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Alicia Torriglia, Imene Jaadane, Cecile Lebon. Mechanisms of cell death in neurodegenerative and retinal diseases: common pathway?. *Current Opinion in Neurology*, 2016, 29 (1), pp.55-60. 10.1097/WCO.0000000000000272 . hal-01292021

HAL Id: hal-01292021

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Submitted on 22 Mar 2016

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Mechanisms of cell death in neurodegenerative and retinal diseases: common pathway?

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The authors declare that they have not conflicts of interest.

Abstract

This review intends to draw the attention of researchers working in retinal degenerations on the fact that classical apoptosis, e.g. apoptosis triggering caspase activation, may not be the main pathway of cellular demise in this tissue.

Former work already showed the difficulty of proving the activation of apoptosis effectors in different models of retinal degeneration. However, these results were not really considered because of the lack of an alternative explanation for cell death. Nowadays, the description of many pathways of cellular demise is filling the gap and other forms of cell death are now described in the retina.

The knowledge on the molecular mechanisms of cell death is very important for the development of new therapeutic strategies, as well as for the evaluation of cell death onset in retinal degeneration.

Key-words: apoptosis, alternative cell death, caspase, TUNEL

Introduction

Neurodegenerative diseases are an increasing health concern in the aging population. They are caused by the death of the neural cells in the Central Nervous System (CNS). Despite massive research, the molecular mechanisms governing this cell death remain a fundamental question. The seminal work of Kerr identifying genomic DNA degradation in oligonucleosomes as a marker of active cell death and the introduction of the terminal deoxynucleotidyltransferase dUTP nick end labeling (TUNEL) method pointed apoptosis as the causative cell demise pathway [1]**. Retina, the photosensitive tissue of the eye, is part of the CNS and apoptosis is also considered as the main pathway by which retinal cells die, leading to vision loss in many blinding conditions.

However, many other forms of cell death exist. Actually, cells can die actively or passively by participating in their own demise or not. If an injury alters the cell in a detrimental manner and poses a threat to the tissue, it may be eliminated through an active regulated cell death process. Contrary to passive cell death, the course of regulated cell death can be altered by pharmacological and genetic interventions; it occurs in a delayed manner and is initiated in the context of adaptive responses that primarily attempt to restore cellular homeostasis. If this attempt fails, the best way to eliminate the damaged cell is apoptosis. Although the notion is less spread than it should be, there are multiple pathways leading to an apoptotic phenotype. Some of them involve the activation of caspase proteases, but some others, while maintaining the morphology of apoptosis, are caspase independent and involve the activation of non caspases proteases [2].

Alternatively, if a cell suffers critical damage, owing to an extreme insult, it loses its plasma membrane integrity, presents organelle and cell swelling, and ultimately passively disintegrates. The most common form of accidental passive cell death is necrosis. The leakage of intracellular components damages neighboring cells and triggers an inflammatory response. While, for many years, necrosis was considered as a strict equivalent of passive cell death and apoptosis was seen as the sole active form of cell death, nowadays the relevance of alternative degeneration mechanisms is becoming increasingly evident. For instance, necroptosis, another regulated form of cell death has

recently become a topic of interest. Necroptosis shares some inductive-phase characteristics with apoptosis but possesses many morphological features of necrosis [3]*.

The cell death pathway activated in a cell depends on several features related to the nature and intensity of the injury, as described above, but also, and this is very important, to the differentiation status of the responding cell. As a matter of fact, upregulation of pro-apoptotic genes expression by transcription factors is commonly observed during development. In *Drosophila*, for instance, the marginal discs of the developing eye, which acquires susceptibility to the pro-apoptotic gene *hid*, differs in a region-specific manner. So that, the same stimulus can induce different cell death pathways in different cell types [4]*.

Cell death in retinal development:

During retina development, as in other parts of the CNS, cell death allows selection of appropriate synaptic connections. Waves of apoptosis sweep through the ganglion cell, inner nuclear and photoreceptor layers as each region undergoes differentiation. This mechanism allows the different parts of the retina to achieve a numerical balance by selecting the right synaptic connexions. This cell death is achieved by the activation of apoptosis [5]. Apoptosis is classically driven by caspases, a family of intracellular cysteine proteases. Once activated, initiator caspases (-8 or -9) can activate effector caspases, (-2, -3, -6 or -7), resulting in the generation of a proteolytic cascade whose downstream targets include integral proteins for cell survival, culminating in cell demise. The intrinsic pathway is initiated by cytochrome C release from the mitochondrion. The association of cytochrome C with Apaf-1 and dATP promotes caspase-9 activation followed by the downstream effector caspases. Actually, caspase 3 KO mice display cerebral overgrowth and retinal hyperplasia, indicating that this set of proteases is important for cell death during retinal differentiation [6].

