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Mini-review

Putative roles of mitochondrial Voltage-Dependent Anion Channel, Bcl-2 family proteins and c-Jun N-terminal Kinases in ischemic stroke associated apoptosis

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Abstract

There is a constant need for better stroke treatments. Neurons at the periphery of an ischemic stroke affected brain tissue remains metabolically active for several hours or days after stroke onset. They later undergo mitochondrion-mediated apoptosis. It has been found that inhibiting apoptosis in the peripheral ischemic neurons could be very effective in the prevention of stroke progression. During stroke associated apoptosis, cytosolic c-Jun N-terminal Kinases (JNKs) and Bcl-2 family proteins translocate towards mitochondria and promote cytochrome *c* release by interacting with the outer mitochondrion membrane associated proteins. This review provides an overview of the plausible interactions of the outer mitochondrial membrane Voltage Dependent Anion Channel, Bcl-2 family proteins and JNKs in cytochrome *c* release in the peripheral ischemic stroke associated apoptotic neurons. The review ends with a note on designing new anti-stroke treatments.
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Keywords: Ischemic penumbra; Mitochondrion-mediated apoptosis; c-Jun N-terminal Kinases; Voltage-Dependent Anion Channel

1. Introduction

Stroke is a major cause of death worldwide. Currently, the only approved therapy for stroke is the administration of tissue plasminogen activator (tPA) but it is beneficial if administered within 3 h of the onset of stroke [1]. Most of the patients exhibit slow evolution of brain injury following the stroke attack and thus there is a need for additional anti-stroke therapies which can be prescribed even after 3 h [1]. This review would aid in overcoming these limitations and the designing of better, new anti-stroke therapies. There are basically two types of stroke: Hemorrhagic and Ischemic. Ischemic stroke is primarily caused by middle cerebral artery occlusion by blood clot or plaque formation which leads to reduced blood supply to the brain tissues [1]. Reduction in blood supply to the brain tissues known as 'cerebral ischemia' results in reduced supply of oxygen and glucose to the affected brain area (ischemic infarct area) (Fig. 1). Furthermore, this reduced supply leads to decreased ATP production and the death of cells in that brain area. Usually, the brain tissue which is closer to the occlusion or clot is affected the most and such brain area is known as 'ischemic core' (Fig. 1). Within a few minutes of ischemia, the cells in the ischemic core undergo death. However, cells at the periphery of the ischemic core region remain metabolically active even after hours or days of ischemia onset and may undergo death then after. This brain area located at the periphery of the ischemic core is known as 'ischemic penumbra' region [1] (Fig. 1). To date there is no full proof therapy available against stroke. Incidentally, preventing cell death in the 'ischemic penumbra' region has been

Abbreviations: VDAC, Voltage Dependent Anion Channel; JNKs, c-Jun N-terminal Kinases; Bif-1, Bax interacting factor-1; MPT pore, Mitochondrial Permeability Transition pore.

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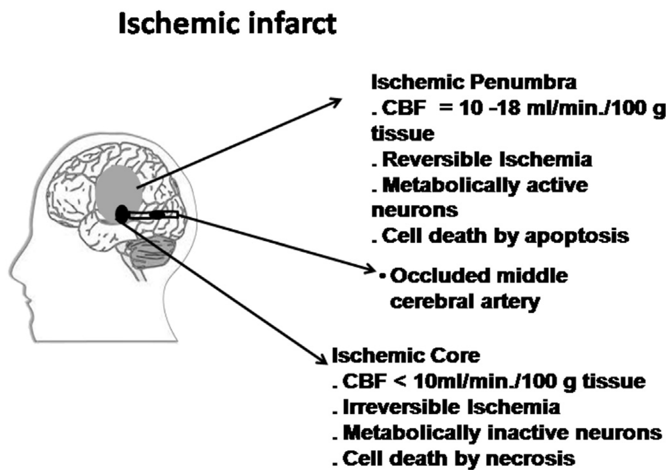

Fig. 1. Schematic representation of the brain tissue suffering from ischemia. Ischemic tissue also known as ischemic infarct has two regions a. Ischemic core in the centre and b. Ischemic penumbra at the periphery surrounding the core. Ischemic infarct involves cell death by necrosis in the core of the tissue and apoptosis in the penumbra. Up to certain time after stroke onset the neurons in the penumbra region remain metabolically active and after that they undergo death by apoptosis.

