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NARRATIVE REVIEW

Extrinsic and intrinsic pathways of apoptosis and related molecules in ischemic stroke

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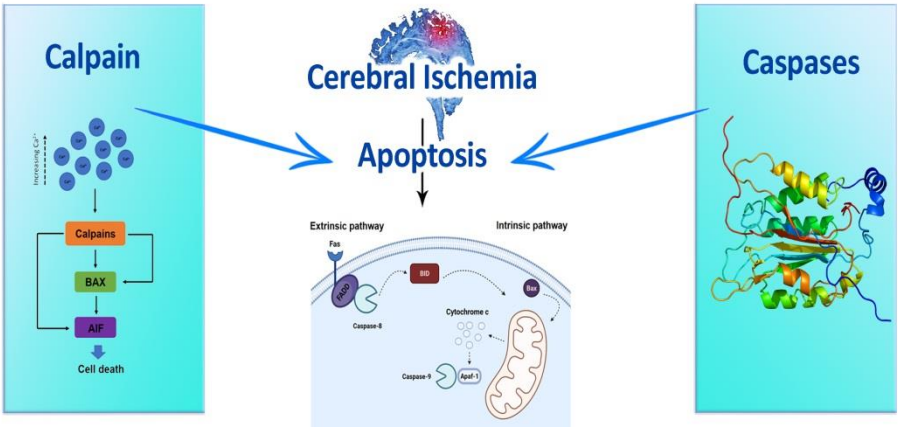
Highlights

- In cerebral ischemia, there is no enough blood flow for the metabolism of brain tissue.
- Apoptosis has a major role in cell death following cerebral ischemia.
- Apoptosis occurs through two general pathways of intrinsic and extrinsic following cerebral ischemia.
- Calpain, caspases, and JNK are among the most important molecules involved in cerebral ischemic-induced apoptosis.

Graphical Abstract



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Abstract

Cerebral ischemia is a disease in which there is no enough blood flow to provide essential nutrients for brain activity and metabolism. Following the stroke, the occurrence of two types of disorders is probable, including ischemia (85%) and haemorrhage (15%). In cerebral ischemia conditions, various mechanisms cause neural death, including increased extracellular glutamate amino acid concentration, inflammation, oxidative stress, apoptosis, and necrosis-induced cell death. Each of these mechanisms can potentially spread into the adjacent cells and tissues through distinct molecular cascades. Although several mechanisms are involved in cerebral ischemia pathogenesis, apoptosis plays a major role in cell death following cerebral ischemia. After the onset of focal cerebral ischemia, the core region in the ischemic brain with severe blood flow is lethally damaged, leading to cell death. Following cerebral ischemia, apoptosis occurs through two general pathways of intrinsic and extrinsic. The role of mitochondria and the released cytochrome C leading to stimulation of caspase-3 is prominent in the intrinsic pathway. But, the extrinsic pathway initiates with the activity of death receptors located on the cell surfaces of neurons, causing stimulation of caspase-8. Calpain, caspases, and c-Jun N-terminal kinases are considered key molecules involved in the cell apoptosis molecular pathway. This study aimed to describe the apoptosis process in cerebral ischemia by the use of key apoptotic molecules.

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Introduction

Cerebral ischemia is a disease in which there is not enough blood flow to the brain to respond to metabolism (1). Stroke includes two types of ischemic stroke with a frequency of approximately 85% and hemorrhagic stroke with a frequency of approximately 15% (2). Stroke is the second most common cause of death and the leading cause of long-term disability (3). Stroke is a common and risky event that leads to death in about 25% of cases (4). The treatments that have been proposed for ischemic stroke so far have not been effective (5), so patients with this type of stroke face treatment problems.

The core of brain tissue that is exposed to decreased blood flow undergoes necrotic cell death. While in the infarct area or penumbra, apoptotic cell death occurs, where the lateral blood flow provides some degree of hypoxia (6). The penumbra area accounts for half of the total volume of injury during the early stages of ischemia, which is affected by apoptosis and efforts, are being made to prevent the spread of injury in this area (7, 8). Due to stroke, various mechanisms cause neuronal death in central nervous system ischemia, including excitotoxicity, increased extracellular glutamate amino acid concentration, oxidative stress, apoptosis, and necrosis-induced cell death. Each of these mechanisms appears to extend through distinct molecular cascades (9). The phenomenon of apoptosis or programmed cell death plays a major role in neuronal death after stroke in and around ischemic areas (10-12).

