# Inhibition of electron flow through complex I of the mitochondrial respiratory chain of Ehrlich ascites carcinoma cells by methylglyoxal

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The effect of methylglyoxal on the oxygen consumption of Ehrlich-ascites-carcinoma (EAC)-cell mitochondria was tested by using different respiratory substrates, electron donors at different segments of the mitochondrial respiratory chain and site-specific inhibitors to identify the specific respiratory complex which might be involved in the inhibitory effect of methylglyoxal on the oxygen consumption by these cells. The results indicate that methylglyoxal strongly inhibits ADP-stimulated α-oxoglutarate and malate plus pyruvate-dependent respiration, whereas, at a much higher concentration, methylglyoxal fails to inhibit succinate-dependent respiration. Methylglyoxal also fails to inhibit respiration which is initiated by duroquinol, an artificial electron donor. Moreover, methylglyoxal cannot inhibit oxygen consumption when the *NNN'N'*-tetramethyl-*p*-phenylenediamine by-pass is used. The inhibitory effect of methylglyoxal is identical on both ADP-stimulated and uncoupler-stimulated respiration. Lactaldehyde, a catabolite of methylglyoxal, can exert a protective effect on the inhibition of EAC-cell mitochondrial respiration by methylglyoxal. We suggest that methylglyoxal possibly inhibits the electron flow through complex I of the EAC-cell mitochondrial respiratory chain.

## INTRODUCTION

It is now well established that methylglyoxal is indeed metabolized in various organisms (Thornalley, 1990; Ray and Ray, 1987). This potentially important biomolecule has significant growth-inhibitory and anti-tumour properties, and it is generally believed that these two properties are interrelated and that methylglyoxal possibly exerts its anti-tumour effect by inhibiting protein synthesis and interacting with nucleic acids (Együd and Szent-Györgyi, 1966; Conroy, 1979; Szent-Györgyi, 1979; Fraval and McBrien, 1980). But methylglyoxal, being a ketoaldehyde, is a highly reactive compound, and is capable of affecting a wide variety of cellular processes. Previous work from our laboratory has shown that methylglyoxal inhibits the respiration of various malignant cells, whereas the respiration of normal cells is not affected (Ray et al., 1991). Moreover, on incubation with methylglyoxal, EAC cells become non-viable within a very short period. We have further observed that methylglyoxal strongly inhibits aerobic glycolysis and mitochondrial respiration of EAC cells, whereas the respiration of mitochondria isolated from liver and kidney of normal mice remains unaffected. As a consequence of the inhibitory effect of methylglyoxal on both glycolysis and mitochondrial respiration of EAC cells, the ATP level of these cells has been found to be drastically decreased (Halder et al., 1993). That cancer cells have defective mitochondrial function resulting in high aerobic glycolysis has long been proposed (Warburg, 1956). The inhibitory effect of methylglyoxal on respiration, specifically of the malignant cells, provides us with an opportunity to understand precisely the possible alteration of mitochondrial functions in malignant cells. The present study was undertaken to identify the involvement of specific respiratory complex, and the results presented in this paper strongly suggest that methylglyoxal specifically inhibits electron flow through complex I of EAC-cell mitochondria.

## MATERIALS AND METHODS

### Chemicals

All the biochemicals were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Rotenone, malonate, antimycin A, duroquinone, TTFA, TMPD and CCCP were also products of Sigma. L- and D-lactaldehyde were prepared from L- and D-threonine, respectively (Huff and Rudney, 1959). All other chemicals were of analytical grade and obtained from local manufacturers.

### Animals and transplantation of tumours

The EAC cells were grown in the abdominal cavity of Swiss albino mice. The cells were maintained by weekly intraperitoneal inoculation of the cells into recipient mice. Each mouse received 0.2 ml of ascites fluid containing approx. $10^7$ cells diluted in sterile normal saline. The cells were harvested after 8–10 days and were initially diluted and washed with 0.9% NaCl. Erythrocytes occasionally present were removed by washing in 35 mM NaCl.