It is important to note that competence to a specific signal that triggers apoptosis can vary depending on the developmental context. For instance, mitotic progenitor cells in *Drosophila* eye are

extremely sensitive to the exogenously induced apoptotic signal. This competence depends on hid expression. Later on, differentiated photoreceptors become resistant to induced apoptosis by accumulating the apoptosis inhibitory protein, DIAP1 [7]. Actually, susceptibility to neuronal apoptosis is controlled at various levels: protection by growth factor signaling, amount of IAP protein, redox regulation, and regulation of the apoptosome machinery and executioner caspases.

The status of the apoptotic machinery in adult retina.

Expression levels of apoptosome components, Apaf-1 and caspase-9 are markedly decreased after development in both Balb/c and C57Bl6 adult retina [8-9]**. This creates a relative deficiency in the activation of caspases pathways in the adult retina. Defective caspase activation due to diminished or absent Apaf-1 has also been reported in cancer cell lines suggesting that the decreased expression of these molecules is a general strategy in the control of apoptosis [10]. Moreover, the main effectors of classical apoptosis, caspases, are also down regulated in adult retina. Caspase-3 expression is also strongly decreased at the end of the differentiation process. Interestingly, the same is seen after brain development indicating a common behavior in different parts of the CNS [11]. Other than this, the IAPs (inhibitor of apoptosis proteins), which are potent inhibitors of caspases are upregulated in the adult retina. Due to this physiologically regulated expression of important pro-apoptotic effectors proteins, it is understandable that caspase activation and caspase dependent cell death is quite spare in the retina. Actually, caspase activation leads to a very rapid, efficient apoptosis which is not adapted to post-mitotic, non replaceable cells, like photoreceptors, explaining why these enzymes are tightly controlled as an strategy to protect valuable cells.

The activation of caspases in retinal apoptosis

Caspases activation is systematically searched when investigating the occurrence of cell death in the retina or in retinal derived cultured cells. Caspase activation is regularly seen in cultured cells. For instance long-term blue light exposure significantly reduces cell viability in RGC-5 cells. This induces a

marked increase in the expression of Bax and active caspase-3 (p17), and is accompanied by Bcl-2 down-regulation. However, this study used proliferating cells in which the differentiation effects on apoptotic effectors have not occurred. In our opinion, this disqualifies this type of cell culture as a method to understand cell death in the retina. Actually, many serious studies show that caspases are not activated in many situations in the adult retina. Instead, other proteolytic enzymes like calpains and cathepsins are involved in cell demise. So that, Comitato et al showed, in three *in vivo* models of retinal degeneration, that the activation of calpains and cathepsins caused activation of BAX (Bcl-2 associated X protein) and the release of AIF (Apoptosis inducing factor) from mitochondria. AIF is a flavoprotein involved in the complex 1 of respiration. Its cleavage by calpain 1 induces its release from mitochondria and its nuclear translocation. Once in the nucleus, AIF condenses the chromatin and promotes DNA cleavage by endonucleases. The importance of this caspase independent pathway in retinal degeneration was shown in several animal models of Retinitis Pigmentosa, like in the P23H mutant rat [12]. AIF seems also to be involved in retinal degeneration caused by proteasomal inhibition [13]. However, the inhibition of this pathway does not completely protect the retina suggesting the activation of other parallel pathways [14].

Interestingly, AIF and mitochondria are not always involved in caspase independent cell death in retina. Myricetin, a vegetable polyphenol, which has been shown to be toxic to ARPE 19 cells, a cell line from the human retinal epithelium, induces a cell death which is insensitive to the inhibition of several members of the caspase pathway: DEVD, an inhibitor of caspase 3, cyclosporin A, inhibitor of the mitochondrial permeability transition, DPQ inhibitor of PARP-1, perindopril, inhibitor of the NADPH oxidase and the uncoupling protein-2/mitochondrial pathways and pinacidil, inhibitor of the mitochondrial KATP channel opener [15]. This clearly indicates that neither caspases, nor the mitochondria are involved in this cell demise. However, authors showed, also in this paradigm, the activation of calpains. Interestingly, if these enzymes are able to release AIF from mitochondria, they also induce lysosome destabilization and permeability as will be discussed later.

