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Project Details

Project Number :	777611
Project Title(English) :	Molecular regulations of reactive nitrogen species inhibitors for preventing thrombolysis-induced blood brain barrier disruption and hemorrhagic transformation in experimental cererbal ischemia-reperfersion models
Project Title(Chinese) :	活性氮自由基抑制劑對缺血性中風溶栓治療腦出血並發症的分子調節研究
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Panel :	Biology & Medicine
Subject Area :	Medicine, Dentistry & Health
Exercise Year :	2011 / 12
Fund Approved :	890,000
Project Status :	Completed
Completion Date :	30-9-2014
Project Objectives :	Objective 1: To test the hypothesis that RNS inhibitors can alleviate t-PA-induced MMPs activations, BBB disruption and hemorrhagic transformation in cerebral ischemia-reperfusion injury Objective 2: To test the hypothesis that cav-1 is a critical target protein of the RNS inhibitors on reducing the BBB disruption and hemorrhagic transformation in the experimental ischemic stroke model with thrombolytic treatment Objective 3: To test the effects of a natural antioxidant on the production of RNS, the expression of cav-1, the activations of MMPs, the BBB permeability and hemorrhagic transformation in the experimental ischemic stroke model with the delayed t-PA treatment.

Abstract as per original application (English/Chinese):	Stroke is the leading cause of disability and the secondary killer in human diseases worldwide. Tissue-plasminogen activator (t-PA) is the only FDA-approved thrombolytic drug for acute ischemic stroke. Early recanalization after t-PA infusion can improve patient outcome. However, systemic thrombolysis with t-PA administered beyond the 3 hour therapeutic window after ischemic stroke carries a potential risk of hemorrhagic transformation and potentiates neuronal damage. The activation of matrix metalloproteinases (MMPs), a proteolytic zinc-containing enzyme family, and the induction of blood brain barrier (BBB) hyper-permeability are critical pathological processes in hemorrhagic transformation during thrombolytic treatment for acute ischemic stroke. Rebuilding circulation by thrombolysis is accompanied by cerebral ischemia-reperfusion injury. Ischemia-reperfusion produces free radicals including reactive oxygen species (ROS) and reactive nitrogen species (RNS). As the major components of RNS, nitric oxide (NO) and peroxynitrite (ONOO-) can activate MMPs, attack tight junction-associated proteins and increase BBB permeability. Since RNS are simultaneously present in the t-PA-treated ischemic brains, it is important to address the questions whether the MMPs activations, the BBB disruption and hemorrhagic transformation are due to the actions of t-PA itself, RNS or the synergistic effects of RNS and t-PA, and how the MMPs activations and related molecular cascades are induced in the process. Our studies revealed that caveolin-1, a 22 kDa integral membrane protein, can inhibit RNS production, MMPs activations and BBB disruption, whereas over-production of NO from the ischemic brains can down-regulate caveolin-1, activate MMPs and increase BBB permeability. Several synthesized and natural RNS inhibitors revealed to inhibit the MMPs activations and reduce BBB permeability in an experimental ischemic stroke animal model with t-PA treatment. Following this line, we will test the hypothesis that the RNS inhibitors can up-regulate caveolin-1 expression, inhibit MMPs activations reduce BBB permeability, and prevent hemorrhagic transformation in t-PA treated ischemic stroke animal model. If the hypothesis can be validated, we logically expect that the antioxidant therapy for inhibiting RNS production can be used to reduce the risk of hemorrhagic transformation and extend the therapeutic time window of t-PA for acute ischemic stroke. Subsequently, the antioxidant therapy can be an add-on therapeutic strategy with t-PA treatment for acute ischemic stroke patients. As t-PA is the only thrombolytic drug clinically used for acute ischemic stroke patients and antioxidants are commonly used healthy supplements, the study will highlight their therapeutic value for stroke treatment. Therefore, the study will be highly innovative and has a multitude of application potentials.

