

Experimental therapy with tissue kallikrein against cerebral ischemia

Julie Chao, and Lee Chao

Department Of Biochemistry And Molecular Biology, Medical University Of South Carolina, Charleston, South Carolina 29425, USA

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1. ABSTRACT

Tissue kallikrein is a serine proteinase capable of cleaving kininogen substrate to produce the potent vasodilator kinin peptide. Kinin mediates a complex set of physiological actions through its receptor signaling. Systemic delivery of the kallikrein gene in an adenoviral vector significantly reduced stroke-induced mortality rate, blood pressure elevation, and aortic hypertrophy in hypertensive Dahl-salt sensitive rats fed a high salt diet. Using a focal cerebral ischemic rat model induced by middle cerebral artery occlusion, intravenous or intracerebroventricular kallikrein gene delivery significantly reduced ischemia/reperfusion (I/R)-induced neurological deficits, cerebral infarction, neuronal and glial cell apoptosis, and inflammatory cell infiltration, while promoting angiogenesis and neurogenesis in the ischemic brain. A continuous infusion of a sub-depressor dose of tissue kallikrein protein through implanted minipump decreased I/R-induced neurological dysfunction and cerebral infarction, inflammation and oxidative stress independent of kallikrein's blood pressure-lowering effect. Moreover, kallikrein offered neuroprotection even when delivered at one day after the onset of stroke. Kallikrein's protective effects were blocked by the kinin B2 receptor antagonist icatibant. The role of the kinin B2 receptor in mediating the protective effect against ischemic brain injury was further confirmed by increases in mortality rate and post-ischemic brain injury in kinin B2 receptor-deficient mice. Taken together, these results suggest a novel function of kallikrein as an anti-inflammatory and anti-oxidative agent in protecting the brain against ischemic stroke-induced injuries. These findings also raise the possibility that tissue kallikrein may have value in the treatment of acute ischemic stroke.

2. INTRODUCTION

Ischemic stroke is the third leading cause of death in the United States and is frequently associated with long-term disability (1). Currently, few therapeutic options are available for the treatment of ischemic stroke and consist mainly of agents that block platelet aggregation or the coagulation cascade (2). Although anti-platelet agents are effective in decreasing the incidence of ischemic stroke, these agents do not reduce cerebral infarct size (2). Stroke-induced neurological deficits and/or mortality are often associated with the timing of treatment after the onset of stroke. The therapy currently approved for the treatment of acute ischemic stroke is intravenous administration of recombinant tissue-type plasminogen activator (tPA) initiated within 3 hours of symptom onset (3). There have been many other clinical trials of thrombolytic drugs and neuroprotective drugs beyond the 3-hour time window, but none of these trials achieved significant improvement over existing procedures. Therefore, the search for suitable regimens to rescue the central nervous system after ischemia has been a major research endeavor. In order to prevent or reduce ischemic brain damage, the development of new interventions for acute stroke therapies is necessary to meet the large need for this important and under-treated disorder.

3. THE TISSUE KALLIKREIN-KININ SYSTEM

All mammals studied thus far have two types of kallikreins: plasma kallikrein and tissue kallikrein, which differ in primary structure, biochemical properties, immunological character, substrate specificity, site of synthesis, and biological function (4). Tissue kallikrein from rat or human processes low molecular weight

kininogen to produce the potent vasoactive kinin peptides bradykinin (BK) or Lys-BK (kallidin), respectively. Kinin is then cleaved by kininase I or II. Intact kinin binds to the kinin B2 receptor, whereas kinin metabolites, such as des-Arg9-BK or des-Arg10-kallidin, bind to the kinin B1 receptor (5). The B2 receptor is constitutively expressed whereas the B1 receptor is expressed at very low levels under normal conditions and is induced by inflammation or under stress (5). Activation of the kinin receptor increases intracellular Ca²⁺ mobilization as well as prostacyclin and cAMP formation. Increased Ca²⁺ enhances endothelial nitric oxide synthase (eNOS) activity and nitric oxide (NO) production by binding of Ca²⁺/Calmodulin (CaM) to the CaM-binding domain of eNOS (6). Activation of Akt by kinin can also lead to Ca²⁺-independent activation of eNOS and thus increased NO and cGMP levels (7, 8). Additionally, cAMP has been shown to enhance NO-cGMP production (9). Activation of kinin receptors modulates a broad spectrum of cellular functions including smooth muscle cell contraction or relaxation, increased vascular permeability, chloride secretion and cell proliferation (4). The system can be blocked at several steps. The B2 receptor can be blocked by the specific B2 receptor antagonist icatibant (also known as Hoe140), while the B1 receptor can be blocked by the specific B1 receptor antagonists des-Arg9-Leu8-BK or des-Arg10-Leu9-kallidin. Kininase II is a dipeptidase and is the same enzyme as angiotensin-converting enzyme (ACE). Therefore, ACE inhibitors not only inhibit conversion of angiotensin I to angiotensin II, but also reduce kinin degradation which leads to kinin accumulation. The beneficial effects of ACE-inhibitor treatment may in part be attributed to elevated levels of kinin.

