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·Minireview·

p53-mediated neuronal cell death in ischemic brain injury

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Abstract: p53 is a key modulator of cellular stress responses. It is activated in the ischemic areas of brain, and contributes to neuronal apoptosis. In various stroke models, p53 deficiency or applications of p53 inhibitors can significantly attenuate brain damage. p53-mediated neuronal apoptosis occurs through various molecular mechanisms. The transcriptional pathway is an important mechanism through which p53 induces neuronal apoptosis by up-regulating the expression of its target gene $p21^{WAF}$, Peg3/Pw1 or p53-up-regulated modulator of apoptosis (PUMA). In addition, p53 disrupts NF-κB binding to p300 and blocks NF-κB-mediated survival signaling. On the other hand, the transcription-independent pathway mechanism is also of great importance. In this pathway, p53 is translocated to mitochondrial and mediates the release of cytochrome *c*. In both pathways, p53 seems to play a key role in post-ischemic brain damage and has become a therapeutic target against stroke pathology.

Keywords: p53; cerebral ischemia; apoptosis

1 Introduction

The crucial processes of neurodegeneration following cerebral ischemia include inflammation and apoptotic cell death as well as regeneration, all of which depend heavily on gene expression. Indeed, in cerebral ischemia, a large repertoire of genes are activated by transcription factors[1]. p53 is one of these transcription factors and regulates the expression of many target genes that induce cell cycle arrest, apoptosis, senescence, DNA repair or metabolism alteration, in response to diverse stress conditions (including DNA damage, overexpressed oncogenes and various metabolic limitations)[2-5]. Current evidence indicates that p53 encodes a sequence-specific transcription factor that controls the expression of genes whose products mediate apoptosis. These products include Bcl-2-associated X protein (Bax)[6], NADPH oxidase activator 1 (Noxa)[7], p53 acetate-induced protein 1 (p53AIP1)[8], and p53-up-regulated modulator of apoptosis (PUMA)[9,10], all of which act directly on mitochondria and induce apoptosis. More recently, p53/Bax and p53/PUMA pathways have been implicated in neuronal apoptosis induced by mitochondrial inhibitor 3-nitropropionic acid in rat striatum[46]. In addition, there has been accumulative evidence for the transcription-independent p53-mediated apoptosis[11]. In some types of cells, a fraction of stabilized p53 is rapidly translocated to mitochondria in response to a death stimulus[12-14]. Exogenous p53 is forcibly targeted to mitochondria in p53 null cancer cells, and mitochondrial p53 is shown to be sufficient to launch apoptosis and suppress colony formation in a transcription-independent way[13]. Moreover, endogenous mitochondrial p53 forms inhibitory complexes with protective Bcl-XL and Bcl-2 proteins, resulting in cytochrome *c*

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release from mitochondria[13], suggesting that p53 contributes to apoptosis by direct signaling at the mitochondria, thereby amplifying transcription-dependent apoptosis of p53. Our research suggests that p53 contributes to neuronal apoptosis induced by excitotoxic injury, mitochondrial dysfunction or 6-hydroxydopamine[46-52]. This review was focused on p53 activation following cerebral ischemia and its contribution to neuronal apoptosis through various molecular mechanisms, including transcription-dependent and transcription-independent pathways.

2 p53 structure and its regulation

p53 is a modular protein consisting of 393 amino acids in human, with 5 proposed domains[15,16], including the transactivation domain (TAD; aminoacid residues 1-40) required for transcriptional activation, the proline-rich domain (PRD; residues 61-94) implicated in the regulation of p53 stability and activity, the DNA-binding domain (DBD; residues 100-300) specifically binding to DNA consensus recognition elements in the promoters of target genes, the tetramerization domain (4D; residues 324-355) and the C-terminal regulatory domain (CTD; residues 360-393) that binds DNA nonspecifically and might regulate specific DNA binding through DBD. PRD contains 5 PXXP motifs (Fig. 1, asterisks) that enable protein–protein interactions, in which P indicates proline and X indicates any other residue. Also noteworthy are the nuclear localization signal (L) located between DBD and 4D, and the nuclear export signal (E) embedded in 4D (Fig. 1).