	中風是人類疾病的第二位殺手，t-PA是FDA批准的唯一溶栓劑，用於缺血性中風治療。早期溶栓能提高病人的治療效果，而中風3小時後的溶栓治療存在腦出血的危險性。研究表明，髓質金屬蛋白酶（MMP）的活化以及血腦屏障高通透性是溶栓治療腦出血的重要環節，同時溶栓以及重建血流供應可產生活性氧和活性氮自由基而造成腦缺血再灌注損傷。一氧化氮和過氧亞硝基是活性氮的重要組成部分，它們能活化MMP，攻擊緊密連接蛋白，增加血腦屏障通透性。由於t-PA和活性氮均能造成上述病理變化，因此，我們提出溶栓治療所致腦出血究竟是t-PA的直接作用的結果還是活性氮自由基所致的問題。我們的實驗證據顯示活性氮自由基清除劑能抑制t-PA引起的MMP的活化，減少血腦屏障通透性。因此，我們設計有關實驗以測試是否活性氮自由基清除劑能減少t-PA引起的血腦屏障破壞和腦出血的形成。如果該假說能成立，抗氧化治療就能作為t-PA溶栓治療的輔助療法，以減少溶栓併發症。由於t-PA是目前唯一的溶栓藥物，而抗氧化劑也是常用的保健品，本研究為抗氧化劑提供了新的治療方向。
Realisation of objectives:	Objective 1. (1)We used mice brain microvascular endothelial cells (BMECs) and investigated the synergistic effect of tissue plasminogen activator (t-PA) and RNS on cell death under oxygen and glucose deprivation (OGD) condition, which mimics cerebral ischemia-reperfusion injury in vitro. (2)We conducted in vivo animal experiments by using SD rat middle cerebral artery occlusion (MCAO)-induced cerebral ischemia-reperfusion injury model. Using this model, we investigated the effect of early and delayed t-PA treatment on hemorrhagic transformation, 3-NT formation and MMPs activation. Then we tested the effect of FeTMPyP, a representative peroxynitrite decomposition catalyst, on hemorrhagic transformation, MMP-9/-2 activation and neurological function in ischemic stroke with delayed t-PA treatment. Objective 2. (1) We investigated the role of caveolin-1 in mediating MMP-9 activation, tight junction disruption and blood brain barrier (BBB) damage in SD rat MCAO model. We further compared the BBB damage in wild type and caveolin-1 knockout mice in MCAO model. (2) We investigated the role of caveolin-1 in regulating MMP-9 by caveolin-1 knockdown in cultured BMEC. (3) We tested the effect of NG-nitro-L-arginine methyl ester [L-NAME, a non-selective nitric oxide synthase (NOS) inhibitor] on caveolin-1 expression, MMP-9 activation, tight junctions' expression and BBB damage in both wild type and caveolin-1 knockout mice. (4) We investigated Calycosin-7-O-β-D-glucoside, an isoflavones derived from AstragaliRadix. We evaluated its effect on nitric oxide/caveolin-1/matrix metalloproteinases pathway mediated BBB damage and brain injury in rat MCAO model. Objective 3 (1) We developed diarylamine-based fluorogenic probes for detection of peroxynitrite. (2) We investigated the direct scavenging effect of baicalin on peroxynitrite by using

	Mass spectroscopy. We further investigated the peroxynitrite scavenging effect of baicalin in human SH-SY5Y neuroblastoma cells under the challenges of 3-morpholinosydnonimine (SIN-1, a peroxynitrite donor). Finally, we investigated the effects of baicalin on 3-nitrotyrosine formation in rat MCAO brains. (3) We studied the effects of baicalin on reducing neurotoxicity induced by peroxynitrite. We further investigated baicalin's effect on brain infarct volume and neuronal apoptosis in rat MCAO model. (4) We investigated the effect of baicalin on attenuating hemorrhagic transformation, improving neurological outcome, reducing mortality rate, inhibiting MMP-9 activation, and preserving tight junction ZO-1expression in rat MCAO model with delayed t-PA treatment. (5) We investigated Sodium Danshensu (SDSS), a representative active compound from salvia miltiorrhiza. We evaluated the potential effects of SDSS on scavenging peroxynitrite (ONOO-) and protecting neuronal cells from ischemia reperfusion injury in human SH-SY5Y cells in vitro and MCAO model in vivo.

