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SHORT COMMUNICATION

Induction of Spermidine/Spermine N^1-Acetyltransferase in Human Cancer Cells in Response to Increased Production of Reactive Oxygen Species

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ABSTRACT. Reactive oxygen species (ROS) are involved in a number of disease states where they are believed to be responsible for cellular damage. In this study we examined the effect of ROS generation on polyamine catabolism. Treatment of human breast cancer cells with either H_2O_2 or hyperoxia increased the activity of spermidine/spermine N^1-acetyltransferase (SSAT). These increases occurred before any significant signs of cellular injury. Agents known to decrease the production of reactive oxygen species such as dimethylthiourea and o-phenanthroline prevented the increase in SSAT activity indicating ROS involvement in the induction process. These results suggest that induction of SSAT may be a protective response to oxidative stress in mammalian cells facilitating removal of polyamines from the cell to prevent their toxic accumulation. BIOCHEM PHARMACOL **55**;7:1119–1123, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. Polyamines; reactive oxygen species; acetylation; cancer cells

The polyamines, spermidine, spermine and their precursor diamine, putrescine, are ubiquitous cellular polycations essential for optimal rates of cell growth and differentiation [1–3]. They have highly regulated pathways of both biosynthesis and degradation and this is one feature which distinguishes them from the metal cations in terms of cellular function and availability. The biosynthetic pathway is regulated by the two decarboxylase enzymes, ornithine decarboxylase (ODC: EC 4.1.1.17) and *S*-adenosylmethionine decarboxylase (AdoMetDC: EC 4.1.1.50). Of these, ODC is the more critical, being the rate limiting enzyme in the biosynthetic part of the metabolic pathway [4]. Both decarboxylases are highly inducible by a range of growth promoting stimuli including the serum growth factors. Both enzymes also have rapid turnover rates. A half-life of as little as 12 min has been recorded for ODC [5]. ODC and AdoMetDC both contain PEST sequences which are commonly present in proteins with rapid turnover rates [6].

The rate limiting enzyme for the catabolic or retroconversion pathway of polyamines is spermidine/spermine N^1-acetyltransferase (SSAT)† which like the two decarboxylases has a rapid turnover rate [7] but unlike ODC and AdoMetDC has no PEST sequence [8]. SSAT is readily induced by a number of growth inhibitory or toxic stimuli [9, 10], with increases in activity of several hundred fold (so called superinduction) being observed in some human cells [11]. Among the most potent inducers of SSAT are the polyamine analogues, a group of compounds which have been synthesised to interfere with polyamine metabolism and transport but not to substitute for the natural polyamines in terms of function [12]. At least one of these analogues is now in clinical trial as a potential antitumour agent. Several other anticancer drugs including doxorubicin and 5-fluorouracil also induce SSAT activity [13] suggesting that increased polyamine catabolism may be a general response to growth inhibition. The fact that SSAT can be induced by such a variety of agents argues for a common signal in the induction process. In some cases it appears that increases in calcium may be a signal for the induction of SSAT [14], but changes in intracellular $[Ca^{2+}]$ cannot explain the inducibility by all agents. Some of the anticancer drugs exert their action by the generation of reactive oxygen species (ROS) or free radicals such as the superoxide radical, and it may be that ROS are involved in the induction of SSAT, possibly before any cellular injury is observed. The aim of this study was therefore to determine if oxidative stress caused by increased production of ROS or free radicals can induce polyamine catabolism via increases in SSAT activity and to determine the temporal relationship between this and cellular injury.

MATERIALS AND METHODS

The human breast cancer cell line (MCF-7) was grown in culture under conditions of 5% CO_2: 95% air at 37°. Cells were cultured in Dulbecco's modification of Eagle's medium

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† *Abbreviations:* ROS, reactive oxygen species; SSAT, spermidine/spermine N^1-acetyltransferase.

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(DMEM) supplemented with 10% (v/v) foetal calf serum. Cells were exposed to either hydrogen peroxide (H_2O_2; 200 μM) or hyperoxia (100% oxygen) for 5 hr and 24 hr respectively. Hyperoxic exposures were carried out in a modular incubator chamber which provided a gas tight environment. Chambers were flushed with 100% oxygen for 3 min at a flow rate of 15 L/min. Control cultures were also enclosed in a modular chamber flushed with air.

