Amino Acids (2007) 33: 231–240
DOI 10.1007/s00726-007-0513-4
Printed in The Netherlands

Amino Acids

Spermine and spermidine mediate protection against oxidative damage caused by hydrogen peroxide

J. E. Rider[1,2], **A. Hacker**[2], **C. A. Mackintosh**[3], **A. E. Pegg**[3], **P. M. Woster**[4], and **R. A. Casero Jr**[1,2]

[1] Department of Environmental Health Sciences, The Johns Hopkins University Bloomberg School of Public Health, Baltimore, MD, USA
[2] The Sidney Kimmel Comprehensive Cancer Center, Johns Hopkins University School of Medicine, Baltimore, MD, USA
[3] Department of Cellular and Molecular Physiology, Pennsylvania State University College of Medicine, Hershey, PA, USA
[4] Department of Pharmaceutical Sciences, Wayne State University, Detroit, MI, USA

Received November 30, 2006
Accepted February 1, 2007
Published online March 30, 2007; © Springer-Verlag 2007

Summary. The polyamines spermidine and spermine have been hypothesized to possess different functions in the protection of DNA from reactive oxygen species. The growth and survival of mouse fibroblasts unable to synthesize spermine were compared to their normal counterparts in their native and polyamine-depleted states in response to oxidative stress. The results of these studies suggest that when present at normal or supraphysiological concentrations, either spermidine or spermine can protect cells from reactive oxygen species. However, when polyamine pools are pharmacologically manipulated to produce cells with low levels of predominately spermine or spermidine, spermine appears to be more effective. Importantly, when cells are depleted of both glutathione and endogenous polyamines, they exhibit increased sensitivity to hydrogen peroxide as compared to glutathione depletion alone, suggesting that polyamines not only play a role in protecting cells from oxidative stress but this role is distinct from that played by glutathione.

Keywords: Polyamines – Spermine – Spermidine – Antioxidants – Hydrogen peroxide – Oxidative damage

Abbreviations: BSO, DL-Buthionine-[S, R]-sulfoximine; CGP48664, 4-amidinoindan-1-one 2′amidinhydrazone; DAPI, 4′,6′-diamidino-2-phenylindole hydrochloride; DFMO, difluoromethylornithine; FITC, fluorescein isothiocyanate; GSH, Glutathione; PBS, phosphate-buffered saline; ROS, reactive oxygen species

Introduction

Spermine (Spm), spermidine (Spd), and the diamine precursor putrescine are ubiquitous, polycationic, aliphatic amines that are absolutely required normal for cell growth. However, only recently have the precise molecular functions in which they are involved begun to be determined. One proposed function for polyamines is that they play a role in protecting cells from reactive oxygen species damage (ROS). Mutant *E. coli* and yeast strains that have a reduced content of polyamines are more sensitive to oxidative damage (Chattopadhyay et al., 2003a, 2006; Jung et al., 2003). Investigations in cell free systems have demonstrated that polyamines protect phospholipid vesicles from both Fenton produced hydrogen peroxide (H_2O_2) and superoxide (Lovaas and Carlin, 1991; Tadolini et al., 1984) and protect DNA from damage caused by various assaulting agents including alkylating compounds (Mackintosh and Pegg, 2000; Rajalakshmi et al., 1978), endonuclease-mediated digestion (Brune et al., 1991), singlet oxygen (Khan et al., 1992), radiation (Chiu and Oleinick, 1997, 1998; Douki et al., 2000; Held and Awad, 1991; Newton et al., 1996, 1997; Spotheim-Maurizot et al., 1995; Sy et al., 1999; Warters et al., 1999), and Fenton produced radicals (Ha et al., 1998a, b). Studies with cells cultured in vitro have been limited to ROS produced by irradiation and these investigations have produced variable results. Some laboratories have found that depletion of endogenous cellular polyamines resulted in increased sensitivity to radiation exposure (Arundel et al., 1988; Courdi et al., 1986; Snyder and Schroeder, 1994; Snyder and Sunkara, 1990; Williams et al., 1994) while other laboratories found no increase in sensitivity (Gerner et al., 1988; Kuo et al., 1987; Seidenfeld et al., 1980). However, these experiments were performed with inhibitors of polyamine biosynthesis that resulted in a decreased putrescine and Spd, but had little effect on endogenous Spm concentrations.

The mechanisms by which polyamines could provide protection include the direct scavenging of reactive agents,

the induction of DNA conformational changes, physical blocking of DNA from interaction with assaulting agents or a combination of these possibilities (Basu et al., 1987; Feuerstein et al., 1986, 1989, 1990; Fujisawa and Kadoma, 2005; Ha et al., 1998a; Hougaard et al., 1988; Warters et al., 1999). Although polyamines may provide protection through multiple mechanisms, little is known about the role of individual polyamines in these processes. Previous work in our laboratory (Ha et al., 1998a) demonstrated that spermine can directly scavenge ROS produced by Fenton reactions in a cell free system. Due to the potential for intimate association of polyamines, especially Spm, with DNA, it is possible that polyamines may provide the primary protection for DNA against exposure to ROS in situ.

The goal of the current study was to investigate the relative requirements for Spm and Spd in protecting cells from exposure to H_2O_2 induced oxidative stress. The effects of such ROS exposure on growth were compared between an embryonic fibroblast cell line lacking the Spm synthase gene (Gy11) and normal fibroblasts (N6) (Mackintosh and Pegg, 2000). Although Gy11 cells are Spm deficient, they contain elevated levels of Spd, resulting in a total polyamine concentration exceeding that of N6 cells. The results of these studies provide in situ evidence supporting a specific role for Spm and Spd in cellular protection from oxidative stress at physiological concentrations and demonstrate differential roles for Spm and Spd in cellular protection from H_2O_2 induced cytotoxicity when intracellular concentration of polyamines are low.

Materials and methods

Chemicals and materials

Glutathione (GSH), DL-Buthionine-[S, R]-sulfoximine (BSO), o-phthaldialdehyde, Spm, Spd, and aminoguanidine (AG) were purchased from Sigma (St. Louis, MO). The ornithine decarboxylase inhibitor α-difluoromethylornithine (DFMO) was a gift from Merrel Dow Research Institute, Cincinnati, OH. The S-adenosylmethionine decarboxylase inhibitor CGP 48,664 was graciously provided by C. W. Porter, Roswell Park Cancer Institute (Buffalo, NY). The polyamine oxidase inhibitor MDL 72,527 was synthesized as previously published (Bey et al., 1985). H_2O_2 (3%) was purchased from Rite Aid Pharmacy (Baltimore, MD). Dulbecco's phosphate-buffered saline (PBS) was purchased from GIBCO (Grand Island, NY).

