grown continuously with medium supplemented with N-hydroxylamines compared with controls. We show that, concomitant with delayed senescence by N-hydroxylamines, the PDL-dependent formation of oxidants was decreased as estimated by DCFH oxidation (Table II), and there was an increase in the GSH/GSSG ratio (Table III). The age-dependent decay in mitochondria was delayed as estimated by Rho123 accumulation (Table II) and by the inhibition of the age-dependent decline in the activity of aconitase (Fig. 4). The level of AP sites in DNA of cells treated with N-hydroxylamines was also 52% lower than that of the control cells. The increase in the ratio GSH/GSSG by treatment with N-hydroxylamines was due to a decrease in the steady-state level of GSSG without changing the concentration of GSH. In addition N-hydroxylamines prevented the age-dependent decline in aconitase activity in IMR90. Aconitase is an enzyme essential for the Krebs cycle and highly abundant in mitochondria compared with cytosol (29). Its iron-sulfur cluster is known to be damaged by superoxide radical and ONOO\(^-\) (25, 30, 31). The mitochondrial enzyme is more sensitive to inhibition by superoxide radical and oxidative modification compared with the cytosolic enzyme (30, 32). These findings provide evidence that N-hydroxylamines lower the endogenous level of oxidants in mitochondria, thus protecting aconitase and causing less GSH to be oxidized to GSSG. Since aconitase plays an important role in the Krebs cycle, changes in its activity will have a large impact on mitochondrial and cellular metabolic pathways. N-Hydroxylamines also protect IMR90 cells from H\(_2\)O\(_2\)-induced senescence, probably by acting as mitochondrial antioxidants. This is further supported by the 79–95% decrease in the rate of DCFH oxidation in N-hydroxylamine-treated cells compared with controls. A higher activity of aconitase in conjunction with low level of GSSG in N-hydroxylamine-treated cells suggests that the difference in the rate of DCFH oxidation between control and treated cells stems from differences in oxidant formation by the cells. The complexity of DCFH oxidation has been discussed (33).

PBN has been shown to protect against oxidative damage in different biological models (5, 34–36). Interestingly, PBN inhibits formation of hydrogen peroxide at the level of complex I in mitochondrial preparations, which suggests a direct interaction with mitochondria in vivo (10). The antioxidative effect of N-t-butyl hydroxylamine can be attributed to a similar, although more efficient, inhibition of superoxide formation by mitochondria in vivo, resulting in less hydrogen peroxide being formed. Further studies of the interaction of N-t-butyl hydroxylamine (as representative of the primary N-hydroxylamines used in this study) with mitochondria in IMR90 cells showed that intracellular N-t-butyl hydroxylamine is maintained in the reduced form by mitochondrial NADH and complex I. Since N-t-butyl hydroxylamine is stable to autooxidation in a cell-free system, this suggests that N-t-butyl hydroxylamine cycles inside the cells between the oxidized and reduced form. Complex I is a mitochondrial site that is implicated in the formation of superoxide radical. Thus a possible mechanism is the interaction of N-t-butyl hydroxylamine with this site to prevent formation of superoxide radical, as with the interaction of PBN with complex I (10).

The age-related increase in oxidative damage to mitochondrial DNA, proteins, and lipids is thought to be a major factor in organismal aging (6, 37–40). Since mitochondria are assumed to play a major role in the formation of superoxide radicals and suggested to contribute to aging, we compared the senescence-dependent changes in mitochondria in control and N-hydroxylamine-treated cells. A PDL-dependent accumulation of Rho123 is observed in IMR90 cells, which reflects a senescence-dependent change in mitochondria (Table II). Although the reason for this change is not clear, it may be due to age-dependent mitochondrial swelling or changes in the mitochondrial inner membrane that elevate the nonspecific binding of Rho123 to this membrane (37, 41). Accumulation of Rho123 was also observed in one fraction of isolated hepatocytes from livers of old rats over hepatocytes from young rats (37). When IMR90 cells were grown in medium supplemented with N-hydroxylamine, a 52–70% slower rate of the age-dependent accumulation of Rho123 was observed when compared with control cells. This suggests, in conjunction with the protective effect on aconitase, that N-hydroxylamines interact with mitochondria and delay the senescence-dependent changes to mitochondria. Since mitochondria are considered a major source for free radical formation, improving the mitochondrial status could cause a significant decrease in the level of oxidants in the cells (Table II).

We also found that cyt C\(^{\text{FeIII}}\) is reduced directly by N-hydroxylamines independently of oxygen or iron, indicating that superoxide radical is not an intermediate in the process. Reduction of cyt C\(^{\text{FeIII}}\) by N-hydroxylamines may have physiological significance and suggests that N-hydroxylamines potentially can interact in vivo also with cytochrome c in addition to mitochondrial NADH. Cyclic-N-hydroxylamines/cyclic nitrooxides are recycled by mitochondrial ubiquinol and cytochrome oxidase (22, 23), a mechanism of regeneration that may be shared by the primary N-hydroxylamines used in the present study. Our primary data show that mitochondrial NADH is involved in keeping the intracellular N-hydroxylamines in reduced form. Although the site of intracellular oxidation of primary N-hydroxylamines is not yet known, it could be either enzyme-mediated, e.g. cytochrome c, or induced by direct inter-

![Graph](https://www.jbc.org/content/172/4/6746/F7)

**Fig. 7. Reduction of cytochrome c by N-hydroxylamines.** A, N-hydroxylamines inhibit autooxidation of cyt C\(^{\text{FeII}}\). Enzymatically reduced cytochrome c (50 μM) by xanthine/xanthine oxidase was incubated with 2 mM N-hydroxylamines in PBS and 0.6 mM DTPA at 25 °C. Autooxidation of cyt C\(^{\text{FeII}}\) in the presence and absence of the three N-hydroxylamines was followed at 550 nm. ○, control; ●, N-hydroxylamine B, rate of reduction of cytochrome c (50 μM) by different concentrations of N-hydroxylamines in PBS, 0.6 mM DTPA at 25 °C. The amount of cytochrome c reduced was calculated based on the millimolar excitation coefficient of 22.9 at 550 nm. Data from one representative experiment is shown. ■, N-t-butyl hydroxylamine; ○, N-benzyl hydroxylamine; ▲, N-methyl hydroxylamine.
action with cellular oxidants. Studies are under way to elucidate the exact mechanism of senescence delay by the N-hydroxylamines.

