Protective effect of creatine against inhibition by methylglyoxal of mitochondrial respiration of cardiac cells

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Previous publications from our laboratory have shown that methylglyoxal inhibits mitochondrial respiration of malignant and cardiac cells, but it has no effect on mitochondrial respiration of other normal cells [Biswas, Ray, Misra, Dutta and Ray (1997) Biochem. J. **323**, 343–348; Ray, Biswas and Ray (1997) Mol. Cell. Biochem. **171**, 95–103]. However, this inhibitory effect of methylglyoxal is not significant in cardiac tissue slices. Moreover, post-mitochondrial supernatant (PMS) of cardiac cells could almost completely protect the mitochondrial respiration against the inhibitory effect of methylglyoxal. A systematic search indicated that creatine present in cardiac cells is responsible for this protective effect. Glutathione has also some protective effect. However, creatine phosphate, creatinine, urea, glutathione disulphide and β-mercaptoethanol have no protective effect. The inhibitory and protective effects of methylglyoxal and creatine respectively on cardiac mitochondrial respiration were studied with various concentrations of both methylglyoxal and creatine. Interestingly, neither creatine nor glutathione have any protective effect on the inhibition by methylglyoxal on the mitochondrial respiration of Ehrlich ascites carcinoma cells. The creatine and glutathione contents of several PMS, which were tested for the possible protective effect, were measured. The activities of two important enzymes, namely glyoxalase I and creatine kinase, which act upon glutathione plus methylglyoxal and creatine respectively, were also measured in different PMS. Whether mitochondrial creatine kinase had any role in the protective effect of creatine had also been investigated using 1-fluoro-2,4-dinitrobenzene, an inhibitor of creatine kinase. The differential effect of creatine on mitochondria of cardiac and malignant cells has been discussed with reference to the therapeutic potential of methylglyoxal.

Key words: cancer, creatine, glutathione, methylglyoxal, mitochondrial respiration.

INTRODUCTION

The results presented in several publications from our laboratory have convincingly demonstrated that methylglyoxal, a normal metabolite, is tumoricidal, and this effect is due to the inhibition of both glycolysis and mitochondrial respiration specifically of malignant cells [1–4]. We have also demonstrated that the effects of methylglyoxal on mitochondrial respiration of both cardiac and malignant cells are strikingly similar. Methylglyoxal inhibits electron flow through complex I of the mitochondrial respiratory chain of Ehrlich ascites carcinoma (EAC) cells [2], leukaemic leucocytes [3] and also of mitochondria of cardiac cells of various normal animals [4]. But it has no effect on mitochondrial respiration of a wide variety of other normal cells [1–4]. Based on these results and especially on the fact that the heart produces an enormous amount of ATP by higher respiratory activity, and also considering the role of ATP in biological systems, we have proposed a hypothesis on cancer which suggests that excessive ATP formation in cells may lead to malignancy [4,5].

The above-mentioned similarity between mitochondria of malignant and cardiac cells, however, is a serious challenge for the development of an effective anti-cancer drug. This is because the drug in question that will effectively kill cancerous cells by its selective ability to attack mitochondria of malignant cells will also have a deleterious effect on cardiac mitochondria and hence on cardiac functions. But during our previous study on the effect of methylglyoxal on cardiac mitochondria and tissue slices, we observed that the inhibitory effect of methylglyoxal on the respiration of cardiac tissue slices was not significant compared with its effect on mitochondrial respiration [4]. Moreover, kymographic experiments with perfused but intact heart had indicated that methylglyoxal had no significant effect on several important functions of heart [4]. These results suggest that in intact cardiac cells outside the mitochondria there is a protective device that counteracts the inhibitory effect of methylglyoxal. In the present work, we have investigated this phenomenon and have observed that creatine present in cardiac cells is responsible for this protective effect. We have further observed that GSH also has a protective function.

EXPERIMENTAL

Chemicals

Methylglyoxal, α-oxoglutarate, glutathione, glutathione disulphide, dithiothreitol, β-mercaptoethanol, Dowex 50 W H^+ and Dowex 1 Cl^- resins, and ADP, were products of Sigma, St. Louis, MO, U.S.A. Creatine, creatine phosphate and 1-fluoro-2,4-dinitrobenzene (FDNB) were from SRL, Mumbai, India. Creatine kinase assay kit was obtained from Bayer Diagnostics India, Baroda, India. All other chemicals were of analytical grade and obtained from local manufacturers.

Abbreviations used: EAC, Ehrlich ascites carcinoma; PMS, post-mitochondrial supernatant; FDNB, 1-fluoro-2,4-dinitrobenzene.

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Preparation of mitochondria and post-mitochondrial supernatant (PMS)

All operations were carried out at 0–4 °C unless mentioned otherwise.

Goat heart

Goat heart was obtained from a local slaughterhouse. The mitochondria were prepared basically by the method of Smith [6]. The heart tissue was made free from fat and connective tissues and was washed several times in buffer (hereinafter referred to as buffer) containing 0.25 M sucrose, 10 mM Tris/HCl, 1 mM sodium succinate and 0.2 mM EDTA, with the pH finally adjusted to 7.8. A 15 g portion was very finely minced with scissors. It was then homogenized in 90 ml of buffer using a loosely fitting glass/teflon Potter–Elvehjem homogenizer. When the heart tissues were no longer discernible, the homogenization was continued up to 10 up-and-down strokes of the pestle. The homogenate, after adjusting the pH to 7.4 by dropwise addition of 1 M KOH, was centrifuged for 20 min at 1200 ***g***. The supernatant was decanted carefully so that the loosely packed fluffy layer was not disturbed. The pellet along with the fluffy layer was retained. The pH of the supernatant was adjusted to 7.4 if necessary with dropwise addition of 1 M KOH. It was then centrifuged for 15 min at 26 000 ***g***. Again, the supernatant was decanted carefully so that the loosely packed fluffy layer was not disturbed, and retained. This is essentially the PMS that was used for further study.