Cell culture and treatment protocols

Immortalized mouse embryo fibroblast cells were derived from 14 day old embryos of spermine-deficient Gy mutant (Gy11) and normal (N6) male mice (Mackintosh and Pegg, 2000). Cells were cultured in Dulbecco's modified eagle medium (DMEM) supplemented with fetal bovine serum (10%), 2 mM L-glutamine, 100 units/ml penicillin, 100 units/ml streptomycin, and 0.1 mM β-mercaptoethanol.

Gy11 and N6 cells were seeded at 5000 cells/well in 96 well plates and incubated at 37 °C for 24 h. Media were removed and cells were subsequently treated with control media containing the serum amine oxidase inhibitor AG (1 mM), or 1 mM AG and 5 mM DFMO (AD) or for the polyamine depeletion/replenishment experiments with a combination of four inhibitors consisting of 1 mM AG, 5 mM DFMO, 10 μM CGP 48,664, 25 μM MDL 72,527 (ADCM), with 10 μM Spm or 10 μM Spd as indicated for 48 h. Multiple combinations of inhibitors in the presence or absence of Spm or Spd were examined in order to determine the best way to deplete cells of polyamines and create cells through replenishment with exogenous polyamines that contain predominantly Spm or Spd (Table 1). The ADCM inhibitor combination demonstrated the best results. The inclusion of MDL 72,527 helped to prevent the back conversion of Spm to Spd thereby aiding in the creation of cells that contained primarily only Spm or Spd.

For H_2O_2 treatment, medium was replaced with media containing the combination of inhibitors indicated and increasing concentrations

Table 1. Polyamine concentrations (nmol/mg protein)[a] in spermine deficient (Gy11) and normal (N6) fibroblast cells after treatments

Media additions[b]	Gy11 cells				N6 cells			
	Putrescine	Spermidine	Spermine	Total	Putrescine	Spermidine	Spermine	Total
AG	<0.05[c]	83.28	<0.05	83.28	<0.05	29.44	15.23	44.66
AG, DFMO	<0.05	29.10	<0.05	29.10	<0.05	0.48	15.50	15.98
AG, DFMO, MDL 72527	<0.05	18.42	<0.05	18.42	<0.05	0.55	11.03	11.59
AG, DFMO, MDL 72527, Spm	<0.05	38.13	8.42	46.54	<0.05	15.98	16.67	32.65
AG, DFMO, MDL 72527, Spd	<0.05	50.05	<0.05	50.05	<0.05	24.47	15.89	40.35
AG, DFMO, CGP 48664, MDL 72527	<0.05	19.69	<0.05	19.69	<0.05	11.30	4.67	15.97
AG, DFMO, CGP 48664, MDL 72527, Spm	0.57	22.89	14.83	38.29	0.94	21.42	19.00	41.36
AG, DFMO, CGP 48664, MDL 72527, Spd	0.81	61.56	<0.05	62.38	0.99	47.83	2.07	50.89
AG, Spm	<0.05	78.71	0.65	79.36	0.89	44.53	22.70	68.12
AG, Spd	<0.05	78.89 ± 11.61	<0.05	78.89	<0.05	46.97	20.06	67.03

[a] Values represent the mean of at least 2 determinations
[b] Cells were treated with combinations of 1 mM AG, 5 mM DFMO, 10 μM CGP 48,664. 25 μM MDL 72,527, 10 μM Spd or 10 μM Spm for 48 h as shown
[c] 0.05 limit of detection

of H_2O_2 for 24 h. For the evaluation of the roles of GSH and polyamines in cellular protection from ROS, cells were seeded as described above and were incubated at 37 °C for 24 h. Medium was removed and cells were next treated with medium containing 1 mM AG or a combination of inhibitors as indicated above for 24 h and then vehicle or 0.10 mM BSO, an inhibitor of GSH synthesis (Griffith and Meister, 1979) was added and cells were treated for an additional 24 h. Cells were then exposed to increasing doses of H_2O_2 followed 24 h later by cell growth analysis. Cell growth was analyzed with the Promega Cell Proliferation Assay (Madison, WI) according to the manufacturer's protocol.

Polyamine concentration and reduced GSH analysis

Polyamine levels were determined by pre-column dansylation reverse-phase high-performance liquid chromatography as previously reported (Kabra et al., 1986). Where GSH levels were measured, Gy11 and N6 cells were plated at 1.0×10^6 cells per T-75 flask for 24 h followed by treatment with increasing concentrations of BSO for 24 h. Viable cells were determined by trypan blue exclusion and frozen at −80 °C for storage prior to analysis. Samples were thawed on ice and analyzed for GSH levels as previously reported (Hissin and Hilf, 1976).

Analysis of apoptopic cells

Gy11 and N6 cells were plated at 1.0×10^5 cells per well in 6 well plates (for all experimental treatment groups with the exception of ADCM) and at 1.0×10^6 cells per T-75 flask (for ADCM treatment groups) and incubated at 37 °C for 24 h. Media were removed and cells were subsequently treated with control media containing 1 mM AG or the indicated combinations and exposed to 0.5 mM or 1.0 mM of H_2O_2 for 24 h. Trypsinized cells and aspirated medium containing detached cells were isolated, counted using trypan blue exclusion, and processed for terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) analysis using the Apo-direct apoptosis detection kit from BD Biosciences Pharmingen, Phoenix Flow Systems (San Diego, CA) using the manufacturer's protocol. Dual fluorescein (FITC) and 4′,6′-diamidino-2-phenylindole hydrochloride (DAPI) stained cells were subsequently fixed to Sigma Poly-Prep slides coated with L-lysine for fluorescent microscopy analysis of DNA damage and apoptosis.

Slides were then photographed using a Nikon Eclipse E800 fluorescent microscope coupled with a Princeton Instrument 5 MHz Fast Line black and white cool coupled device. Data were analyzed using MetaMorph 5.0 (Universal Imaging, PA). Cells were scored as normal when both DAPI and FITC staining were negative. Cells were scored as DNA damaged when DAPI staining was negative for apoptotic morphology and FITC staining was positive. Cells were scored apoptotic when both DAPI and FITC stained positive. Results represent 10 slides per treatment group ($\geq$250 cells per treatment).

Statistical analysis

Statistical comparisons between treatment groups were performed using one-way ANOVA analyses or Student's *t*-test. Differences were considered significant at $p < 0.05$.

