Reversible sensitization and desensitization of carnitine palmitoyltransferase I to inhibition by malonyl-CoA in isolated rat liver mitochondria

Significance for the mechanism of malonyl-CoA-induced sensitization

Victor A. ZAMMIT

Hannah Research Institute, Ayr, Scotland KA6 5HL, U.K.

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Preincubation of rat liver mitochondria with 5,5'-dithiobis-(2-nitrobenzoic acid) (Nbs₂) followed by removal of excess reagent by washing the mitochondria with 0.5 mM-reduced glutathione resulted in a desensitization of carnitine palmitoyltransferase (CPT) I activity to malonyl-CoA inhibition. The effect was not observed if mitochondria were washed with 0.5 mM-dithiothreitol. The desensitization effect of Nbs₂ could be reversed by a second incubation in the presence of 8 μ M-malonyl-CoA. In addition, malonyl-CoA, when present simultaneously with Nbs₂, protected CPT I activity against the desensitization effect of the thiol-group reagent. These results suggest that malonyl-CoA exerts an effect on one or more thiol groups of the enzyme, and that this effect is related to the ability of the metabolite to sensitize CPT I to malonyl-CoA inhibition.

The sensitivity of the overt activity of CPT and, consequently, the rate of long-chain fatty acid oxidation, in rat liver mitochondria to inhibition by malonyl-CoA varies with the physiological state of the animals from which the mitochondria are isolated (Ontko & Johns, 1980; Cook *et al.*, 1980; Saggerson & Carpenter, 1981; Robinson & Zammit, 1982; McGarry & Foster, 1981; Stakkestad & Bremer, 1983). When studied under conditions in which the enzyme is sensitive to inhibition by physiological concentrations of malonyl-CoA, the sensitivity of CPT I to malonyl-CoA is directly and quantitatively related to the concentration of malonyl-CoA *in vivo* (Robinson & Zammit, 1982). This relationship can be mimicked *in vitro* by preincubation of mitochondria with malonyl-CoA (Zammit, 1983). The sensitization of the enzyme so induced is retained after separation of the mitochondria from the malonyl-CoA-containing medium (Zammit, 1983). The concentrations of malonyl-CoA required to elicit such sensitization *in vitro* are in the same range as those found *in vivo* (0.4–6.5 nmol/g; Zammit, 1981). Therefore it was suggested that a direct malonyl-CoA-mediated sensitization of CPT I to competitive inhibition of the enzyme by the same metabolite could explain the relationship between CPT I sensitivity and malonyl-CoA *in vivo* (Robinson & Zammit, 1982) and, more

importantly, that it may operate *in vivo* to produce an amplification of the response of the enzyme to small changes in hepatic malonyl-CoA concentrations (Zammit, 1983).

If CPT I sensitivity to malonyl-CoA is at least partly determined by direct effects of malonyl-CoA, it should be possible to induce reversible changes in sensitivity of the enzyme *in vitro* if appropriate sensitizing and desensitizing conditions are used. Such observations would have major physiological relevance in that they would indicate that changes in sensitivity similar to those observed *in vivo* could be reproduced in the absence of hormonal and other stimuli in the intact cell.

Previous work has shown that two conditions that have opposite effects on CPT I sensitivity are preincubation of mitochondria with malonyl-CoA (Zammit, 1983) and incubation with the thiol-group reagent Nbs₂ (Saggerson & Carpenter, 1981). Therefore in the present study the effects of successive exposure of mitochondria to these two conditions have been used to investigate whether the sensitivity of CPT I inherent in mitochondria from rats in any one given physiological condition can be changed reversibly *in vitro*.

Materials and methods

Rats

The source and maintenance of the female Wistar rats used were as described previously (Zammit,

Abbreviations used: CPT, carnitine palmitoyltransferase (EC 2.3.1.21); Nbs₂, 5,5'-dithiobis-(2-nitrobenzoic acid); GSH, reduced glutathione.

1980, 1981). The animals weighed 180–200 g when used.

Chemicals

The sources of biochemicals and other reagents were as described by Robinson & Zammit (1982). In addition, Nbs₂ was from Sigma (London) Chemical Co. (Poole, Dorset BH17 7NH, U.K.) and L-[methyl-¹⁴C]carnitine hydrochloride was from Amersham International (Amersham, Bucks. HP7 9LL, U.K.).

Preparation and preincubation of mitochondria

Rats were killed at 09:30h by cervical dislocation, and the livers were quickly excised, rinsed in ice-cold homogenization buffer, minced and homogenized in a medium containing 250 mM-sucrose, 5 mM-Tris/HCl and 1 mM-EGTA, pH 7.4 at 0–2°C. Mitochondria were prepared as described by Chappell & Hansford (1972) and washed once. Mitochondria from one liver were resuspended in 9 ml of the sucrose medium, and three equal samples (containing approx. 40 mg of mitochondrial protein) were preincubated under various conditions.