Toledo and Wahl have described a model of p53 regulation based on *in vitro* and transfection data (Fig. 1)[15]. In unstressed cells (Fig. 1A), p53 is kept inactive and at a low level due to the action of MDM2, a negative regulator of p53. MDM2 inhibits p53 in mainly 2 ways: by occluding the p53 TAD, thereby then preventing the recruitment of transcriptional co-activators such as p300, or through ubiquitination of p53 CTD at the lysine sites via its ubiquitin-ligase activity, to promote p53 degradation by the proteasome. On the other hand, under stress conditions including DNA damage and possibly ischemia (Fig. 1B), p53 can be stabilized, up-regulated and activated. The phosphorylation of p53 by stress-induced kinases reduces the affinity of p53 for MDM2 and enables a tighter association between p53 and its co-activators, such as the histone acetyl transferase (HAT) p300, which binds PRD through PXXP motifs. This leads to the acetylation at lysines in CTD to promote p53 stabilization and increase specific DNA binding at target genes. p300 can also acetylate histones at the promoter of target gene, inducing promoter opening and transcription activation to induce different cellular responses. This in turn enables the transcriptional activation of p53 and transactivation of its target genes, inducing cell-cycle arrest, apoptosis, DNA repair and senescence. Although activated p53 is preferentially located in the nucleus, some studies also suggest that following stress, a fraction of p53 molecules remain in the cytoplasm and induces apoptosis by direct signaling at the mitochondria (Fig. 1B).

3 p53 mediates neuronal cell death following cerebral ischemia

Accumulating findings demonstrate that p53 is involved in neuronal death in both *in vivo* cerebral ischemic animal models[17,22,24,25,31,32] and *in vitro* hypoxic conditions[22,27] (Table 1). Many studies have demonstrated that p53 functions as a pro-apoptotic factor and its expression is up-regulated in apoptotic lesions after cerebral ischemia. Besides, p53-deficiency or applications of p53 inhibitors could potentially reduce the infarct volumes in various stroke models.

Increases in mRNA and protein levels of p53 have been observed in the ischemic area in various cerebral ischemic models, including focal cerebral ischemia, global cerebral ischemia (GCI) and transient forebrain ischemia[18,19,20,28,29]. Our recent study has revealed that in the ischemic cortex in permanent middle cerebral artery occlusion (pMCAO) animals, the mRNA level of p53 significantly increases at 3 h, reaching a peak at 12 h, and then decreases at 24 h after ischemia (unpublished data). Besides, p53 has been shown to be localized in neurons and astroglia, and accumulates in both the nucleus and the cytoplasm[18,28,44]. Similarly, in cultured neurons, hypoxia increases the expression of neuronal p53[27]. More recently, in cultured astrocytes, mRNA expression level of p53 is up-regulated at 12 h after oxygen-glucose depriva-

