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Structural Specificity of Polyamines and Polyamine Analogues in the Protection of DNA from Strand Breaks Induced by Reactive Oxygen Species

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Reactive oxygen species are known to induce strand breaks and/or base modifications in DNA. DNA strand breaks are associated with many pathologies and programmed cell death. We have examined the ability of the polyamines and their analogues to protect ϕX-174 plasmid DNA from strand breakage induced by a oxygen-radical generating system. Spermine and several unsymmetrically substituted polyamine analogues reduced the amount of strand breakage at a physiologically relevant concentration of 1 mM. However, putrescine, spermidine, N^1-acetylspermine, N^1-acetylspermidine and symmetrically alkylated polyamine analogues were not able to reduce strand breakage at the same concentration. Thus, the unsymmetrically alkylated polyamine analogues and natural spermine can protect DNA against strand breakage induced by Cu(II)/H_2O_2 generated ROS similar to other more classical antioxidants. © 1998 Academic Press

The naturally occurring polycationic polyamines spermine, spermidine, and their diamine precursor putrescine are found in all eukaryotic cells. Intracellular polyamines are essential for cell proliferation and differentiation, and the intracellular concentration of these ubiquitous molecules is highly regulated by their metabolic pathway (1, 2). Spermine and spermidine are known to stabilize chromatin and nuclear enzymes by forming complexes with organic polyanions (3). Some of the roles ascribed to polyamines include preventing endonuclease-mediated DNA fragmentation (4) and inhibiting damage caused by alkylating agents (6), singlet oxygen (7) and radiation (8, 9). The natural polyamines have also been shown to inhibit lipid peroxidation (10). Depletion of intracellular polyamines by inhibiting polyamine biosynthesis with 2-difluoromethylornithine or by inducing polyamine catabolism with polyamine analogues results in alteration of chromatin and DNA structure and may in some cases result in programmed cell death (11- 15). Both the natural polyamines and their analogues (Fig. 1) contain amine groups similar to sodium azide (NaN_3) which is known to act as a scavengers of singlet oxygen (16).

DNA damage by reactive oxygen species (ROS) can result in strand breakage and/or base modifications. Such DNA damage is linked to many pathologies and occurs in programmed cell death (17-22). ROS include singlet oxygen ($^1O_2$), superoxide anion ($O_2^{-\bullet}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($OH^\bullet$) that are produced by enzymatic and non-enzymatic reactions in cells. H_2O_2 and $^1O_2$ are relatively stable oxidants whose actions are not limited to effects at their sources. However, the hydroxyl radical is very reactive and reacts with its substrates at diffusion-limited rates. Hydroxyl radicals can be generated by the reaction of hydrogen peroxide with DNA associated copper in a Fenton type mechanism, $H_2O_2 + Cu(I) \rightarrow OH^\bullet + OH^- + Cu(II)$ (23, 24, 25). Hydroxyl radical formation mediated by transition metals is thought to be responsible for the oxidative toxicity *in vivo* rather than H_2O_2 itself (23, 24, 26, 27).

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The abbreviations used are: BCS, bathocuproinedisulfonic acid; BENSpm, N^1, N^{11}-bis(ethyl)norspermine; BESpm, N^1, N^{12}-bis(ethyl)-spermine; CPENSpm, N^1-ethyl-N^{11}-((cyclopropyl)methyl)-4,8-diazaundecane; CHEXENSpm, N^1-ethyl-N^{11}-((cyclohexyl)methyl)-4,8-diazaundecane; CHENSpm, N^1-ethyl-N^{11}-((cycloheptyl)methyl)-4,8-diazaundecane; DMPO, 5,5-dimethyl-1-pyrroline-N-oxide; GSH, reduced glutathione; NAC, N-acetyl-L-cysteine; ROS, Reactive oxygen species.