### Preparation of mitochondria

Mitochondria from EAC cells were prepared essentially by the digitonin permeabilization method with minor modifications (Moreadith and Fiskum, 1984). The EAC cells were collected and washed in normal saline rather than in the wash medium suggested by Moreadith and Fiskum (1984). Erythrocytes present were removed as described above. The cells were then transferred to H-medium (Moreadith and Fiskum, 1984) and mitochondria

Abbreviations used: EAC, Ehrlich ascites carcinoma; TTFA, 2-thenoyltrifluoroacetone; TMPD, *NNN'N'*-tetramethyl-*p*-phenylenediamine; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; α-OG, α-oxoglutarate.

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were prepared. However, in our study the BSA concentration was 0.02% (w/v) in both H-medium and in H-medium (minus EGTA), instead of 0.5% (w/v) as used in the method of Moreadith and Fiskum (1984).

For the preparation of liver or kidney mitochondria, the tissues were suspended in 0.25 M sucrose and homogenized in a Teflon/glass Potter–Elvehjem homogenizer, and mitochondria were prepared by differential centrifugation (Johnson and Lardy, 1967).

### Measurement of respiration

Oxygen consumption was measured with an oxygraph (Gilson, Villiers-le-Bel, France) equipped with a Clark electrode. The respiratory medium for mitochondria of EAC cells contained, in a total volume of 2.2 ml, 125 mM sucrose, 50 mM KCl, 5 mM Hepes buffer (pH 7.2), 2 mM $KH_2PO_4$, 1 mM $MgCl_2$ and respiratory substrates (10 mM α-OG or 10 mM succinate or 5 mM malate plus 5 mM pyruvate or 0.75 mM duroquinol) and mitochondria containing 2–3 mg of protein suspended in 210 mM mannitol/70 mM sucrose/0.02% BSA/5 mM Hepes buffer, pH 7.2. Other additions are mentioned in the Figure legends. After the indicated period of time ADP (0.5 mM final concn.) was added to the reaction mixture in order to start phosphorylating respiration. Duroquinone was converted into duroquinol just before use as a respiratory substrate (Nelson and Gellerfors, 1978).

Methylglyoxal was measured by 2,4-dinitrophenylhydrazine–alkali colour reaction (Cooper, 1975). The concentration of lactaldehyde was determined precisely enzymically by using cytosolic aldehyde dehydrogenase and $NAD^+$ (S. Ray and M. Ray, 1984). However, for routine purpose, lactaldehyde was also measured as follows: to 1 ml of lactaldehyde solution was added 0.9 ml of water and 0.33 ml of 0.1% 2,4-dinitrophenylhydrazine solution in 2 M HCl. After incubation for 15 min at 30 °C, 1.67 ml of 10% (w/v) NaOH was added and the $A_{420}$ of the yellow colour was measured after a further period of 15 min. Under these conditions, 1 μmol of lactaldehyde is shown to have an absorbance of 3.8, which agreed well with the enzymic estimation. Protein was measured with BSA as a standard (Lowry et al., 1951).

## RESULTS

Figure 1 shows the effect of methylglyoxal on mitochondrial respiration of EAC cells with α-OG and succinate as respiratory substrates. When 2.5 mM methylglyoxal was added to α-OG-dependent ADP-stimulated respiring mitochondria, there was almost complete inhibition of respiration within 5 min. However, when succinate was added to the system, oxygen consumption started immediately, which could be further inhibited by malonate (Figure 1a). The inhibitory effect of methylglyoxal on the oxygen consumption of EAC-cell mitochondria was identical in both ADP-stimulated and uncoupler-stimulated respiration. Methylglyoxal has been found to be inhibitory for α-OG-dependent respiration which has been initiated by the uncoupler CCCP instead of ADP. Moreover, similarly to the results presented above, when succinate was added oxygen consumption started immediately (Figure 1b).

