

Tumour necrosis factor-alpha uncouples respiration in isolated rat mitochondria

Sílvia Busquets^a, Xavier Aranda^b, Miquel Ribas-Carbó^c,
Joaquim Azcon-Bieto^b, Francisco J. López-Soriano^{a,*}, Josep M. Argilés^a

^a*Cancer Research Group, Departament de Bioquímica i Biologia Molecular, Facultat de Biologia, Universitat de Barcelona, Diagonal 645, Barcelona 08028, Spain*

^b*Departament de Biologia Vegetal, Universitat de Barcelona, Barcelona, Spain*

^c*Departament de Biologia, Universitat de les Illes Balears, Mallorca, Spain*

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Abstract

Recent studies have demonstrated the existence of an intracellular (associated with mitochondria) tumour necrosis factor- α (TNF) binding protein. In an attempt to elucidate if this receptor could be involved in TNF action, we have incubated liver isolated mitochondria in the presence of recombinant murine TNF. The results show that the addition of TNF at concentrations as low as 10^{-6} U/ μ l resulted in a clear uncoupling respiration of liver isolated mitochondria, therefore suggesting that TNF can indeed exert intracellular effects, which are possibly linked with its cytotoxic mechanism.

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1. Introduction

The pleiotropic cytokine tumour necrosis factor- α (TNF), primarily produced by activated macrophages and blood monocytes, exerts a wide range of inflammatory and immunomodulatory activities [1] and exerts diverse metabolic effects (see Ref. [2] for a review). In addition, TNF (particularly in combination with other cytokines such as interferon- γ) has been shown to be cytotoxic for a number of different cell types including tumour cells. The mechanism of its cytotoxicity seems to be associated with the induction of reactive oxygen intermediates (ROIs), presumably produced in the mitochondria [3] as a result of interference with the normal electron flow. In addition, it has been demonstrated that in L929 cells, TNF leads to an early degeneration of the mitochondrial ultrastructure without any pronounced damage to other cellular organelles [4].

TNF initiates its biological effects by its binding to high affinity cell surface receptors. It binds as homotrimer to two kinds of receptors: TNFR1 (p55) and TNFR2 (p75) [5]. The expression of the genes encoding the two receptors has been detected in almost all types of nucleated cells and it is differentially regulated. Thus TNFR1 is expressed constitutively and plays a central role in many biological processes, whereas less is known about TNFR2 expression (see Ref. [6] for a review). Recently, in addition to the two receptors localized in the cell surface, Ledgerwood et al. [7] have identified a 60 kDa TNF mitochondrial-binding protein as well as an intracellular trafficking pathway for the cytokine. Indeed, by using TNF conjugated to gold particles, the authors reported that intracellular TNF was localized predominantly to lysosomal structures and trafficking vesicles, and a proportion of it was delivered to morphologically normal mitochondria [7].

In relation to TNF action, Lancaster et al. [8] have reported the inhibition of cell mitochondrial electron transfer by TNF, and suggested that a bioenergetic dysfunction may be involved in the cytotoxic mechanism of the cytokine. Others have also shown that the cytotoxic

* Corresponding author. Tel.: +34-9340-34-609; fax: +34-9340-21-559.

E-mail address: fsoriano@porthos.bio.ub.es (F.J. López-Soriano).

activity of TNF upon L929 cells is associated with an inhibition of cell respiration, in particular using succinate and NADH as substrates [4].

It was therefore the main aim of the present work to elucidate if the action of TNF on mitochondrial respiration is direct at the mitochondrial level, possibly by interacting with the aforementioned mitochondrial TNF-binding protein. To accomplish this objective, we measured oxygen consumption in isolated mitochondria from liver in the absence or presence of different concentrations of the cytokine.

2. Results and discussion

In order to elucidate the effects of TNF on mitochondrial oxygen consumption, the cytokine was added ($1 \text{ U}/\mu\text{l}$ – $10^{-6} \text{ U}/\mu\text{l}$) to mitochondria 5 min before the substrate (10 mM succinate) was added. Oxygen consumption was assessed after this period and the cytokine was not added again.