The centrifuge tubes containing the pellet along with the fluffy layer were gently shaken and the solution was decanted to remove the loosely packed damaged mitochondria. After wiping out the adhering materials at the top of the centrifuge tube with tissue paper, the tightly packed bottom layer containing mitochondria was suspended in 30 ml of buffer and gently homogenized by hand with a Potter–Elvehjem homogenizer and retained (suspension A).

The pellet along with the fluffy layer obtained after 1200 ***g*** centrifugation as mentioned earlier was suspended in 60 ml of buffer and homogenized in a Potter–Elvehjem homogenizer with five up-and-down strokes. The suspension was centrifuged at 1200 ***g*** for 20 min and the pellet was discarded. The supernatant was centrifuged at 26 000 ***g*** for 15 min. The supernatant along with the loosely packed pellet was discarded. The tightly packed mitochondria were suspended in 20 ml of buffer and homogenized by hand in a Potter–Elvehjem homogenizer and mixed with the above-mentioned suspension A. The mixed suspension was centrifuged at 26 000 ***g*** for 15 min. After discarding the supernatant along with the loosely packed pellet, the tightly packed pellet was suspended in the minimum volume of buffer possible and this mitochondrial suspension was used for respiratory studies. This mitochondrial preparation could also be stored in small aliquots at −20 °C for further use. We usually performed experiments for measuring respiration with freshly prepared mitochondria. For repeat experiments, once-thawed mitochondria were used. The results were more or less similar.

Rat heart

Heart tissue from a freshly killed animal was rinsed in the buffer and minced. The minced tissue was suspended in 6 vol. (w/v) of the buffer and homogenized in a Potter–Elvehjem homogenizer with 10 up-and-down strokes. The homogenate was centrifuged at 800 ***g*** for 10 min. The pellet and the turbid suspension at the bottom of the centrifuge tubes were rejected. The supernatant was centrifuged at 8000 ***g*** for 10 min. The clear supernatant (PMS) was retained. After rejecting the loosely packed pellet, the mitochondrial pellet was suspended in the buffer and centrifuged at 8000 ***g*** for 10 min. After rejecting the supernatant, the mitochondria were suspended in a minimum volume of the buffer and were used for respiratory study. Mouse and chicken heart mitochondria and PMS were also prepared by the method similar to that of rat heart.

EAC mitochondria were prepared as described previously [2]. For the preparation of EAC cell PMS, 2 ml of packed cells were suspended in 12 ml of 20 mM Tris/HCl buffer, pH 7.4 and homogenized in a Potter–Elvehjem homogenizer with 15 up-and-down strokes and the suspension was centrifuged at 2000 ***g*** for 10 min and the pellet was rejected. The supernatant was centrifuged at 15 000 ***g*** for 15 min. After rejecting the pellet, the supernatant was retained and used as EAC cell PMS.

Preparation of muscle PMS

Skeletal muscle of goat was minced, suspended in 6 vol. (w/v) of the buffer and homogenized in a Potter–Elvehjem homogenizer with 15 up-and-down strokes. The homogenate was centrifuged at 2000 ***g*** for 10 min and the pellet was rejected. The supernatant was centrifuged at 20 000 ***g*** for 20 min. After rejecting the pellet, the supernatant was saved and used as PMS.

The rat and chicken muscle PMS were prepared in an identical fashion. The goat liver, kidney and spleen PMS are essentially the supernatants that were obtained after the preparation of mitochondria of the respective organs, the preparations of which were described previously [4].

Partial purification of the ‘protecting factor’ from heart PMS

A portion of the PMS was heated in a boiling-water bath for 1.5 min, cooled and centrifuged for 10 min at 10 000 ***g***. After rejecting the denatured protein, the supernatant was treated with activated charcoal (35 mg/ml of the supernatant), mixed and centrifuged. The fine charcoal particles present in the supernatant was removed by filtration. The filtrate was retained and used for further study.

For resin treatment, to a 2 ml portion of the above-mentioned charcoal-treated PMS of goat heart, 0.2 g of either Dowex 50 W H^+ or Dowex 1 Cl^- was added and mixed. The suspensions were filtered and used for further study. The PMS was dialysed in the buffer for a total period of 6 h; three times with 200 ml of the buffer/1 ml of PMS.

Preparation of tissue slices

Thin sections of cardiac tissue of goat was made with a razor blade in a cold room at 4 °C. The preparation took 2–3 min [7].

Measurement of respiration

Oxygen consumption was measured with a Gilson oxygraph fitted with a Clark electrode. The respiratory medium for mitochondria contained, in a total volume of 2 ml, 125 mM sucrose, 50 mM KCl, 5 mM Hepes buffer (pH 7.2), 2 mM KH_2PO_4, 1 mM $MgCl_2$ and the respiratory substrate, usually 10 mM α-oxoglutarate, and mitochondria. The mitochondrial protein in the medium was 0.15–0.25 mg or 0.5–0.8 mg for cardiac and EAC cell mitochondria respectively. The temperature of the incubation medium was 30 °C. Other additions are mentioned in the Figure legends. After the indicated periods of time, ADP (0.5 mM, final concentration) was added to start phosphorylating respiration. The respiratory control ratio for mitochondria was usually 7.

