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Signaling Pathways in Mitochondrial Dysfunction and Aging

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Abstract

Mitochondria are central players in the determination of cell life and death. They are essential for energy production, since most cellular ATP is produced in their matrix by the oxidative phosphorylation pathway. At the same time, mitochondria are the main regulators of apoptotic cell death, mediating both extrinsic (cell-surface receptor mediated) and intrinsic apoptotic pathways. Reactive oxygen species (ROS) accumulate as side products of the electron transport chain, causing mitochondrial damage. Non-functional mitochondria accumulate in aged individuals, and cell homeostasis is maintained by removing damaged mitochondria by an autophagic process called “mitophagy”. In addition, mitochondrial ROS represent signaling molecules leading to autophagy, consisting in the bulk degradation of cytosolic portions. When cell homeostasis is perturbed, and cytosolic components are damaged, autophagy represents a defense mechanism aimed at removing non functional proteins and organelles. If this is not sufficient, cell death occurs with distinct morphological hallmarks from apoptosis. This binary choice integrates a number of critical information converging on a number of common regulatory elements. In this review, the focus will be placed on the central role of mitochondria in the cross-talk between autophagy and apoptosis, highlighting the signaling pathways and molecular machinery impinging on these organelles.

Mitochondria and Aging

During aging, a general decline in cellular function occurs, due to accumulation of damaged macromolecules and organelles which are not readily removed. Targeted deletion of specific genes has demonstrated that multiple components of the Igf-1/insulin signalling pathway play a role in the aging process spanning from nematodes to rodents (Bartke, 2008). Caloric restriction is the only non-genetic mechanism known to extend lifespan, most likely activating multiple mechanisms (Canto and Auwerx, 2009). Back in 1956, Herman proposed a theory of aging according to which damage caused by ROS leads to senescence (Harman, 1956). He hypothesized that the introduction of anti-oxidant agents in the diet would prolong life span as a result of reduced oxidative damage to key cellular components. By feeding different mouse strains with various anti-oxidant agents, he could demonstrate the validity of his revolutionary idea (Harman, 1972). Since then, a great amount of work, described in exhaustive reviews (Beckman and Ames, 1998), has been done to unravel the role of ROS in aging.

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Since ROS are generated mainly as by-products of mitochondria respiration it appears evident that mitochondria play a fundamental role in aging and represent putative targets of anti-aging strategies. Nevertheless, the relationship between ROS-induced damage, mitochondria function and involved regulators, and aging is far to be clarified. In mitochondria ROS cause mutations of mtDNA which affect the electron transport chain function triggering increased ROS generation and accumulation of mtDNA damage over time. Whether this process affects the decrease in respiration function observed in aging is still matter of debate (Chan, 2006). Indeed, animal models depleted for the proofreading function of DNA-polimerase-γ (POLG) show increase in mtDNA mutations, classical sign of aging and reduced life span (Kujoth et al., 2005; Trifunovic et al., 2004). However, no increase in hydrogen peroxide and in oxidized macromolecules was detected in these mice, in contrast with the putative direct interconnection between ROS production, mtDNA mutations, and respiration. Moreover, mtDNA mutations affect only a proportion of senescence cells, suggesting that age-related mitochondria dysfunction only partially develop by mtDNA mutations. Caloric restriction extends life span in many organisms but, in contrast to what one could imagine at a first glance, reducing food intake does not lower respiration rate but rather it increases mitochondria function, and this opens a number of unanswered questions on the physiological role of such response (Guarente, 2008). What is clear is that during aging, together with aberrant macromolecules, giant non-functional mitochondria, defective in ATP production, accumulate especially in post-mitotic organs, such as the nervous system and the cardiac and skeletal muscle. Moreover, defects in autophagy are detected in age-related disorders such as neurodegenerative diseases and sarcopenia (Levine and Kroemer, 2008). Indeed a general decline of autophagy accompanies aging (Bergamini et al., 2007) and strategies that prolong life-span, such as caloric restriction, imply induction of autophagy. On the other side, overexpression of autophagy genes, for example Atg8, in drosophila brain, greatly prolongs life-span (Simonsen et al., 2008). The fundamental role of autophagy is demonstrated by experiments in nematodes, in which the life-span extension effect of single gene mutations (such as the Insulin/Igf-1 receptor DAF-2 or TOR) or of caloric restriction require the expression of autophagy genes (Hansen et al., 2008; Melendez et al., 2003).

Mitophagy

Autophagy consists in the bulk degradation of cytosolic portions that is active in various conditions either at basal or induced level. The engulfed cytosolic content is surrounded by a double-membrane structure (the autophagosome) and delivered to the lysosome where it is degraded by lysosomal proteases.

