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INDUCTION OF SPERMIDINE/SPERMINE N^1-ACETYLTRANSFERASE IN HUMAN BREAST CARCINOMA CELLS

A POSSIBLE ROLE FOR CALCIUM

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Abstract—Methylglyoxal bis(guanylhydrazone) (MGBG) a structural analogue of spermidine produced a dose-dependent induction of cytosolic spermidine/spermine N^1-acetyltransferase (N^1-SAT) in the human breast carcinoma cell line, T47-D. Intracellular accumulation of MGBG was found to be saturable and the drug produced characteristic effects on intracellular polyamines, decreasing spermidine and spermine content, with concomitant increases in putrescine levels. The MGBG-induced increase in N^1-SAT activity was potentiated by both tetronasin, a calcium ionophore, and felodipine, a calcium channel blocking agent. Only tetronasin was an active inducer of the enzyme when used alone. Both drugs influenced intracellular MGBG content but in opposite directions: tetronasin increased MGBG content while felodipine decreased it. Therefore, the potentiation of N^1-SAT induction is not simply the result of increased intracellular accumulation of MGBG but is more likely to be related to the concentration of intracellular free calcium in these cells.

The naturally occurring polyamines, putrescine, spermidine and spermine, are essential for cellular growth and differentiation in both eukaryotic and prokaryotic cells [1, 2]. The intracellular polyamine content and the activity of the enzymes of the biosynthetic pathway are regulated precisely according to growth status, such that high polyamine concentrations are associated with rapid rates of cell proliferation whilst low concentrations are found in quiescent cells [3]. Polyamines are also essential mediators of hormonally stimulated breast cancer cells *in vivo* and *in vitro* [4, 5] with the activity of ornithine decarboxylase, a key polyamine biosynthetic enzyme, being stimulated by oestrogens in human breast cancer cells *in vivo* and *in vitro* [6]. Elevated concentrations of the polyamines, and in particular acetylspermidine, have been found in malignant breast tissue in comparison to normal tissue [7]. This suggests that polyamine metabolism, specifically polyamine acetylation, may be altered in breast cancer.

In cancer chemotherapy, agents which are able to manipulate intracellular polyamine content may be useful antitumour agents. A number of polyamine analogues which are able to affect polyamine metabolism without substituting for the polyamines in their growth function have been developed recently with promising results [8–10]. Methylglyoxal bis(guanylhydrazone) (MGBG†), a structural analogue of spermidine, is one such agent. It is a potent antiproliferative drug thought to act via effects on polyamine metabolism, although its use in man is limited by its toxicity [11]. MGBG is a competitive, reversible inhibitor of *S*-adenosylmethionine decarboxylase decreasing spermidine and spermine synthesis with a concomitant increase in putrescine levels [12]. MGBG is known to inhibit competitively spermidine and spermine uptake by virtue of the fact that it shares the same transport system(s) [13, 14]. In addition, MGBG has effects on polyamine catabolism, including inhibition of diamine oxidase [15] and induction of cytosolic spermidine/spermine N^1-acetyltransferase (N^1-SAT†), the rate-limiting enzyme in the degradation and interconversion of the polyamines [16, 17]. The mechanism of this N^1-SAT induction has been reported to be a combination of *de novo* RNA and protein synthesis, and stabilization of the enzyme protein against proteolytic degradation [17, 18]. The inducibility of N^1-SAT in response to a number of growth inhibitory or toxic insults including carbon tetrachloride, thioacetamide and dialkylnitrosamines suggests there may be a common intracellular signal to increase the enzyme activity. However, the cellular signal responsible for N^1-SAT induction is not known.

One intracellular second messenger which has been studied as a potential mediator of the induction stimulus is calcium [19]. A rise in cytosolic free calcium (0.01–1.0 to approx. 10 μM) is an early activation event in a wide variety of cellular responses, including increases in metabolism, growth and differentiation [20]. There have also been a

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† Abbreviations: MGBG, methylglyoxal bis(guanylhydrazone); N^1-SAT, spermidine/spermine N^1-acetyltransferase.

number of observations suggesting a role for the polyamines in voltage-dependent and receptor-mediated signal transduction and calcium mobilization [21].

The aim of this study was to investigate the mechanism by which polyamine acetylation is induced by polyamine analogues, namely MGBG, and to determine whether agents which influence intracellular calcium concentrations play a role in the induction process in breast cancer cells.

