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INHIBITION OF LONG-CHAIN FATTY ACID OXIDATION BY METHYLGLYOXAL BIS(GUANYLHYDRAZONE)

Pirjo Nikula, Leena Alhonen-Hongisto, Pauli Seppänen and Juhani Jänne

Department of Biochemistry, University of Helsinki,
SF-00170 Helsinki 17, Finland

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SUMMARY: Methylglyoxal bis(guanylhydrazone) (MGBG), an inhibitor of spermidine and spermine biosynthesis and clinically used anti-cancer drug, powerfully inhibited carnitine-dependent fatty acid oxidation in heart muscle homogenates. Equipotent inhibition was also produced by spermine whereas spermidine and putrescine were less effective. MGBG appeared to act as a competitive inhibitor in respect to carnitine. Even though MGBG and spermine equally effectively depressed palmitate oxidation in muscle homogenates in vitro, a striking difference existed between the compounds as regards their effects on fatty acid oxidation in cultured tumor cells. Micromolar concentrations of MGBG distinctly impaired palmitate utilization also in cultured L1210 leukemia cells, whereas similar concentrations of spermine markedly enhanced the oxidation of the fatty acid. The inhibitory effect of MGBG in cultured tumor cells was, at least partly, reversed upon addition of exogenous carnitine. The finding indicating that MGBG impairs fatty acid utilization may be an explanation for the known hypoglycemic effect produced by the drug in most animal species as well as for some of the side-effects associated with its clinical use, most notably severe myalgia.

Methylglyoxal bis(guanylhydrazone), an inhibitor of S-adenosylmethionine decarboxylase (EC 4.1.1.50) (1) and hence of the biosynthesis of spermidine and spermine, is a strongly antiproliferatively acting compound, which is currently under clinical re-evaluation for its usefulness as an anti-cancer drug. It is widely accepted that even though the compound is a potent inhibitor (at least under cell culture conditions) of spermidine and spermine formation, the prevention of polyamine accumulation unlikely is solely responsible for the strong growth-inhibitory effect exerted by the drug on most mammalian cells.

In addition to the inhibition of adenosylmethionine decarboxylase, MGBG also inhibits diamine oxidase activity (2,3), utilizes the putative polyamine carrier for its cellular entry (4) and produces an early and profound mitochondrial damage in proliferating cells (5,6).

The early animal experiments and clinical trials already indicated that administration of MGBG can also lead to fatal hypoglycemia in several animal species, including man (7). The mechanism of the development of hypoglycemia is not known, although a relationship to the reported depression of oxidative phosphorylation by the drug (8) has been suggested (7).

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The side-effects of MGBG when used clinically today include, in addition to antiproliferative type, severe myalgia and other muscle disturbances occurring in high percentage of the patients (9,10). There may be a common metabolic denominator for hypoglycemia and muscle symptoms produced by MGBG. Impaired long-chain fatty acid oxidation could conceivably be the link between hypoglycemia and muscle pain. In Jamaica, there is a disease called "vomiting sickness" produced by the ingestion of the unripe fruit of a tropical plant (ackee fruit). Severe hypoglycemia is associated with the illness. The toxic principle of the unripe fruit has been shown to be hypoglycin, an amino acid, the metabolites of which are powerful inhibitors of fatty acid oxidation (11,12). Furthermore, impaired fatty acid utilization, such as associated with a hereditary deficiency of carnitine acyltransferase (needed for the transport of long-chain fatty acids into mitochondria), leads to striking skeletal muscle symptoms also in man (13).

We show here that MGBG, at concentrations easily achievable during drug treatments in vivo, inhibits carnitine-dependent oxidation of palmitate in tissue homogenates. The drug likewise depressed the utilization of long-chain fatty acids in cultured L1210 leukemia cells at micromolar concentrations.

MATERIALS AND METHODS

Animals and cells: Heart muscle tissue was obtained from elderly (about 350g) male Sprague-Dawley rats. Excised hearts were homogenized with 20 volumes of PBS (phosphate buffered saline, pH 7.4).

