Ligustrazine monomer against cerebral ischemia/reperfusion injury

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Abstract

Ligustrazine (2,3,5,6-tetramethylpyrazine) is a major active ingredient of the Szechwan lovage rhizome and is extensively used in treatment of ischemic cerebrovascular disease. The mechanism of action of ligustrazine use against ischemic cerebrovascular diseases remains unclear at present. This study summarizes its protective effect, the optimum time window of administration, and the most effective mode of administration for clinical treatment of cerebral ischemia/reperfusion injury. We examine the effects of ligustrazine on suppressing excitatory amino acid release, promoting migration, differentiation and proliferation of endogenous neural stem cells. We also looked at its effects on angiogenesis and how it inhibits thrombosis, the inflammatory response, and apoptosis after cerebral ischemia. We consider that ligustrazine gives noticeable protection from cerebral ischemia/reperfusion injury. The time window of ligustrazine administration is limited. The protective effect and time window of a series of derivative monomers of ligustrazine such as 2-[(1,1-dimethylethyl)oxidoimino]methyl]-3,5,6-trimethylpyrazine, CXC137 and CXC195 after cerebral ischemia were better than ligustrazine.

Key Words: nerve regeneration; ligustrazine; ischemia; cerebral ischemia/reperfusion injury; neural regeneration

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Introduction

Cerebral ischemia/reperfusion injury refers to cerebral ischemia-induced brain cell damage that is aggravated by restoring the blood supply. The type of injury (focal or whole-brain, complete or incomplete, temporary or permanent), the spatial and temporal changes in the molecular mechanism of injury, and the interaction between these factors complicate analysis. Recent studies have mainly focused on cerebral ischemia/reperfusion injury. To select reasonable neuroprotective strategies would include choosing the drug type, its administration mode, and time window proved most effective in preventing and treating nerve injury after cerebral ischemia/reperfusion injury.

Traditional Chinese medicine has some advantages in the comprehensive treatment of multi-site, multi-target conditions and overall regulation. Ligustrazine (2,3,5,6-tetramethylpyrazine) is the main active ingredient of Szechwan lovage rhizome and can be used to promote blood flow, the circulation of *Qi*, and to remove blood stasis, thus it has been extensively used to treat ischemic cardiovascular and cerebrovascular diseases, such as atherosclerosis, hypertension and stroke (Guo et al., 1983). It has good therapeutic effects in preventing and treating ischemic cerebrovascular disease (Mu et al., 2014). Ligustrazine is an alkaloid monomer isolated and extracted from the Umbelliferae Szechwan lovage rhizome, and its molecular formula is $C_8H_{12}N_2$ (Li et al., 2011). The therapeutic effects of ligustrazine on ischemic brain disease have been verified in the clinic and in experiments (Liao et al., 2004).

Ligustrazine has been shown to suppress caspase-3 activation and cell apoptosis induced by middle cerebral artery occlusion-induced cerebral ischemia and to reduce the infarct volume after cerebral ischemia and reperfusion (Chang et al., 2007). Ligustrazine has anti-apoptotic effects by increasing bcl-2 expression and diminishing Bax expression in rabbits with spinal cord ischemia (Fan et al., 2006). It has also been shown to exert a protective effect on hypoxia- and excitotoxicity-induced neuronal injury in the hippocampus (Shih et al., 2006). The rate of elimination of ligustrazine is fast after intravenous administration. Its blood level is almost zero

after 120 minutes. Thus, intraperitoneal injection is the optimal method to perform ligustrazine studies. Clinical studies with ligustrazine have been increasingly valued by patients as its injection has had dramatic therapeutic effects on acute cerebral infarction and the recovery of neurological function (Wang et al., 2011, 2014; Wei et al., 2012). This has stimulated more clinical studies on this new therapeutic strategy and experiments on how ligustrazine acts in the treatment of ischemic cerebrovascular disease.

The mechanism of action of ligustrazine is very complicated. There has been very little discussion on the overall protective effects of ligustrazine on cerebral ischemia-induced ischemia/reperfusion injury. Reports mainly focused on one or other unilateral action mechanism. In view of the special physical characteristics of ligustrazine, variation of its administration time can alter its effect. We summarize the protective mechanisms, existing problems, time window of administration, administration mode, and clinical application of ligustrazine after cerebral ischemia/reperfusion injury. We hope a wider audience will appreciate the approach of traditional Chinese medicine as well as specifically providing valuable data for the clinical treatment of ischemic cerebrovascular disease.