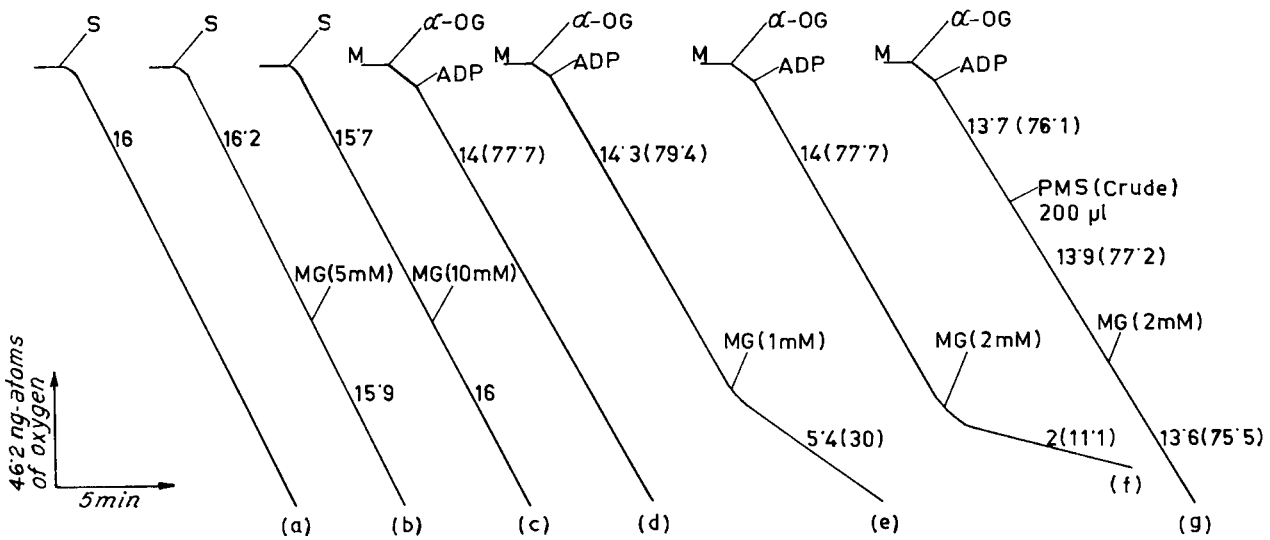

Figure 1 Effect of methylglyoxal on the respiration of tissue slices and mitochondria of goat heart

The figure shows the direct oxygraph tracings of typical experiments. Details of the incubation conditions are described in the Experimental section. Addition of compounds is indicated in the respective tracings. The numbers along the tracings without parentheses and within parentheses represent the rate of oxygen consumption (ng-atom/min and ng-atom/min per mg of protein respectively). Abbreviations: S, slices; M, mitochondria; α-OG, α-oxoglutarate; MG, methylglyoxal.

Oxygen consumption by tissue slices ($\approx$ 13 mg) was measured in the above-mentioned respiratory medium with 3 mM D-glucose as respiratory substrate, but containing no ADP.

Estimation of methylglyoxal, creatine, GSH and protein

Methylglyoxal was measured by 2,4-dinitrophenylhydrazine-alkali colour reaction [8]. Creatine was measured by α-naphthol-diacetyl colour reaction [9]. Glutathione was measured according to method I as described by Akerboom and Sies [10]. Protein was estimated by the method of Lowry et al. as described by Layne [11].

Assay of glyoxalase I and creatine kinase

Glyoxalase I was assayed by monitoring the formation of *S*-D-lactoylglutathione at 240 nm [12]. Creatine kinase was assayed by monitoring the formation of NADPH at 340 nm as per the instructions of the manufacturer of the assay kit (Bayer Diagnostics India). The reaction mixture contained, in a total volume of 1 ml, 25 μmol of Tris/HCl buffer, pH 7.2, 2.5 μmol of magnesium acetate, 5 μmol of *N*-acetyl-L-cysteine, 0.5 μmol of ADP, 1.25 μmol of AMP, 0.5 μmol of NADP, 5 μmol of D-glucose, 2.5 nmol of diadenosine pentaphosphate, 0.5 μmol of EDTA, 7.5 μmol of creatine phosphate, 8.5 units of hexokinase and 5 units of glucose-6-phosphate dehydrogenase. After 2 min of incubation at 30 °C an appropriately diluted aliquot of different PMS was added and the change in absorbance was noted from the end of first minute to the end of fifth minute.

The activity of both glyoxalase I and creatine kinase is defined as the amount of the enzyme which forms 1 μmol of the product/min under standard condition of the assay. The specific activity is the units of activity/mg of protein.

The inhibitory effect of FDNB on mitochondrial creatine kinase of both goat heart and EAC cells was tested in the same assay system for creatine kinase as described above. In the assay medium, either goat heart mitochondria (4 μg of protein) or EAC cell mitochondria (0.26 mg of protein) was added. FDNB was dissolved in 80% ethanol. Appropriate controls with the assay medium containing FDNB but no mitochondria were maintained. Moreover, the amount of ethanol that had been transferred in the assay medium had no effect.

RESULTS

Protection of mitochondrial respiration by goat heart PMS against methylglyoxal inhibition

As mentioned before, methylglyoxal strongly inhibits mitochondrial respiration of EAC cells [1,2] and leukaemic leucocytes [3], but it has no inhibitory effect on mitochondrial respiration of several types of normal cell [1–4]. However, similar to malignant cells, methylglyoxal strongly inhibits the mitochondrial respiration of cardiac cells [4]. In contrast to mitochondria, methylglyoxal has no significant inhibitory effect on the respiration of cardiac tissue slices. Moreover, it has no effect on several important functions of perfused toad heart [4].