MATERIALS AND METHODS

Dulbecco's modification of Eagle's medium, foetal calf serum, penicillin and streptomycin were all from Life Technologies (Paisley, U.K.). Tissue culture plastics were from Sterilin Ltd (Middlesex, U.K.).

Methylglyoxal bis(guanylhydrazone) dihydrochloride (MGBG), acetyl-coenzyme A and insulin were from the Sigma Chemical Co. (Poole, U.K.). The calcium ionophore, tetronasin, was a kind gift from Coopers Animal Health Ltd. Felodipine was supplied by AB Astra Molndal (Sweden). [^{3}H]-Acetyl-coenzyme A (sp. radioact. 2.5 Ci/mmol) was purchased from Du Pont U.K. Ltd (Herts, U.K.).

Cell culture. T47-D cells, a human breast carcinoma line (European collection of animal cell cultures, Porton Down, U.K.), were grown routinely at 37° in monolayer culture in Dulbecco's medium supplemented with 10% (v/v) foetal calf serum and insulin 8 μg/mL, in a humidified atmosphere of air/CO_2 (19:1). For experiments, the cells were seeded at a density of 2.5×10^4 cells/cm^2 on 10 cm diameter plates and grown for 16 hr. All drug/vehicle treatment were given for 48 hr. Cells were harvested mechanically with a "rubber policeman". Cell cultures were checked routinely for bacterial and fungal contamination.

Measurement of polyamines and protein. Polyamines were extracted in 0.2 M $HClO_4$ and quantified by HPLC as described previously [22]. The acid-insoluble cell pellet was resuspended in 0.3 M NaOH, and used for the determination of protein using bovine serum albumin as a standard [23].

Measurement of N^1-SAT. Acetyltransferase activity was determined as described previously [24]. Cytosolic extracts were prepared and assayed immediately for acetyltransferase activity. Each assay contained in final concentration 3 mM spermidine; 100 mM Tris–HCl, pH 7.8; 25 μM [^{3}H]-acetyl-CoA and 0.2–1.0 mg of protein. Separation and identification of the individual products of the acetyltransferase assay was achieved using the HPLC method with radiomatic detection.

Basal cytosolic SAT activity is the uninduced activity and consists of both N^1- and N^8-SAT activities with the latter predominating. Inducible cytosolic SAT activity is almost exclusively N^1-SAT.

Measurement of intracellular MGBG content. MGBG content was measured by HPLC after acid extraction with 0.2 M $HClO_4$ according to the method of Wallace and Cameron [25].

RESULTS

T47-D cells treated with MGBG (10–100 μM) produced a dose-dependent increase in cytosolic SAT activity at 48 hr, with a maximal 100-fold stimulation of N^1-SAT activity after exposure to 50 μM MGBG (Fig. 1). Low concentrations of MGBG (0.1 and 1 μM) had little effect on cytosolic SAT activity (Fig. 1). MGBG was detected intracellularly at all concentrations tested with a concentration gradient of approximately 13-fold forming across the cell membrane. The maximum intracellular content at 48 hr was 47.25 ± 4.17 nmol/mg of protein, after exposure to 50 μM MGBG. Higher concentrations of MGBG had no further effect on the accumulation of the drug (Table 1). Treatment with 100 μM MGBG caused 39% inhibition of cell growth, as determined by a reduction in cellular protein content (Table 1). Characteristically, the total polyamine content of MGBG-treated cells decreased compared to untreated cells, with reduction in the intracellular content of spermidine and spermine. An increase in intracellular putrescine was observed with MGBG treatment (0.1–10 μM); higher concentrations of the drug decreased putrescine content (Table 1).