Ligustrazine Inhibited the Release of Excitatory Amino Acids

Glutamate is the most common excitatory neurotransmitter with the strongest effects and widest distribution in the central nervous system. After depolarization in the nerve ending, excitatory amino acids in the synaptic vesicle are released into the synaptic cleft and bind to specific receptors on the postsynaptic membrane to perform a specific physiological function. When cerebral ischemia occurs, a large amount of glutamate is released into the extracellular fluid (Takano et al., 2001), resulting in nerve cell degeneration. This massive release of glutamate further aggravates secondary brain injury (Bullock et al., 1998).

Glutamate receptors are divided into two categories: (1) ionotropic receptors: either NNMDA, kainate, or AMPA receptors, all relatively fast-acting; and (2) metabotropic glutamate receptors: couple with G-protein in the membrane, exert effects by a cascade of reactions that modulate the excitation state *via* a second messenger, producing a slow physiological effect. Arcella et al. (2005) confirmed that voltage-dependent Ca^{2+} channels and metabotropic glutamate receptors play an important role in neuronal excitotoxicity.

Cerebral ischemia causes glutamate release from synaptic endings, activates ionotropic receptors (NMDA, kainate and AMPA receptors) in the postsynaptic membrane, leads to membrane depolarization, membrane excitability increase, cation channel opening, great Ca^{2+} influx, intracellular Ca^{2+} overload, and starts a series of enzyme-induced cell damage. Activated nitric oxide synthase catalyzes and produces an excess of nitric oxide and forms peroxynitrite ion and hydroxyl with a superoxide anion, which destroys mitochondrial membranes and opens mitochondrial permeability transition

pores, disrupting the ion balance (Gomi et al., 2000).

Ligustrazine and ionotropic receptors: ligustrazine enhances glutamate uptake by inhibiting glutamate biosynthesis and secretion, it suppresses Ca^{2+} influx-induced Ca^{2+} overload, and diminishes the injury of glutamate excitotoxicity to neurons (Fu et al., 2008). A previous study showed that kainic acid-induced slow neurotoxicity differed from the direct cytotoxic effects of glutamate and aspartic acid on neurons (Rothman and Olney, 1995). Shih et al. (2002) demonstrated that ligustrazine has a protective effect on kainate-induced excitotoxicity in hippocampal neurons by regulating the affinity and the number of kainate receptors. This blocks extracellular Ca^{2+} influx and intracellular Ca^{2+} release, maintaining mitochondrial membrane potential, reducing the generation of free radicals and increasing the intracellular free radical scavenging rate.

Ligustrazine and metabotropic glutamate receptors: Glutamate alters cell permeability by activating metabotropic glutamate receptors, induces a large amount of Na^+ and Cl^- into the cells, and results in cell swelling, necrosis and apoptosis (Lewen et al., 2000). Nevertheless, metabotropic glutamate receptor activity can differ by cell type and metabolic environment and the lack of a specific receptor antagonist results in the lack of a specific detection index in the study of ligustrazine, so a paucity of studies concerning metabotropic glutamate receptors points to a possible future direction of study.

Ligustrazine Promoted Migration, Differentiation and Proliferation of Endogenous Neural Stem Cells

Cerebral ischemia can induce the migration and differentiation of endogenous neural stem cells to the ischemic focus, where proliferating neuronal cells can replace necrotic cells. This is considered to be a compensatory and adaptive response of the body and is the key link in neural regeneration after cerebral ischemia (Ikeda, 2008), contributing to the recovery of neurological function.

Endogenous neural stem cells are mainly present in the subventricular zone and subgranular zone of the dentate gyrus of the hippocampus of adult brain tissue (Xiao et al., 2010; Du et al., 2014; Jiang et al., 2014). After cerebral ischemia, proliferated neural stem cells move to the ischemic lesion site, such as the olfactory bulb, cerebral cortex, dentate gyrus and corpus striatum, then differentiate into neuronal cells in those specific areas, and exert specific neurological functions.

Tanaka et al. (2004) demonstrated that early regenerating neural stem cells, which were primarily located in the subgranular zone of the dentate gyrus of the hippocampus of adult Mongolian gerbils with middle cerebral artery occlusion, had moved to the subgranular zone and expressed mature neuronal markers with identical functions as normal granular cells 30 days post injury. Nakatomi et al. (2002) labeled cells in the subventricular zone with DiI and labeled newly regenerating neural stem cells in the subventricular zone with 5-bromo-2′-deoxyuridine (BrdU) in models of

middle cerebral artery occlusion. Fourteen days after ischemia, DiI/BrdU-labeled cells were detected in the peri-infarct region of the corpus striatum or cortex, indicating that neural stem cells in the subventricular zone moved to the infarct region of corpus striatum or cortex (Nakatomi et al., 2002). Iwai et al. (2003) verified that newly regenerating neural stem cells in the subventricular zone moved to the olfactory bulb and differentiated into mature neuronal cells at 60 days after cerebral ischemia.