These observations prompted us to investigate the mechanism of this protective action against methylglyoxal inhibition in intact cardiac cells. In Figure 1, traces a–c clearly show that methylglyoxal up to a concentration of 10 mM has no effect on slices of goat heart. But methylglyoxal at 1 and 2 mM inhibits the mitochondrial respiration of goat heart to the extents of 60 and 85% respectively (Figure 1, traces e and f). Interestingly, in the presence of 200 μl of PMS of goat heart, 2 mM methylglyoxal almost completely failed to inhibit the mitochondrial respiration (Figure 1, trace g).

Effect of different tissue PMS on the inhibition of heart mitochondrial respiration by methylglyoxal

The effect of PMS of different tissues on the inhibition of goat heart mitochondrial respiration by methylglyoxal was investigated. Table 1 shows that the maximum protective effect was observed with PMS of goat skeletal muscle. An almost complete protective effect had been observed by goat skeletal muscle PMS against methylglyoxal inhibition. Protection by goat

Table 1 Effect of different tissue PMS on the inhibition of goat heart mitochondrial respiration by methylglyoxal

After addition of ADP, the oxygen consumption was monitored for at least 10 min. The final concentration of methylglyoxal was 3 mM where added. A 200 μl aliquot of PMS was added to 2 ml of the incubation mixture. The data are means ± S.D. from four experiments. Other conditions of the assay are described in the Experimental section.

	Rate of oxygen consumption (ng-atom of oxygen/min)	
Addition of PMS	Before methylglyoxal addition	After methylglyoxal addition
No PMS	18.2 ± 1.3	1.2 ± 0.2
Goat		
Skeletal muscle	19.2 ± 1.6	18.7 ± 1.0
Heart	18.6 ± 1.2	17.0 ± 1.1
Liver	18.3 ± 1.6	8.9 ± 0.9
Kidney	19.0 ± 1.7	4.7 ± 0.7
Spleen	18.9 ± 1.3	3.8 ± 0.4
Rat		
Skeletal muscle	18.8 ± 1.8	17.5 ± 1.3
Heart	18.5 ± 1.1	16.3 ± 1.1
Liver	19.3 ± 1.4	8.2 ± 1.0
Chicken		
Skeletal muscle	19.3 ± 1.7	18.5 ± 1.2
Heart	19.3 ± 1.2	17.5 ± 1.5
EAC	19.5 ± 1.4	1.5 ± 0.3

heart PMS was also nearly to the same extent. The effect of PMS of skeletal muscle and heart of rat and chicken was also quantitatively similar to the effect of goat heart and skeletal muscle PMS. However, PMS of liver, kidney and spleen had moderate to little protective effect. Moreover, PMS of EAC cells had no protective effect (Table 1).

A similar experiment was performed with rat heart mitochondria and PMS of different tissues of rat and similar results were obtained. Moreover, PMS of goat heart and skeletal muscle could completely protect the mitochondrial respiration of rat heart against methylglyoxal inhibition (results not shown).

Attempts to identify the protecting factor(s) in tissue PMS

In order to identify the protecting factor(s) present in PMS of different tissues, especially in heart and skeletal muscle, we subjected the PMS to heat, charcoal and resin treatment and also dialysed the PMS; the details of this are described in the Experimental section. When these differently treated PMS were tested for their possible protecting effects of mitochondrial respiration against methylglyoxal inhibition, it was observed that the PMS, after heat, charcoal and H^+ resin treatment, could retain almost full protective activity. But the PMS that were subjected to dialysis and Cl^- resin treatment lost their protective abilities almost completely (Table 2).

The above-mentioned experiment suggests that the protecting factor is a non-protein, heat-stable, low-molecular-mass compound with a positive charge, which tempted us to search for compounds with these properties, which are abundant in cardiac and skeletal muscle tissue [13].

Protective effect of creatine against methylglyoxal inhibition of mitochondrial respiration

The above-mentioned properties of PMS prompted us to investigate whether creatine and/or creatine phosphate is/are responsible for the protecting effect of PMS, since these two compounds

Table 2 Protective effect of goat heart PMS after different treatments

The assay conditions were similar to those in Table 1. Where indicated, the final concentration of methylglyoxal in the assay medium was 2 mM. The volume of differently treated PMS solutions was 200 μl in each case. The total volume of the reaction mixture was 2 ml. The data are means ± S.D. from four experiments.

	Rate of oxygen consumption (ng-atom of oxygen/min)	
Addition of PMS	Without addition of methylglyoxal	After addition of methylglyoxal
None	15.2 ± 1.1	3.5 ± 0.3
PMS, untreated	17.0 ± 1.3	16.8 ± 1.1
PMS, after heat treatment	17.3 ± 1.1	17.0 ± 1.3
PMS, after heat and charcoal treatment	16.8 ± 1.0	16.5 ± 1.1
PMS, after heat, charcoal and H^+ resin treatment	16.2 ± 1.1	15.8 ± 0.9
PMS, after dialysis	16.0 ± 0.9	4.5 ± 0.5
PMS, after heat, charcoal treatment and dialysed	15.8 ± 0.9	4.0 ± 0.3
PMS, after heat, charcoal and Cl^- resin treatment	15.6 ± 1.0	3.6 ± 0.2

Table 3 Protective effect of creatine against inhibition of goat heart mitochondrial respiration by methylglyoxal

Assay conditions were similar to those in Table 1. Where indicated, 1.25 mM methylglyoxal was present in the assay medium. Data are means ± S.D. from four experiments.