4. BRAIN KALLIKREIN-KININ SYSTEM IN BLOOD PRESSURE REGULATION

The kallikrein-kinin system components are present in the brain, suggesting a physiological relevance in the central nervous system (10-12). We have shown that anti-sense inhibition of the endogenous kininogen and B2 receptor mRNA increased blood pressure of spontaneously hypertensive rats (SHR), indicating a role of kinin B2 receptor in central regulation of the cardiovascular system (13). In contrast, anti-sense inhibition of the B1 receptor mRNA in the brain of these rats caused blood pressure reduction (14). Moreover, we have demonstrated that enhanced kallikrein/kinin levels by intracerebroventricular (ICV) injection of the human tissue kallikrein gene into rats caused a significant and prolonged blood pressure reduction (15). Taken together, these results indicate that the brain KKS, through the kinin B1 and B2 receptors, plays a role in the central regulation of blood pressure.

5. TISSUE KALLIKREIN-KININ SYSTEM IN HEMORRHAGIC STROKE

There are two types of stroke: pressure-induced hemorrhagic stroke and ischemic stroke. Hypertension is a critical factor in the development of hemorrhagic stroke in humans. In stroke-prone spontaneously hypertensive rats (SHR-SP), high-salt intake accelerates the development of

malignant hypertension (16). In the brains of SHR-SP, fibrinoid necrosis and associated thrombosis primarily affect cerebral arterioles, leading to their obstruction and infarction, whereas cerebral hemorrhage is caused by microaneurysms (17). A recent study showed that a high dose of cerivastatin, a 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitor (collectively known as statins), protected against hypertension-induced stroke and ameliorated stroke-associated symptoms, which was accompanied by reduced superoxide production and inflammatory cell infiltration to the stroke lesion in SHR-SP (18). Lovastatin and simvastatin have also been shown to reduce brain injury during cerebral ischemia via up-regulation of eNOS (19). We showed that a single injection of adenovirus carrying the human tissue kallikrein gene in Dahl salt-sensitive (DSS) rats fed a high salt diet at an early age significantly reduced blood pressure elevation, stroke-induced mortality, hemorrhages and aortic hypertrophy, which was accompanied by increased cGMP levels, an indicator of NO formation (20). These findings indicate that kallikrein, through NO/cGMP formation, may play a protective role in pressure-induced stroke.

6. NEUROPROTECTION BY LOCAL DELIVERY OF THE KALLIKREIN GENE

Focal brain ischemia is the most common event leading to ischemic stroke in humans. We showed that ICV delivery of adenovirus containing the kallikrein gene in rats after middle cerebral artery occlusion (MCAO) for 60 minutes reduced locomotor deficit scores and cerebral infarction (21). Kallikrein gene delivery also markedly reduced I/R-induced apoptosis and enhanced the survival and migration of glial cells into the ischemic penumbra and core. Similarly, in primary cultured glial cells, kinin stimulated cell migration, but inhibited hypoxia/reoxygenation-induced apoptosis; these effects were blocked by icatibant. The ability of kallikrein gene delivery to enhance cell survival is most likely due to reduced reactive oxygen species (ROS) formation and caspase-3 activation, as well as increased NO levels and Akt phosphorylation. These results indicate that kallikrein gene delivery protects against cerebral injury after ischemic stroke by promoting glial cell survival and inhibiting apoptosis through suppression of oxidative stress and activation of the Akt signaling pathway.

