Minireview

Cells depleted of mitochondrial DNA (ρ^0) yield insight into physiological mechanisms

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Abstract A resurgence of interest in mitochondrial physiology has recently developed as a result of new experimental data demonstrating that mitochondria function as important participants in a diverse collection of novel intracellular signaling pathways. Cells depleted of mitochondrial DNA, or ρ^0 cells, lack critical respiratory chain catalytic subunits that are encoded in the mitochondrial genome. Although ρ^0 cells contain petit mitochondria, they cannot support normal oxidative phosphorylation and must survive and replicate using ATP derived solely from glycolysis. Without a functional electron transport chain, ρ^0 cells cannot normally regulate redox potential and their mitochondria appear to be incapable of generating reactive oxygen species. Emerging evidence suggests that these signals are important components in a number of mitochondria-initiated signaling pathways. The present article focuses on how ρ^0 cells have contributed to an understanding of the role that mitochondria play in distinct physiological pathways involved with apoptosis, glucose-induced insulin secretion, and oxygen sensing.

Key words: Mitochondrion; Apoptosis; Insulin; Hypoxia; Oxygen sensing; Rho zero cell; Reactive oxygen species

1. Introduction

The mitochondrion has long been referred to as the 'powerhouse' of the cell because of its ability to generate adenosine-5′-triphosphate (ATP) from adenosine-5′-diphosphate (ADP) and inorganic phosphate (Pi) via respiratory chain phosphorylation. However, the classical viewpoint that mitochondria simply function as organelles responding to changes in ATP demand has recently given way to a more complex picture as data have emerged indicating that mitochondria also function as active signaling organelles in a number of important intracellular signaling pathways. A critical contribution to this growing understanding has come from an experimental approach using cells depleted of their mitochondrial DNA, or ρ^0 cells. Mammalian cell lines can be mutated into ρ^0 cells by long-term exposure to ethidium bromide and are found to be dependent on uridine and pyruvate for growth [1,2]. The mammalian mitochondrial DNA encodes 13 polypeptides including critical catalytic subunits for complex I (NADH dehydrogenase), complex III (bcl complex), complex IV (cytochrome *c* oxidase) and the F_1F_0 ATP synthase [3]. Thus, ρ^0 cells are not competent to carry out normal electron transport or ATP synthesis and must rely solely on ATP derived from anaerobic glycolysis for survival and growth. The fact that ρ^0 cells can survive without oxidative phosphorylation has allowed investigators to gain additional insight into the novel roles that mitochondria play in a number of signaling systems. For example, because they lack an electron transport system, ρ^0 cells should be impaired in any signaling processes that are linked to mitochondrial redox potential or that involve the generation of mitochondrial superoxide via electron transfer to molecular O_2. This article focuses on how ρ^0 cells have helped to identify the role that mitochondria play in the intracellular signaling mechanisms involved in physiological processes as diverse as apoptosis, glucose-induced insulin secretion and cellular oxygen sensing functions.

2. Apoptosis

Apoptosis is a morphologically distinct form of programmed cell death that plays essential roles in development, tissue homeostasis and a wide variety of diseases including cancer, AIDS, stroke, myopathies and various neurodegenerative disorders [4]. Apoptosis occurs after the activation of an intrinsic cell suicide program that is expressed in most mammalian cells. Moreover, the key components of the apoptotic pathway have been conserved throughout evolution in species ranging from worms to man. The central components of the intrinsic apoptotic pathway appear to include caspases and mitochondria [5]. The caspases are a family of proteases that cleave proteins at specific aspartic acids. Caspases have been established as the apoptotic executioners responsible for the morphological hallmarks of apoptosis including cell shrinkage, membrane blebbing, and chromatin degradation [6]. By contrast, the mitochondrial aspect of apoptosis has been more controversial [7]. Early evidence suggested that mitochondria might play a role in apoptosis because the anti-apoptotic proteins Bcl-2 and Bcl-xL appeared to localize to the mitochondria [8]. However, the observation that ρ^0 cells could still undergo apoptosis in response to growth factor withdrawal seemed to suggest that normal mitochondria were not required for apoptosis [9]. A clearer picture of the mitochondrial involvement in the apoptotic process emerged when Wang and colleagues demonstrated that cytochrome *c* was one of the factors required for activation of the cell death protease, caspase-3, in a cell-free system [10]. Subsequent experiments demonstrated that intact cells were capable of releasing cytochrome *c* from mitochondria in response to diverse apoptotic stimuli [11–13]. At about the same time, Kroemer and colleagues demonstrated that a 50-kDa protein

