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Mini review

Apoptosis, programmed cell death and the hypersensitive response

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Abstract

Apoptosis is typically a morphologically identifiable form of programmed cell death in mammals that is regulated by genes with homologues in other animal Phyla. Although both plants and fungal plant pathogens exhibit forms of developmental programmed cell death, demonstrated morphological or genetic homologies with mammalian apoptosis are still generally lacking. Because of its ubiquity and the involvement of signal transduction pathways in its induction, a strong case is developing that the hypersensitive response is a specific form of plant programmed cell death evolved as a defense against microbial parasites. Data suggest that separate signalling pathways may lead to the cell death and the defense gene activation that characterize this response and that parasite-specific resistance genes represent only one of many types of genes involved in response regulation. However, despite some biochemical similarities between the hypersensitive response, forms of developmental programmed cell death in plants, and animal apoptosis, no unique and consistent markers for the hypersensitive response (or plant programmed cell death in general) have yet been found. Whether any of these forms of plant cell death should be called apoptosis depends on how the term is defined. Assuming the hypersensitive response is a form of programmed cell death and is the 'default state' upon pathogen entry into a cell, it seems likely that intracellular biotrophic plant pathogens resemble some animal viruses in being able to suppress this response in susceptible hosts.

Abbreviations: AOS – active oxygen species; HR – hypersensitive response; ICE – interleukin-1β-converting enzyme; PARP – poly(ADP-ribose) polymerase; PCD – programmed cell death; PR – pathogenesis-related; Rb – retinoblastoma.

Introduction

Cell death that is brought about by 'suicide' processes within the cell is known as programmed cell death (PCD). The best studied is apoptosis in mammalian cells, a form of PCD characterized by its striking morphological features. However, forms of what might be considered PCD also can be found in unicellular organisms (Ameisen, 1996), invertebrates (Hale et al., 1996), fungi (Leslie and Zeller, 1996), and higher plants. In recent years, it has become popular to assume that one type of plant PCD is the hypersensitive response (HR), a rapid cell death in plants associated with disease resistance. This review examines the evidence for this assumption and compares the HR with indisputable forms of PCD in animals and plants. Because there is increasing evidence that cell death and disease resistance may not be as tightly linked as previously thought, this review focuses on the cell death part of the response and defines the HR as rapid cell death that results from an incompatible interaction between plant and microbe.

Apoptosis in animal cells

The recognition of apoptosis in mammalian cells has been aided by its usual (although not mandatory) association with a characteristic set of morphological features such as chromatin condensation, nuclear blebbing, and the fragmentation of the cell into apoptotic

bodies (Chinnaiyan and Dixit, 1996). These features generally accompany biochemical markers such as DNA cleavage into nucleosomal fragments, the degradation of the DNA repair enzyme, poly(ADP-ribose) polymerase (PARP), and the activation of a specific family of cysteine proteases (caspases) related to ICE (interleukin-1β-converting enzyme) (Chinnaiyan and Dixit, 1996). Apoptosis can be triggered by a wide variety of different signals, many of which induce cell differentiation and proliferation in other situations (Hale et al., 1996). This relationship between apoptosis and cell cycle is emphasized by the fact that apoptosis is generally found in proliferating tissues (Meikrantz and Schlegel, 1995) and by the morphological similarities between apoptosis and normal or aberrant mitoses (King and Cidlowski, 1995). Furthermore, both apoptosis and cell proliferation may be regulated by the same genes, and many genes involved in the induction or suppression of apoptosis act as tumour suppressor genes or proto-oncogenes, respectively (Hale et al., 1996).

Some of the genes that regulate mammalian apoptosis have homologues in nematodes and insects (Chinnaiyan and Dixit, 1996), suggesting considerable evolutionary conservation within the animal kingdom. However, the typical mammalian cytological features of apoptosis may not be present during PCD in invertebrates (Lockshin and Zakeri, 1994), in which autophagy and autolysis may be prominent (Bowen, 1984).

The primary role of mammalian apoptosis is to kill unwanted, damaged, or pathogen-invaded cells in such a manner that they are phagocytosed and do not cause an inflammatory response (Hale et al., 1996). Apoptosis also has been suggested to be the default state in mammalian cells, so that even healthy cells will die if they do not receive survival signals (Raff, 1992).