Summary of objectives addressed:

	Objectives	Addressed	Percentage achieved
1.	Objective 1: To test the hypothesis that RNS inhibitors can alleviate t-PA-induced MMPs activations, BBB disruption and hemorrhagic transformation in cerebral ischemia-reperfusion injury	Yes	100%
2.	Objective 2: To test the hypothesis that cav-1 is a critical target protein of the RNS inhibitors on reducing the BBB disruption and hemorrhagic transformation in the experimental ischemic stroke model with thrombolytic treatment.	Yes	100%
3.	Objective 3: To test the effects of a natural antioxidant on the production of RNS, the expression of cav-1, the activations of MMPs, the BBB permeability and hemorrhagic transformation in the experimental ischemic stroke model with the delayed t-PA treatment.	Yes	100%

Research Outcome	
Major findings and research outcome:	1. We developed a highly sensitive probe for peroxynitrite detection in vitro and in vivo In this study, we developed diarylamine-based fluorogenic probes for detection of peroxynitrite which only generate fluorescence in presence of peroxynitrite without cross-reaction with other free radicals like hydroxyl radical. The results have been published in J Am Chem Soc, 136(33), 11728-11734. 2. Peroxynitrite plays an important role in mediating delayed tissue plasminogen activator (t-PA) treatment induced hemorrhagic transformation (HT) in ischemic brains via regulating MMPs activation The study proves that early t-PA reduces 3-NT (a peroxynitrite biomarker) formation, while delayed t-PA treatment exacerbates 3-NT formation in ischemic brains and induces HT. FeTMPyP, a representative peroxynitrite decomposition catalyst, significantly reduces HT via inhibiting MMP-9/-2 activation. These results have been published in CNS Neurosci Ther 21(7):585-590, 2015. 3. Caveolin-1 plays an important role in mediating blood brain barrier damage in ischemic stroke via regulating MMPs activity We found that caveolin-1 was down regulated in ischemic stroke, leading to activation of MMPs, disruption of tight junction ZO-1 and subsequently BBB damage. L-LAME treatment partially restored the expression of caveolin-1 and down regulated MMP-9 activity, preserved tight junction ZO-1, thus protected blood brain barrier. These results have been published in J Neurochem. 120(1):4-6, 2012. 4. Calycosin-7-O-β-D-glucoside regulates nitric oxide/caveolin-1/matrix metalloproteinases pathway and reduced BBB damage in ischemic stroke The study found that Calycosin-7-O-β-D-glucoside inhibited nitric oxide, secured expression of caveolin-1 and inhibited MMP-9/-2 activity in rat MCAO model and in cultured BMECs, hence, reduced brain infarct volume and BBB damage in vivo and attenuated BMECs death in vitro. These results have been published in J Ethnopharmacol, 155(1), 692-701, 2014. 5. Baicalin could directly scavenge peroxynitrite and reduce neuronal apoptosis in ischemic stroke The study found that baicalin, a flavonoid compound isolated from Scutellaria baicalensis G, could directly scavenge peroxynitrite in vivo and in vitro. Moreover, baicalin attenuated peroxynitrite induced neurotoxicity in vitro and reduced the brain infarct volume in vivo. These results have been published in Journal of Ethnopharmacology 150(1):116-124, 2013. 6. Baicalin co-treatment significantly reduced hemorrhagic transformation induced by delayed t-PA treatment in ischemic stroke, via inhibiting peroxynitrite mediated MMP-9 activation The study found that baicalin co-treatment with t-PA significantly attenuated HT, ameliorated brain edema, reduced mortality rate, and improved the neurological