extensively investigated in the prevention of stroke progression. To understand the mechanisms of cell death in human ischemic stroke injury various *in vivo* animal and *in vitro* cell culture models have been developed [1]. The three main classes of animal stroke models are 1) global ischemia, 2) focal ischemia, and 3) hypoxia/ischemia. The last method involves combination of vessel occlusion with breathing a hypoxic gaseous mixture and is exclusively used in young animals. Global ischemia models used are 1) rat four vessel occlusion (4-VO) or two-vessel occlusion (2-VO) combined with hypotension; 2) gerbil-2-VO; and 3) mouse-2-VO. 4-VO rat model involves permanent coagulation of the vertebral arteries, and temporary ligation of the two common carotid arteries while 2-VO rat model involves bilateral occlusion of the common carotid arteries along with a blood pressure reduction to 50 mm Hg using different methods [1]. Focal ischemic stroke models involves occlusion of one middle cerebral artery (MCA). There are two models of focal ischemic stroke, 1) transient focal ischemia and 2) permanent focal ischemia. In transient focal ischemia, vessels are blocked for periods of up to 3 h, followed by prolonged reperfusion; whereas, in permanent focal ischemia, the arterial blockage is maintained throughout an experiment, usually for one or more days [1]. *In vitro* cell culture ischemic stroke model is oxygen-glucose deprivation followed by re-oxygenation (OGD-R) model [1]. OGD-R model involves culturing of neurons in glucose-free medium followed by exposure to hypoxic chamber (PO_2 < 50 mm Hg) and then re-oxygenation in a glucose containing medium.

Mitochondrion plays an important role in ATP production and keeping the cells metabolically active [2]. It has been observed that if we can prevent mitochondrial machinery from dying in the ‘ischemic penumbra’ neurons then stroke progression can be hampered [2]. Generally, there are two pathways of cell death in penumbral cells: the intrinsic apoptotic pathway (also known as mitochondria-mediated pathway) and the extrinsic apoptotic pathway (death receptor pathway) [3]. In this review we have highlighted the plausible role of the mitochondrion-mediated apoptotic pathway in penumbral cell apoptosis. Death by the mitochondrion-mediated apoptotic pathway involves the release of a mitochondrial intermembranous space protein ‘cytochrome *c*’ to the cytosol. In the cytosol cytochrome *c* activates caspase proteases which degrade cytosolic proteins leading to apoptosis [2]. Mitochondrion-mediated pathway in penumbral cells involves activation of cytosolic c-Jun N-terminal Kinases (JNKs) by phosphorylation [3]. JNKs are stress-activated serine–-threonine kinases and activated JNKs translocate toward mitochondria and promote cytochrome *c* release [3]. It has been established that JNKs promote cytochrome *c* release by interacting with the outer mitochondrial membrane associated proteins, but the mechanisms of these interactions in the penumbral cells needs to be explored. In this review we have dealt with some of the questions which could be relevant in understanding the mechanisms of cytochrome *c* release in penumbral cells. What are all the plausible pathway(s) of cytochrome *c* release during mitochondrion-dependent apoptosis? What all pathways have been experimentally validated to be important in penumbral cell apoptosis? By what all means cytochrome *c* release can be prevented in ischemic penumbral cells and what all ways have been already tested? And finally how much preventing cytochrome *c* release in penumbral cells can aid in overcoming the current limitations in the designing of full proof therapy against stroke? We would like to mention here that there are reviews on mitochondrial apoptotic neuronal death pathways in stroke, but those highlight mainly the established pathways involved in this process [4,5]. Here we discuss all the putative mechanisms of cytochrome *c* release through mitochondrial outer membrane that could be relevant in penumbral cell apoptosis. Especially, the putative mechanisms of cytochrome *c* release involving the outer mitochondrial membrane protein, Voltage-Dependent Anion Channel (VDAC) and its interactions with pro-apoptotic proteins, JNKs have been highlighted.

2. Does VDAC play role in penumbral cell apoptosis?

VDAC is the most abundant protein present at the outer mitochondrion membrane with VDAC-1 as the predominant isoform (Fig. 2). It transports ions (preferably anions), water, adenine nucleotides like ATP and anionic metabolites across the outer mitochondrial membrane by opening-closing and its malfunctioning can lead to apoptosis [6]. To the best of our knowledge, the role of VDAC in penumbral cell apoptosis has not been explored but it is very likely that it plays a key role. Thus, we have tried to understand the plausible role of VDAC in penumbral cell apoptosis. Mitochondrial permeability transition (MPT) pore is a complex of mitochondrial proteins that is formed under pathological conditions involving cell death like stroke and allows transport of molecules from the mitochondrial matrix and inner membrane to the cytosol. MPT pore formation is believed as the major mechanism for

