Programmed cell death dynamics during hyperglycemia and ischemic brain injuries.

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Abstract

Ischemia-reperfusion injuries of the brain are serious conditions that involve a high degree of programmed cell death. The two major pathways of programmed cell death which are widespread among mammalian cells, apoptosis and necroptosis, drive the damage of these injuries. In this mini-review, the signaling that underlies both of these programmed cell death pathways is broken down, highlighting important overlap and differences between the two pathways. The roles of both apoptosis and necroptosis in driving the pathology of ischemic brain injuries are also outlined. Special attention is paid to the balance between apoptosis and necroptosis in ischemic brain injuries and recent findings that demonstrate that hyperglycemia upregulates necroptosis and may shift apoptosis to necroptosis. The significance of these recent findings are discussed with regard to their role in ischemia-reperfusion injury of the brain and possible underlying mechanisms and avenues for further research on the topic are considered.

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Introduction

Ischemic injuries of the brain, including stroke and neonatal hypoxia-ischemia, are characterized by widespread cell death of neurons in the affected tissue [1-3]. The cell death that occurs includes two related programmed cell death (PCD) pathways, apoptosis and necroptosis [2,4,5]. These pathways overlap in their initial signaling steps but diverge to produce two very different outcomes. In general, cell death by apoptosis is largely non-inflammatory while necroptosis stimulates robust inflammation [6]. Both pathways and their roles in ischemic brain injuries are discussed below.

Apoptosis and Ischemic Brain Injury

Apoptosis may be activated by the extrinsic or intrinsic pathways [7,8]. In the extrinsic pathway, apoptosis is induced by cytokines of the TNF family after they bind to their cognate receptors on the cell surface. These cytokines include TNF-α, Fas ligand (FasL), and TNF-related apoptosis-inducing ligand (TRAIL). Following receptor binding, cytosolic signaling complexes are formed which function to set apoptosis signaling in motion. These cytosolic complexes differ slightly depending on the receptor: ligand stimulus but each lead to many of the same signaling events [7,8]. The best studied, and therefore best understood, extrinsic pathway is that induced by TNF-α [9]. The interaction of TNF-α with its receptor results in the formation of complex I, a membrane-proximal complex bound to the cytosolic portion of TNF receptor [8,9]. Complex I includes TNF receptor-associated death domain (TRADD), cellular inhibitors of apoptosis 1 and 2 (cIAP1/2), and receptor-interacting protein kinase 1 (RIP1) [9,10]. Within this complex, RIP1 is in a polyubiquitylated state in which it serves as the docking site for transforming growth factor beta activated kinase 1 (TAK1) in the TNF-α survival pathway [11,12]. However, once RIP1 is deubiquitylated, usually by cylindromatosis protein (CYLD), this complex is internalized further into the cell [13,14]. Following this, Fas-associated death domain (FADD) and inactive caspase-8 join resulting in the formation of complex II [7,10]. The presence of several molecules of caspase-8 in complex II leads to proximity activation of these signaling proteins. Once activated, caspase-8 cleaves and activates executioner caspases [including caspase-3,6,7] which go on to cleave multiple substrates resulting in apoptotic cell death [7,10].

Intrinsic apoptosis may be activated on its own in response to internal cellular stress or may be activated downstream of extrinsic apoptosis [7,15]. In either case, BH3-only proteins (which include Bad, Bid, and Bim) are activated to inhibit the anti-apoptotic Bcl-2 proteins [16]. This leads to the formation of channels in the outer membrane of the mitochondria by BH123 proteins. The channels formed by BH123 proteins allow for the release of cytochrome c (cyt c) into the cytosol [16]. Once released from the mitochondria, cyt c binds apoptosis protease-activating factor-1 (Apaf1) [7,10,16]. Several cyt *c*-bound Apaf1 molecules oligomerize via their caspase recruitment domains (CARDs) [7,10]. Subsequently, inactive caspase-9 molecules bind the oligomerized Apaf1 molecules via their CARD domains forming the apoptosome complex. Within the apoptosome, several molecules of caspase-9 induce self-cleavage due to proximity activation. Similar to caspase-8, once caspase-9 is activated it cleaves and activates executioner caspases which continue the signaling that leads to apoptotic cell death [7,10].