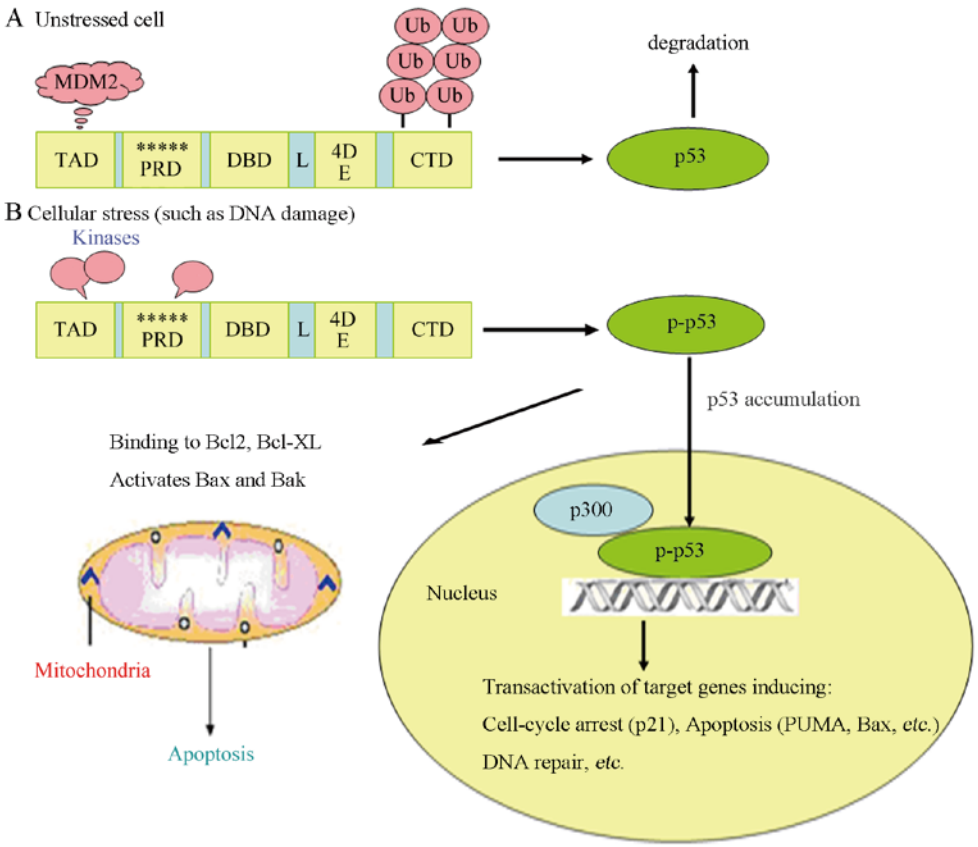

Fig. 1 p53 structure and its regulation (modified from Toledo F, Wahl GM, 2006)[15]. Human p53 consists of 5 proposed domains, including TAD, PRD, DBD, 4D, and CTD. A: In unstressed cells, p53 is kept inactive and at a low level due to the action of MDM2. B: Under various cellular stress, p53 is phosphorylated at serines and threonines in TAD and at threonines in PRD, inhibiting the binding of MDM2. Reduced MDM2 binding induces p53 accumulation, to form tetramers. The phosphorylation in TAD also favors the interaction with HATs such as p300, which binds PRD through PXXP motifs. This leads to the acetylation at lysines in CTD to promote p53 stabilization and to increase specific DNA binding at target genes. p300 can also acetylate histones at the promoter of target gene, inducing promoter opening and transcription activation to induce different cellular responses. In addition, p53 could also remain in the cytoplasm to bind anti-apoptotic Bcl-2 or Bcl-xL and promote apoptosis through mitochondrial outer membrane permeabilization. Ub: ubiquitylated lysine.

tion (OGD) (unpublished data). In addition, Cheng *et al.* find that p53 is activated in hypoxic human brain endothelium[30]. These findings indicate that p53 is activated in different types of cells with ischemic injury.

Levels of p53 and its products increase during early hours after ischemia onset and are especially high in the infarct border zone in focal cerebral ischemia models[18,29], in vulnerable hippocampal CA1 neurons after transient global cerebral ischemia (tGCI)[25], and in transient forebrain ischemia models[29] where cells are more likely to die from apoptosis. Furthermore, a strong correlation between the increase in p53 activity and ischemic injury degree and duration has been found[27]. A direct way to explore the pathophysiological importance of p53 under *in vivo* conditions is the specific deletion of *p53* gene. Crumrine *et al.*[17] permanently ligated the middle cerebral artery in p53-deficient mice and have observed that the decrease in infarct volume is more significant in heterozygous than in homozygous animals. A recent study by Yonekura *et al.*[24] has demonstrated that neuronal death in hippocampal CA1 region is significantly reduced in $p53^{-/-}$ mice after GCI, compared with that in $p53^{+/+}$ wild-type mice. Consistent with the *in vivo* results, cultured neurons deficient in p53 exhibit little or even no damage in response to excitotoxin, compared with $p53^{+/+}$ or $p53^{+/-}$ neurons[31]. In

addition, in cultured neurons, injection of antisense oligonucleotides against p53 can significantly increase the number of surviving neurons during hypoxic exposure[27]. These findings demonstrate a direct relationship between p53 expression and loss of neuronal viability in the central nervous system following ischemia.