Table 1 shows the effects of the cytokine ($1 \text{ U}/\mu\text{l}$ – $10^{-6} \text{ U}/\mu\text{l}$) on the respiratory behaviour of isolated liver mitochondria. Treatment with the cytokine resulted in a significant decrease in respiratory control ratio (RCR), suggesting a potent uncoupling effect. The RCR is an indication of how fast the rates of O_2 consumption are for each experiment and also how well-coupled the mitochondria are (a lower RCR showed that the reaction was not as well-coupled). This decrease was observed at all concentrations tested, being as low as $10^{-6} \text{ U}/\mu\text{l}$, clearly in the pathophysiological range [9,10]. Interestingly, other proinflammatory cytokines (but not involved in apoptosis) such as interleukin-1 α (IL-1 α) and interleukin-1 β (IL-1 β) had no effects in the respiratory behaviour of isolated liver mitochondria, therefore suggesting that the effects of TNF are clearly specific of this

Table 1
Effect of TNF on respiratory control ratio (RCR)

Addition	RCR
None	3.82 ± 0.10 (11)
TNF $1 \text{ U}/\mu\text{l}$	2.83 ± 0.05 (2)**
TNF $10^{-1} \text{ U}/\mu\text{l}$	3.13 ± 0.18 (5)**
TNF $10^{-2} \text{ U}/\mu\text{l}$	2.94 ± 0.02 (3)***
TNF $10^{-3} \text{ U}/\mu\text{l}$	3.11 ± 0.17 (8)***
TNF $10^{-4} \text{ U}/\mu\text{l}$	2.89 ± 0.55 (3)*
TNF $10^{-5} \text{ U}/\mu\text{l}$	3.08 ± 0.17 (3)**
TNF $10^{-6} \text{ U}/\mu\text{l}$	3.30 ± 0.10 (6)**
IL-1 α $10^{-1} \text{ U}/\mu\text{l}$	3.69 ± 0.11 (6)
IL-1 β $10^{-1} \text{ U}/\mu\text{l}$	3.70 ± 0.10 (3)

For more details, see Section 3. Mitochondria were incubated for 5 min in the absence or presence of TNF ($1 \text{ U}/\mu\text{l}$ – $10^{-6} \text{ U}/\mu\text{l}$) or IL-1 α and IL-1 β at 25°C . Oxygen consumption was determined after this period. Results are RCR (state III/state IV) and are mean \pm S.E.M. (number of experiments). Statistical significance of the results (by Student's *t* test). None versus TNF: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

cytokine. The results presented in Fig. 1 are representative of the effects induced by TNF in oxygen consumption in fresh liver mitochondria preparations.

Several mechanisms have been suggested as responsible for the physiological uncoupling on the basis of an increase of proton conductivity of the inner mitochondrial membrane, including adenine nucleotide translocase and the aspartate/glutamate carrier [11]. These mechanisms coexist with the specialized uncoupling proteins (UCPs) described in different tissues. It has been reported that the expression of the gene coding for one of these proteins (UCP2) is initiated in liver after TNF