Programmed cell death in plants and fungi

Cell death that is executed by intra-organismal signals, rather than by external toxic factors, is widespread in higher plants and increasing evidence points to its existence in fungi. Examples of PCD that occur during a vascular plant's life cycle are the differentiation of xylem tracheary elements (Fukuda, 1997; Groover et al., 1997), the death of various tissues during different stages of sexual reproduction (Bell, 1996; Havel and Durzan, 1996a; Orzaez and Granell, 1997a), and the controlled senescence of specific plant parts such as petals and leaves (Buchanan-Wollaston, 1997). However, attempts to identify genes in plants that are homologous to those intimately involved in animal PCD have generally failed (Dangl et al., 1996). An exception is the *dad*-1 (*defender against apoptotic death*) gene that prevents PCD in nematodes and mammals, which has a homologue in pea (Orzaez and Granell, 1997b) and other plants. Nevertheless, the significance of this homology is questioned by the recent identification of *dad*-1 as a subunit of the mammalian oligosaccharyltransferase (Kelleher and Gilmore, 1997). The key role of this enzyme in the synthesis of N-linked glycoproteins raises the possibility that the plant-animal similarity may reflect homology in protein processing pathways rather than similarities in PCD. Assuming that PCD in plants resembles animal PCD in its links to the cell cycle (Gilchrist, 1997; Havel and Durzan, 1996b), it may be of greater significance that plants contain homologues of the retinoblastoma (Rb) family of proteins (Grafi et al., 1996; Xie et al., 1996) that control cell cycle progression and the suppression of apoptosis in mammalian cells (Hale et al., 1996). However, any role of these proteins in plant PCD remains to be proven.

Despite models that relate plant PCD and animal apoptosis (Havel and Durzan, 1996b), hard data are still relatively sparse. Overall, there is no strong evidence of a consistent 'apoptotic' morphology in plants but, instead, a diversity of cellular processes among different forms of PCD. For example, degradation of DNA into oligonucleosomal fragments (seen as 'ladders' on agarose gels) occurs in senescing pea carpels (Orzaez and Granell, 1997a) and salt-stressed roots (Katsuhara and Kawasaki, 1996) but not during xylem differentiation (Groover et al., 1997). DNA-containing 'apoptotic bodies' are not found in xylem elements (Groover et al., 1997) but have been seen in root cap cells and can be induced in tomato protoplasts by fungal toxins (Wang et al., 1996). The same toxins also cause an enrichment of phosphatidylserine on the outer surface of the protoplast plasma membrane (as detected by fluorescein-conjugated annexin V binding) (D.G. Gilchrist, pers. comm.), another feature typical of mammalian apoptosis (Hale et al., 1996). Assuming that these toxins are triggering PCD, the fact that they are sphinganine analogs suggests that, as in animal apoptosis (Hale et al., 1996), ceramide-linked signalling systems may be involved (Gilchrist, 1997). The strong morphological similarity of the protoplast responses to mammalian apoptosis also suggest that unwalled plant cells may

exhibit a more animal-like PCD than do walled cells in intact plants.

The significance of this diversity in plant PCD remains to be determined and it may not be as great as it first appears, given that even some of the hallmark features of mammalian apoptosis can vary depending on the cellular environment (Hampton et al., 1996; Pandy et al., 1994). However, as in animal PCD, a feature that seems to characterize all forms of plant PCD in which it has been looked for is the activation of cysteine (and other) proteases (Buchanan-Wollaston, 1997; Fukuda, 1997). It may turn out that these enzymes are the most fundamental link between all forms of PCD, regardless of organism.

In fungi, there is an increasing awareness of the existence of PCD (e.g. Leslie and Zeller, 1996) and evidence that some parts of the apoptosis pathway may be conserved between vertebrates and yeast (Tao et al., 1997). Fungal PCD can have some significance in plant disease since good examples can be found among the obligately biotrophic pathogens. Germ tubes of rust fungi eventually die if they do not receive a plant signal to form infection structures, and haustorial mother cells die rapidly if they do not receive a plant signal to form a haustorium (Heath and Perumalla, 1988). Interestingly, haustorial mother cell PCD (Heath and Stumpf, 1986), and death after cell cycle arrest in yeast (Motizuki et al., 1995), seem to involve autophagy of cell contents, as in invertebrate PCD and in the early stages of PCD in differentiating xylem tracheary elements (Groover et al., 1997).