Cells were harvested mechanically and reduced glutathione and polyamines were extracted in 0.2 M of perchloric acid. Acid-soluble fractions were stored at −20° until analysed. Reduced glutathione content was determined by the fluorimetric method of Hissin and Hilf [15] and polyamines were quantified by HPLC as described previously [10]. Protein content was determined by the method of Lowry *et al.* [16]. SSAT activity was measured in cytosolic fractions as the amount of [^{3}H]acetylspermidine formed as described previously [17]. Individual *N*-acetyl derivatives were identified after separation by HPLC with radiomatic detection [13]. Samples for radiomatic detection were pooled in order to give sufficient radioactivity when individual polyamines were separated in the control samples.

RESULTS

Exposure of MCF-7 cells to hyperoxia (100% oxygen) for 24 hr or to 200 μM of H_2O_2 for 5 hr resulted in significant increases in the total activity of SSAT within the cells (Fig. 1a). Hyperoxia produced approximately a 3-fold increase in total SSAT activity, whereas H_2O_2 produced 6–7 fold increase in total activity. This estimate of the induction of SSAT is always an underestimate as the amount of N^1-acetyltransferase activity in the total activity measured in control cells is less than 100%. In untreated cells, the proportion of acetyltransferase activity attributable to N^1-SSAT was approximately 55% of the total (Fig. 1A). Induction of the SSAT resulted in the proportion of the total acetyltransferase activity being N^1-SSAT increasing to 88% after treatment with H_2O_2 and 98% after hyperoxia (Fig. 1A). Thus in these cells the induction of N^1-SSAT caused by the treatments is closer to 5- and 10-fold respectively (Fig. 1). Little change was observed in the activity of the N^8-SAT (Fig. 1A).

These increases in SSAT activity preceded any cellular injury as measured by changes in protein, reduced glutathione and polyamine content (Table 1). No acetyl polyamines were detected at any time. Toxicity was however observed at longer exposure times to hyperoxia (Fig. 2).

In order to determine whether the induction of SSAT activity was due to the generation of ROS or free radicals, dimethylthiourea (DMTU), a free radical scavenger, and *o*-phenanthroline, an iron chelating agent, were added to the cultures at the same time as H_2O_2. DMTU (10 mM) alone produced a small increase in total acetyltransferase activity but this was not due to increases in N^1-SSAT activity (Fig. 1B). *o*-Phenanthroline (50 μM) did increase N^1-SSAT activity alone by about 2.5-fold but the reason for this is unclear at present. Both agents were able to prevent completely the induction of SSAT activity caused by exposure to H_2O_2 (Fig. 1B). Neither DMTU nor o-phenanthroline had any significant effect on cell growth over the exposure time (Table 1).

