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Research update

Current mechanistic insights into the CCCP-induced cell survival response

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ABSTRACT

The ring-substituted derivatives of carbonyl cyanide phenylhydrazone, CCCP and FCCP, are routinely used for the analysis of the mitochondrial function in living cells, tissues, and isolated mitochondrial preparations. CCCP and FCCP are now being increasingly used for investigating the mechanisms of autophagy by inducing mitochondrial degradation through the disruption of the mitochondrial membrane potential ($\Delta\Psi m$). Sustained perturbation of $\Delta\Psi m$, which is normally tightly controlled to ensure cell proliferation and survival, triggers various stress pathways as part of the cellular adaptive response, the main components of which are mitophagy and autophagy. We here review current mechanistic insights into the induction of mitophagy and autophagy by CCCP and FCCP. In particular, we analyze the cellular modifications produced by the activation of two major pathways involving the signaling of the nuclear factor erythroid 2-related factor 2 (Nrf2) and the transcription factor EB (TFEB), and discuss the contribution of these pathways to the integrated cellular stress response.

Contents

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1. Introduction

Oxidative phosphorylation (OXPHOS) involves the coupling of nutrient oxidation to ATP production through the cycling of protons across the mitochondrial inner membrane (MIM). Oxidative phosphorylation is achieved by four electron transport complexes (Complexes I, II, III and IV) and the ATP-synthase complex (Complex V). The electron transfer through Complexes I, III and IV is coupled to the extrusion of protons from the matrix to the inter-membrane space. These protons re-enter the matrix through complex V to phosphorylate ADP in ATP [1,2]. The proton pumps of the electron transport chain, together with the ATP-synthase, cycle the protons across the MIM with a proton motive force (Δp). According to Mitchell's chemiosmotic theory, Δp consists of two components, the electrical membrane potential ($\Delta\Psi m$) and the pH gradient (ΔpH) across the MIM. The maintenance of the integrity of Δp is essential to various aspects of mitochondrial physiology, including ATP synthesis, transfer of calcium, and other ion exchanges, as well as the import of proteins and metabolites [3]. In physiological conditions, mitochondria are not fully coupled. During oxidative phosphorylation, there is a natural leak of protons across the mitochondrial inner-membrane. This leak is thought as a protective mechanism to minimize the production of reaction oxygen species (ROS). This endogenous uncoupling is ascribed to the presence of mitochondrial anion carriers superfamily which counts, the aspartate/glutamate carriers [4], the mitochondrial permeability pore, mPTP [5], the adenine nucleotide translocase, ANT and the uncoupling proteins, UCPs. These uncoupling proteins (UCPs) contribute to the regulation of mitochondrial ROS production associated with various metabolic and neurodegenerative disorders [6,7]. For a longtime among the five members that comprise the family, only UCP2 and UCP3 were extensively reviewed for their implication in ROS regulation [8,9], but later studies have also involved UCP4 and UCP5 in the adaptive response of cells to metabolic and oxidative stress [10,11]. Overexpression of these four UCPs in traumatic brain injuries, ischemia and neurodegenerative diseases such as Parkinson's disease, decrease ROS production and leads to improved tissue sparing [12–14]. Mitochondrial UCPs expression is inducible by various metabolic insults including hypoxia, high fat diet or caloric restriction and can be activated by fatty acids and free radicals [9,15–17].

Mitochondrial uncoupling can also be achieved by means of pharmacological tools, which either interfere with the generation of Δp or cause its dissipation. Uncouplers, which mimic the action of UCPs count among its members the carbonyl cyanide phenylhydrazones, the subject of this review.

CCCP and FCCP, members of the lipophilic weak acid class, are known as proton shuttling compounds because they selectively increase the permeability of lipid membranes to protons. With regard to mitochondria, each of these compounds dissolves in the lipids of the MIM before crossing the membrane to release a proton in the mitochondrial matrix, which is slightly alkaline. In this process, the ΔpH is disrupted. The ionized negatively charged compound then diffuses across the membrane, down the electric field gradient, dissipating the $\Delta\Psi m$ [18]. CCCP and FCCP may also uncouple mitochondria because of their high reactivity with thiol groups. Studies have shown that different thiol-combining agents uncouple OXPHOS at low concentrations and inhibit respiration at high concentrations by the chemical modification of a small but significant number of mitochondrial thiol groups. The dissipation or collapse of the $\Delta\Psi m$ signals mitochondrial degradation and apoptosis. However, studies carried out on both CCCP and FCCP show that cultured cells resist treatment with these compounds and survive, even after the loss of $\Delta\Psi m$ and cessation of mitochondrial ATP production. These observations suggest that, depending on the time of exposure and the concentration of the compounds, cells are able to adapt by developing protective mechanisms against CCCP/FCCP-induced apoptosis. One of the main pathways of this adaptive response is the activation of mitochondrial degradation through autophagy and the activation of an anti-oxidant response. Indeed, several studies have shown that CCCP induces the production of reactive oxygen species (ROS) [19–21]. Treatment with CCCP or FCCP induces the depletion of the antioxidant glutathione in mitochondria with subsequent cell death [21]. Glutathione depletion is believed to occur on account of excessive oxidation or because of a possible conjugation with FCCP. Recent studies have shown that the depletion of glutathione is due to its complexation with the uncoupling agent, with a time and concentration dependency [22]. The depletion of glutathione leads to an increase in ROS production. In turn, the elevation of ROS triggers: (i) the activation of various protein kinases and phosphatases by oxidizing the thiol groups of several cysteine residues; (ii) the disruption of protein trafficking with subsequent ER stress; (iii) DNA damage; and (iv) the promotion of Ca^{2+} signaling. To return to cellular homeostasis following CCCP or FCCP exposure, these initial stresses initiate and coordinate the stabilization and activation of Nrf2 and TFEB with subsequent cytoprotective responses [23–25]. In this review, we discuss how CCCP and FCCP mediate the activation of Nrf2 and TFEB directly through their electrophilic properties, or indirectly through ROS or Ca^{2+} signaling.

2. CCCP and FCCP activate the Nrf2 pathway

Under unstressed conditions, the kelch-like ECH-associated protein 1 (Keap1) sequesters and facilitates the ubiquitination and degradation of Nrf2 in the cytoplasm, thus repressing the nuclear activation of the antioxidant response elements by Nrf2. Indeed, Keap1 is a cysteine rich, homodimeric zinc-finger protein that functions as an adapter for the Cul3-Rbx E3 ubiquitin ligase complex, thereby targeting Nrf2 for proteasomal degradation under basal conditions (Fig. 1). However, under oxidative stress the Nrf2-Keap1 complex dissociates, allowing the translocation of Nrf2 to the nucleus and the subsequent expression of the antioxidant response element (ARE) pathway, which includes expression of the antioxidant genes and the transcription factors involved in mitochondrial turnover [26].

2.1. CCCP and FCCP react with Keap1 thiol groups, inducing the dissociation of Nrf2 from the Keap1-Nrf2 inhibitory complex

CCCP and FCCP react covalently with thiols relatively to their potencies [27,28]. Keap1 has at least 25 reactive thiols (Cys-SH), most of which are found in the intervening linker region (IVR) redox-sensing domain making Keap1 an ideal sensor of electrophiles and oxidation stresses. Kinetic, radiolabeling, and UV spectroscopic studies of the Keap1 interaction with several inducers show that four of the 25 reactive cysteines of Keap1 participate