Characteristics of the hypersensitive response

The HR was defined by Stakman (1915), and more recently by Goodman and Novacky (1994) as the rapid death of plant cells associated with disease resistance. It occurs in resistant plants in response to pathogenic viruses, bacteria, fungi or nematodes, and is associated with a multitude of biochemical processes that make these dead cells, and the adjacent living cells, an inhospitable environment for microbes (Kombrink and Somssich, 1995). Which of these processes actually causes the cessation of pathogen growth has rarely been unequivocally proven, and it probably varies among different plant - pathogen combinations (Heath, 1997).

As with developmental PCD in plants, there are many differences, and some tantalizing similarities, between the HR and mammalian apoptosis. Notwithstanding some claims to the contrary (Levine et al., 1996), plant cells dying due to the HR do not clearly show the morphological cellular changes that characterize mammalian apoptosis (Bestwick et al., 1995; Littlefield and Heath, 1979; Mittler et al., 1997). Furthermore, despite numerous light and electron microscope studies of the HR, no morphological features have emerged that clearly define and distinguish the HR from cell death caused by other means (Heath, 1976; Heath and Skalamera, 1997; Heath et al., 1997). In fact, the considerable diversity in morphology in hypersensitively-dying cells is echoed by diversity in other features such as the timing of irreversible membrane damage during the death process (e.g. compare Bennett et al., 1996 with Heath et al., 1997). Plant DNA cleavage has been reported in examples of the HR triggered by a virus (Mittler et al., 1997), bacteria (Levine et al., 1996; Mittler et al., 1997) and a fungus (Ryerson and Heath, 1996), but its timing during the death process varies and it results in oligonucleosomal fragments only with the fungus. As typical of mammalian apoptosis, the involvement of cysteine proteases that can cleave exogenous PARP has been indicated in an HR shown by cowpea leaves to the cowpea rust fungus (I. D'Silva and M.C. Heath, unpubl.) but these dying cells do not show an enrichment of phosphatidylserine on the outer surface of the plasma membrane as detected by fluorescein-conjugated annexin V binding (M.J.R. Mould and M.C. Heath, unpubl.).

Quite different from plant developmental PCD, and from the role of mammalian apoptosis in limiting inflammatory responses of surrounding tissue, is the association of the HR with defense responses. Unlike many of the features discussed above, this association is highly consistent between different examples of the HR. Defenses include the modification of plant cell walls, the accumulation of potentially antimicrobial molecules such as pathogenesis-related (PR) proteins (chitinase, glucanase) or phytoalexins (Kombrink and Somssich, 1995), and the eventually autofluorescence and browning of the dead cell due to the accumulation of oxidized phenolic compounds (Nicholson and Hammerschmidt, 1992). The upregulation of 'defense genes' that code for antimicrobial molecules or the enzymes that produce them is not restricted to the cells destined to die, since this also occurs in adjacent living cells. In addition, the HR typically induces systemic changes throughout the plant, including induced resistance to a variety of previously compatible pathogens (Kombrink and Somssich, 1995).

Although the association of cell death with some of these gene products and responses has been used to define the HR in the absence of a pathogen (Dangl et al.,

1996), the validity of this approach is debatable. Many defense responses can be triggered by bacteria (Bestwick et al., 1995; Jakobek and Lindgren, 1993), or by fungal or plant products (elicitors) (Hargreaves and Bailey, 1978; Perez et al., 1997) in the absence of cell death, indicating that defense responses alone are not a reliable indicator of the HR. Similar responses may also accompany cell death caused by known biocides such as heavy metals (Lummerzheim et al., 1995), by the expression of a bacterial ribonuclease in transgenic plants (Strittmatter et al., 1995), or by certain types of physical damage (Hargreaves and Bailey, 1978). It seems likely, therefore, cells dying from a variety of causes can release signals that trigger localized or systemic defensive responses in the plant. To define the HR as cell death that is associated with such responses is to define the HR by these 'after death signals'; the danger is that this may identify as an HR forms of cell death that have little resemblance in induction or execution to that triggered in resistant plants by pathogen infection.