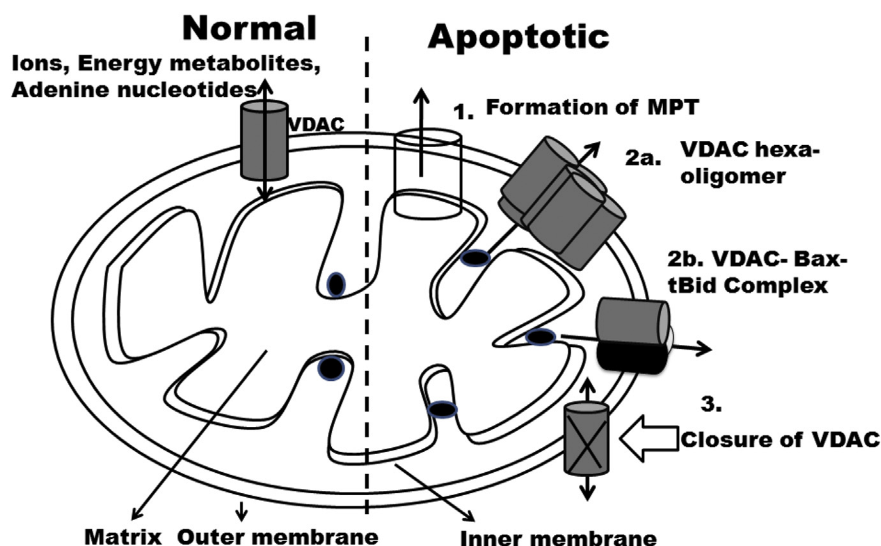

Fig. 2. Schematic representation of the mitochondrion in the normal and in the apoptotic cells present in the ischemic 'penumbra' region. Three different models have been proposed for the release of cytochrome *c* during penumbral cell apoptosis. First model is that during apoptosis mitochondrial permeability transition (MPT) pores are formed through which cytochrome *c* passes out into the cytosol. The exact composition of MPT pore is unknown till date. VDAC at the outer mitochondrial membrane, adenine nucleotide translocator (ANT) protein present at the inner mitochondrial membrane, inorganic phosphate carrier (PHC) of the inner mitochondrial membrane and cyclophilin-D (CYPD) protein present in the mitochondrial matrix were earlier believed to be essential molecular components of MPT pore but knock down experiments have shown that MPT is possible without them. Mitochondrial F_1-Fo-ATP synthase, in particular the c subunit of the Fo domain, and SPG7, an integral protein of the inner mitochondrial membrane with metalloprotease activity have been recently identified as MPT components. In particular, F_1-Fo-ATPase dimers have been proposed to constitute the long-sought pore-forming component of the MPT pore. Bcl-2 family proteins, mitochondrial creatinine kinase 1 (CKMT1), hexokinase isoforms 1, 2 (HXK1, HXK2), glycogen synthase kinase 3β (GSK3β), p53 protein, peripheral benzodiazepine receptor (PBR) also known as translocator protein (TSPO) and protein kinase C epsilon isoform (PRKCε) have been shown to modulate the activity of the MPT pore. Second model is that during penumbral cell apoptosis VDAC might form homo- or hetero-oliomers. VDAC1 monomers and lower-order homo-oligomers (dimers, trimers, tetramers) form higher-order homo-oligomers (hexamers, octamers) through inter-channel contacts which act as cytochrome *c* conducting channel or there is association of Bax and truncated Bid (t-Bid) pro-apoptotic proteins with mitochondrial VDAC and formation of Bax-tBid-VDAC complexes/hetero-oligomers on the outer mitochondrial membrane. Formation of large pore size Bax-tBid-VDAC complexes allows passage of cytochrome *c* into the cytosol through them. Third model is that VDAC closes down during apoptosis. In the normal cell VDAC transports ions, adenine nucleotides and energy related metabolites across the mitochondria and cytochrome *c* remains bound to the inner mitochondrial membrane. It is proposed that during ischemic stroke associated penumbral cell apoptosis, there might be closure of VDAC by different mechanisms such that all the transport through VDAC is blocked. Blockage of VDAC leads to accumulation of ions and water in the mitochondrial matrix leading to matrix swelling, rupture of the outer mitochondrial membrane and cytochrome *c* release.