Regulating migration, differentiation and proliferation of endogenous neural stem cells can increase brain plasticity and improve the recovery of neurological function after cerebral ischemia. Xiao et al. (2010) measured the infarct area with 2,3,5-triphenyl-tetrazolium chloride assay and analyzed cell proliferation and differentiation using an immunohistochemical method in rat models of middle cerebral artery occlusion. Results showed that ligustrazine had a protective effect against cerebral ischemia/reperfusion injury in that it reduced the infarct volume. It contributed to the proliferation and differentiation of neural stem cells in the subventricular zone and subgranular zone of the hippocampal dentate gyrus by inhibiting the synthesis of neuronal nitric oxide synthase. Another study (Qiu et al., 2007) used an immunohistochemical method to observe the migration of doublecortin-labeled neuronal precursor cells in the subventricular zone and rostral migratory stream of rat models of left middle cerebral artery occlusion after cerebral ischemia/reperfusion injury. Results revealed that at 3 days after ischemia, doublecortinpositive cells generated in the subventricular zone migrated through the rostral migratory stream to the olfactory bulb for 21 days. At 7 days, doublecortin-positive cells generated in the subventricular zone migrated through the rostral migratory stream to the corpus striatum adjacent to the ischemic area, reaching a peak at 14 days. Twenty one days later, a few doublecortin-positive cells had migrated through the corpus callosum to the ischemic cortex. Compared with the ischemia model group, doublecortin-positive cell migration pathways were significantly enhanced in the subventricular zone of the ligustrazine group. The above findings suggest that ligustrazine promotes direct migration of neuronal precursor cells to the ischemic cortex and corpus striatum, and plays a protective effect on the recovery of brain function after cerebral ischemia.

Ma et al. (2008) confirmed that ligustrazine contributes to neural regeneration by stimulating brain-derived neurotrophic factor and fibroblast growth factor expression. An animal study demonstrated that ligustrazine could improve the microenvironment of cerebral ischemic lesions and promote endogenous neural stem cell migration by up-regulating the expression of vascular endothelial growth factor (VEGF), brain-derived neurotrophic factor and fibroblast growth factor (Chen et al., 2005). They also found that a guidance factor, Slit, and a large secreted extracellular matrix glycoprotein, Reelin, could alter the migration of neural stem cells (Zhang et al., 2005). A previous study (Gu et al., 2011) showed that Slit2 expression increases in models of focal middle cerebral artery occlusion, reaches a peak at 7 days, followed by a plateaus for 30 days, and then gradually declines. Their results demonstrated that Slit2 accelerates neural stem cells, generated in the subventricular zone, to migrate longitudinally to the olfactory bulb along the rostral migratory stream through the Robo2-RhoA pathway in the manner of repulsive guidance. Simultaneously, some neural stem cells migrate transversely to the cortex and corpus striatum, lateral to brain tissue along the corpus callosum, which has a protective effect on ischemic neurons. Courtes et al. (2011) used two lesion models of lysolecithin-induced focal demyelination of the corpus callosum, and unilateral cortical ischemia induced by thermocoagulation of pial arterioles, and demonstrated that Reelin expression is increased at the lesion site. Reelin contributed to the migration of neural precursor cells/neural stem cells to the lesion site through the subventricular zone-rostral migratory stream-olfactory bulb, and then protected the recovery of neurological function after ischemia. Nevertheless, the links between the protective action of ligustrazine on cerebral ischemia and the mechanisms of action of Slit and Reelin remain unclear.

Ligustrazine Accelerated Vascular Regeneration after Cerebral Ischemia

Cerebral ischemia-induced cerebral infarction is composed of a central necrotic zone where necrotic cells and apoptotic cells coexist and the ischemic penumbra where apoptotic cells are mainly found (Pan et al., 2013; Shen et al., 2013). Recent studies suggest that promoting the regeneration of vascular endothelial cells in the ischemic region is the key to accelerate neovascularization, to restore the blood supply in the ischemic lesion, to rescue dying neurons and glial cells, and to improve the recovery of neurological function after cerebral ischemia (Zhang et al., 2014c).