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in the intermembrane space of mitochondria (termed apoptosis inducing factor, or AIF) caused isolated nuclei to undergo apoptotic changes including chromatin condensation and internucleosomal DNA fragmentation in an in vitro system [14]. These studies also demonstrated that mitochondrial membrane potential can either hyperpolarize or hypopolarize following exposure of the cells to pro-apoptotic stimuli. Moreover, this change in mitochondrial membrane potential precedes the release of cytochrome *c* or AIF from mitochondria. The anti-apoptotic proteins Bcl-2 and Bcl-xL can prevent the change in mitochondrial membrane potential and thereby prevent the release of cytochrome *c* from mitochondria. Since neither cytochrome *c* nor AIF is encoded by mitochondrial DNA, it would be expected that ρ^0 cells should still be able to undergo apoptosis following release of these factors to the cytosol. But the story became more confusing when it was demonstrated that ρ^0 cells do not undergo apoptosis in response to all stimuli that activate cell death in wild-type cells [15], which raises the question of why ρ^0 cells are more resistant to some forms of apoptotic stimuli than others. To answer this question it is essential to recognize that changes in mitochondrial membrane potential likely precede cytochrome *c* release both in normal and in ρ^0 cells. It is therefore imperative to understand how mitochondrial membrane potential is maintained in ρ^0 cells lacking an electron transport system, and how various pro-apoptotic stimuli affect the maintenance of this potential.

Normal cells generate mitochondrial membrane potential by pumping protons across the inner membrane via a process coupled to electron transfer along the electron transport chain. So how do ρ^0 cells still maintain a mitochondrial transmembrane potential when they lack key essential components of the electron transport chain? Interestingly, they maintain transmembrane potential by a different mechanism that can explain their different sensitivity to pro-apoptotic stimuli. Recently, Buchet and Godinot demonstrated that components of the ATP synthase, along with a functional adenine nucleotide transporter (ANT), are required to maintain mitochondrial membrane potential in ρ^0 cells [16]. Although ρ^0 cells do not possess F_0 subunits 6 and 8 of the ATP synthase encoded by mitochondrial DNA, they do possess a functional F_1-ATPase and a functional ANT. Within the mitochondrial matrix, ATP^{4-} hydrolysis is catalyzed by the F_1 subunit, yielding ADP^{3-} and Pi. The escape of ADP^{3-} from the matrix to the cytosol via the ANT is coupled to the uptake of ATP^{4-} generated in the cytosol by anaerobic glycolysis. This concentration-driven exchange is electrogenic, and generates a sufficient mitochondrial membrane potential to prevent osmotic swelling of the matrix and subsequent cytochrome *c* release after rupture of the outer membrane. Thus, ρ^0 cells maintain their membrane potential by virtue of ATP consumption in their mitochondria.

Based on Buchet and Godinot's observations, factors that inhibit the exchange of adenine nucleotides across the inner mitochondrial membrane should initiate apoptosis in ρ^0 cells. For example, the ANT inhibitor bongkrekic acid and the F_1-ATPase inhibitor aurovertin both induce growth arrest and membrane depolarization in ρ^0 cells. By contrast, ρ^0 cells are insensitive to mitochondrial electron transport inhibitors such as rotenone and antimycin [17] that are potent initiators of apoptosis in wild-type cells. In cell lines that are dependent on growth factors, withdrawal of interleukin-3 has been shown to inhibit the inner membrane exchange of adenine nucleotides in wild-type cells [18]. The loss in ADP supply to the mitochondrial matrix results in a membrane hyperpolarization that causes osmotic rupture and cytochrome *c* release. Similarly, growth factor withdrawal in ρ^0 cells should lead to an inhibition of adenine nucleotide exchange, thereby preventing the exchange of glycolytic ATP for mitochondrially generated ADP. The subsequent loss of mitochondrial membrane potential would result in osmotic swelling and cytochrome *c* release. By contrast, apoptotic stimuli that affect other aspects of mitochondrial function appear not to induce apoptosis in ρ^0 cells. For example, unlike wild-type cells, ρ^0 cells are insensitive to tumor necrosis factor α (TNFα) [15,19]. This observation suggests that the mechanism of cell death after exposure to TNFα does not involve a primary disruption of the ANT. It is conceivable that TNFα could initiate a loss of mitochondrial membrane potential in wild-type cells by disrupting electron transport; previous investigations have also suggested that ceramide, a complex III electron transport inhibitor, may act as a signaling mediator in the mitochondrial dysfunction induced by TNFα [19,20]. In any case, the sensitivity of ρ^0 cells to different apoptotic stimuli has yielded insight into the mechanisms of mitochondrial participation in the apoptosis pathway in normal cell lines.