Following an ischemic stroke, apoptosis begins in two general ways. One is the intrinsic pathway that occurs primarily in the mitochondria, releases cytochrome C, and is associated with caspase-3 stimulation. Another is the extrinsic pathway that begins with the activity of death receptors on the surface of neurons and leads to the stimulation of caspase-8 (7). The caspase family of proteases is important in inducing apoptosis in neurons after stroke. Recent studies have shown that in addition to caspase-3, another protease is involved in apoptosis. Studies show that by removing caspase-3, calpains play a prominent role in the process of apoptosis (13). Calpains are active proteolytic enzymes of calcium that are located in the cell cytoplasm. Calpain in the cytoplasm is an inactive proenzyme that is activated in the presence of high levels of calcium (14). The studies also show that this cysteine protease is involved in neuronal cell death (15).

Other evidence to elucidate the role of calpains suggests that the apoptosis-inducing factor (AIF) is known as one of the calpain substrates (16). This evidence suggests an interaction between calpain and mitochondria, in which calpain activity regulates the release of proteins from the inner membrane of mitochondria to the cytoplasm. These proteases play a unique role in the regulation of caspase-independent apoptosis (17). On the other hand, studies have shown that calpain is activated by DNA damage and activates P53. Calpain inhibitors reduce the activity of P53, indicating that calpain regulates apoptosis due to DNA damage (18, 19). Another mechanism that leads to cell death by activated calpain is the cleavage of several proteins in the axonal cytoskeleton of neurons (20). This cysteine protease is secreted by leukocytes, endothelial cells, and parathyroid cells and plays a vital role in inflammation/immunity. Few studies have shown that calpain is present partially outside the cell, but the secretion and function of external calpain are less known (21). However, in addition to calpains, some other proteins such as caspases and c-Jun N-terminal kinases play key roles in the apoptosis process. These molecules are also involved in the pathophysiology of stroke.

This study aimed to describe the intrinsic and extrinsic apoptotic pathways involved in stroke and the corresponding molecules in each of these pathways.

Stroke disorder

Stroke is the second leading cause of death after a heart attack, accounting for 9% of worldwide deaths (22). Stroke includes two main types of ischemia and haemorrhage (23). Ischemic stroke has a complex nature, multiple causes and variable clinical manifestations. The cause of cerebral ischemia is approximately 45% of small or large vascular thrombosis cases, 20% of embolic and other cases unknown. When cerebral ischemia occurs, blood flow to the brain is disrupted, and brain cells are deprived of the glucose and oxygen they need to function (24). Neurons are vulnerable to glial and vascular cells and, when exposed to ischemic hypoxia,

rapidly lose their function and die (25). Cerebral ischemia is divided into three categories: focal and global, and multifocal ischemia. Global ischemia occurs when cerebral blood flow (CBF) is reduced in most or all parts of the brain. Following complete global ischemia, blood flow is completely cut off, while with incomplete global ischemia, blood flow is severely reduced to the point where it is not sufficient to maintain metabolism and brain structure. In global cerebral ischemia, there is not enough CBF in any brain area, which initially leads to neuronal damage to the brain, and if it continues, all neurons will be destroyed. In multifocal ischemia, which is limited to a specific brain area, there is an irregular pattern of reduced CBF in most or all parts of the brain (26).

Molecular mechanisms of stroke

In cerebral ischemia, various mechanisms cause neuronal death, including increased extracellular glutamate amino acid concentration, inflammation, oxidative stress, apoptosis, and necrosis-induced cell death. Each of these mechanisms appears to extend through distinct molecular cascades (27). In cerebral ischemia, hypoxia occurs due to the temporary cessation of blood flow, and hypoxia has devastating effects on brain tissue. For example, in these areas, there is a wave of depolarization that leads to the release of neurotransmitters, weak ion pumps, leukocytes activate and start to the secretion of inflammatory mediators and free radicals, and finally, lead to inflammation of neurons, neuronal damage, and apoptosis (28).