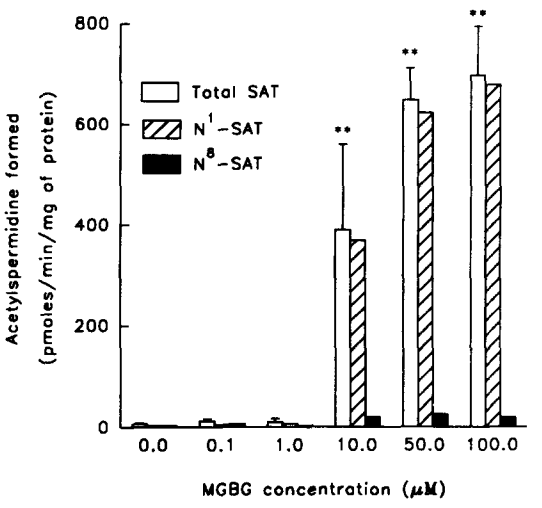

Fig. 1. Effect of MGBG on the SAT activity in T47-D cells. Cells were seeded at a density of 2.5×10^4 cells/cm^2 and grown for 16 hr then exposed to either 0.9% saline or 0.1–100 μM MGBG for 48 hr. The cells were harvested mechanically and the cytosolic extract assayed for acetyltransferase activity. Triplicate products of the assay were pooled and analysed by HPLC with radiomatic detection to determine the proportion of N^1- and N^8-acetylspermidine formed as described in the experimental section. Results are means ± SD (N = 3). In comparison with control, $P < 0.05$*, $P < 0.01$**, using ANOVA with Dunnett's test.

The calcium ionophore, tetronasin (0.1–2.5 μg/mL), produced a dose-dependent increase in total SAT activity after 48 hr (Fig. 2). A maximal 20-fold stimulation of N^1-SAT activity was achieved with 2.5 μg/mL tetronasin (Fig. 2). N^8-SAT activity, at all the concentrations of tetronasin examined, represented <25% of the total cytosolic SAT activity, in comparison to untreated cells where it represented

Table 1. Intracellular MGBG and polyamine content in T47-D cells

Treatment (μM MGBG)	MGBG content (nmol/mg protein)	Protein content (mg/plate)	Polyamine content			
			Putrescine	Spermidine (nmol/mg of protein)	Spermine	Total polyamines
0	0	0.65 ± 0.04	2.97 ± 0.49	8.25 ± 0.71	14.28 ± 1.18	25.50 ± 2.38
0.1	4.82 ± 1.28	0.57 ± 0.02	20.58 ± 0.72†	9.01 ± 0.61*	12.28 ± 0.73†	41.87 ± 2.06†
1.0	6.12 ± 0.77	0.61 ± 0.06	18.87 ± 0.69†	3.89 ± 0.25†	8.27 ± 0.11†	31.03 ± 1.05†
10	36.15 ± 2.11	0.43 ± 0.01	3.71 ± 0.28*	3.35 ± 0.08†	10.18 ± 0.26†	17.24 ± 0.62†
50	47.25 ± 4.17	0.38 ± 0.03	0.81 ± 0.20†	3.12 ± 0.33†	9.28 ± 0.94†	13.21 ± 1.47†
100	48.90 ± 2.86	0.40 ± 0.04	ND†	3.33 ± 0.47†	9.04 ± 1.23†	12.37 ± 1.70†

Cells were grown for 16 hr then exposed to 0.1–100 μM MGBG for 48 hr. Control cells were treated with 0.9% saline. Cells were harvested and the polyamines and MGBG were extracted in 0.2 M $HClO_4$, and quantified by HPLC [22, 25].
Results are means ± SD (N = 4).
ND, not detected.
In comparison with control, $P < 0.05$*, $P < 0.01$†, using ANOVA with Dunnett's test.

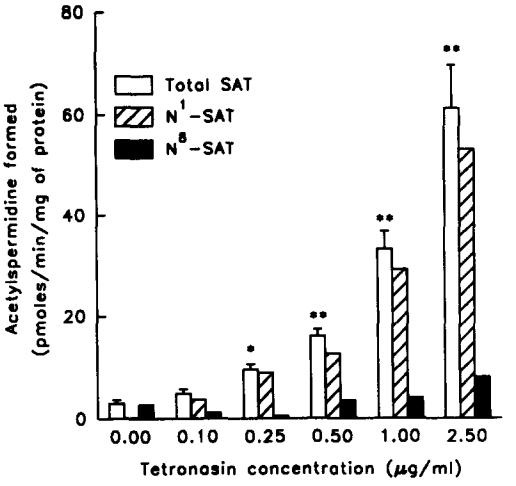

Fig. 2. Effect of tetronasin on the SAT activity in T47-D cells. Cells were seeded at a density of 2.5×10^4 cells/cm^2 grown for 16 hr then exposed to either absolute ethanol or 0.1–2.5 μg/mL tetronasin for 48 hr. The cells were harvested mechanically and the cytosolic extract assayed for acetyltransferase activity. Triplicate products of the assay were pooled and analysed by HPLC with radiomatic detection to determine the proportion of N^1- and N^8-acetylspermidine formed as described in Materials and Methods. Results are means ± SD (N = 3). In comparison with control, $P < 0.05$*, $P < 0.01$**, using ANOVA with Dunnett's test.