Angiogenesis is a complex process, and can be affected by many factors, including the vascular endothelium, vascular extracellular matrix, proteolytic enzymes, pro-angiogenesis factor and shear stress. Of them, the pro-angiogenesis factor involved in signal transduction has a crucial effect on the endothelial system and angiogenesis. There are more than 20 kinds of pro-angiogenesis factors, such as VEGF, basic fibroblast growth factor and angiopoietin. Marti et al. (2000) observed a noticeable increase in VEGF and its receptor expression in the ischemic penumbra in rat models of permanent middle cerebral artery occlusion. Shen et al. (2008) revealed many new vessels in the penumbra in mouse models of transient middle cerebral artery occlusion after VEGF expression was controlled, as detected by double-labeled immunostaining. This confirmed the importance of VEGF in the repair of blood vessels after ischemia. Issa et al. (2005), using a western blotting assay, found that basic fibroblast growth factor increases in the cerebral infarct region and ischemic penumbra of patients. They also showed that basic fibroblast growth factor mRNA is highly expressed in vascular endothelial cells in the ischemic penumbra, accompanied by the formation of new blood capillaries. It is assumed that basic fibroblast growth factor may be an important factor for angiogenesis after cerebral infarction.

Research has focused on investigating whether ligustrazine
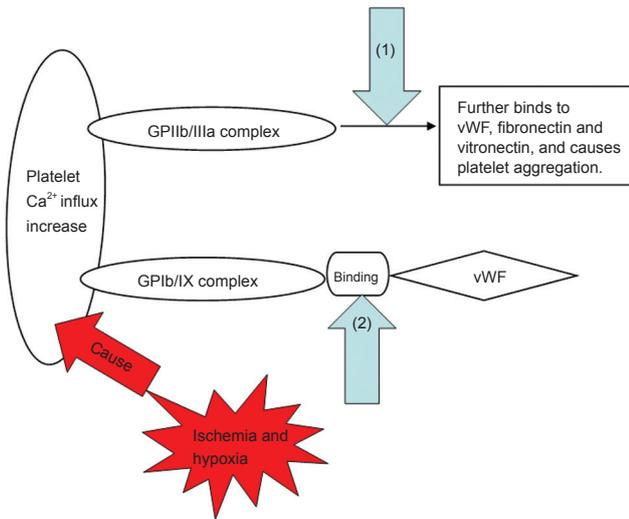
Figure 1 Targets for an inhibitory effect of ligustrazine on platelet aggregation.

Target (1): Inhibiting vWF factor binding to GPIb/IX complex; target (2): inhibiting GPIIb/IIIa complex binding to adhesion protein, which can form platelet aggregates. GP: Glycoprotein; vWF: von Willebrand factor.
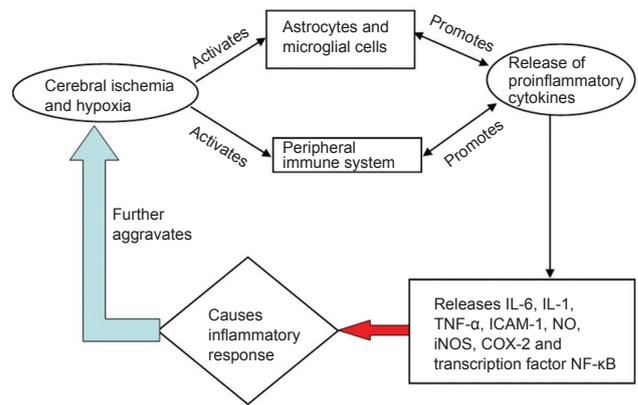
Figure 2 Inflammatory responses after cerebral ischemia.

IL: Interleukin; TNF-α: tumor necrosis factor-α; ICAM-1: intercellular adhesion molecule-1; NO: nitric oxide; iNOS: inducible nitric oxide synthase; COX-2: cyclooxygenase-2; NF-κB: nuclear factor κB.
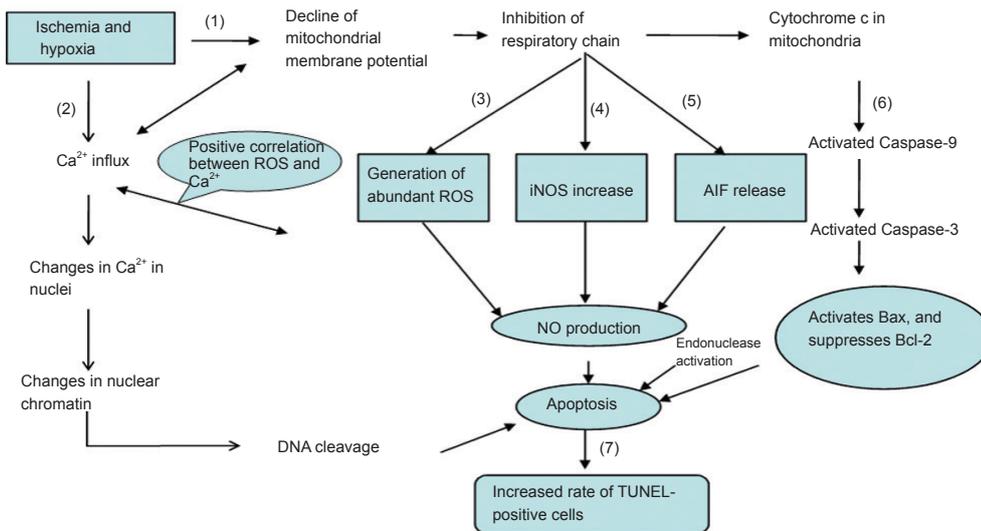
Figure 3 Targets of ligustrazine for cerebral ischemia.