Results

Effect of polyamine content on growth and sensitivity to ROS produced by H_2O_2

As reported previously for cells grown in media without AG (Mackintosh and Pegg, 2000), the control cultures of Gy11 cells had no Spm but a substantial increase in Spd such that the total polyamine concentration in the Gy cells was greater (Table 1). When Gy and N6 cells were treated with increasing concentrations of H_2O_2 for 24 h, Gy11 cells exhibited increased sensitivity. This difference was statistically significant in sensitivity in cells exposed to 1.0 and 2.5 mM H_2O_2 (Fig. 2).

Gy11 and N6 cells were treated for 48 h with the combination of inhibitors ADCM, with no addition, or the addition of 10 μM Spm, or 10 μM Spd to produce cells that contained predominantly Spm or Spd (Fig. 1 and Table 1) and then exposed for an additional 24 h to increasing concentrations of H_2O_2. Gy11 and N6 cells depleted of polyamines by exposure to ADCM showed profound increased sensitivity to exposure to H_2O_2 (Fig. 3). Co-treatment with either Spm or Spd restored cell sensitivity to control levels in both Gy11 (Fig. 3a) and N6 (Fig. 3b) cell lines. As a control, cells were also treated with 1 mM AG, and either 10 μM Spm or 10 μM Spd, followed by cell growth assessment. Treatment of cells with polyamines in this manner did not alter intracellular polyamine concentrations (Table 1) or inhibit growth, and the treated cells exhibited similar sensitivity to ROS as control cells.

Depletion of polyamines by growth in AD (1 mM AG and 5 mM DFMO) was found to be the best combination to produce Gy11 and N6 cells with low levels of only Spd or Spm (Table 1). When treated with this combination, Gy11 cells contained low concentrations of Spd alone whereas N6 cells contained low concentrations of only Spm (Table 1). Gy11 and N6 cells depleted of polyamines by AD demonstrated increased sensitivity to H_2O_2, similar to the response of cells treated with ADCM (Fig. 3). However, N6 cells containing only low levels of Spm were less sensitive to low concentrations of H_2O_2 (= 0.5 mM) than Gy11 cells containing only low levels of Spd suggesting at low concentrations, Spm is more effective than Spd at protecting cells from H_2O_2 effects. It is not possible to interpret experiments involving polyamine add-back to cells in these conditions since polyamine catabolic pathways allow for interconversion of Spm and Spd. Therefore, the ADCM combination was used for the follow-up experiments as the best alternative to achieve cells with predominantly Spm or Spm.

Comparison of the effect of polyamines and GSH on sensitivity to H_2O_2

Gy11 and N6 cells were treated for 24 h with increasing doses of BSO, an inhibitor of gamma glutamyl cysteine

Fig. 1. The polyamine biosynthetic pathway and inhibitors. *ODC* Ornithine decarboxylase; *AdoMetDC* S-adenosylmethionine decarboxylase; *MTA* 5′-methlylthioadenosine; *SSAT* spermidine/spermine N^1-acetyltransferase; *APAO* polyamine oxidase; *SMO* spermine oxidase; *DFMO* α-difluoromethylornithine

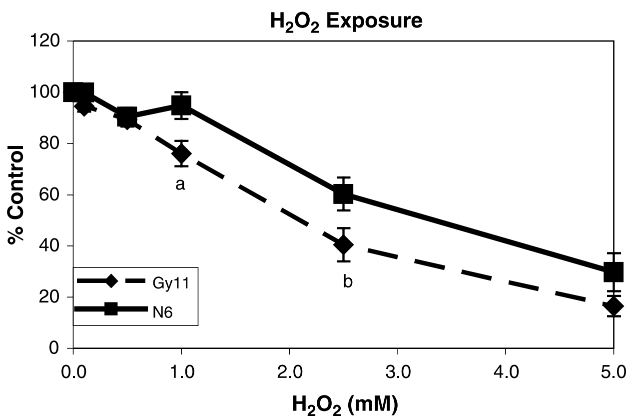

Fig. 2. Cell viability of Gy11 and N6 cells after exposure to H_2O_2. Results are shown for Gy 11 (◆) and N6 (■) cells. Points represent the mean of at least 3 determinations ± standard deviation. [a,b]Indicate a statistical significant difference between Gy11 cells and the N6 in response to the indicated concentrations of H_2O_2 ($p < 0.05$)

synthetase, the rate-limiting enzyme in the synthesis of GSH, followed by GSH analysis. The concentrations of BSO used were not cytotoxic in either cell type (data not shown). BSO treatment resulted in a dose-dependent depletion of GSH to below detectable levels at concentrations ≥0.10 mM (Fig. 4a). Therefore, Gy11 and N6 cells were treated for 48 h with combinations of ADCM in the presence or absence of 10 μM Spm or 10 μM Spd and at 24 h prior to treatment with H_2O_2, cells were treated with 0.10 mM BSO, exposed to increasing doses of H_2O_2 for 24 h, and assessed for growth. Cells depleted of GSH with BSO exhibited increased sensitivity to H_2O_2 exposure similar to the increased sensitivity observed in cells depleted of polyamines. Cells depleted of both GSH and polyamines demonstrated greater sensitivity to H_2O_2 treatment compared with cells depleted of either GSH or polyamines alone (Fig. 4b). Since Gy11 and N6 cells