	Rate of oxygen consumption (ng-atom of oxygen/min)	
Addition	Before addition of methylglyoxal	After addition of methylglyoxal
None	12.5 ± 1.0	4.0 ± 0.4
Creatine (10 mM)	12.3 ± 1.2	12.0 ± 1.0
Creatine phosphate (10 mM)	12.0 ± 1.3	3.5 ± 0.4
Creatinine (10 mM)	12.8 ± 1.5	3.9 ± 0.5
Urea (10 mM)	12.8 ± 1.1	3.9 ± 0.3
GSH (3.5 mM)	12.8 ± 1.3	9.1 ± 0.7
Glutathione disulphide (3.5 mM)	13.0 ± 1.6	4.2 ± 0.5
Dithiothreitol (3.5 mM)	12.4 ± 1.4	3.4 ± 0.4
β-Mercaptoethanol (3.5 mM)	12.9 ± 1.1	4.4 ± 0.3

are known to be abundantly present in tissues of heart and skeletal muscle. We also tested other compounds, e.g. creatinine, urea, GSH etc., for their possible protective ability. Table 3 shows that creatine strongly protected mitochondrial respiration against methylglyoxal inhibition. Of all the other compounds tested, GSH has a significant protective effect. Methylglyoxal at a concentration of 1.25 mM inhibits the respiration to about 70 %. This inhibition was almost completely protected by 10 mM creatine; whereas, GSH reduced this inhibition to the extent of 30 %. Other compounds tested had no effect on the oxygen consumption by goat heart mitochondria or on the inhibition by methylglyoxal (Table 3).

Tests for the possible utilization of methylglyoxal in the presence of GSH and reaction of methylglyoxal with creatine

With GSH as the co-substrate, glyoxalase I could convert methylglyoxal to *S*-D-lactoylglutathione [14], thereby reducing the inhibitory effect of methylglyoxal. So we tested the utilization of methylglyoxal in the presence of GSH under conditions that

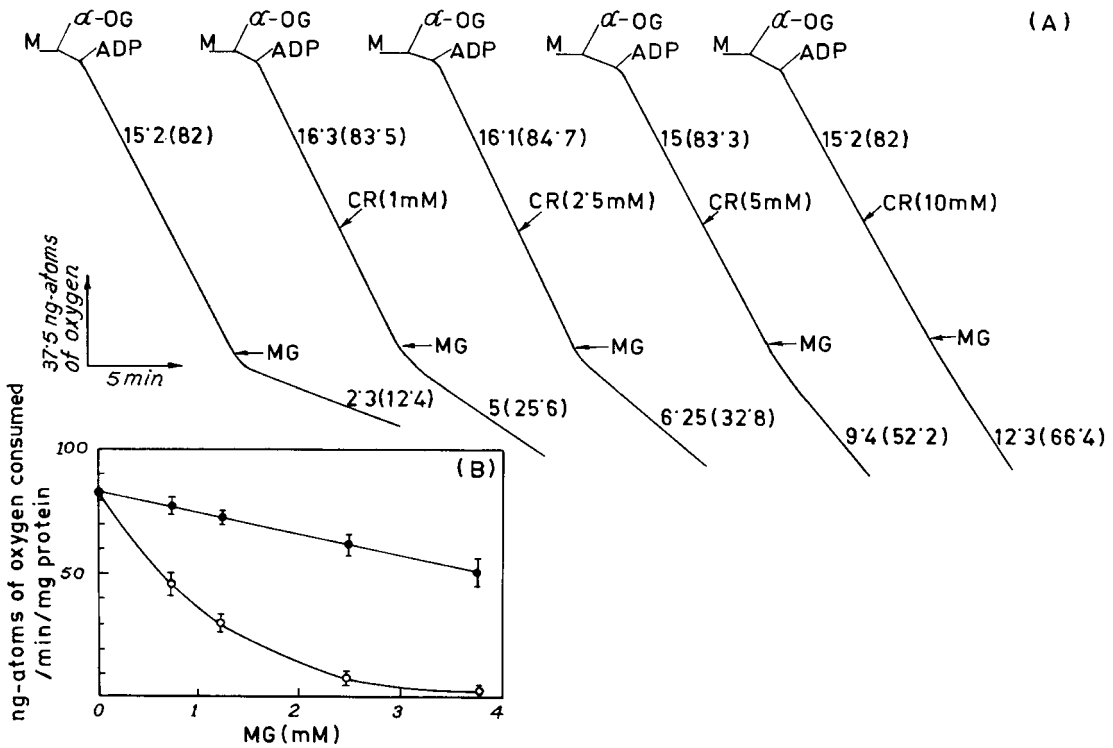

Figure 2 Protective effect of creatine on methylglyoxal inhibition of mitochondrial respiration of goat heart

(**A**) Direct oxygraph tracings of a typical experiment. The final concentration of methylglyoxal (MG) in the incubation medium was 2 mM; CR, creatine. (**B**) Oxygen consumption in the absence (○) and presence (●) of 10 mM creatine with various concentrations of methylglyoxal as indicated. The results are means ± S.D. from four experiments. The other conditions of the incubation are described in the Experimental section and in the legend of Figure 1. Abbreviations are given in Figure 1.

were identical to the conditions for monitoring the mitochondrial respiration of cardiac cells, except for the addition of α-oxoglutarate and ADP. Only 5–10% of methylglyoxal was utilized for a period of 15 min, indicating that the protective effect of GSH was not due to possible utilization of methylglyoxal in the presence of GSH.

There are numerous reports in the literature of the glycation of proteins by glucose, methylglyoxal and several other saccharide derivatives and oxoaldehydes [15,16]. This glycation, producing advanced glycation endproducts ('AGEs'), is usually due to the reaction of arginine, lysine and N-terminal amino acid residues of proteins with these above-mentioned metabolites. Since creatine and arginine both have two guanidino amino groups, we tested whether creatine could react with methylglyoxal and may thereby counteract the inhibitory effect. So, we incubated methylglyoxal with creatine under conditions that were identical to the conditions for monitoring mitochondrial respiration (except for the addition of α-oxoglutarate and ADP) and estimated methylglyoxal [8]. The amount of methylglyoxal remained unchanged for up to 15 min. Moreover, the differential effects of creatine on the respiration of cardiac and EAC cell mitochondria further suggest that the protective effect of creatine on cardiac mitochondrial respiration is not due the reaction of methylglyoxal with creatine (see below).

