Chapter 7
Apoptosis-Inducing Factor Translocation to Nuclei After Transient Global Ischemia

Yang Sun, Tuo Yang, Jessica Zhang, Armando P. Signore, Guodong Cao, Jun Chen, and Feng Zhang

Abstract As a common human disorder, global ischemia causes long-term cognitive dysfunction. Selective death of hippocampal CA1 neurons underlies the cognitive impairment. After global ischemia, CA1 neuronal death occurs in a delayed manner, suggesting a type of programmed cell death. Apoptosis-inducing factor (AIF) is a mitochondrial protein with an important role in energy metabolism under physiological conditions. Following ischemia, AIF leaves mitochondria, translocates into nuclei, and induces DNA cleavage and chromatin condensation, therefore playing critical roles in inducing caspase-independent programmed cell death. In this chapter, we summarize the roles of AIF in CA1 nearonal death following global ischemia, highlighting recent progress.

Keywords AIF · Apoptosis · Stroke · Neuropretection

7.1 Introduction

In humans, global cerebral ischemia occurs following cardiac arrest and resuscitation, shock, or hypoxia, which produces neuronal cell death in the brain and reduced cognitive function if the patients recover. The loss of mitochondrial membrane integrity and the subsequent release of apoptogenic factors are critical in mediating the intrinsic, or mitochondrial, apoptotic pathway (Goto et al. 2002; Cao et al. 2003; Fujimura et al. 1999; Sugawara et al. 1999). Both caspase-dependent and -independent pro-death pathways can be initiated by the intrinsic pathway (Graham and Chen 2001; Zhang et al. 2004). The key signaling molecule to initiate the caspase-independent route is apoptosis-inducing factor (AIF), which is released by the mitochondria. AIF is a mitochondrial-specific flavoprotein that normally resides in the intermembrane space (Krantic et al. 2007). Following global ischemia, AIF is

Y. Sun · T. Yang · J. Zhang · A. P. Signore · G. Cao · J. Chen (✉) · F. Zhang (✉)
Department of Neurology, Pittsburgh Institute of Brain Disorders and Recovery,
University of Pittsburgh, Pittsburgh, PA, USA
e-mail: zhanfx2@upmc.edu; chenj2@upmc.edu

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truncated by calpain, allowing it to translocate from compromised mitochondria to the nucleus, where it degrades the nuclear genome (Cao et al. 2003, 2007).

Global ischemia can be induced in rodents using several models. The essential feature of these models is a delayed (48–72 h) but selective loss of neurons in the CA1 region of the hippocampus (Zhang and Chen 2008; Zhang et al. 2006, 2004). Neuronal death in these models occurs at least in part via an apoptotic mechanism (Jin et al. 1999; Endo et al. 2006; Xu et al. 2016). The traditional view of cell death in mammalian cells largely envisioned two distinct processes with mechanisms that shared little in common: programmed cell death (PCD) and unprogrammed cell death. PCD includes apoptosis, autophagy and paraptosis, and unprogrammed cell death mainly denotes necrosis (Krantic et al. 2007). In order to identify potential therapeutic targets and to develop successful treatments, it is crucial to understand the specific contribution of the signaling pathways activated by each of the cell death mechanisms in ischemic neuronal death.

All of the death processes mentioned above are involved in ischemic neuronal death (Xue et al. 2016; Zhao et al. 2016; Wei et al. 2015), whereas only apoptosis and necrosis are mediated by AIF (Cao et al. 2003, 2007; Xu et al. 2016). Apoptosis is a controlled, energy-dependent, and well-orchestrated degradation of cellular structures. It was first described in development, where apoptosis removes extraneous tissue to mold body and organ structures. Apoptosis is exemplified by the condensation of the nucleus and the active dismantling of cellular components. The second broadly defined process is necrosis, usually referred to as an uncontrolled or nonregulated death of cells because of sudden and accidental irreversible damage. The distinction between these modes of death has recently been blurred, and cell death is now described as a continuum of programmed cell death pathways that show characteristics from each type of cell death (Boujrad et al. 2007; Bredesen et al. 2006; Golstein and Kroemer 2007; Xu et al. 2016).