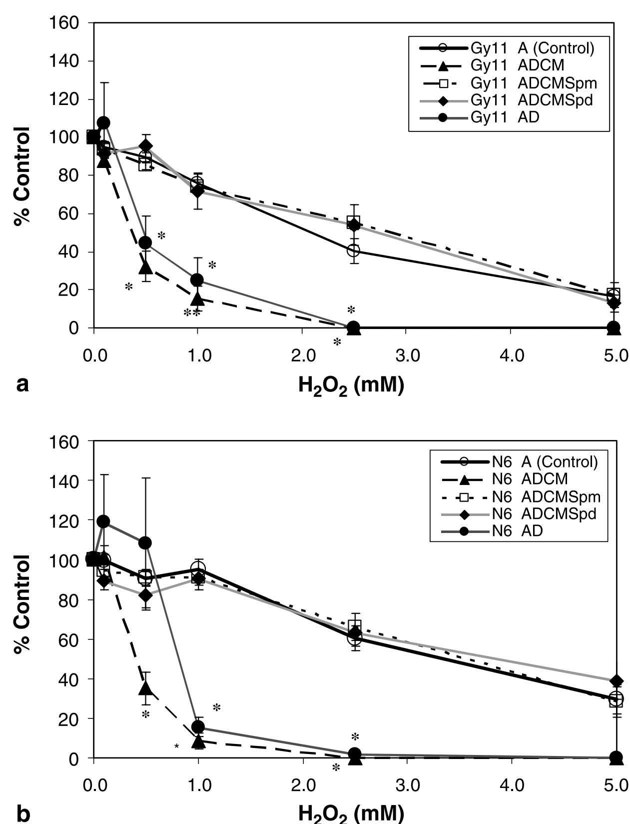

Fig. 3. Effects of polyamine depletion in Gy11 and N6 cells on sensitivity to H_2O_2 exposure. Cells were treated for 48 h with a combination of 1 mM AG (○), 5 mM DFMO + 1 mM AG (AD) (●), 10 μM CGP 48,664, 25 μM MDL 72,527, 5 mM DFMO +1 mM AG (ADCM) (▲) in the absence or presence of 10 μM Spm (□) or 10 μM Spd (◆) as indicated followed by exposure to H_2O_2 for 24 h. **a** Results for Gy11, **b** results for N6 cells exposed to increasing concentrations of H_2O_2. *Indicate statistically significant differences ($p < 0.05$) in **a** between Gy11 cells treated with ADCM (▲) or AD (●) compared to control Gy11 cells (Gy11 A, ○); and in **b** N6 cell treated with ADCM (▲) compared to control N6 cells (N6 A, ○). Note that N6 cells treated with only AD (●) are not significantly different from control cells exposed to 0.5 mM H_2O_2

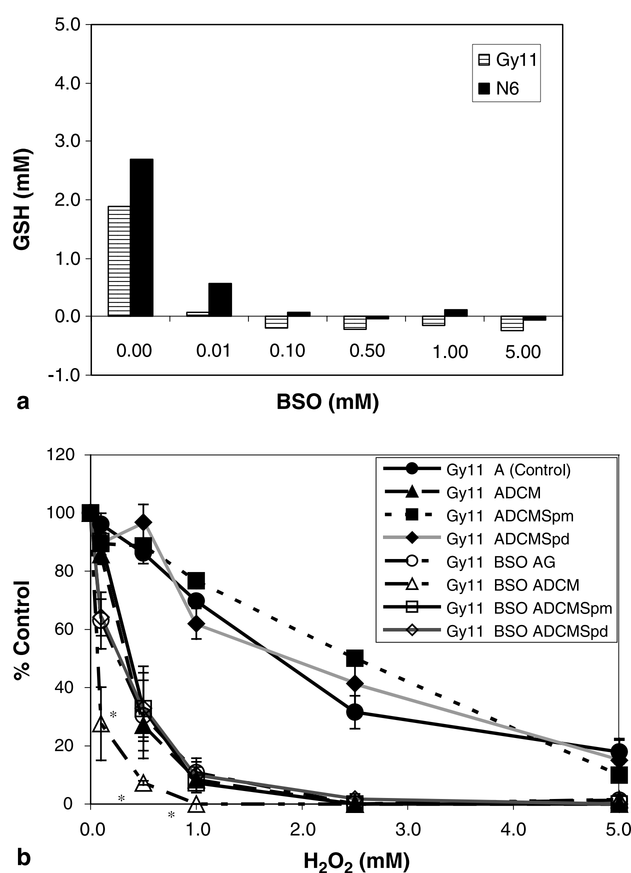

Fig. 4. Effects of GSH and/or polyamine depletion on Gy11 cells after exposure to H_2O_2. **a** GSH levels in control cell cultures treated for 24 h with increasing doses of BSO. Values represent the mean of two determinations. **b** Cell viability of Gy11 cells treated with indicated inhibitors ± BSO followed by exposure to H_2O_2. Cells were treated for 48 h with the ADCM combination in the absence of additional polyamines or the presence of 10 μM Spd or 10 μM Spm as indicated. Twenty-four hours prior to treatment with H_2O_2, cells were treated with 0.1 mM BSO (open symbols) or vehicle (closed symbols) followed by exposure to increasing doses of H_2O_2 for 24 h. Each point represents the mean of 4 determinations ± standard error. *Indicates a statistically significant difference ($p < 0.05$) between cells depleted of both polyamines and GSH with BSO and ADCM (△) compared to cells only depleted of GSH (BSO AG, ○) or compared to cells only depleted of polyamines (ADCM, ▲)

displayed similar results, only Gy11 data are shown. Co-treatment with either Spm or Spd resulted in a restoration of cell viability to the control levels in cells depleted of polyamines, cells depleted of GSH, and cells depleted of both polyamines and GSH. Replenishment with Spm or Spd did not rescue cell growth in GSH depleted cells exposed to H_2O_2.

DNA damage and apoptosis in Spm deficient and normal fibroblasts

In order to determine if the observed changes in growth rates of ROS assaulted cells were accompanied by evidence of DNA damage and/or apoptosis, cells were treated as described above for the specific depletion/replenishment of polyamines and exposed for 24 h to increasing concentrations of H_2O_2. Both DAPI and TUNEL staining were performed for each treatment group; however, only TUNEL stained cells are shown in Fig. 5. Quantification of the DNA damage is shown in Fig. 6. Cells that demonstrated apoptotic DAPI morphology and positive TUNEL staining were scored as apoptotic and cells that demonstrated normal DAPI morphology (not shown) and positive TUNEL staining were scored as DNA damaged. Polyamine depletion results in significant induction in DNA damage