Apoptosis and its role in stroke

The word apoptosis was first introduced by researchers in 1972. Apoptosis, the Greek word for leaf fall from a tree, signified a new type of cell death in the liver and, because of its characteristics, distinguished it from other types of cell death (29). Apoptosis is a systematic process of cell death dependent on energy that destroys excess cells (6). Apoptosis is involved in the pathogenesis of many disorders such as cancer, AIDS and other immune disorders, cardiovascular disease and many neurodegenerative diseases, including Alzheimer's, Parkinson's and stroke (29, 30). Although several mechanisms are involved in the pathogenesis of cerebral ischemia, apoptosis plays a major role in cell death after cerebral ischemia (31, 32). Some minutes after the onset of focal cerebral ischemia, brain tissue in the core region under severe blood flow is fatally damaged, leading to cell death. An area surrounds the core with less severely affected tissue (penumbra), which is functionally marginal due to reduced blood flow but is metabolically active. It is known as ischemia penumbra and represents an area for which there is a chance of salvation through post-stroke treatment. Therefore, preventing and targeting apoptosis in the penumbra is essential as a rational therapeutic target to limit the volume of cerebral infarction after stroke. Following cerebral ischemia, apoptosis begins through two general pathways. One is the internal pathway that occurs mainly in the mitochondria, releases cytochrome C, and is involved in stimulating caspase-3. The other is the external pathway that begins with the activity of death receptors on the surface of neurons and leads to the stimulation of caspase-8.

The intrinsic pathway of apoptosis

The onset of cerebral ischemia limits the delivery of substrates, primarily oxygen and glucose, and disrupts the energy required to maintain a neuronal ion gradient. Rapid depletion of energy after cerebral ischemia leads to loss of membrane potential and neuronal depolarization. In the intrinsic pathway, increasing the level of extracellular concentration of the glutamate amino acid leads to excessive activity of the NMDA glutamate receptor subset, which leads to the accumulation of intracellular Ca^{2+}. Thus, Ca^{2+} dependent enzymes are activated, including calpain protease, caspases, nitric oxide-producing enzymes, free radicals, and arachidonic acid metabolites (33). By increasing Ca^{2+} through the internal pathway of apoptosis and stimulating caspase-8 through the external pathway of apoptosis, the activity of calpain leads to the breakdown of BID into its active form, tBID. BID is a cytosolic member of the Bcl-2 family of pro-apoptotic proteins, which is transmitted to the mitochondria when it receives a lethal signal (34). BID is an essential mediator of ischemia-induced cell death within neurons (35). The tBID targets the outer membrane of the mitochondria and induces structural changes

in other pre-apoptotic proteins such as Bax, BAD, Bak, and caspases. These pro-apoptotic proteins can react with tBID and anti-apoptotic proteins. The mechanism that induces the release of apoptotic factors from the periplasmic space by pre-apoptotic proteins has not been established; however, it is believed that this mechanism occurs through the opening of mitochondrial transport pores. After the mitochondrial transporter pores open, two groups of pre-apoptotic protein are released from the periplasmic space into the cytosol. The first group contains cytochrome C and serine protease, and the second group contains apoptosis-inducing factors (AIF) and endonuclease G (36). When the first group of proteins is released, they activate the caspase-dependent mitochondrial pathway. After release from the inner mitochondrial membrane, cytochrome C forms the apoptosome complex with the cytosolic protein Apaf-1 and the precursor caspase-9, followed by activating caspase-9, a primer of the caspase cascade dependent on cytochrome C, and then caspase-3. Caspase 3 breaks down many substrate proteins, leading to DNA damage and cell death through apoptosis (37).