Whether induced by the extrinsic pathway, intrinsic pathway, or both, the outcome of cell death by apoptosis is largely non-inflammatory [6]. This is due to the controlled cleavage and packaging of cellular components during the execution phase of apoptosis [8,10,17]. In this phase, caspase-3 cleaves and inactivates the inhibitor of caspase-activated DNase (ICAD) resulting in DNA fragmentation [8,10]. Executioner caspases also cleave lamins A, B, and C which induces nuclear fragmentation. Additionally, the Golgi, ER, and mitochondria are similarly subject to fragmentation [8,10]. Cytoskeletal integrity is lost due the cleavage of gelosin (an actin nucleating factor) by caspases [10]. Not only does this prevent actin

polymerization but gelosin fragments also cleave polymerized actin filaments in the presence of calcium [10]. Caspases also activate membrane scramblases, the major of which is Xkr8, which leads to the externalization of phosphatidylserine (PS) [17,18]. All of these events lead to the breakdown of the cell into fragments called apoptotic bodies which display PS on their surface [8,10]. The PS on apoptotic bodies is recognized by macrophages and the cells are normally removed before any cellular contents are released resulting in the non-inflammatory phenotype [8,10].

Apoptosis is active following ischemia-reperfusion of the brain and is responsible for areas of cell death following this injury [2-4]. A minor percentage of cells lost in the ischemic core die via apoptosis [19]. However, in tissue areas surrounding the ischemic core, apoptosis is prevalent. Apoptosis is noticeably prominent in the region just outside of the ischemic core, referred to as the periinfarct region. Additionally, there is increased expression of FasL in these regions. This could suggest that FasL instigates post-ischemic apoptosis of the periinfarct region, prolonging the tissue damage induced by ischemia. The cells in these regions have been measured as actively undergoing apoptosis shortly after the ischemic event. That these cells have not undergone complete demise raises the possibility that tissue damage may be limited post-injury by the inhibition of apoptosis [19]. Such therapy could prove valuable in the recovery of affected individuals.

Necroptosis and Ischemic Brain Injury

Necroptosis, or programmed necrosis, is a major PCD pathway that occurs across diverse cell types including neurons, leukocytes, and erythrocytes among many other cells [20-22]. Necroptosis shares identical steps with extrinsic apoptosis during its initiation phase [20,23]. In fact, the very same stimuli, cytokines of the TNF family, induce both extrinsic apoptosis and necroptosis. As with extrinsic apoptosis, the best-studied stimulus of necroptosis is TNF-α. Following receptor binding, complex I forms identically to that in extrinsic apoptosis [20,23]. As with extrinsic apoptosis, RIP1 must be deubiquitylated by CYLD for complex I to be further internalized into the cell [13]. Also identical to extrinsic apoptosis, complex II forms following this with the recruitment of FADD and inactive caspase-8 [20,23]. At this point, depending on the conditions, the balance may tip toward apoptosis or necroptosis. In the case of the balance shifting to apoptosis, caspase-8 acts on downstream caspases to induce the signaling of non-inflammatory apoptosis, as described above [7,10]. Once activated, caspase-8 also cleaves RIP1 and a related kinase, RIP3, and inactivates them [24-26]. In cases where caspase-8 activity is compromised or RIP1 activity outweighs caspase-8, the balance is shifted to necroptosis [20,23]. This begins with the phosphorylation of RIP1 which is followed by the recruitment and phosphorylation of RIP3 and mixed lineage kinase-domain like (MLKL) protein [23,27]. The inclusion of all three of these phosphokinases results in a cytosolic complex referred to as the necrosome, which is the central signaling complex of necroptosis [20,23]. This is followed by the activation of a number of downstream effectors which results in the outcome of cellular lysis by necroptosis, which is highly inflammatory [28]. A key event downstream of necrosome formation is the translocation of MLKL to the membrane [29-31]. Once this occurs, MLKL oligomerizes into a membrane pore, contributing to the increased membrane permeability that is characteristic of necroptosis [29-31]. It should not be overlooked, however, that as caspase-8 cleaves and inactivates RIP1 and RIP [24,25], it antagonizes necroptosis while activating apoptosis [20,23].