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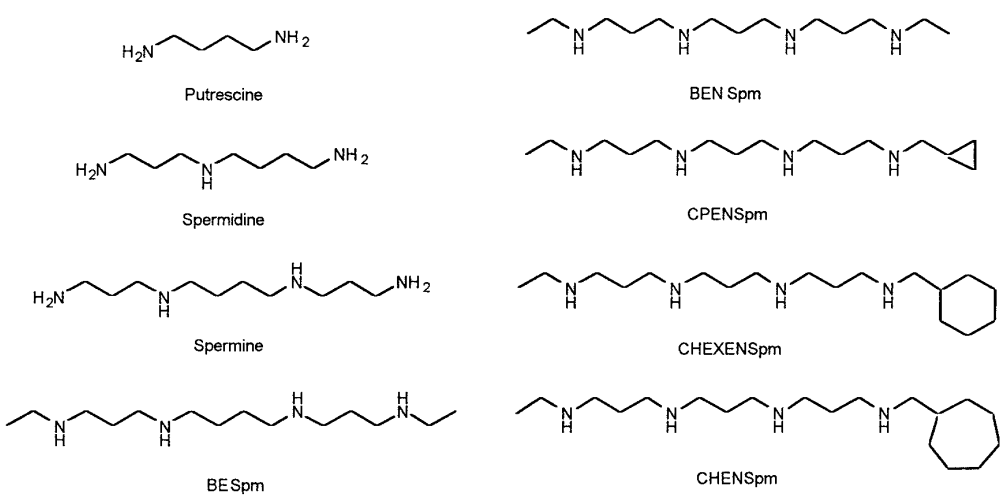

FIG. 1. Structure of polyamines and polyamine analogues. Putrescine, Spermidine, Spermine BESpm, N^1, N^{12}-bis(ethyl)spermine; BENSpm, N^1, N^{11}-bis(ethyl)norspermine; CPENSpm, N^1-ethyl-N^{11}-((cyclopropyl)methyl)-4,8-diazaundecane; CHEXENSpm, N^1-ethyl-N^{11}-((cyclohexyl)methyl)-4,8-diazaundecane; CHENSpm, N^1-ethyl-N^{11}-((cycloheptyl)methyl)-4,8-diazaundecane.

The goal of the current study was to determine whether the polyamines or their analogues were capable of protecting against ROS-induced strand breakage of ϕX-174 plasmid DNA caused by a Cu(II)/H_2O_2 dependent oxygen-radical generating system (28). Our results indicate that both spermine and unsymmetrically alkylated polyamine analogues possess the ability to protect DNA against strand breakage and demonstrate a potentially critical protective role for the natural polyamines as antioxidants.

MATERIALS AND METHODS

Materials. The HCl salts of the natural polyamines putrescine, spermine, spermidine, N^1-acetylspermine, and N^1-acetylspermidine, along with bovine liver catalase, bathocuproinedisulfonic acid (BCS), cupric chloride, reduced glutathione (GSH), N-acetyl-L-cysteine (NAC), sodium azide (NaN_3), triethylamine ($(CH_3CH_2)_3N$), and 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) were purchased from Sigma Chemical Co. (St. Louis, MO). The unsymmetrically substituted polyamine analogues N^1-ethyl-N^{11}-((cyclopropyl)methyl)-4,8-diazaundecane (CPENSpm), N^1-ethyl-N^{11}-((cyclohexyl)methyl)-4,8-diazaundecane (CHEXENSpm), and N^1-ethyl-N^{11}-((cycloheptyl)methyl)-4,8-diazaundecane (CHENSpm) were synthesized as previously reported (29). The symmetrically substituted polyamine analogues N^1, N^{12}-bis(ethyl)spermine (BESpm) and N^1, N^{11}-bis(ethyl)norspermine (BENSpm) were kindly provided by Dr. R. Bergeron (Gainesville, FL) (30) and Parke-Davis (Ann Arbor, MI), respectively. The stock solution of the natural polyamines and their analogues were prepared at a concentration of 10 mM in ddH_2O. Hydrogen peroxide (30 %) in water was purchased from Fisher Scientific Co. (Fair Lawn, NJ), Dulbecco's phosphate-buffered saline (PBS) from GIBCO (Grand Island, NY), and ϕX-174 RF1 double-stranded plasmid DNA from New England Biolabs (Beverly, MA).