Murine L1210 leukemia cells were cultured in RPMI 1640 medium supplemented with 5% pooled human serum (Finnish Red Cross, Helsinki, Finland), penicillin and streptomycin. For the determination of fatty acid oxidation, subconfluent ($0.6 - 0.8 \times 10^6$ cells/ml) cultures were used.

Analytical methods: 1-[^{14}C] Palmitate was bound to albumin as follows: Defatted albumin was dissolved in water and mixed a solution of palmitate in 99.5% ethanol to give a mixture containing defatted albumin 50 mg/ml and palmitate 0.73 mg/ml. This mixture was incubated at 4°C for at least 3 days before use.

The oxidation of palmitate in vitro was measured in the presence of 0.39 ml (19.5 mg of tissue) homogenate, 2mM ATP and 0.5 µ Ci of albumin-bound palmitate in a total volme of 0.5 ml. Evolved labeled carbon dioxide was trapped into Soluene-350 (Packard). The incubation was carried out at 37°C for 10 min. Oxidation was halted with 2 M citric acid whereafter the incubation was continued for a further 15 min. For the determination of the oxidizing capacity of the tumor cells, about 10^6 cells were used for each assay. The cell suspensions (in RPMI 1640 medium) were incubated in the presence of 0.5 µCi of albumin-bound palmitate for 2 h at 37°C. The oxidation was stopped with 50% (wt./vol.) trichloroacetic acid whereafter the incubation was continued for a further 15 min.

The content of MGBG was determined by the enzyme inhibition assay of Seppänen et al. (14). Cell densities were measured with the aid of an electronic particle counter (Coulter Counter).

Chemicals: 1-[^{14}C] Palmitate (specific radioactivity 56 Ci/mole) was obtained from Amersham International (Amersham, Bucks., U.K.). MGBG was synthesized by and obtained from Orion Pharmaceutical Company (Espoo,

Finland). L-Carnitine and defatted albumin were purchased from Sigma (St. Louis, MO, U.S.A.) and the polyamines (as their hydrochloride salts) from Calbiochem (Lucerne, Switzerland).

RESULTS

As shown in Table 1, MGBG and the three natural polyamines depressed palmitate oxidation in heart muscle homogenates. MGBG and spermine were equipotent in this respect and more effective than spermidine or putrescine (Table 1). When carnitine (1 mM) was added into the reaction mixture, the basal oxidation of palmitate was enhanced by a factor of more than 5, and, interestingly, carnitine appeared to protect the oxidation from the inhibition exerted by MGBG but not by spermine (Table 1).

The effect of increasing concentrations of MGBG, in the absence or presence of carnitine, on palmitate oxidation in vitro is depicted in Fig.1. While the oxidation steadily decreased with increasing concentrations of the drug in the absence of carnitine, there was no or little changes up to a drug concentration of 5 mM when exogenous carnitine was included in the assay (Fig.1).

The nature of the inhibition of fatty acid oxidation by MGBG is shown in Fig.2. MGBG obviously acted as a competitive inhibitor in respect to carnitine with an apparent Ki value of 2.4 mM. This kind of intracellular drug levels are easily achievable in living cells, since the drug is extremely effectively concentrated by animal cells (15).

The fact that sufficiently high intracellular drug concentrations were reached in cultured cells exposed to the drug is shown in Table 2. In these experiments, the fatty acid oxidizing capacity was measured using whole tumor cells grown overnight in the presence of 5 μM or 2.5 μM (Table 2) MGBG. An interesting finding is likewise included in Table 2.

Table 1. Effect of MGBG and the natural polyamines on palmitate oxidation by heart muscle homogenate. The concentration of carnitine in the incubation mixture was 1 mM. The incubations were carried out in the presence of 19.5 mg tissue.

