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EFFECT OF METHYLGLYOXAL BIS(GUANYLHYDRAZONE) ON HEPATIC, HEART AND SKELETAL MUSCLE MITOCHONDRIAL CARNITINE PALMITOYLTRANSFERASE AND β-OXIDATION OF FATTY ACIDS

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Abstract—Methylglyoxal bis(guanylhydrazone) (MGBG) is an antileukemic agent and a structural polyamine analogue which inhibits *S*-adenosyl methionine decarboxylase. However, MGBG also produces profound mitochondrial structural damage and inhibition of fatty acid oxidation. Carnitine palmitoyltransferase-A (CPT-A) is located on the outer surface of the inner mitochondrial membrane and is the putative rate-controlling enzyme for mitochondrial long-chain fatty acid oxidation. The present experiments were designed to determine if MGBG inhibits CPT-A. Liver, heart and skeletal muscle mitochondria were isolated from rats following 24 hr of starvation. Measuring the reaction in the direction of palmitoylcarnitine plus CoA formation from palmitoyl-CoA plus carnitine ("forward reaction"), MGBG was competitive with *l*-carnitine. The MGBG CPT-A K_i values were (mM): liver, 5.0 ± 0.6 (N = 15); heart 3.2 ± 1.2 (N = 3); and skeletal muscle, 2.8 ± 1.0 (N = 3). Lysis of hepatic mitochondria with Triton X-100 yielded a K_i of 4.0 ± 2.0, which was not significantly different from intact mitochondria or inverted vesicles (4.9 mM). Purified hepatic CPT had a K_i of 4.2 mM. MGBG did not inhibit purified CPT in the "reverse reaction" (palmitoyl-CoA plus carnitine formation from palmitoylcarnitine plus CoA). Spermine and spermidine, which are structurally similar to MGBG, did not inhibit either CPT activity or acid-soluble product formation from 1-[^{14}C]palmitoyl-CoA. MGBG inhibited mitochondrial state 3 oxidation rates of palmitoyl-CoA and palmitoylcarnitine, as well as of glutamate. However, the fatty acid substrates were considerably more sensitive than glutamate to MGBG inhibition. MGBG also increased hepatic mitochondrial aggregation which was reversed by *l*-carnitine. Fluorescence polarization, using 1,6-diphenyl-1,3,5-hexatriene (DPH) as a probe, indicated that MGBG increased membrane rigidity in a dose-dependent manner. This effect was not altered by *l*-carnitine. MGBG also inhibited purified pigeon breast carnitine acetyltransferase (CAT; K_i = 1.6 mM). While MGBG appeared to be competitive with *l*-carnitine for both CPT and CAT, MGBG also exhibits a number of effects which may be mediated through membrane interaction and which are not reversed by carnitine.

Methylglyoxal bis(guanylhydrazone) (MGBG) is an antiproliferative agent which acts as an inhibitor of *S*-adenosylmethionine decarboxylase (EC 4.1.1.50) and is, thus, an inhibitor of spermine and spermidine synthesis. In addition, MGBG produces mitochondrial damage in proliferating cells [1] and decreases mitochondrial substrate oxidation [2, 3]. The side-effects of the clinical use of MGBG include muscle disturbances similar to those seen in hypoglycin poisoning and carnitine palmitoyltransferase deficiency [4, 5]. Nikula *et al.* [4] have demonstrated that both MGBG and polyamines decrease fatty acid oxidation in heart homogenates. This decrease could be partially prevented by concurrent addition of *l*-carnitine to the incubation medium. *l*-Carnitine prevents early mitochondrial damage caused by MGBG in L1210 leukemia cells [5] and stabilizes energy-linked processes in rat liver mitochondria [6].

The only known metabolic role of carnitine is as a substrate in the acyltransferase reactions. The effects of *l*-carnitine in ameliorating MGBG effects on mitochondria and the reports of muscle-related side-effects of drug use in humans led us to examine the effects of MGBG on carnitine palmitoyltransferase (EC 2.3.1.21) and fatty acid oxidation in hepatic, heart and skeletal muscle mitochondria. Because MGBG may be considered a polyamine analog, we also explored the effects of polyamines on these parameters.

EXPERIMENTAL

Male and female Sprague–Dawley rats (75–150 g) were obtained from the Washington State University breeding colony. They received water and a stock diet (Wayne Lab Blox) *ad lib.* Lights were on 12 hr, off 12 hr. Rats were killed at 7:30–8:00 a.m. Food was withdrawn 24 hr before killing.