Pharmacological inhibition of p53 can also exert strong neuroprotecive effects against cerebral ischemia. In a mouse model of pMCAO or a rat model of transient MCAO (tMCAO), treatment with pifithrin α (PFT), a novel specific inhibitor of p53, can reduce the cortical infarct[22,32]. Besides, PFT can protect against ischemic brain damage when applied at 3 h after onset of ischemia. Even applied at 6 h after ischemia onset, PFT can still exert a cerebroprotective effect[33]. PFT also protects hippocampal neurons against OGD[22].

Further studies demonstrate that p53 is involved in neuronal apoptosis after cerebral ischemia. An early study has demonstrated that in cultured neurons exposed to hypoxia, antisense oligonucleotides for p53 can reduce the number of apoptotic cells[27]. More recently, it has been found that intravenous administration of PFT can significantly inhibit DNA fragmentation or decrease the number of TUNEL-positive cells after tMCAO or tGCI[25,32].

Table1. Protection against neuronal injury by pharmacological inhibition or genetic inactivation of p53 *in vitro* and *in vivo*

Experimental model	Inducer of injury	Mode of p53 inhibition	Effect of p53 inhibition	Reference
Mouse	pMCAO	$p53^{-/-}$phenotype, $p53^{-/-}$phenotype	Reduced infarction size	[17]
Mouse hippocamapal neurons	Kainic acid or glutamate	$p53^{-/-}$phenotype	Reduced neuronal death	[31]
Neocortical neurons	Hypoxia	Antisense oligonucleotides to p53	Reduced neuronal death	[27]
Mouse	pMCAO	PFT	Reduced infarction size	[22]
Rat or mouse hippocampal neurons	OGD	PFT	Reduced neuronal cell death	[22]
Rat	tMCAO	PFT	Reduced infarction site; Reduced TUNEL-positive cells	[22]
Mouse	tGCI	$p53^{-/-}$phenotype	Reduced neuronal death	[24]
Rat	tGCI	PFT	Reduced DNA fragmentation and TUNEL-positive cells	[25]

4 Molecular mechanisms of p53-mediated neuronal apoptosis following cerebral ischemia

p53 can mediate apoptosis caused by death stimuli, through transcriptional activation of pro-apoptotic genes and transcription-independent mechanisms. More recently, increasing evidence shows that p53 is also involved in neuronal apoptosis induced by cerebral ischemia, via transcriptional activation of pro-apoptotic genes and transcription-independent mechanisms.

4.1 p53 mediates neuronal apoptosis by transcriptional pathway following cerebral ischemia Translocation of resident p53 into the nucleus and binding to its specific DNA sites are early events in p53-induced apoptosis in ischemic brain cells, and prevention of this early translocation could reduce brain damage[32].

4.1.1 p53 induces expression of its target gene $p21^{WAF}$ $p21^{WAF}$ is a target gene of p53, and is believed to participate in the control of cell fate in apoptosis. The level of $p21^{WAF}$ increases after cerebral ischemia[29]. Leker *et al.* find that PFT can reduce the number of apoptotic cells in the ischemic brain by inhibiting the binding of p53 to its target DNA sites, through reducing the expression of the p53-related gene $p21^{WAF}$, without changing the amount of p53 protein itself[32].