Autophagy permits clearance of misfolded proteins and non functional organelles, that accumulate especially in post-mitotic cells such as those of the nervous system and of skeletal muscle, thus delaying aging of those tissues; autophagy is induced during fasting, in order to provide aminoacids which can be used by the liver for gluconeogenesis. It is also active during development, in immune responses and in tumor suppression.

Mitochondria are both the major source of intracellular ROS and, at the same time, targets of ROS. Damaged mitochondria are degraded by a specialized form of autophagy called mitophagy (Figure 1), ensuring the maintenance of a functional mitochondria population. During aging autophagy declines and aberrant mitochondria accumulate. Increasing autophagic activity in the elderly may thus be beneficial in that it contributes to the maintenance of a functional mitochondria population. Whether autophagy is a nonspecific degradation process, or rather some proteins or organelles may be specifically targeted for autophagic degradation is still debated. Growing evidence suggests that a certain level of

specificity is achieved, and mitochondria represent privileged targets when their degradation is required, both in physiological and in pathological conditions.

In yeast, protein localized in mitochondria, such as Uth1p (Kissova et al., 2004) and Aup1p (Tal et al., 2007), are required for efficient mitophagy. In mammalian cells, initial studies suggested that serum withdrawal promotes autophagic degradation preferentially of those mitochondria carrying mtDNA mutations (Gu et al., 2004). Direct evidence that damaged mitochondria are removed by autophagy in mammalian cells came from experiments based on starvation and laser-induced photodamage of hepatocytes mitochondria (Kim et al., 2007). In these experiments, hepatocytes of GFP-LC3 transgenic mice were loaded with TMRM, a positively charged fluorescent dye which accumulates in the mitochondria matrix down the membrane potential generated across the inner mitochondrial membrane by the respiratory chain ($\Delta\psi_m$). After nutrient deprivation a fraction of the mitochondria population was sequestered in GFP-LC3 positive vesicles. After sequestration, mitochondria lost their TMRM fluorescence, indicating that depolarization occurred. In a second experiment, after exposure to a laser light, mitochondria lost again their membrane potential. Also in this case damaged mitochondria were eventually sequestered in GFP-LC3-labeled autophagosomes (Kim et al., 2007).

A physiological example of mitophagy is given by the maturation process of reticulocytes. During this event, mitochondria are completed eliminated by autophagy-dependent and independent pathways (Zhang et al., 2009). As described in the “common players” section, BH3-only proteins, such as Binp3 and Nix (Bnip3L), induce autophagy by releasing Beclin-1 from its interaction with members of the Bcl2 family. The autophagic degradation of reticulocytes mitochondria requires Nix (Bnip3L), since mitochondria autophagy is defective in Nix −/− reticulocytes (Schweers et al., 2007). Nix-dependent collapse of the $\Delta\psi_m$ appears to be an essential event for erythroid mitophagy and maturation (Sandoval et al., 2008). Moreover, Nix is specifically required for mitophagy, since ribosomes were normally eliminated in the absence of Nix (Sandoval et al., 2008). Erythroid maturation is also regulated by the serine threonine kinase Ulk1, the mammalian homolog of yeast atg1p, which, in complex with Atg13, is responsible for autophagosome formation regulated by mTOR activity. In contrast to Nix, Ulk1 regulates clearance of both ribosomes and mitochondria during this process (Kundu et al., 2008). Remarkably, an Atg5/Atg7-independent macroautophagy system has been identified, which partially accounts for mitochondrial removal during erythrocyte maturation (Nishida et al., 2009; Zhang et al., 2009).

Removal of aberrant mitochondria has been shown to play a protective role in age-related neurodegenerative disorders, such as Parkinson disease. Specifically, the ubiquitin-ligase Parkin, the loss-of-function of which causes Parkinson's disease, is involved. Parkin is selectively recruited to dysfunctional mitochondria with low membrane potential in mammalian cells and causes their autophagy-mediated degradation (Narendra et al., 2008), suggesting that Parkinson's disease may be at least in part associated with failure to eliminate dysfunctional mitochondria. The translocation of Parkin to mitochondria requires PINK1, which is a protein kinase, ubiquitously expressed in the human brain, and localized to the intermembrane space, as well as in membranes of the mitochondria. At the mitochondria, Parkin and PINK1 are localized in close proximity, but no evidence of mutual post-trascriptional regulation was found (i.e. phosphorylation of Parkin by PINK1 or ubiquitination of PINK1 by Parkin). However, co-expression of Parkin and PINK1 caused formation of mitochondrial aggregates surrounded by autophagic vacuoles (Vives-Bauza et al., 2009), suggesting that impairment of mitochondria turnover by either Parkin or PINK1 mutations may cause accumulation of defective mitochondria and neurodegeneration in Parkinson's disease. In a different setup, represented by SH-SY5Y cell line, loss of PINK1