Genes involved in the HR

Despite its shortcomings, the lack of any better markers of the HR means that looking for mutations that mimic the response in both cell death and defense gene activation is one of the few approaches available to identify genes involved in HR regulation in pathogen-free plants. Encouragingly, a role in the HR for at least some of these 'lesion mimic mutants' is supported by the mutated genes mapping to loci known to control HR-resistance to specific pathogens (Dangl et al., 1996). Few lesion mimic genes have yet been sequenced, but the wild-type allele of the *lls1* gene in maize is predicted to code for a aromatic ring-hydroxylating dioxygenase; this had led to the suggestion that the enzyme may suppress the HR in uninfected plants by degrading a phenolic mediator of cell death (Gray et al., 1997). The implication from this conclusion is that the HR (rather than other forms of plant PCD) is the default state of the cell and must be constantly prevented in uninfected plants; if true, this is of considerable conceptual significance, particularly in terms of the role of pathogens in plant evolution. However, only time will tell whether the plant cells die in these lesion mimic mutants because of activation (or lack of suppression) of the HR or because of some metabolic catastrophe that results in dead cells which then trigger HR-mimicking defense responses.

The HR typically occurs in 'gene-for-gene' plant-pathogen interactions in which resistance in a host cultivars is controlled by parasite-specific resistance (*R*) genes that have to be 'matched' by avirulence (*Avr*) genes in the pathogen. Most of the *R* genes that have been cloned so far seem to be involved in signal transduction pathways (Baker et al., 1997, Feuillet et al., 1997, Hammond-Kosack and Jones, 1997). Historically, plant pathologists have placed considerable importance on *R* genes, but it must be remembered that their discovery reflects the fact that they segregate during crosses with susceptible plants in which the active *R* allele is missing (Flor, 1971). With the advent of molecular genetics, it is becoming apparent that *R* genes are part of a battery of genes involved in signalling and defense expression, most of which are present in all genotypes of a plant species. This fact presumably explains why disease resistance, and the HR, can be expressed in nonhost plants in the absence of parasite-specific *R* genes (Heath, 1997).

One of the currently best-studied *R* genes is *Pto*, which confers resistance to bacterial speck disease in genotypes of tomato. This gene codes for a cytoplasmic serine/threonine protein kinase which, after binding to the bacterial *Avr* gene product, interacts with a family of transcription factors that bind a *cis*-element found in genes that code for PR proteins (Zhou et al., 1997). Significantly, the Pto kinase also interacts with a different gene product (another serine/threonine kinase) that is involved in triggering hypersensitive cell death (Zhou et al., 1995).

The relationship between cell death and defense gene activation in the HR

This separation of defense gene activation from cell death is now being reported in a number of situations other than in the HR controlled by *Pto*. It has also been suggested in barley containing the *Mlo* gene, a recessive allele of which conditions resistance to powdery mildew as well as cell death in uninfected plants (Büschges et al., 1997). It also been shown for a group of cell death-inducing oomycete proteins known as elicitins in which the defense-gene-activating part of the proteins are distinct from that which causes cell death (Perez et al., 1997). In addition, transgenic lesion mimic (*lsd*) mutants of *Arabidopsis* unable to accumulate salicylic acid still exhibited cell death although they showed reduced PR gene expression and resistance to *Peronospora parasitica* (Hunt et al., 1997). These results suggest that, although there may be