cytochrome *c* release in ischemic stroke [7]. The exact composition of MPT pore is unknown till date. VDAC, adenine nucleotide translocator (ANT) protein present at the inner mitochondrial membrane, inorganic phosphate carrier (PHC) of the inner mitochondrial membrane and cyclophilin-D (CYPD) protein present in the mitochondrial matrix were earlier believed to be essential molecular components of MPT pore but knock down experiments have shown that MPT is possible without them [7] (Fig. 2). Mitochondrial F_1-Fo-ATP synthase, in particular the c subunit of the Fo domain, and SPG7, an integral protein of the inner mitochondrial membrane with metalloprotease activity have been recently identified as MPT components (Fig. 2). In particular, F_1-Fo-ATPase dimers have been proposed to constitute the long-sought pore-forming component of the MPT pore [8]. However, these findings require further investigations. Furthermore, Bcl-2 family proteins, mitochondrial creatinine kinase 1 (CKMT1), hexokinase isoforms 1, 2 (HXK1, HXK2), glycogen synthase kinase 3β (GSK3β), p53 protein, peripheral benzodiazepine receptor (PBR) also known as translocator protein (TSPO) found on the outer mitochondrial membrane and protein kinase C epsilon isoform (PRKCε) have been shown to modulate the activity of the MPT pore [7]. MPT formation involves following steps during ischemic stroke: Within seconds of cerebral ischemia, local cortical activity ceases. This massive shutdown of neural activity is induced by K^+ efflux from neurons, mediated initially by the opening of voltage-dependent K^+ channels and later by ATP-dependent K^+ channels, leading to transient plasma membrane hyperpolarization. A few minutes later, an abrupt and dramatic redistribution of ions occurs across the plasma membrane, associated with membrane depolarization (efflux of K^+ and influx of Na^+, Cl^- and Ca^{2+}). This "anoxic depolarization" results in the excessive extracellular release of neurotransmitters, in particular, excitatory neurotransmitter glutamate, promoting further spatial spread of cellular depolarization, depletion of energy stores and advancement of injury cascades. Extracellular glutamate over stimulates postsynaptic NMDA, AMPA and Kainite receptors promoting Na^+ and Ca^{2+} ion influx and K^+ efflux through these channels. The gating of these channels effectively achieves membrane shunting, which spreads in waves (spreading depression) from the ischemic core out toward the margins of the ischemic penumbra. Spreading depression increases metabolic demand and energy failure, thus further enhancing glutamate release. This process is known as 'Glutamate induced excitotoxicity'.

In an 11 vessel occlusion rat model of ischemic stroke which involves uni/bilateral occlusion of the common carotid arteries and/or the middle cerebral artery it has been shown that accumulated extracellular glutamate triggers an uptake of Ca^{2+} by postsynaptic cells [9]. High Ca^{2+} concentration in cells leads to the formation of MPT pore in the inner mitochondrial membrane [9]. The activation of the MPT pore disrupts the selective permeability of the inner mitochondrial membrane, causing uncoupling of oxidative phosphorylation, accumulation of Ca^{2+} ions and water in the mitochondrial matrix which leads to osmotic swelling and rupture of the outer mitochondrial membrane and the release of cytochrome *c* into the cytosol. Although, MPT pore formation can promote cell death both by necrosis as well as by apoptosis, however, it is a major mechanism of necrosis in the ischemic core. Thus, there remains a great deal of interest in understanding the other mechanisms of cell death which could be relevant in the ischemic penumbral cell apoptosis. It has been widely seen that overexpression of VDAC-1 in cells leads to cytochrome *c* release into the cytosol and apoptosis [6]. Thus, VDAC pore plays an important role in cytochrome *c* release. VDAC pore resembles a slightly elliptical cylinder with horizontal dimensions of approximately 3.1 by 3.5 nm and a height of approximately 4 nm [10]; NMR studies have indicated a diameter of about 2.5 nm for the open state [11], while the X-ray-based structure suggested maximal inner dimensions of 2.7 by 2.4 nm [12]. High-resolution atomic force microscopy (height, 3.8 nm; diameter, 2.7 nm) [13,14] and electron microscopy (diameter, ≈ 3 nm) [15] in the native state derived from *Saccharomyces cerevisiae* in the natural membrane composition yielded similar dimensions for VDAC pore although these structures have been questioned whether they represent native VDAC structure or not [16,17]. All these structural findings suggest that VDAC pore cannot pass cytochrome *c* (~diameter, 3.4 nm) through it in the native state. Thus various models have been put forward to understand how VDAC promotes cytochrome *c* release during apoptosis [18]. VDACs are present as monomers, dimers, trimers, and higher oligomers in a dynamic equilibrium on the outer mitochondrial membrane under normal physiological conditions. First proposed model is that apoptosis induction promotes association of these VDAC monomers and higher oligomers through inter-channel contacts at the native outer mitochondrial membrane shifting the dynamic equilibrium towards the increased formation of higher order homo-oligomers, preferably hexamers & octamers [19–21] (Fig. 2). These higher order homo-oligomers form hydrophilic channels (diameter ~ 4 nm or higher depending upon the oligomer composition) for the passage of cytochrome *c*. Furthermore, it has been shown that during apoptosis there is increase in intracellular $[Ca^{2+}]i$ level which induces VDAC-1 expression and its oligomerization. Increase in mitochondrial $[Ca^{2+}]m$ level also leads to increased VDAC-1 oligomerization [22,23]. In a different set of experiments, VDAC-1 oligomerization inhibitors as DIDS, DPC [24] or VBIT-3 and VBIT-4 [25] have been shown to inhibit apoptosis in various cell lines. Thus, VDAC-1 oligomers might take part in penumbral cell apoptosis. Second proposition is that VDAC forms large pore size complexes with pro-apoptotic molecules on the outer mitochondrial membrane leading to the passage of cytochrome *c* into the cytosol through these complexes (Fig. 2). Third proposal is that apoptosis induction closes down VDAC (Fig. 2). Partial closure of VDAC leads to inhibition of transport through VDAC and accumulation of ions, water, energy-related metabolites and adenine nucleotides in the mitochondrial matrix which cause matrix swelling, rupture of the outer mitochondrial membrane and leakage of cytochrome *c* into the cytosol and apoptosis [18]. Also, closure of VDAC hampers energy related metabolite transport into the cytosol for important cytosolic processes, thus leading to cell death [18] (Fig. 2). Using these models and the available experimental data we have tried to explore the putative role of VDAC in penumbral cell apoptosis.