Addition	Concn. (mM)	Palmitate oxidation (cpm/assay) -Carnitine	+Carnitine
None	-	33200	179000
MGBG	2.5	18500	181000
	5.0	14200	164000
Spermine	2.5	17300	136000
	5.0	12200	89400
Spermidine	5.0	18500	153000
Putrescine	5.0	23800	202000

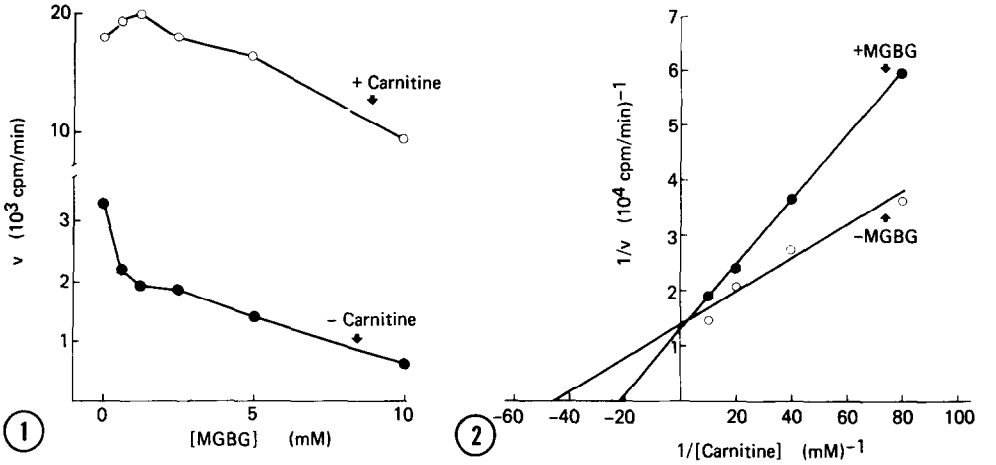

Fig.1. Effect of increasing concentrations of MGBG on palmitate oxidation by heart muscle homogenates. Experimental details as in Table 1.

Fig.2. Double reciprocal presentation of the effect of carnitine concentration on MGBG inhibition of palmitate oxidation. Experimental details as in Table 1 except that the concentration of carnitine was varied.

Even though MGBG and spermine equally effectively inhibited the oxidation of palmitate in tissue homogenates (Table 1), the effects of these compounds on palmitate utilization in cultured tumor cells were just the opposite: instead of inhibition, spermine doubled the oxidation of the fatty acid. Under these conditions, carnitine partially reversed the inhibition produced by MGBG (Table 2). The reversal only occurred when the drug-induced inhibition was moderate (less than 50%); in case of profound depression at high MGBG concentrations carnitine was without effect (results not shown). The partial reversal by carnitine of MGBG inhibition was not based on an effect on drug transport, since intracellular concen -

Table 2. Effect of MGBG and spermine on palmitate oxidation in cultured L1210 leukemia cells. Triplicate or duplicate cultures were grown overnight in absence or presence of MGBG or spermine without or with 1 mM carnitine and assayed for palmitate oxidation.

Treatment	Palmitate oxidation (cpm ± S.D./10^6 cells)	
	-Carnitine	+Carnitine
Expt 1		
None	6220 ± 172	9210 ± 1390
MGBG (5 μM)	3940 ± 892*	5130 ± 964 *
Spermine (5 μM)	13000 ± 2340**	17800 ± 4570*
Expt 2		
None	2290	3680
MGBG (2.5 μM)	1380	2030

* P<0.05; ** P<0.01 (as compared with untreated controls)

trations of MGBG were similar in cells grown without or with carnitine (results not shown). The growth-inhibitory effect exerted by MGBG on cultured L1210 leukemia cells was not influenced by an inclusion of 1 mM carnitine in the culture medium to any appreciable extent (results not shown).

DISCUSSION

There is an array of circumvential evidence suggesting that MGBG could influence lipid metabolism. The observed inhibition of fatty acid oxidation by MGBG may explain some of the puzzling side-effects produced by the drug. By analogy with the fruit poison, hypoglycin (11,12), the well-documented hypoglycemic effect of MGBG could be based on the same mechanism i.e. on an inhibition of carnitine-dependent oxidation of long-chain fatty acids. Since the clinical symptoms associated congenital carnitine acyltransferase deficiency mainly manifest as muscle disorders, such as myalgia occurring under conditions (starvation,strenuous exercise) where fatty acid oxidation is the major energy source for the muscle (13), it is tempting to speculate that the frequently reported muscle disorders in connection of MGBG treatment (9,10,) would likewise be based on the observed inhibition of carnitine-dependent long-chain fatty acid oxidation.