Downstream of the necrosome, cellular lysis by necroptosis may be influenced by a number of effectors. These include Ca^{2+}-activated calpains, sphingomyelinase-produced ceramide, advanced glycation end products (AGEs), and reactive oxygen species (ROS) [20,23]. By no means do all of these effectors need to be involved in every case of necroptosis. In fact, the effectors involved seem to differ from cell type to cell type. In most cases of necroptosis, however, AGEs and ROS are central to cell death [20,32,33]. Both of these are toxic byproducts of metabolism [34-36]. AGEs are formed due to the synthesis of the toxic derivative, methylglyoxal, from the fragmentation of glyceraldehyde-3-phosphate and dihydroxyacetone phosphate (DHAP) during glycolysis [34,35]. The production of ROS is predominantly due to premature release of partially reduced oxygen from the electron transport chain of oxidative phosphorylation [36]. Another major source of ROS is the NADPH oxidase (NOX) family of proteins, which are of wide tissue distribution [37]. As oxidative phosphorylation depends on the metabolism of pyruvate to NADH in the citric acid cycle [36], glycolysis is viewed as a regulator of ROS production. Similarly, NADPH utilized by NOX is produced via breakdown of glucose-6-phosphate, the product of the first step of glycolysis, in the pentose phosphate pathway [37] further underscoring a critical role for glycolysis in the production of ROS. Necroptosis results in AGE formation as RIP1 stimulates glycolysis via glycogen phosphorylase (PYGL) [20]. As stated, the stimulation of glycolysis will consequently lead to stimulation of oxidative phosphorylation and the production of ROS [36], thereby linking induction of necroptosis to these toxic molecules. RIP1, RIP3, and MLKL have been linked to ROS production in other, more direct ways, as well. RIP3 and MLKL activate the mitochondrial protein phosphoglycerate mutase 5 (PGAM5) and recruit it to the necrosome [38,39]. Following its phosphorylation, PGAM5 dephosphorylates and activates the mitochondrial fission protein Drp1 [39]. This causes mitochondrial fission and ROS production [39]. Additionally, MLKL polymerizes pores in the mitochondrial membrane which promotes the release of ROS into the cytosol [40]. RIP1 translocates to the mitochondria and can modulate ADP/ATP exchange and ROS production during necroptosis as well [41]. RIP1 has also been shown to increase the expression of peroxisome proliferator-activated receptor c coactivator-1α (PGC-1α) [42]. This is of significance as PGC-1α stimulates the production metabolic and mitochondrial genes which, consequently, lead to ROS production [42].

Necroptosis is responsible for the majority of cell death in ischemia-reperfusion injury of the brain, kidneys, and heart and has been particularly well-studied in ischemic brain injuries [5]. Inhibition of RIP1 with the pharmacologic inhibitor, necrostatin-1 (nec-1), drastically reduces infarct size in murine stroke models of cerebral ischemia-reperfusion injury [43] and reduces cell death and pathology following traumatic brain injury in mice [44]. Also, RIP1 inhibition prevents damage of brain tissue due to neonatal hypoxia-ischemia [45,46]. The protective effect of nec-1 in neonatal hypoxia-ischemia is in part due to prevention of mitochondrial dysfunction in neurons and astrocytes [47]. That

nec-1 protects cells and tissues of the brain from these necrotic injuries indicates that RIP1-dependent necroptosis is central to the pathology of ischemic injuries of the brain. Additionally, necroptosis is a highly inflammatory PCD pathway as a result of its cellular lysis endpoint [20,28]. Therefore, it is linked to the production of inflammation in a number of injuries which can lead to bystander cell death exacerbating tissue damage [48]. This may well be the case in ischemic brain injuries as the production of pro-inflammatory cytokines drives the pathology of these conditions [49]. The connection between necroptosis, inflammation, and pathology needs to be investigated further in the context of ischemic brain injuries in order to establish a solid connection between the exacerbation of these injuries and necroptosis-produced inflammation. Lending support to the potential role of necroptosis-induced inflammation in bystander damage during injuries of the central nervous system is the fact that necroptosis of microglia leads to astrocyte death in spinal cord injury [50,51].