Following cerebral ischemia and reperfusion, features of both apoptosis and necrosis appear (Muller et al. 2007; Pagnussat et al. 2007; Zhang et al. 2004). The pathological process of an individual neuron depends on the type, intensity, and duration of the cell death stimuli (Pagnussat et al. 2007). The harmful release of AIF depends in large part upon the health status of the mitochondria. Therefore, the greater the mitochondrial injury, the more likely AIF will be released and actively involved in inducing cell death. This chapter now focuses on AIF activation and the mechanisms in the model of global cerebral ischemia.

7.1.1 Structures and Functions of AIF

The *AIF* gene is located at X chromosome, and AIF protein is synthesized as a ~67 kDa precursor, with N-terminal prodomain containing two mitochondrial localization sequences. After AIF precursor is imported into mitochondria, it is processed to a ~62 kDa mature protein (Susin et al. 1999). The mature form of AIF has three

structural domains: a FAD-binding domain, a NADH-binding domain, and a C-terminal domain (Mate et al. 2002). Under normal conditions, AIF is confined to the internal mitochondrial membrane, with its N-terminal region exposed to the matrix and the C-terminal to the inter-membrane space (Otera et al. 2005). Using multiple biochemical and immunogold electron microscopic analyses of mouse brain mitochondria, a recent study showed that about 30% of AIF loosely associates with the outer mitochondrial membrane (Yu et al. 2009). It is worth noting that the primary structure of AIF's C-terminal region shares about 30% identity with several bacterial NADH-oxidoreductases, suggesting its function (Miramar et al. 2001).

The discovery of AIF's normal functions was revealed by a mutation in the mouse. The AIF-deficient mouse, known as Harlequin, harbors a viral insertion in AIF, which diminished AIF expression to 20% or less in mutant Hq/Hq mice compared to wild type mice (Klein et al. 2002). Neuronal degeneration is a hallmark of the Hq/Hq mouse. Study of Hq/Hq mice determined several physiological functions of AIF, including an important NAD oxidase activity that uses nicotinamide adenine dinucleotide (NAD+) as a cofactor (Susin et al. 1999). A deficit in AIF expression causes mitochondrial complex I dysfunction and impaired oxidative phosphorylation, evidenced by increased dependence on glycolytic glucose metabolism and progressive multifocal neuropathology (El Ghouzzi et al. 2007; Vahsen et al. 2004). Loss of central neurons was due to reduced neuronal survival during brain development and increased oxidative radical activity (Cheung et al. 2006). Increased sensitivity to peroxides occurs in Hq/Hq neurons, and neurons can aberrantly re-enter the cell cycle (Klein et al. 2002). The loss of cells in Harlequin mice is specific to the brain and retina and does not appear to occur in the heart or liver, despite little AIF expression in these tissues as well (Vahsen et al. 2004). In addition, AIF plays an important role in maintaining the integrity of mitochondrial structure via preserving the complex I and III subunits, most likely duo to post-translational mechanisms (Vahsen et al. 2004).

Given that caspase-independent cell death requires AIF activation, several studies have reported neuroprotective effects of AIF inhibition by either neutralizing intracellular AIF or genetically reducing the expression of AIF (Cao et al. 2007; Culmsee et al. 2005; Yu et al. 2002; Xu et al. 2016). In transient global ischemia, total AIF expression levels per se are not significantly altered (Cao et al. 2003; Xu et al. 2016). Instead, AIF is cleaved at position G102/L103 (in mouse) by activated calpains and/or cathepsins, resulting in a mature isoform via its N-terminal truncation (Otera et al. 2005; Cao et al. 2007). Due to a nuclear localization sequence at AIF's C-terminal domain, this isoform is then translocated into the nucleus, resulting in large-scale DNA fragmentation and chromatin condensation (Dalla Via et al. 2014). The discharge of AIF from mitochondria is also dependent on the pro-apoptotic Bcl-2 family members, Bax and Bid (Cregan et al. 2002; Culmsee et al. 2005; Van Loo et al. 2002). Through direct interaction with genomic DNA along with the activity of endonuclease G (EndoG), AIF causes chromatin condensation (Cande et al. 2002).