Other forms of cell death activated in the retina

A review of the literature indicates that cell death mechanisms in the retina are poorly understood. In a set of interesting studies, the group of Paquet-Durand in Switzerland gave some light in this issue by reasoning in terms of time scale. They noted that the different cell death pathways run on different time-scales, so they used the evaluation of the duration of photoreceptors cells death to have some insight on the underlying cell death mechanisms. They reasoned that necrosis is a rapid and chaotic, destruction of the cell taking about an hour to be completed, whereas apoptosis may take 6 to 18 h [16-17]**. By using rd1 mice, they estimated the time elapsed between the rise in cGMP, that they used as the inducer of cell death, and the TUNEL labeling, used as the final point of cell death before cells are cleared away. They calculated a total cell death duration of about 80 h. These results been incompatible with the execution of necrosis or classical apoptosis, they concluded that we may be facing alternative cell death mechanisms. Some of them that have already been described in the literature, as shown below, may have a greater weight in retinal degeneration than currently accepted.

Necroptosis: In RGC-5 cells submitted to high hydrostatic pressure, the cell death is inhibited by the use of necrostatin 1 or by calpain inhibition [18]*, suggesting that cells are dying by necroptosis. Necroptosis is also executed in the retina after ischemia-reperfusion [19] and contributes to retinal disorders such as retinal detachment, retinitis pigmentosa and age-related macular degeneration (AMD) [20]. Although the molecular mechanisms of this type of cell demise are not completely understood, we know that it depends on the kinase activity of receptor-interacting protein kinase 1 and 3 (RIP1 or RIPK1; RIP3 or RIPK3). Both kinases have been detected in the photoreceptors of rats exposed to the light produced by light emitting diodes (LED) [21]*. In addition, pharmacological inhibition of RIP1 and RIP3 activity contributes to delayed cone cell death in the zebrafish mutant pde6cw59 [20]. All these studies suggest the involvement of necroptosis in retinal degeneration.

Parthanatos: The Glutamate-mediated excitotoxicity both in diabetic rat retina and in Glutamate-treated R28 cells shows an unchanged state of cleaved and uncleaved caspase-3 while a cell death related to parthanatos (involving PARP-1 activation) is triggered [22].

Paraptosis: This form of active cell death is characterized by caspase independence, lack of DNA degradation and lack of membrane blebbing. It has been described in the retina for the first time several years ago by ourselves when studying the toxicity of glucocorticoids on the retina [23]. Recent studies describe this type of cell death also in ganglion cells [24].

Autophagy: Autophagic cell death occurs in the absence of chromatin condensation but is accompanied by massive autophagic vacuolization of the cytoplasm. Knocking down genes required for autophagy or inhibiting the process by chemicals reduces cell death [25]. While being able to act as a cell death mechanism, autophagy is basically a cell survival process activated by starvation. Autophagy allows the recycling of intracellular material and damaged organelles, including permeabilized mitochondria. This is important because it has been shown that mitochondrial permeabilization is incomplete most of the time. Some mitochondria remain intact and can repopulate the cell enabling cell survival [3]. In the absence of caspase activity, mitochondrial permeabilization is associated with enhanced autophagy through upregulation of ATG12. In this context, and in others [26]*, autophagy acts as a pro-survival mechanism [27]. However, extensive autophagy may turn into a cell death mechanism. It is even possible that this form of cell death represents just an over activation of a survival process. This switch of autophagy from cell survival to cell death has been clearly shown in ARPE-19 cells [28]*. Cell death by autophagy in the retina has been described in several models including chemical treatments like ethambutol and ROCK inhibitors as well as in retinal detachment [29-31].

Pyroptosis: Pyroptosis is an inflammatory-mediated cell death, defined as being dependent of caspase-1. During pyroptosis, caspase-1 is activated in a multiprotein platform termed inflammasome or pyroptosome, which is formed by the adaptor protein ASC (Apoptosis-associated

Specklike protein containing a CARD) and NLRs (NOD-like receptors), a cytosolic sensor of either DAMPS (Danger Associated Molecular Patterns) or PAMPS (Pathogen Associated Molecular Patterns). Active caspase-1 has been detected in retinal and vitreal samples from diabetic patients. This mechanism is probably involved in the demise of Müller cells, which present a nuclear accumulation of GAPDH, a feature that could be used as a marker of this type of cell death [25].

Other forms of cell death might be important in the retina?