Hepatic mitochondria were prepared by differential centrifugation [7]. We have described previously the preparation of inverted submitochondrial vesicles [8]. In brief, inverted vesicles were prepared

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by nitrogen compression/decompression after Fleischer *et al.* [9]. To further remove right-side-out vesicles, vesicles were washed, batch-wise, with cytochrome *c*-Sepharose-4B [10], prepared according to Godinot and Gautheron [11], prior to final pelleting.

Total skeletal muscle mitochondria were prepared from mixed hindlimb muscle of rats by a modification of the method of Tomec and Hoppel [12]. Muscle was immediately excised and placed into ice-cold modified Chappell–Perry [13] buffer: 100 mM KCl, 50 mM morpholinopropane sulfonic acid (MOPS), 1 mM EDTA, 1 mM ATP, 5 mM $MgSO_4$, 0.2% bovine serum albumin (BSA) fraction V, pH 7.4. Fat and connective tissue were removed, and the muscle was blotted, minced, and suspended in 100 mM KCl, 50 mM MOPS, 1 mM EDTA. To a suspension of 10 g minced muscle in 100 ml of this medium was added 50 mg nagarse (subtilisin). The suspension was kept on ice and stirred for 10 min. The resulting slurry was homogenized in a glass vessel with a loose-fitting Teflon pestle, followed by a tight pestle. The homogenate was centrifuged at 9800 *g* for 10 min and the supernatant fraction was discarded. The pellets were resuspended in the modified Chappell–Perry buffer and centrifuged at 700 *g*. The 9800 *g* pellet was extracted twice in this manner, and the combined supernatant fractions were filtered through four layers of cheesecloth, and centrifuged at 7700 *g*. The resulting mitochondrial pellets were resuspended and repelleted twice. Final suspension (30–50 mg of mitochondrial protein/ml) was in 100 mM KCl, 50 mM MOPS. Total heart mitochondria derived from ventricles were prepared in the same way as skeletal muscle. However, since fewer grams of heart were available, volumes were adjusted accordingly.

Carnitine palmitoyltransferase activity of intact mitochondria (CPT-A, "outer enzyme") was measured in the direction of palmitoylcarnitine formation using [^{14}C-*methyl*]-*l*-carnitine as substrate (the "forward reaction" of Tomec and Hoppel [12]). The CPT assay has been presented previously [8]. The assay contained, in a final volume of 0.25 ml: 80 mM KCl, 50 mM MOPS, 1 mM ethyleneglycolbis(amino-ethylether)tetra-acetate (EGTA), 1 mg/ml bovine serum albumin (defatted and dialyzed), 4 mg/ml sodium tetrathionate, 5–60 μM palmitoyl-CoA, and 0.01–10 mM total *l*-carnitine (labeled + unlabeled), pH 7.0. The reaction was initiated by addition of 50 μg of mitochondrial protein, and allowed to proceed for 2 min at 30° unless otherwise noted. Linearity of kinetic and inhibitor plots has been confirmed [8], and linearity was verified in the presence of MGBG ($r^2 > 0.99$ for kinetic plots). The "reverse reaction" was measured in the direction of palmitoyl-CoA formation from palmitoyl[^{14}C-*methyl*]carnitine as described previously [10]. In brief, the CPT activity in the reverse direction was assayed using a kinetic modification of the assay II of Tomec and Hoppel [12]. The final assay volume of 0.2 ml contained: 80 mM KCl, 50 mM MOPS, 1 mM EGTA, 1 mg/ml defatted and dialyzed BSA, 5 mM CoA, and 40 or 10 μM palmitoyl-*l*-[^{14}C-*methyl*]carnitine. MGBG was added to achieve a final concentration of 0–20 mM. These concentrations are comparable to levels used by others to produce mitochondrial effects [2–4]. After a 5-min preincubation at 37°, the reaction was initiated by the addition of 5 μg of enzyme protein. The reaction was terminated after 3 min by addition of 0.2 ml of 6% perchloric acid. The reaction was linear up to 10 μg protein and for 4 min. Following perchloric acid addition, samples were cooled on ice for 30 min and then centrifuged at 12,000 g_{av} for 5 min in an Eppendorf Microfuge; an aliquot of the supernatant fraction, containing the liberated [^{14}C-*methyl*]-*l*-carnitine, was counted. Carnitine acetyltransferase (CAT) activity (0.7 μg/ml) was measured spectrophotometrically with acetyl-CoA plus *l*-carnitine (0.5 or 6 mM *l*-carnitine) as substrates using 5,5-dithio-2-bis-nitrobenzoate (DTNB) to measure CoA release. MGBG was varied 0–100 mM.