2.1. Phosphorylation of VDAC by JNKs might be important in penumbral cell death

Phosphorylation controls the gating behavior of VDAC to a great extent in cells and is an important regular of apoptosis [26–28]. As mentioned in the Introduction cerebral ischemia involves activation and mitochondrial translocation of JNKs. Thus, we hypothesize that phosphorylation of VDAC by JNKs might play an important role in penumbral cell apoptosis [29,30]. JNKs regulate normal physiological functions like immune responses; cell and tissue morphogenesis and also they are involved in pathological processes. Transient focal cerebral ischemia induced by 60 min of middle cerebral artery occlusion in rats showed that the level of JNK activity and phospho-JNK levels were significantly increased 2.4 and 6.7 fold 3 and 6 h after reperfusion, respectively, compared with the non-ischemic controls [31]. Furthermore, it has been found that following ischemic stroke the profile of JNK isoforms in mitochondria gets completely changed; normally present JNK1 almost completely disappears from mitochondria. In striking contrast activated JNK2 and even more pronounced JNK3 substantially increase in mitochondria. These changes in mitochondria associated JNKs initiate mitochondria-mediated apoptosis [32,33]. JNK3 is the neuronal isoform of JNKs and is an important intracellular mediator of penumbral cell apoptosis [34]. Mice with disruption of the gene coding for JNK3 enzyme are resistant to ‘Glutamate induced excitotoxicity’ [34]. In another report it has been proved that JNK dependent cytochrome *c* release from brain mitochondria is independent of MPT [35]. Thus VDAC might take part in penumbral cell apoptosis by either through the closure mechanism or by homo/hetero-oligomerization process (Fig. 2). With this hypothesis in one of our work we checked the phosphorylation of rat brain purified mitochondrial VDAC by JNK3 and also studied the electrophysiological properties of JNK3 phosphorylated VDAC on Bilayer Lipid Membranes (BLMs) [36]. Briefly, purified rat brain mitochondrial VDAC reconstituted in a BLM was phosphorylated with the JNK3 enzyme. We observed that *in vitro* phosphorylation by JNK3 leads to partial closure of VDAC (Fig. 3). Closure of VDAC

might lead to cell death by the mechanism discussed above [18]. Thus, we propose that closure of VDAC as a result of phosphorylation by JNK3 could be an important means of penumbral cell apoptosis. This is a hypothesis which needs to be tested *in vivo*.

2.2. VDAC can form hetero-oligomeric pores with pro-apoptotic Bcl-2 family proteins which might be important in penumbral cell death

Bcl-2 family is classified into three subgroups: the pro-apoptotic proteins (Bax, BAD, Bak and Bok etc.); the anti-apoptotic proteins (Bcl-2, Bcl-xL and Bcl-w etc.) and the BH3 only members including Bad, Bid, Bim, Noxa, PUMA (p53 upregulated modulator of apoptosis) [37]. VDAC is considered as a mitochondrial receptor for Bcl-2 family proteins. Recombinant pro-apoptotic proteins like Bax and Bak have been shown to accelerate the opening of mitochondrial VDAC whereas anti-apoptotic protein Bcl-x(L) closes it on artificial bilayers [38]. A higher anti-apoptotic to pro-apoptotic protein ratio of expression is essential for cell survival. After cerebral ischemia, both Bax and Bcl-2 mRNA levels are reported to increase in the penumbral neurons. However, Bax levels appear to increase more than Bcl-2 and thus Bax is responsible for the ischemia-induced penumbral cell death [39,40]. In general, Bax is found in the cytosol in an inactive state in healthy cells. Cellular ischemia causes Bax to translocate towards outer mitochondrial membrane [41,42]. There are reports which suggest that during cerebral ischemia cytosolic Bax gets phosphorylated by JNKs and phosphorylation activated Bax then translocates towards mitochondria and causes cytochrome *c* release [43,44]. JNK3 has been shown to phosphorylate Bax *in vitro* [45]. During apoptosis Bax associates with Bid protein [46,47] (Fig. 4). In healthy cells, Bid protein is found in the cytoplasm. During mitochondria-mediated apoptosis Bid protein is cleaved by caspase 8 protease and truncated Bid (tBid) protein translocates to mitochondria [48]. tBid has been shown to close down VDAC on artificial bilayers [49]. Furthermore, it has been shown by our group on bilayer lipid membranes that mitochondrially translocated monomeric Bax and tBid proteins associates with rat brain purified mitochondrial VDAC and increase its pore size. It was hypothesized that this increase in VDAC pore size might cause leakage of cytochrome *c* into the cytosol during mitochondria-mediated apoptosis *in vivo* [50,51]. There are no reports available suggesting the formation of VDAC-Bax-tBid complexes during penumbral cell apoptosis and needs to be tested in future.