3. Glucose-induced insulin secretion

Glucose-stimulated insulin release from pancreatic beta cells involves a complex series of signaling pathways. Glucose, the main physiological secretagogue, results in an increase in the cytosolic ATP/ADP ratio as it is metabolized by the beta cell [21]. The rise in ATP/ADP ratio results in the closure of ATP-sensitive potassium channels (K_{ATP}) and subsequent depolarization of the plasma membrane. This leads to Ca^{2+} influx through voltage-gated Ca^{2+} channels and a rise in cytosolic Ca^{2-} concentration ($[Ca^{2-}]c$) [22]. The increase in $[Ca^{2+}]c$ is the main trigger for exocytosis, the process by which insulin-containing secretory granules fuse with the plasma membrane [23]. A fundamental question underlying glucose-induced insulin secretion relates to the source of the increased [ATP] in response to glucose uptake. Some investigators have argued that glycolytic, but not Krebs cycle metabolism of glucose is critically involved in this signaling process [24,25]. Studies have demonstrated that inhibitors of glycolysis suppress glucose-stimulated insulin secretion, whereas methyl pyruvate can generate a normal secretory response. Pancreatic islets exposed to exogenous pyruvate failed to respond in terms of a stimulation of insulin secretion, and did not demonstrate K_{ATP} channel closure even though islet cells can oxidize pyruvate as effectively as glucose. Based on these observations it could be concluded that ATP derived from mitochondrial pyruvate metabolism does not substantially contribute to the regulation of K_{ATP} channels in response to a glucose challenge.

To determine the relative importance of mitochondrial versus glycolytic ATP for the insulin secretion response to glucose, Soejima et al. tested whether ρ^0 cells could respond to a glucose challenge [26]. If glycolytic stimulation were sufficient to augment [ATP] and to trigger K_{ATP} channel closure leading to insulin secretion, then ρ^0 cells should augment insulin secretion in response to glucose challenge. By contrast, if ATP from the mitochondria is required then ρ^0 cells should fail to

respond. Using ρ^0 cells derived from a cultured mouse pancreatic beta cell line MIN6, Soejima et al. showed that, unlike wild-type MIN6 cells, the MIN6 ρ^0 cells failed to stimulate insulin secretion and failed to increase their $[Ca^{2+}]c$ in response to the glucose stimulus. The possible involvement of a nuclear genome-encoded factor in this response was excluded by showing that the insulin secretion response could be restored in MIN6 ρ^0 cells by re-populating them with foreign mitochondria containing normal DNA. These results were corroborated by Kennedy et al. who demonstrated that ρ^0 cells from the highly differentiated insulin-secreting cell line INS-1 failed to increase [ATP] in response to glucose [27]. In addition, there was no effect of glucose on K_{ATP} yet depolarization of the plasma membrane with KCl could still elicit insulin secretion. Also, Hayakawa et al. demonstrated that cells treated with ethidium bromide for 6 days, which presumably limited mitochondrial DNA replication, could no longer stimulate insulin secretion in response to elevated glucose, while the K_{ATP} channel blocker glibenclamide still did [28]. Collectively, these results indicated the importance of functional mitochondria in the insulin secretion response to glucose, but they did not resolve the paradox regarding the failure of pyruvate to secrete insulin. Specifically, if increased [ATP] is required for the response, why does pyruvate fail to activate secretion even though it can be metabolized by islet cells? Recently, Eto et al. suggested that the cytosolic pool of NADH may be an important participant in the signaling required for insulin secretion [29]. Those investigators found that the glucose-induced increases in ATP content and insulin secretion were abrogated if the glycerol phosphate and the malate-aspartate shuttles responsible for the transfer of cytosolic reducing equivalents into the mitochondria were inhibited. These results suggest that glycolysis-derived NADH may be an important source of mitochondrial energy production and metabolism in beta cells, and that mitochondria therefore function as critical signal transduction organelles in this response.