Results similar to those described above were also obtained with malate plus pyruvate as respiratory substrates instead of α-OG, and TTFA as an inhibitor instead of malonate (results not shown). Both α-OG and malate plus pyruvate donate electrons at complex I, whereas succinate donates electrons at complex II, by-passing complex I. Malonate inhibits succinate dehydrogenase, and TTFA inhibits electron transfer from iron–sulphur centres to ubiquinone; both these inhibitors thereby block the electron transfer from complex II to complex III (Hatefi, 1985). So the results presented in Figure 1 strongly suggest that methylglyoxal inhibits electron flow through complex I of the mitochondrial respiratory chain.

The effect of methylglyoxal on the specific complex of the mitochondrial respiratory chain was further investigated by using several mitochondrial complex-specific inhibitors and artificial electron donors. The results presented in Table 1 indicate that methylglyoxal (5 mM) has no effect on the oxidation of duroquinol, which donates electron directly to complex III, further suggesting that electron flow through complex III and complex IV of EAC-cell mitochondria was not affected by methylglyoxal.

To eliminate further the possibility of involvement of complex IV in methylglyoxal inhibition, the TMPD by-pass was used. It is well known that ascorbate plus TMPD can donate electrons directly to complex IV (Lee et al., 1967). It has been reported that TMPD can also accept electrons from other components of complex I and complex II, i.e. from NAD-dependent or FAD-dependent substrates after oxidation (Lee et al., 1967; Sanadi and Jacobs, 1967). We tested whether methylglyoxal has any inhibitory effect on the electron flow through this TMPD bypass. Table 1 shows that α-OG-dependent respiration is strongly inhibited by rotenone, but in the presence of TMPD this inhibition is completely relieved. Moreover, TMPD could also relieve α-OG-dependent respiration which has been inhibited by methylglyoxal. However, the electron flow through this TMPD bypass could, as usual, be readily inhibited by azide (results not

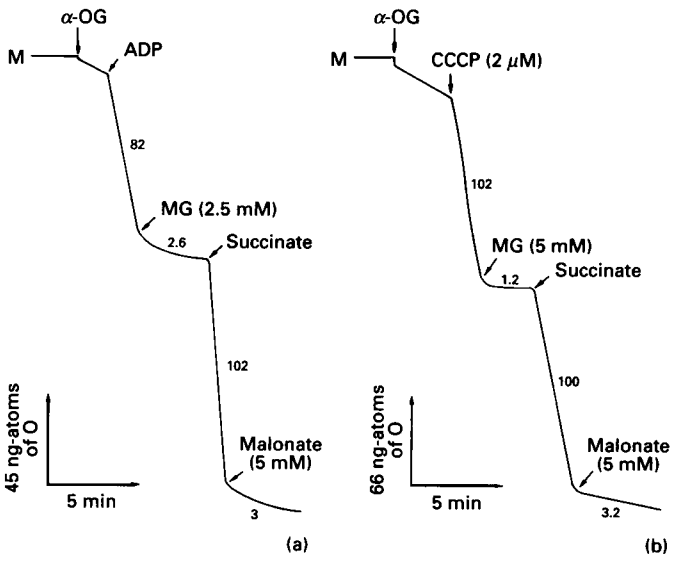


**Figure 1 Effect of methylglyoxal on the respiration of EAC-cell mitochondria with α-OG or succinate as respiratory substrate**