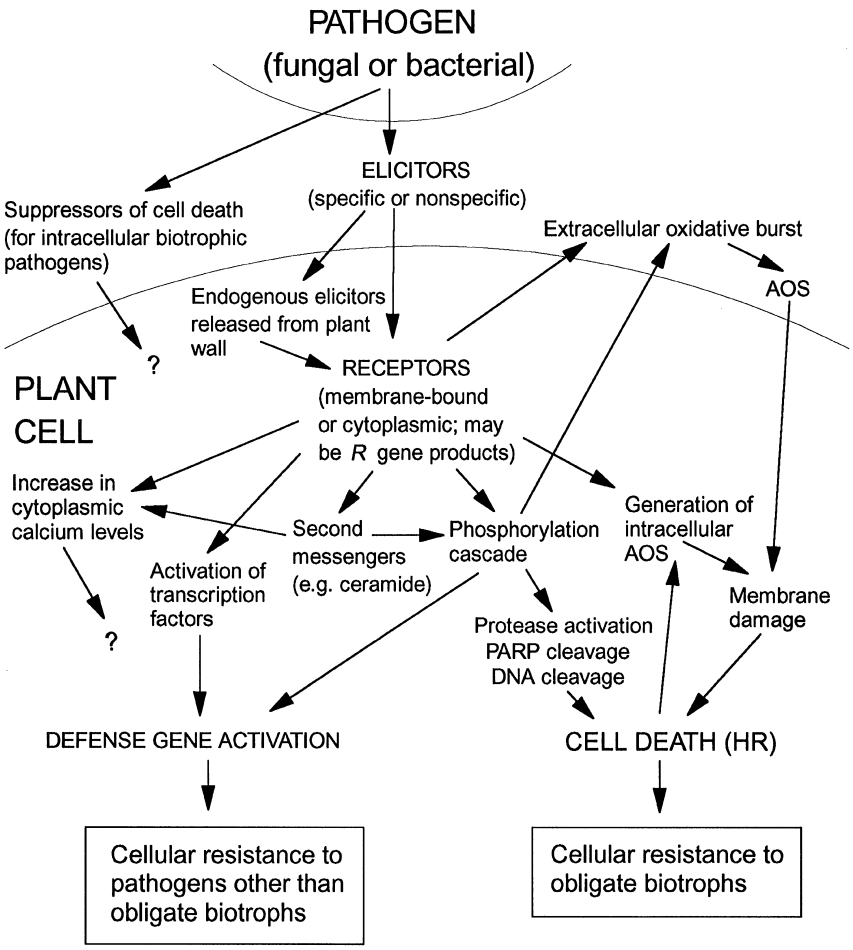

Figure 1. An overview, based on currently available data, of the processes that accompany, and/or may lead to, the hypersensitive response (HR) within a resistant plant cell. It is likely that different receptor-ligand combinations activate different combinations of these processes. The possibility that cell death during some examples of the HR is caused by directly toxic pathogen products is not illustrated. Also ignored are the signals released by the dying cell that activate defense genes in neighbouring, living, cells or trigger systemic acquired resistance in the plant.

'cross-talk' between signal transduction pathways, the one(s) that leads to cell death becomes separated at some point from the one(s) which leads to defense gene activation (Figure 1). It then becomes pertinent to question why cell death is such a common component of resistance. For obligately biotrophic parasites such as viruses and rust fungi, cell death itself may be the primary defense, and the activation of defense genes could merely be a 'back-up' to protect against saprophytic invaders. However, for pathogens that can grow in dead tissue, it seems likely that defense gene activation is more directly related to disease resistance than cell death. Since plant defenses tend not to be 'tailor made' for the inducing microbe (Heath, 1997), one possibility is that cell death acts as an endogenous 'secondary signal', re-enforcing the induction of localized defenses and releasing signals that trigger systemic defense (Figure 1).

Signals involved in the HR

Current models suggests that the HR involves a G-protein-mediated oxidative burst that generates extracellular active oxygen species (AOS) as a consequence of nonspecific or specific (*Avr* gene products) elicitors binding to plant receptors (Wojtaszek, 1997) (Figure 1). In the case of specific elicitors, these receptors may be the *R* gene products (Chandra et al., 1996). However, oxidative bursts do not necessarily lead to cell death (Baker and Orlandi, 1995) suggesting that AOS may

act as another signal, rather than be the direct cause of the HR. Hydrogen peroxide, in particular, has been suggested to trigger cell death in some systems (Tenhaken et al., 1995), but despite original conclusions to the contrary (Chen and Heath, 1994), more recent cytochemical and inhibitor studies have revealed no evidence of an oxidative burst, or the involvement of H_2O_2, in triggering an HR in cowpea by the cowpea rust fungus (Heath, unpubl.). Similarly, extracellular generation of AOS does not seem to be important in the death of cultured parsley cells invaded by *Phytophthora infestans* although AOS within the cell may be responsible for eventual membrane damage (Naton et al., 1996). The apparently variable role of AOS in the HR reflects that in mammalian apoptosis, with evidence from studies in highly hypoxic conditions that AOS are not always required for the latter (Hale et al., 1996). Interestingly, an oxidative burst also does not seem to be mandatory for developmental plant PCD, as it does not occur during the death of xylem tracheary elements (Groover at al., 1997).