elevated superoxide production, induced mitochondria fragmentation, $\Delta\psi_m$ collapse, autophagy and mitophagy (Dagda et al., 2009). Activation of mitochondria fission and increased mitochondrial oxidant production were required for autophagy in PINK1-deficient cells. Moreover, overexpression of Parkin enhanced the mitophagy response. Thus, PINK1 appears to play an important role for the maintenance of mitochondrial networks which, together with Parkin-induced mitophagy, may serve to reduce toxicity associated with dysfunctional mitochondria in PD. Indeed, morphological changes of the mitochondrial network occur continuously and regulate mitochondrial and cellular functions. By tracking photolabeled mitochondria, it has been shown that fission and fusion events permit the segregation of dysfunctional mitochondria which are eventually degraded by autophagy (Twig et al., 2008). Work on the pro-fission protein Fis1 demonstrates that fission events trigger autophagy per se, but only when associated with mitochondrial dysfunction (Gomes and Scorrano, 2008).

Even if there is now evidence for selective autophagy-dependent degradation of mitochondria, the mechanism of mitochondrial selection has been started to be elucidated only very recently thanks to studies carried in budding yeast. A first indication came from the recognition that the cargo adaptor protein Atg11 is essential for mitophagy (Kanki and Klionsky, 2008). A mitochondria-anchored receptor, Atg32, which confers selectivity during mitophagy was subsequently identified (Kanki et al., 2009; Okamoto et al., 2009). Atg32 does not have homologues in higher eukaryotes, leaving open the question of how selectivity is reached in other species.

Mitochondrial ROS in the regulation of autophagy and apoptosis

Excessive ROS production represents a death threat for the cell, which reacts to this insult by activating defence mechanisms, including autophagy. ROS act as signalling molecules in the early events of autophagy induction. If the pro-survival attempt fails, ROS cause cell death which, depending on the experimental context, involves either the autophagic or the apoptotic pathway, or both (Figure 1). Whether autophagy activation can cause death per se in physiological conditions is still matter of debate (Kroemer and Levine, 2008).

Signaling pathways mediated by ROS play a role in physiological situations in which autophagy is induced. Indeed, during starvation mitochondrial ROS production is enhanced and autophagy increased (Scherz-Shouval et al., 2007). ROS production is essential for autophagy induction, as treatment with antioxidant agents abolishes autophagosome formation upon starvation, and the effect of ROS is partially PI3K dependent. The authors suggested that upon starvation, ROS, and in particular H_2O_2, oxidize and inhibit Atg4, which is a protease responsible for LC3 de-lipidation promoting autophagosome maturation (Scherz-Shouval et al., 2007). In this case no sign of programmed cell death was observed, in agreement with the well known pro-survival function of starvation-induced autophagy.

In a different setup, ROS-induced autophagy was linked to cell death, rather than survival. This effect was observed specifically in cancer cell, in which the role of autophagy is still debated. Indeed, whether autophagy represents a tumor suppressor mechanism (by removing aberrant mitochondria, therefore lowering ROS production and genome instability) (see below) or rather promotes tumorigenesis (exerting a protective role on cancer cells), is not clear, and most likely is context-dependent. Autophagy-dependent death was observed in cancer cell lines treated with inhibitors of complex I and II of the mitochondrial electron transport chain (rotenone and TTFA respectively). These cells displayed increased ROS production, cell death and autophagy (Chen et al., 2007). Cell death was decreased by treatment with siRNA against autophagy genes or with the autophagy inhibitor 3-

methyladenine (3-MA). Moreover, autophagy and cell death were decreased when the ROS scavenger tiron was added to rotenone or TTFA treated cells.

The dual role of ROS to induce either apoptosis or autophagy depends on the cell context and on specific modulators of ROS activity. One of these factors is TIGAR, which is a p53-target gene. Similarly to other proteins involved in DNA damage repair, the tumor-suppressor p53 plays an essential role in life span determination and has been implicated in the regulation of the senescence process (reviewed in (Rodier et al., 2007)). Its target gene TIGAR contributes to the regulation of intracellular ROS levels by modulation of the glycolytic pathway (Bensaad et al., 2006). By decreasing glycolytic rate and redirecting glycolytic intermediates to the oxidative branch of the pentose phosphate pathway, TIGAR causes an increase in NADPH production, which contributes to the scavenging of ROS by reduced glutathione. In this manner, TIGAR lower the sensitivity of cells to p53-dependent apoptosis induced by oxidative stress (Bensaad et al., 2006). At the same time, TIGAR inhibits autophagy induced by nutrient starvation and metabolic stress, independently of mTOR or p53 modulation (Bensaad et al., 2009).