7.2 AIF Translocation Mechanism and Therapeutic Targets

7.2.1 The Time Course of AIF Translocation

AIF-mediated cell death is an energy-dependent process. After the period of greatest energy depletion during ischemia, neuronal death occurs during the recovery of energy after reperfusion (Pagnussat et al. 2007). The time course for AIF nuclear translocation after experimental stroke varies with the severity of injury. AIF translocation into the nucleus does not occur until after 6 h of reperfusion following short (30 min) MCAO in mice, but was seen following as little as 20 min of reperfusion after longer (1 h) MCAO. When 2 h of MCAO was used instead, AIF translocation was again delayed until 6 h reperfusion (Li et al. 2007; Plesnila et al. 2004). Interestingly, AIF is a larger protein than cytochrome c, but its translocation precedes cytochrome *c* release. The small pool (30%) of AIF on the outer mitochondrial membrane may play an important part in this phenomenon. It was discovered that the outer mitochondrial membrane accounts for the rapid release of a small pool of AIF, as 20% of the uncleaved AIF rapidly translocated to the nucleus and caused death following NMDA treatment (Yu et al. 2009). However, more studies are needed to determine the different mechanisms involved in the release of the two AIF pools.

7.2.2 Mechanism of AIF Release

7.2.2.1 Poly (ADP-ribose) Polymerase-1 (PARP-1) and AIF

The release of AIF after global ischemia occurs upon a variety of stimuli, and several cascades contribute to AIF neurotoxicity, which include the inappropriate activation of DNA reparative enzymes. Our study showed that DNA single-strand breaks is a form of DNA damage induced early in neurons following cerebral ischemia (Chen et al. 1997; Stetler et al. 2010). PARP-1 is an abundant and very active chromatin-associated enzyme involved in DNA repair, as well as histone and other nuclear protein modifications. The enzyme relies on consumption of NAD+ to form ADP-ribose polymers (for reviews see Ame et al. 2004; Ha 2004; Ame et al. 2004; Ha 2004). Enzyme activity of PARP-1 is in fact activated by DNA strand breaks, and thus PARP-1 functions as a sensor of DNA damage (Demurcia and Demurcia 1994). Reperfusion following cerebral ischemia induces the generation of oxidative stimuli such as reactive oxygen species, which can lead to DNA damage, with activation of PARP-1 (Demurcia and Demurcia 1994; Eliasson et al. 1997). PARP-1 is a crucial part of both apoptosis and necrosis because its pathological over-activation can lead to cell death, generally by depleting cellular NAD+ and ATP (Szabo and Dawson 1998; Ha and Snyder 1999; Herceg and Wang 1999; Shall and de Murcia 2000; Yu et al. 2002).

Therefore, inhibition of PARP-1 over-activation should ameliorate cell death under these toxic conditions. Indeed, PARP-1 knockout animals demonstrate resistance to stroke (Eliasson et al. 1997; Endres et al. 1997; Goto et al. 2002). It is possible to prevent the death of even highly sensitive hippocampal CA1 neurons after transient ischemia by administering the PARP inhibitor PJ34 as late as 8 h after ischemia (Hamby et al. 2007). 3-AB, a PARP-1 inhibitor, protects neurons against necrosis, which is dependent on the duration of the ischemic-reperfusion episode (Strosznajder and Walski 2004). Our previous work showed that when adequate cellular NAD+ levels were maintained (Nagayama et al. 2000) or supplied (Wang et al. 2008), inhibition of PARP-1 diminished neuronal survival in the transient global ischemia model or neuronal cultures (Nagayama et al. 2000; Wang et al. 2008). Since caspase-3-mediated cleavage of PARP-1 blocks DNA repair and concomitantly prevents a depletion of cellular NAD+ pool by PARP activity (Boulares et al. 1999; Herceg and Wang 1999), the exact role that PARP-1 plays in cell death remains contentious, and may depend on which pathway is preferentially activated in individual neurons.