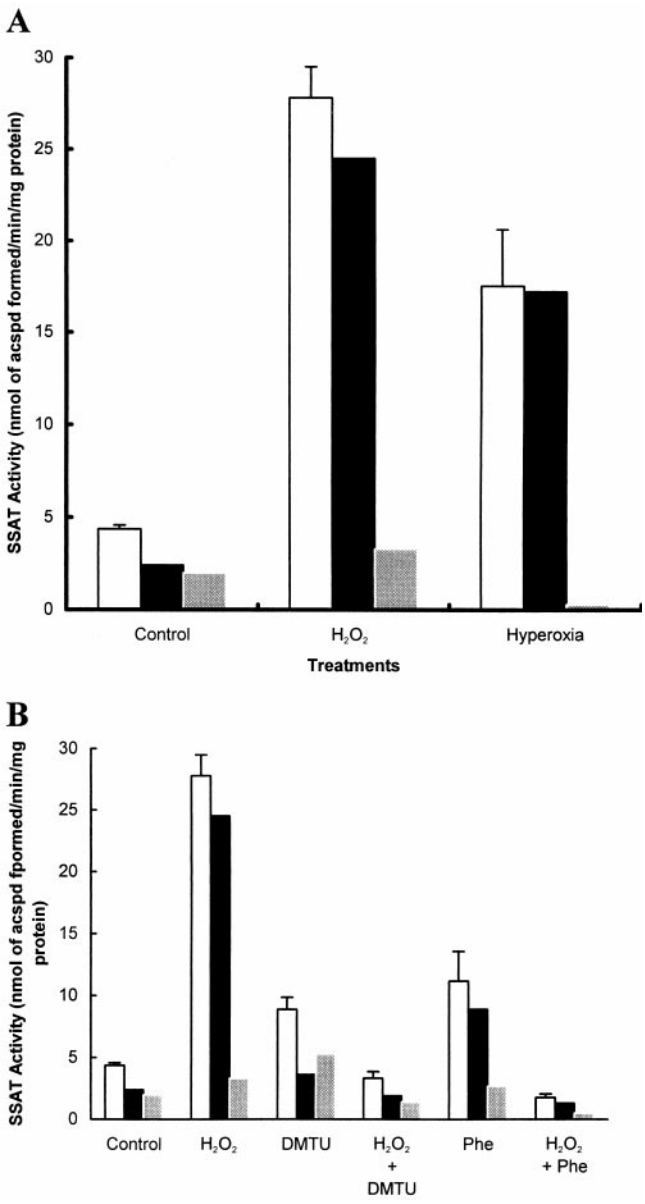

FIG. 1. Effect of treatment of MCF-7 cells with hydrogen peroxide (200 μM), with or without DMTU and o-phenanthroline, for 5 hr or hyperoxia (100% oxygen) for 24 hr on SSAT activity. Cells were seeded at 2.5 × 10^4 cells/cm^2 and grown for 96 hr before treatment with 200 μM of H_2O_2 or hyperoxia. Cells were exposed to treatments for 5 hr and 24 hr respectively in the presence or absence of 10 mM of DMTU or 50 μM of *o*-phenanthroline (Phe). At the appropriate time cells were mechanically harvested and cytosolic fractions prepared. Total SSAT activity (□) was measured in the cytosol and N^1- (■) and N^8- (▨) acetyltransferase activities were determined in pooled samples after separation of the individual acetyl polyamines by HPLC [29]. Results are mean ± SD (N = 3 with 3 replicates per experiment).

TABLE 1. Effect of hyperoxia, exposure to H_2O_2, DMTU and *o*-phenanthroline on growth of MCF-7 cells

	Treatment							
Growth Parameter	None	Hyperoxia	None	H_2O_2 (200 μM)	DMTU	*o*-phe	H_2O_2 + DMTU	H_2O_2 + *o*-phe
Protein content (mg/culture)	1.6 ± 0.1	1.5 ± 0.1	1.2 ± 0.06	1.2 ± 0.02	1.07 ± 0.06	1.22 ± 0.10	1.10 ± 0.07	1.28 ± 0.05
GSH content (nmol/mg protein)	46.5 ± 4.9	59.4 ± 6.7	34.5 ± 1.4	35.7 ± 7.1	41.3 ± 7.0	49.6 ± 7.6	40.8 ± 6.0	39.1 ± 5.9
Polyamine content (nmol/mg protein)	31.3 ± 3.9	29.3 ± 8.8	27.2 ± 2.2	21.7 ± 5.6	ND	ND	ND	ND

Cells were seeded at 2.5×10^4 cells/cm^2 in Hepes buffered medium and allowed to attach for 16 hr. Cells were grown for a further 96 hr before exposure to either normoxia (21% oxygen), hyperoxia (100% oxygen) for 24 hr or 200 μM H_2O_2 for 5 hr either in the presence or absence of DMTU or *o*-phenanthroline (*o*-phe). Intracellular protein, glutathione and polyamine content were determined as described in the materials and methods. Results are mean ± SD ($N = 4$). ND = not determined.

DISCUSSION

In order to inhibit growth, cells must effectively lose growth factors. The way in which this can be achieved in terms of polyamines is by excretion of acetylated polyamines from the cells to the culture medium [18]. Before this can happen, however, the higher polyamines, spermidine and spermine, need to be acetylated in the N^1-position by the intracellular enzyme, spermidine/spermine N^1-acetyltransferase and, in the case of spermine, oxidised by polyamine oxidase (PAO). Therefore an increase in the activity of SSAT may be an initiating signal for the cessation of cell growth. Indeed the short half life of this enzyme and its inducibility in response to treatment with cytotoxic agents such as carbon tetrachloride [19] suggests that this is the key enzyme in regulating the polyamine response to down regulation of growth. There are other polyamine acetyltransferases in mammalian cells [20] including the acetyltransferase with specificity for the N^8-amino group of spermidine but as the activity of this enzyme does not change significantly in this study it seems to be of less importance in the regulation of cell growth. In one study in melanoma cells it has been suggested that the relative inducibility of SSAT is a determinant of the sensitivity of the cells to toxic agents [21]. More recently, induction of SSAT has been associated with cell death by apoptosis [22] implying a role for the polyamines in the regulation of cell death as well as cell growth processes. The agents used in that study do however influence polyamine metabolism in their own right and so in the present study we set out to investigate the effect of toxic stimuli, which are not reported to specifically interfere with polyamine metabolism, namely ROS, on the induction of SSAT and its relationship to cellular injury.