4. Cellular oxygen sensing

Oxygen is needed for the survival of virtually all mammalian cells due to its requirement for ATP synthesis. Accordingly, organisms have developed adaptive strategies at the organ level and at the cellular/molecular level to protect against the consequences of decreased oxygen availability (hypoxia, $p_{O_2} < 40$ Torr). Adaptive responses to hypoxia at the organismal level include pulmonary vasoconstriction (helping to maintain pulmonary gas exchange) and carotid body chemotransduction (helping to stimulate lung ventilation) [30]. Cellular and molecular adaptive responses to hypoxia include the activation of various transcription factors including hypoxia inducible factor 1 (HIF-1) [31]. The HIF-1 complex is involved in the transcriptional activation of several genes that are responsive to the lack of oxygen, such as erythropoietin (EPO), vascular endothelial growth factor (VEGF) and glycolytic enzymes [32–34]. The HIF-1 complex is composed of two b-HLH proteins: HIF-1β that is constitutively expressed, and HIF-1α, a protein whose rapid degradation by the proteasome pathway is inhibited during hypoxia [35]. Although much progress has been made in understanding the molecular mechanisms underlying the transcriptional activation of glycolytic enzymes, EPO, and VEGF genes during hypoxia, no consensus has evolved with respect to the identification of the cellular O_2 sensor responsible for initiating the response to hypoxia. Early progress in understanding molecular mechanisms underlying mammalian oxygen sensing came from the observation that EPO mRNA can be induced under normoxic conditions in the human hepatoma Hep3B cell line by incubation with transition metals such as cobalt [36]. This led to the proposal that the sensor is a heme protein capable of interacting with O_2. Subsequently, mechanisms including O_2-sensitive potassium channels and NADPH oxidase have been proposed as possible oxygen sensors [37,38]. Since mitochondria are the main site of oxygen consumption, they have long been considered as a possible site of O_2 sensing. But studies using inhibitors of mitochondrial electron transport such as cyanide or azide have failed to elicit either an activation during normoxia or an inhibition of HIF-1 activity during hypoxia, leading some to question the feasibility of their role in oxygen sensing [39].

We examined the role of mitochondria in the transcriptional response by assessing the ability of ρ^0 cells to activate gene expression in response to hypoxia or cobalt [40]. Hypoxia and cobalt independently increased HIF-1 DNA binding and EPO mRNA levels in wild-type Hep3B cells. However, hypoxia failed to induce EPO mRNA expression or HIF-1 DNA binding in ρ^0-Hep3B cells. Yet surprisingly, ρ^0-Hep3B cells maintained their ability to activate HIF-1 DNA binding and EPO mRNA expression in response to cobalt chloride [40]. Collectively, these results indicate that a functional mitochondrial respiratory chain is required for the transcriptional response to hypoxia. Cobaltous ion, however, appears able to bypass this requirement, possibly by acting downstream of the mitochondria. What aspect of mitochondrial function, i.e. ATP, calcium, reactive oxygen species, or mitochondrial membrane potential, is required for hypoxic activation? We focused on the possible role of reactive oxygen species (ROS) as signaling molecules required for the hypoxic stabilization of HIF-1 and subsequent induction of EPO mRNA. Previous reports had indicated that ROS can serve as signaling molecules by activating transcription factors [41], although one might expect the intracellular generation of ROS to decrease when O_2 availability is lowered during hypoxia. Paradoxically, wild-type Hep3B cells increased their generation of ROS during hypoxia (1–2% O_2), as evidenced by the increased rate of oxidation of 2,7-dichlorofluorescein (DCFH) dye [40]. This ROS generation requires electron transfer through mitochondrial complex III, based on the observation that pharmacological inhibitors of electron transport acting upstream of that site attenuated oxidation of the DCFH dye in wild-type cells, and that increased oxidation of DCFH dye was not detected in Hep3B ρ^0 cells during hypoxia [40]. Interestingly, cobalt chloride increased the intracellular generation of ROS in both wild-type and ρ^0 cells during normoxia, as evidenced by an increase in DCFH oxidation. The antioxidant compounds pyrrolidine dithiocarbamate and ebselen attenuated the ROS response and abolished the transcriptional activation of EPO and HIF-1 DNA binding during hypoxia in wild-type Hep3B cells. These antioxidants attenuated the ROS signal and abolished the transcriptional activation response to cobalt in both cell types [40]. These findings reveal that hypoxia activates gene transcription via a mitochondria-dependent signaling process involving increased ROS. By contrast, cobalt chloride appears to activate the same signaling system by

catalyzing the generation of ROS via a mechanism that does not require mitochondrial electron transport.

5. Concluding remarks and perspectives

The recent advances in diverse cell signaling pathways summarized above have enhanced our understanding of the roles that mitochondria play in the early intracellular events associated with apoptosis, glucose-induced insulin secretion, and O_2 sensing in diverse cell types. Although there is no question regarding the importance of mitochondria in maintaining the cellular bioenergetic state, these findings collectively underscore our growing realization that mitochondria also function as initiators of intracellular signaling systems that are critically important for cell survival and specialized cell functions. Because the bioenergetic functions of mitochondria are normally essential for cell survival, it has been difficult or impossible in the past to uncover these additional roles using pharmacological studies because the acute inhibition of electron transport leads to rapid cell death. By creating a system that is not critically dependent on oxidative phosphorylation for survival, studies with ρ^0 cells have contributed to our understanding of these novel signaling functions.

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