The patho-physiological importance of ROS production and autophagy induction was studied in skeletal muscle. Muscle atrophy is a hallmark of amyotrophic lateral sclerosis (ALS) and is associated to increased oxidative stress. However, whether oxidative stress triggers muscle atrophy per se, or it is a consequence of muscle atrophy was not clear. In order to answer this question and to dissect the pathways involved, Musarò and colleagues developed a mouse model in which expression of mutated superoxide dismutase 1 ($SOD1^{G93A}$) was restricted to skeletal muscle (Dobrowolny et al., 2008). Accumulation of oxidative stress in the muscles of these mice triggered progressive atrophy associated to increased autophagy and FoxO3 expression, a transcription factor which is required for the induction of autophagy in skeletal muscle (Mammucari et al., 2007; Zhao et al., 2007).

The consequences of ROS-mediated damage due to lack of active autophagy are evident in the liver. Liver-specific autophagy-deficient mice display liver dysfunction, in particular hepatomegaly with concomitant accumulation of ubiquitin-positive aggregates (Komatsu et al., 2005). Proteomic analysis of autophagy-deficient liver demonstrates that autophagy is required to reduce oxidative stress, and hence cellular damage (Matsumoto et al., 2008). Such analysis showed that, although autophagy impairment leads to a general increase in total protein mass with no changes in overall protein composition, a series of oxidative stress-inducible proteins, including glutathione S-transferase families, protein disulfide isomerase and glucose regulated proteins, were specifically increased in autophagy-deficient liver (Matsumoto et al., 2008). Interestingly, the nuclear levels of the transcription factor Nrf2, which translocates to the nucleus in response to oxidative stress and activates the transcription of various detoxifying enzymes, were increased in autophagy-deficient liver (Komatsu et al., 2007). p62/SQSTM1 is an adaptor protein which binds LC3 and polyubiquitinated proteins to form inclusion bodies which are degraded by autophagy and that accumulate when autophagy in inhibited (Bjorkoy et al., 2005; Pankiv et al., 2007). Loss of p62 suppresses inclusion formation in autophagy-deficient hepatocytes. Moreover induction of detoxifying enzymes and NRF2 nuclear translocation in autophagy-deficient liver were suppressed by loss of p62 (Komatsu et al., 2007). Altogether these results indicate that autophagy deficiency causes liver dysfunction and cell stress, with concomitant p62-dependent Nrf2 activation (Komatsu et al., 2007).

A similar link among autophagy, p62 and oxidative stress has been identified in tumor cells (Mathew et al., 2009). p62 accumulation in autophagy-deficient tumor cells upon metabolic stress is sufficient to cause accumulation of damaged mitochondria, elevated oxidative stress and DNA damage response. Such effects are suppressed by knockdown of p62, indicating

that elevated oxidative stress is due directly to p62 accumulation. Thus p62 contributes directly to tumor growth when autophagy is inhibited (Mathew et al., 2009).

Control of life and death by mitochondrial calcium pathways

Mitochondria are essential mediators of apoptotic stimuli, playing a fundamental role in the execution of both intrinsic and extrinsic apoptosis pathways. The vastness of the literature in the field is such that a reasonable comment of it is beyond the scope of this review.

Thus, here we will focus on the role of signalling mediated by the second messenger Ca2+ in the regulation of apoptosis and on recent data on the role of p66shc in mediating the response of mitochondria to ROS-induced apoptosis. Next we will briefly summarize data linking mitochondrial calcium regulation to longevity in a model of Leigh syndrome, a mitochondrial disease associated with cytochrome c oxidase deficiency.