Other than the forms of non apoptotic cell death described above, other forms of cell death, not described in the retina so far, exist. This is the case for mitotic catastrophe, cornification, anoikis, entosis or dark degeneration. Among this forms only dark degeneration, a non-characterized form of cell death described essentially in the brain, could be investigated in the retina since the other forms of cell death correspond mostly to proliferating or epithelial cells. However, it is not impossible that, in the future, other forms of cell death could be described and found to be important in some retinal pathologies.

Why the literature on alternative forms of cell death in the retina is so rare?

As said before, apoptosis and caspase activation is systematically investigated in retinal degeneration excluding the investigation of all other forms of cell death, most of the time because of lack of precise markers. Moreover, as DNA fragmentation is one of the key markers of apoptosis, the TUNEL assay is the most used method to identify dying cells. This assay is based on the detection of 3'-OH termini that are labeled with dUTP by the terminal deoxynucleotidyltransferase. Although the test is very reliable and sensitive in caspase-dependent apoptosis, it is completely useless when cell death is mediated by pathways involving DNA degradation generating 3'-P ends. This is the case for the LEI/L-DNase II pathway. L-DNase II is a nuclease whose activation has been reported in the retina [32]. We

have shown, for instance, that light exposure activates calpains. The activation of calpain 1 permeabilizes the lysosomal membrane by cleaving the lysosomal associated membrane protein 2 (LAMP2) [33]. This permeabilization releases, among other proteases, cathepsin D. This enzyme activates L-DNase II that degrades genomic DNA and induces caspase independent cell death. Actually, this pathway is associated with different types of phenotypic cell death like apoptosis, necroptosis and paraptosis and could be the common effector of the pro-death activity of PKC zeta. Recent work performed in our laboratory showed that the nuclear translocation of L-DNase II, and therefore its pro-apoptotic activity may be inhibited by this kinase. PKC zeta is also an activator of the NFkB pathway, known to have a role in modulating apoptosis through the synthesis of anti-apoptotic factors. PKC zeta also interacts with p62 (sequestosome), involved in autophagy, and with RIP1, involved in necroptosis [34]. These data place PKC zeta at the interface of apoptosis (caspase dependent and independent), oxidative stress response and necroptosis, becoming the master of the cell signaling orchestra and probably linking L-DNase II to different forms of cell death (Fig 1).

Conclusion:

Apoptosis is usually thought to be the main cell death mechanism in the retina and other cell death mechanism are often neglected. These last few years several, groups have shown that alternative pathways of cell death could be activated (Table 1). Some reports even suggest that cells in the retina die mostly by alternative pathways. Moreover, several pathways may be active at the same time. The use of techniques detecting almost exclusively classical apoptosis or very late stages of some other pathways results in an underestimation of cell demise. This could be extremely critical in experiments designed to evaluate a drug safety, for example, because a lack of toxicity could be erroneously inferred.

Key bullets points:

- Retinal cells are protected against classical apoptosis by down regulation of its molecular effectors.
- Non-apoptotic caspase-independent cell death like necroptosis, pyroptosis or paraptosis were identified in many models of retinal degeneration.
- Calpains and lysosome emerges as important effectors of retinal cell death.

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Acknowledgements

We would like to thank Dr Patricia Crisanti-Lassiaz for critical reading of the manuscript.

Financial support and sponsorship

This work was supported by the INSERM

Conflicts of interest

The authors have no conflicts of interest.

Legends to figures:

Table 1: Non apoptotic cell death in the retina: Some non apoptotic pathways of cell death described in the retina and the model in which they have been described. The column “markers” does not represent systematically the molecular markers that have been used by the authors to assess this type of cell death but markers that are simple to use and easily available.

Figure 1: Different mechanisms of cell death activated in retinal cells: A mild stress generates a protective response in which kinases, like PKC zeta, participate. Under an extremely important stress unregulated necrosis arises, while a less intense stress induces different death pathways depending on the intensity of the stimulus and on the metabolic state of the cell. These include caspase dependent apoptosis. However, this type of cell death is not favored due to the under regulation of caspases and other factors of the caspase cascade. Among the different effectors activated L-DNase II is involved in some of them as the DNA degrading enzyme (plain arrows) and is also suspected to participate to other pathways (dotted arrows). This enzyme interacts and cooperates with AIF also involved in alternative cell death in the retina.