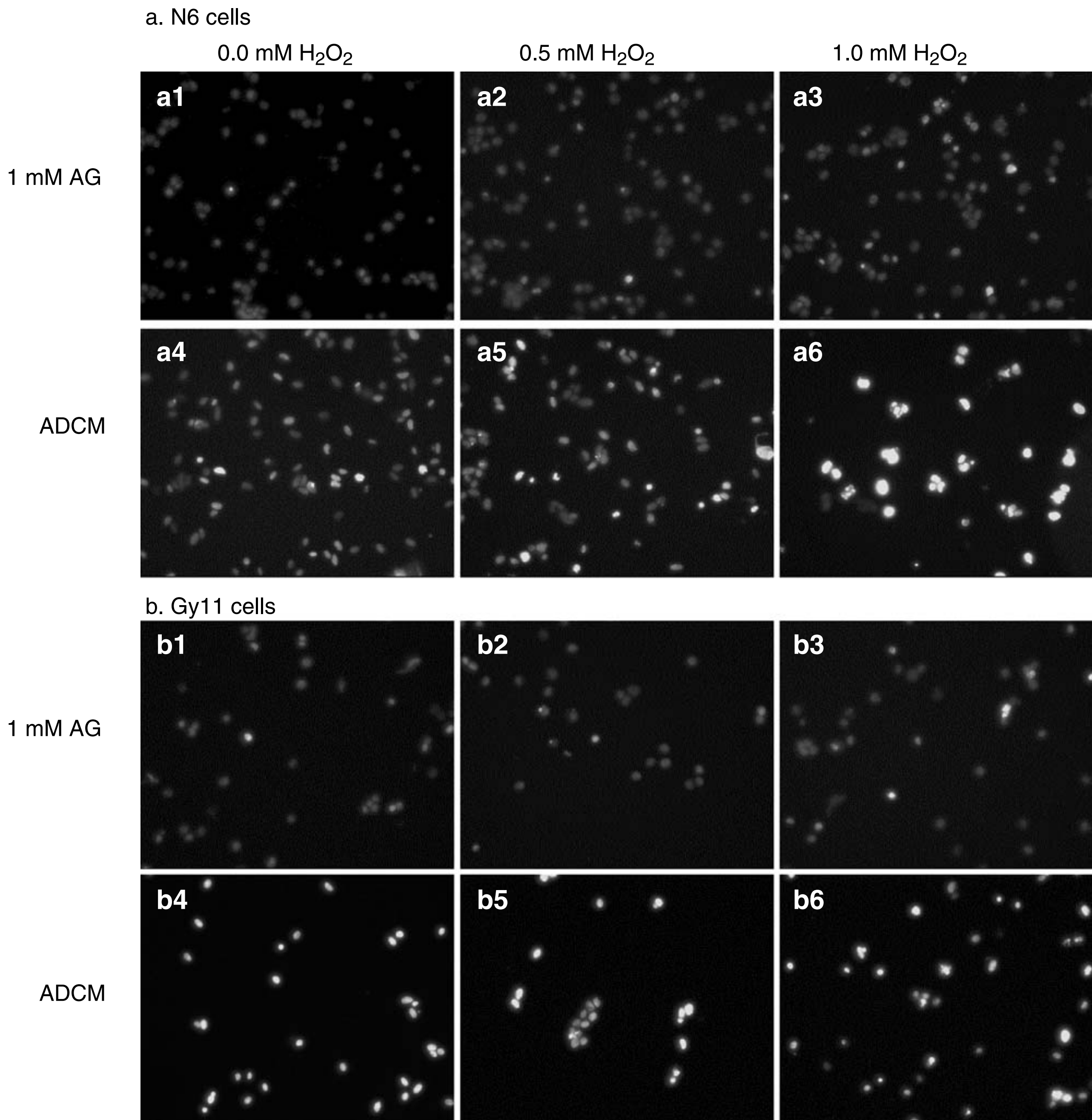

Fig. 5. TUNEL assessment of polyamine depleted N6 and Gy11 cells exposure to H_2O_2. Cells were treated for 48 h with combinations of 1 mM AG (*1–3*) or ADCM (*4–6*) followed by exposure to 0 mM, (*1*, *4*) 0.5 mM H_2O_2 (*2*, *5*) or 1.0 mM H_2O_2 (*3*, *6*) for 24 h as indicated. **a** TUNEL staining of control and polyamine depleted N6 cells following exposure to H_2O_2. **b** TUNEL staining of control and polyamine depleted Gy11 cells following exposure to H_2O_2

and apoptosis in both N6 and Gy11 cells, in concurrence with cell growth data; however Gy11 cells exhibited a differential morphology of damage as compared to N6 cells (not shown). Gy11 cells prior to polyamine depletion exhibited a small increase in TUNEL staining as compared to N6 cells following H_2O_2 exposure. However, polyamine depleted Gy11 cells demonstrated very different apoptotic and DNA damage profiles as compared to the N6 cells.

N6 control cells exposed to increasing doses of H_2O_2 demonstrated a dose-dependent increase in apoptotic cells (Figs. 5a and 6a). N6 cells depleted of polyamines by ADCM exhibited increased sensitivity to increasing doses of H_2O_2, consistent with the growth data presented above.

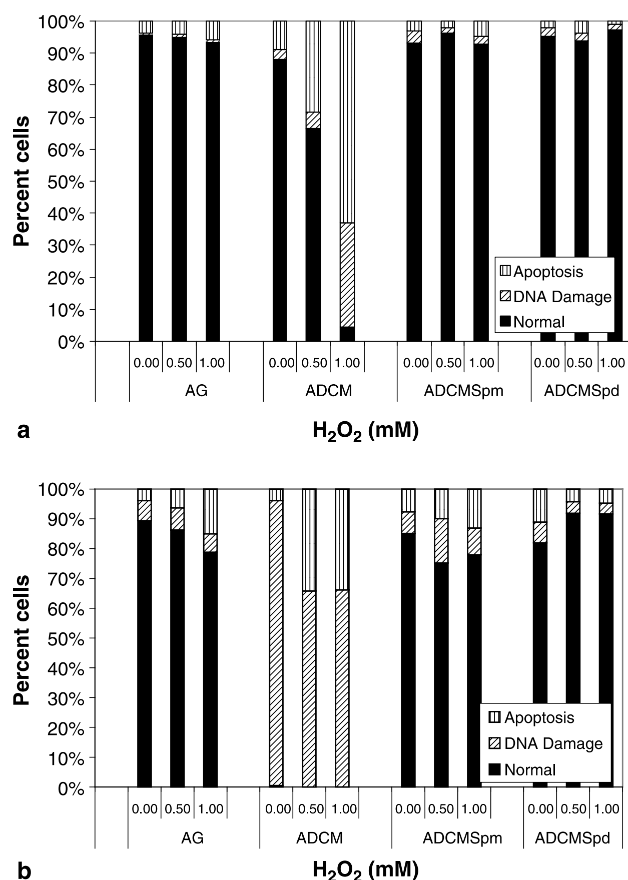

Fig. 6. Apoptosis and DNA damage quantified in polyamine depleted N6 and Gy11 cells following exposure to H_2O_2. **a** N6 cells, **b** Gy11 cells. Treatment with inhibitors, polyamines and H_2O_2 was as described in Fig. 5. Data represent the results of ten slides quantified per treatment group (>250 cells total)

The polyamine depleted N6 cells demonstrated dose dependent increases in both apoptotic and DNA damage scored cells. Interestingly, polyamine depleted N6 cells that were not exposed to exogenous oxidative stress also demonstrated an increased number of apoptotic cells (~10% of cells scored positive for apoptosis) (Fig. 6a). Gy11 cells generally exhibited increased scoring for DNA damage/apoptosis as compared to their N6 counterparts. Control Gy11 cells also exhibited a dose dependent increase in the number of apoptotic and DNA damage scored cells when exposed to H_2O_2 and this was greater than that seen in the comparable N6 cells (Fig. 6a), which was also consistent with the cell growth data presented above. Remarkably, when the Gy11 cells were depleted of polyamines by ADCM, there was greater incidence of DNA damage than in N6 cells and even the polyamine depleted Gy11 cells that were not exposed to exogenous oxidative stress exhibited a near universal staining for DNA damage (~96%) (Fig. 6b). The addition of either Spm or Spd prevented DNA damage and apoptosis and restored cell sensitivity to control levels in both cell types [only quantification data is shown (Fig. 6)].