7. SYSTEMIC KALLIKREIN GENE DELIVERY PROTECTS AGAINST ISCHEMIC BRAIN INJURY

Kallikrein gene delivery via the ICV route protects against ischemic brain injury (21). However, mechanical trauma due to direct injection of the gene into the brain may exacerbate the ischemic cerebral damage after I/R surgery. We thus employed a more practical approach by intravenous injection of the kallikrein gene. Our results showed that intravenous kallikrein gene delivery at 8 hours after I/R significantly reduced I/R-induced neurological deficit scores, cerebral infarction and inflammatory cell accumulation, while promoting the proliferation of endothelial and neuronal cells in the ischemic brain. Kallikrein gene delivery resulted in

increased cerebral NO levels and Akt phosphorylation, as well as reduced NADPH oxidase activity and superoxide production. Kallikrein's effects were blocked by icatibant. These results show that systemic kallikrein gene delivery after cerebral I/R can protect against ischemic brain injury by inhibiting inflammation and promoting angiogenesis and neurogenesis. Like local gene delivery, the protective effects of systemic kallikrein gene delivery are mediated by suppression of oxidative stress and activation of the Akt signaling cascades.

8. DIRECT NEUROPROTECTIVE EFFECT OF KALLIKREIN VIA PROTEIN INFUSION

Infusion of tissue kallikrein at a sub-depressor dose via minipumps implanted under the skin of rats after MCAO can achieve a continuous and stable supply of kallikrein. Use of osmotic minipumps enabled us to control the precise time and rate of tissue kallikrein infusion for a defined period of time, thus allowing us to determine the appropriate time window to administer tissue kallikrein following MCAO. Kallikrein protein infusion, at one day after the onset of ischemic stroke, reduced cerebral I/R-induced locomotive deficit scores, cerebral infarct size and inflammation without affecting blood pressure. Kallikrein's protective effects were mediated by the kinin B2 receptor as icatibant abolished these effects. These results showed that delayed infusion of exogenous kallikrein/kinin has a direct effect on neurological recovery from stroke-induced locomotor disability independent of its blood pressure-lowering effect.

9. ROLE OF KININ B2 RECEPTOR IN NEUROPROTECTION AGAINST ISCHEMIC STROKE

The role of kinin B2 receptor in mediating kallikrein's protective effects in ischemic brain injury was further verified using kinin B2 receptor-deficient mice. Infarct volume, neurological deficit scores and apoptosis in kinin B2 receptor-deficient mice were more pronounced than wild-type mice after cerebral I/R. The accumulation of neutrophils in the ischemic brain of B2 receptor-deficient mice decreased at 1 day but increased at 3 days after MCAO when compared with wild-type mice. This result indicates that the B2 receptor has a detrimental effect in increasing inflammatory cell infiltration in the early stage of ischemic stroke, but has a protective effect in the later stage. In addition, the survival rate of kinin B2 receptor-deficient mice was significantly reduced at 7 days after MCAO when compared with wild-type mice. Interestingly, our results are in contrast to a recent study that implicates a role for the B2 receptor in reducing survival after MCAO (22). The reason for this discrepancy is not clear at the present time, yet one explanation may be that we used four times more animals in the survival rate study compared to theirs. However, our results are consistent with those of Groger *et al.* in regards to increased brain edema in kinin B2 receptor-deficient mice 24 hours after MCAO (22). Therefore, there is strong evidence for the role of the kinin B2 receptor in inducing vascular permeability in the early

stages after I/R injury. Moreover, we have demonstrated that these alterations in kinin B2 receptor-deficient mice one week after MCAO were associated with reduced Akt phosphorylation, as well as increased NADH oxidase activity and superoxide anion formation. Increased mortality and post-ischemic brain injury in kinin B2 receptor-deficient mice support a role of the kinin B2 receptor in protection against ischemic cerebral injury.