Hepatic mitochondrial oxygen consumption using palmitoyl-CoA + carnitine + malate, palmitoylcarnitine + malate or glutamate as substrates was determined as described by Brady and Hoppel [14]. Acid-soluble products of hepatic mitochondrial palmitoyl-CoA oxidation were also determined in separate experiments at 30° using [1-^{14}C]palmitoyl-CoA in the presence of 5 mM malonate, 80 mM KCl, 50 mM MOPS, 5 mM KPO_4, 1 mM EGTA, 1 mg/ml bovine serum albumin (defatted and dialyzed), 2 mM ADP, 0.01 to 10 mM *l*-carnitine and 10 or 40 μM palmitoyl-CoA. Under these conditions with hepatic mitochondria, acetoacetate should be the major product when malonate is used and citrate when malate is used [15]. Mitochondrial protein was 0.5 to 1.0 mg/ml. The rate of accumulation of acid-soluble product was linear for 3 min. The reaction was terminated after 1 min with 10% perchloric acid (v/v in water). Acid-soluble products were counted in the supernatant fraction after centrifugation for 5 min at 12,000 *g* in an Eppendorf microfuge.

Hepatic carnitine palmitoyltransferase was purified from mitochondria isolated from rats fed the stock diet containing 2% diethylhexylphthalate for 2 weeks as described previously [10]. Polyacrylamide gel electrophoresis-sodium dodecylsulfate (PAGE-SDS) yielded a single band with a molecular weight of 68,000 daltons. The specific activity was 21–28 μmoles/min per mg of protein. Activity was determined either as described above or spectrophotometrically using DTNB as described by Bieber *et al.* [16].

Fluorescence polarization was determined using 1,6-diphenyl-1,3,5-hexatriene (DPH) as probe after Shinitsky and Barenholz [17]. Intact mitochondrial or inverted vesicle protein was adjusted to 0.5 mg/ml and incubated with DPH for 1 hr prior to introducing the various compounds of interest.

Mitochondrial swelling was measured spectrophotometrically at 25° as the increase (swelling) or decrease (aggregation) in absorbance at 540 and 800 nm. Mitochondria (150–250 μg of mitochondrial protein) were suspended in 1 ml of 10 mM Tris, 250 mM sucrose, pH 7.4 [2, 3]. A stable baseline was established (1–3 min) prior to the addition of substrates.

Data were analyzed by analysis of variance for a factorial design with the potential to accommodate unbalanced data [18] or for a completely randomized design [19]. The standard error of the mean was derived from the mean square error of the analysis