Assays for DNA strand breaks. DNA strand breakage was measured by the conversion of supercoiled ϕX-174 RF1 double-stranded DNA to open circular and linear forms. To assess DNA cleavage, 0.2 μg DNA was incubated for 1 hr in the presence of 30 μM H_2O_2 and 10 μM $CuCl_2$ in PBS (pH 7.4) in a total volume of 30 μL as described previously (28). Polyamines, analogues, or antioxidants were co-incubated as indicated. Following incubation the samples were separated by electrophoresis in a 1 % agarose gel containing 40 mM Tris-acetate and 1 mM EDTA in a horizontal slab gel apparatus using Tris/acetate gel buffer. The gel was stained with ethidium bromide (2 μg/ml) for 10 min, followed by destaining in water for 10 min and was photographed by UV translumination. The gels were photographed using an Eagle Eye digital camera (Stratagene, La Jolla, CA) and the digital image was quantified using ImageQuant software (Sunnyvale, CA). A single strand break in supercoiled double-stranded DNA results in the formation of open circular DNA, and double strand breaks result in the formation of linear DNA (31). Two single strand breaks in opposite strands but near each other can also result in the formation of linear DNA (32). In agarose gel electrophoresis of untreated ϕX-174 plasmid DNA supercoiled DNA migrates faster than open circular DNA (32) Which is less compact (33). It should be noted that the commercially available ϕX-174 DNA contained approximately 32 % open circle DNA prior to treatment.

RESULTS

Effects of the natural polyamines and known antioxidants on Cu(II)/H_2O_2-induced strand breakage. When ϕX-174 plasmid DNA was incubated in the presence of 10 μM $CuCl_2$/30 μM H_2O_2 for 1 hour, no remaining supercoiled DNA was observed, and open circular and linear DNA represented 71±9% and 29±9%, respectively (Fig. 2 and Table 1). Neither 10 μM $CuCl_2$ nor 30 μM H_2O_2 alone induced DNA strand breaks. In the absence of $CuCl_2$/H_2O_2 (negative control) the supercoiled and open circular DNA were 69±5% and 32±5% respectively (n=11).

The addition of spermine effectively inhibited the Cu(II)/H_2O_2-mediated formation of linear DNA and partially protected the supercoiled DNA in a time and dose dependent manner (Fig. 3 a & b). Spermine

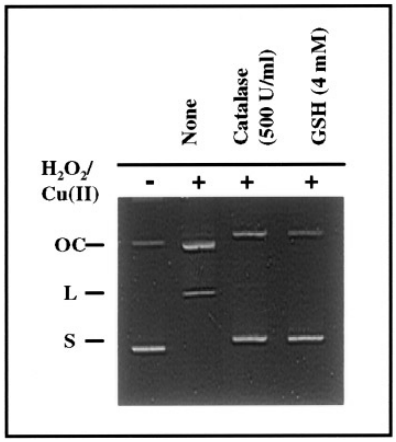

FIG. 2. Protection of DNA strand breaks by antioxidants. Two hundred ng ϕX -174 plasmid DNA was incubated for 1 hour with the indicated additions in the presence of 30 μM H_2O_2 and 10 μM $CuCl_2$. Abbreviations used are: GSH, reduced glutathione; OC, open circular; L,: linear; S, supercoiled.

provided some protection at concentrations as low as 0.1 mM. However, the efficacy of spermine decreased gradually with time, leading to the decrease of supercoiled DNA and the accumulation of circular DNA. However, no increase in linear DNA was observed. Spermidine, putrescine, N^1-acetylspermine, and N^1-acetylspermidine were not effective in protecting supercoiled DNA at concentrations of 1 mM (Table 1). At the concentration of 10 mM spermidine provided protection similar to 1 mM spermine (Table 1). The acetylated polyamines provided little protection in the radical system used here. The inorganic cation, Mg^{2+} (as $MgCl_2$), was also not capable of protecting DNA from strand breaks with the concentration range from 1 mM to 10 mM (Table 1).

DNA strand breakage was completely blocked by 500 units/ml of catalase (Fig. 2 and Table 1), 4 mM NAC (Table 1), 4 mM GSH (Fig. 2 and Table 1), and 40 μM BCS, a Cu(I) chelator. DMPO, a hydroxyl *free* radical scavenger, had no inhibitory effect at 100 mM (Table 1). NaN_3, an efficient quencher of $^1O_2$, inhibited strand breakage at 10 mM but not at 1 mM (Table 1). $(CH_3CH_2)_3N$ at 1 mM had no inhibitory effect on DNA damage and degraded DNA at higher concentrations ($>$ 5 mM) (Table 1), producing a DNA smear without evidence of remaining supercoiled, open circular, or linear DNA.