Preincubations were performed essentially as described by Zammit (1983). Each period of preincubation lasted 10 min and was performed either in sucrose medium or in KCl medium (120 mM-KCl, 5 mM-Tris/HCl, 1 mM-EGTA, 0.5 mM-GSH and 10 mg of dialysed fatty-acid-free albumin/ml, pH 7.4 at 0–2°C) as indicated in the legends to Figures. In all experiments the mitochondrial pellets obtained after sedimentation from the final incubation in KCl medium were washed with fresh KCl medium without resuspension, and were then resuspended in 5 ml of the same medium and the mitochondria separated through a layer of oil into sucrose medium as described previously (Zammit, 1983). The tubes and surfaces of the oil layers were rinsed as described previously (Zammit, 1983), and the pellets were resuspended in 3 ml of sucrose medium (protein concentration approx. 10 mg/ml) and kept on ice. Assays were performed within 30 min of final resuspension of the mitochondria.

Assay of enzyme activity

CPT I activity was assayed over 2 min as described previously (Robinson & Zammit, 1982; Zammit, 1983). The assay medium (pH 7.1) contained (in 2 ml) 220 mM-sucrose, 40 mM-KCl, 10 mM-Tris/HCl, 1 mM-EGTA, 1 mM-GSH, 10 mg of dialysed fatty-acid-poor albumin/ml, 80 μM-palmitoyl-CoA, the appropriate amount of malonyl-CoA and approx. 0.3 mg of mitochondrial protein.

Expression of results

The 'control' activity of CPT I for mitochondria treated according to any one particular protocol refers to the activity measured in the absence of added malonyl-CoA from the assay medium. Throughout, the text the terms 'sensitivity', 'sensitization', 'resensitization' and 'desensitization' refer to the sensitivity (and the changes thereof) of CPT I to inhibition by malonyl-CoA during assay of the enzyme activity.

Results and discussion

Desensitization effect of Nbs₂

These experiments were designed to investigate the effect of preincubation of mitochondria with Nbs₂ and, after reaction of excess (bulk phase) reagent with GSH, to study the sensitivity of CPT I to inhibition by malonyl-CoA during assay. Therefore it must be stressed that Nbs₂ was not added to the assay medium, and any effects of the reagent must have been due to its continued effect on the mitochondria after their washing and separation through an oil layer.

Preincubation of mitochondria from fed rats with 0.9 mM-Nbs₂ resulted in the desensitization of CPT I to malonyl-CoA inhibition (Fig. 1). Routinely 0.5 mM-GSH was used to wash mitochondria after Nbs₂ treatment, but use of a 10-fold higher concentration of GSH did not alter the degree of desensitization observed. However, the Nbs₂-induced desensitization was reversible by 0.5 mM-dithiothreitol. These observations suggest that the effect of Nbs₂ was reversible and that it was due to the formation of an Nbs–enzyme disulphide linkage, which could be reduced by dithiothreitol but not by GSH, the former having a much lower redox potential (Cleland, 1964). Nbs₂ did not have any effect on 'control' activity of CPT I.

Reversal of Nbs₂-induced desensitization by malonyl-CoA

The observation that Nbs₂-induced desensitization persisted after reaction of excess reagent with GSH enabled the study of the ability of malonyl-CoA to reverse the effects of Nbs₂. When mitochondria were exposed to a second period of incubation with malonyl-CoA (8 μM), the Nbs₂-induced desensitization of CPT I to malonyl-CoA inhibition was reversed (Fig. 1). This reversal restored the sensitivity of the enzyme almost to that found in 'native' mitochondria (i.e. those treated simultaneously in an identical manner, but in the absence of any added malonyl-CoA and/or Nbs₂; see the Materials and methods section). Qualitatively similar Nbs₂-induced desensitization and malonyl-CoA-mediated resensitization character-

Fig. 1. Desensitization effect of Nbs_2 on CPT I activity and its reversal by malonyl-CoA in mitochondria from livers of fed virgin female rats

Mitochondria (40 mg of protein) were incubated for successive 10 min periods in 90 ml of sucrose medium either in the absence of any additions (○) or in the presence of 0.9 mM- Nbs_2 (●), or 0.9 mM- Nbs_2 in sucrose medium followed by 8 μM-malonyl-CoA in 90 ml of KCl medium (Δ). After separation from the incubation medium (see the Materials and methods section) the activity of CPT I was measured in each type of mitochondria either in the absence ('control') activity = 100%) or in the presence of different concentrations of malonyl-CoA. 'Control' activities were 0.79 ± 0.05 , 0.76 ± 0.07 and 0.72 ± 0.14 nmol/min; per mg of mitochondrial protein, at 37°C, respectively. Values are means $\pm$ S.E.M. for four determinations on separate mitochondrial preparations.

istics were found for mitochondria from livers of starved pregnant rats (results not shown), in which the sensitivity of CPT I was initially lower (see also Robinson & Zammit, 1982; Zammit, 1983).