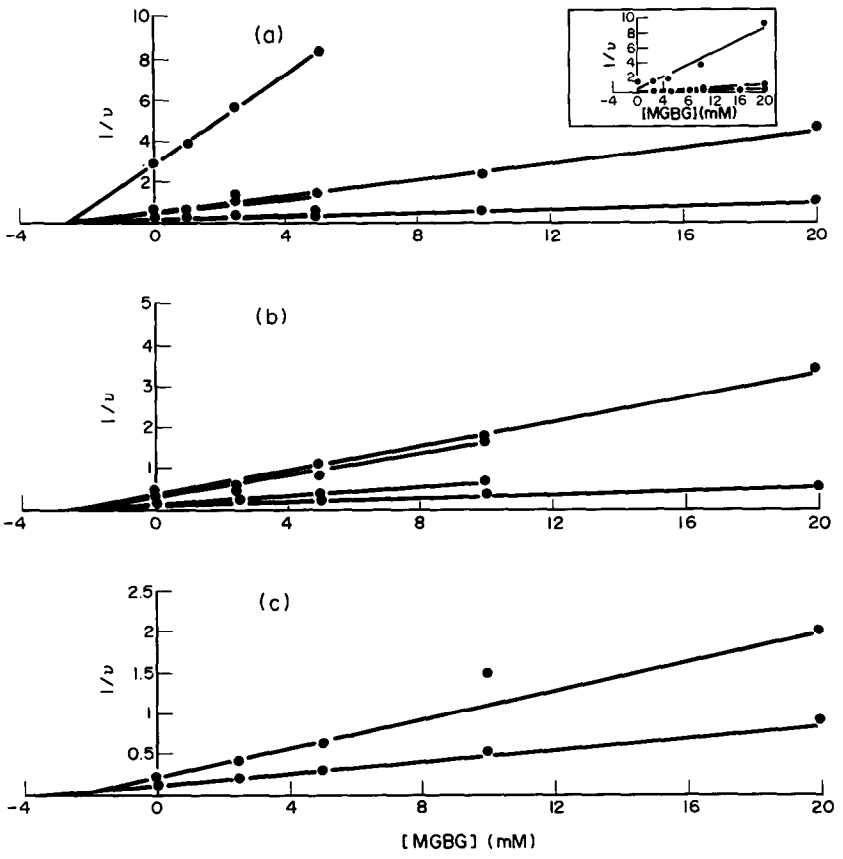

Fig. 1. Competitive inhibition of CPT-A by MGBG. Mitochondria were isolated and CPT-A activity was determined as described (Experimental). Palmitoyl-CoA was present at 40 μM and $1/v$ is expressed as (nmoles/min per mg)$^{-1}$. (a) Liver: *l*-carnitine was present at 2, 0.5, 0.1 and 0.05 mM. MGBG was present at 0, 2.5, 5, 10 and 20 mM. The insert is the Dixon plot for purified hepatic CPT. *l*-Carnitine was present at 2, 0.2 and 0.02 mM; MGBG was present at the same levels as used for intact mitochondria. (b) Heart: *l*-carnitine was present at 10, 5, 2 and 0.5 mM. MGBG was present at 0, 2.5, 5, 10 and 20 mM. (c) Skeletal muscle: *l*-carnitine was present at 10 and 2 mM. MGBG was present at 0, 2.5, 5, 10 and 20 mM.

of variance. Linear regression analysis was determined by least squares techniques [19].

Materials. MGBG was initially obtained from Dr. P. Nikula and J. Janne, University of Helsinki, and, thereafter, from the Sigma Chemical Co. (St. Louis, MO, U.S.A.). Spermine and spermidine were obtained as trihydrochloride salts from Sigma Chemical. Nagarse (subtilisin) and carnitine acetyltransferase (pigeon breast muscle) were also obtained from Sigma. Palmitoyl-CoA was synthesized as described by Seubert [20]. *l*-Carnitine was the gift of Sigma-Tau, Rome, Italy. [^{14}C-*methyl*]-*l*-Carnitine was synthetized using [^{14}C-*methyl*]iodide (Dupont-New England Nuclear) [21]. [1-^{14}C]Palmitoyl-CoA was purchased from DuPont-New England Nuclear, Boston, MA. Bovine serum albumin, type V (Sigma), was defatted [22] and dialyzed [23]. All other chemicals were reagent grade or better.

RESULTS

MGBG inhibition of CPT. Representative plots of MGBG inhibition of intact mitochondrial CPT-A are presented in Fig. 1. MGBG K_i values were assessed using MGBG as an inhibitor in competition with *l*-carnitine (CPT forward reaction). MGBG was not competitive with palmitoyl-CoA. Liver (Fig. 1a) gave a K_i of 5.0 ± 0.6 mM (mean ± SEM; N = 15). This value was not altered if hepatic mitochondria were first lysed with Triton X-100 (4.0 ± 2.0 mM; N = 3) or if inverted vesicles were used (K_i = 4.9 mM). In one series of studies, *d*-carnitine was varied over the range of 0–2 mM in the presence of 0.5 mM *l*-carnitine and 20 mM MGBG. In these studies, MGBG inhibition was not altered. Heart mitochondria (Fig. 1b) yielded a K_i of 3.2 ± 1.2 mM (N = 3). Skeletal muscle CPT-A (Fig. 1c) was inhibited by MGBG much the same as liver with a K_i of 2.8 ± 1.0 mM (N = 3). Thus, all of these tissues yielded comparable sensitivity of the CPT-A forward reaction to MGBG. The purified hepatic CPT had a comparable K_i of 4.2 mM (Fig. 1a insert). This value was the same whether purified CPT was assayed in the presence of albumin in the radiochemical assay or without albumin in the presence of 0.1% Triton in the spectrophotometric assay. MGBG did not compete with palmitoylcarnitine in the "reverse reaction" using the purified CPT. CAT purified from pigeon breast muscle had a K_i for MGBG of 1.6 mM with MGBG competing with carnitine.