3. Does Bax play role in penumbral cell apoptosis?

3.1. Bax forms homo-oligomeric pores during penumbral cell apoptosis that play an indispensible role in cytochrome c release

Bax apart from forming hetero-oligomeric channels with VDAC, can also form homo-oligomeric large-conductance ion pores on the outer mitochondrial membrane and promote cytochrome *c* release [52] (Fig. 4). Ion pores formed by oligomeric Bax vary in conductance from picosiemens to

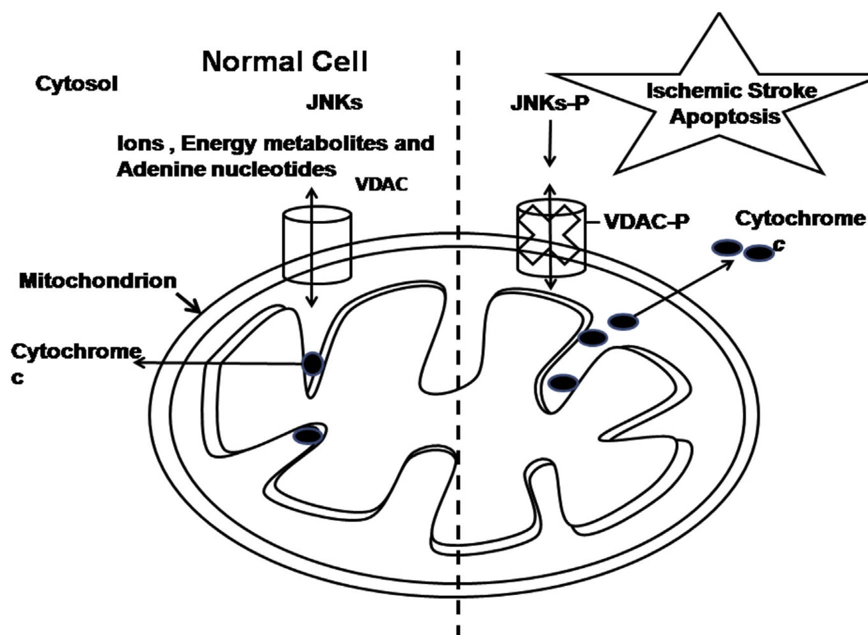

Fig. 3. Schematic representation showing the plausible interaction of c-Jun N-terminal Kinases (JNKs) with mitochondrial VDAC in penumbral neurons undergoing apoptosis. In the normal cell VDAC transports ions, adenine nucleotides and energy related metabolites across the mitochondria and cytochrome *c* remains bound to the inner mitochondrial membrane. Upon induction of apoptosis, JNKs translocate towards mitochondria and it is proposed that they phosphorylate VDAC. Phosphorylation of VDAC by JNKs leads to partial closure of VDAC such that ion, nucleotide and metabolite transport through VDAC is blocked. Blockage of VDAC leads to accumulation of ions and water in the mitochondrial matrix leading to swelling, rupture of the outer mitochondrial membrane and cytochrome *c* release. Cytochrome *c* activates caspase proteases in the cytosol dismantling the cells.