Target (1): Slowing the decline in mitochondrial membrane potential; target (2): inhibiting Ca^{2+} influx; target (3): suppressing ROS production; target (4): inhibiting iNOS production; target (5): resisting AIF release; target (6): suppressing Caspase family and Bax, increasing Bcl-2; target (7): inhibiting the increased number of TUNEL-positive cells.

ROS: Reactive oxygen species; iNOS: inducible nitric oxide synthase; AIF: apoptosis-inducing factor; NO: nitric oxide; TUNEL: transferase-mediated deoxyuridine triphosphate-biotin nick end labeling.

had any effect on these angiogenesis factors. Zhang et al. (2014c) concluded that ligustrazine at a low concentration enhances VEGF expression in the mouse microvascular cell line bEnd.3 through the Fas-FasL pathway, and then accelerates angiogenesis. Ma et al. (2008) confirmed that ligustrazine could promote basic fibroblast growth factor expression in the brain tissue of rat models of severe brain injury, and has a protective effect on Nissl body and neuron functions. VEGF binding to its receptor facilitates angiogenesis and increases vascular permeability, and induces endothelial cell proliferation and neovascularization to improve the blood supply in ischemic brain tissue. However, VEGF simultaneously increases vascular permeability that may increase the local cerebral edema and aggravate brain tissue necrosis in the ischemic area. These recent studies confirm that the angiopoietin family is of great significance in balancing angiogenesis and permeability. Angiopoietin-1 binding to its Tie-2

receptor plays a key role in late-stage angiogenesis. It recruits surrounding support cells, promotes the complete formation of the vascular wall by antagonizing endothelial cell apoptosis and promoting endothelial cell sprouting and branching (Kim et al., 2000). This lessens the increased vascular permeability induced by VEGF and relieves blood-brain barrier leakage (Iivanainen et al., 2003). The angiopoietin-1/Tie-2 system and VEGF/VEGF receptor system play a cooperative and complementary role in neovascularization following cerebral ischemia/reperfusion injury. The above findings suggested that ligustrazine could exert an effect on angiogenesis through VEGF after cerebral ischemia. There are no reports addressing possible effects of ligustrazine on blood-brain barrier leakage due to VEGF-induced vascular permeability increase. An increase in blood-brain barrier permeability will aggravate regional brain edema that would delay the recovery of neurological function after ischemia. Angiopoietin

antagonizes cerebral edema caused by vascular permeability increase. Thus, it is necessary to clarify the relationship between ligustrazine and angiopoietin and how they act during cerebral ischemia to fully understand the role of ligustrazine in angiogenesis after cerebral ischemia.

Ligustrazine Suppressed Platelet Aggregation and Resists Thrombosis

Blood is in a hypercoagulable state after cerebral ischemia, which encourages the formation of platelet thrombi. Platelet activation and thrombus formation directly participate in the occurrence and development of cerebral ischemia (Okada et al., 1994; Siesjo et al., 1995; Serebruany et al., 2004).The aggregation and adhesion of activated platelets are important stages of thrombosis, which may result in secondary brain injury (Gachet et al., 1997). Thus, inhibiting thrombosis by suppressing platelet aggregation could protect post-ischemic brain injury.

The most abundant platelet membrane glycoprotein is the adhesion receptor glycoprotein IIb/IIIa (GPIIb/IIIa) complex which is involved in platelet aggregation and thrombosis. After platelet activation induced by cerebral ischemia, various adhesion proteins (including fibrinogen, von Willebrand factor (vWF), fibronectin, and vitronectin) and various inducers (such as thrombin, collagen, and thromboxane A2) bind to receptors on the platelet, which change the GPIIb/IIIa steric configuration, which results in platelet activation, aggregation, and release. GPIIb/IIIa has become a molecular marker of platelet activation (Gleerup et al., 1993).