Effects of the symmetrically and unsymmetrically substituted polyamine analogues on Cu(II)/H_2O_2 induced strand breakage. Since spermine exhibited significant protection of DNA from ROS damage, we examined the ability of a new class of antitumor polyamine analogues to protect DNA since it was suspected they may not have the same protective capabilities. The unsymmetrically substituted spermine analogue CPENSpm was capable of significantly reducing Cu(II)/H_2O_2-induced DNA strand breakage at 1 mM (Fig. 4). Similar to spermine, the protection by CPENSpm diminished with time. Interestingly, the symmetrically substituted polyamine analogues, BESpm and BENSpm,

TABLE 1

Effects of Polyamines, Polyamine Analogues, and Antioxidants on ϕX-174 Supercoiled Plasmid DHA Exposed to Cu(II)/H_2O_2 Oxygen Radical Generating System

	Percent DNA in the three forms		
Treatment[a]	Supercoiled	Open circle	Linear
None	69 ± 5	32 ± 5	NQ[b]
Cu(II)/H_2O_2	NQ	71 ± 9	29 ± 9
100 mM DMPO	NQ	84 ± 11	19 ± 6
10 mM NAC	60 ± 7	37 ± 3	NQ
1 mM $MgCl_2$	NQ	76 ± 8	23 ± 8
5–10 mM $MgCl_2$	NQ	77 ± 3	19 ± 5
4 mM GSH	64 ± 12	31 ± 9	5 ± 5
500 u/ml CAT	55 ± 20	42 ± 18	NQ
1 mM NaN_3	NQ	81 ± 2	16 ± 2
10 mM NaN_3	56 ± 2	39 ± 2	5 ± 2
1 mM $(C_2H_5)_3N$	NQ	44 ± 6	56 ± 6
>5 mM $(C_2H_5)_3N$	ND[c]	ND	ND
1 mM Put	NQ	64 ± 7	32 ± 5
1 mM Spd	NQ	78 ± 5	22 ± 7
2 mM Spd	2 ± 2	77 ± 12	21 ± 10
5 mM Spd	15 ± 5	73 ± 4	13 ± 1
10 mM Spd	30 ± 2	63 ± 4	7 ± 3
1 mM Spm	**24 ± 11**	**73 ± 11**	**NQ**
1 mM N^1-AcSpd	NQ	71 ± 7	26 ± 8
2 mM N^1-AcSpd	4 ± 1	25 ± 4	71 ± 3
5 mM N^1-AcSpd	5 ± 4	76 ± 1	19 ± 5
10 mM N^1-AcSpd	5 ± 3	79 ± 3	16 ± 6
1 mM N^1-AcSpm	NQ	73 ± 6	25 ± 5
2 mM N^1-AcSpm	3 ± 2	76 ± 6	21 ± 5
5 mM N^1-AcSpm	5 ± 1	77 ± 9	17 ± 7
10 mM N^1-AcSpm	12 ± 2	80 ± 3	8 ± 1
1 mM BESpm	NQ	77 ± 7	20 ± 10
1 mM BENSpm	5 ± 6	80 ± 7	15 ± 10
1 mM CPENSpm	29 ± 5	68 ± 6	5 ± 2
1 mM CHENSpm	24 ± 6	72 ± 6	NQ
1 mM CHEXENSpm	24 ± 6	70 ± 6	6 ± 1