The Figure shows the direct oxygraph tracings of two typical experiments. Tracings (**a**) and (**b**) represent ADP-stimulated and uncoupler (CCCP)-stimulated respirations respectively. Details of the incubation media are described in the Materials and methods section. Respiration was measured at 30 °C with EAC-cell mitochondria containing approx. 2.8 mg (tracing **a**) or 3.1 mg (tracing **b**) of protein. Additions of other compounds are indicated in the respective tracings. Numbers along the tracings represent the rate of oxygen consumption (ng-atoms/min). In a control set of experiments where no methylglyoxal or inhibitor was added, the added amount of mitochondria can utilize the full amount of oxygen (approx. 990 ng-atoms) present in the incubation media within a period of 15 or 12 min with α-OG or succinate as respiratory substrate respectively, after addition of ADP. For CCCP-stimulated respiration, the same amount of oxygen can be consumed by the mitochondria within 12 min with α-OG as substrate (results not shown). Abbreviations: M, mitochondria; MG, methylglyoxal.

**Table 1 Effect of methylglyoxal on ADP-stimulated respiration of EAC-cell mitochondria with various substrates at 30 °C**

Respiration was measured by monitoring the oxygen consumption of EAC-cell mitochondria containing approx. 2.5–3 mg of protein.

| Respiratory substrates | Methylglyoxal (mM) | Addition of inhibitors (complex-specific) | Oxygen consumed (ng-atoms/10 min) |
|---|---|---|---|
| α-OG (10 mM) | None | Malonate (5 mM) | 426 ± 18 |
| α-OG (10 mM) | 2.5 | Malonate (5 mM) | 21 ± 4 |
| Succinate (10 mM) | None | Rotenone (4 μM) | 477 ± 21 |
| Succinate (10 mM) | 10 | Rotenone (4 μM) | 471 ± 19 |
| Duroquinol (0.75 mM) | None | Rotenone (4 μM) + malonate (5 mM) | 270 ± 17 |
| Duroquinol (0.75 mM) | 5 | Rotenone (4 μM) + malonate (5 mM) | 276 ± 16 |
| α-OG (10 mM) + TMPD (60 μM) | 10 | None | 456 ± 21 |
| α-OG (10 mM) | None | Rotenone (5 μM) | 26 ± 4 |
| α-OG (10 mM) + TMPD (60 μM) | None | Rotenone (5 μM) | 442 ± 22 |