N-Hydroxylamines (5–10 mM) inhibit the reduction of cyt CFeIII by superoxide radical, which was generated with xanthine/xanthine oxidase. N-Hydroxylamines do not inhibit the catalytic activity of xanthine oxidase since the formation of uric acid (obligatory product with superoxide radical) was not inhibited. This suggests that in vivo, primary N-hydroxylamines (or their corresponding nitrooxides), react with superoxide radical, as is known for the cyclic hydroxylamines/cyclic nitroxides. We find that N-t-butyl hydroxylamine rapidly enters cells and is concentrated by approximately 5-fold. To test the contribution of superoxide scavenging to the mechanism of senescence delay we tested three cyclic nitroxides as typical nonsomod SOD mimics. The three cyclic nitroxides tested (3-CP, TEMPO, and 4-OH-TEMPO) that form the cyclic hydroxylamines in the cell did not delay the replicative senescence of the cells (at 25 mM) and, at higher concentrations (100 mM), were even toxic. This suggests that there are some differences in the mode of action between cyclic hydroxylamines and the primary N-hydroxylamines. Consequently we suggest that mitochondria are a potential primary target for N-hydroxylamines due to their ability to slow the senescence-dependent changes to mitochondria and lower oxidants and delay senescence of IMR90 cells. Ex vivo and in vivo studies are currently under way to uncover the molecular details of the interaction of N-hydroxylamines with mitochondrial components. Our initial results provide further evidence that mitochondria are the primary target of N-hydroxylamines.

Nitric oxide was proposed as a product of PBN decomposition and, thus, was suggested to possess a role in the activity of PBN in vivo (11, 13). N-t-Butyl hydroxylamine has also been shown to be oxidized by UV photolysis to produce tNB, which further decomposes to give nitric oxide (11, 13). The in vivo evidence for the formation of N-t-butyl hydroxylamine-dependent (or PBN-dependent) nitric oxide has not been demonstrated, and the evidence is circumstantial or based on in vitro experiments (42, 43). To assess if tNB contributes to the effect of N-t-butyl hydroxylamine on IMR90 cells, the cells were grown in a medium supplemented with tNB. We found that tNB is toxic at 50 mM and has no effect on the cells at much lower concentrations (10 mM). Thus, it seems that tNB plays a negligible role in the mechanism underlying the biological effect of N-t-butyl hydroxylamine, although it is possible that a small fraction of tNB is formed in our system by the effect of other oxidants.

The three N-hydroxylamines used in this study possess different side chains, two alkyl groups and one benzyl group. All the three N-hydroxylamines exhibit the ability to delay senescence of IMR90 cells, and thus, the N-hydroxylamine (R-NHOH) functional group possesses the biological activity. Cyclic N-hydroxylamines (R2NOH) and their respective nitrooxides enhance the clinical recovery of damaged brains in closed-head injury (44) and protect against oxidative damage induced by H2O2 (45) but did not delay senescence of IMR90 cells. This emphasizes the remarkable feature of the primary N-hydroxylamines as antioxidants. Harman in 1961 (46) showed that HNHOH (hydroxylamine) possesses anticancer activity and delayed senescence in mice. On the other hand, O-hydroxylamines, which possess a different functional group (R-O-NH2) but the same alkyl groups (and benzyl group) as N-hydroxylamines, do not affect the rate of senescence, the level of oxidants, or the changes in mitochondria in IMR90 cells. This further indicates that the N-hydroxylamine functional group (R-NHOH) is involved in the effect of delaying senescence in IMR90 cells. It is likely that the alkyl and aromatic groups of the primary N-hydroxylamines could affect their oxidation-reduction potential, as is the case with cyclic nitrooxides/cyclic hydroxylamines (22). This ratio is also determined by the oxygen status of the cell (24, 47). In addition, the alkyl groups and their different hydrophobicities may determine the intracellular location of the N-hydroxylamines. We observed differences in the activity of each N-hydroxylamine toward each factor that was measured only at the late PDLs. These topics still need to be studied further.

In summary, the anti-senescent effect of PBN on IMR90 cells can be mimicked efficiently by N-t-butyl hydroxylamine, and other N-hydroxylamines, which suggests that the functional compound in the PBN preparation is the N-hydroxylamine rather than PBN itself. Other N-hydroxylamines were also effective in delaying senescence and protecting IMR90 cells. The results of this study strongly suggest that more studies should be done to assess the relative contribution of PBN and N-hydroxylamines in the protective effect of PBN on different systems (1, 3–5, 7–9). The biological activity of the N-hydroxylamines appears to be due to an antioxidant effect on mitochondria. The use of N-hydroxylamine also avoids the benzaldehyde formed when PBN decomposes (43). The low doses of N-hydroxylamine required make them desirable compounds for delaying aging and protecting from oxidative damage. This is the first time that an anti-aging activity has been attributed to a group of chemicals that share a common functional group.

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REFERENCES

N-Hydroxylamines as Mitochondrial Antioxidants