Discussion

Polyamines have previously been implicated in protecting DNA from oxidative damage in cell free systems. However, the mechanisms underlying this protection and the relative requirements for Spm versus Spd have not been elucidated. The results of the present study suggest that either Spm or Spd can protect against ROS assault when present in normal physiological concentrations, but may function differently when present in lower concentrations with Spm being more potent. This is consistent with studies showing that Spm is a more active scavenger of peroxy radicals (Fujisawa and Kadoma, 2005) and studies with reconstituted systems indicating that Spd could only replace the protective role of Spm only when present at higher molar concentrations (Ha et al., 1998b; Newton et al., 1997). Previous eukaryotic cell studies using polyamine-depleted irradiated cells have provided conflicting results with both increased sensitivity (Arundel et al., 1988; Courdi et al., 1986; Snyder and Schroeder, 1994; Snyder and Sunkara, 1990; Williams et al., 1994), or no effect being reported (Gerner et al., 1988; Kuo et al., 1987; Seidenfeld et al., 1980). These conflicting results may be due to the variable degree of polyamine depletion achieved and to the fact that Spm pools were largely unaltered.

Our studies used fibroblast cells (Gy11) that lack spermine synthase and are devoid of Spm, while containing three times the concentration of Spd resulting in a higher total polyamine concentrations than control (N6) cells (Mackintosh and Pegg, 2000). Before manipulation of their endogenous polyamine pools, the Spm deficient Gy11 cells exhibited a small increase in sensitivity to H_2O_2 compared to their normal counterparts cells. Previous experiments by Nilsson et al. suggested that Spm deficient primary skin fibroblast cells from Gy mice were less sensitive to a single H_2O_2 treatment and more sensitive to UV-C irradiation exposure than normal fibroblast cells (Nilsson et al., 2000). The data presented here with immortalized embryonic fibroblasts suggests Spm deficient Gy11 cells demonstrate similar sensitivity to low doses of H_2O_2 but are more sensitive at higher doses of H_2O_2 than N6 cells. This trend was observed both in cell growth analysis and through TUNEL staining. Overall, the results of the current study suggest that total polyamine concentration, not the Spm level alone, is critical in determining cellular sensitivity to ROS.

To examine the individual requirements of Spm and Spd in cellular protection from ROS, cells were depleted of endogenous polyamine pools using specific combinations of inhibitors of the metabolic pathway followed by exposure to ROS. Gy11 and N6 cells depleted of polyamines by ADCM both demonstrate similar increases in sensitivity to H_2O_2 exposure as evaluated by both cell growth assessment and TUNEL analysis for DNA damage and apoptosis. Gy11 cells exhibited a general increase in DNA damage scored cells as compared to the N6 controls exhibiting nearly universal staining for DNA damage in the absence of exogenous oxidative stress. These data suggest that low levels of Spm are not replaceable by low levels of Spd, even in the absence of exogenous oxidative stress and support the concept that Spm deficiency is leading to continued DNA damage.

The results presented here also suggest that the polyamines play a role in the protection of cells from ROS that is independent of protection provided by GSH. GSH peroxidases have been shown to afford the primary cellular protection from H_2O_2 in many cell types (Agostinelli et al., 1996; Chu et al., 2004; de Haan et al., 1998; Hiraishi et al., 1993; Pietarinen et al., 1995). However, when Gy11 and N6 cells were depleted of both GSH and polyamines they exhibited an increased sensitivity to H_2O_2 exposure over that observed in cells depleted of polyamines or GSH alone indicating that polyamines possess roles distinct from those of GSH in protection from H_2O_2.

The suggestion that spermine oxidase SMO/PAOh1 (Vujcic et al., 2002; Wang et al., 2001) may play a role in the etiology *H. pylori*-induced gastric cancer and potentially other inflammation associated cancers (Babbar and Casero, 2006; Xu et al., 2004) is even more appealing when considered in the context of the results presented here. In the case of *H. pylori*-induced SMO/PAOh1, there is both an increase in H_2O_2 production and a decrease in spermine, both a direct result of the oxidase activity. Combined with the results of Chu et al. (2004) and Lee et al. (2006) demonstrating increased bacteria-induced intestinal cancers in mice with disrupted GSH peroxidase 1 and 2 genes, it seems that polyamines may not only serve as a guard against ROS, but in specific instances may also serve as the source of damaging ROS. The positive charge of polyamines at physiological pH allows close association with negatively charged, nucleophilic macromolecules that typically are the target of electrophilic and oxidative attack. The close association of polyamines with DNA, phospholipids and charged proteins could explain their important and separate role from those provided by GSH in cellular protection from H_2O_2.

It should be noted that although Spd is essential for viability in eukaryotes, lower eukaryotes such as yeast (Chattopadhyay et al., 2003b) and even mice (Lorenz et al., 1998; Mackintosh and Pegg, 2000) and plants (Imai et al., 2004) can survive without Spm. Mice lacking Spm synthase (Gy mice) have a variety of defects including significant growth retardation, a very short life span, deafness and neurological defects including circling behavior, which are corrected by restoring Spm synthase showing that Spm has important but not essential functions in mammals (Lorenz et al., 1998; Wang et al., 2004). The exact targets affected by spermine to bring about these effects are currently unknown but our studies do not support an increased level of oxidative damage since the excess Spd in these mice should compensate for the lack of Spm.

In summary, the data presented support the hypothesis that both Spm and Spd can fulfill the important role of protecting cells from exposure to ROS. Cellular polyamines provide protection from H_2O_2 damage that is distinct from the protective role of GSH, strongly supporting a unique role for polyamines in protection from oxidative stress. DFMO is currently being investigated for use as a chemopreventive agent for multiple types of cancer (Gerner and Meyskens, 2004; Meyskens and Gerner, 1999; Wallace and Caslake, 2001). Other strategies for polyamine depletion have also been suggested for chemoprevention. Although elevated levels of polyamines are associated with promotion during tumorigenesis, our data suggest that low levels of polyamines may make cells more susceptible to oxidative stress and therefore may augment the initiation step of carcinogenesis. Consequently, caution is necessary when considering chemopreventive strategies that include depletion of polyamines particularly Spm. The discovery of the precise roles polyamines play in protection from oxidative stress will further our understanding of the importance of polyamines in cellular growth and differentiation and potentially result in improved clinical efficacy of agents that alter polyamine function and metabolism.