[a] The standard strand break assay was performed with 30 μM H_2O_2 and 10 μM $CuCl_2$ with 0.2 μg ϕX-174 DNA for 1 hour at 37°C. DNA was then separated on a 1% agarose gel, stained with ethidium bromide and the fluorescent image was digitized and quantified using ImageQuant software. Values represent the mean of at least 3 separate experiments ± standard deviation, except where noted. Abbreviations used are: CAT, catalase; Put, putrescine; Spd, spermidine; Spm, spermine; N^1-AcSpd, N^1-acetylspermidine; N^1-AcSpm, N^1-acetylspermine; BENSpm, N^1, N^{11}-bis(ethyl)norspermine; BESpm, N^1, N^{12}-bis(ethyl)spermine; CPENSpm, N^1-ethyl-N^{11}-((cyclopropyl)methyl)-4,8-diazaundecane; CHEXENSpm, N^1-ethyl-N^{11}-((cyclohexyl)methyl)-4,8-diazaundecane; CHENSpm, N^1-ethyl-N^{11}-((cycloheptyl)methyl)-4,8-diazaundecane; DMPO, 5,5-dimethyl-1-pyrroline-N-oxide.

[b] NQ, < 5%, not quantifiable.

[c] Represents the complete degradation of DNA.

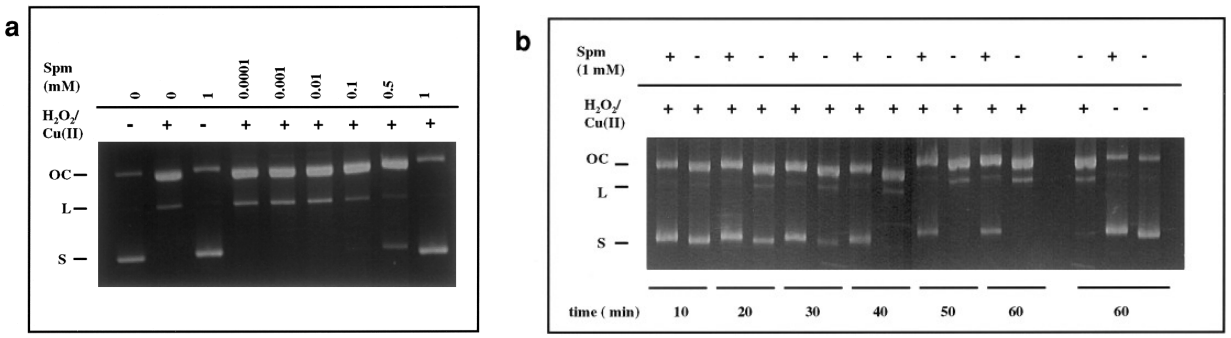

FIG. 3. **a.** Dose dependent protection of ϕX -174 DNA strand breaks by spermine. Two hundred ng ϕX -174 plasmid DNA was incubated for 1 hour with the indicated additions in the presence (+) of 30 μM H_2O_2 and 10 μM $CuCl_2$. Abbreviations used: Spm, spermine; OC, open circular; L,: linear; S, supercoiled. **b.** Time dependent protection of ϕX -174 DNA strand breaks by spermine. ϕX -174 DNA was incubated as above for the indicated times in the presence (+) or absence (−) of 1 mM spermine.

did not provide similar protection at 1 mM (Table 1). BESpm was found to be incapable of protecting DNA even at 10 mM (Table 1). The unsymmetrically substituted polyamine analogues, CHEXENSpm and CHENSpm, demonstrated protection similar to that observed with CPENSpm at 1 mM. These results suggest a stringent structural specificity is required for the protection of DNA from Cu(II)/H_2O_2-induced DNA strand breakage.

DISCUSSION

Several polyamine analogues have been shown to induce rapid cell death in a phenotype-specific manner in a series of important human solid tumor cell types (14, 15, 34-36). This cell death is often associated with a rapid and large increase in polyamine catabolism (37-39) which generates ROS and depletes cells of the natural polyamines (14). It has been proposed that the natural polyamines protect DNA from ROS by altering chromatin structure, making it less susceptible to be attacked by ROS, or possibly by acting as free radical scavengers (40). In the present study the ability of the natural polyamines to protect DNA from ROS was compared to the protective capacity of polyamine analogues. Spermine, at physiologically relevant concentrations, demonstrated significant ability to prevent DNA damage in this system. Surprisingly, the unsymmetrically substituted analogues were as effective as spermine and the classical antioxidants in protecting DNA.