Protection by malonyl-CoA against Nbs_2 -induced effects on CPT I

To investigate whether malonyl-CoA could protect CPT I against the Nbs_2 -induced effects, the

Fig. 2. Protection by malonyl-CoA against the desensitization effect of Nbs_2 on CPT I in mitochondria from fed virgin rats

CPT I activity was measured either in the absence ('control') or in the presence of 0.2 μM-malonyl-CoA. Mitochondria were preincubated with concentrations of Nbs_2 in sucrose either after preincubation with, and in the presence of, 8 μM-malonyl-CoA (●) or in the absence of malonyl-CoA (○). 'Control' activities of CPT I for mitochondria preincubated in the presence of 1.2 mM- Nbs_2 alone, 1.2 mM- Nbs_2 plus 8 μM-malonyl-CoA, 8 μM-malonyl-CoA alone, or in the absence of any additions, were 0.71 ± 0.06 , 0.75 ± 0.08 , 0.72 ± 0.06 and 0.74 ± 0.07 nmol/min per mg respectively. 'Control' activities were determined for all the other Nbs_2 concentrations used, and they were within the range of the examples given.

dependence of desensitization on Nbs_2 concentration was studied in the presence or absence of malonyl-CoA (Fig. 2). Inclusion of malonyl-CoA (8 μM) in the incubation medium protected CPT I against desensitization by Nbs_2 . The effect of Nbs_2 on the enzyme showed saturation characteristics such that the relationships between the extent of desensitization and Nbs_2 concentration in the presence or absence of malonyl-CoA could be linearized in plots of the form $100 \times (\text{desensitization})^{-1}$ versus $[Nbs_2]^{-1}$

(correlation coefficients for the regression lines were >0.99) (results not shown). When desensitization was expressed as a percentage of sensitivity in the absence of Nbs_2 (i.e. 'initial' activity; see the Materials and methods section), the effect of malonyl-CoA was to raise the concentration of Nbs_2 required to produce half-maximal desensitization from 0.7 mM- to 1.4 mM- Nbs_2 .

Physiological considerations

Two main observations have emerged from the present study: first, that malonyl-CoA can reverse Nbs_2 -induced desensitization of CPT I, and second, that malonyl-CoA protects CPT I against desensitization by Nbs_2 . The data are comparable with the suggestion that Nbs_2 interacts with a thiol group(s) which is (are) essential for the expression of the malonyl-CoA-induced sensitization of CPT I.

The present work provides some indications about the mechanism through which the malonyl-CoA-induced sensitization of the enzyme is achieved. The protection by malonyl-CoA of CPT I against the effects of Nbs_2 suggests that sensitization of CPT I by malonyl-CoA *in vitro* results either in an alteration in the availability of one or more thiol groups of the enzyme for reaction with Nbs_2 or in the establishment of a state of CPT I, the formation of which is antagonized by modification of thiol groups. Therefore these observations suggest a role

for malonyl-CoA in affecting the possible involvement of thiol groups in the modulation of CPT I sensitivity (Saggerson & Carpenter, 1981).

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References

- Chappell, J. B. & Hansford, R. G. (1972) in *Subcellular Components; Preparation and Fractionation*, 2nd edn. (Birnie, G. D., ed.), pp. 77–91, Butterworths, London
- Cleland, W. W. (1964) *Biochemistry* **3**, 480–482
- Cook, G. A., Otto, D. A. & Cornell, N. W. (1980) *Biochem. J.* **192**, 955–958
- McGarry, J. D. & Foster, D. W. (1981) *Biochem. J.* **200**, 217–223
- Ontko, J. A. & Johns, M. L. (1980) *Biochem. J.* **192**, 959–962
- Robinson, I. N. & Zammit, V. A. (1982) *Biochem. J.* **206**, 177–179
- Saggerson, E. D. & Carpenter, C. A. (1981) *FEBS Lett.* **129**, 229–232
- Stakkestad, J. A. & Bremer, J. (1983) *Biophys. Acta* **750**, 244–252
- Zammit, V. A. (1980) *Biochem. J.* **190**, 293–300
- Zammit, V. A. (1981) *Biochem. J.* **198**, 75–83
- Zammit, V. A. (1983) *Biochem. J.* **210**, 953–956