Evidence indicates that the release of AIF from mitochondria is also dependent on PARP-1-initiated nuclear signals (Cipriani et al. 2005; Yu et al. 2002). Reperfusion accelerates the appearance of nuclear AIF after 1 h of transient focal brain ischemia compared with permanent ischemia, consistent with the possibility that early oxidant stress triggers the signaling pathways that stimulate AIF translocation (Li et al. 2007). In concert with it, inhibition of PARP-1 reduces nuclear AIF translocation (Culmsee et al. 2005). The PAR polymer, generated when PARP-1 is over-activated, is now known as a key signaling molecule in the PARP1 mediated cell death (Andrabi et al. 2006; Komjati et al. 2005). They reach a toxic level when PARP-1 becomes over-activated and translocates to the cytosol, inducing AIF nuclear translocation (Yu et al. 2006). AIF is a PAR polymer-binding protein and a physical interaction between PAR and AIF is required in inducing the release of AIF from the mitochondria (Gagne et al. 2008; Wang et al. 2011).

PARP-1 might also contribute to the gender differences found in cerebral ischemia that are not directly attributable to the neuroprotective effects of the female hormones. In the immature male brain, neurons display greater caspase-independent translocation of AIF after hypoxic ischemia, whereas female-derived neurons exhibit stronger activation of caspase-3 (Zhu et al. 2006). In addition, while male PARP-1 knockout mice were protected from ischemia, female brain showed exacerbated histological injury after MCAO (McCullough et al. 2005).

The importance of PARP-1 is highlighted by recent reports, which showed that the activation of PARP-1 is necessary for calpain-mediated AIF cleavage (Cao et al. 2007; Vosler et al. 2009). In addition to PARP-1, a recent study showed that PARP-2 also contributed to nuclear translocation of AIF after transient focal cerebral ischemia in male mice (Li et al. 2010). The activation of AIF by PARP signaling may occur with a significant number of variables including length of insult, moment-to-moment levels of cell energy levels, cell-specific expression, and gender. The final activation due to cellular damage can thus be highly variable and may spare some neurons for reasons not readily apparent.

7.2.2.2 Direct Activation of AIF: Truncation by Calpain

A number of cysteine proteases, including the caspases, cathepsins, and calpains, are activated in neurons after ischemic injury (Graham and Chen 2001; Windelborn and Lipton 2008; Vosler et al. 2009). In AIF-mediated neuronal death, two families of cysteine proteases play a key role: calpains and cathepsins. Calpain I, also known as u-calpain, requires micromolar order of calcium to be activated, while calpain II or m-calpain requires millimolar calcium, as measured *in vitro*. Nevertheless, calcium is not involved in the cathepsin-mediated control of AIF activation. The cellular distribution of mRNA for calpain I and calpastatin, the endogenous calpain inhibitor, are relatively uniform throughout the mouse brain. In contrast, calpain II gene expression is selectively higher in specific neuronal populations, including pyramidal neurons of the hippocampus (Li et al. 1996), which are the most sensitive population of neurons to global ischemia.

While calpains are normal complements of cellular enzymatic activities, their inappropriate-activation or over-activation will lead to pathophysiological activity and contribute to cell death following cerebral ischemia. A major target of calpains is AIF. Calpain I cleaves AIF in a caspase-independent cell death manner in liver mitochondria (Polster et al. 2005; Cao et al. 2007) and the PC12 neuronal cell line (Liou et al. 2005). Our previous work shows that calpain I induces AIF release in the nervous system after both oxygen-glucose deprivation (OGD) *in vitro* and transient global ischemia *in vivo* (Fig. 7.1) (Cao et al. 2007). In fact, N-terminal truncation by calpain I was found to be required for AIF activation. When neurons express a mutant form of AIF that could not be cleaved by calpain, AIF was not released from mitochondria and was not found in the nucleus after OGD or global ischemia (Cao et al. 2007). This important finding shows that calpain I is a direct activator of AIF release, and is specifically involved in caspase-independent cell death.