Acknowledgements

This work was supported by NIH grants CA51085, CA98454, ES07141, The Irene and Bernard Schwartz Scholarship, The Patrick C. Walsh Prostate Cancer Research Fund (RAC) and GM 26290 (AEP).

References

Agostinelli E, Przybytkowski E, Averill-Bates DA (1996) Glucose, glutathione, and cellular response to spermine oxidation products. Free Radic Biol Med 20: 649–656

Arundel CM, Nishioka K, Tofilon PJ (1988) Effects of alpha-difluoromethylornithine-induced polyamine depletion on the radiosen-

sitivity of a human colon carcinoma cell line. Radiat Res 114: 634–640
Babbar N, Casero RA (2006) Tumor necrosis factor-α increases reactive oxygen species by inducing spermine oxidse in human lung epithelial cells: a potential mechanism for inflammation-induced carcinogenesis. Cancer Res 66: 11125–11130
Basu HS, Shafer RH, Marton LJ (1987) A stopped-flow H-D exchange kinetic study of spermine-polynucleotide interactions. Nucleic Acids Res 15: 5873–5886
Bey P, Bolkenius FN, Seiler N, Casara P (1985) N-2,3-Butadienyl-1,4-butanediamine derivatives: potent irreversible inactivators of mammalian polyamine oxidase. J Med Chem 28: 1–2
Brune B, Hartzell P, Nicotera P, Orrenius S (1991) Spermine prevents endonuclease activation and apoptosis in thymocytes. Exp Cell Res 195: 323–329
Chattopadhyay MK, Tabor CW, Tabor H (2003a) Polyamines protect Escherichia coli cells from the toxic effect of oxygen. Proc Natl Acad Sci USA 100: 2261–2265
Chattopadhyay MK, Tabor CW, Tabor H (2003b) Spermidine but not spermine is essential for hypusine biosynthesis and growth in Saccharomyces cerevisiae: spermine is converted to spermidine in vivo by the FMS1-amine oxidase. Proc Natl Acad Sci USA 100: 13869–13874
Chattopadhyay MK, Tabor CW, Tabor H (2006) Polyamine deficiency leads to accumulation of reactive oxygen species in a spe2Delta mutant of Saccharomyces cerevisiae. Yeast 23: 751–761
Chiu S, Oleinick NL (1997) Radioprotection against the formation of DNA double-strand breaks in cellular DNA but not native cellular chromatin by the polyamine spermine. Radiat Res 148: 188–192
Chiu S, Oleinick NL (1998) Radioprotection of cellular chromatin by the polyamines spermine and putrescine: preferential action against formation of DNA-protein crosslinks. Radiat Res 149: 543–549
Chu FF, Esworthy RS, Chu PG, Longmate JA, Huycke MM, Wilczynski S, Doroshow JH (2004) Bacteria-induced intestinal cancer in mice with disrupted Gpx1 and Gpx2 genes. Cancer Res 64: 962–968
Courdi A, Milano G, Bouclier M, Lalanne CM (1986) Radiosensitization of human tumor cells by alpha-difluoromethylornithine. Int J Cancer 38: 103–107
de Haan JB, Bladier C, Griffiths P, Kelner M, O'Shea RD, Cheung NS, Bronson RT, Silvestro MJ, Wild S, Zheng SS, Beart PM, Hertzog PJ, Kola I (1998) Mice with a homozygous null mutation for the most abundant glutathione peroxidase, Gpx1, show increased susceptibility to the oxidative stress-inducing agents paraquat and hydrogen peroxide. J Biol Chem 273: 22528–22536
Douki T, Bretonniere Y, Cadet J (2000) Protection against radiation-induced degradation of DNA bases by polyamines. Radiat Res 153: 29–35
Feuerstein BG, Pattabiraman N, Marton LJ (1986) Spermine-DNA interactions: a theoretical study. Proc Natl Acad Sci USA 83: 5948–5952
Feuerstein BG, Pattabiraman N, Marton LJ (1989) Molecular dynamics of spermine-DNA interactions: sequence specificity and DNA bending for a simple ligand. Nucleic Acids Res 17: 6883–6892
Feuerstein BG, Pattabiraman N, Marton LJ (1990) Molecular mechanics of the interactions of spermine with DNA: DNA bending as a result of ligand binding. Nucleic Acids Res 18: 1271–1282
Fujisawa S, Kadoma Y (2005) Kinetic evaluation of polyamines as radical scavengers. Anticancer Res 25: 965–969
Gerner EW, Meyskens FL Jr (2004) Polyamines and cancer: old molecules, new understanding. Nat Rev Cancer 4: 781–792
Gerner EW, Tome ME, Fry SE, Bowden GT (1988) Inhibition of ionizing radiation recovery processes in polyamine-depleted Chinese hamster cells. Cancer Res 48: 4881–4885
Griffith OW, Meister A (1979) Potent and specific inhibition of glutathione synthesis by buthionine sulfoximine (S-n-butyl homocysteine sulfoximine). J Biol Chem 254: 7558–7560
Ha HC, Sirisoma NS, Kuppusamy P, Zweier JL, Woster PM, Casero RA Jr (1998a) The natural polyamine spermine functions directly as a free radical scavenger. Proc Natl Acad Sci USA 95: 11140–11145
Ha HC, Yager JD, Woster PA, Casero RA Jr (1998b) Structural specificity of polyamines and polyamine analogues in the protection of DNA from strand breaks induced by reactive oxygen species. Biochem Biophys Res Commun 244: 298–303
Held KD, Awad S (1991) Effects of polyamines and thiols on the radiation sensitivity of bacterial transforming DNA. Int J Radiat Biol 59: 699–710
Hiraishi H, Yajima N, Yamaguchi N, Ishida M, Katoh Y, Harada T, Terano A, Ivey KJ (1993) Antioxidant protection against oxidant-induced damage in cultured gastric mucosal cells. Gastroenterol Jpn 28 [Suppl 5]: 132–138
Hissin PJ, Hilf R (1976) A fluorometric method for determination of oxidized and reduced glutathione in tissues. Anal Biochem 74: 214–226
Hougaard DM, Del Castillo AM, Larsson LI (1988) Endogenous polyamines associate with DNA during its condensation in mammalian tissue. A fluorescence cytochemical and immunocytochemical study of polyamines in fetal rat liver. Eur J Cell Biol 45: 311–314
Imai A, Akiyama T, Kato T, Sato S, Tabata S, Yamamoto KT, Takahashi T (2004) Spermine is not essential for survival of Arabidopsis. FEBS Lett 556: 148–152
Jung IL, Oh TJ, Kim IG (2003) Abnormal growth of polyamine-deficient Escherichia coli mutant is partially caused by oxidative stress-induced damage. Arch Biochem Biophys 418: 125–132
Kabra PM, Lee HK, Lubich WP, Marton LJ (1986) Solid-phase extraction and determination of dansyl derivatives of unconjugated and acetylated polyamines by reversed-phase liquid chromatography: improved separation systems for polyamines in cerebrospinal fluid, urine and tissue. J Chromatogr 380: 19–32
Khan AU, Di Mascio P, Medeiros MH, Wilson T (1992) Spermine and spermidine protection of plasmid DNA against single-strand breaks induced by singlet oxygen. Proc Natl Acad Sci USA 89: 11428–11430
Kuo WL, Deen DF, Marton LJ, Shafer RH (1987) Filter elution analysis of the effect of alpha-difluoromethylornithine on X-ray-induced DNA damage in 9L cells. Radiat Res 109: 68–77
Lee DH, Esworthy RS, Chu C, Pfeifer GP, Chu FF (2006) Mutation accumulation in the intestine and colon of mice deficient in two intracellular glutathione peroxidases. Cancer Res 66: 9845–9851
Lorenz B, Francis F, Gempel K, Boddrich A, Josten M, Schmahl W, Schmidt J, Lehrach H, Meitinger T, Strom TM (1998) Spermine deficiency in Gy mice caused by deletion of the spermine synthase gene. Hum Mol Genet 7: 541–547
Lovaas E, Carlin G (1991) Spermine: an anti-oxidant and anti-inflammatory agent. Free Radic Biol Med 11: 455–461
Mackintosh CA, Pegg AE (2000) Effect of spermine synthase deficiency on polyamine biosynthesis and content in mice and embryonic fibroblasts, and the sensitivity of fibroblasts to 1,3-bis-(2-chloroethyl)-N-nitrosourea. Biochem J 351: 439–447
Meyskens FL Jr, Gerner EW (1999) Development of difluoromethylornithine (DFMO) as a chemoprevention agent. Clin Cancer Res 5: 945–951
Newton GL, Aguilera JA, Ward JF, Fahey RC (1996) Polyamine-induced compaction and aggregation of DNA – a major factor in radioprotection of chromatin under physiological conditions. Radiat Res 145: 776–780
Newton GL, Aguilera JA, Ward JF, Fahey RC (1997) Effect of polyamine-induced compaction and aggregation of DNA on the formation of radiation-induced strand breaks: quantitative models for cellular radiation damage. Radiat Res 148: 272–284
Nilsson J, Gritli-Linde A, Heby O (2000) Skin fibroblasts from spermine synthase-deficient hemizygous gyro male (Gy/Y) mice overproduce