	outcomes. Moreover, baicalin co-treatment reduced 3-NT formation, attenuated MMP-9 activation and preserved ZO-1 expression in ischemic brains with delayed t-PA treatment. The manuscript is in preparation.
Potential for further development of the research and the proposed course of action:	Based on the achievements of our studies, we expect following potential directions for further studies: (1) To further explore the molecular mechanism of how t-PA reduced or exacerbated 3-NT formation at different treatment time points in ischemic stroke model; (2) To compared the effect of baicalin with baicalein on attenuating hemorrhagic transformation induced by delayed t-PA treatment in ischemic stroke; (3) To investigate the potential roles of peroxynitrite in mediating hyperglycemia induced hemorrhagic transformation in ischemic stroke. Due to the limitation of funding and research period, we could not achieve the goals. We plan to seek for more funds from RGC GRF grants or other funding sources to continue our studies in this direction.
Layman's Summary of Completion Report:	Stroke is a leading cause of disability in human diseases and is one of the major disease burdens worldwide. Tissue plasminogen activator is the only FDA approved agent for acute ischemic stroke treatment, with limited time window of 4.5 hours. Treatment beyond the time window significantly induces hemorrhagic transformation (HT), which is a severe complication resulting in worsened outcomes. Thus, developing approaches to reduce HT is timely important. With the support of RGC GRF grant, we conducted a series of experiments to test our hypothesis that peroxynitrite plays an important role in mediating hemorrhagic transformation induced by delayed t-PA treatment in ischemic stroke. We demonstrate that peroxynitrite is increased in ischemic brains by delayed t-PA treatment and scavenging peroxynitrite significantly reduces hemorrhagic transformation. Caveolin-1 may be the downstream of peroxynitrite, and mediated blood brain barrier (BBB) damage in ischemic stroke. Baicalin is a natural compound isolated from Traditional Chinese Medicine. Baicalin directly scavenges peroxynitrite, significantly reduces hemorrhagic transformation and improves the overall outcomes. Therefore, peroxynitrite is an important target for reducing hemorrhagic transformation in ischemic stroke with delayed t-PA treatment. This project yields 7 original articles, 2 articles in preparation, 6 review articles and trained 5 postgraduate students.

Research Output

Peer-reviewed journal publication(s) arising directly from this research project : (* denotes the corresponding author)

Year of Publication	Author(s)	Title and Journal/Book
2012	Gu Y, Zheng GQ, Xu MJ, Li Y, Chen	Caveolin-1 regulates nitric oxide mediated matrix

	XM, Zhu WZ, Tong Y, Chung SK, Liu KJ, Shen JG*	metalloproteinases activity and blood-brain barrier permeability in focal cerebral ischemia and reperfusion injury. Journal of Neurochemistry 120(1):147-156, 2012
2012	Shen JG*	Reactive nitrogen species: Dual roles for blood brain barrier disruption and brain repairs in cerebral ischemia and reperfusion injury. Acta Biophysica Sinica 2012, 28(4) 295-306
2013	Chen XM, Chen HS, Xu MJ, Shen JG*	Targeting reactive nitrogen species: A promising therapeutic strategy for cerebral ischemia-reperfusion injury. Acta Pharmacologica Sinica 34(1):67-77, 2013
2014	Shen JG*, Gu Y.	Insights into mechanisms of blood Brain barrier permeability: Roles of free radicals, matrix metalloproteinases and caveolin-1. Systems Biology of Oxidative Stress. (Eds.Ismail Laher),Springer pp 2049-2067
2013	Xu MJ, Chen X, Gu Y, Peng T, Yang D, Chang R, So KF, Liu KJ, Shen JG*	Baicalin can scavenge peroxynitrite and ameliorate endogenous peroxynitrite-mediated neurotoxicity in cerebral ischemia-reperfusion injury. J Ethnopharmacol, 150(1), 116-124.
2013	Yang LP, Shen JG*, Xu WC,Li J, Jiang JQ*.	Secondary metabolites of Genus Astragalus : Structure and biological activity update. Chemistry & Biodiversity 10: 1004-1054, 2013
2014	Gu Y, Lee W, Shen J*.	Site-2 protease responds to oxidative stress and regulates oxidative injury in mammalian cells, Sci