Calpain activity was previously thought to be limited to the cytosol; however, there is ample evidence showing that a mitochondrial calpain exists and that it cleaves AIF, thus allowing AIF to leave the mitochondria (Garcia et al. 2005). A new form of calpains has been discovered and named calpain-10 (Arrington et al. 2006). Calpain-10 is located at the mitochondrial outer membrane, intermembrane space, inner membrane, and matrix region, and is an important mediator of mitochondrial dysfunction via the cleavage of Complex I subunits and activation of the mitochondrial-permeable transition pore (Arrington et al. 2006). The mitochondrial calpain most closely resembles m-calpain, as anti-m-calpain antibodies can also stain the mitochondrial calpain, and the calcium dependency of m-calpain and mitochondria calpain are similar (Ozaki et al. 2007). Thus, the discovery of calpain activity in the mitochondria itself shows that there is a direct link between mitochondrial dysfunction, elevated calcium levels, and protease activity.

Calpains also cleave a number of other substrates crucial in the cell death process, illustrating how intertwined the different cell death pathways are in neurons. For example, calpain I can cleave Bid (Chae et al. 2007); and calpain II can trigger the ischemia-induced lysosomal release of cathepsins in brain (Windelborn and

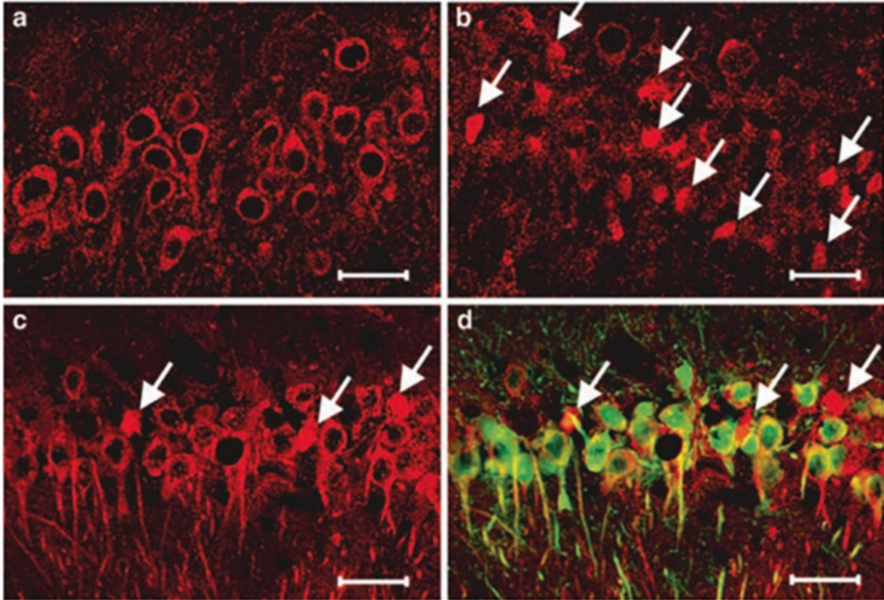

Fig. 7.1 AIF translocation in vivo following global ischemia is prevented by overexpression of calpastatin. Representative immunofluorescence of AIF (red) from non-ischemic CA1 (**a**) or 72 h after global ischemia (**b**–**d**). AAV–Cps (**c**, **d**) or the empty vector (**b**) was infused 14 days before ischemia, and brain sections were double-label immunostained for AIF (red) and hemagglutinin (HA) (green, **d**). Note that majority of CA1 neurons lost normal localization of AIF after ischemia (**b**, arrows), but AIF translocation was rare in Cps-overexpressed CA1 (**c**, **d**, arrows). Scale bars, 50 μm. From Cao et al. (2007)

Lipton 2008), which brings about the truncation of procaspase-3 into its active form (Blomgren et al. 2001; Mcginnis et al. 1999). This latter pathway is particularly interesting, as caspase-3 in turn reduces the activity of calpastatin, the endogenous calpain inhibitor, to form a positive feedback loop, which can result in the further activation of calpains and subsequent release of AIF (Kato et al. 2000; Porn-Ares et al. 1998; Wang et al. 1998).