Changes in cytosolic Ca2+ concentration are observed in many circumstances. Cellular Ca2+ overload represents a threat for cell life, since many proteases and phospholipases are activated by Ca2+ and can lead to necrotic cell death. This is not the only effect of changes in cytosolic Ca2+. Indeed apoptotic cell death relies on increased Ca2+ concentrations (Murgia et al., 2009), mediated both by endoplasmic reticulum (ER) calcium release and by capacitative Ca2+ influx through Ca2+ release-activated Ca2+ channels (Pinton and Rizzuto, 2006). Depending on the range of the insult, and consequently on the amount of Ca2+ increase, either necrosis or apoptosis is activated. Within the cell Ca2+ can establish local concentrations, due to its low rate of diffusion in comparison with other second messengers, and the dynamic sequestration of this ion by several organelles (Rizzuto and Pozzan, 2006). This unique property permits the cell to decipher various signals triggering different outcomes through a single molecule. ER plays a fundamental role in the regulation of calcium concentration and in the sensitivity to apoptosis. Ca2+ accumulated in the ER can be released upon apoptotic stimuli coupled to IP3, and being detected by mitochondria. Bcl-2 family proteins, strategically located at the ER and mitochondria surfaces, are important regulator of this process. Bcl-2 overexpressing cells showed a significant reduction in the Ca2+ levels within the ER and the Golgi apparatus. Consequently, reduced Ca2+ concentration increases upon stimuli coupled to IP3 generation were detected both in the cytosol and in the mitochondria (Pinton et al., 2000). The same effect was observed in cells in which the pro-apoptotic members of Bcl-2 family, Bax and Bak were deleted (Danial and Korsmeyer, 2004). At the same time, Bax and Bak double knockout cells are protected against apoptotic stimuli (Scorrano et al., 2003). In these cells, silencing of Bcl-2 partially restored ER Ca2+ values to control levels (Danial and Korsmeyer, 2004).

Stimuli that induces cytosolic Ca2+ rise elicit large Ca2+ influxes in the mitochondria matrix, despite the low affinity of mitochondrial Ca2+ transporters for this ion. This is explained by the existence of mitochondria-ER contacts, where microdomains of high Ca2+ concentrations are present and trigger rapid accumulation of Ca2+ in the matrix (Hayashi et al., 2009). Mitochondria Ca2+ uptake causes a variety of responses, from stimulation of metabolism (ATP production) when they are subject to a transient stimuli, to apoptosis in case of a more persistent or excessive Ca2+ increase. Moreover, mitochondria Ca2+ accumulation triggered by apoptotic stimuli causes swelling and fragmentation, accompanied by cytochrome c release. Permeability transition pore (PTP) opening was also observed upon ceramide-induced apoptosis, which sensitizes mitochondria to the otherwise physiological IP3-mediated Ca2+ signal (Szalai et al., 1999) (Figure 1). As for ER calcium concentrations, Bcl-2 family members control this apoptosic pathway. In particular Bax and Bak localize at the outer mitochondria membrane upon apoptotic stimuli and trigger

mitochondria outer membrane permeabilization and release of apoptotic factors in the cytosol (Danial and Korsmeyer, 2004).

Impairment of mitochondrial function caused by ROS-induced mtDNA damage, lipid peroxidation or protein oxidation may also lead to apoptotic cell death. In this scenario, the redox enzyme p66shc plays a fundamental role. Ablation of p66shc enhances cellular resistance to apoptosis upon oxidative stress and life span extension (Migliaccio et al., 1999). When exposed to oxidative conditions, p66shc is phosphorylated by PKCβ and translocates to mitochondria after being recognized by the prolyl isomerase Pin1, where it causes alterations of the mitochondrial calcium responses (Pinton et al., 2007). Moreover, the mitochondrial fraction of p66shc behaves as redox enzyme that utilizes reducing equivalents derived from the mitochondrial electron transport chain to produce H_2O_2 in the intermembrane space (Giorgio et al., 2005). Alteration of mitochondrial three-dimensional structure, calcium responses and ROS generation eventually lead to apoptosis (Giorgio et al., 2005; Pinton et al., 2007) (Figure 1).

The role of mitochondrial calcium regulation in the determination of life span extension has been explored in the context of a Leigh syndrome model, a lethal mitochondrial disorder associated with COX decifiency. Mice in which one of the genes responsible for the disease, Surf1, which encodes a putative COX assembly factor, was deleted, showed COX defect, although milder then in humans (Dell'agnello et al., 2007). These mice did not show spontaneous neurodegeneration, and were protected from Ca2+-dependent neurotoxicity induced by exposure to kainic acid. Moreover, similarly to deletion of p66shc, Surf1 deficiency caused increased longevity in mice (Dell'agnello et al., 2007). The difference between the effects of Surf1 deletion in human, where it is fatal in childhood, and mice may be due to the difference in severity of COX deficiency. These results are anyway surprising since absence of COX activity should cause increase of ROS and accelerated aging.

Common players

Autophagic and apoptotic responses to stress are intimately interconnected. A number of different outcomes have been described, depending on the various experimental contexts. When apoptosis is impaired, the cell may respond by activating autophagy and vice-versa. However, in some circumstances, the physiological relevance of the observation made is not obvious. Readers are referred to exhaustive reviews on the topic (e.g.(Maiuri et al., 2007b)), while here we will focus on the description of regulatory elements that impinge on both pathways at the mitochondrial level.