>90% of the total SAT activity (Fig. 2). Higher concentrations of tetronasin also induced SAT activity but were cytotoxic. Tetronasin at 1 μg/mL was the chosen concentration for further studies. When used in combination with MGBG, tetronasin potentiated the induction by approximately 2-fold (Table 2). Induction of N^1-SAT activity by MGBG was also potentiated by the dihydropyridine calcium channel antagonist, felodipine, but in contrast to the ionophore, felodipine had no effect on the enzyme activity (Table 3).

Analysis of intracellular MGBG content showed that 1 μg/mL tetronasin increased the accumulation of MGBG by 80%, while 25 μM felodipine resulted in a 20% reduction in accumulation (Table 4).

Total intracellular polyamine content was decreased by all treatments in comparison to untreated, control cells (Table 5). In the presence of felodipine and MGBG there was a further decrease in polyamine content in comparison to MGBG alone. In contrast, tetronasin plus MGBG had no additional effect on spermidine and spermine levels; however, the intracellular putrescine content was further increased in comparison to MGBG alone (Table 5).

DISCUSSION

The inducibility of SAT has been well documented [2]. However the cellular signal(s) for this induction are not well understood. In this study we examined the possible involvement of calcium in the induction

Table 2. Effect of tetronasin on the SAT activity by MGBG in T47-D cells

Treatment	SAT activity (pmol/min/mg of protein) Total SAT	N^1-SAT	N^8-SAT
Saline	7.64 ± 1.99	3.04	4.60
Ethanol	11.36 ± 2.42	1.13	10.23
Tetronasin	25.89 ± 6.42	25.89	0.00
MGBG	348.61 ± 32.11	325.24	23.37
MGBG/tetronasin	600.01 ± 73.45*	577.14	22.87

Cells were seeded at a density of 2.5×10^4 cells/cm^2 and grown for 16 hr then exposed to either 1 μg/mL tetronasin, 50 μM MGBG or the combination for 48 hr. Two separate controls were analysed, one with 0.9% saline the other with absolute ethanol. The cells were harvested mechanically and assayed immediately for cytosolic acetyltransferase activity. Triplicate products of the assay were pooled and analysed by HPLC with radiomatic detection to determine the proportion of N^1- and N^8-acetylspermidine formed [24].

Results are means ± SD (N = 3).

In comparison with MGBG alone, $P < 0.01$*, using ANOVA with Dunnett's test.

Table 3. Effect of felodipine on the induction of SAT activity by MGBG in T47-D cells

Treatment	SAT activity (pmol/min/mg of protein) Total SAT	N^1-SAT	N^8-SAT
Saline	9.75 ± 1.14	8.66	1.09
Felodipine	4.38 ± 1.16	1.58	2.80
MGBG	450.60 ± 45.09	450.60	0.00
MGBG/felodipine	786.63 ± 44.73*	774.81	11.82

T47-D cells were seeded at a density of 2.5×10^4 cells/cm^2 and grown for 16 hr then exposed to either 25 μM felodipine, 50 μM MGBG or the combination for 48 hr. Control cells were treated with 0.9% saline. The cells were harvested mechanically and assayed immediately for acetyltransferase activity. Triplicate products of the assay were pooled and analysed by HPLC with radiomatic detection to determine the proportion of N^1- and N^8-acetylspermidine [24].

Results are means ± SD (N = 3).

In comparison with MGBG alone, $P < 0.01$*, using ANOVA with Dunnett's test.

process, using a number of pharmacological agents which influence calcium homeostasis.

MGBG is a potent inducer of cytosolic N^1-SAT activity in T47-D cells. However, low concentrations of MGBG had no effect on SAT activity after 48 hr, despite detectable intracellular accumulation of the drug (Fig. 1, Table 1). This implies that there may be a critical threshold value required for the initiation of the induction process. The intracellular accumulation of MGBG was shown to be a saturable process (Table 1). This is in agreement with previous reports, suggesting uptake is via a carrier-mediated transport system.