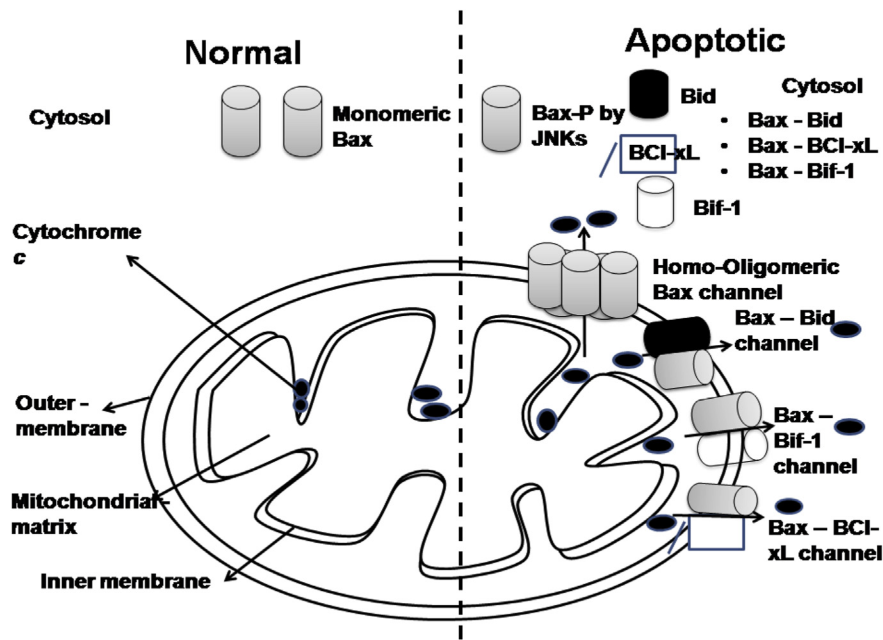

Fig. 4. Schematic representation showing the plausible roles of BCl_2 family proteins in penumbral cell apoptosis. Upon induction of apoptosis Bax gets activated by phosphorylation by c-Jun N-terminal Kinases (JNKs) or by interaction with other proteins like t-Bid, ΔN BCl-xL or Bax interacting factor (Bif-1) leading to the formation of homo or hetero-oligomers. At the mitochondrial outer membrane these oligomers form pores through which cytochrome *c* leaks out into the cytosol and activates caspases leading to cell demise.

nanosiemens depending upon the oligomerization status [52]. Oligomeric Bax pore inhibitors (Bci1 and Bci2) have been shown to prevent cytochrome *c* release from ischemic hippocampal cells in gerbils [53].

3.2. Bax forms hetero-oligomeric pores with other Bcl-2 family proteins during penumbral cell apoptosis that play an indispensible role in cytochrome c release

Bcl-x_L is an anti-apoptotic Bcl-2 family member. It has been shown that during cerebral ischemia it is cleaved by caspases and one of its cleavage products (ΔN-Bcl-x_L) forms different oligomers which promote cytochrome *c* release from the liposomes [54–56]. It has been hypothesized that either oligomeric ΔN-Bcl-x_L alone or in association with Bax can form large-conductance cytochrome *c* releasing pores at the outer mitochondrial membrane (Fig. 4). This hypothesis could be important in understanding the mechanisms of ischemic stroke associated penumbral cell death by the mitochondrion-mediated pathway. Moreover, it has been observed that ischemic preconditioning followed by after 48 h ischemia and reperfusion prevents cleavage of Bcl-x_L to generate ΔN-Bcl-x_L and formation of large-conductance pores in the outer mitochondrial membrane [57]. This knowledge could be important in preventing penumbral cell death.

3.3. Bax complex with non Bcl-2 family proteins like Bif-1 might form cytochrome c releasing pores during penumbral cell apoptosis

Bax interacting factor-1 (Bif-1) is a cytosolic protein and it is required for neuronal viability [58]. In middle cerebral artery occlusion (MCAO) model of ischemic stroke in mice, it has been shown that Bif-1 expression is decreased in the penumbra. The decrease was greater for neuron-specific isoforms (Bif-1b/c) than for Bif-1a. This decrease at day 2 post-MCAO occurred with no neuronal death. Furthermore, Bif-1-deficient mice developed larger infarcts and an exaggerated astrogliosis response following ischemic stroke and the drop in Bif-1 expression preceded the penumbral cell death. Thus, Bif-1 might play a prosurvival role for penumbral neurons and decreased Bif-1 expression is causal to increased vulnerability of penumbral neurons to apoptotic stress [58]. Bif-1 overexpression has also been suggested to promote neuronal survival and reduce the apoptotic rate in ischemic penumbral cortical neurons [59]. However, further studies suggested that Bif-1 function is highly cell specific. For instance, it promotes mitochondria-mediated apoptosis in non-neuronal cells. It has been shown that during apoptosis Bif-1 translocates towards mitochondria and co-immunoprecipitates with Bax protein (Fig. 4). So far as the role of Bif-1 in mitochondrial apoptosis is concerned some reports suggest that Bif-1 activates Bax and loss of Bif-1 suppresses apoptosis [60,61], while others believe that Bax activates Bif-1 oligomerization [62] and oligomeric Bif-1 can cause lipid membrane vesiculation, thus helps apoptosis. In order to have a better understanding of the mechanisms of Bif-1 action, in one of our studies we co-incubated an equimolar mixture of monomeric Bax and Bif-1 proteins and tested them for oligomerization in gel. We found that co-incubation did not cause oligomerization of any of the two proteins. Furthermore, the mixture of these proteins on the artificial bilayers formed large-conductance ion pores (open state conductance 4.96 nS–5.41 nS), which was not seen when monomeric Bax or Bif-1 protein was added to the bilayer alone

[63]. It is likely that these large-conductance pores formed by Bax-Bif-1 interaction might act as channels for cytochrome *c* release during penumbral cell apoptosis.