Bcl-2 family members

Proteins belonging to the Bcl-2 family are well known regulators of apoptosis. Bcl-2, Bcl-X_L and MCL-1 suppress apoptosis, while Bax, Bak and the BH3 only proteins are proapototic factors. Their role in autophagy regulation emerged when Beclin 1, which is a BH3-only protein (Oberstein et al., 2007) that interacts with Bcl-2 and Bcl-X_L, was identified as an essential autophagy gene (Liang et al., 1999; Yue et al., 2003). Beclin 1 is a haploinsufficient tumor-suppressor gene frequently deleted in breast, ovarian and prostate cancer (Maiuri et al., 2009). It is part of a Class III PI3K dynamic multimeric complex that participates in autophagosome formation (Kihara et al., 2001). Beclin 1 was found to interact with Bcl-2 in yeast two hybrid screening (Liang et al., 1998). In such way Bcl-2, in particular the Bcl-2 fraction localized at the ER, inhibits Beclin 1 dependent autophagy, thus exerting a dual role of antiapoptotic and antiautophagic protein (Pattingre et al., 2005). This binding may regulate autophagy levels and cell survival (Pattingre et al., 2005) and is modulated by nutrient status. Beclin 1 contains a BH3 domain essential for its interaction with the BH3 groove of Bcl-2 and Bcl-X_L, and thus for its regulation (Sinha and Levine,

2008). The regulation of this interaction and, consequently, of autophagy, can be explained by two mechanisms. In the first case, starvation causes JNK1 activation, which leads to phosphorylation of Bcl-2 and dissociation of Bcl-2 and Beclin 1 (Wei et al., 2008), and the starvation-induced dissociation is abrogated in cells lacking JNK1 or in which JNK1 is inhibited. The second mechanism involves the competitive binding of the BH3 domain of Beclin 1 to BCl-2 by other BH3-containing proteins. BH3-only proteins such as Bnip3 (Zhang et al., 2008), Bad, EGL-1 and the BH3 domain peptidomimetic (Maiuri et al., 2007a) induce autophagy by competitively disrupting the interaction between Beclin 1 and Bcl2 or Bcl-X_L. Moreover, other BH3 only proteins, such as Bik (Rashmi et al., 2008), Noxa and Puma (Abedin et al., 2007) have been shown to induce autophagic cell death. The mechanism for such induction may be similar to the one described for other BH3-only proteins or, alternatively, may involve different effectors. For example, the BH3-only protein Puma is a central mediator of p53-dependent apoptosis and functions by activating Bax and mitochondrial outer membrane permeabilization. In response to mitochondrial perturbations Puma can also induce autophagy through Bax, leading to selective removal of mitochondria, and this is not due to release of Beclin 1 from Bcl-2, since this event would not be influenced by Bax or Bak expression (Yee et al., 2009). Moreover, inhibition of autophagy diminishes Puma and Bax mediated apoptosis, suggesting that, at least in some circumstances, selective autophagic targeting of mitochondria can enhance apoptosis (Yee et al., 2009). For what concern their physiological relevance in the cross-talk between apoptosis and autophagy induction, the best characterized BH3 only proteins are Bnip3 and Bnip3L (Nix). Bnip3L is required for normal erythrocyte differentiation, as mentioned earlier (Sandoval et al., 2008; Schweers et al., 2007), and both Bnip3L and Bnip3 play a role in cellular responses to ischemia/reperfusion injury in the heart (Galvez et al., 2006; Hamacher-Brady et al., 2007). Bnip3 levels are induced after stress such as hypoxia in a HIF-1-dependent manner (Zhang et al., 2008) and consequently it localizes at the mitochondria causing increase in ROS, opening of the permeability transition pore and loss of mitochondrial membrane potential (Chen et al., 1999), leading to cell death, most likely both via apoptosis and via autophagy. In cardiomyocytes, localization of Bnip3 at mitochondria causes the release of cytochrome and caspase-dependent apoptosis (Regula et al., 2002). Although the regulation of Bnip3 at mitochondria is unclear, Bax and Bak appear to be downstream effectors of Bnip3-mediated mitochondrial dysfunction (Kubli et al., 2007). In parallel to apoptosis, Bnip3 can also induce autophagy (Hamacher-Brady et al., 2007; Mammucari et al., 2007; Tracy et al., 2007; Zhang et al., 2008). In addition to the mechanism reported earlier, involving differential interactions among Bnip3, Beclin 1 and Bcl2 (Zhang et al., 2008), Bnip3 may induce autophagy by two other ways. In the first case the damage that Bnip3 causes to mitochondria in cardiomyocytes as a result of ischemic injury leads to induction of autophagy (Hamacher-Brady et al., 2007). In a different setting Bnip3 has been found to block signalling by mTOR, which is a master regulator of autophagy, through interaction with Rheb (Li et al., 2007). In the setting of heart failure, Bnip3 expression is up-regulated during ischemia and is maintained elevated during reperfusion in rat ventricular myocytes (Pitts et al., 2008), causing cell death and cardiac remodelling (Diwan et al., 2007). How Bnip3 causes cell death in I/R injury is not completely clear, and apoptosis, autophagy and necrosis could occur simultaneously. Opening of the mitochondrial permeability transition pore (PTP) seems to be an essential event in Bnip3-mediated cell death (Vande Velde et al., 2000). In this respect, besides its well known role in apoptosis, autophagy activation has been observed as a consequence of PTP opening. For example, in cultured hepatocytes inhibition of the mitochondrial permeability transition with CsA blocks both mitochondrial depolarization and the proliferation of autophagosomes (Rodriguez-Enriquez et al., 2004).