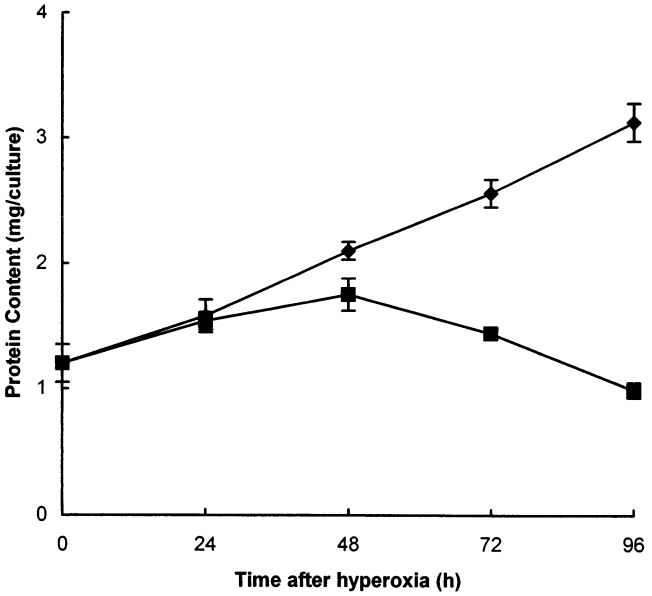

FIG. 2. Effect of long term hyperoxic exposure on the growth of MCF-7 cells. Cells were seeded at 2.5×10^4 cells/cm^2 and grown for 96 hr before exposure to hyperoxia for 24 hr. Cells were exposed to treatment in Hepes buffered medium with a medium change every 48 hr. Cells were harvested mechanically and protein extracted and dissolved in alkali and quantified by the method of Lowry *et al.*, [16]. Values are mean ± SD ($N = 4$). (◆) Control; (■) Hyperoxia.

ROS and the derived free radicals have been implicated in the pathogenesis of a number of disease states including both breast and colon cancer [23, 24] and are known to cause injury to cells when present in excess [25]. Hyperoxia (100% oxygen) is thought to produce increased amounts of superoxide radical and H_2O_2 at the intracellular sites of ROS formation [25, 26] and it is these species which produce the ensuing cellular injury. Although H_2O_2 *per se* is not a free radical, radicals can be generated by both Haber Weiss and Fenton reactions. Under conditions where no obvious cellular injury was present (Table 1) both hyperoxia and H_2O_2 produced significant induction of SSAT (Fig. 1a). Since both *o*-phenanthroline and DMTU prevented the increase in SSAT activity induced by H_2O_2 (Fig. 1b) it suggests strongly that ROS are responsible for the induction of SSAT activity and that increases in SSAT are an early indication of potential cellular injury. Preliminary work from our laboratory indicates that treatment of these cells with H_2O_2 results in a significant increase in DNA fragmentation which is one of the classic signs of

apoptotic cell death thus supporting a role for H_2O_2 and SSAT induction in cell death. The induction of SSAT by *o*-phenanthroline alone may be due to the toxicity of this compound. Although short exposures, as used in this study, were not toxic *o*-phenanthroline was toxic after longer exposures (results not shown).

It is worth noting that H_2O_2 is a product of the other polyamine catabolic reaction catalysed by polyamine oxidase (PAO). It has been suggested in embryonic development that H_2O_2 produced via PAO activity is responsible for programmed cell death [27]. Therefore increases in SSAT activity will produce increased acetyl polyamine derivatives which are the preferred substrates for PAO. Oxidation of these acetyl polyamines by PAO will produce H_2O_2 which in turn will induce SSAT activity (Fig. 1a) thus PAO and SSAT activities essentially produce a cell death generating system. Effectively this cycle of acetylation and oxidation will amplify the oxidative stress induced in the cell and hence produce a high local concentration of H_2O_2, possibly high enough to induce an apoptotic type of cell death.

In summary therefore, ROS derived from hyperoxia or H_2O_2 induce a rise in the activity of SSAT in human breast cancer cells. This increase may be an initiating signal for apoptosis and is likely to be protective to the cell in that it will prevent the over accumulation of polyamines under conditions where cell growth is prevented. Increased SSAT activity will increase the acetyl polyamine derivatives produced and since these were not detected within the cells they are presumably further metabolised or released into the extracellular medium. In this way the cell is protected from the otherwise cytotoxic effects of polyamine overload [28].

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