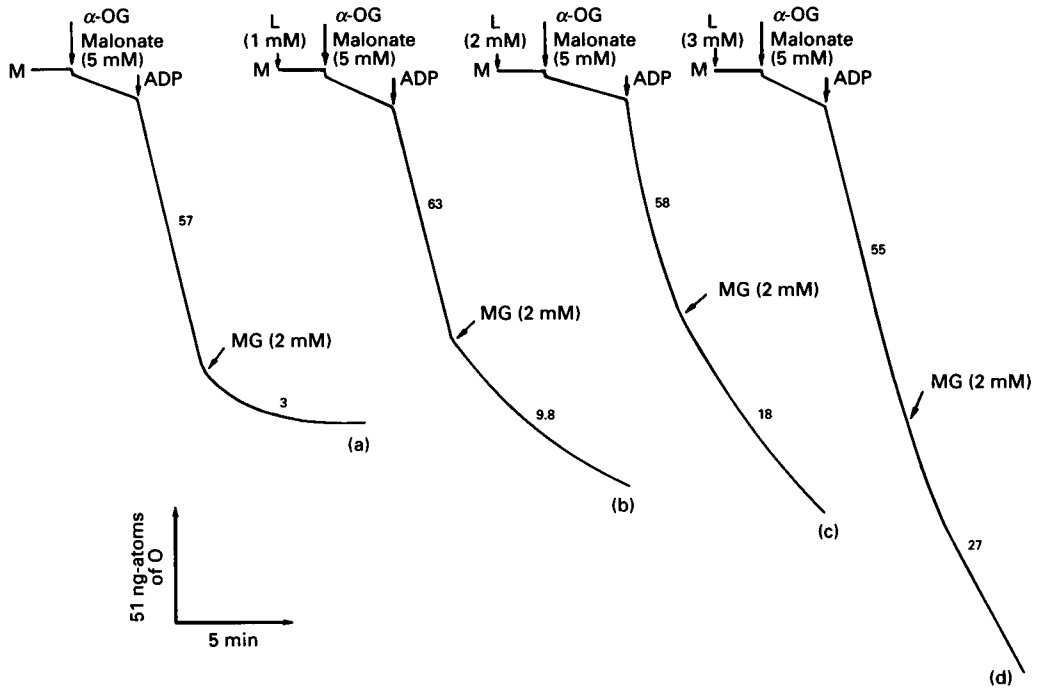


**Figure 2 Effect of L-lactaldehyde on methylglyoxal inhibition of EAC-cell mitochondrial respiration with α-OG as respiratory substrate**

In each case, respiration was measured at 30 °C with EAC-cell mitochondria (M) containing approx. 2.0 mg of protein. The Figure shows the direct oxygraph tracings of a typical experiment. Tracing (**a**) represents oxygen consumption in the absence of L-lactaldehyde, and tracings (**b**), (**c**) and (**d**) represent oxygen consumption in presence of 1 mM, 2 mM and 3 mM L-lactaldehyde (L) respectively. Addition of methylglyoxal (MG) and other compounds is also indicated in the tracings. Numbers along the traces represent the rate of oxygen consumption (ng-atoms/min). A control was maintained without methylglyoxal or L-lactaldehyde, where the added amount of methylglyoxal could utilize the full amount of oxygen (approx. 990 ng-atoms) present in the incubation media within 20 min with α-OG as substrate after addition of ADP (results not shown).

shown). All these studies confirm that the electron flow through complex IV was not inhibited by methylglyoxal. We could not use ascorbate plus TMPD for the possible reaction of methylglyoxal with ascorbate, as previously reported by other investigators (Fodor et al., 1979; Szent-Györgyi, 1979).

We have also observed that the inhibitory effect of methylglyoxal on α-OG-dependent respiration of EAC-cell mitochondria is irreversible. After incubation of EAC-cell mitochondria for 15 min with methylglyoxal (5 mM), washing in PBS (40 mM NaCl, 2.7 mM KCl, 6.5 mM $Na_2HPO_4$, 1.5 mM $KH_2PO_4$, adjusted to pH 7.3 with NaOH) and monitoring of the oxygen consumption, the tumour cell mitochondria did not regain their respiratory ability with α-OG as substrate. But when succinate was added to that system, mitochondrial respiration immediately started, further confirming that no other complex of the respiratory chain except complex I was affected by methylglyoxal. However, in the control set of experiments, in which mitochondria had been incubated without methylglyoxal, there was both α-OG and succinate-dependent respiration (results not shown).

We had previously observed that, in contrast with EAC-cell mitochondrial respiration, the respiration of mitochondria isolated from liver and kidney of normal mice was not affected by methylglyoxal (Halder et al., 1993). To test the possibility that methylglyoxal was differently utilized by mitochondria of normal and malignant cells, methylglyoxal was incubated separately

with EAC-cell mitochondria and mitochondria of liver or kidney of normal mice. It was observed that methyglyoxal remained completely un-utilized by both normal and malignant-cell mitochondria (results not shown).

We previously reported the purification and characterization of the enzyme methylglyoxal reductase from goat liver (M. Ray and S. Ray, 1984). This enzyme forms L-lactaldehyde from methylglyoxal and NAD(P)H. We have also observed that in the presence of lactaldehyde the inhibitory effect of methylglyoxal on the respiration, viability and transplantability of EAC cells is significantly decreased (Ray et al., 1991). This protective effect of lactaldehyde against methylglyoxal inhibition has been investigated further in the present study with EAC-cell mitochondria.