Autophagic cell death has been described in the case of Bax/Bak double knockout fibroblasts. These cell are resistant to apoptosis but they still undergo a non-apoptotic death

which is dependent on essential autophagy genes (Atg5 and Beclin 1), that was suppressed by autophagy inhibitors, such as 3-methyl adenine, and that is modulated by Bcl-X_L (Shimizu et al., 2004). Thus autophagy appear to play not only a pro-survival, but also a pro-death role, although in many cases this occurs when apoptosis is inhibited. Whether autophagy plays a role in cell death is physiological condition in not completely clear.

Atg5 and calpain

Atg5 is an essential autophagy gene involved in the early stages of autophagosome formation (Mizushima et al., 1998). Yousefi et al. demonstrated that Atg5 over-expression sensitizes tumour cells to various apoptotic stimuli. When breast cancer cells expressing increased levels of Atg5 were implanted in vivo, tumour growth was reduced and high levels of apoptosis were observed. Apoptosis is favoured by calpain-mediated cleavage of Atg5, which leads to an amino-terminal cleavage product. The cleaved form of Atg5 translocates from the cytosol to the mitochondria where it associates to Bcl-x_L and triggers cytochrome c release and caspase activation (Yousefi et al., 2006), although it is not clear whether this association, by releasing Bax from the inhibitory interaction with Bcl-x_L, leads to Bax activation.

Calpain not only triggers apoptosis, but appears to be essential for autophagy, too. Indeed, in calpain-deficient cells autophagy is impaired and apoptotic cell death is highly increased and CAPNS1-deficient cells are more sensitive to apoptosis induced by several autophagic stimuli (Demarchi et al., 2006). From this results it appears clear that calpain plays a role in the switch between autophagy and apoptosis, but its role in regulating these two processes is not completely clear yet.

p53 pathway

The tumour-suppressor p53 plays a complex role in response to stresses, exerting both nuclear and cytosolic functions. The transactivation and transcriptional repression functions of p53 are well documented, and regulate the expression of genes involved in various processes such as cell cycle arrest, apoptosis and others (Menendez et al., 2009). By depleting stem cell pools, p53-induced cell cycle arrest can lead to aging phenotypes (Rodier et al., 2007). In addition to this, p53 can trigger apoptosis independently of its transcriptional activity (Caelles et al., 1994; Haupt et al., 1995; Kakudo et al., 2005). Indeed, upon various cell-death stimuli p53 translocates to mitochondria where it causes mitochondrial outer membrane permeabilization and consequent release of proapoptotic factors. p53 interacts with Bcl2 family members and alternative interaction with anti- or pro-apoptotic proteins may regulate its function at the mitochondrial surface (Speidel, 2009). At the same time, p53 regulates autophagy, both by transcriptional activation of target genes and by non-transcriptional mechanisms. For example, DRAM (damage-regulated autophagy modulator) is a p53 target genes which encodes a lysosomal protein. p53 induces autophagy in a DRAM dependent manner. Moreover, DRAM is essential for p53-mediated apoptosis (Crighton et al., 2006). Accordingly, autophagy was not induced by etoposide in p53−/− MEFs (Feng et al., 2005). In a model of lymphoma in cells carrying inducible p53, activation of p53 causes increased apoptotic cell death, while autophagy was activated in the surviving cells. Tumour cell death was induced in p53 overexpressing cells when autophagy was inhibited (Amaravadi et al., 2007).