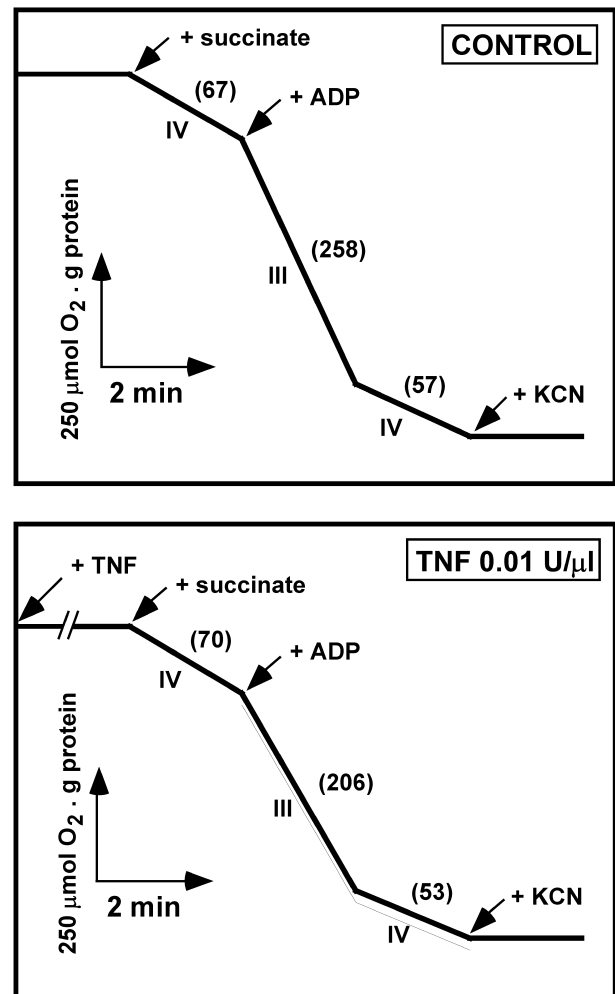


Fig. 1. Effects of TNF on the respiratory behaviour of isolated liver mitochondria. For more details, see Section 3. TNF was added where indicated. Rates are expressed as $\mu\text{mol O}_2/\text{min/g protein}$. Oxygen uptake was measured by mixing the isolated mitochondria in a total volume of 2 ml of buffer (0.3 M sucrose, 5 mM KH_2PO_4 , 10 mM TES, 10 mM NaCl, 2 mM MgSO_4 , 0.1% fatty acid free bovine serum albumin at pH 7.2) and the paper speed was 1 cm/min. Mitochondria were incubated for 5 min in the absence or presence of TNF ($1 \text{ U}/\mu\text{l}$ – $10^{-6} \text{ U}/\mu\text{l}$) or IL-1 α and IL-1 β at 25°C . Succinate was used as a substrate. ADP (100 μM) was added where indicated. The reaction was stopped with 2 mM KCN. Each plot is representative of at least three different experiments with similar results. III: state III; IV: state IV.

challenge [12]. In addition, TNF administration results in an increased expression of both UCP2 and UCP3 in skeletal muscle [13] as well as in a cancer cachexia in an experimental model associated with high levels of plasma TNF [14].

The results presented here clearly indicate that TNF is capable of exerting direct effects on mitochondria. This is the first report that demonstrates this direct action and agrees with the presence of an intracellular (mitochondria-linked) TNF-binding protein as described by Ledgerwood et al. [7]. Indeed, these authors have identified a 60 kDa TNF mitochondrial-binding protein as well as an intracellular trafficking pathway for the cytokine, suggesting that the cytotoxic effects of TNF could well follow this mode of action. Thus, it is now widely recognized that mitochondria have a central role in the cell death process [15] and mitochondrial involvement has been reported in TNF-induced cytotoxicity [3]. Further, Hennet et al. [16] have shown that TNF cytotoxicity was associated with decreased activity of succinate dehydrogenase in L929 cells. Interestingly, using L929 cells, Sánchez-Alcazar et al. [17] have shown that by partially blocking mitochondrial respiration there is a down-regulation of cell surface receptors, suggesting that this is possibly accomplished by changing the affinity of receptors for the cytokine.

In conclusion, the results presented here suggest that some of the TNF effects do not need a mediator to transmit the cytosolic TNF signal to the mitochondria but rather internalized intact TNF is able to exert direct effects on mitochondria. This may contribute to the further understanding of the molecular mechanism underlying TNF-induced cytotoxicity.

3. Materials and methods

3.1. Materials

Recombinant murine TNF- α , IL-1 α and IL-1 β were obtained from PeproTech (London, UK). All other biochemicals were reagent grade and obtained from Sigma Chemical Co. (St Louis, MO, USA) or from Roche Diagnostic SL (Barcelona, Spain).

3.2. Isolation of mitochondria

Mitochondria isolation was performed essentially as described [18]. Briefly, fresh liver tissue was minced in a medium containing 70 mM sucrose, 210 mM mannitol, 2 mM HEPES and 1 mM EGTA (pH 7.2). Bovine serum albumin (free of free fatty acid) was present at a concentration of 0.5 g/l. After homogenization, the supernatant was sequentially centrifuged to obtain mitochondria. Activity assays for plasma membrane (alkaline phosphodiesterase), lysosomes (β -hexosaminidase)

and cytosol (lactate dehydrogenase) confirmed the purity of the mitochondrial preparations used.

3.3. Oxygen consumption studies

The rate of O₂ uptake was determined with an oxygen electrode (Rank Brothers, Bottisham, Cambridge, UK) in a closed cuvette kept at 25 °C. The O₂ concentration of the solution in equilibrium with air was considered to be 254 μ M. All measurements refer to 500 μ g of mitochondrial protein determined according to the method of Bradford [19]. Succinate (10 mM) was used as substrate in all the experiments.

3.4. Statistical analysis

Statistical analysis of the data was performed by means of the Student's *t* test.

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