Changes in cytoplasmic calcium levels commonly accompany signal transduction in plants and animals, and increases have been reported in some, but not all, forms of induced mammalian apoptosis (Hale et al., 1996). There have been few comparable studies of the HR, but a role for cytoplasmic calcium seems likely as calcium channel blockers delay the HR caused by bacteria (Levine et al., 1996) and the cowpea rust fungus (H. Xu and M.C. Heath, unpubl.). In addition, fluorescence ratio imaging shows a transient increase in cytosolic calcium levels prior to the onset of the HR in a resistant cowpea cells invaded by this rust fungus (H. Xu and M.C. Heath, unpubl.).

Is the HR a form of PCD and should it be called apoptosis?

Despite the lack of any unique markers to characterize the HR, its ubiquity in pathogen-infected, resistant plants has led to the widespread assumption that it is a form of PCD (Greenberg et al., 1994). Until recently, the best supporting evidence has been the fact that the HR, like PCD in animals, requires metabolism (Goodman and Novacky, 1994; Heath et al., 1997). Now this assumption is further substantiated by the revelation that most cloned *R* genes, which regulate pathogen-specific HR, seem to be part of signalling systems. The ability to disassociate the HR from defense gene activation as discussed above, as well as evidence of differential reaction cascades to different exogenous elicitors (e.g. Bohland et al., 1997), suggest that plants, like animals, have multiple signal transduction pathways and that there may be a distinct, and programmed, pathway leading to cell death. However, it is also clear from the previous sections of this review that although some forms of HR show some similarities to developmental PCD in plants and apoptosis in animals, there is considerable diversity in induction and expression. Also, the ubiquitous association of cell death and defense gene activation during a normal HR suggests that pathogens trigger a specific, defensive, type of PCD rather than eliciting one of the forms of plant developmental PCD. Overall, the indications that the HR is a form of PCD seem strong. Nevertheless, *proof* requires evidence of a commonality in regulation and execution downstream from the signalling systems. Such information is also needed to reveal just how much conservation in PCD there is between animals and plants.

Whether the HR, or other forms of PCD in plants, should currently be called apoptosis depends on how one defines the term. 'PCD' has the benefit of implying no specific genetic or cellular features and if 'PCD' and 'apoptosis' are considered synonymous there are no problems. However, if one equates apoptosis with the morphological features of mammalian PCD used to originally define the term (Chinnaiyan and Dixit, 1996), then 'apoptosis' does not seem appropriate for plants. It also is problematical if apoptosis is defined by the genes and biochemistry that are conserved across animal Phyla, since it is debatable whether there is as yet sufficient evidence of homologies in plants to warrant using the term in most examples of plant PCD.

Suppression of the HR by intracellular biotrophic pathogens

Fungal pathogens that are biotrophic and do not cause cell death during intracellular growth in susceptible hosts will still cause an HR when they penetrate cells of nonhost species (Heath and Skalamera, 1997). Therefore, if the HR is a form of PCD, it appears to be the default state with respect to pathogen penetration, irrespective of the presence of parasite-specific *R* genes. This implies that biotrophic, intracellular parasites must be adapted not to trigger, or to suppress, the HR in susceptible host species (Figure 1), and that *R* genes re-establish a signalling system that leads to this response in resistant genotypes (Heath et al., 1997). Comparable cellular 'manipulation' of hosts by animal pathogens is well documented. For example, DNA tumour viruses of mammals which cannot multiply in quiescent cells

produce proteins that bind to the Rb protein, driving the cell into S-phase (Hale et al., 1996). A similar situation for plant viruses is indicated by the discovery of Rb homologues in plants, and Rb-binding domains in the RepA early protein of some plant geminiviruses (Xie et al., 1996). These observations strengthen the possibility that plant viruses may also mimic animal viruses by producing inhibitors of PCD (Chinnaiyan and Dixit, 1996). If viruses can control the HR, then it is highly likely that biotrophic eukaryotic pathogens, such as the rust and powdery mildew fungi, can do the same. Understanding how they do so will not only provide insight into the complex plant-fungal interactions that typify resistance and susceptibility to these pathogens (Heath and Skalamera, 1997), but should also reveal critical information of how the HR can be regulated.

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