4. Discussion and conclusions

In this review we have summarized all the plausible ways by which cytochrome *c* can be released from the intermembrane space into the cytosol during penumbral cell apoptosis. In particular the role of the outer mitochondrial membrane VDAC and Bcl-2 family members have been reviewed. In penumbral cell apoptosis two plausible mechanisms of cytochrome *c* release involving mitochondrial VDAC are suggested; a. Inhibition of mitochondrial 'VDAC' as a result of phosphorylation by JNKs leading to mitochondrial swelling and rupture of outer mitochondrial membrane causing leakage of cytochrome *c* release into the cytosol; b. Formation of large-conductance homo or hetero-oligomeric pores by interaction of VDAC with Bax and tBid proteins which make outer mitochondrial membrane porous for cytochrome *c* release. Furthermore, we observed that phosphorylation of cytosolic Bax by JNKs is important for its mitochondrial translocation and formation of pores on the outer mitochondrial membrane. Thus, JNKs can affect both VDAC as well as the Bax mediated pathways of cytochrome *c* release by phosphorylation of these proteins. This could be the reason why selected inhibitors of JNKs are effective in preventing penumbral cell apoptosis for the relatively long therapeutic window that is up to six hours after stroke onset [33]. The major disadvantage of using JNK inhibitors in stroke therapy is that normal functioning of JNKs would get disturbed that way later on after the ischemic injury is over. We think if JNK inhibitors with an effective half-life paralleling the ischemic injury process can be designed then they can act as important tools for the prevention of penumbral cell apoptosis in stroke. Furthermore, we observed that oligomeric Bax, oligomeric ΔN-Bcl-x_L, Bax-ΔN-Bcl-x_L complex and Bax-Bif-1 complex can form cytochrome *c* releasing pores during penumbral cell apoptosis. Further we mentioned our work in which we reported the formation of large conductance pores by Bax-Bif-1 mixture on artificial bilayer membranes which suggest that this complex might promote cytochrome *c* release *in vivo* during penumbral cell apoptosis [63]. Thus, if we can prevent Bax-Bif-1 interaction or inhibit the pore formed by the Bax-Bif-1 mixture, penumbral cell apoptosis should be reduced. All this knowledge would help therapists in deciding at what level cellular application of the inhibitors would be most effective in stroke treatment. A variety of pharmacological compounds/inhibitors like JNK inhibitors, Bax channel inhibitors developed utilising the knowledge of apoptotic mechanisms obtained from the bench experiments have proved to be effective against cerebral ischemia in animal and cell culture models [33,53]. VDAC-1 oligomerization inhibitors have proven to be effective in preventing mitochondrion–mediated apoptosis in animal and cell culture models of many neurodegenerative diseases and their role in ischemic stroke is an important topic for future investigations [24,25]. Despite significant knowledge of mechanistic aspects of cerebral ischemia and potential therapeutic compounds an effective therapy for this disorder is not available till date [64]. Applying results of experimental models of cerebral ischemia to the clinical situations of stroke in humans has a lot of drawbacks. As discussed earlier JNKs inhibitors applied to block the injury process at the initial stage may block the physiological process at a later stage. Designing of an inhibitor which has a half-life corresponding to the time of the injury process is most essential. Second drawback is that the compounds which prove to be very good candidates for stroke treatment in animal and cell culture models commonly fail to succeed at the human level because the level of cellular, molecular and genetic complexity encountered in human subjects is not seen in animal models. Third, all the therapeutic compounds are to be delivered systemically in humans and they have to cross the blood–brain barrier before entering the brain. Unlike animal studies the compounds cannot be directly injected into the human brain. Fourth, we must remember that along with the mitochondria-mediated pathway there are other cell death pathways operating simultaneously, e.g. necrosis, autophagy, death receptor apoptotic pathway, post-ischemic inflammation and the newly recognised necroptosis (programmed necrosis) [65]. All these pathways may or may not get activated at the same time, thus a particular treatment would only be effective in a certain time window and assessment of that time window for a particular treatment is absolutely essential. Moreover, different cells in different regions of an ischemia affected area (the penumbra and the core) undergo death by different pathways. Therefore, suggesting a therapy for stroke prevention based on mitochondrial apoptotic pathway alone may not be a good idea. In future a combined therapy for ischemic stroke which can inhibit all these pathways might be more effective. Development of new treatment strategies like stem cell therapy, growth factor therapy and non-invasive instrumentation has helped a lot in the treatment of this disorder [66]. Although it is an uphill task, we believe a combination of all the above-mentioned aspects would help designing a good therapy for stroke taking care of the needs of individual patients.

Conflict of interest

Both the authors i.e.; Dr. Rajeev Gupta [Research Associate, All India Institute of Medical Sciences (AIIMS) (DRF-031/2015/RS)] and Prof. Subhendu Ghosh [Professor, Dept. of Biophysics, University of Delhi South Campus (RC/2015/9677 & DU DST PURSE Grant Phase II)] declare no financial or academic conflict of interest.

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