4.1.2 p53 up-regulates Peg3/Pw1 expression Peg3/Pw1 was originally identified as a paternally expressed gene, and its protein is a large molecule containing 12 zinc finger-like domains and 2 proline-rich periodic repeat domains[21]. During development, Peg3/Pw1 mRNA is ubiquitously expressed in all the tissues at low levels in mice, while in adult mice,

Peg3/Pw1 is abundantly expressed in the central nervous system and skeletal muscle. In human, Peg3/Pw1 mRNA is expressed at high levels in ovary, testis, and placenta, and at modest levels in brain and pancreas. The subcellular localization of Peg3/Pw1 is in the nucleus, and Peg3/Pw1 has been shown to be involved in the tumor necrosis factor-NF-κB signal transduction pathway, through the interaction with TRAF2 in some experimental systems. Recently, it has been reported that Peg3/Pw1 is involved in p53-mediated apoptosis through translocating Bax from cytosol to mitochondria or interacting with seven in absentia homology (Siah) family members in mouse fibroblast cells[21]. Besides, Peg3/Pw1 is specifically activated during p53/c-Myc- and p53/E2F-1-mediated apoptosis, but not during p53-dependent G1 arrest period. Moreover, inhibition of Peg3/Pw1 function can block p53/c-Myc-mediated cell death, whereas Peg3/Pw1 expression alone does not induce apoptosis, suggesting that Peg3/Pw1 is involved in the downstream of p53-mediated cell death pathway[21]. Yamaguchi *et al.*[21] have found that Peg3/Pw1 expression is enhanced in peri-ischemic neurons in a rat model of MCAO by *in situ* hybridization analysis, and p53 expression is also induced. Further studies using double staining method reveal that p53 is co-localized with Peg3/Pw1 in brain ischemia/hypoxia. In human neuroblastoma-derived SKN-SH cells, Peg3/Pw1 mRNA expression is enhanced remarkably at 24 h post-hypoxia, and p53 protein expression is also enhanced at a high level. Subcellular localization of Peg3/Pw1 is in the nucleus. Adenovirus-mediated high dose p53 overexpression can induce the expression of Peg3/Pw1 mRNA. Overexpression of Peg3/Pw1 reduces cell viability under hypoxic conditions, whereas deletion of its C-terminal and application of anti-sense Peg3/Pw1 can inhibit hypoxia-induced cell death. These results suggest that Peg3/Pw1 acts as a downstream effector of p53-mediated cell death in brain ischemia/hypoxia. However, the precise mechanism how Peg3/Pw1 is induced in p53-mediated neuronal death remains unclear.

4.1.3 p53 up-regulates the expression of PUMA Mitochondrial outer membrane permeabilization (MOMP) is governed by pro- and anti-apoptotic Bcl-2 family members, which are characterized by the Bcl-2 homology (BH) domains. The anti-apoptotic members, such as Bcl-2, Bcl-xL and Mcl-1, contain BH1-4 domains. The pro-apoptotic members can be divided into 2 subclasses: the ultimate MOMP effectors Bax and Bak containing BH1-3 domains, and the BH3-only class (Bim, Bad, Bid, Bik, PUMA, NADPH oxidase activator, and Hrk) carrying a single BH3 domain. Upon activation, Bax and Bak insert into the outer mitochondrial membrane and oligomerize to form dynamic lipid pores that release lethal proteins from the mitochondrial intermembranous space. Functionally, BH3-only proteins (more than one dozen exist) are further divided into 2 subgroups: "activators" and "derepressors", based on how they trigger apoptosis. Activators (tBid and Bim) trigger MOMP by direct stimulation of Bax and Bak oligomerization, while derepressors such as PUMA, Noxa and Bad inhibit the function of anti-apoptotic Bcl-2 family members and induce the release of pro-apoptotic members (e.g. Bax or tBid from Bcl-xL, and Bak from Mcl1)[58].

PUMA was initially identified as a gene activated by p53 in cells undergoing p53-induced apoptosis[9]. In p53-induced cell death, PUMA is shown to be localized in mitochondria. It interacts with Bcl-2, Bcl-XL and Bax, and induces cytochrome *c* release, thereby activating caspases-9 and -3[43]. Niizuma *et al.* find that after 5 min of tGCI induced by bilateral common carotid artery occlusion combined with hypotension, PUMA is up-regulated in vulnerable hippocampal CA1 neurons and is localized to mitochondria. Besides, PUMA is bound to Bcl-xL and Bax in the hippocampal CA1 subregion after tGCI. Moreover, PFT can inhibit the up-regulation of PUMA, suggesting that PUMA is partly controlled by p53 transcriptional pathway after tGCI[39].