The platelet glycoprotein GPIb/IX complex mainly binds to vWF. This binding to GPIb/IX leads to intracellular Ca^{2+} increase, and activates GPIIb/IIIa to become the receptor of vWF, leading to platelet aggregation (Goto et al., 1995). GPIb binding to vWF exerts an activating effect, and GPIIb/IIIa binding to vWF is necessary to form the stable platelet aggregation (Ikeda et al., 1993). Li et al. (2001) considered that ligustrazine reduces shear-induced platelet aggregation, at a high shear rate of 10,800/s, by inhibiting the binding of vWF to GPIbα and GPIIb/IIIa.

Ligustrazine suppresses platelet aggregation at two targets after cerebral ischemia (**Figure 1**), which provides new treatment ideas and concepts for the early treatment of ischemic encephalopathy. However, many previous studies have focused mainly on *in vitro* experiments, which do not account for environmental factors. There have been very few studies examining the interaction of ligustrazine and platelet aggregation *in vivo* after cerebral ischemia. So far, studies on the effects of ligustrazine on platelet activation and ischemia-induced platelet release remain unclear. Clarifying the role and mechanisms of ligustrazine would provide valuable references for its multidisciplinary application in the clinic.

Ligustrazine Could Inhibit the Release of Inflammatory Cytokines and Further Inhibit the Inflammatory Response

The inflammatory response is a major reason for sustained brain damage after cerebral ischemia (Zheng and Yenari, 2004), especially during cerebral ischemia and reperfusion. Controlling the cascade of reactions of inflammation after ischemia (**Figure 2**) is a key strategy of relieving cerebral ischemia/reperfusion injury (Liao et al., 2001).

Microglia and astrocytes are activated after cerebral ischemia. Proliferated microglia and astrocytes release a large number of proinflammatory mediators and neurotoxic molecules, which participate in the inflammatory response induced by cerebral ischemia (Chen and Swanson, 2003). In turn, these inflammatory mediators can stimulate the activation and proliferation of microglia and astrocytes, which elevate the inflammatory response, forming a positive feedback loop (Barone and Feuerstein, 1999). Liao et al. (2004) clarified that ligustrazine suppresses the activation, migration, and aggregation of microglia (labeled by ED-1), astrocytes (labeled by glial fibrillary acidic protein), and other inflammatory cells (labeled by myeloperoxidase), playing a protective role after cerebral ischemia/reperfusion injury.

Chang et al. (2007) verified that ligustrazine inhibits the expression of hypoxia-inducible factor-1α and tumor necrosis factor-α after cerebral ischemia, suppresses cell apoptosis, and has a protective effect on neurons. Nuclear factor κB is a key transcription factor, involved in the inflammatory response after ischemia. Cells stimulated by hypoxia and ischemia trigger a series of signal transductions, cause nuclear factor κB inhibitor IκB phosphorylation degradation, and activate nuclear factor κB to enter nuclei and stimulate target gene transcription. These trigger positive feedback, finally leading to an overwhelming inflammatory response, and accelerate cerebral ischemia/reperfusion injury. Recent studies demonstrated that DNA binding activity of nuclear factor κB is dramatically enhanced within 6–12 hours after ischemia, and gradually declines between 24–72 hours. This indicates that NF-κB translocation occurs in a time-dependent manner after cerebral ischemia (Kunz et al., 2008; Rodriguez-Yanez and Castillo, 2008). Ligustrazine suppresses the production of lipopolysaccharide-induced nitric oxide and inducible nitric oxide synthase. It thus plays a protective effect by inhibiting nuclear factor κB activation, mitogen-activated protein kinase and serine threonine kinase (Akt) phosphorylation and suppressing the formation of intracellular reactive oxygen species (Liu et al., 2010). Ligustrazine has been shown to downregulate the expressions of tumor necrosis factor-α and intercellular adhesion molecule-1 by inhibiting nuclear factor κB activation. Thus, it can lessen the inflammatory response after cerebral ischemia/reperfusion injury, and plays a protective role (Wu et al., 2012).

Interleukin-1β, a common inflammatory factor, is an endogenous pyrogen that stimulates the expressions of other proinflammatory mediators by facilitating its own activation. It activates microglia and astrocytes, increases the tolerance of neuronal cells to hypoxia, accelerates the expression of adhesion molecules, chemokines and E/P-selectin on vascular endothelial cells, and destroys the blood-brain barrier. Zhu et al. (2011) used real-time fluorescence PCR in rat models of cerebral ischemia/reperfusion injury and verified

Table 1 Precise studies concerning the targets of ligustrazine for cerebral ischemia published from 2000 to 2015