Cell death	marker	model
Necroptosis	RIP increase AIF nuclear translocation	RGC-5, pressure, ischemia reperfusion, retinal detachment, LED induced retinal degeneration, pde6cw59 mutant
Parthanatos	PAR synthesis	Excitotoxicity
Autophagy	LC3	Chemical toxicity
Pyroptosis	IL-1 increase	Diabetes
Caspase independent apoptosis	L-DNase II activation AIF nuclear translocation	Light induced retinal degeneration, steroids toxicity
Paraptosis	No DNA degradation Cellular vacuolization	Steroids on RPE

Table 1

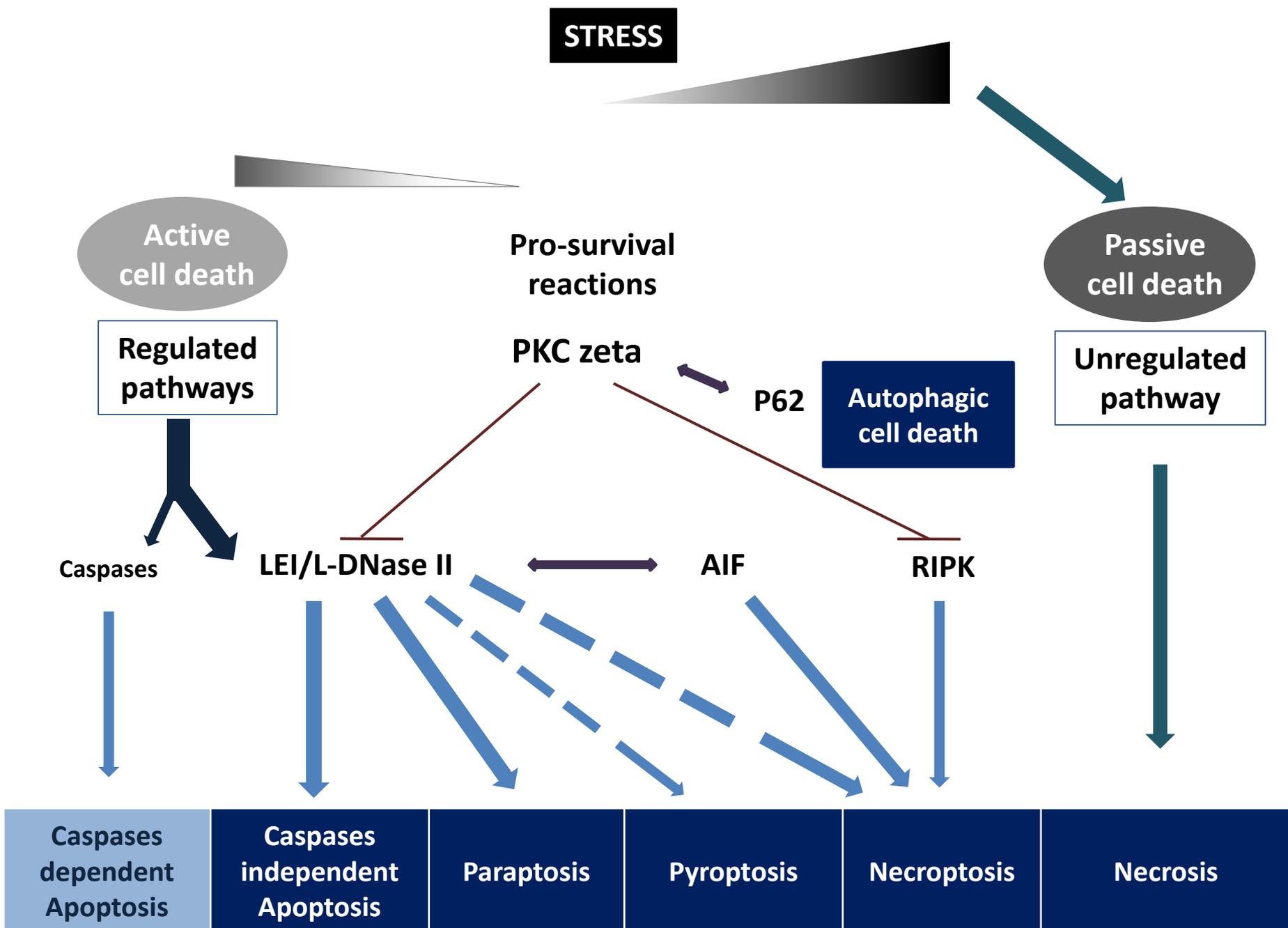


Figure 1