4.1.4 p53 blocks NF-κB-mediated survival signaling The role of p53 in blocking the activities of other transcription factors, such as NF-κB, has been proposed as a crucial mechanism for p53-mediated cell death[33-35]. However, controversy still exists on the role of the transcription factor NF-κB in p53-mediated apoptosis, due to the differences in cell types and experimental conditions. In neurons, NF-κB supports the survival signaling by inducing the expression of anti-apoptotic factors, such as anti-apoptotic Bcl-2 family members, manganese superoxide dismutase (MnSOD), and inhibitors of apoptosis (IAP)[36-38,45,63]. However, some reports

also indicate that NF-κB supports apoptosis and is substantially involved in p53-mediated neuronal death[1,40-42].

In cultured neurons deprived of oxygen and glucose and in brain tissue with focal cerebral ischemia in mice, p53 inhibitor PFT can prevent ischemia-induced repression of NF-κB activity and reduce the brain damage, in a dose-dependent manner. This may be attributed to the balanced competitive interaction of p53 and NF-κB with the transcriptional cofactor p300. Exposure to lethal stress activates p53 and disrupts the binding between NF-κB and p300, thereby blocking NF-κB-mediated survival signaling. On the other hand, p53 inhibitors can provide pronounced neuroprotective effects, since they can block p53-mediated induction of cell death, and concomitantly enhance NF-κB-induced survival signaling[22].

4.2 p53 mediates neuronal apoptosis by transcription-independent pathway following cerebral ischemia The first hint of transcription-independent apoptotic activity of p53 dates back to 1994-1995, when researchers found that p53-dependent apoptosis occurred in the presence of transcriptional or translational inhibitors, and that p53 truncation mutants lacking transcriptional activity could still trigger apoptosis[53-55]. Later studies suggest that direct p53 signaling participates in caspase activity regulation[56,57]. However, the mechanisms of transcription-independent pro-apoptotic function of p53 remain unclear until recent years. Under stress conditions, cytoplasmic p53 rapidly translocates to mitochondria, and interacts with multidomain members of the anti- and pro-apoptotic Bcl-2 family, to either inhibit or activate them. This direct action of p53 results in robust MOMP, unleashing the enzymatic apoptotic machinery of caspases and inducing chromatin degradation.

The relevance of direct mitochondrial p53 program to the overall p53-mediated stress response *in vivo* has been investigated recently. Accumulating evidence for the important role of mitochondrial p53 death pathway, specifically in ischemic cerebral injury, has been reported. Mitochondrial, rather than nuclear, accumulation of p53 in hypoxia-sensitive hippocampal CA1 neurons strongly correlates with CA1 cytochrome *c* release and neuronal death within the first 24 h of tGCI, while occasional nuclear p53 staining can be detected only later, at 72 h. Conversely, i.v. injection of pan-p53 small molecule inhibitor PFTα can suppress p53 mitochondrial translocation and block apoptosis by 70% *in vivo*. On the other hand, mitochondrial p53 accumulation can not be seen in hypoxia-resistant CA3 neurons[25,59]. Moreover, in C6 glioma cells and primary cultured rat astrocytes with exposure to oxidative H_2O_2 stress for 1 h followed by 6-24-h recovery, p53 level continues to rise. Of note, p53 is localized overwhelmingly in mitochondrion, coincident with caspase-3 activation and apoptosis[60]. Conversely, the neuronal protection against ischemic pre-conditioning correlates with the suppression of mitochondrial p53 translocation during the subsequent ischemia-reperfusion challenge[61].

5 Conclusion

p53 up-regulation in ischemic areas in various stroke models and in p53-mediated signaling cascades remarkably contributes to ischemic neuronal cell death. Through transcription-dependent and transcription-independent pathways, p53 inhibitors can block p53-mediated neuronal apoptosis, thus being potential candidates for reducing the infarct volumes in various stroke models (Fig. 2). However, since p53 plays a crucial role in tumor suppression by sensing genotoxic stress and preventing proliferation of damaged cells through inducing cell cycle arrest, senescence, or apoptosis[62], clinical application of p53 inhibitors for stroke treatment may also induce an increased risk of cancer. Thus, further studies are still needed to clarify this problem.