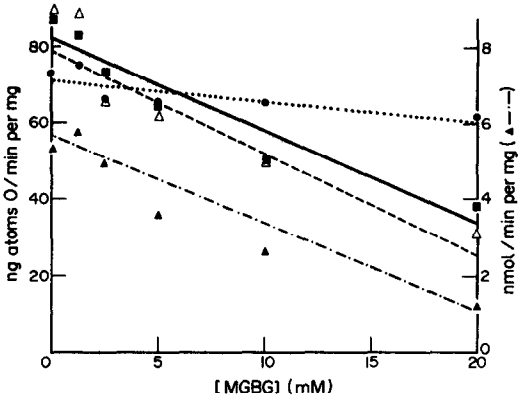

Fig. 2. Effect of [MGBG] on hepatic mitochondrial state 3 oxidation and CPT-A activity. Isolation of mitochondria, state 3 oxidation and CPT-A measurements are described in Experimental. MGBG was varied from 0 to 20 mM. Key: palmitoyl-CoA, ng atoms 0/min per mg (■——); palmitoylcarnitine (△---); glutamate (●....); and CPT-A, nmoles/min per mg (▲—.—).

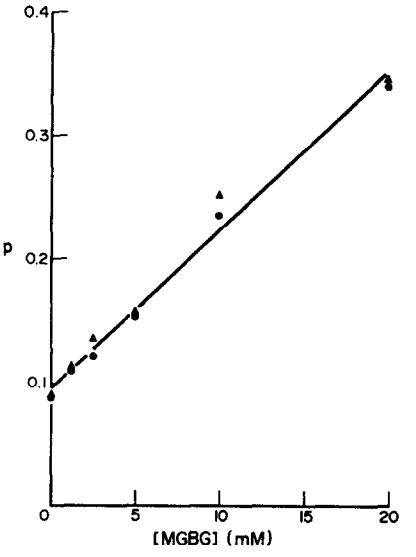

Fig. 3. Effect of MGBG and *l*-carnitine on fluorescence polarization ratio (*p*). Intact hepatic mitochondria (0.5 μg/ml) were incubated for 1 hr with DPH prior to the addition of MGBG (●) as described in Experimental. *l*-Carnitine was added to the cuvette to provide a 2 mM final concentration following the determination of fluorescence polarization with MGBG (▲). Each value is the mean of three rats. The standard error of the mean, 0.021, was derived from the error mean square in the analysis of variance.

There was no effect of spermine or spermidine, at the levels used (0–10 mM), on CPT-A activity or fatty acid oxidation (data not shown), although others have reported inhibition of heart homogenate fatty acid oxidation by these compounds [4]. Thus, the MGBG effect appears to be specific for MGBG and not for the polyamines after which MGBG was designed.

Fatty acid oxidation. Hepatic mitochondrial oxygen consumption as a function of MGBG concentration is presented (Fig. 2). Although MGBG did reduce palmitoyl-CoA and palmitoylcarnitine state 3 oxidation rates, glutamate oxidation was also somewhat reduced. Thus, in this case, it was unlikely that MGBG action on intact mitochondria was restricted to interaction with the CPT active site above. Since palmitoylcarnitine oxidation was depressed in parallel with palmitoyl-CoA oxidation, MGBG may have some mechanism of action beyond the interaction with CPT-A (outer CPT). It was possible that the MGBG effect was mediated by general membrane effects as will be discussed below.

Membrane changes with MGBG. In support of a broad effect of MGBG on the mitochondrial membrane, and in addition to CPT inhibition, it was noted that MGBG caused aggregation of mitochondria (i.e. increased absorption of 0.01–0.02 units at 540 or 800 nm with 5 mM MGBG), as did spermine and spermidine. Interestingly, *l*-carnitine tended to reverse this process, resulting in mitochondrial swelling (decreased absorption at 540 or 800 nm). However, the magnitude of absorbance change was dependent on concentration and order of addition. Byczkowski *et al.* [2, 3] have observed previously that MGBG and polyamines induce aggregation of rat liver mitochondria in a dose-dependent manner. Further, MGBG resulted in increased DPH fluorescence polarization, indicating that the mitochondrial membrane may have become more rigid (Fig. 3). This phenomenon was not noticeably reversed by carnitine administration. We have observed previously that increased mitochondrial membrane rigidity was associated with decreased activity of both CPT-A and CPT-B [8, 24].