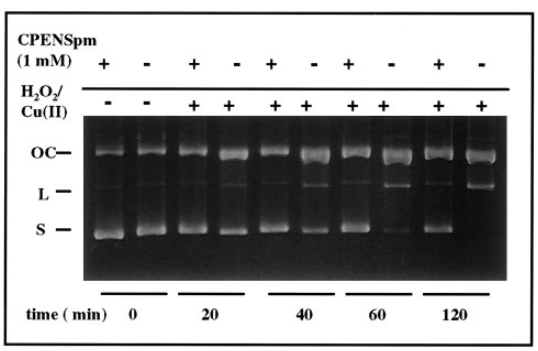

FIG. 4. Time dependent protection of ϕX -174 plasmid DNA by CPENSpm. ϕX -174 DNA was incubated as in Figure 4 for the indicated times in the presence (+) or absence (−) of 1 mM CPENSpm.

It should be noted that the hydroxyl free-radical scavenger, DMPO, did not protect DNA from strand breakage even at a concentration as high as 100 mM suggesting the *free* hydroxyl radical may not be the ROS directly causing DNA strand breakage in the system used here. It is also possible that DMPO is unable to protect DNA in this system because it is not capable of positioning itself sufficiently near the hydroxyl radical generation site on or near DNA. Imlay *et al.* suggested that the hydroxyl radical is complexed either to its precursory metal, representing a copper-peroxide complex, or to its substrate (41).

NaN_3 (10 mM), a $^1O_2$ scavenger, inhibited DNA strand breakage, suggesting that $^1O_2$ was generated and participated in the Cu(II)/H_2O_2-induced DNA strand break formation as demonstrated by Yamamoto (24). Although the data suggest the involvement of hydroxyl radicals and $^1O_2$, the precise nature of the ultimate ROS causing DNA strand breaks remains uncertain.

There are several mechanisms by which the polyamines and the analogues could protect DNA from Cu(II)/H_2O_2-mediated oxidative damage including: 1) alteration of DNA structure making less susceptible to attack; 2) chelation of transition metal ions reducing the amount of free transition metals to participate in the ROS formation; 3) acting as free radical scavengers to inactivate ROS.

It is unlikely that the protection afforded by the polyamines is based soley on charge. Mg^{++} had no protective effect. Also, while each of the symmetrically and unsymmetrically alkylated polyamine analogues has a net positive charge of 4, only the unsymmetrically alkylated polyamine analogues protected DNA effectively against strand breaks at the concentrations tested. These data demonstrate that the protection of DNA by polyamines and polyamine analogues is not solely due to polycationic properties but also involves structural specificity. This is consistent with the work of Basu et al. (42, 43) demonstrating that small structural changes, while maintaining identical charge, could profoundly effect polyamine analogue interactions with DNA.

Løvaas *et al.* suggested that spermine chelates transition metal ions, thus preventing the generation of ROS (40). However, spermine is able to block DNA strand breakage induced by radiation and by $^1O_2$, processes which do not involve transition metal ions (7, 8). Additionally, the symmetrically substituted polyamine analogues would be expected to be equally effective in chelating copper as spermine, but they afforded little protection in this system.

The protonation of ROS by the amine groups leading to neutralization represents a possible mechanism for protection. The amine group of the polyamines and the analogues are similar to NaN_3,which is known to act as scavengers of $^1O_2$ (16), suggesting a similar mechanism of radical scavenging. However, it is reasonable to assume that the polyamines and polyamine analogues can protect DNA strand breaks by a combination of mechanisms.

In summary, the results presented here suggest that spermine plays a critical role in the protection of DNA from oxidative damage and from the potential mutations arising from such damage. The results of the current study demonstrate that spermine and its unsymmetrically alkylated analogues can protect DNA at physiologically relevant concentrations against strand breakage induced by a $Cu(II)/H_2O_2$ dependent oxygen-radical generating system. DNA damaging ROS can be generated by the Fenton-type reaction in the nucleus where the transition metal ions are bound to DNA. Thus, depletion of intracellular polyamines, especially nuclear spermine, may enhance either the efficacy of radiation therapy or the susceptibility of normal cells to oxidative stress, thus leading to cell death and pathogenesis. Therefore, it is necessary to consider these factors in the continued design of new polyamine analogues, regardless of whether the intention is to create radiosensitizers or more effective chemotherapeutic agents.

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