Targets for inhibitory effect	Action mode after cerebral ischemia	Author and publication year
Receptor and ion channels	(1) Ionic Ca^{2+} channel: inducing an increase in Ca^{2+} influx. (2) Receptor-dependent cation channel: glutamate binding to ionic N-methyl aspartate receptor leads to the increase in Ca^{2+} influx.	Bie et al., 2006; Wan et al., 2007; Fu et al., 2008; Tan, 2009; Li et al., 2010; Shao et al., 2010; Tang et al., 2012; Chen et al., 2013
Nitric oxide or inducible nitric oxide synthase	Inducible nitric oxide synthase catalyzes the production of nitric oxide from L-arginine under oxidative stress. Inducible nitric oxide synthase is the only rate-limiting enzyme of nitric oxide synthesis.	Hsiao et al., 2006; Liu et al., 2010; Yang et al., 2012; Zheng et al., 2013
Nitric oxide synthase	(1) Mitochondrial transmembrane potential decline; mitochondrial respiratory chain produces massive active nitric oxide synthase to oxidize cardiolipin on inner mitochondrial membrane, finally induces apoptosis. (2) Nitric oxide synthase is positively correlated with Ca^{2+}.	Shih et al., 2002; Lee et al., 2005; Kang et al., 2009; Li et al., 2010; Liu et al., 2010; Ou et al., 2010
Mitochondrial membrane potential	Ischemia, hypoxia and oxidative stress decrease the mitochondrial transmembrane potential.	Shih et al., 2002; Li et al., 2010; Xu et al., 2014
Caspase-3	(1) Intracellular Ca^{2+} accumulated. Cytochrome C released by mitochondrion binds to Caspase-9 precursor, and forms apoptosome complexes with the presence of adenosine triphosphate. Thus, Caspase-3 is activated and subsequent Caspase cascade reaction is triggered. (2) Intracytoplasmic Ca^{2+} accumulation causes the changes in Ca^{2+} levels in the nuclei. The latter interacts with DNA and genes, which results in the changes in chromatin structure and activates endonuclease, and finally leads to DNA cleavage.	Zhang et al., 2003; Kao et al., 2006; Chang et al., 2007; Tang et al., 2012; Yang et al., 2012
Bax, Bcl-2	Proteolysis mediated by caspases family makes Bcl-2 inactive and activates Bax.	Tang et al., 2012; Yang et al., 2012; Kao et al., 2006; Ding et al., 2013; Cheng et al., 2007; Gong et al., 2014; Zhang et al., 2014a, b
TUNEL-positive neuronal cells	Mitochondria releases apoptosis-inducing factor, which may be a hydrolase. Activated hydrolase moves in the cytoplasm, and cuts DNA into small pieces.	Yang et al., 2005; Tang et al., 2012; Ding et al., 2013

TUNEL: Transferase-mediated deoxyuridine triphosphate-biotin nick end labeling.

that ligustrazine could suppress interleukin-1β expression and protect brain tissue against ischemia.

Intracellular adhesion molecule-1 and cyclooxygenase-2 are targets of ligustrazine for treating ischemia/reperfusion injury. Wu et al. (2012) confirmed that ligustrazine apparently suppresses intracellular adhesion molecule-1 expression and protects human umbilical vein endothelial cells.

Ligustrazine has an inhibitory effect on the migration of inflammatory cells towards the ischemic zone and the expression of inflammatory factors. However, there are no reports on any anti-inflammatory effects by ligustrazine on interleukin-6, intracellular adhesion molecule-1 and cyclo-oxygenase 2 *in vivo* during cerebral ischemia/reperfusion injury. These provide a new direction for future studies.

Ligustrazine Inhibited Apoptosis

Apoptosis is the process of programmed cell death (Wood and Bristow, 1998). Apoptosis is strongly associated with cerebral ischemia/reperfusion injury (Kao et al., 2006). Inhibiting apoptosis can lessen the degree of ischemic brain injury and has a protective effect on brain tissue (Kao et al., 2006). In recent years, the studies regarding the inhibitory effect of ligustrazine on apoptosis have become a hot topic; its effect can act on a number of targets (**Figure 3**).

Ischemic brain injury is a complex pathophysiological process (Zheng et al., 2014). Ligustrazine protects neurons in many ways, such as slowing the decrease in mitochondrial

membrane potential, lessening mitochondrial damage, inhibiting Ca^{2+} influx, suppressing the production of reactive oxygen species, inducible nitric oxide synthase and nitric oxide, suppressing caspase family expression, suppressing Bax, promoting Bcl-2 expression, and suppressing the number of TUNEL-positive neurons (summarized in **Table 1**). The above studies suggest that ligustrazine suppresses cerebral ischemia-induced apoptosis. We will focus on the mitochondrial membrane potential to thoroughly investigate the mechanisms of action of ligustrazine on anti-apoptosis following cerebral ischemia.