spermidine and exhibit increased resistance to oxidative stress but decreased resistance to UV irradiation. Biochem J 352: 381–387

Pietarinen P, Raivio K, Devlin RB, Crapo JD, Chang LY, Kinnula VL (1995) Catalase and glutathione reductase protection of human alveolar macrophages during oxidant exposure in vitro. Am J Respir Cell Mol Biol 13: 434–441

Rajalakshmi S, Rao PM, Sarma DS (1978) Studies on carcinogen chromatin – DNA interaction: inhibition of N-methyl-N-nitrosourea-induced methylation of chromatin – DNA by spermine and distamycin A. Biochemistry 17: 4515–4518

Seidenfeld J, Deen DF, Marton LJ (1980) Depletion of intracellular polyamine content does not alter the survival of 9L rat brain tumour cells after X-irradiation. Int J Radiat Biol Relat Stud Phys Chem Med 38: 223–229

Snyder RD, Schroeder KK (1994) Radiosensitivity of polyamine-depleted HeLa cells and modulation by the aminothiol WR-1065. Radiat Res 137: 67–75

Snyder RD, Sunkara PS (1990) Effect of polyamine depletion on DNA damage and repair following UV irradiation of HeLa cells. Photochem Photobiol 52: 525–532

Spotheim-Maurizot M, Ruiz S, Sabattier R, Charlier M (1995) Radioprotection of DNA by polyamines. Int J Radiat Biol 68: 571–577

Sy D, Hugot S, Savoye C, Ruiz S, Charlier M, Spotheim-Maurizot M (1999) Radioprotection of DNA by spermine: a molecular modelling approach. Int J Radiat Biol 75: 953–961

Tadolini B, Cabrini L, Landi L, Varani E, Pasquali P (1984) Polyamine binding to phospholipid vesicles and inhibition of lipid peroxidation. Biochem Biophys Res Commun 122: 550–555

Vujcic S, Diegelman P, Bacchi CJ, Kramer DL, Porter CW (2002) Identification and characterization of a novel flavin-containing spermine oxidase of mammalian cell origin. Biochem J 367: 665–675

Wallace HM, Caslake R (2001) Polyamines and colon cancer. Eur J Gastroenterol Hepatol 13: 1033–1039

Wang X, Ikeguchi Y, McCloskey DE, Nelson P, Pegg AE (2004) Spermine synthesis is required for normal viability, growth, and fertility in the mouse. J Biol Chem 279: 51370–51375

Wang Y, Devereux W, Woster PM, Stewart TM, Hacker A, Casero RA Jr (2001) Cloning and characterization of a human polyamine oxidase that is inducible by polyamine analogue exposure. Cancer Res 61: 5370–5373

Warters RL, Newton GL, Olive PL, Fahey RC (1999) Radioprotection of human cell nuclear DNA by polyamines: radiosensitivity of chromatin is influenced by tightly bound spermine. Radiat Res 151: 354–362

Williams JR, Casero RA, Dillehay LE (1994) The effect of polyamine depletion on the cytotoxic response to PUVA, gamma rays and UVC in V79 cells in vitro. Biochem Biophys Res Commun 201: 1–7

Xu H, Chaturvedi R, Cheng Y, Bussiere FI, Asim M, Yao MD, Potosky D, Meltzer SJ, Rhee JG, Kim SS, Moss SF, Hacker A, Wang Y, Casero RA Jr, Wilson KT (2004) Spermine oxidation induced by Helicobacter pylori results in apoptosis and DNA damage: implications for gastric carcinogenesis. Cancer Res 64: 8521–8525

Authors' address: Robert A. Casero Jr., Johns Hopkins Cancer Research Building, 1650 Orleans St. Room 551, Baltimore, MD 21231, USA, Fax: +1-410-614-9884, E-mail: rcasero@jhmi.edu