Hyperglycemia and the Exacerbation of Ischemic Brain Injury

As described above, glucose (and its metabolism) has a central role in driving necroptosis as this is how toxic AGEs and ROS are produced during this PCD [20,23,52]. Inevitably, this leads one to question how the situation is affected in conditions of high levels of cellular glucose, as in hyperglycemia and diabetes. This has been addressed at the biochemical level recently *in vitro* as well as in an *in vivo* model of neonatal cerebral hypoxia-ischemia [52]. In three different cell types, necroptotic cell death was upregulated in hyperglycemic conditions *in vitro* in response to three different stimuli of necroptosis, including bacterial pore-forming toxins, TNF-α, and FasL. It was confirmed that the hyperglycemic upregulation of cell death was indeed due to necroptosis as inhibition of RIP1, with the pharmacologic inhibitor necrostatin-1s (nec-1s) or siRNA, prevented such exacerbation. The hyperglycemic upregulation of necroptosis depended on glycolysis as the non-metabolizable 2-deoxy-D-glucose prevented it while the addition of exogenous pyruvate restored this exacerbation. The inhibition of AGEs and ROS blunted the upregulation of necroptosis in hyperglycemic conditions which suggested that these toxic molecules are at least partially involved in this phenomenon. Unexpectedly, total protein levels of RIP1, RIP3, and MLKL increased robustly during the hyperglycemic upregulation of necroptosis. Currently, it is not known how the levels of these proteins increased but evidence points to this being a post-translational event as mRNA transcript levels were unaffected and as the ribosome inhibitor cycloheximide was used in these experiments. It is clear, however, that increased levels of these kinases could account for the increase in necroptosis during hyperglycemia [52].

While hyperglycemic conditions upregulated necroptosis, the same was not true of extrinsic apoptosis or eryptosis (a PCD unique to erythrocytes) [52]. This showed that the hyperglycemic exacerbation of extrinsic cell death was specific to necroptosis. In fact, in the case of extrinsic apoptosis, which was induced by the exact same stimuli as necroptosis in this study (TNF-α and FasL), hyperglycemia inhibited this PCD. Interestingly, while hyperglycaemic conditions inhibited caspase-dependent extrinsic apoptosis, cell death itself was not inhibited. In other words, significant levels of caspase-independent cell death remained under hyperglycemic conditions [52]. This raises the possibility that hyperglycaemia may potentiate a shift from apoptosis to necroptosis. This would be significant as it would represent a shift from non-inflammatory to inflammatory cell death. More research is necessary to fully establish whether or not hyperglycemia truly shifts apoptosis to necroptosis, however. Lending support to this argument, is the fact that total protein levels of RIP1, RIP3, and MLKL increase robustly in hyperglycemic conditions following treatment of cells with the apoptotic stimulus, TNF-α [52].

In addition to demonstrating the upregulation of necroptosis in hyperglycemic conditions at the cellular level, this work connected this phenomenon to neonatal hypoxia-ischemia in the brain [52]. Using a mouse model of neonatal brain hypoxia-ischemia, this work showed that cerebral infarcts were exacerbated significantly in hyperglycemic mice. Importantly, the exacerbation of cerebral infarcts in hyperglycemic mice was prevented completely by the administration of the RIP1 inhibitor, nec-1s [52]. This is significant as it clearly connects the hyperglycemic exacerbation of neonatal brain hypoxia-ischemia with increased RIP1-dependent necroptosis. That hyperglycemic conditions upregulate necroptosis at the cellular level certainly provides an attractive explanation for the exacerbation of this injury during hyperglycemia [52]. It is also possible that, in addition to exacerbated necroptosis, the apoptosis that occurs in this injury [2-4] shifts to necroptosis as a result of hyperglycemia. It is tempting to speculate that this may play a role as inhibition of RIP1 resulted in a significant improvement in tissue damage so much so that brain tissue from hyperglycemic mice that received nec-1s were of a much smaller infarct size than even normal mice that received hypoxia-ischemia injury [52].

Hypotheses for the Mechanism of Hyperglycemic Exacerbation of Ischemic Brain Injury via Necroptosis