		Rep, 4:6268
2014	Gu Y, Chen J, Shen J*	Herbal Medicines for Ischemic Stroke: Combating Inflammation as Therapeutic Targets, J Neuroimmune Pharmacol, 9(3):313-39
2014	Peng T, Wong NK, Chen X, Chan YK, Ho DH, Sun Z, Hu JJ, Shen J, El-Nezami H, Yang D*	Molecular imaging of peroxynitrite with HKGreen-4 in live cells and tissues, J Am Chem Soc, 136(33), 11728-11734
2014	Xingmiao Chen, Hansen Chen, Ruixia Deng, Jiangang Shen*	Pros and Cons of Current Approaches for Detecting Peroxynitrite and Their Applications, Biomedical journal, 37(3), 120
2014	Fu S, Gu Y, Jiang JQ, Chen X, Xu M, Chen X, Shen J*.	Calycosin-7-O-β-D-glucoside regulates nitric oxide /caveolin-1/matrix metalloproteinases pathway and protects blood-brain barrier integrity in experimental cerebral ischemia-reperfusion injury, J Ethnopharmacol, 155(1), 692-701
2015	Gong J, Sun F, Li Y, Zhou X, Duan Z, Duan F, Zhao L, Chen H, Qi S, Shen J*	Momordica charantia polysaccharides could protect against cerebral ischemia/reperfusion injury through inhibiting oxidative stress mediated c-Jun N-terminal kinase 3 signaling pathway, Neuropharmacology, 91, 123-134
2015	Chen HS, Chen XM, Feng JH, Liu KJ, Qi SH, Shen JG*	Peroxynitrite Decomposition Catalyst Reduces Delayed Thrombolysis-induced Hemorrhagic Transformation in Ischemia-reperfused Rat Brains, CNS neuroscience and

			therapeutics 21(7):585-590

Recognized international conference(s) in which paper(s) related to this research project was/were delivered :

Month/Year/City	Title	Conference Name
10/2011/Beijing	Danshensu is a novel peroxynitirte scavenger and has neuroprotective effects against cerebral ischemia-reperfusion injury.	17th International Biophysics Congress &12th Chinese Biophysics Congress
08/2012/Macau	Baicalin protects neural cells against cerebral ischemia reperfusion injury by scavenging peroxynitrite.	The 11th Meeting of the Consortium for Globalization of Chinese Medicine
08/2012 /Lanzhou	Reactive nitrogen species: Dual roles for blood brain barrier disruption and brain repairs in cerebral ischemia and reperfusion injury	The 1st International Chinese Symposium on Free Radical Research, the 5th Symposium for Three Distracts of Cross-straits on Free Radical Research
08/2012/Xiamen	Current progress in medical imaging diagnosis and therapeutic strategies for stroke: Opportunities and challenges for Chinese medicine	The 9th National Integrated Western and Chinese Medical Congress in Neurology.
05/2013 /Shanghai	Caveolin-1 is a crucial molecular target in blood-brain-barrier (BBB) disruption and infarction enlargement during cerebral ischemia-reperfusion injury	XXVth International Symposium on Cerebral Blood Flow, Metabolism and Function& XIth International Conference on Quantification of Brain

			Function with PET
	09/2013/Vienna	Caveolin-1 phosphorylation plays an important role in inhibition of oxygen-glucose-deprivation-induced endothelial cell apoptosis via regulating Stat3 pathway	XXI World congress of neurology
	09/2013/Beijing	Nitric oxide/Caveolin-1/MMP pathway: a novel therapeutic strategy for drug discovery from herbal medicine targeting blood-brain-barrier disruption during cerebral ischemia-reperfusion injury	International Conference for Microcirculation and the 13th Annual Conference of the Professional Committee for Microcirculation, Chinese Association of Integrative Medicine
Other impact (e.g. award of patents or prizes, collaboration with other research institutions, technology transfer, etc.):	Patent: Diarylamine-Based Fluorogenic Probes for Detection of Peroxynitrite Inventors: Dan Yang, Tao Peng, Jiangang Shen, Xingmiao Chen. US application or PCT International Application No. 13/754,499.		

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