Calcium influx in the apoptosis

Alteration of Ca^{2+} concentration stimulates and regulates many important cellular functions, including muscle contraction and secretion of neurotransmitters. Ca^{2+} also plays an important role in regulating mitochondrial physiology and cell death. Regulation of stable endoplasmic calcium levels seems an important regulator of Ca^{2+} dependent apoptotic cell death (38). The concentration of free Ca^{2+} in the cytoplasm of resting neurons is deficient (100 nM), while its extracellular concentration is estimated to be 1-2 mM. Neural levels of Ca^{2+} are maintained by: 1) Extracellular Ca^{2+} entry through ligand-dependent receptors or voltage-dependent Ca^{2+} channels. 2) Release of Ca^{2+} from the endoplasmic reticulum by stimulation of InsP3R receptors or the mitochondria via Ca^{2+}-Na^{+} exchange. 3) Extraction of Ca^{2+} via Ca^{2+}-ATPase or Ca^{2+}-Na^{+} exchange in the plasma membrane. 4) Binding of Ca^{2+} to target proteins. 5) Transfer of Ca^{2+} to the endoplasmic reticulum via Ca^{2+}_ATPase or through electrophoretic mechanisms. Thus, energy loss in hypoxic ischemia leads to neuronal accumulation of free Ca^{2+} by increasing the entry and release of Ca^{2+}. The Ca^{2+} entry via NMDA receptors affects a large proportion of Ca^{2+} uptake after hypoxic ischemia. The continued increase in intracellular Ca^{2+} leads to the catabolic process of vital molecules and neuronal cell death through various mechanisms involving the activity of Ca Ca^{2+}- dependent influencing proteins (9). Calcium accumulation activates catabolic processes mediated by proteases, lipases, nucleases, and other Ca^{2+}- dependent enzymes, leading to NO, arachidonic acid metabolites, and superoxides, which ultimately lead to cell death (39).

The extrinsicpathway of apoptosis induced by cell death receptors

Death receptors on the cell's plasma membrane are involved in the extrinsic mechanism of apoptosis, also known as the "death receptor pathway". Death receptors on the cell membrane belong to the TNFR receptor superfamily and include TNFR-1, FAS, and p75NTR.Forkhead1 is a member of the Fork head family and is a transcription factor that stimulates the expression of target genes such as FasL used in the extracellular receptor pathway. FasL initiates apoptosis by binding to the Fas receptor, leading to the invocation of the FADD adapter protein. FADD has an effective death region in its N-terminal region that interacts with a similar region in the caspase-8 precursor. This FasL-Fas-FADD-procaspase-8 complex is referred to as the death-inducing signalling complex. This signal complex catalyzes the proteolytic breakdown and activation of caspase-8 precursor to produce caspase-8, thereby converting procaspase-8 to activated caspase-8. When caspase-8 is activated, it is released from the complex into the cytosol and initiates the downstream breakdown of caspase-3 directly or through a mitochondrial-dependent mechanism (40). The differences of extrinsic and intrinsic apoptosis pathways are shown in Figure 1.

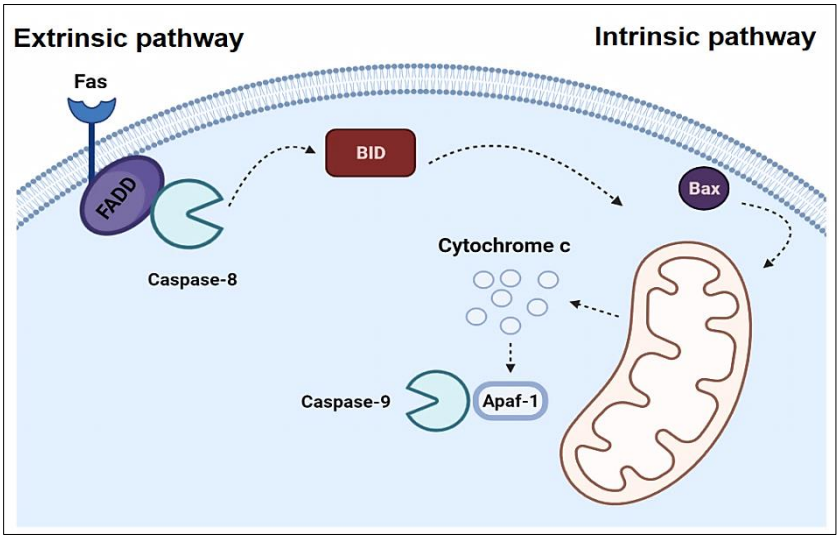

Figure 1. Difference between intrinsic and extrinsic apoptosis pathways. The surface receptors of the cell membrane are involved in the extrinsic pathway while the mitochondria are involved in the intrinsic pathway.

