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Stimulation by pro-apoptotic valinomycin of cytosolic NADH/cytochrome c electron transport pathway—Effect of SH reagents

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ABSTRACT

Intrinsic and extrinsic apoptosis are both characterised by the presence of cytochrome c (cyto-c) in the cytosol. We present data on the extra-mitochondrial NADH oxidation catalysed by exogenous (cytosolic) cyto-c, as a possible answer to the paradox of apoptosis being an energy-dependent program but characterized by the impairment of the respiratory chain. The reduction of molecular oxygen induced by the cytosolic NADH/cyto-c pathway is coupled to the generation of an electrochemical proton gradient available for ATP synthesis. Original findings show that SH reagents inhibit the NADH/cyto-c system with a conformational change mechanism. The mitochondrial integrity-test of sulfite oxidase unequivocally demonstrates that this enzyme (120 kDa) can be released outside but exogenous cyto-c (12.5 kDa) does not permeate into mitochondria. Valinomycin at 2 nM stimulates both the energy-dependent reversible mitochondrial swelling and the NADH/cyto-c oxidation pathway. The pro-apoptotic activity of valinomycin, as well as to the dissipation of membrane potential, can be also ascribed to the increased activity of the NADH/cyto-c oxidation pathway useful as an additional source of energy for apoptosis. It can be speculated that the activation of the NADH/cyto-c system coupled to valinomycin-induced mitochondrial osmotic swelling may represent a strategy to activate apoptosis in confined solid tumours.

1. Introduction

Permeabilization of the mitochondrial outer membrane (MOM) remains one of the first events of both the intrinsic and extrinsic pathways of apoptosis driving the release of pro-apoptotic proteins, including cytochrome c (cyto-c), from the mitochondrial intermembrane space (MIS) (Ow et al., 2008). The discovery of the involvement of cytosolic cyto-c remains a milestone in the elucidation of the programmed cell death (Liu et al., 1996). It was definitively shown that cyto-c is not present exclusively in the MIS but mobilized by cleavage of OPA1 protein (Frezza et al., 2006) participates in fundamental processes inside the cytosol.

Cumulative data are consistent with the direct oxidation of cytosolic NADH catalysed by cyto-c molecules present outside the mitochondria. This system promotes the reduction of molecular oxygen, generates an electrochemical proton gradient available for ATP synthesis and is inhibited by cyanide but not by other respiratory chain inhibitors (Bodrova et al., 1998; Gorgoglione et al., 2007; La Piana et al., 1998, 2005; Lemeshko et al., 2003; Lofrumento et al., 1991; Marzulli et al., 1995). The main components of the system may reside at specific contact points between the two mitochondrial membranes indicated as "respiratory contact sites" (Gorgoglione et al., 2010; La Piana et al., 2005; Marzulli et al., 1999). The exogenous NADH oxidation supported by the malate-aspartate shuttle is comparable to that of the NADH/cyto-c system (Abbrescia et al., 2012). Note that the malate-aspartate shuttle is an indirect pathway since the reducing equivalents are first transferred to oxaloacetate, converted into malate which is relocated to and oxidized inside the mitochondria generating NADH in the matrix to be oxidized by Complex I. Then electrons are sent via the

Abbreviations: cyto-c, cytochrome c; MOM, mitochondrial outer membrane; MIS, mitochondrial intermembrane space; MIM, mitochondrial inner membrane; $\Delta\Psi m$, mitochondrial membrane potential; Δp, electrochemical proton gradient; porin, voltage dependent anion channel; Sox, sulfite oxidase; NEM, *N*-ethylmaleimide; Mrs, mersalyl.

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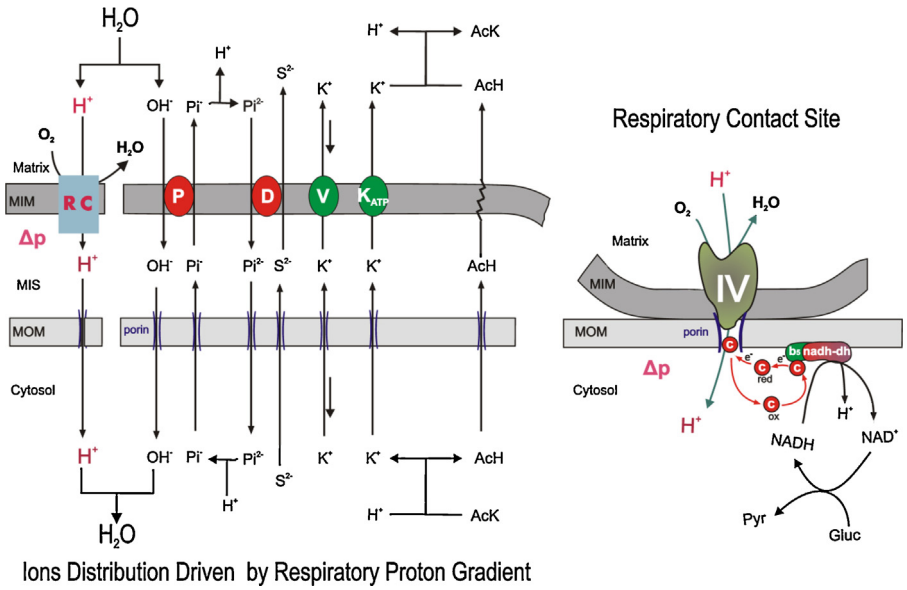