Figure 2 indicates that L-lactaldehyde can exert a similar protective action against inhibition of EAC-cell mitochondrial respiration by methylglyoxal. That the protective effect of L-lactaldehyde was specific against methylglyoxal inhibition was indicated by the fact that L-lactaldehyde could not provide any protective effect against rotenone-induced inhibition of mitochondrial respiration (results not shown). A similar protective effect was also observed with D-lactaldehyde instead of L-lactaldehyde (results not presented).

## DISCUSSION

Although the precise biological function of methylglyoxal has still remained uncertain, the anti-tumour and growth-inhibitory properties of this compound have been known for a long time (Együd and Szent-Györgyi, 1966, 1968; Apple and Greenberg, 1968). It is generally believed that methylglyoxal exerts its anti-tumour effect through its growth-inhibitory property, which is mediated by inhibiting protein synthesis and interacting with nucleic acids (Együd and Szent-Györgyi, 1966; Conroy, 1979). But whether these effects of methylglyoxal are qualitatively different in normal and malignant cells has not been seriously investigated. On the other hand, our previous work with several normal and malignant cells and tissues have shown that methylglyoxal has a strong inhibitory effect on the respiration specifically of the malignant cells (Ray et al., 1991). We have also found that EAC-cell mitochondrial respiration was inhibited by methylglyoxal, whereas mitochondria isolated from liver and kidney of normal mice were not affected (Halder et al., 1993).

The results in the present paper have extended our earlier findings on the effect of methylglyoxal on respiration and strongly suggest that methylglyoxal inhibits electron flow through the complex I of EAC-cell mitochondria. This was evident from our observation that methylglyoxal inhibited specifically the α-OG- or malate plus pyruvate-dependent respiration, whereas succinate-dependent respiration was completely unaffected. We have also observed that the respiration of EAC-cell mitochondria with glutamate plus malate or glutamine as substrates is inhibited by methylglyoxal (S. Ray, S. Dutta and M. Ray unpublished work). The involvement of complex I was further confirmed by other studies using various artificial substrates and inhibitors of the mitochondrial respiratory chain.

It was reported long ago that methylglyoxal at a very high concentration (46 mM) inhibited succinate-dependent oxidation in rat liver mitochondria, and the inhibition required time-dependent incubation of the mitochondria with methylglyoxal before addition of the substrate succinate (Kun, 1950). But our studies indicate that, up to a concentration of 10 mM, methylglyoxal has no effect on succinate-dependent respiration, whereas the α-OG- or malate plus pyruvate-dependent respiration is almost instantaneously inhibited even with 2.5 mM methylglyoxal, suggesting the possible inhibition of succinate dehydrogenase by methylglyoxal to be of limited physiological significance.

The possible involvement of various metabolite transporters of mitochondria in the inhibitory effect of methylglyoxal has not been investigated in the present work. Different respiratory substrates are transported in mitochondria through specific transporters (Klingenberg, 1979). From the results in the present paper, it appears that methylglyoxal inhibits the oxygen consumption of EAC-cell mitochondria with a variety of respiratory substrates, e.g. α-OG, malate plus pyruvate, glutamate plus malate, and glutamine. All these substrates are NAD-linked and donate electrons to complex I. But these substrates are transported to mitochondria through some different, as well as some common, metabolite transporters. On the other hand, the oxidation of succinate, a FAD-linked substrate which donates electrons at complex II, is not inhibited by methylglyoxal. It is unlikely that methylglyoxal is inhibitory to the transport of all the substrates which donate electrons at complex I, leaving unaffected the transport of succinate, which donates electrons at complex II. Succinate, incidentally, has a common transporter with malate. However, a more definite answer to this question should come from the direct study of the transport of various respiratory substrates and/or from the isolation and assay of complex I.

If methylglyoxal is inhibitory for complex I, it seems rational to identify the specific component of this complex and its possible alteration in the malignant cells. Results in the present and in our previous papers (Ray et al., 1991; Halder et al., 1993) strongly suggest the importance of this study in understanding the specific biochemical difference between a normal and a cancerous cell.

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