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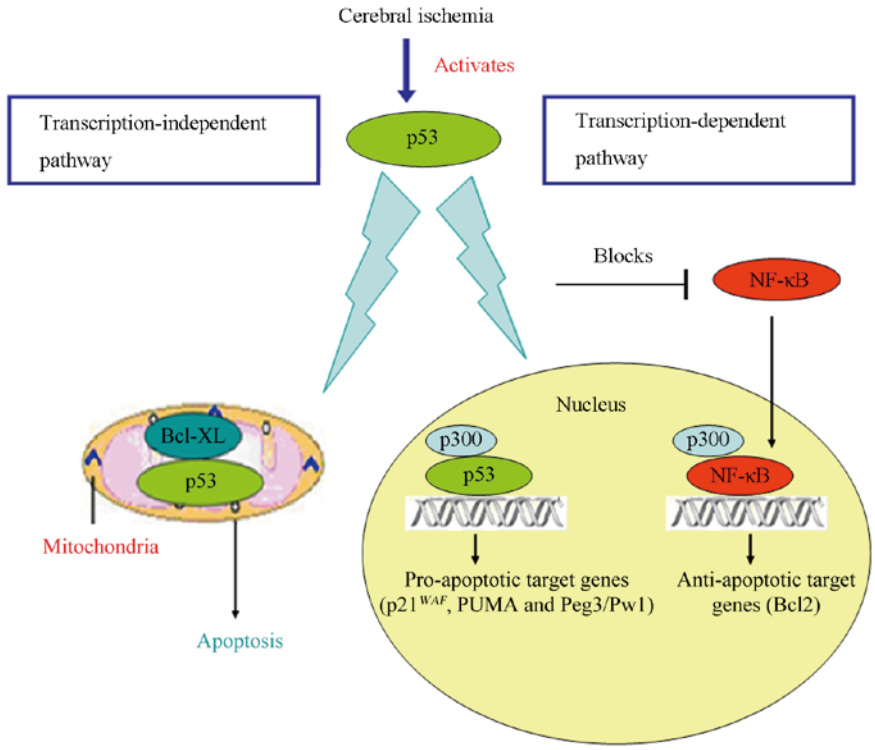

Fig. 2 p53-mediated neuronal apoptotic signaling cascades after cerebral ischemia. p53-mediated neuronal apoptosis can be initiated through transcription-dependent and transcription-independent pathways following cerebral ischemia. The transcription-dependent pathways involve the up-regulation of $p21^{WAF}$, Peg3/Pw1 or PUMA, and p53-induced disruption of NF-κB binding to p300 and blockage of NF-κB-mediated survival signaling. Alternatively, the transcription-independent pathways involve the direct translocation of p53 to mitochondrion and p53-mediated cytochrome *c* release.

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p53 介导缺血性脑损伤的神经元死亡

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摘要：p53 是调节细胞应激反应的关键因子。脑缺血激活 p53，后者导致神经元凋亡。对于多种动物中风模型的研究均表明，p53 缺陷或 p53 抑制剂均能显著减轻脑损伤。p53 介导神经元凋亡的机制主要有两种，包括转录依赖和转录非依赖途径。在转录依赖途径中，p53 通过上调其靶基因 $p21^{WAF}$, Peg3/Pw1 或 PUMA（受 p53 基因上调表达的凋亡调控基因）诱导神经元凋亡。此外，p53 还干预 NF-κB 与胸苷激酶 p300 的结合，进而阻断 NF-κB 介导的生存信号。在转录非依赖途径中，p53 进入线粒体并介导细胞色素 C 的释放。因此，p53 有可能在缺血后脑损伤过程中起关键作用，并有望成为脑中风药物治疗的靶点。

关键词：p53; 脑缺血; 凋亡