DISCUSSION

While a number of inhibitors of CPT exist, the majority of these act as inhibitors of acyl-CoA binding. Most prominent among this class are malonyl-CoA, the putative *in vivo* modulator of CPT-A activity, bromopalmitoyl-CoA and tetradecylglycidyl-CoA, a putative irreversible inhibitor. However, malonyl-CoA, and possibly tetradecylglycidyl-CoA may function at sites distinct from the CPT catalytic site [25–28]. MGBG competes with *l*-carnitine rather than the CoA derivatives. Further, unlike malonyl-CoA and tetradecylglycidyl-CoA, there was no question that sensitivity to inhibition was retained even when the membrane was solubilized or as the enzyme was purified, and regardless of the presence or absence of albumin in the incubation media.

Although Nikula *et al.* [4] indicated that MGBG and carnitine are not obviously similar, we feel that there may be structural similarities with carnitine and acetylcarnitine. A recent structural hypothesis [29] for the mode of action of carnitine in acyl transfers provides the framework from which to speculate about how MGBG serves as a competitive inhibitor for carnitine. The guanidium group occupies the quaternary ammonium site and the chain extends in a similar fashion to carnitine with an imine group occupying a position similar to the acyl carbonyl. In this location, MGBG can form a thioaminal with either CoA or cysteine present in the active site.

Although this is a covalent linkage, the reaction is readily reversible. Support for this explanation comes from the chemistry of imines and the biochemistry of aldehydes as competitive inhibitors of proteases. Thiols form stable tetrahedral addition compounds with imines [30–33]. Aldehydes form similar tetrahedral addition compounds with hydroxyl or thiol groups in the active sites of proteases [34]. In general, the equilibrium constants for formation of these tetrahedral addition compounds are greater for thiol than hydroxy as well as for aldehyde than imine. However, the equilibrium constants for hydroxy addition to aldehydes are of similar magnitude to thiol addition to imines. Therefore, in the same fashion that aldehydes are proposed to function as competitive inhibitors of proteases, MGBG could be functioning in the carnitine acyltransferases.

MGBG and, indeed, *l*-carnitine may have a number of effects which do not directly relate to interaction with CPT but may involve non-specific membrane effects. DiLisa *et al.* [6] have shown that the addition of *l*-carnitine to mitochondria exposed to stressing conditions delays mitochondrial de-energization. They presumed that this was due to removal of long chain acyl-CoA from the membrane by carnitine. However, our data indicate that *l*-carnitine addition also results in increased swelling, counteracting the MGBG effect (not shown). We have examined *d*-carnitine inhibition of CPT and found that this compound was not a CPT inhibitor (data not shown). We reasoned that if inhibition of CPT-A by MGBG were mediated by non-specific membrane effects, then it was probable (but not certain) that *d*-carnitine should compete equally as well as *l*-carnitine with MGBG. The failure of *d*-carnitine to compete with MGBG provides further evidence that MGBG interacts with the CPT active site as a carnitine analogue.

The slight depression of glutamate oxidation observed may be mediated by non-specific membrane effects. However, the glutamate oxidation was not affected as profoundly as the long-chain fatty acid oxidation. We have observed communication between CPT-A and the activity of the inner surface of the inner membrane, CPT-B [10]. CPT-A and -B have been proposed to be the same protein [35]. If the activities were contained in a single membrane-spanning protein with an active site on each surface, communication might be explained.

It is possible that MGBG has general membrane effects which result in impaired fatty acid oxidation. It is also possible that MGBG depresses transport of palmitoylcarnitine into the mitochondria, perhaps through an effect on the acylcarnitine translocase. MGBG did not inhibit the CPT reverse reaction in purified CPT. Thus, it was not likely that the inhibition of CPT-B (inner enzyme) was responsible for inhibition of palmitoylcarnitine oxidation. However, it has been suggested that CAT is important in maintaining adequate free CoA levels within the mitochondrial matrix [36]. Since CAT was inhibited by MGBG, it may be that the inhibition of palmitoylcarnitine oxidation relates to the sequestration of intramitochondrial CoA, since a major function of CAT is believed to be the formation of acetylcarnitine from acetyl-CoA. The significance of this will require further study. There was, however, some effect on β-oxidation, in general, and this may also be mediated through membrane alterations.

In summary, MGBG was competitive with *l*-carnitine for CPT and for CAT. This was not altered by solubilization of the mitochondria or purification of the CPT. While this specific inhibition may be responsible for the decrease in β-oxidation, MGBG has other effects less directly related to CPT-A inhibition. Among these effects, MGBG caused mitochondrial aggregation, as did spermine and spermidine (which have no effects on β-oxidation). This effect was reversed by carnitine. Further, MGBG caused a stepwise increase in membrane rigidity. In this case, the effect was not altered by carnitine.

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