Fig. 1. Schematic representation of a "respiratory contact site" and ions distribution driven by the respiratory proton gradien.
Abbreviations: MIM, mitochondrial inner membrane; MIS, mitochondrial intermembrane space; MOM, mitochondrial outer membrane; RC, respiratory chain; Δp, electrochemical proton gradient; porin, voltage dependent anion channel (VDAC); P, Pi translocator; D, dicarboxylic acids translocator; V, valinomycin K^+ translocator; K_{ATP}, ATP-dependent K^+ translocator; S, succinate; AcK, potassium acetate; AcH, acetic acid; IV, cytochrome oxidase (complex fourth); b_5-nadh-dh, NADH-cytochrome b_5 oxido–reductase complex of MOM; C, cytochrome c; Pyr, pyruvate; Gluc, glucose.

respiratory chain to cytochrome oxidase for the reduction of molecular oxygen. In the NADH/cyto-c system only electron are directly transferred from cytosolic NADH to cytochrome oxidase (Fig. 1) but not protons. The unfeasible proposal, which is sometimes resurrected is that not exogenous but endogenous cyto-c would be able to shuttle electrons from the NADH/cytochrome b_5 reductase sited on the external leaflet of MOM to the cytochrome oxidase of the mitochondrial inner membrane (MIM) (Bernardi and Azione, 1981; Nicholls et al., 1969). Such a mechanism would run in the absence of exogenous cyto-c, but this is not the case.

Apoptosis is an ATP-dependent process, converted to necrosis in the presence of energy shortage (Eguchi et al., 1999). At the early stages of apoptosis, with the release of cyto-c from mitochondria, not only the respiratory chain but also the glycolytic activity is impaired despite an expected increase in the cytosolic NADH oxidation by pyruvate reduction to lactate. A drop in the cellular ATP is also expected. The NADH/cyto-c electron transfer pathway becomes functional in the reactivation of cytosolic NADH oxidation. The synthesis of ATP can be restored by both improved glycolysis and the activity of the system. The results obtained with two well-known pro-apoptotic compounds, ceramide (Gorgoglione et al., 2010) and valinomycin (Lofrumento et al., 2011), support this view. Valinomycin, a well known potassium ionophore, has been shown to promote apoptosis in a large range of concentrations (Abdalah et al., 2006; Furlong et al., 1998; Morrison et al., 2005). Recently, functionalised valinomycin analogues have been obtained and tested on isolated mitochondria in a general strategy of drug delivery to cancer cells (Annese et al., 2013).

Before starting with experiments on cultured cells, we addressed our effort to revealing the functional activity and the role of components involved in the cytosolic NADH/cyto-c oxidation pathway utilizing liver mitochondria. To maximize the activity of the system we induced an energy-dependent and reversible swelling in isolated mitochondria similar to that linked to the energy state of the cell (Hackenbrock, 1968). With the increased volume of the matrix, the interaction between the two membranes and the frequency of the respiratory contact sites are both expected to increase and the activity of NADH/cyto-c system should be stimulated. The effect of activators and/or inhibitors can be better appreciated under these conditions. Fig. 1 illustrates the correlation between the data on NADH oxidation and the rationale behind mitochondrial swelling induced by ion movement in the presence and the absence of pro-apoptotic valinomycin.

2. Materials and methods

2.1. Materials

All reagents were of analytical grade obtained from Sigma-Aldrich (Milan, Italy).

2.2. Isolation and incubation of mitochondria

Rat liver mitochondria were isolated by the differential centrifugation method in a mannitol-sucrose medium (Marzulli et al., 1995). Incubations were carried out at 25 °C in a standard medium at pH 7.4 containing 220 mM sucrose, 20 mM KCl, 1 mM EDTA, 20 mM Hepes-Tris and 3 μM rotenone. Oxygen consumption was determined with a Clark-type electrode with 5 mM succinate as substrate, 2 mM Pi and 4 mM $MgCl_2$. The P/O ratio was 1.9 ± 0.2 (S.D.) and the ratio of State 3/State 4 respirations was always higher than 5 with ADP and not lower than 9 with the uncoupler carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone (FCCP). Intactness of mitochondrial membranes was routinely determined by four different but convergent and already described integrity tests (Gorgoglione et al., 2007, 2010; La Piana et al., 2008; Lofrumento et al., 2011) based on the following activities: (a) the oxidation of exogenous NADH in the absence of both rotenone and exogenous cyto-c to assess the impermeability of NADH through the MIM; (b) the insensitivity of intermembrane adenylate kinase to proteolytic attack by impermeant trypsin to reveal the increased permeability, if any, of MOM in the course of incubation; (c) the reduction of exogenous cyto-c by sulfite oxidase (Sox—present in the MIS) coupled to its sensitivity to trypsin to assess the impermeability of exogenous cyto-c through the MOM; (d) the succinate/exogenous cyto-c oxido-reductase activity to reveal the