Effect of different concentrations of creatine on inhibition of goat heart mitochondrial respiration by a fixed concentration of methylglyoxal and *vice versa*

Because creatine has been found to protect cardiac mitochondrial respiration against inhibition by methylglyoxal we measured the respiration with various concentrations of creatine at a fixed concentration of methylglyoxal. Figure 2(A) shows that creatine protected mitochondrial respiration in a dose-dependent manner. When creatine was not added, 2 mM methylglyoxal inhibited the mitochondrial respiration by 85%. With 1 mM creatine this inhibition was 70%; increasing the concentration of creatine to 10 mM reduced this inhibition to only 20%.

Figure 2(B) shows the effect on mitochondrial respiration of a fixed concentration of creatine with various concentrations of methylglyoxal.

Effect of creatine on the inhibition of mitochondrial respiration of EAC cells by methylglyoxal

As mentioned in the Introduction, methylglyoxal inhibits the electron flow through complex I of the mitochondrial respiratory chain of both cardiac and malignant cells, suggesting a similarity between mitochondria of these two cell types. So we investigated whether creatine, similar to its effect on cardiac mitochondria, could protect the mitochondrial respiration of EAC cells against methyglyoxal inhibition. Figure 3 represents the results of such a study.

Traces b and c in Figure 3 (top panel) show that methylglyoxal at 1 and 2 mM inhibited the mitochondrial respiration of EAC cells by 65 and 85% respectively. But in contrast to the results obtained with cardiac mitochondria, creatine completely failed to protect mitochondrial respiration against methylglyoxal inhibition (Figure 3, top panel, traces d and e).

Figure 3 (bottom panel) shows that, similar to creatine, both crude and heat- and charcoal-treated goat heart PMS solutions, EAC cell PMS solution and also GSH completely failed to counteract the inhibitory effect of methylglyoxal on EAC cell mitochondrial respiration.

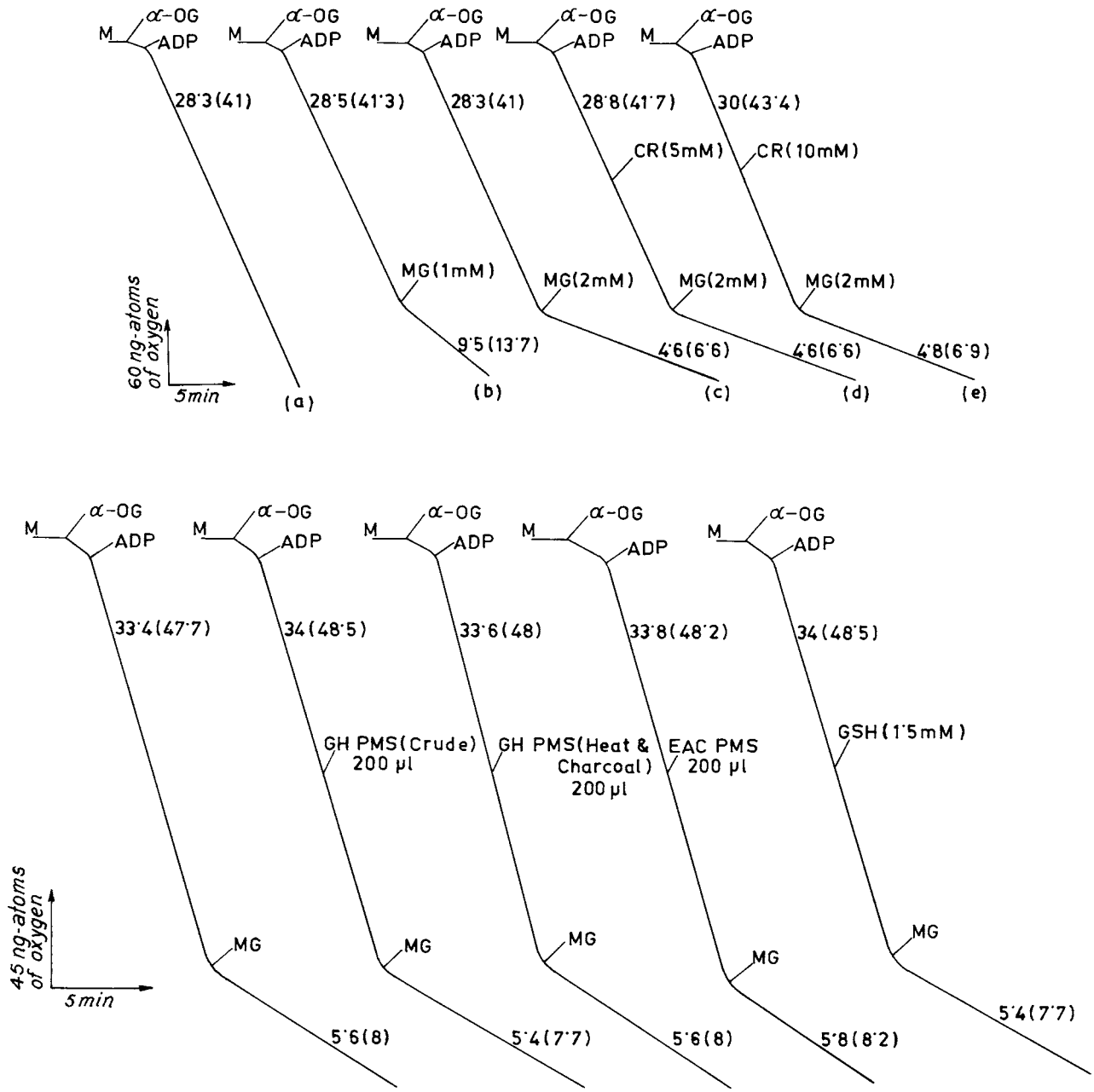

Figure 3 Creatine, GSH and goat heart and EAC PMS on the inhibition of mitochondrial respiration of EAC cells by methylglyoxal