Exacerbation of ischemic brain injury due to increased necroptosis and a possible shift from apoptosis to necroptosis as a result of hyperglycemia are novel concepts. The initial evidence provided in this recent work highlights a role for increased necroptosis in the exacerbation of these injuries [52], however, more work must be done on this topic. In particular, further work must be conducted to elucidate the underlying mechanisms of the hyperglycemic upregulation of necroptosis. Additionally, more investigation must be completed to determine whether or not hyperglycemia promotes a shift from apoptosis to necroptosis. However, much is known about the mechanisms of necroptosis and apoptosis already and this information can provide us with some possibilities regarding the mechanisms of these phenomena. At this point, this is speculation but can provide some logical starting points for this exciting avenue of research. Regarding the increase in protein levels of RIP1, RIP3, and MLKL during the hyperglycemic upregulation of necroptosis [52], it is possible that this may be due to increased necrosome formation. The necrosome has been shown to be of an amyloid nature, therefore, increased formation of this complex could protect these proteins from degradation thereby resulting in their increased levels [53]. Additionally, recent evidence has shown that RIP1, RIP3,

and MLKL are ubiquitylated in the necrosome and that this modification stabilizes these kinases and their interaction in the necrosome complex [54-56]. Ubiquitylation of these kinases also appears to promote cell death by necroptosis [54-56]. Stabilization of RIP1, RIP3, and MLKL by ubiquitylation may account for their increased levels during hyperglycemic priming of necroptosis. Ubiquitylation of RIP1 and RIP3 have been shown to be promoted by cellular inhibitor of apoptosis protein 1 (cIAP1) and heme-oxidized IRP2 ubiquitin ligase 1L (HOIL-1L) [54-56]. Therefore, increased activity of these enzymes in hyperglycemic conditions may account for the increase in the levels of these kinases. With regard to a potential shift from apoptosis to necroptosis in hyperglycemia, glucose metabolism is known to slow activation/cleavage of procaspase-8 due to increased levels of the endogenous inhibitor of caspase-8, cFLIP [57-59]. Similarly, high glucose leads to increased levels of the endogenous caspase-3/7 inhibitor X-linked inhibitor of apoptosis (XIAP) [60]. Inhibition of caspases by these molecules could certainly promote a shift from apoptosis to necroptosis as caspase inhibition is a known trigger of necroptosis [20,23]. Additionally, there may be a role for Bad, a pro-apoptotic protein of the Bcl family [61,62]. Normally, Bad functions to inhibit anti-apoptotic Bcl family proteins thereby promoting apoptosis. Bad serves another role separate from apoptosis, however. When phosphorylated on Ser155 by protein kinase A, it no longer promotes apoptosis but instead interacts with glucokinase to stimulate glycolysis and oxidative phosphorylation [61,62]. This may have a role in hyperglycemia potentially promoting a shift to necroptosis as high glucose has been shown to activate protein kinase A [61,62]. In this context, protein kinase A would phosphorylate Bad shutting off its role in promoting apoptosis so that it can serve a role in initiating glycolysis (and oxidative phosphorylation) of the excess glucose. As apoptosis would be effectively shut off in this situation, the cell would default to necroptosis.

Conclusions and Significance

Necroptosis and apoptosis are two major PCD pathways with roles in ischemic brain injuries. New evidence showing that hyperglycemia upregulates necroptosis and may promote a shift from apoptosis to necroptosis provides an explanation for the exacerbation of these injuries in hyperglycemia and diabetes [52]. In particular, an attractive hypothesis is that hyperglycaemia exacerbates neonatal hypoxia-ischemia injury of the brain by promoting a shift from apoptosis to wide-scale necroptosis of cells of the brain. It is logical to conclude that a shift from apoptosis to necroptosis would lead to more tissue damage as necroptosis is a highly inflammatory mode of PCD associated with the increased release of damaging agents and damage-associated molecular patterns [28,48]. Thus far, there is clearly a role for RIP1-dependent mechanisms in the hyperglycaemic exacerbation of neonatal hypoxia-ischemia brain injury [52]. This points to dependence of this exacerbation on necroptosis and raises the possibility that the increased necroptosis seen in hyperglycemia during this injury is, at least in part, due to a shift in PCD dynamics from apoptosis to necroptosis [52]. If true, this would provide an explanation at the cellular/molecular level for the exacerbation of ischemic brain injuries during hyperglycemia and diabetes [63-66]. An increased understanding of these mechanisms can also lead to new therapeutic approaches to combat such exacerbation. Moreover, the study of hyperglycemia as a specific condition in which PCD dynamics shift can uncover important molecular switches that govern the major PCD pathways. This knowledge can then be applied to other situations to better understand precise cell death responses. This is an exciting connection but more work must be done to elucidate the underlying cellular mechanisms that are involved and to truly establish hyperglycemia as a condition in which the balance of PCD shifts from non-inflammatory apoptosis to inflammatory necroptosis.

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