Somehow surprisingly, Kroemer and co-workers found that autophagy is stimulated also in the absence of p53 (Tasdemir et al., 2008), thus acting as an endogenous repressor of autophagy. The cytosolic fraction of p53 is the one responsible for inhibition of autophagy. In glucose starvation conditions, ATP levels are drastically reduced in wild type cells while, in the absence of p53, ATP levels are maintained high. The resistance of p53 −/− cells to

metabolic stress is dependent on autophagy, since suppression of autophagy by depletion of AMPKalpha or Beclin 1 reduced the capacity of p53−/− cells to maintain ATP levels during starvation (Tasdemir et al., 2008). It is not clear which mechanism allows p53 to function either as autophagy inhibitor or apoptosis inducer, but the two processes may be co-ordinately regulated by cell death inducers.

The tumor suppressor ARF (murine $p19^{ARF}$ and human $p14^{ARF}$) inhibits the p53 suppressor Mdm2, thus causing activation of p53. A mitochondrial short form of $p19^{ARF}$ (smARF) causes dissipation of mitochondrial membrane potential in a p53- and Bcl2-independent manner, eventually inducing autophagy and caspase-independent cell death, which was blocked by knockdown of endogenous autophagic proteins, such as Atg5 and Beclin 1 (Reef et al., 2006). In contrast, the nucleolar $p19^{ARF}$ was incapable of inducing p53-independent autophagy (Reef and Kimchi, 2008), suggesting the existence of two different tumour suppressor pathways activated by the two ARF isoforms. The first one relies on a rapid p53-mediated nuclear response and the second one implicating mitochondrial-mediated autophagy induction. smARF levels are controlled by its interaction with p32, a protein predominantly localized to the mitochondrial matrix whose functions are not completely clear yet. Suppression of p32 protein levels reduces smARF levels by increasing its turnover and reduces the ability of ectopically expressed smARF to induce autophagy and mitochondrial membrane potential dissipation. In contrast, the levels of nucleolar full-length $p19^{ARF}$ were not affected by p32 depletion (Reef et al., 2007).

Conclusions

Age-related disorders such as neurodegenerative diseases, stroke, sarcopenia are propagating in developed countries due to the exponentially increase in aged population. Trying to combat aging, in the attempt not only to prolong life, but especially to preserve physiological functions, represents an important strategy to ameliorate life quality. At the cellular level, homeostasis is maintained when renewal of essential macromolecules is warranted and organelles such as mitochondria are kept at maximum efficiency. Systems such as the ubiquitin-proteasome and the autophagylysosome pathways are responsible for the recognition and the degradation of non-functional cytosolic components. In the worst case, apoptosis is activated. The cross-talk among these systems, two of which we have analyzed here, is finely regulated. Future research will shed further light on how these systems are regulated during senescence, with the aim of ensuring a healthy aging to the future populations.

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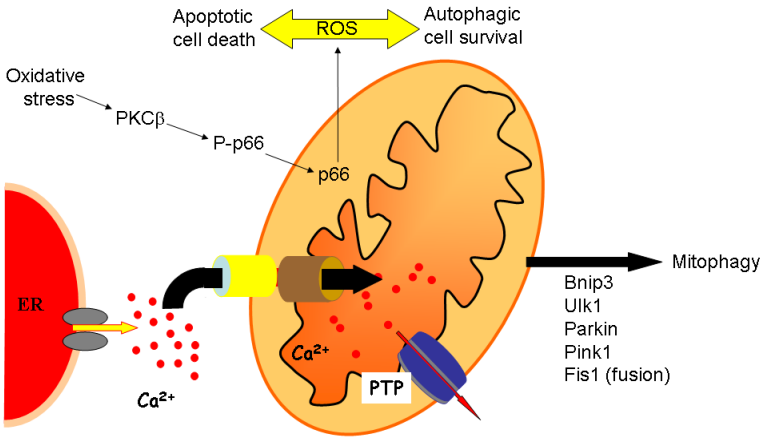

Figure 1. Signaling pathways regulating mitochondrial function

Mitochondria calcium uptake is tightly regulated and occurs at ER-mitochondria contacts where microdomains of high calcium concentration are present. This event causes a variety of responses depending on the amount of Ca2+ increase, from stimulation of metabolism and ATP production to PTP opening and apoptosis. Mitochondria represent also the privileged site of ROS production. ROS may act as signaling molecules, inducing a pro-survival autophagic response, or may cause damage to cell components and apoptotic cell death. ROS production is induced by oxidative stress, which activates a signaling cascade involving the PKCβ-dependent phosphorylation of p66shc and its translocation to the mitochondrial matrix. Mitochondria are also targets of ROS damage. Damaged mitochondria are removed by mitophagy, a specialized form of autophagy, which is regulated by different players, some of which are listed here.