Both panels represent direct oxygraph tracings of typical experiments. Conditions of the assay are described in the Experimental section and in the legend of Figure 1. MG, methylglyoxal; CR, creatine; α-OG, α-oxoglutarate, GH, goat heart.

Creatine, GSH and protein contents of different tissue PMS

As presented in Table 1, heart and skeletal muscle PMS could protect against methylglyoxal inhibition of cardiac mitochondrial respiration. A moderate protective effect was observed with other tissue PMS, whereas EAC cell PMS failed to afford any protection. Therefore we estimated the creatine and GSH contents of PMS of different tissues and EAC cells and also their protein contents (Table 4).

With regard to the creatine contents of different tissues, our results agree well with what is available in the literature [13]. In tissues that have maximum creatine content, the corresponding PMS have a maximum protective effect. On the other hand, there was no such correlation between the GSH content and the protective effect of PMS. Moreover, a much higher amount of GSH than its content in PMS is needed to observe any protective effect by this metabolite.

In consonance with the small amount of creatine and GSH present in PMS of EAC cells, this PMS has no protective effect (Tables 1 and 4).

Creatine kinase and glyoxalase I activities in different tissue PMS solutions

We have also measured in PMS the activities of two important enzymes, glyoxalase I and creatine kinase, which act upon methylglyoxal plus GSH and creatine respectively. The results,

Table 4 Creatine, GSH and protein content of different tissue PMS solutions

The methods for the preparation of different PMS solutions and estimation of creatine, GSH and protein are described in the Experimental section. The data are means ± S.D. from four experiments.

PMS	Creatine (μg/ml)	GSH (μg/ml)	Protein (mg/ml)
Goat			
Skeletal muscle	638 ± 48	45 ± 4	6.9 ± 0.7
Heart	300 ± 34	119 ± 11	7.2 ± 0.1
Liver	9.5 ± 0.3	237 ± 30	13.5 ± 1.5
Kidney	30 ± 2	200 ± 32	9.0 ± 1.1
Spleen	32.5 ± 0.7	216 ± 21	9.8 ± 0.3
Rat			
Skeletal muscle	659 ± 30	15 ± 2	10.2 ± 1.4
Heart	252 ± 11	37 ± 5	11.8 ± 1.1
Chicken			
Skeletal muscle	585 ± 43	62 ± 6	13.4 ± 1.3
Heart	190 ± 20	152 ± 17	13.5 ± 1.2
EAC	16.7 ± 1.9	10.6 ± 0.8	2.64 ± 0.24

Table 5 Specific activities of creatine kinase and glyoxalase I in different tissue PMS solutions

The details of the preparation and assay methods are described in the Experimental section. The data are means ± S.D. from four experiments.

	Specific activity (units/mg of protein)	
PMS	Creatine kinase	Glyoxalase I
Goat		
Skeletal muscle	49 ± 0.3	0.8 ± 0.05
Heart	18 ± 2	0.42 ± 0.08
Liver	0.16 ± 0.04	1.5 ± 0.1
Kidney	0.3 ± 0.05	0.66 ± 0.04
Spleen	0.5 ± 0.06	0.3 ± 0.06
Rat		
Skeletal muscle	77 ± 10	1.1 ± 0.12
Heart	16 ± 1.5	0.39 ± 0.06
Chicken		
Skeletal muscle	51 ± 8	0.82 ± 0.1
Heart	11 ± 2	0.45 ± 0.05
EAC	0.11 ± 0.01	0.24 ± 0.03

which are presented in Table 5, indicate that the tissues that have higher creatine contents also have higher creatine kinase activities. The maximum activity of creatine kinase and the maximum amount of creatine are present in skeletal muscle, which agree well with results presented in the literature [13].

Effect of FDNB on (i) mitochondrial creatine kinase of goat heart and EAC cells and (ii) the protective effect of creatine against methylglyoxal inhibition of mitochondrial respiration

To investigate whether mitochondrial creatine kinase might be involved in the protective effect of creatine against methylglyoxal inhibition we used FDNB, a potent and specific inhibitor of creatine kinase. We tested the effect of FDNB on creatine kinase of mitochondria of both goat heart and EAC cells. We observed that FDNB (30 μM) inhibited the goat heart creatine kinase by about 75%, which is similar to the results available in the literature [17]. Creatine kinase of mitochondria of EAC cells was inhibited by 60% with 30 μM FDNB.

Table 6 Effect of FDNB on the protective effect of creatine against methylglyoxal inhibition of mitochondrial respiration of goat heart and EAC cells

After addition of ADP the oxygen consumption was monitored for at least 15 min. The final concentration of methylglyoxal was 1.5 mM where added. FDNB was dissolved in 80% ethanol. Appropriate controls indicated that the amount of ethanol that had been transferred in the respiratory media had no effect. The data are means ± S.D. from four experiments. Other assay conditions are described in the Experimental section.