7.2.2.3 Release of AIF from Mitochondria: Formation of the Mitochondrial Outer Membrane Pore

In addition to AIF and EndoG, mitochondria can release a variety of death-promoting molecules, including cytochrome c, Smac/Diablo, and Omi/HtrA2. The ability of mitochondria to release these molecules depends largely on the formation of large and nonselective pores or channels through the two layers of membrane systems in mitochondria. The inner membrane forms the mitochondrial-permeable transition

pore, which is a calcium-dependent process and uses the proteins cyclophilin (Cyp) D, voltage-dependent anion channel and adenylate nucleotide translocase. The Bcl-2 family proteins, including Bid, Bax, and Bak and very likely others as yet unidentified proteins, form the outer membrane permeabilization pore, termed the mitochondrial apoptosis channel (for complete review, see Belizario et al. 2007). Many of these proteins need to undergo proteolytic cleavage before they can form any type of channel or pore, and the cleavage is usually induced by many stressors, including ischemia. For example, Bid can be truncated by caspase-8 and translocates to the outer mitochondrial membrane in ischemic brain (Gross et al. 1999). The understanding of the mechanisms behind the formation of the mitochondrial death channels is still incomplete and under debate. In general, Bid is activated and leads to BAK and BAX oligomerization, mitochondrial outer membrane permeabilization, and AIF release following stressors (for complete review, see Chipuk et al. 2010). The prevention of unwanted mitochondrial channels during normal physiological conditions is critical for maintaining cellular function and health. Regulation occurs via several anti-apoptogenic members of the Bcl-2 protein family, including Bcl-2 and Bcl-xL (Breckenridge and Xue 2004). In fact, AIF translocation and cleavage is inhibited by Bcl-2 and Bcl-xL overexpression (Cao et al. 2003; Otera et al. 2005).

7.2.3 Regulation of AIF Activity in the Cytoplasm

Once released from mitochondria to the cytosol, AIF then translocates into the nucleus. This process is regulated positively by Cyp A and negatively by heat shop protein 70 (Hsp70).

7.2.3.1 Cyp A Induces AIF Nucleus Translocation

Cyclophilins were first identified as the intracellular receptors for the immunosuppressant drug cyclosporin A (Handschumacher et al. 1984). Previous studies have shown that cyclophilins are involved in degradation of the genome during apoptosis (Montague et al. 1997; Cande et al. 2004; Zhu et al. 2007). For instance, CypA can form a complex with AIF to act as a co-factor for AIF nuclear translocation and AIF-dependent chromatinolysis following cerebral ischemia (Cande et al. 2004; Zhu et al. 2007). Elimination of Cyp A confers neuroprotection in vivo, suggesting that the lethal translocation of AIF to the nucleus requires interaction with Cyp A (Zhu et al. 2007).

7.2.3.2 Hsp70 Inhibits AIF Nucleus Translocation

Previous studies have shown that Hsp70 over-expression protects cells from death induced by various insults that cause either necrosis or apoptosis, including hypoxia and ischemia/reperfusion, by inhibiting multiple cell death pathways (Giffard and Yenari 2004). One of the mechanisms by which Hsp70 may be neuroprotective is the sequestration or neutralization of AIF by Hsp70, as evidenced by Hsp70 overexpression (Gurbuxani et al. 2003; Ravagnan et al. 2001; Wang et al. 2015). This process is associated with increased cytosolic retention of AIF when bound to Hsp70, limiting the entry of activated or cytotoxic AIF into the nucleus (Gurbuxani et al. 2003; Kroemer 2001).