Time Window of Administration and Administration Mode

Middle cerebral artery occlusion has been the most commonly used model for cerebral ischemia/reperfusion injury (Longa et al., 1989; Sun et al., 2014). There has been a gradual realization of the protective effect of ligustrazine against cerebral ischemia. Nevertheless, due to the special physical characteristics of ligustrazine, it is necessary for the correct administration mode and time window to be ascertained for clinical and experimental studies.

Cai et al. (1989) analyzed the pharmacology of ligustrazine using high-performance liquid chromatography on samples from six healthy volunteers after they took a ligustrazine capsule (174.5 mg) orally. Results showed that ligustrazine could be rapidly absorbed and distributed all over the body,

but would be scavenged very quickly. Its bioavailability is low. Thus, oral administration is not ideal in the study addressing biological effects of ligustrazine. Tsai and Liang (2001) selected intravenous administration of ligustrazine (10 mg/kg) in Sprague-Dawley rats. High-performance liquid chromatography-infrared method demonstrated that blood drug level reached a peak at 10–20 minutes, but was rapidly eliminated; by 120 minutes, ligustrazine could not be detected (Tsai and Liang, 2001). Therefore, intravenous administration is also not fit for our study. Other studies (Tsai and Liang, 2001; Liao et al., 2004) confirmed that intraperitoneal injection is convenient to perform. The area of peritoneum is large. The peritoneum is covered by blood vessels and lymphatic vessels, and its absorptive capacity is strong. Moreover, the time of intraperitoneal fluid infusion is rapid and short, so it places little load on the heart. Intraperitoneal injection has been the ideal administration mode in the study of protective effects of ligustrazine in Sprague-Dawley rats with cerebral ischemia (Tsai and Liang, 2001; Liao et al., 2004). There has been active research recently into the pharmacokinetics of ligustrazine with the analysis of percutaneous administration (Zhao et al., 2011) and nasal administration (Zhu et al., 2009) using microdialysis, which provides a new method for clinical administration.

Zhu et al. (2009) and Jia et al. (2009) selected intraperitoneal administration of ligustrazine 20 mg/kg at 1, 2, 4, and 6 hours after reperfusion in male Sprague-Dawley rat models of transient focal cerebral ischemia. Transient focal cerebral ischemia was produced by right middle cerebral artery occlusion for 120 minutes, followed by 72-hour reperfusion, as described previously (Longa et al.,1989). Results demonstrated that the protective effect of ligustrazine against ischemia and reperfusion was in a time-dependent manner. The protective effect peaked at 4 hours, and then gradually declined, and disappeared at 6 hours, suggesting that the use of ligustrazine at an early stage of cerebral ischemia best reflects its protective effect. Derivatives of ligustrazine such as TBN (Sun et al., 2012), CXC137 (Chen et al., 2014) and CXC195 (Zhang et al., 2014b) have better effects than ligustrazine in regard to protective effects and the time window of administration, but still reserve their pharmacological properties associated with the pyrazine ring. These derivatives provide new opportunities for future studies.

However, the mechanisms underlying neuronal cell injury induced by cerebral ischemia involve multiple factors and pathways that will impact the pathophysiological process and final results (Kao et al., 2006). Thus, we should further investigate the time window of administration.

Application in the Clinic

In recent years, ligustrazine has been widely used in the clinic with the gradual recognition of its mechanism of action. Ran et al. (2014) evaluated the prognosis of early cerebral ischemia in elderly patients after intracranial aneurysm clipping using the Glasgow Outcome Scale and Chinese Stroke Scale. Results revealed that vascular resistance was significantly lower, cardiac output, cardiac index, and stroke volume index were better in the administered group compared with the conventional group; scores of Glasgow Outcome Scale and Chinese Stroke Scale were significantly higher in the administered group than in the conventional group. Patients' conditions improved to different degrees. These findings indicate that ligustrazine has an obvious protective effect against cerebral ischemia. At present, clinical effects of ligustrazine lack some specific detection indicators, which could provide a reference and guidance for our future investigations.

Conclusions

The protective effect of ligustrazine against ischemic brain injury, especially ischemia and reperfusion injury has been gradually recognized in the wider world. Numerous studies have shown that ligustrazine is effective, safe, and non-toxic in the treatment of cerebral ischemia. Its limitation of the effective time window of administration may be overcome by some derivatives of ligustrazine.

A deep understanding of the pharmacology of ligustrazine action on ischemic brain injury, especially ischemia/reperfusion injury, will provide valuable clinical guidelines in the prevention and treatment of ischemic brain disease, in promoting the recovery of neurological function after cerebral ischemia, and improving its prognosis.

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