Increasing intracellular calcium concentration with a specific calcium ionophore induced cytosolic N^1-SAT activity (Fig. 2). This finding is in agreement with previous reports that the calcium ionophore, A23187 (0.1 μg/mL), was able to induce an approximately 4-fold increase in SAT activity in bovine lymphocytes [19]. Whether the induction is attributable to the effect of high levels of intracellular calcium, or is a direct effect of calcium on the regulation of the enzyme is not known. The potentiation of MGBG-induced N^1-SAT activity by both tetronasin and felodipine is difficult to explain

Table 4. Effect of felodipine and tetronasin on the accumulation of MGBG in T47-D cells

Treatment	Intracellular MGBG content (nmol/mg of protein)	(% of control)
MGBG	56.28 ± 2.83	100
MGBG/felodipine	45.29 ± 2.45*	81
MGBG/tetronasin	100.80 ± 8.83†	180

T47-D cells were grown for 16 hr then exposed to 50 μM MGBG alone or in combination with either 25 μM felodipine or 1 μg/mL tetronasin for 48 hr. MGBG was extracted in 0.2 M $HCLO_4$ and quantified by HPLC [25].

Results are means ± SD (N = 4).

In comparison with MGBG alone, $P < 0.05$*, $P < 0.01$†, using ANOVA with Dunnett's test.

Table 5. Effect of MGBG alone and in combination with felodipine and tetronasin on the polyamine content of T47-D cells

Treatment	Polyamine content			
	Putrescine	Spermidine	Spermine	Total polyamines
	(nmol/mg of protein)			
Control	1.89 ± 0.57	7.65 ± 2.01	16.55 ± 1.49	26.09 ± 4.07
MGBG	1.13 ± 0.04	3.74 ± 0.14†	11.91 ± 1.24*	16.78 ± 1.42†
MGBG/felodipine	0.66 ± 0.44†	2.70 ± 0.35†	8.23 ± 1.23†	11.59 ± 2.02†
MGBG/tetronasin	2.27 ± 0.40‡	4.40 ± 0.25†	11.28 ± 2.97†	17.95 ± 3.62†

T47-D cells were grown for 16 hr then exposed to 50 μM MGBG alone or in combination with either 25 μM felodipine or 1 μg/mL tetronasin for 48 hr. Control cells were treated with 0.9% saline. Cells were harvested and the polyamines extracted and quantified by HPLC [22].

Results are means ± SD (N = 4).

In comparison with control, $P < 0.05$*, $P < 0.01$†; in comparison with MGBG alone, $P < 0.01$‡, using ANOVA with Dunnett's test.

(Tables 2 and 3). One possibility is that induction of N^1-SAT activity is the result of enhanced MGBG accumulation. However, analysis of the intracellular MGBG content of the treated cells revealed that whilst tetronasin did enhance the accumulation of MGBG, it was decreased in the presence of felodipine (Table 4). This conflicting result indicates that the induction is not purely related to drug accumulation and may be attributable to other effects of these agents most likely on calcium availability. Tetronasin is known to effect directly intracellular calcium [26]. The calcium channel antagonist, felodipine, in contrast reduces intracellular calcium availability. However, felodipine has also been reported to possess calmodulin inhibitory activity [27, 28]. Therefore, the cellular response to felodipine may not be to decrease calcium availability through blocking calcium channels but may be to increase intracellular calcium indirectly as a result of inhibiting calmodulin-dependent calcium efflux. Interestingly, tetronasin also increased the accumulation of MGBG which suggests that calcium may facilitate MGBG uptake; the opposite may be true for felodipine. It may be that by altering the availability of intracellular calcium it is possible to regulate the polyamine transporter and thus influence the accumulation of the polyamines and their structural analogues as suggested previously [29].

The increase in acetyltransferase activity by both tetronasin and felodipine did not produce an increase in either spermidine or putrescine. In addition, no acetylpolyamines were detected in the treated cells suggesting that efflux may be the fate of the acetylated derivatives formed (Table 5). This is in agreement with previous reports indicating a link between polyamine acetylation and efflux [30].

The present study provides evidence that the activity of cytosolic N^1-SAT in human breast carcinoma cells can be altered by agents which change intracellular free calcium concentrations. The effect of both tetronasin and felodipine on MGBG-induced N^1-SAT activity is not dependent on increased intracellular MGBG accumulation and therefore it seems more likely that the potentiation of N^1-SAT activity by these agents in combination with MGBG is attributable to their effects on intracellular calcium concentrations. If N^1-SAT is regulated by intracellular calcium then induction of this enzyme may be a general response of cells to injury, toxic insult or other growth limiting agents which are known to increase intracellular calcium in cells.

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