Calpain apoptotic protease

After global cerebral ischemia, neuronal death often occurs hours or days late. This interval indicates the best time for treatment. Research shows that disruption of Ca^{2+} neuronal homeostasis plays an essential role in this process. The increase in cytosolic Ca^{2+} during ischemia is often reversible and occurs in neurons that have been destroyed and those that are alive (41). However, a secondary increase in intracellular Ca^{2+} in neurons causes them to be destroyed. Research also suggests that this secondary increase in Ca^{2+} accelerates neuronal death. Elevation of cytosolic Ca^{2+} causes the activity of calpain, a family of rare nonlysosomal cysteine proteases (41). Calpain is a calcium-dependent neutral cysteine protease with two iso-enzyme forms: m-calpain and calpain-μ. Calpain is present as a proenzyme in an inactive form in the cytosol, in the normal range of 50-100 nM concentration of Ca^{2+} in resting cells. An increase in free cytoplasmic Ca^{2+} leads to calpain activity, which depends on the amount of calcium and its activity site. The calpain-μ needs millimolar levels of Ca^{2+} to activate it. Thus, calpain is a major protease of apoptosis involved in neurodegenerative diseases. Calpains are activated in apoptotic and necrotic conditions, while caspase-3 is activated only in apoptotic neurons (42). Physiological roles of clumps in the brain including regulating neurite outcomes and long-term amplification and synaptic regeneration. After focal and global ischemia, brain calpain activity increases and calpain inhibitors provide varying levels of neuroprotective activity in animal models. However, the mechanisms involved by calpain in neuronal death after ischemia have been estimated. Calpains often break down a large number of the cytoskeleton and regulatory proteins, leading to alterations and loss of function. Thus, calpain activity contributes to the apoptotic death of neurons (41). The effect of increasing calcium on calpain function is illustrated in Figure 2.

Caspases enzymes

Caspases are proteases that are synthesized as inactive zymogenes and undergo proteolytic breakdowns during apoptosis. These active proteases are responsible for the coordinated, non-inflammatory, and efficient destruction of cells. Efficient removal of apoptotic cells plays an important role in the formation of three-dimensional structures, homeostasis, and abnormal, harmful, and dysfunctional cells. Inefficient removal of apoptotic cells has been associated with many autoimmune and chronic inflammatory diseases. As a result of these fractures, small and large subunits are produced from them, and finally, the active form of the enzyme is obtained. Family members of caspases have been categorized in different ways, for example, Marie Mancini and colleagues during their research in 1998 (43) have divided them into three groups based on the specificity of the substrate but usually divide them into two general groups; 1) initiator/signalling caspases: whose primary

function is to activate members of the second group. This group includes caspases 2, 8, 9, and 10. 2) Executor/effective caspases are responsible for the proteolytic breakdown of specific target proteins in the cell and are caspases 3, 6, 7. Caspase enzymes were first identified as the cause of programmed cell death or apoptosis in a study of Caenorhabditis elegans worms. This study found that a death agent called CED had to be expressed during the embryonic development of these worms for apoptosis to occur (44). It has also been suggested that the structure of CED is very similar to that of humans in ICE, now called caspase-1.

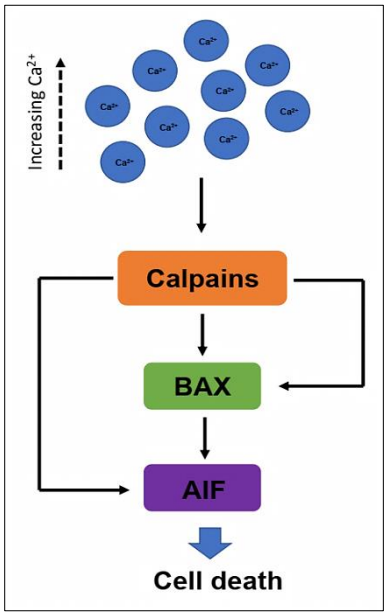

Figure 2. Cell death mechanisms. The calcium-calpain pathway has a main role in the apoptosis procedure. Increasing calcium incytoplasm trigger calpainproteases which, by acting on AIF, result in the cell death via activation of BAX.