The competition between MGBG and carnitine in palmitate oxidation is difficult to explain, since little structural similarities exist between the two compounds. Nevertheless, carnitine is able to partially reverse the inhibition exerted by MGBG on fatty acid oxidation not only in muscle homogenates but also in cultured cells.

As recently reported, spermine, and to lesser extent also spermidine and putrescine, inhibit the formation of palmityl carnitine at millimolar concentrations in mitochondria obtained from human platelets (16). However, as indicated by our experiments (Table 1), the inhibition of fatty acid oxidation by spermine only occurred in homogenates in vitro, when supplied for growing cells this polyamine produced a highly significant and reproducible enhancement of palmitate oxidation (Table 2). Whether this phenomenon reflects a "true" biologic function of spermine is not known. Apart from the known stabilizing effect exerted by polyamines on cells and cell organelles, these compounds, especially spermine, may be involved in the regulation of some mitochondrial functions. Spermine is reported to restore oxidative phosphorylation and respiration of heat-aged mitochondria at low concentrations (17). It has been also described (18) that very small changes of spermine concentrations significantly alter respiratory control ratio and rate of respiration in rat liver mitochondria.

It is thus possible that spermine, "the polyamine of the eukaryotes", plays some special role in the mitochondrial metabolism and function, which are counteracted by MGBG resulting in mitochondrial damage.

From a practical point of view, the observation that MGBG competes with carnitine in fatty acid oxidation may offer a way to protect experimental animals (and human patients) from the potentially fatal hypoglycemia and muscle disorders produced by the drug. In fact, our preliminary (unpublished) results have indicated that administration of carnitine to starved mice treated with lethal doses of MGBG indeed protected the animals from drug-induced death.

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REFERENCES

1. Williams-Ashman, H. G. and Schenone, A. (1972) Biochem. Biophys. Res. Commun. 46, 288-295.
2. Hölttä, E., Hannonen, P., Pispa, J. and Jänne, J. (1973) Biochem. J. 136, 669-676.
3. Pegg, A. E. and McGill, S. M. (1978) Biochem. Pharmacol. 27, 1625-1629.
4. Dave, C. and Caballes, L. (1973) Fedn. Proc. 32, 736. (abstr.)
5. Mikles-Robertson, F., Feuerstein, B., Dave, C. and Porter, C. W. (1979) Cancer Res. 39, 1919-1926.
6. Porter, C. W., Mikles-Robertson, F., Kramer, D., and Dave, C. (1979) Cancer Res. 39, 2414-2421.
7. Mihich, E. (1963) Cancer Res. 23, 1375-1389.
8. Pine, M. J. and DiPaolo, J. A. (1966) Cancer Res. 26, 18-25.
9. Zeffren, J., Yagoda, A., Watson, R.C., Natale, R.B., Blumenreich, M.S., Chapman, R. and Howard, J. (1981) Cancer Treatment. Rep. 65, 525-527.
10. Todd, III, R.F., Garnick, M.B., Canellos, G.P., Richie, J.P., Gittes, R.B., Mayer, R. J. and Skarin, A. T. (1981) Cancer Treatment Rep. 65, 17-20.
11. Entman, M. and Breseler, R. (1967) Mol. Pharmacol. 3, 333-340.
12. Billington, D. and Sherratt, H.S.A. (1981) Meth. Enzymol. 72, 610-616.
13. Engel, W.K., Vick, N.A., Glueck, C.J. and Levy, R.I. (1970) New Engl. J. Med. 282, 697-704.
14. Seppänen, P., Alhonen-Hongisto, L., Pösö, H. and Jänne, J. (1980) FEBS Lett. 111, 99-103.
15. Seppänen, P., Alhonen-Hongisto, L. and Jänne, J. (1981) Eur. J. Biochem. 118, 571-576.
16. Villanueva, V.R. Advances in Polyamine Research, vol. 4 (Bachrach, U., Kaye, A. and Chayen, R., eds.) Raven Press, New York, 1983, pp. 297-306.
17. Phillips, J.E. and Chaffee, R.R.J. (1982) Biochem. Biophys. Res. Commun. 108, 174-181.
18. Chaffee, R.R.J., Arine, R.M. and Rochelle, R.H. (1979) Biochem. Biophys. Res. Commun. 86, 293-299.