	Rate of oxygen consumption (ng-atom of oxygen/min)	
Addition	Before methylglyoxal addition	After methylglyoxal addition
Goat heart		
None	17.6 ± 1.1	4.5 ± 0.6
Creatine (5 mM)	17.4 ± 1.3	13.8 ± 0.8
Creatine (10 mM)	16.8 ± 1.0	15.3 ± 1.1
FDNB (30 μM)	17.2 ± 1.3	4.6 ± 0.5
Creatine (5 mM) + FDNB (30 μM)	16.8 ± 1.2	15.6 ± 1.3
Creatine (10 mM) + FDNB (30 μM)	16.6 ± 1.0	15.8 ± 1.2
EAC		
None	16.2 ± 1.2	4.2 ± 0.7
Creatine (10 mM)	16.0 ± 1.3	4.3 ± 0.5
FDNB (30 μM)	16.7 ± 1.1	4.6 ± 0.8
Creatine (10 mM) + FDNB (30 μM)	16.5 ± 1.5	4.2 ± 0.4

We then tested the effect of FDNB on the protective ability of creatine against methylglyoxal inhibition of mitochondrial respiration of goat heart and EAC cells. Table 6 indicates that FDNB at a concentration of 30 μM may have very little augmenting effect on the protective ability of creatine. This may be due to the inhibitory effect of FDNB on mitochondrial creatine kinase. However, a detailed study with various concentrations of creatine, FDNB and methylglyoxal is required to arrive at a definite conclusion.

DISCUSSION

The results presented in this paper show clearly that PMS of cardiac cells can almost completely protect the cardiac mitochondria from the potential deleterious effect of methylglyoxal. Moreover, it appears that creatine present in high amounts in cardiac cells is mainly responsible for this protective effect. GSH also has some protective effect. There may be other unidentified compounds that have some protective ability.

As mentioned, previous publications from our laboratory have demonstrated that methylglyoxal specifically inhibited mitochondrial respiration of malignant and cardiac cells by inhibiting electron flow through complex I, whereas mitochondrial respiration of a wide variety of other normal cells remained completely unaffected [1–4]. Interestingly as shown in the present paper, creatine could not protect the mitochondrial respiration of malignant cells from the inhibitory effect of methylglyoxal, suggesting a possible difference in complex I of cardiac and malignant cells.

Mitochondrial complex I is probably the most complex protein/enzyme in a cell, consisting of at least 42 polypeptides and the coenzyme ubiquinone [18,19]. The number of binding sites for both ubiquinone and inhibitors in mitochondrial complex I is an unresolved question and has been the subject of intense controversy [20–22]. Unique and different as well as overlapping binding sites for ubiquinone and inhibitors have been suggested.

Results presented in this and previous publications strongly point to differences that may be present in mitochondrial complex I of cardiac, other normal and malignant cells; since we have observed that methylglyoxal inhibits mitochondrial complex I of cardiac and malignant cells but not of other normal cells. Moreover creatine could protect the mitochondrial respiration of only cardiac cells and not of malignant cells. It seems likely that the effects of methylglyoxal and creatine on mitochondrial complex I can be used to understand the site and nature of binding of the coenzyme and inhibitors and also to understand the difference in complex I in these three cell types.

There are several creatine analogues; most of them are synthetic but some are natural. Some of these analogues are cyclocreatine, ethyl creatine, 3-guani-dinopropionic acid and phosphinic creatine [13]. These analogues can be used to understand the mechanism of the protective action of creatine against methylglyoxal inhibition. However, the mechanism of the protective effect of creatine is at present not clear to us.

To explain the difference between mitochondria of cardiac and EAC cells regarding the effect of methylglyoxal and creatine, the role of mitochondrial creatine transporter cannot be ruled out. It may be speculated that methylglyoxal specifically inhibits the mitochondrial transporter of EAC cells, thereby blocking the entry of creatine into mitochondria and preventing it from reaching the target site for methylglyoxal. Since very few studies had been made previously [13,23] on the mitochondrial creatine transporter it was difficult to study the effect of methylglyoxal on mitochondrial creatine transport. However, in a recent publication, Walzel et al. [24] have demonstrated a mitochondrial creatine transporter activity. It will be interesting to study the effect of methylglyoxal on the activity of this transporter.

The protective effect of glutathione against metyhylglyoxal inhibition does not seem unusual considering the recent study that glutathione depletion results in inhibition of the activity of mitochondrial complex I [25]. It appears that the protective effect of glutathione is not only due to the presence of thiol groups, because other thiol-group-containing reagents such as dithiothreitol and β-mercaptoethanol were found to have no effect.

The protective effect of creatine appears to be a windfall for the prospective treatment of cancer. This is because an anti-cancer drug that inhibits mitochondrial respiration of specifically malignant cells may also inhibit mitochondrial respiration of cardiac cells. But from the results presented in this paper it appears that cardiac cells have an inherent protective device to counteract this possible deleterious effect.

The anti-tumour effect of cyclocreatine has been studied extensively [13]. It has been observed that a combination of cyclocreatine and another anti-cancer drug produced an additive synergistic effect against cellular proliferation [13,26]. Moreover cyclocreatine has a unique mechanism of anti-tumour activity and is well tolerated by cancer patients [13]. Interestingly, normal cell lines that express high levels of creatine kinase were not inhibited by cyclocreatine [27].

A methylglyoxal-based anti-cancer formulation is at present undergoing clinical trials [28]. On the other hand, creatine supplementation in the diet is now popular among sports persons to enhance performance. The use of creatine has also been extended to the medical field for the treatment of energy and neuromuscular-related diseases. No major adverse side effect due to creatine supplementation has been reported [13,29,30].

All these observations suggest that creatine and/or cyclocreatine supplementation with methylglyoxal-based formulation seems worth exploring for the treatment of cancer patients.

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