This study led to the recognition of other members of this family of proteases called caspases and their role in causing inflammation and apoptosis. So far, 14 members of this family have been identified (45). Caspases are very similar in terms of amino acid sequence and structure. They are all single-chain protostomes with three regions of the N segment, a large subunit and a small subunit. Caspases are divided into two groups based on their function in apoptosis. It is believed that the first group, called an activator, upstream or apical (caspases 2, 8, 9, and 10) is responsible for the onset of apoptotic activator cascades, while the second group, called final, downstream, or practical (caspases 3, 6, and 7) is responsible for the final destruction of the cell (46).

This destruction includes the destruction of cellular structures, disruption of cell metabolism, inactivation of proteins that inhibit cell death, and other destructive enzymes (47). According to a study by Zheng et al., in 2003 (48), it can be concluded that in most forms of apoptosis in mammals, the expression and activity of caspases occur. The exact mechanism of action of caspases is not yet known. However, most stimuli that lead to apoptosis appear to activate caspases in three ways (48); 1) death receptor pathway, 2) cytotoxic T lymphocyte pathway (CTL)/natural killer (CTL/NK-derived gr-anzyme B), and 3) Mitochondrial stress pathway/apoptosis.

We will study this enzyme further, considering the role of caspase-3 as the main destroying enzyme in all three possible pathways. Other names for Caspase 3 are CPP32 / YAMA / APOPAIN. This enzyme is responsible for breaking down many essential proteins such as PARP. Using the DNA sequencing technique, it was found that the human caspase-3 sequence encodes a 32 kDa cysteine protease called CPP32 (49). Two other research groups independently identified it as YAMA (Hindi word meaning "god of death") and Apopain (50). Caspase 3 is highly expressed in cells of lymphocytic origin. As mentioned, the enzyme caspase 3 breaks down polyribosome polymerases or PARP. A substance similar to PARP was made as a substrate based on the fracture site of this polymerase called Asp-Glu-Val-Asp (DEVD). The name of this substrate is p-nitroanilide,

and the enzyme caspase breaks it down like PARP. This phenomenon is the basis of testing the activity of caspase-3. Another substance is called Ac-DEVD. CHO was developed as a specific inhibitor of PARP, which could be used to purify caspase-3 protease or as a caspase-3 inhibitor for use in the negative control of caspase-3 assay. The active enzyme caspase-3 has two subunits of 17 and 12 kDa (51). During the destruction phase in apoptosis, caspase-3 is responsible for the proteolysis of all or most substrates that have an Asp-Xaa-Xaa-Asp motif, and their breaking mechanism is similar to that described for PARP (52).

c-Jun N-terminal Kinase

The c-Jun N-terminal kinases (JNKs) are a subset of MAP kinases. Kinases are a family of signal proteins and are mainly activated by cytokines such as TNF-α and IL-1, and JNK is activated by exposure to environmental stresses such as radiation and oxidative stress. Also known as stress-activated protein kinase (SAPK), it is activated by several environmental stresses. There are three genes for JNK: JNK1, JNK2, JNK3 (53-55). It also contains 10 isoforms derived from three genes JNK1: four isoforms, JNK2 has four isoforms, and JNK3 has two isoforms. JNK1, 2 are expressed everywhere, but JNK3 is mainly expressed in cardiac, neuronal, and testis tissues (56). Each JNK is expressed as two subunits (46 kDa) and (54 kDa) (57). The JNK pathway plays a major role in apoptosis. The signal pathways that initiate apoptosis are divided into two groups: 1) external pathways initiated by death receptors. 2) internal pathways initiated by mitochondrial events. JNK has been shown to play a central role in both directions. JNK was first identified as a protein kinase that phosphorylates the c-Jun transcription factor at the N-terminal activation site in serins 63 and 73 (57, 53).

JNK induces apoptosis through transcription-dependent and non-transcriptional mechanisms. In addition, mitochondria are a primary target for JNK pre-apoptotic signalling (58). JNK is activated by a kinase cascade. Dual phosphorylation is involved in the JNK activation mechanism. This phosphorylation is carried out by two specific MAPK kinases or MKK. JNK is phosphorylated and activated by mkk4 and mkk7 at the residue site of tyrosine and threonine. The diversity of the upstream mediators mkk4 and mkk7 may activate the JNK pathway by a range of external stimuli (59). These upstream kinases are activated by the MAPKKK5 family, including MLK1, and MEKK7 (60).