10. NEUROPROTECTIVE EFFECT OF DELAYED KALLIKREIN GENE DELIVERY OR PROTEIN INFUSION

Stroke-induced neurological deficits and mortality are often associated with timing of treatment after the onset of stroke. Previous studies have shown that cerebral I/R injury induced by MCAO may disrupt and open up the blood-brain-barrier (BBB) by increasing cerebral vascular permeability for up to 72 hours (23, 24). Therefore, systemic delivery of therapeutic foreign gene or protein may penetrate through the BBB and reach the damaged area of the brain to exert protective actions against cerebral vascular insult. Tissue plasminogen activator is effective for the treatment of stroke if administered intravenously within 3 hours after the onset of stroke (3). However, our studies showed that delayed intravenous kallikrein gene delivery at 8 hours and 3 days after the onset of ischemic stroke reduced neurological dysfunction, cerebral infarction and inflammation, as well as promoting cell survival in the ischemic brain (Table 1). Similarly, delayed kallikrein protein infusion at one day after ischemic injury also exerted neuroprotection (Table). These findings have uncovered important new information regarding the time window of kallikrein gene delivery or kallikrein protein infusion in the treatment in acute ischemic stroke.

11. NOVEL ROLES OF KALLIKREIN AND KININ AS ANTI-INFLAMMATORY AND ANTI-OXIDATIVE AGENTS

It is intriguing to observe that kallikrein gene delivery or kallikrein protein infusion reduced I/R-induced inflammatory cell accumulation. Our data indicate that kallikrein/kinin may function as an anti-inflammatory agent in protection against I/R-induced injury. Our results appear to contradict previous findings regarding the role of kinin as a pro-inflammatory agent (25). This discrepancy may be attributed to the amounts of kinin produced locally and the distinct inflammatory roles of kinin receptors in the acute versus chronic phases. Previous studies have shown that early activation of the kallikrein-kinin system after cerebral ischemia increased brain vessel permeability, edema and spread of the ischemic lesion (26, 27). Also, an early administration of a kinin B2 receptor antagonist improved neurological recovery after focal cerebral I/R (28). Thus it appears that kinin aggravates cerebral injury at an early stage by promoting edema. However, kinin may have a protective role in the later stages, as indicated by our recent studies. Our results showed that delayed kallikrein protein infusion at one day after the onset of ischemic stroke reduced infarct size and decreased inflammatory cell

Table 1. Time windows of kallikrein administration in protection against ischemia brain injury before and after middle cerebral artery occlusion

	Kallikrein gene delivery			Kallikrein protein infusion
	-2 Days	8 Hours	72 hours	24 Hours
Infarct Size	↓	↓	↓	↓
Neurological Deficit	↓	↓	nd	↓
Apoptosis	↓	↓	↓	↓
Inflammation	nd	↓	nd	↓
Angiogenesis	nd	↑	nd	nd
Neurogenesis	nd	↑	nd	nd
Oxidative Stress	↓	↓	↓	↓

↑: increased, ↓: decreased, nd: not determined

accumulation in the ischemic brain. Similarly, a recent study showed that delayed treatment of VEGF exerted neuroprotection after focal cerebral ischemia, whereas early administration of VEGF induced edema (29, 30). A possible explanation for the early versus late interventions is that the timing of brain inflammation occurs within several days/weeks after stroke, including early injury and late post-ischemic repair processes (31). The early inflammatory response contributes to brain damage after I/R injury. Therefore, late intervention after the onset of the injury with exogenous kallikrein/kinin could exert neuroprotection by facilitating repair and recovery of regeneration processes through suppression of oxidative stress and inflammation.

12. FUTURE ASPECT OF TISSUE KALLIKREIN IN STROKE THERAPY

Using gene delivery and protein infusion approaches, we have shown that delayed kallikrein treatment 2 days before ischemic injury, or 8 hours, 1 and 3 days after the onset of ischemic stroke can improve neurological function (Table). Kallikrein, through the kinin B2 receptor, exhibited beneficial effects including the reduction of stroke-induced mortality, cerebral infarct size and neurological deficit scores independent of its blood pressure-lowering effect. Enhanced kallikrein/kinin levels by kallikrein gene delivery or protein infusion also promoted neurological recovery and cell survival by reducing apoptosis and inflammation, and promoting angiogenesis and neurogenesis in the ischemic brain (Table). These results indicate that tissue kallikrein may have a significant therapeutic value for the treatment of acute ischemic stroke.

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Send correspondence to: Dr Julie Chao or Lee Chao, Department of Biochemistry and Molecular Biology, Medical University of South Carolina, 173 Ashley Avenue, PO Box 250509, Charleston, SC 29425, Tel: 843-792-9927, Fax: 843-792-4850, Email: chaoj@musc.edu or chaol@musc.edu

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