7.2.3.3 Ubiquitination of AIF Via XIAP

X-linked inhibitor of apoptosis (XIAP) is an inhibitor of caspases and apoptosis (Suzuki et al. 2001). XIAP is also involved in the signal transduction and regulation of ubiquitin-ligase activity in the cellular system (Reffey et al. 2001; Yamaguchi et al. 1999; Yang et al. 2000). Previous work shows that XIAP may participate in the ubiquitinization of AIF (Wilkinson et al. 2008), which leads to AIF proteosomal degradation (Wilkinson et al. 2008). Further study shows that lysine 255 of AIF is critical to bind DNA and degrade chromatin, and the target of XIAP is this lysine residue (Lewis et al. 2011).

7.2.4 AIF-Induced DNA Fragmentation

Although AIF is involved in the breakdown of neuronal DNA, AIF itself is devoid of any nuclease activity (Susin et al. 2000, 1999). AIF translocates to the nucleus where it directly interacts with DNA by virtue of positive charges, which are clustered on the surface of AIF. DNA binding is therefore required for the death-promoting action of AIF (Ye et al. 2002). The binding of AIF to DNA induces chromatin condensation by interacting directly with DNA and possibly displacing chromatin-associated proteins. AIF could then disrupt normal chromatin structure, leading to the appearance of nuclear condensation. The remodeling of chromatin upon AIF modulation may increase the susceptibility of DNA to nucleases (Ye et al. 2002). The binding site within AIF is the same for distinct nucleic acid species, with no clear sequence specificity (Vahsen et al. 2006).

EndoG is another death-promoting factor released from mitochondria along with AIF (Susin et al. 1999). In the cerebral cortex, 4 h after ischemia, endoG level is significantly increased in the nucleus, correspondingly with decreased mitochondrial endoG content. EndoG may also interact with AIF in the nucleus after cerebral ischemia (Lee et al. 2005). In these ways, AIF truncation and release lead to the disruption of neuronal DNA via both direct (DNA binding) and indirect (EndoG) mechanisms.

7.2.4.1 Cyclophilins

Cyp A has been stated as a co-factor for AIF nuclear translocation and AIF-dependent chromatinolysis. Cyp D, on the other hand, is thought to be one of the components that forms the inner permeability transition pore and is thus involved in the release of death-promoting factors from the mitochondria (see above). A clearer understanding of how Cyp D participates in the formation of the mitochondrial permeability transition pore will provide important answers to the role these proteins play in caspase independent cell death.

7.2.4.2 Histone H2AX

Histone H2AX is another key factor in AIF-mediated apoptosis. As a member of the histone H2A family, H2AX participates in forming the histone nucleosome core. Previous studies have shown that the function of H2AX is primarily associated with DNA damage repair. On exposure of cells to inducers of double-strand breaks DNA damage, H2AX is phosphorylated at Ser139 in the nucleosomes surrounding the break point (Thiriet and Hayes 2005). Phosphorylated H2AX (γH2AX) renders damaged DNA sites accessible to repairing factors (Pilch et al. 2003). On the other hand, H2AX is crucial for AIF- mediated neuronal death. After DNA alkylating agent treatment, H2AX Ser139 phosphorylation is required for AIF mediated cell death (Artus et al. 2010; Baritaud et al. 2012).

7.3 Conclusion

Current knowledge about caspase-independent, AIF-induced neuronal death is incomplete. Though a large part of programmed cell death pathways involve the activation of caspases, inhibition of caspases alone as a therapeutic strategy is not sufficient to rescue damaged neurons. The caspase-independent pathway mediated by AIF provokes a compound network of signaling cascades that in and of themselves, can account for some of the specific cell death seen in the hippocampus following global ischemia. In vivo, the caspase-dependent and caspase-independent death pathways are, however, highly interconnected and often not easily distinguished from each other. The over-activation of calpains, the specific activator of AIF, maybe a promising target for drug intervention using calpain-specific inhibitors.

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