Upon activation by upstream kinases, phosphorylated JNK is transported to the nucleus, where it activates c-jun at serine residues 13 and 73 and induces the transcription-dependent signal pathway JNK. It also phosphorylates several other transcription factors, including JunD, ATF82, and ATF3. Thus, JNK nuclear activity could potentially increase the expression of pre-apoptotic genes or decrease the expression of pro-survival genes (61). In addition to nuclear signalling, which leads to the upregulation of pre-apoptotic genes and the down-regulation of anti-apoptotic genes, JNK plays an essential role in modulating the function of pro-apoptotic proteins located in the mitochondria through separate phosphorylation events. Accordingly, following activation by an apoptotic stimulus, JNK is readily transported to the mitochondria. Several studies have shown that JNK can both phosphorylate and regulate their precursor and anti-apoptotic proteins. For example, JNK can phosphorylate Bcl-2 and Bcl-xL and reduce their anti-apoptotic activity, and it can also phosphorylate Bim and Bmf pre-apoptotic proteins and increase their pre-apoptotic effect.

Phosphorylation of Bim and Bmf by JNK releases them (most pre-apoptotic proteins are inactivated and bound to their binding protein in the cytoplasm. However, after ischemia or other stress stimuli) Are activated and distributed from the cytoplasm to the mitochondria). Released Bim and Bmf can activate Bax. Activated Bax forms membrane channels for the release of apoptotic proteins such as cytochrome C. Another protein targeted by the JNK to increase apoptosis is bad. JNK phosphorylates explicitly Bad at Ser128. Bad increases cell death by interacting and inhibiting Bcl-2 proteins (54, 61).

Recently, the JNK signalling pathway has been considered in studies of neuronal apoptosis in cerebral ischemia. Studies show that the JNK pathway plays a vital role in the pathogenesis of many neurological disorders, including ischemic stroke, Parkinson, and Alzheimer (54). The JNK signalling pathway is a potential cascade that mediates neuronal apoptosis induced by focal and global ischemia. Inhibition of JNK reduces

neuronal apoptosis in the ischemic region. Several neuroprotectants, including SP600125, Edaravone, and Emodin, exert their protective effect by inhibiting the JNK pathway. D-JNKI1 is a JNK inhibitor that prevents neuronal loss in both transient and permanent middle cerebral artery occlusion models and has a strong neuroprotective effect even when given 1 to 12 hours after ischemia. This is the first drug that provides such strong protection with delayed administration. JNK is, therefore an important therapeutic target for the prevention of ischemic neuronal death (62). However, some papers are investigating the role of apoptosis in the pathology of stroke, which can help better understand these contents (63, 64).

Conclusion

Stroke is a major health threat with a high mortality rate. Various mechanisms cause neuronal death in ischemia of the central nervous system due to stroke. The phenomenon of apoptosis or programmed cell death plays a major role in neuronal death after stroke in and around ischemic areas. Apoptosis occurs through the intrinsic and extrinsic pathways. Calpains are calcium-active proteolytic enzymes located in the cell cytoplasm and lead to apoptotic cell death through the intrinsic pathway. Caspases are divided into two categories: initiator and effector. The exact mechanism of action of caspases is not yet known. However, most stimuli that lead to apoptosis appear to activate caspases in three ways: 1) death receptor pathway, 2) cytotoxic T lymphocyte pathway (CTL)/natural killer (CTL/NK-derived granzyme B), and 3) mitochondrial/apoptosome pathway. The c-Jun N-terminal kinases are a subset of MAP kinases. MAP kinases are a family of signal proteins and are mainly activated by cytokines such as TNF-α and IL-1, and JNK is activated by exposure to environmental stresses such as radiation and oxidative stress. Also known as stress-activated protein kinase (SAPK), it is activated by several environmental stresses. Identifying the cellular and molecular mechanisms involved in stroke, such as apoptosis, will significantly aid in therapeutic approaches to this disorder.

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